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PROTEIN COMPOSITION OF INTACT AND FRACTIONATED MEMBRANES ISOLATED FROM DARK AND LIGHT GROWN CELLS OF A BLUE GREEN MUTANT OF *RHODOSPIRILLUM RUBRUM* (BG_1)*

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SUMMARY

1. By means of different fractionation procedures we have separated two major protein fractions present in photosynthetically grown cells of the blue-green mutant of *Rhodospirillum rubrum* (BG_1). These proteins are found in differing amounts in the two subchromatophore fragments produced by the detergent Triton X-100. A more extensive breakdown caused by digestion of intact photosynthetic membrane with Triton X-100 and trypsin produced four main protein fractions which were separated by means of gel electrophoresis. Some of these bands originated from proteins that were intimately associated with bacteriochlorophyll in the intact membrane.

The other protein fractions seem to derive from a group of proteins whose function could be a supporting one. These protein fractions seem to be a somewhat degraded form of the basic membrane core that cannot be further attacked by the combined action of the detergent and the trypsin.

2. Electron micrographs of the fractions obtained by the digestion with Triton X-100 and trypsin are presented, showing a very different structure for the degraded basic membrane and a unique structure for the aggregated units of the bacteriochlorophyll-containing fraction.

INTRODUCTION

Pancreatin readily breaks down *Rhodospirillum rubrum* chromatophores in the presence of Triton X-100[1]. It appears that the detergent alters the structure of the chromatophores in such a way that the digestive enzymes are then able to interact with their substrate molecules within the organized photosynthetic membranes. This treatment produces a series of protein complexes which contain the pigment molecules bacteriochlorophyll and carotenoids[1]. The bacteriochlorophyll-containing fraction, on the basis of its absorption properties, represents a state of bacteriochlorophyll resembling that found in the intact membrane.

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It is also known that Triton X-100 acts upon chromatophores from *Chromatium*[2] and *R. rubrum*[3] to produce two derived fragments clearly separable by sucrose density centrifugation. The effect of the detergent is different in each case. In *Chromatium* the Triton X-100 extracts part of the bulk bacteriochlorophyll from the chromatophores resulting in the formation of a heavy fraction containing the reaction center (P890) as well as the remaining bulk bacteriochlorophyll. In *R. rubrum*, on the other hand, although the detergent produces subchromatophore fractions, the reaction center is equally distributed between them.

The near infrared absorption spectra of the bacteriochlorophyll-containing fractions, obtained from photosynthetic bacteria using different treatments[1-3], show the close similarity of the absorption bands of these fractions to the absorption spectrum detected *in vivo*. This could be due to the fact that the environment of the bacteriochlorophyll molecules in those complexes is not far from the environment *in vivo*.

We thought it would be of interest to obtain information on this matter by studying the protein composition of some of these bacteriochlorophyll-containing fractions in order to be able to obtain more insight into one of the factors that may contribute to their near-infrared spectrum. This information is also necessary if one is to describe the changes in the protein composition of the membrane that take place when cells growing under aerobic nonphotosynthetic conditions are subsequently grown anaerobically in the light. With this is mind we have studied the protein composition of photosynthetic membranes and fractions derived from them. To carry out these experiments we have chosen the blue-green mutant of *R. rubrum*, since the lack of carotenoids might produce a slower rate of incorporation of the free form of bacteriochlorophyll into the membrane, as has been described by CELLARIUS *et al.*[4]. This parameter may become important when studying the repigmentation process.

MATERIALS AND METHODS

Cells of the blue-green mutant of *R. rubrum* (BG_1) were grown in a medium which is equally suitable for aerobic and photosynthetic growth[5]. The depigmentation of the cells was carried out by aerobic growth in the dark with shaking and vigorous bubbling with air (30°). After 10 generations of such growth, at which time no bacteriochlorophyll was detectable by spectroscopic methods, a culture in log phase growth was used to inoculate a 400-ml volume of medium in an erlenmeyer flask equipped with a sintered glass aerator. This culture was allowed to grow at 30° using both shaking and vigorous air bubbling.

After growth, the cells were collected by centrifugation and washed twice with 0.05 M Tris buffer (pH 7.9).

Disruption of cells—osmotic shock

The method of ROBRISH AND MARR[6], slightly modified, was used for this purpose. The pellet of cells (approx. 3 g) was resuspended in 30 ml of 3.0 M glycerol. After standing 1 h at 5°, the mixture was slowly poured into 10 vol. of mechanically stirred 0.05 M Tris buffer (pH 7.9). At this step Mg^{2+} was absent, which resulted in a low RNA concentration in the membrane fraction[7]. This ensures that there is very little contamination with insoluble ribosomal proteins. The viscous suspension was treated

with 0.5 μg of deoxyribonuclease per ml and allowed to stand at room temperature for 30 min. The suspension was then centrifuged for 30 min at 2000 $\times$ *g* and the supernatant was again centrifuged for 45 min at 20000 $\times$ *g*.

The precipitated membranes were washed twice with 0.05 M Tris buffer (pH 7.9). This yielded a preparation of membranes which was used without further purification. This method of preparation will result in the simultaneous precipitation of the wall material.

The ratio μg of bacteriochlorophyll/mg of protein of these membranes usually was between 35 to 40.

Preparation of H and L subfractions from membranes

The membranes were suspended in 0.05 M Tris buffer (pH 7.9) and sufficient Triton X-100 was added to obtain a ratio of mg detergent/mg bacteriochlorophyll = 70. Higher detergent ratios resulted in partial degradation of the extracted bacteriochlorophyll. The final concentration of bacteriochlorophyll was adjusted to give 1 mg per 9.0 ml of suspension, which was centrifuged in a sucrose gradient as previously described[3].

Tryptic digestion of the membranes—preparation of fractions H_t and L_t

The membranes were suspended in 0.05 M Tris buffer (pH 7.9) also containing Triton X-100 (2 mg of detergent/mg of protein) and crystalline trypsin at a final enzyme concentration of 1 mg/ml. The final concentration of bacteriochlorophyll was 1.5 mg/9.0 ml of reaction mixture. The suspension was incubated for 15 h at 38° and the mixture was then centrifuged in a sucrose gradient as previously described[1]. This resulted in the formation of a heavy nonpigmented band (H_t) and a blue bacteriochlorophyll-containing light fraction (L_t). Close to 95 % of the bacteriochlorophyll originally present in the membrane remained associated with the L_t fraction.

Both fractions were taken off the gradient by means of a needle and a hypodermic syringe, centrifuged at 144000 $\times$ *g* for 1 h, resuspended in 0.05 M Tris buffer (pH 7.9) and repurified by a new gradient centrifugation. The purified H_t and L_t fractions were then ready for further characterization.

Disc gel electrophoresis

The method of TAKAYAMA *et al.*[8] was used with slight modification. The concentration of acrylamide used was 6 %.

Treatment with urea

The fractions to be dispersed by urea were suspended in 8.0 M urea in 0.01 M Tris buffer (pH 9.6) and slowly frozen and rapidly thawed at least 10 times. The resulting suspension was used for further characterization.

Protein determination

This was done by the method of LOWRY *et al.*[9].

Bacteriochlorophyll determination

The fraction was extracted with acetone–methanol and the concentration of bacteriochlorophyll determined by using the millimolar extinction coefficient of 75 as previously published by CLAYTON[10].

Structure

Suspensions of heavy and light fractions derived from membranes of *R. rubrum* (wild type) were examined after Triton and trypsin digestion. The blue crystals (L_t) were viewed by spraying onto carbon-coated grids in a 1 % solution of potassium phosphotungstate, pH 6.0. The thin membrane fraction (H_t) was applied to similar grids and stained by washing with 1 % vanadyl molybdate, made by dissolving $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ to 0.8 % in a 0.2 % solution of vanadyl chloride and adjusting the pH to 3.2.

This is a modification of the method described by CALLAHAN AND HORNER[11] to post stain thin sections.

Abbreviations employed to designate the different membrane fractions used

M, intact photosynthetic membrane from *R. rubrum* (BG_1).

L fraction, light fraction isolated from the photosynthetic membrane by means of Triton X-100 treatment.

H_1 fraction, heavy fraction isolated from the photosynthetic membrane after a single treatment with Triton X-100.

L_t fraction, light fraction isolated from photosynthetic membrane by the combined action of Triton X-100 and trypsin.

H_t fraction, heavy fraction isolated from photosynthetic membrane by the combined action of Triton X-100 and trypsin.

H_f fraction, heavy fraction isolated from the photosynthetic membrane by the repetitive treatment with Triton X-100.

H_{1t} fraction, protein fraction obtained from H_1 incubated in the presence of trypsin and in the absence of Triton X-100.

H_{1tH}, H_{1tM} and H_{1tL} fraction, heavy, medium and light fractions, respectively, obtained from H_{1t} by treatment with alkaline urea.

M_0, intact membrane derived from dark grown *R. rubrum* (BG_1).

M_{0t} fraction, protein fraction obtained from M_0 incubated in the presence of trypsin and Triton X-100.

RESULTS

R. rubrum chromatophores readily break down in the presence of the detergent Triton X-100[3], producing a heavy (H) and a light (L) fraction. It has also been shown[12] that the mutant BG_1 exhibits a similar behaviour. The repetitive extraction of the cytoplasmic membrane isolated from BG_1 using a constant ratio of detergent to bacteriochlorophyll, produces successive H and L fractions. RIVAS *et al.*[12] have postulated that the last H fraction obtained in this manner (H_f) (after this last extraction no additional L fraction is extracted by the detergent) represents a basic membrane protein to which the complex fraction L is hydrophobically bound. This basic membrane would contribute to the structural properties of the whole cytoplasmic membrane in these bacteria.

Fig. 1 shows the protein composition of some of those fractions compared to the intact membrane (M). In fractions H_1 and M it is possible to detect two major bands (protein fractions I and II) apart from minor components. Protein fraction II is not seen in fraction L and protein fraction I is not detected in fraction H_f. Since

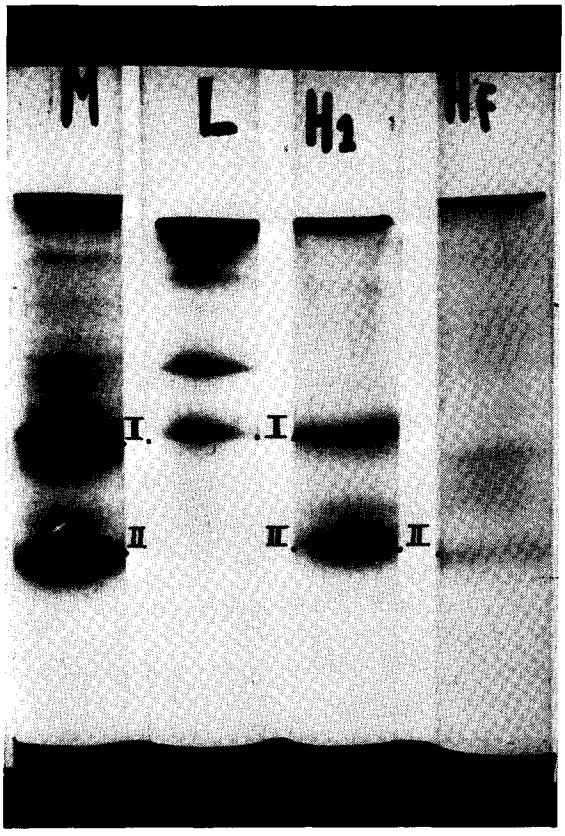

Fig. 1. Gel electrophoresis of intact membranes (M), the light fraction obtained by washing the membranes with Triton X-100 (L) and the corresponding heavy fraction (H_1). H_f represents the heavy fraction obtained after washing the membranes twelve successive times with Triton X-100. Approximately 400 μg of proteins were used for M, L and H_1 and 280 μg for H_f. The proteins were stained with amido black. Other experimental details are included in MATERIALS AND METHODS.

the optical absorption properties of the L fraction indicate that the environment of the bacteriochlorophyll molecules has not been substantially altered, it is reasonable to assume that the protein composition of the *in vivo* state of the bacteriochlorophyll complexes is similar to that found in the L fraction. Protein fraction I and all of the other components present in fraction L could be intimately related to the bacteriochlorophyll in the membrane.

In this context we tend to favor protein fraction I as being one of the bacteriochlorophyll-bearing proteins, since this fraction is present not only in fraction L, but also in fraction H_1, where the bacteriochlorophyll is still present and where slower proteins than I are not detected. Further, the absorption spectrum of the bacteriochlorophyll in fraction H_1 was identical to that detected in fractions L and M indicating that in all these cases the environment of the bacteriochlorophyll and most particularly its protein carriers were closely similar.

Digestion with trypsin

Incubation of the cytoplasmic membrane in the presence of Triton X-100 and

trypsin, followed by density gradient centrifugation, produces a heavy nonpigmented fraction (H_t) and a blue, bacteriochlorophyll-containing, light fraction (L_t) (Fig. 2). It should be mentioned that the presence of Triton X-100 is necessary for the enzymatic action to take place. It seems, therefore, reasonable to assume that one of the effects of the detergent is to remove from the surface of the membrane certain lipids that prevent the trypsin action. Furthermore, we have shown before that Triton X-100 alone separated protein fractions I and II, which could be hydrophobically bound when present in the intact membrane.

If fraction H_1 was incubated in the presence of trypsin and in the absence of detergent and then submitted to a density centrifugation, a single pigmented band was obtained (H_{1t}), as shown in Fig. 3a. The absorption spectrum of this band was identical to that of fraction L_t.

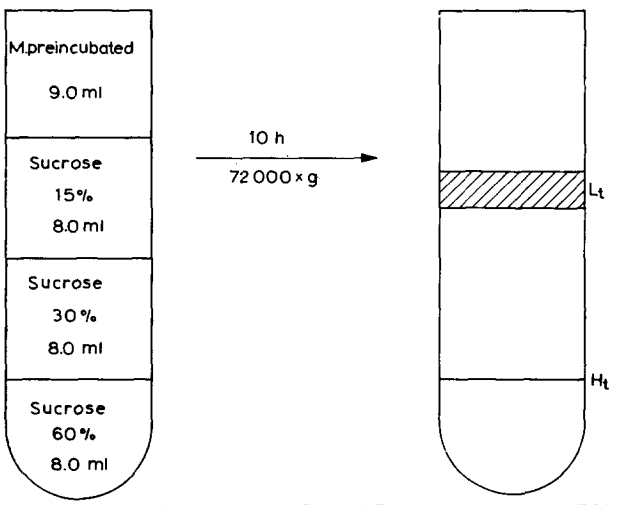

Fig. 2. Pattern of separation of H_t and L_t from membranes (M_0) previously incubated with trypsin and Triton X-100. All details are included in MATERIALS AND METHODS.

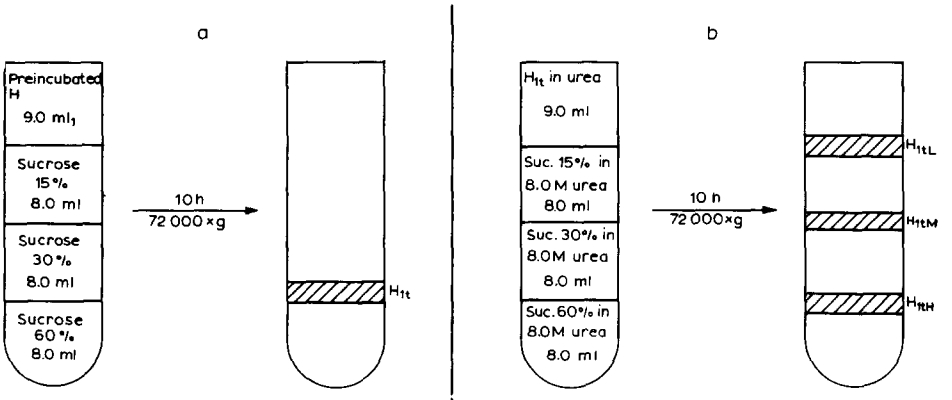

Fig. 3. (a) Pattern of separation of H_{1t} from H_1 previously incubated in the presence of trypsin and in the absence of Triton X-100. (b) Pattern of separation of fractions H_{1tH}, H_{1tL} and H_{1tM} obtained from fraction H_{1t} (see Fig. 4a) previously frozen and thawed in the presence of 8 M urea. All other details are included in MATERIALS AND METHODS.

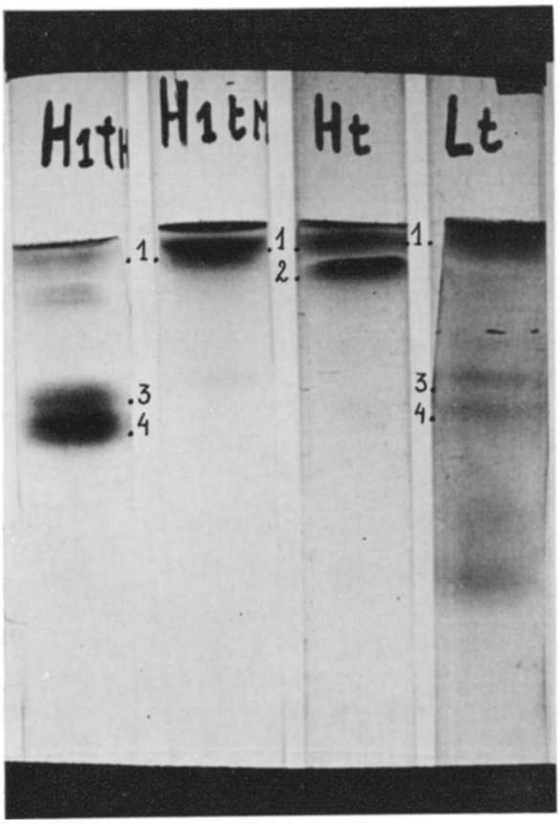

Fig. 4. Comparison of the protein components present in H_t, L_t, H_{1tH} and H_{1tM}. 450 μg of protein were used in each case. Fractions H_{1tL} and H_{1tM} show no difference in their protein composition. All other details are included in MATERIALS AND METHODS.

Treatment with alkaline urea

When fraction H_{1t} was suspended in 0.05 M Tris buffer (pH 9.6) and 8 M urea, frozen and thawed ten times and then submitted to a new gradient centrifugation, the result shown in Fig. 3b was obtained. We further studied the protein composition of bands H_{1tH} and H_{1tM}. Fig. 4 shows the protein composition of fractions H_{1tH} H_{1tM}, H_t and L_t. We should mention that the protein composition of fraction H_{1tL} was identical to that found for H_{1tM}. The difference in density between fraction H_{1tM} and H_{1tL} could reflect differences in their specific content of lipids.

The protein composition of fraction H_{1tH} shows the presence of two major and two minor bands. The two lower bands have hydrodynamic properties similar to bands 3 and 4 present in fraction L_t. On the other hand the slower bands in fraction H_{1tH} presented the same mobility as band 1 and 2 detected in fraction H_t. The major band present in fraction H_{1tM} was a slow running bands with similar electrical properties to band 1 present in both fractions H_t and L_t.

Since the only band present in fraction H_t and not present in fraction L_t is protein band 2 we think that it is possible that this is the only band that did not originate from a protein that binds to bacteriochlorophyll in the original membrane.

On the other hand the presence of bacteriochlorophyll, in both fractions H_{1tH} and H_{1tM} reinforces the idea that at least band 1 is able to bind bacteriochlorophyll. We should also point out that protein band 2 was again present only in the heavy fraction, H_{1tH}. From Fig. 4 it is also evident that the action upon the digested material of alkaline urea and Triton X-100 was different. This could reflect a multiple effect of Triton X-100. One of these effects could be due to its lipid binding ability perhaps resulting in a weakening of the lipid–protein and/or lipid–lipid interactions.

Origin of protein band 2

We have shown above that although the protein compositions of fractions M and L were somewhat similar, the relative intensity of protein fractions I and II was different in each case. Protein fraction II is more abundant in the intact membrane than in the L fragment, where it is almost undetectable. We have therefore incubated fractions M and L in the presence of Triton X-100 and trypsin and analysed the relative concentration of heavy (H_t) and light (L_t) bands obtained from each after density gradient centrifugation. Quantitative determination of the protein obtained from the incubation of fraction M in the presence of Triton X-100 and trypsin shows that the H_t fraction contains twice as much protein as the corresponding L_t fraction. On the other hand, if the same experiment is carried out using the L fraction instead of M only traces of H_t fraction are found and most of the protein is transformed into fraction L_t. Since the protein composition of the L_t fraction obtained from either fraction M or L was identical, it is obvious that protein fractions 1, 3 and 4 did not originate from protein fraction II and it seems likely that this fraction was transformed into protein band 2 upon digestion in the presence of trypsin and Triton X-100.

In Fig. 5 it is possible to see the protein composition of membranes prepared from dark and light grown cells. It is seen that growing the cells under very different physiological conditions produces drastic changes in the electrophoretic pattern of the membrane proteins. No counterparts of protein fractions I and II are detected in the dark grown membranes.

Tryptic digestion of the membrane isolated from dark cells in the presence of Triton X-100 followed by density gradient centrifugation produced only one major band in the gradient, at the same position where the H_t fragment was found. Protein analysis of this heavy fraction (M_{0t}) shows that apart from minor bands, only three major bands were detected by gel electrophoresis, some of them having approximately the same electrical mobility as the protein bands 1 and 2 present in fragment H_t. These results are shown in Fig. 5.

Electron micrographs of fractions L_t and H_t

As can be seen in Fig. 6a, the H_t fraction appears as large, smooth structureless sheets. This is in contrast to what has been shown before for the intact photosynthetic membrane in *Chromatium*[2] and *R. rubrum*[3], where individual units approx. 6 to 7 nm in diameter are arrayed in the chromatophore in a repeating fashion with rows of subunits separated by approx. 10 to 15 nm. Since no bacteriochlorophyll is left in the H_t fraction, this observation could be interpreted by saying that the subunits seen in the membrane contain most of the bacteriochlorophyll originally present, together with other components.

The L_t particles (Fig. 6b) present a crystalline structure which is an aggregate

of bacteriochlorophyll–protein and whose dimensions are 33 nm thick with a 7 nm lumen. This is in contrast to the structure of the bacteriochlorophyll–protein complex in the native membrane, and of course must indicate some significant change in its structure being produced by the removal of the lipids and perhaps some lipophilic portions of the original membrane through the combined action of the detergent and the trypsin.

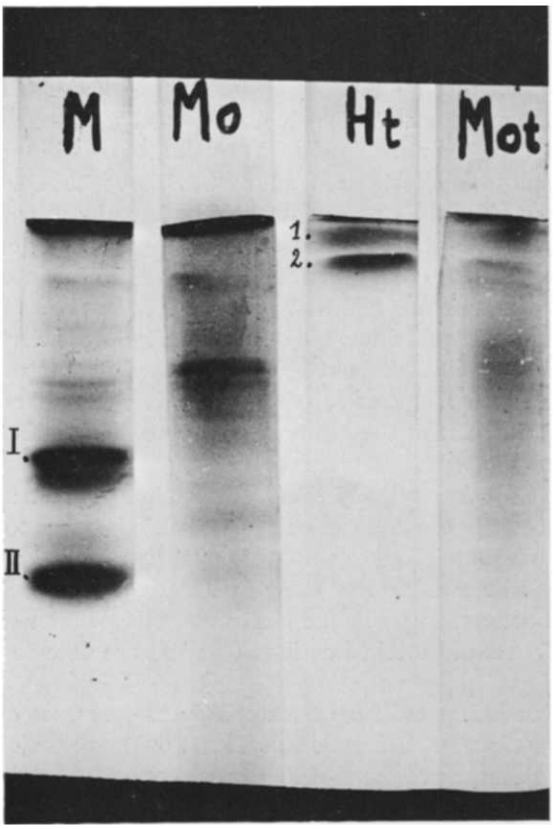

Fig. 5. Comparison of the protein components present in H_t, M, M_0 and M_{ot}, 400 μg of protein were used in the case of M and M_0, 450 μg in the case of H_t and 350 μg for M_{ot}.

DISCUSSION

By means of different methods we have been able to fractionate the photosynthetic membrane from *R. rubrum* (BG_1) cells. Both 8 M urea and Triton X-100 seem to interact with this membrane preparation by breaking down its structure. The intact membrane shows, by means of gel electrophoresis, the presence of two major bands (protein fractions I and II).

Treatment of the photosynthetic membrane with Triton X-100 produces two chromatophore fractions. The protein composition of the L fragment shows that it has been enriched in protein fraction I, whereas the H_f fragment has been enriched in protein fraction II.

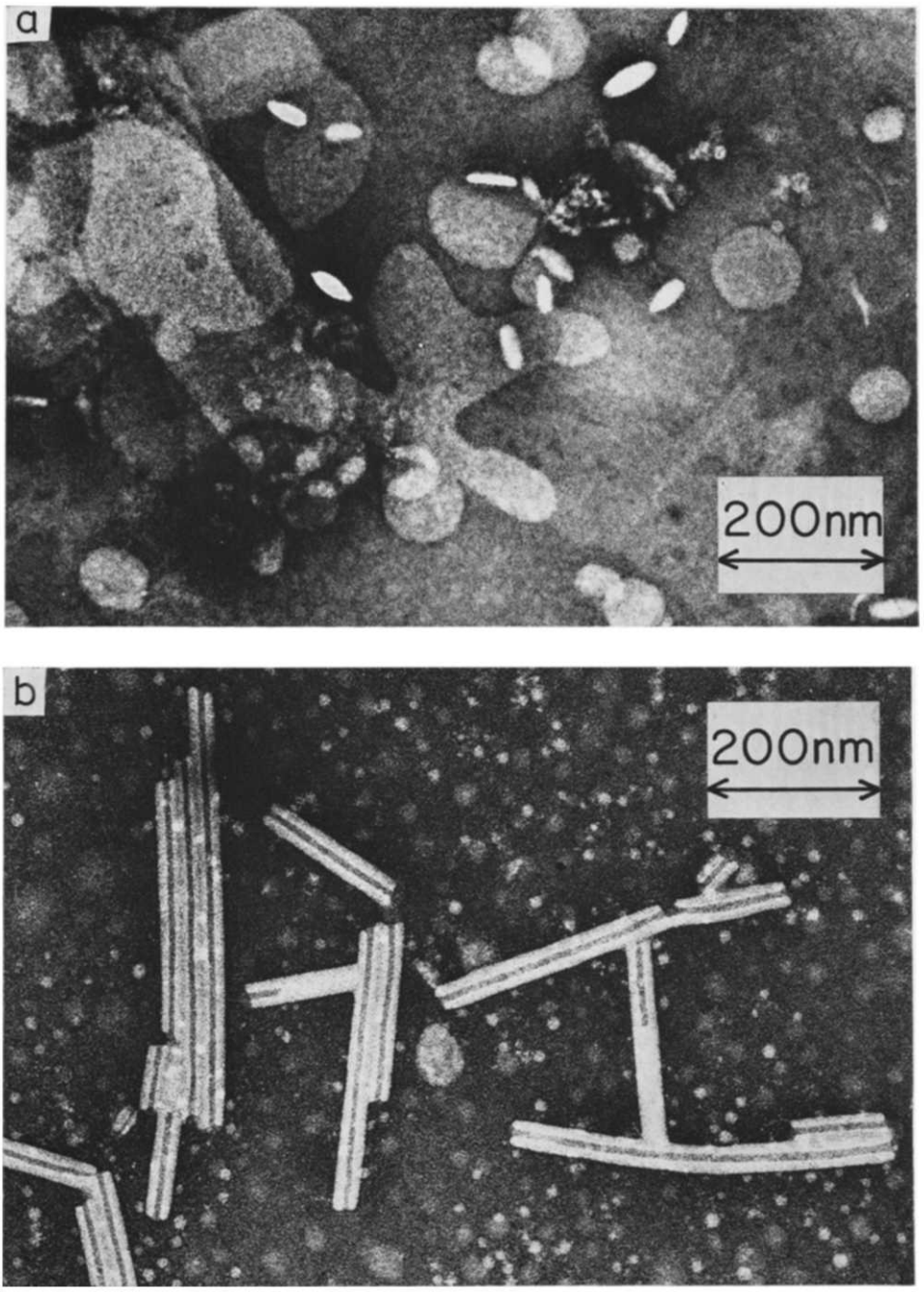

Fig. 6. (a) Heavy fraction (H_t) of *R. rubrum*, wild type, stained with 1% vanadyl molybdate, pH 3.2. These are thin sheets and chromatophore sized discs, some light structured. (b) Light fraction (L_t) of *R. rubrum*, wild type, stained with 1% phosphotungstate, pH 6.0. These are thin bacteriochlorophyll–protein tubes averaging 33 nm in diameter with a 7-nm lumen. Although these preparations were obtained from wild type *R. rubrum*, they are similar to those obtained from the blue-green mutant.

The detergent treatment is specific in extracting those proteins closely associated to bacteriochlorophyll. The remaining proteins in fragment H_f, although they may have been bound to bacteriochlorophyll in the intact membrane, did not contribute to the characteristic infrared spectrum of this bacterium.

When the membranes are first washed once with Triton X-100 and the resulting H_1 fraction incubated in the presence of trypsin and in the absence of detergent and the extract submitted to a density gradient centrifugation, one band is detected in the gradient (H_{1t}). This band, however, may be further fractionated by treatment with alkaline urea, somewhat mimicing the effect of Triton X-100 and producing different subfractions. The protein composition of these subfractions demonstrates that it is possible to separate a light pigmented fraction (H_{1tL}) which contains mainly protein band 1 and is present in both fraction H_t and L_t. Two more bands are also obtained in the gradient, both pigmented and both heavier than H_{1tL}. The protein composition of band H_{1tM} is identical to that of H_{1tL}, being mainly band 1. The protein composition of H_{1tH} shows the presence of bands 1, 3 and 4 present in L_t and band 2 present in H_t. The amount of band 1 present in H_{1tH} is low, and this band was concentrated mainly in fractions H_{1tM} and H_{1tL}.

The results presented in Fig. 4 also make clear the different action of Triton X-100 and urea. At variance with what was found for H_t obtained by treatment with trypsin and Triton X-100, protein bands 1 and 2 have been somewhat separated by the urea treatment, while protein fractions 3 and 4 remain together with protein fraction 2 in the heavy fraction H_{1tH} and not in H_t. It is possible that this difference may reflect a multiple action of Triton X-100 due to its lipid binding capacity.

By comparing the protein composition of membranes obtained from dark and light grown cells it is possible to see that this bacterium adapts itself to different physiological conditions by altering the protein composition of its membrane.

When membranes obtained from dark grown cells were incubated in the presence of trypsin and detergent and the extract submitted to a density gradient centrifugation, only one protein band was detected in the gradient, and its protein composition showed the presence of three slow-running proteins and some faster-running components. Two of these slow-running proteins have somewhat similar hydrodynamic properties to bands 1 and 2 present in H_t. This result reinforces the idea of the presence of a group of proteins present in both M and M_0 which produce, after tryptic digestion, heavy nonpigmented fractions (H_t or M_{0t}) with similar electrical properties. This does not necessary mean that the proteins that gave origin to those slow-running components, detected in H_t and M_{0t}, are the same, or that they should have the same electrical properties.

We would like to put forward the idea that in the photosynthetic membranes the protein fractions obtained in L would be related to the photochemical apparatus. We do not as yet know how many of the proteins present in L are the bacteriochlorophyll bearing proteins, although we must not forget that the L fraction is a heterogenous complex in which cytochromes and the proteins related to NAD^+ photoreduction are present[3]. Without discarding other possibilities we think that protein fraction I is one of the proteins that bind to the bacteriochlorophyll and that upon tryptic digestion it gives rise to either protein 1, 3 or 4.

RIVAS *et al.*[12] have postulated that the H_f fraction or, as they called it, "basic membrane", could have a support function. The protein composition of H_f shows the

presence of protein fraction II as a major component. It could therefore be that protein fraction II is the protein upon which the bacteriochlorophyll-containing proteins and all of the other protein and lipid components of the photosynthetic membrane are bound.

An ornithine-containing lipid has been detected in some dark and light grown cells. GORCHEIN[13] suggests that this lipid could be a membrane structural component. The H_t fraction contained the ornithine-containing lipid as the only polar lipid. We have analyzed (unpublished experiments) the lipid distribution in H_t and L_t fragments from *R. rubrum* (wild type and BG_1) and have obtained similar results. The ornithine-containing lipid is only present in H_t, and no lipid phosphorus could be detected in this fraction. On the other hand the L_t fraction contained only phospholipids and only traces of ornithine-containing lipid. In these experiments the ratio nmoles ornithine/mg protein in H_t was higher than that found in both the intact membrane and H_f. This result would also indicate that the remaining protein in H_t represents the core of the "basic membrane" that cannot be further degraded.

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