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LOCALIZATION OF PHOTOSYNTHETIC REACTION CENTERS BY ANTIBODY BINDING TO CHROMATOPHORE MEMBRANES FROM *RHODOPSEUDOMONAS SPHEROIDES* STRAIN R26

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SUMMARY

Rabbit antiserum against highly purified reaction center preparations was shown to react specifically with a single component of chromatophore membranes from *Rhodopseudomonas spheroides* strain R-26. The conjugate of purified gamma globulin and ferritin prepared with toluene diisocyanate was used to determine the localization of reaction centers in the chromatophore membranes. Virtually no antibody was bound by intact membranes. After removing the 9 nm ATPase from these membranes by dilute EDTA treatment, a considerable amount of antibody was bound to the exposed outer membrane surface. The reaction center binding sites were estimated to be uniformly distributed with approx. 1 reaction center per 200 nm^2 of membrane surface. These results indicate that the reaction centers are located near the outer membrane surface but below the ATPase particles. Since the distribution of reaction centers and particles on rough faces seen by freeze-fracture electron microscopy are similar, it is suggested that the freeze-fracture particle may be a complex of a reaction center and other electron transfer components localized within the hydrophobic region of the membrane.

INTRODUCTION

Asymmetry of chromatophore membranes from *Rhodopseudomonas spheroides* strain R-26 has been indicated by studies of the fractionation of these membranes into functional protein components [1,2]. Three components have been released from the membrane sequentially by detergent treatments. Washing with dilute Triton X-100 or with EDTA removed a 9 nm-ATPase particle from the outer membrane surface [3]. Higher Triton X-100 concentrations solubilized the protein complex which contained the photosynthetic reaction center and other components of the electron transfer system. The large particles which remained contained the 5 nm-light-har-

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vesting bacteriochlorophyll proteins [3, 4]. A model of the chromatophore membrane structure has been proposed which is consistent with the physical and chemical properties of the chromatophore membranes and the subchromatophore particles [4].

Reaction center preparations of much smaller particle size have been isolated using lauryl dimethyl-amine-N-oxide [5, 6]. These reaction centers do not contain the cytochrome components and other secondary electron transfer components present in the large reaction center complex isolated with Triton X-100. The small molecular weight reaction center preparations contain a pigment complex of four bacteriochlorophyll *a* and 2 bacteriopheophytin *a* molecules which functions as the primary electron donor [7, 8, 9] and a nonheme iron component which is most likely the primary electron acceptor [10].

In this investigation, immunochemical techniques have been used to localize the reaction centers within the chromatophore membrane. Some of these results have previously been communicated in preliminary form by us [11], and Steiner et al. [12] have recently communicated in preliminary form related experiments on reaction center localization.

METHODS

Rps. spheroides strain R-26 was grown photosynthetically in modified Hutner medium and used immediately. Chromatophores were isolated and purified by the procedure described previously [2]. The ATPase was removed from chromatophores by washing with 0.001 M EDTA (pH 7.5) as reported elsewhere [3, 4]. Concentrations of light-harvesting and reaction center bacteriochlorophyll were determined spectrophotometrically [2] and protein was measured by the procedure of Lowry et al. [13] using bovine serum albumin as the standard.

The low molecular weight reaction center was isolated by Triton X-100 treatment of chromatophores to solubilize the complex of reaction center and cytochromes [2]. DEAE-cellulose (Reeve Angel DE52) chromatography, in the presence of lauryl dimethylamine-N-oxide, LDAO (Onyx Chem. Co.), was utilized to separate the reaction center from the other electron transfer components of the complex. The photosynthetic reaction center complex preparation was placed on a DEAE column and the column was eluted sequentially with 0.01 M Tris/HCl (pH 8.0) to remove Triton X-100, with a continuous KCl gradient (0.05 M–1.0 M) to remove polar proteins, with Tris buffer to remove the salt and then with a continuous KCl gradient (0.0 M – 0.5 M) in buffer containing 0.2 % LDAO to elute the reaction centers. The purity of the photosynthetic reaction center preparation was established by sedimentation in the ultracentrifuge and by polyacrylamide gel electrophoresis.

Preparation of antiserum to the reaction centers, immunodiffusion and immunoelectrophoresis were carried out by the general methods of Campbell et al. [14]. New Zealand rabbits were injected subcutaneously three times at weekly intervals with a mixture of 1–2 mg photosynthetic reaction center protein in 0.12 M NaCl and an equal volume of Freund's adjuvant (Calbiochem). After a two week rest, blood was removed from an ear vein, stored overnight at 4 °C and centrifuged to remove the clot. The antiserum was used immediately or after storage at −10 °C.

The gamma-globulin fraction was purified by chromatographing the antiserum on DEAE-cellulose according to the method of Fahey [15]. The protein fractions

which were eluted from the DEAE column by washing with 0.001 M potassium phosphate (pH 8.0) and with buffer containing 0.01 M KCl were combined and concentrated by ultrafiltration on an Amicon M50 membrane. The purity of the gamma globulin was established by polyacrylamide gel electrophoresis using the procedure of Davis [16].

The purified gamma globulin was conjugated with ferritin (Calbiochem, A grade) using toluene-2,4-diisocyanate (Matheson) according to the method of Rifkind et al. [17]. The ferritin-antibody was separated from unconjugated gamma globulin by centrifugation for 2 h at 150000 $\times g$. The supernatant containing unconjugated gamma globulin was discarded. The pellet of ferritin-antibody conjugate was suspended in 0.05 M potassium phosphate (pH 7.5) and stored at 4 °C, and was subsequently used to label the sites of the reaction center protein complex in chromatophore membrane preparations.

After reaction with ferritin-antibody, the purified chromatophore and EDTA-washed membrane preparations were separated from unreacted ferritin-antibody by sucrose density gradient centrifugation and examined by electron microscopy. Mixtures of 0.1 ml of membrane preparation, 0.25 ml of 0.01 M sodium borate (pH 8.0) containing 0.85 % NaCl and 0.05 ml of ferritin-antibody were incubated at 30 °C for 15 min. EDTA-washed chromatophores were also incubated similarly with 0.025 ml of antiserum before reaction with ferritin-antibody or incubated separately with an equivalent concentration of unconjugated ferritin. The incubation mixtures were layered onto linear sucrose gradients prepared from 2.4 ml each of 0.5 and 1.2 M sucrose in borate (0.01 M, pH 8.0) saline (0.85 %) buffer and centrifuged for 90 min at 120000 $\times g$. The membranes which reacted with the antibody or ferritin-antibody were recovered in pellets at the bottom of the tubes separate from excess ferritin or ferritin-antibody in the upper one-third and unreacted membranes near the middle of the gradients.

Chromatophore suspensions for negative staining were applied to carbon-coated grids and washed with either 0.5 % uranyl acetate or 1 % potassium phosphotungstate, pH 6.0. The former stains ferritin well, while the latter resolves chromatophore surfaces better. The membranes were fixed for sectioning as follows; Treatment for 1½ h in ice with 1 % glutaraldehyde in 0.01 M sodium borate, pH 8.0 containing 0.85 % NaCl, or in 0.05 M collidine, 0.06 M sucrose, pH 7.3, then washed with buffer, post-fixed 1 h in 1 % osmium tetroxide in the same buffer, dehydrated in a graded ethanol series, and embedded either in Mollenhauer's Araldite-Epon mixture # 1 [18] or Spurr's medium-hard, low-viscosity, epoxy mixture [19]. Sections were normally post-stained with uranyl acetate and Reynold's lead citrate [20]. For exposing membrane detail, chromatophore sections were post-stained 10 min with 1 % barium permanganate and washed for 45 sec with 0.025 % citric acid [21].

Antibody reaction for negative staining was performed in borate buffer after application of the membranes to grids coated with carbon films. Grids were floated for 1 min in 0.05 % bovine serum albumin in buffer, blotted, incubated for 5 min in X10 diluted ferritin-labelled antibody, washed five times for 1 min in buffer, and stained. The unlabelled control was exposed for 5 min to gamma-globulin antibody before the ferritin-label treatment.

RESULTS

Reaction of the antiserum with the purified reaction center and with a chromatophore membrane component is shown by the immunodiffusion plate in Fig. 1. The formation of one precipitin band indicates that the antiserum and the reaction center preparations comprise a single antigen-antibody system. One major reactive component is present in the solubilized chromatophore membrane fraction and the convergence of the precipitin bands indicates that the reaction center protein is immunologically identical to this chromatophore membrane component. Comparison of the positions of the precipitin bands at several photosynthetic reaction center concentrations in the circumferential wells indicates that the reactivity of the purified photosynthetic reaction center and components in the solubilized chromatophore membrane preparations are very similar.

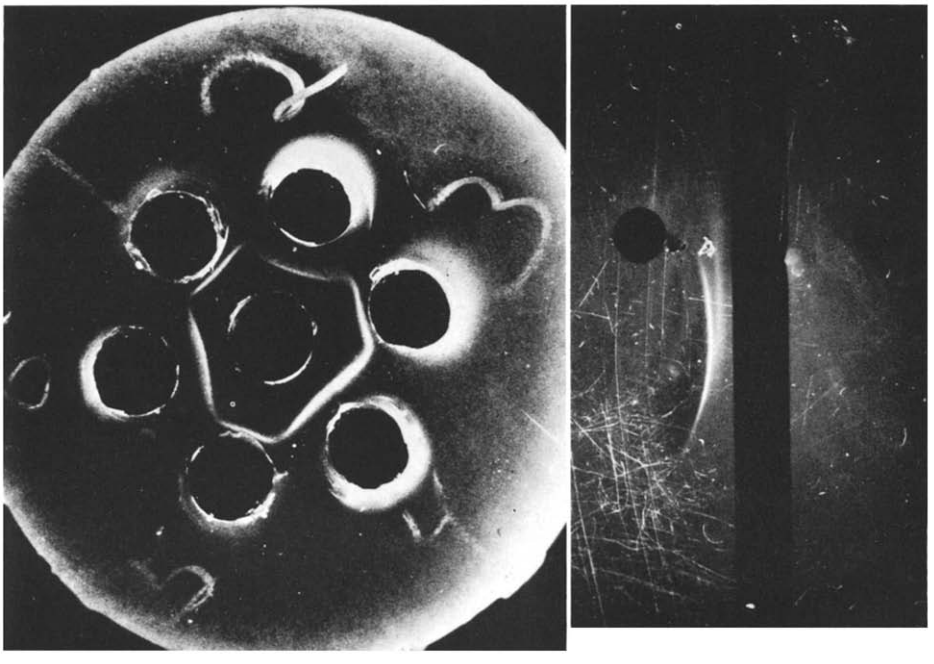


Fig. 1. Ouchterlony plate showing precipitin bands from antiserum to reaction center from *Rps spheroides* strain R26. Undiluted antiserum was placed in the center well. Wells numbered 2,4 and 6 contained reaction center protein with P_{870} absorbance calculated to obtain 6 μM, with the amounts in the respective wells at 6, 13 and 7.5 μl. Wells 1, 3 and 5 contained chromatophores (P_{870} equivalent at 20 μM) with respectively 23 μl of undiluted preparation, 20 μl of X2 diluted and 27 μl of $\times$ 5 diluted preparation. The final volume of fluid in all wells was adjusted to 100 μl with the Amonyx L-O detergent in borate buffer at pH 8. The plate was photographed after 24 h. Note that only one continuous precipitin profile is exhibited even in wells containing chromatophores and attesting to photosynthetic reaction center-protein isolation as a homogeneous preparation.

Fig. 2. Immunoelectrophoresis of photosynthetic reaction center protein. The reaction center-well contained P_{870} equivalent of 10 μM. The control well contained 100 μg of bovine serum albumin, both wells filled to 100 μl capacity. Undiluted antiserum was placed in the center well. Electrophoresis was carried out for 4 h at 4 V/cm across the slide. A smaller band (presumably aggregated protein) can be seen just below the main band. No reaction was obtained with bovine serum albumin.

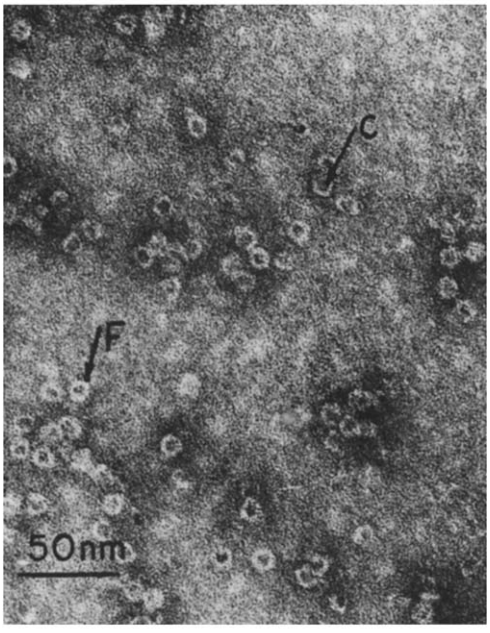


Fig. 3. Ferritin-labelled anti-reaction center gamma globulin conjugate (× 262 000). The conjugates were negatively stained with 1 % potassium phosphotungstate. The ferritin part of the complex is visible, 11.8 nm diam. (F) with a 5.5 nm electron-dense core (C).

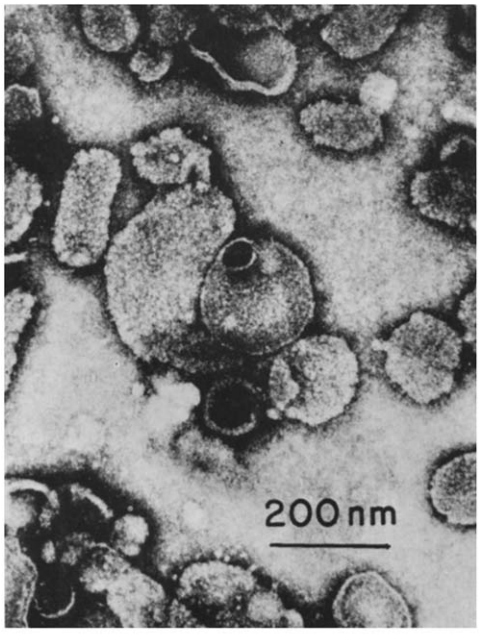


Fig. 4. Purified chromatophores (× 79 900), negatively stained with 1 % potassium phosphotungstate. Note the textured surfaces of the membranes.

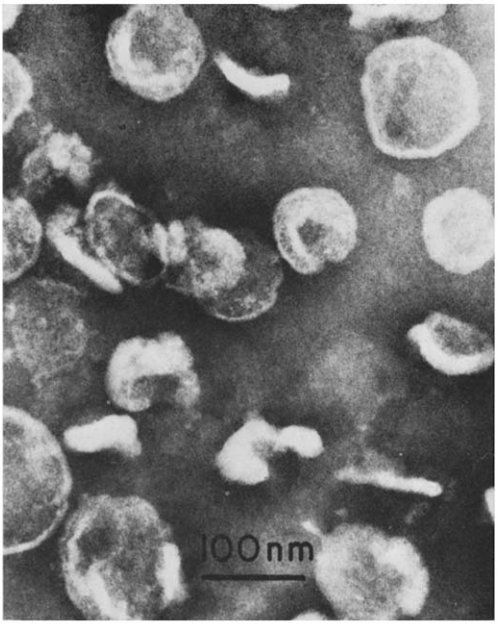


Fig. 5. EDTA-washed chromatophores (× 138 000). The membranes were negatively stained with 1 % KPT. Membrane surfaces are quite smooth compared with those in Fig. 4.

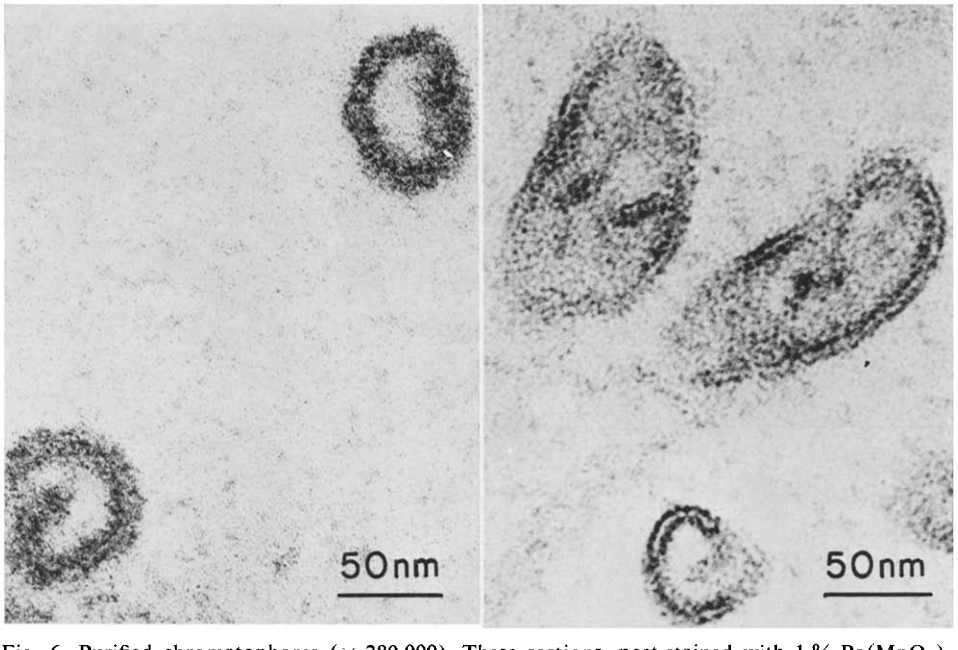


Fig. 6. Purified chromatophores (× 280 000). These sections, post-stained with 1 % $Ba(MnO_4)_2$, show a granular, and often irregular, outline. Membrane thickness varies from 12 to 16 nm.

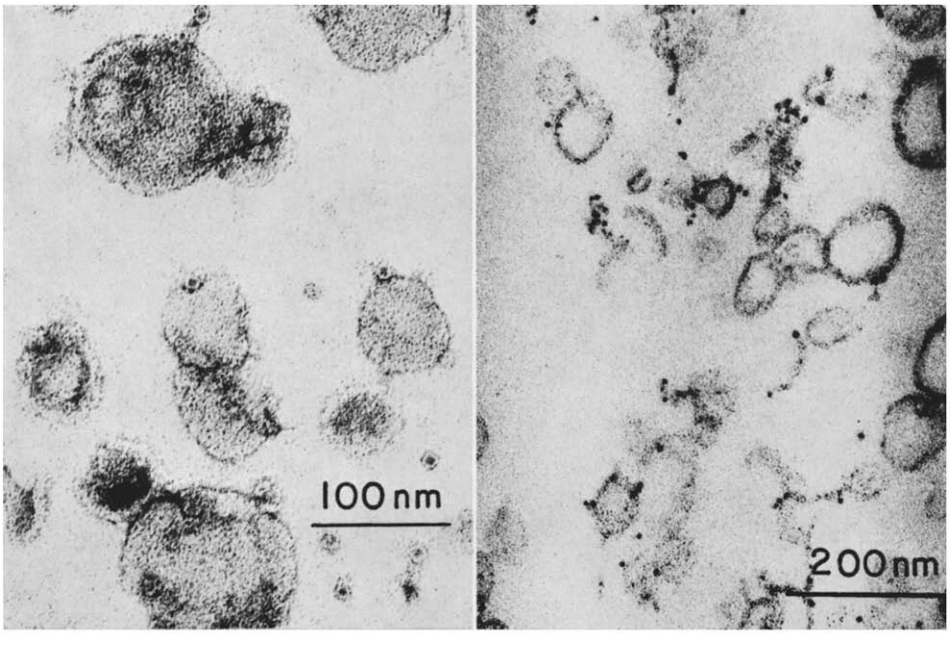
Fig. 7. EDTA-washed chromatophores (× 280 000). These membrane sections, post-stained with 1 % $Ba(MnO_4)_2$, have a thinner, smoother profile about 10 nm thick.

Additional evidence for the homogeneity of the reaction center and antiserum system is shown by immunoelectrophoresis in Fig. 2. The reaction center preparation migrates as a major reactive component with a minor component of lower mobility. The minor component probably results from some aggregation of the hydrophobic photosynthetic reaction center-protein.

In electron micrographs, the ferritin-antibody conjugate appears as a uniform preparation of ferritin molecules. They are linked to the much smaller gamma globulin molecules which are not visible (Fig. 3). Antibody binding is easily detected by the characteristic 5.5 nm electron-dense iron core which is apparent in membranes prepared by negative staining techniques, as well as those examined after pelleting and sectioning.

Electron micrographs of purified chromatophores prepared by differential centrifugation and linear sucrose density centrifugation of French-pressed cells [2] are shown in Fig. 4 and of EDTA-washed chromatophores in Fig. 5. The chromatophores are vesicles of 100–300 nm diameter, enclosed by one bilayer membrane.

The purified chromatophores (Fig. 4) have textured, knobbed surfaces with 9–10 nm bodies in or protruding from membrane surfaces. EDTA-washed chromatophores (Fig. 5) have smooth surfaces bearing only an indication of the textures evident in Fig. 4. The bounding membrane of the purified chromatophores, when embedded and sectioned, is 12 to 16 nm thick (Fig. 6). Globular substructure can be seen within the membrane at high magnification. Chromatophores which have been washed with EDTA to remove the surface ATPase are smoother and thinner,

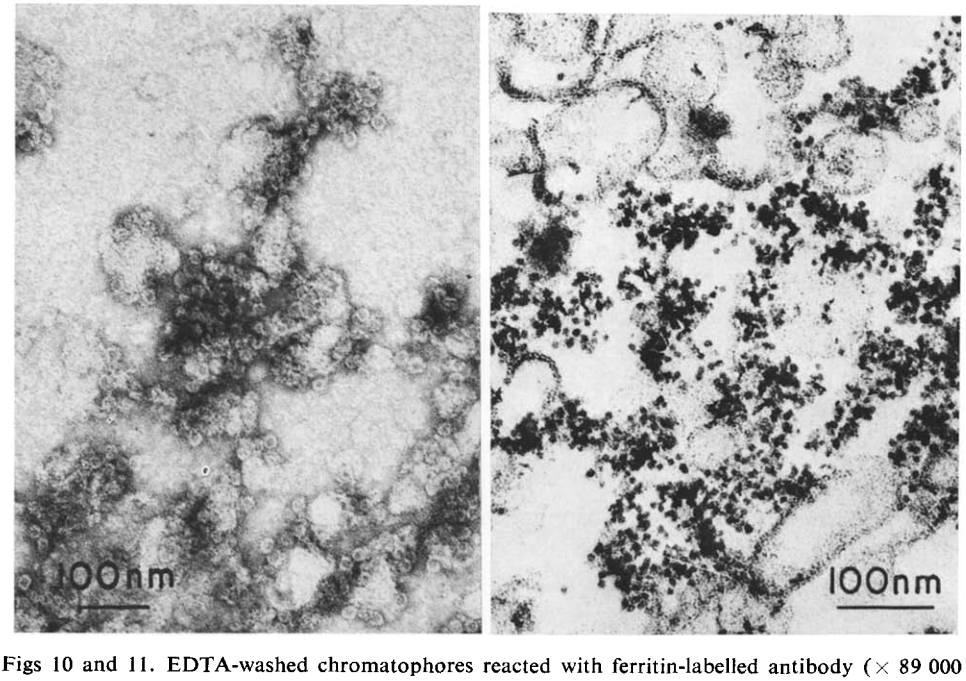


Figs 8 and 9. Purified chromatophores treated with ferritin-labelled reaction center antibodies (× 186 000 and 105 000). Fig. 8 is negatively stained with 0.7 % uranyl acetate: Fig. 9 shows sectioned chromatophores. The reaction center antibody reacts with chromatophore membranes in a widely scattered pattern, suggestive of membrane sites damaged during isolation.

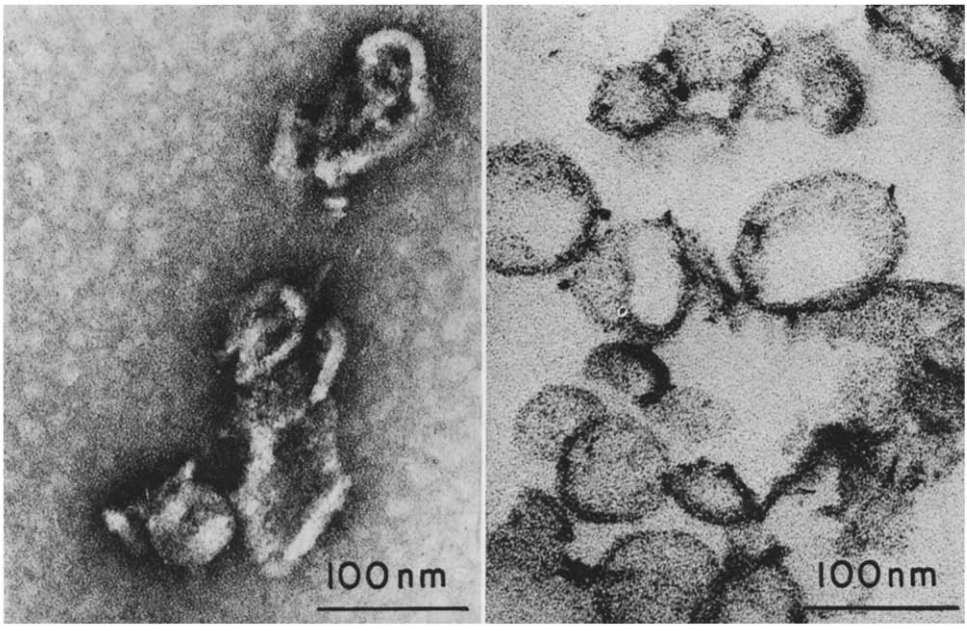
the bounding membrane being 9–11 nm thick, and having less apparent substructure (Fig. 7).

Electron micrographs of purified chromatophore preparations which have been incubated with the ferritin-antibody conjugate are shown in Figs 8 and 9. A comparatively small number of ferritin molecules of the ferritin-antibody are detectable in negatively stained (Fig. 8) or in sectioned preparations (Fig. 9). These membranes bind a small and scattered amount of the antibody prepared against the purified reaction centers. For negative staining an aliquot of the chromatophore suspension was applied to the carbon-film and antibody binding is indicated by single ferritin molecules on the background and by small groups at membrane sites (Fig. 8). Sectioned chromatophores reflect the same scattered distribution (Fig. 9). Examining both the surfaces and sections of chromatophores indicates adherence of the label to membrane strips and at discontinuities in the membrane surface. Since chromatophores are prepared by the high-pressure rupture of intracytoplasmic tubules, it is possible that some deep-seated molecules would be uncovered and that the pattern of antibody binding seen in Figs 8 and 9 is the result.

After removing the ATPase from the chromatophores by EDTA treatment, the membranes are extensively labelled by the ferritin-antibody as shown by Figs 10 and 11. A large number of ferritin molecules adhere to membrane surfaces in negatively stained preparations (Fig. 10). In thin section (Fig. 11) these washed membranes show a pattern of ferritin label suggesting chromatophore size and outline, despite the dense image of closely packed ferritin molecules and the difficulty in seeing thin



Figs 10 and 11. EDTA-washed chromatophores reacted with ferritin-labelled antibody (× 89 000 and 129 000). Negative staining with 0.5% uranyl acetate (Fig. 10) shows the labelled antibody crowded onto membrane surfaces. The thin section (Fig. 11) shows the close binding of antibody label over chromatophore-sized membranes.



Figs 12 and 13. EDTA-washed chromatophores, unlabelled antibody control (· 204 000). Washed chromatophores were treated with unlabelled reaction center antibody, then with ferritin-labelled antibody. The membranes, which were negatively stained with 0.7 % uranyl acetate (Fig. 12) or sectioned (Fig. 13), indicate that pretreatment with unlabeled antibody interferes with subsequent binding of the label.

EDTA-washed membranes. The distribution of the label indicates that the antigenic reaction centers are exposed for antibody binding after the ATPase is removed. On many micrographs the reaction centers, as shown by the ferritin label, are uniformly distributed on exposed membrane surfaces with 12–15 nm between centers at a density of approx. 1 antibody per 200 nm^2 (the calculated figure is 1 antibody per 175 nm^2). The specificity of the ferritin antibody binding is demonstrated by two sets of micrographs. Pictures of EDTA-washed chromatophores incubated with the protein, ferritin, which had not been conjugated to antibody, shows the virtual absence of labelling by ferritin. The micrographs in Figs 12 and 13 show an absence of significant binding to membranes which were pretreated with antiserum in order to occupy the antigenic sites before incubation with the ferritin antibody.

DISCUSSION

The asymmetric chromatophore membrane from *Rps spheroides* strain R-26 contains the ATPase on the outer membrane surface. The 9 nm ATPase particles are removed by treating the membranes with EDTA [3] and the recovery of soluble ATPase is consistent with a 1:1 stoichiometry of ATPase per reaction center in the intact membrane [4]. Upon removing the ATPase, the thickness of the chromatophore membrane in sectioned preparations decreases from approximately 14 nm to 10 nm (Figs. 6 and 7) and the reaction centers are exposed for ferritin-antibody binding.

The configuration of the ATPase in the intact chromatophore membrane has not been clearly established but the ATPase is most likely somewhat thinner than the 9 nm diameter of the particles in purified ATPase preparations. Only swollen membranes have 9-nm particles protruding from the outer surfaces in negatively stained preparations [3] and particles which appear similar are only occasionally detected in sectioned preparations. The difference of approximately 4 nm between the thickness of intact chromatophores and of membranes without the ATPase is considerably less than 9 nm. If the ATPase is spherical, much of the particle must be submerged into the membrane structure.

The reaction centers in the chromatophore membrane are located below the ATPase on the outer membrane surface. Antibody which reacts specifically with the photosynthetic reaction center component of chromatophores (Fig. 1) does not bind significantly to the surface of purified chromatophore membranes (Figs 8 and 9) but the ferritin-antibody extensively labels the membranes after the ATPase has been removed (Figs 10 and 11). The estimated reaction center distribution of 1 per 175 nm^2 determined by antibody binding to this exposed membrane surface agrees closely with the particle density of 1 per 200 nm^2 on the rough faces obtained by freeze-fracture through the hydrophobic interior of the chromatophore membrane [3]. The localization and distribution of the reaction centers within the chromatophore membrane provides additional support for our suggestion that the freeze-fracture particle within the chromatophore membrane may be an electron transfer complex containing the photosynthetic reaction center and other electron transfer components.

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