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SOME PROPERTIES OF THE ATPase FROM CHROMATOPHORES OF *RHODOPSEUDOMONAS SPHEROIDES* AND ITS STRUCTURAL RELATIONSHIP TO THE BACTERIOCHLOROPHYLL PROTEINS*

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SUMMARY

Chromatophores from *Rhodopseudomonas spheroides* exhibit adenosine triphosphatase activity which is Mg^{2+}-dependent and stimulated maximally by dithiothreitol and Triton X-100. Dilute EDTA or Triton X-100 solutions remove the ATPase from the membranes concomitant with the release of the 9-nm negative stain particles from the outer surfaces. The larger 13-nm freeze–fracture particles within the membranes are unchanged by these treatments. The ATPase purified from the wash solutions appears as uniform 9-nm particles with an apparent molecular weight near $3 \cdot 10^5$. The results indicate that the asymmetric chromatophore membranes contain the 9-nm ATPase on the outer surface and suggest that the 13-nm freeze–fracture particles within the membranes may be reaction center complexes containing the reaction center bacteriochlorophyll and other electron transfer components.

INTRODUCTION

Protein complexes containing the two functional forms of bacteriochlorophyll from chromatophores of *Rhodopseudomonas spheroides* strain R-26 have previously been characterized[1-3]. The physical and chemical properties of these preparations suggest that the bacteriochlorophyll proteins occupy an asymmetric layered arrangement within the chromatophore membranes. Triton X-100 releases the 12-nm reaction center complex particles containing P870 and the other electron transfer components from the residual membrane containing the 5-nm light-harvesting bacteriochlorophyll proteins. In addition to proteins which capture and transfer light energy or transfer electrons, a detailed formulation of the chromatophore membrane structure must also consider the localization of the proteins which catalyze the reactions of photophosphorylation. The synthesis of ATP by the phosphorylation of ADP constitutes a major function and presumably also occupies a major portion of the membrane proteins.

Preparations from mammalian mitochondria[4], heterotrophic bacteria[5], higher plant chloroplasts[6] and chromatophores of photosynthetic bacteria[7] which act as

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coupling factors for phosphorylation also catalyze the hydrolysis of ATP (the ATPase reaction) to a greater or lesser degree. In the present investigation, we have determined properties of the ATPase activity of *Rps. spheroides* chromatophores and of purified preparations. Some of these results have previously been reported in preliminary form[8]. Procedures have been developed to remove the majority of the ATPase from the chromatophore membranes and to isolate this protein complex in high yield. The ATPase has been localized on the chromatophore membranes by comparing the physical and chemical properties of the purified preparations to those of the intact chromatophores.

METHODS

Absorption spectra were measured at 25 °C with a Cary 14 recording spectrophotometer equipped with a scattered transmission attachment. Concentrations of light-harvesting bacteriochlorophyll, B870, were determined from the absorbance at 860 nm using the extinction coefficient of 127 $mM^{-1} \cdot cm^{-1}$ (ref. 9). Light-induced absorbance changes were measured with an Aminco-Chance dual beam spectrophotometer equipped for actinic illumination at a right angle to the measuring beam. Actinic light from a tungsten-iodide lamp (Sylvania Sun Gun) was passed through a Bausch and Lomb 4-96 filter and an 865-nm interference filter was placed in the measuring beam. Concentrations of reaction center bacteriochlorophyll, P870, were determined from the light-induced absorbance decrease at 865 nm using the differential extinction coefficient of 93 $mM^{-1} \cdot cm^{-1}$ (ref. 10).

Protein was measured by the method of Lowry *et al.*[11] using bovine serum albumin as the standard and inorganic phosphate was measured by the method of Chen *et al.*[12]. ATPase activity was determined by measuring the inorganic phosphate formed from ATP (Sigma grade) in the dark at 25 °C. The reaction mixtures contained 60 mM Tris–HCl buffer, pH 8.1, 1 mM ATP, 1 mM $MgCl_2$, 2 mM dithiothreitol and 0.15 ml of purified chromatophores containing 59 nmoles of B870. To measure the total ATPase activity of membrane preparations, sufficient Triton X-100, 0.04% (v/v), was included to give maximal reaction rates.

Rps. spheroides, strain R-26, was grown photosynthetically in succinate medium. The cells were harvested by centrifugation, rinsed with 0.1 M Tris–HCl buffer (pH 7.5) and stored as a frozen paste at −10 °C. All remaining steps were carried out at 0–10 °C in 0.01 M Tris–HCl buffer (pH 7.5). The bacteria were suspended in approximately 4 vol. of buffer and broken by passage twice through an Aminco French pressure cell at 20000 lb/inch^2. The broken cell suspension was diluted with an equal volume of buffer, and cell debris was removed by centrifugation for 15 min at 17000 × *g*. Crude chromatophores were isolated from the supernatant by centrifugation for 60 min at 240000 × *g*. The pellets were suspended in buffer and diluted to 0.72 mM B870.

Chromatophores were purified from the crude fraction by an additional sedimentation on a linear sucrose density gradient. 4-ml portions were layered onto 34-ml gradients formed from 0.5 and 1.5 M sucrose and the tubes were centrifuged for 12 h at 95000 × *g* (25000 rev./min in the Spinco SW-27 rotor). The supernatants containing some soluble proteins and the bottom opalescent bands of principally cell wall material were discarded. The chromatophore bands approximately 2 cm from the top of the gradients were pooled, diluted with an equal volume of buffer and sedi-

mented by centrifugation for 60 min at 240000 × *g*. The pellets were suspended in buffer and diluted to 0.39 mM B870.

Similar conditions were used to compare the effects of Triton X-100 on the ATPase activity and on the release of proteins from the chromatophore membranes. One third volume of buffer containing the appropriate Triton X-100 was added slowly with stirring to a sample of purified chromatophores. A portion, 0.2 ml, of the mixture was diluted into a reaction mixture and the ATPase activity measured. A larger 0.5-ml sample was layered onto 4.5 ml of 0.5 M sucrose and centrifuged for 90 min at 105000 × *g* (35000 rev./min in the SW-39 rotor) to separate the soluble components recovered in the supernatant from the residual membranes recovered in the pellets.

The ATPase was removed from chromatophores by washing the membranes with dilute solutions of EDTA or Triton X-100. In the EDTA wash, purified chromatophores were diluted with ten volumes of 0.001 M EDTA (pH 7.5) and passed twice through the French pressure cell at 20000 lb/inch^2. The samples were made to 2 mM ATP and centrifuged for 60 min at 200000 × *g*. The ATPase was purified further by concentrating the supernatant on a Amicon XM-100 membrane filter at 20 lb/inch^2 and chromatographing 2.0-ml samples on a 2.2 cm × 35 cm Agarose A50m column in 10 mM Tris–HCl buffer, pH 7.5, containing 1 mM ATP and 5 mM EDTA. With Triton X-100, 0.13 ml of a 5 % (v/v) solution was slowly added to a stirring 2.0-ml sample of chromatophores. The mixture was layered onto 10 ml of 0.5 M sucrose and centrifuged at 200000 × *g* for 60 min. The pellet was suspended in buffer, sedimented and resuspended.

The ultrastructure of most preparations was examined by negative staining. Usually a carbon-coated grid was wet with the particle suspension and washed with 1.5–2 % methyl-modified phosphotungstic acid adjusted to pH 6.4–6.5 with KOH. Replicas of chromatophore membranes were made by the freeze–fracture method of Moor and Mühlethaler[13]. The membranes were pelleted from a mixture of 0.1 M KCl and 20 % glycerol (w/v), applied to a copper disc and quick-frozen in Genetron 22 (monochlorodifluoromethane, Allied Chemical Corp., Morristown, N.J.) kept at its freezing point by liquid nitrogen. The fractured membranes were shadowed with platinum at an angle of 45° and replicated with carbon in a Balzers BA 360 M freeze etch apparatus. All specimens were examined with a Philips EM 200 electron microscope. Particle diameters were measured at a right angle to the direction of shadowing and were not corrected for thickness of the platinum.

RESULTS

The pH optimum for the ATPase activity of chromatophore preparations from strain R-26 of *Rps. spheroides* is near pH 8.1 (Fig. 1) and the activity is similar in Tris–HCl or Tricine– HCl buffers. Addition of Mg^{2+} or Ca^{2+} without EDTA produces only a small increase in the reaction rate (Fig. 2). However, in the presence of 1 mM EDTA, a strict requirement of the reaction for divalent metal ion is apparent. The metal ion requirement is not highly specific although the reaction rate is somewhat higher with Mg^{2+} rather than with Ca^{2+} both before and after activation of the ATPase (Table I).

The ATPase is stimulated by the addition of both dithiothreitol and Triton

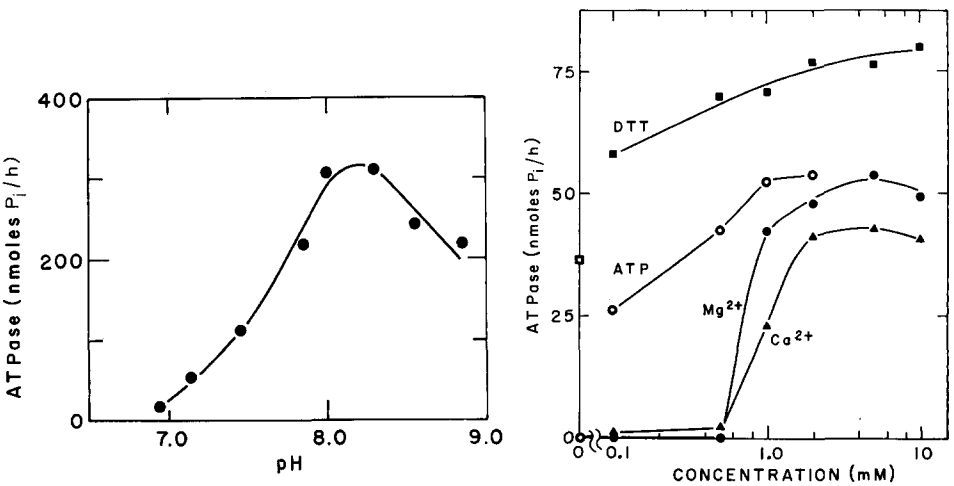

Fig. 1. Effect of pH on the ATPase activity of chromatophores from *Rps. spheroides*, strain R-26. The reaction conditions are described in the text except that the pH of the Tris–HCl buffer was varied.

Fig. 2. Effect of substrate and cofactor concentrations on the chromatophore ATPase activity. Reaction mixtures contained chromatophores with 29 nmoles B870 in 60 mM Tris–HCl buffer, pH 8.1, with the following additions: 1 mM ATP (□); 1 mM ATP, 2 mM $MgCl_2$ and various concentrations of dithiothreitol (■); 2 mM $MgCl_2$ and various concentrations of ATP (○); or 1 mM ATP, 1 mM EDTA and various concentrations of $MgCl_2$ (●), or $CaCl_2$ (▲).

TABLE I

STIMULATION OF THE ATPase ACTIVITY OF *Rps. spheroides* CHROMATOPHORES AND ITS METAL ION SPECIFICITY

Addition	*Mg^{2+}-ATPase (nmoles P_i/h)*	*Ca^{2+}-ATPase (nmoles P_i/h)*
None	38	34
2 mM dithiothreitol	58	42
0.04% Triton X-100	56	50
0.04% Triton X-100 + 2 mM dithiothreitol	68	59

X-100 and the effects are somewhat additive (Table I). The activation does not increase significantly when chromatophores are preincubated with these reagents for times longer than those required for mixing. Dithiothreitol stimulates both chromatophore and soluble ATPase preparations and the activation is maximal at 2 mM dithiothreitol (Fig. 2). The effect of Triton X-100 depends primarily on the relative concentrations of detergent and chromatophores with maximal activation at approximately 0.15 % Triton X-100 and 14.7 μM reaction center bacteriochlorophyll (Fig. 3). At a constant ratio of detergent to chromatophores, varying their concentrations over a 10-fold range has little effect on the percentage activation.

Several effects of treating chromatophores with different Triton X-100 concentrations are summarized in Fig. 3. The ATPase activity increases in the presence of low detergent concentrations with half maximal activation at 0.1 % Triton X-100. A higher concentration of 0.22 % is required for half maximal recovery of soluble

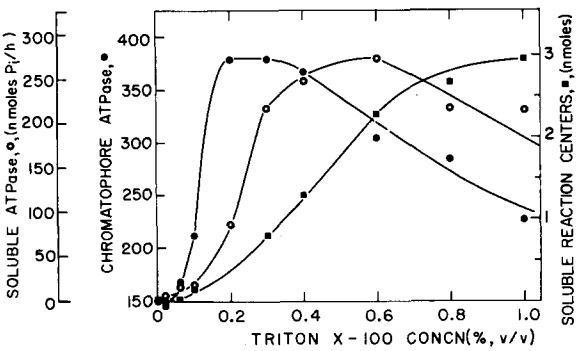

Fig. 3. Effects of different Triton X-100 concentrations on chromatophore membrane components. Samples of chromatophores containing 0.29 mM B870, 2 mM dithiothreitol, 10 mM Tris–HCl buffer, pH 7.5, and the appropriate Triton X-100 concentrations were either diluted into reaction mixtures for ATPase measurements or centrifuged to recover soluble components. ATPase activities and soluble component recoveries from 0.2-ml aliquots of chromatophore mixtures are presented in the vertical scale.

ATPase following differential centrifugation. At still higher detergent concentrations, the reaction center bacteriochlorophyll is released into the small particle fraction and the yield of reaction centers is 50 % near 0.5 % Triton X-100.

The purified chromatophores appear quite uniform in electron micrographs of negatively stained preparations (Figs 4 and 5). The flattened vesicles were 100–300 nm

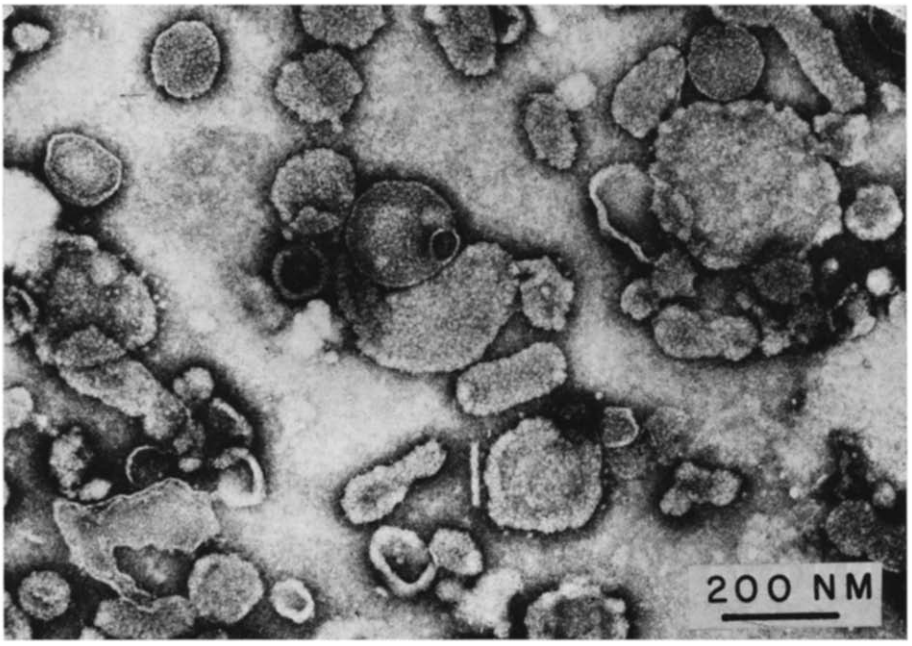

Fig. 4. Compact purified chromatophores washed with 2% methyl-modified phosphotungstic acid, pH 6.5. The chromatophores are flattened vesicles, 100–300 nm across. The surfaces are closely covered with 9-nm particles. × 74900.

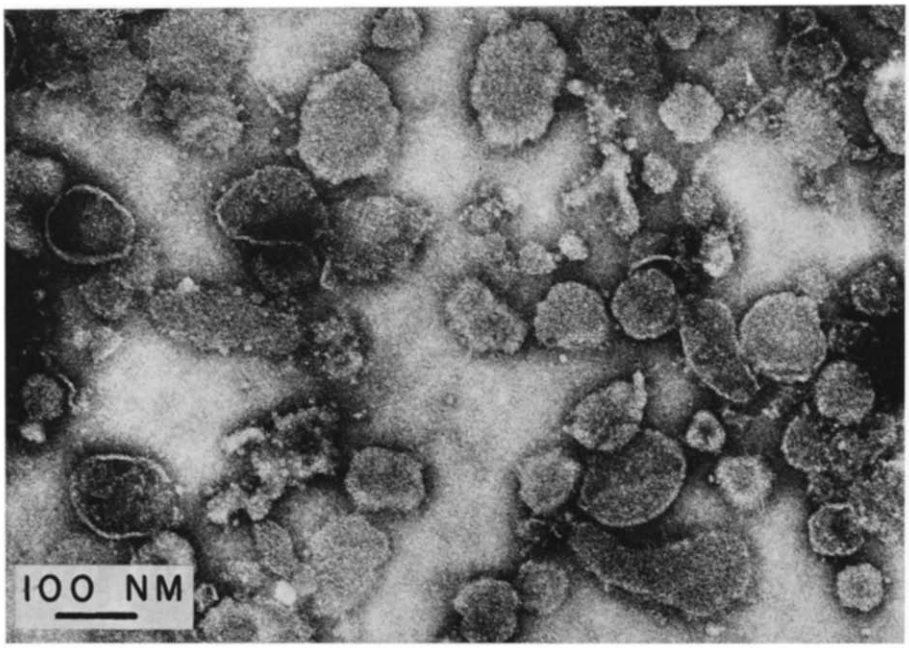

Fig. 5. Swollen purified chromatophores. They were put into 0.1% bovine serum albumin and washed with 2% methyl-modified phosphotungstic acid, pH 6.5. The particles protruding from the edges and surfaces of the membranes are 10-nm diameter. × 102000.

Fig. 6. Freeze-fracture replica of chromatophores. Most faces are covered with 13-nm particles and some faces are smooth or nearly smooth. × 69200.

across with 9-nm-diameter particles on the outer membrane surfaces. When the membranes were compact (Fig. 4), the surface particles were closely spaced on the membranes and in more swollen preparations (Fig. 5) the particles frequently protruded from the surface and periphery of the membranes and appeared slightly larger (10 nm).

The particulate substructure of the chromatophores is made more apparent by two techniques for electron microscopy. Fracture of the membranes by the freeze–fracture technique reveals smooth and rough faces (Fig. 6). The rough faces contain 42 particles/10^4 nm^2 with diameters of about 13 nm and approximately 15 nm between centers. The smooth faces show very few particles larger than the limit of resolution on this replica (approx. 5 nm). Dispersion of chromatophores in dilute Triton X-100 solution before applying the mixture to a grid shows the membranes dissociated into a mixture of particles and lipid droplets (Fig. 7). Most of the small individual particles have diameters of either 9 or 12 nm. The large sheets of lipid exhibit the typical appearance of myelin figures and the other large structures probably contain membrane proteins. These large structures have very few of the 9-nm surface particles seen on intact chromatophores. Most appear quite smooth and somewhat thinner but some faces are rough having larger particles which appear similar to those seen on the rough faces in freeze–fracture preparations.

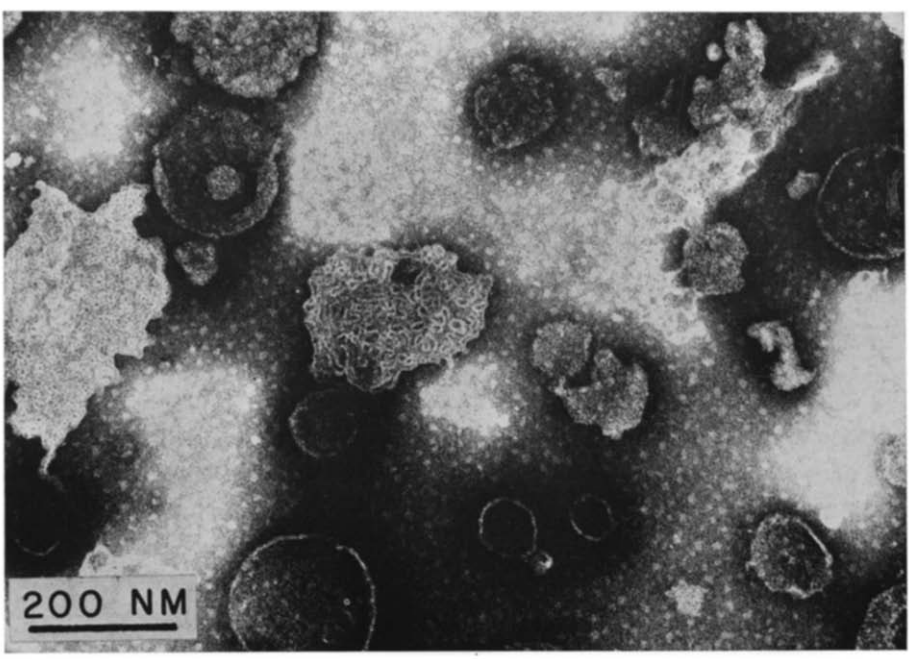

Fig. 7. Chromatophores incubated with Triton X-100. One volume of diluted chromatophores (29.5 mM B870) was incubated 5 min with 5 vol. 10^{-4} M Triton X-100 and washed on the grid with 1.5% methyl-modified phosphotungstic acid, pH 6.4. Visible are components of disintegrating vesicles, scattered 9- and 12-nm particles, and myelin-like exudates. × 93800.

Results of removing the ATPase from chromatophores by treating the membranes with dilute solutions of EDTA or Triton X-100 are summarized in Table II. Approximately 70–80 % of the chromatophore bacteriochlorophyll is recovered in the washed membranes following these treatments with 20–30 % of the total ATPase remaining associated with the membranes.

TABLE II

TREATMENTS TO REMOVE THE ATPase FROM CHROMATOPHORE MEMBRANES

Treatment	*Mg^{2+}-ATPase activity (nmoles P_i/h per nmole P870)*
None	120
0.3% Triton X-100	25
1 mM EDTA	39

TABLE III

PURIFICATION OF THE CHROMATOPHORE ATPase BY EDTA EXTRACTION

Fraction	*Mg^{2+}-ATPase*		*Recovery (%)*	*Protein*	
	Total activity (μmoles P_i/h)	*Specific activity (μmoles P_i/h per mg)*		*Total (mg)*	*Relative (g/μmole P870)*
Chromatophore	4.00	0.109	100	36.6	1.09
Extracted membranes	1.49	0.048	37	31.4	0.94
EDTA extract	1.89	0.50	47	3.7	0.12

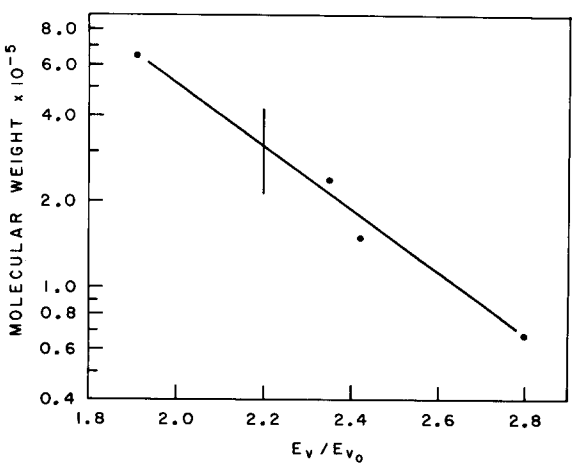

Fig. 8. Apparent particle weight of the ATPase complex from strain R-26 of *Rps. spheroides* determined by gel filtration on a 2.2 cm × 35 cm Agarose A50m colum in 10 mM Tris–HCl buffer, pH 7.5, containing 1 mM ATP and 5 mM EDTA. Samples of 20–30 mg in 4 ml were chromatographed at a flow rate of 40 ml/h. Proteins were: ATPase complex (vertical bar); thyroglobulin, catalase, rabbit muscle aldolase and hemoglobin.

Results of ATPase isolation by a less rigorous EDTA treatment are listed in Table III. When chromatophores in 1 mM EDTA are passed four times through a 24-gauge syringe needle essentially only the ATPase is recovered in the soluble fraction following centrifugation. Approximately 11 % of the total chromatophore protein with 47 % of the total ATPase activity is recovered in this EDTA supernatant fraction. These recoveries and the increase in specific activity of 2.2 times correspond to an ATPase content in the untreated chromatophores of approximately $2.6 \cdot 10^5$ g per mole of reaction center P870. The apparent molecular weight of the ATPase determined by gel filtration is approximately $3 \cdot 10^5$ (Fig. 8).

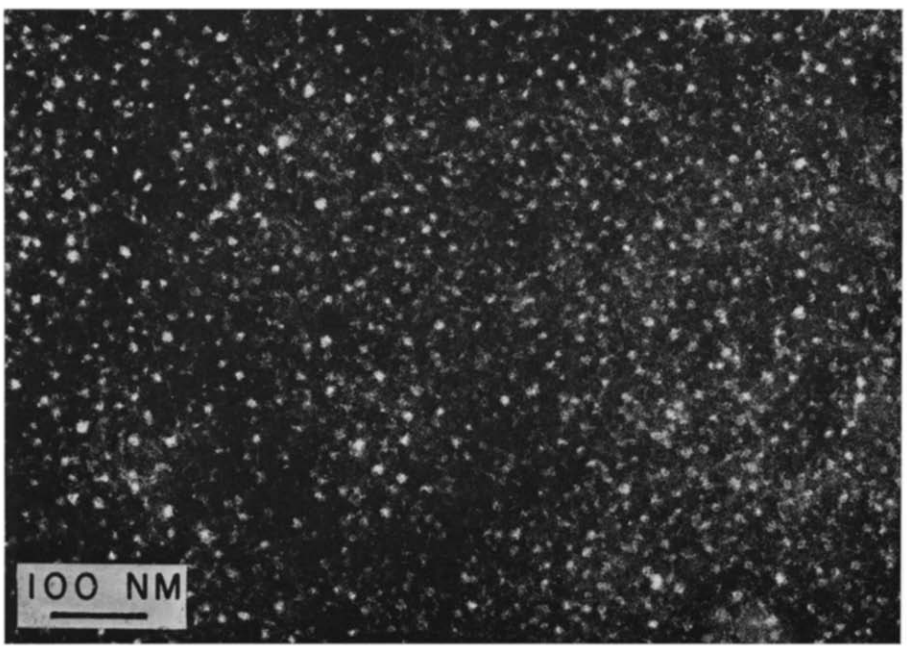

Fig. 9. Isolated ATPase particles. Washed with 1.5% methyl-modified phosphotungstic acid, pH 6.4, the small particles are 8–10 nm diameter with occasionally larger 12–13-nm ones. × 127000.

Electron micrographs of the soluble ATPase preparations (Fig. 9) show particles which are near 9 nm in diameter. There is some variation but the average particle size is consistent with the apparent molecular weight of the ATPase (Fig. 8) and is essentially identical to the size of particles seen bound to the surfaces of the intact chromatophore membranes (Figs 4 and 5). Electron micrographs of the chromatophores which have been washed with EDTA (Fig. 10) still have some surface texture and apparent thickness although they have lost much of their ATPase. Electron micrographs of membranes which have been washed with Triton X-100 (Fig. 11) show smooth membranes which appear thinner and devoid of surface particles. However, when the ATPase-less chromatophores prepared by EDTA washing are freeze–fractured (Fig. 12), their rough and smooth faces appear very similar to those of intact chromatophores (Fig. 6). The rough faces contain 38 particles/10^4 nm^2 with diameters near 13 nm.

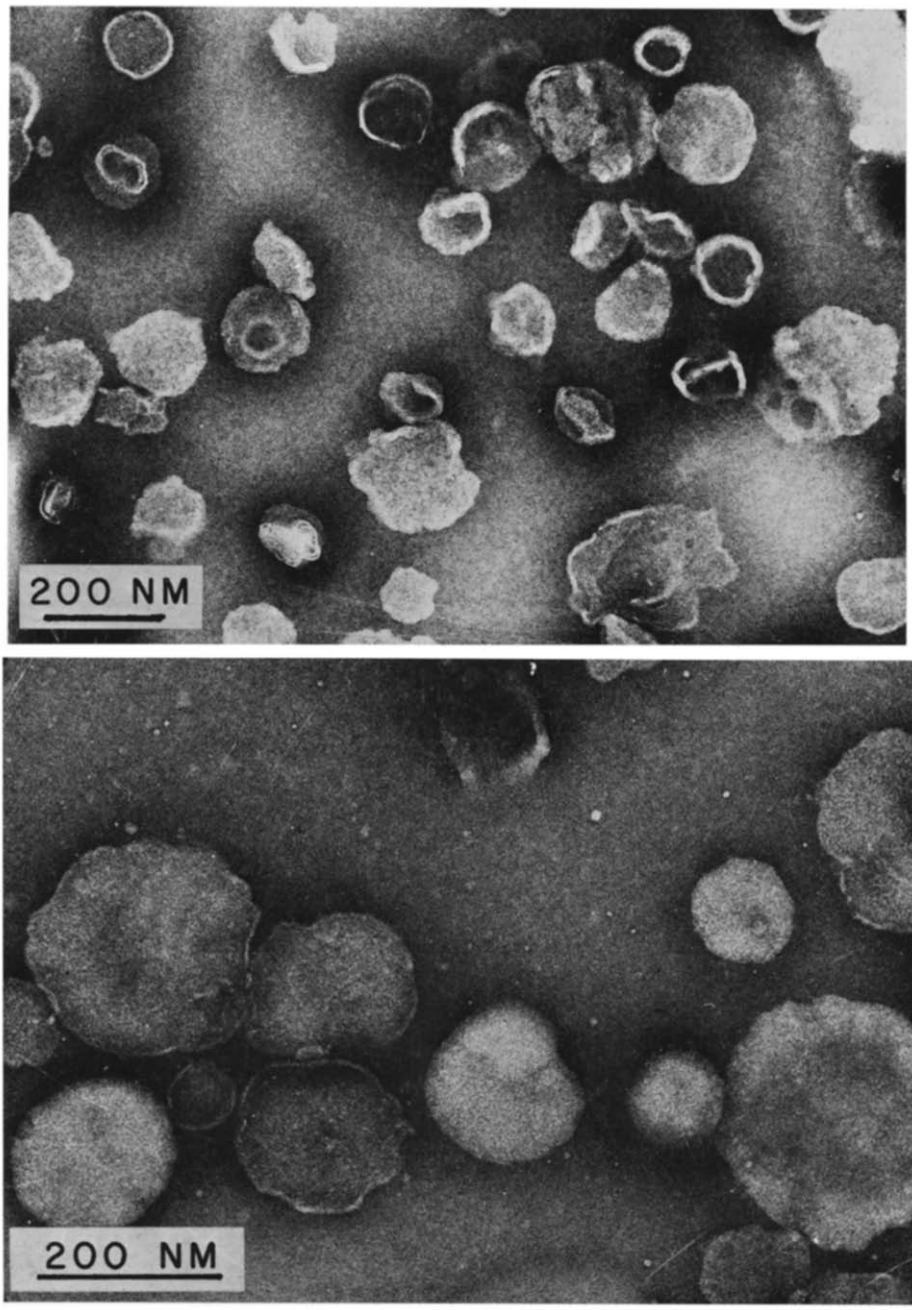

Fig. 10. ATPase-less chromatophores washed with 10^{-3} M EDTA. The suspension was mixed to 1% methyl-modified phosphotungstic acid, pH 6.3, and sprayed as fine droplets onto a carbon-coated grid. The vesicles maintained texture and thickness though they have lost most of their ATPase. × 77000.

Fig. 11. ATPase-less chromatophores, Triton X-100 washed. These membranes stained on the grid with 1.5% methyl-modified phosphotungstic acid, pH 6.4, are noticeably smoother and thinner than those in Figs 4 and 10. × 119600.

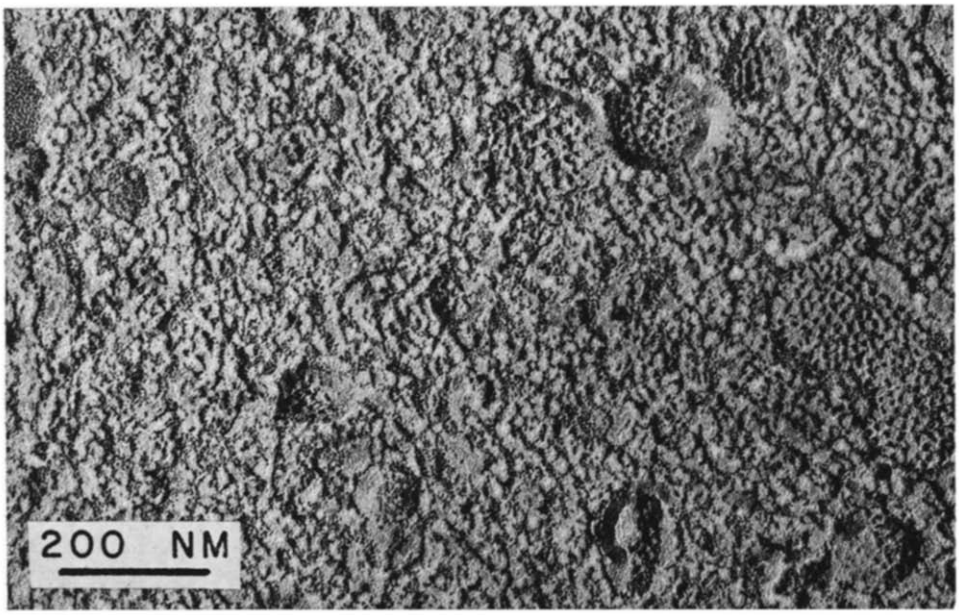

Fig. 12. Freeze–fracture replica of EDTA-washed chromatophores. The membrane faces are similar to those of Fig. 6, with the rough ones covered by 13-nm particles. × 99500.

DISCUSSION

The functional protein components in chromatophore membranes from *Rps. spheroides* strain R-26 are arranged asymmetrically with the ATPase on the outside. By negative staining the great majority of the chromatophore membranes appear to be oriented similarly with 9-nm-diameter particles on the outer surfaces. Splitting the membranes through their hydrophobic interior by freeze fracturing[14] reveals larger 13-nm particles on the rough fracture faces. The surface particles seen by negative staining are removed by washing the membranes with dilute solutions of EDTA or Triton X-100 concomitant with the loss of the ATPase activity from the membranes. The ATPase preparations purified from the wash solutions contain uniform 9-nm-diameter particles and exhibit an apparent molecular weight of $3 \cdot 10^5$ consistent with this particle size.

Removing the surface ATPase has no apparent effect on the integrity of the other protein components within the chromatophore membranes. No differences between washed and intact chromatophores were detected in the infrared absorption bands arising from the specialized bacteriochlorophyll environment. The essentially identical particle size and distribution on the rough fracture faces on freeze–fracture replicas of the membranes also indicates that little change has occurred in the molecular organization giving rise to the freeze–fracture particles.

The asymmetry of the chromatophore membrane is also indicated by the sequential effects of Triton X-100 on the membrane components. ATPase activation occurs at low detergent concentrations. Somewhat higher Triton X-100 is required to release the ATPase from the membranes and still higher concentrations solubilize the reaction

centers. Maximal ATPase activation requires approximately 160 Triton X-100 molecules per reaction center. This amount of detergent which occupies 0.48–0.54 nm^2 per molecule in a monolayer[15] is enough to form a monolayer having a circular cross section per reaction center of near 10 nm diameter. The majority of the ATPase is released from the membranes by Triton X-100 equivalent to a monolayer with a 15-nm diameter cross section and the reaction centers are solubilized by a Triton X-100 concentration which is nearly equal to the concentration of the total membrane lipids.

The properties of the chromatophores and of the particles solubilized from these membranes suggest that the proteins containing the reaction center bacteriochlorophyll P870 and the other electron transfer components are organized into a functional reaction center complex located within the interior of the membrane. Chromatophores exhibit the 9-nm particles of the ATPase complex on the negatively stained outer surface and can be appropriately treated to raise or remove these particles. The effects of dispersing chromatophores in dilute Triton X-100 indicates a successive dissociation from the membrane of 9-nm particles and 12-nm particles which are both seen in the background of Fig. 7. The purified ATPase consists essentially of 9-nm particles (Fig. 9) and the purified reaction centers are 12-nm particles[3]. On some faces of the detergent-treated membranes are fields of 12-nm particles which appear similar to the 13-nm particles on the rough fracture faces of freeze–fracture replicas (Figs 6 and 12). Splitting the chromatophore membrane through its hydrophobic interior by the freeze–fracture process[14] reveals rough faces which may be composed of particles of the reaction center complex situated at regular intervals on an apparently smooth surface of the light-harvesting component[3] composed of a tightly appressed sheet of 5-nm light-harvesting bacteriochlorophyll proteins. However, additional evidence is necessary to completely establish the relationship between the freeze–fracture particles and the reaction center complex.

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