BBA 45807

# BACTERIOCHLOROPHYLL PHEOPHYTINIZATION IN CHROMATOPHORES AND SUBCHROMATOPHORES FROM *RHODOSPIRILLUM RUBRUM**

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(Received December 9th, 1968)

SUMMARY

1. Spectral changes of *Rhodospirillum rubrum* chromatophores and subchromatophores at low pH have been investigated both in the absence and presence of the nonionic detergent, Triton X-100.

2. Bchl 800 readily undergoes pheophytinization below pH 3, whereas Bchl 880 is not easily converted to bacteriopheophytin until Triton X-100 is added.

3. In appropriate concentrations of Triton X-100, Bchl 880 is transformed to Bph 755 *via* some intermediates absorbing at 770–790 nm. The Bph 755 is subsequently converted to Bph 850.

4. These changes are more clearly observed with subchromatophores at different pH values. When subchromatophore particles reaggregate upon removal of Triton X-100, Bchl 880 again becomes resistant to acid treatment.

5. Treatment of chromatophores with Triton X-100 at low pH also causes a considerable decrease of absorbance in the wavelength region where carotenoids absorb.

6. The disintegrating action of Triton X-100 is enhanced by lowering pH, and a carotenoid complex can be separated from a heavier bacteriopheophytin complex.

## INTRODUCTION

THOMAS *et al.*[1] reported pH-induced changes of the infrared absorption spectra of photosynthetic purple bacteria. They found that different absorption bands, namely 800, 850 and 880 nm, are differently affected by low pH, and suggested that the pH influence is based upon changes in pigment–protein interactions or pigment–pigment interactions. Very recently, the accumulation of intermediates prior to the formation of bacteriopheophytin has been shown by OLSON and co-workers[2,3] in their bacteriochlorophyll–protein complex exposed to low pH. This was attributed to a conformational change of the protein.

Abbreviations: Bchl 800 and Bchl 880, bacteriochlorophyll absorbing at 800 and 880 nm, respectively; Bph 755 and Bph 850, bacteriopheophytin absorbing at 755 and 850 nm, respectively.

* This work was presented at the Fifth International Congress on Photobiology, Hanover, N.H., U.S.A., August 26–31, 1968.

The state of bacteriochlorophyll *in vivo*, which determines its spectral characteristics, could be influenced by the aggregation of bacteriochlorophyll or by the association of bacteriochlorophyll with other molecules such as proteins and lipids. Some bacteriochlorophyll molecules may be exposed to an aqueous environment, while others could be buried inside a lipoprotein matrix. Lowering the pH of aqueous media possibly induces changes in protein conformation. It is also expected that the rate of pheophytinization of bacteriochlorophyll will depend on the state of bacteriochlorophyll.

This paper suggests a possible difference in environment of Bchl 800 and Bchl 880 in *Rhodospirillum rubrum* chromatophores and subchromatophores. This investigation reveals not only the modification of bacteriochlorophyll–protein environment before pheophytinization, but also the rearrangement of bacteriopheophytin after pheophytinization.

It will also be shown that pH-induced disintegration of chromatophores, coupled with the action of Triton X-100, results in the formation of a bacteriopheophytin complex separated from the carotenoid complex.

## METHODS

Cells of *R. rubrum* grown for 3–5 days in a tryptone–dextrose–yeast extract medium were harvested by centrifugation, washed with a phosphate buffer (pH 7) and ruptured by sonication for 3 min using a Bronson Instruments, sonifier Model S-110.

Chromatophores were obtained by centrifugation collecting the fraction sedimenting between 20 000 × *g* (30 min) and 144 000 × *g* (1 h). Washed chromatophores were resuspended in an appropriate buffer or in distilled water and subsequently homogenized. For the study of pH-dependent spectral changes as a function of time, portions of the suspension in distilled water were added to the buffer solutions.

Subchromatophores were prepared from chromatophores by essentially the same method used by GARCIA *et al.*[4]. Separation into heavy and light fragments was not achieved in our sucrose density-gradient centrifugation after 4 h. Triton X-100 (10%) was added to a chromatophore suspension (phosphate buffer (pH 7)) having an absorbance of about 50 units/cm at 880 nm, and allowed to stand overnight at 4°. Portions of this suspension were placed on the top of centrifuge tubes containing a discontinuous sucrose-density gradient consisting of 6, 12, 24, 35, 40, 50 and 60% sucrose in phosphate buffer (pH 7). The tubes were centrifuged for 4 h at 120 000 × *g* in the swinging-bucket type SW-39 rotor of a Spinco Model L ultracentrifuge. The fraction from the second red band was collected and dialyzed against distilled water at 4° with daily change of distilled water. The same method was applied to sucrose density-gradient centrifugation at different pH's.

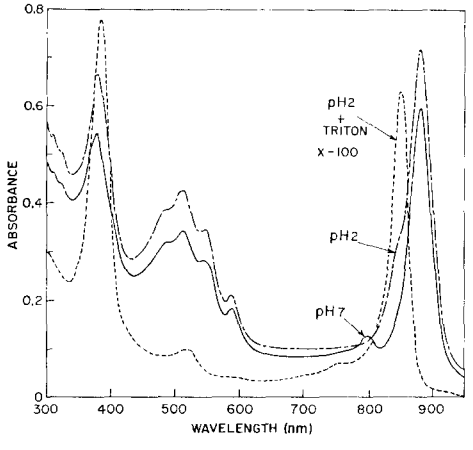
Absorption spectra were recorded using a Cary-14 spectrophotometer equipped with a phototube of near S-20 photosurface or in some cases with the infrared attachment. Buffer solutions with an ionic strength of 0.1 were used in all experiments. Triton X-100 was obtained from Rohm and Haas (Philadelphia).

## RESULTS

When a chromatophore suspension was maintained at pH 2 (glycine–HCl buffer) for several days at 4°, the absorption spectrum of a 1:30 dilution of this suspension

(Fig. 1) demonstrated the survival of Bchl 880. Bchl 800 disappeared and a shoulder was formed at about 850 nm. These changes are similar to those observed at pH 1 by THOMAS *et al.*[1] except that the increase of absorption at 850 nm was greater in this case. Triton X-100 (3%) was added to the original suspension at pH 2 and the mixture was allowed to stand overnight. As also shown in Fig. 1, this treatment caused the complete disappearance of Bchl 880, accompanied by the appearance of a peak at 850 nm. Carotenoid absorption bands were considerably reduced.

The chromatophore suspension in the presence of Triton X-100 (3%) at pH 2 was centrifuged in a sucrose-density gradient. Two distinctly separate, colored bands were obtained in the upper portion of the centrifuge tube after 4 h of centrifugation.



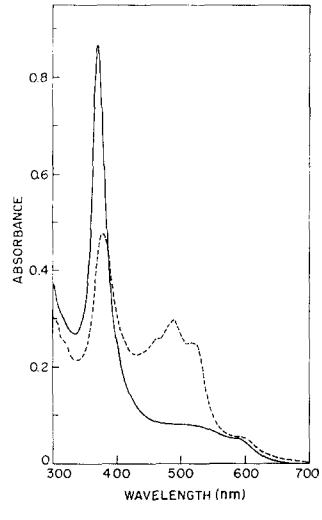




Fig. 1. Absorption spectra of *R. rubrum* chromatophores at pH 7 (phosphate buffer) (———) and at pH 2 (glycine–HCl buffer) without (– – – –) and with Triton X-100 (- - - - - -).

Fig. 2. Absorption spectra of the carotenoid complex in an aqueous suspension (—— –) and in methanol (- - - - - -). A suspension containing the carotenoid complex was diluted (1:37.5) with a buffer solution (glycine–HCl (pH 2)) or methanol. The methanol extract was centrifuged before taking its absorption spectrum.

The upper, reddish-brown fraction exhibited a sharp absorption peak at 370 nm and weak diffuse bands in the visible region, as shown in Fig. 2 (solid curve). A methanol extract of this fraction showed the usual carotenoid absorption (Fig. 2, dotted curve). This unusual form of the carotenoid complex is similar to that obtained by VERNON AND GARCIA[5] from treatment of *R. rubrum* chromatophores with pancreatin in the presence of Triton X-100. Fig. 3 shows the absorption spectra of the lower, dark-greenish fraction as well as its methanol extract. This fraction, absorbing at 850 nm, contains bacteriopheophytin. These results indicate that in the presence of Triton X-100 chromatophores at pH 2 yield two complexes, one containing carotenoids and the other bacteriopheophytin.

The results thus obtained at pH 2 motivated further investigation of the detailed spectral changes occurring with time both in the absence and presence of Triton X-100 at different pH's. Fig. 4 shows the disappearance of Bchl 800 as a function of

time at both pH 1 and 3. The solid curve in Fig. 4 represents the absorption spectrum at pH 7. The absorption spectrum taken immediately at pH 1 (HCl–KCl buffer) (Fig. 4, dotted curve) shows that the 800-nm band rapidly disappears producing a new band at 755 nm. After 15 min, an increase of absorption around 850 nm became evident with the decrease of the 755-nm band. Bchl 880 was not affected during this period. Only after a longer period of standing at pH 1 did the decrease of Bchl 880 and the increase of Bph 850 become apparent. At pH 3 (glycine–HCl buffer), the disappearance of Bchl 800 occurred very slowly (Fig. 4).

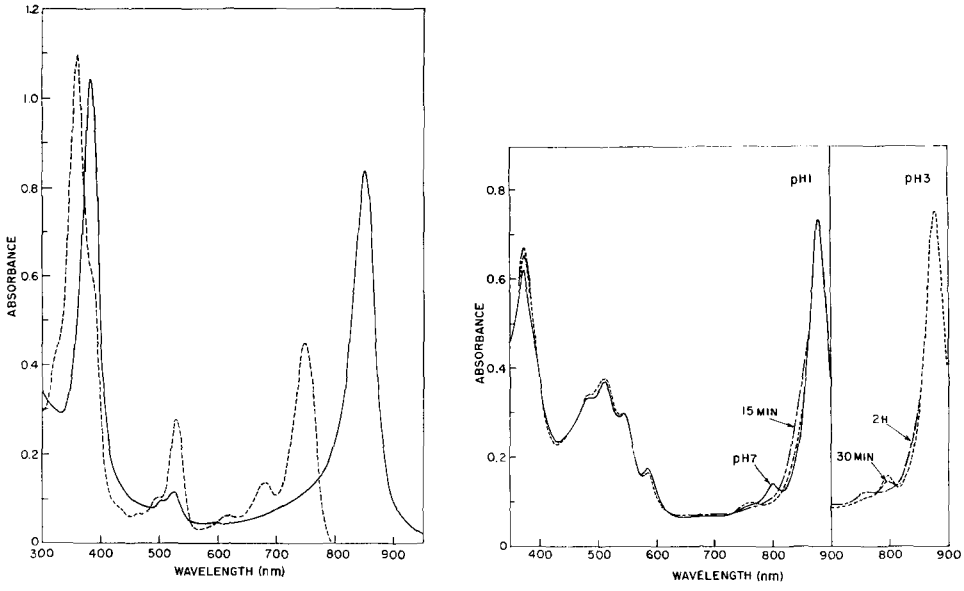



Fig. 3. Absorption spectra of the bacteriopheophytin complex in an aqueous suspension (———) and in methanol (-------). A suspension containing the bacteriopheophytin complex was diluted (1:150) with a buffer solution (glycine–HCl (pH 2)) or methanol. The methanol extract was centrifuged before taking its absorption spectrum.

Fig. 4. Spectral changes of chromatophores at pH 7 (phosphate buffer) (———); at pH 1 (HCl–KCl buffer) (-------, immediately; –·—·–, after 15 min); and at pH 3 (glycine–HCl buffer) (------, immediately; –·—·–, after 30 min; — — —, after 2 h).

When chromatophores kept at pH 2 (absorbance of about 0.6 unit/cm at 880 nm) were treated with 0.1% Triton X-100, both the 880-nm band and carotenoid bands were immediately reduced as shown in Fig. 5 (dotted curve). New bands at 755 and 850 nm appeared, along with a shoulder at about 790 nm. Absorption spectra taken after 5 and 30 min show the disappearance of the 790-nm band and the remarkable increase of the 850-nm band at the expense of the 755-nm band (Fig. 5). These changes indicate that the 790-nm band appears due to the formation of intermediates during the conversion of Bchl 880 to Bph 755 and that Bph 850 is formed from Bph 755. The intermediates found here are similar to those observed by OLSON and his co-workers[2,3] in their bacteriochlorophyll–protein complex.

As shown in Fig. 4, the pheophytinization of Bchl 800 to Bph 755, followed by the transformation to Bph 850, occurs readily even in the absence of Triton X-100. On the other hand, Bchl 880 is resistant to pheophytinization. The addition of Triton

X-100 accelerates the pheophytinization of Bchl 880. However, the formation of Bph 755 or Bph 850 is dependent upon the concentration of Triton X-100 used. Increasing the Triton X-100 concentration to 1% markedly accelerates the conversion of Bchl 880 to Bph 755, but most of the bacteriopheophytin remains in the Bph 755 form. When the Triton X-100 concentration is decreased to 0.01%, the pheophytinization is retarded and much of the Bchl 880 appears to be transformed slowly and directly to the Bph 850 form.

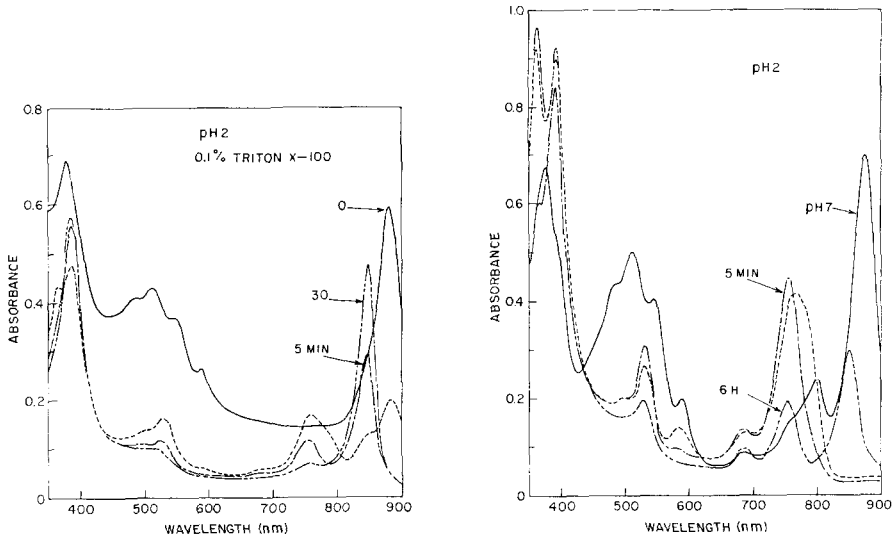



Fig. 5. Spectral changes of chromatophores suspended at pH 2 (glycine–HCl buffer) (— · — · —) and treated with 0.1% Triton X-100 (-------, immediately; – – – –, after 5 min; — · — · —, after 30 min).

Fig. 6. Spectral changes of subchromatophores at pH 7 (phosphate buffer) (———); and at pH 2 (glycine–HCl buffer) (-------, immediately; ———, after 5 min; ———, after 6 h).

Sucrose density-gradient centrifugation at pH 2 revealed that 0.01% Triton X-100 disintegrates chromatophores into heavier fragments which appear in the lower portion of the centrifuge tube. Triton X-100 in a concentration of 0.1% produced two separate bands in the upper portion of the tube. Their absorption spectra were similar to those shown in Figs. 2 and 3. A Bph 755-containing fraction obtained with 1% Triton X-100 was lighter than the Bph 850-containing fraction formed with 0.1% Triton X-100. At pH 7, however, heavier fragments were obtained with 0.1% Triton X-100 treatment, while 1% Triton X-100 produced lighter subchromatophores whose absorption spectra are similar to those of chromatophores. These results indicate that the separation into the carotenoid complex and the Bph-850 complex is a pH-induced action occurring in cooperation with an appropriate concentration of Triton X-100.

The effect of pH was also investigated for subchromatophores prepared from chromatophores treated with Triton X-100 at pH 7. The subchromatophore suspension was dialyzed for 3 days against distilled water. The absorption spectrum of this sample at pH 7 (phosphate buffer) is shown by the solid curve in Fig. 6. The subchromatophore suspension, when transferred to a glycine–HCl buffer of pH 2, showed

the immediate disappearance of both Bchl 800 and Bchl 880, accompanied by the appearance of Bph 755 and the intermediates absorbing at 770–790 nm (Fig. 6, dotted curve). This spectrum also exhibits a considerable decrease of carotenoid bands as well as a slight blue-shift of the orange bacteriochlorophyll band from 590 to 585 nm, giving rise to another band at 525 nm of Bph 755. The band at 585 nm is attributable to an orange band of intermediate forms of bacteriochlorophyll. Two new bands in the near ultraviolet region also appeared at 368 and 393 nm. The 368-nm band is a Soret band of Bph 755, but the origin of the 393-nm band is not certain. It might be due to some form of carotenoid complex, probably an intermediate to the final form of carotenoid complex absorbing at 370 nm (Fig. 2). As shown also in Fig. 6, this initial reaction was followed by further conversion of the Bchl 770–790 intermediates to Bph 755 and then to Bph 850.

Sucrose density-gradient centrifugation experiments also showed pH-induced disintegration of subchromatophores. The sample of subchromatophores used in the spectral study described above appeared at pH 7 in the lower part of the centrifuge tube after centrifugation, indicating a certain degree of reaggregation after dialysis. The centrifugation at pH 2 resulted in the appearance of lighter fractions in the upper part of the tube. When the subchromatophore suspension was further exhaustively dialyzed against distilled water for several days, further reaggregation occurred and caused the particles to sediment to the bottom of the centrifuge tube. The spectral change of Bchl 880 was found to be much slower in this sample at pH 2. It is concluded, therefore, that the Triton X-100 in the sample of subchromatophores used in Fig. 6 and in the following experiments at various pH's has not been completely removed.

The subchromatophores at pH 3 (glycine–HCl buffer) show that the conversion of Bchl 880 through the intermediate bacteriochlorophyll forms to bacteriopheophytin is slowed down, but the transformation of Bph 755 to Bph 850 is accelerated. The gradual blue-shift of the absorption bands of the intermediates, as well as the marked increase of absorbance in Bph 850, can now be seen. Increasing the pH to 3.6 (citrate buffer), the slow pheophytinization of Bchl 880 is followed by the faster conversion to Bph 850, which proceeds even before all of the bacteriochlorophyll is converted to Bph 755. This is manifested by the appearance of two peaks at 850 and 880 nm after 5 h of standing. Further increase to pH 4.2 (acetate buffer) shows slight changes in Bchl 880 and carotenoid absorption and very gradual changes of Bchl 800 to Bph 755 *via* intermediates. After 3 h, two bands are observed at 795 and 755 nm. At pH 5.2 (acetate buffer), no specific changes were found except a minuscule and gradual decrease of absorption over the entire visible region.

## DISCUSSION

The present investigation demonstrates that different forms of bacteriochlorophyll in *R. rubrum* chromatophores, Bchl 800 and Bchl 880, differ in their conversion to bacteriopheophytin. Even when Bchl 800 is completely converted to bacteriopheophytin, Bchl 880 does not show any change. This indicates the difference between Bchl 800 and Bchl 880 in their environment around bacteriochlorophyll molecules. Apparently, Bchl 800 is in an aqueous environment, while the bulk of Bchl 880 is not. A similar conclusion was drawn by CLAYTON *et al.*[6] for the major Bchl component

*in vivo*. From this study it is not clear whether or not a photoactive component (p 870) associated with a specialized bacteriochlorophyll molecule (p 800)[7,8] is also easily pheophytinized.

Bchl 880, probably buried in a lipoprotein environment while in the chromatophore, is exposed by the action of Triton X-100 to the acidic aqueous media and then can be converted to bacteriopheophytin. Appropriate concentrations of Triton X-100 also induce separation of the bacteriopheophytin complex from the carotenoid complex. In neutral media, however, chromatophores are merely broken down by Triton X-100 to subchromatophore particles which do not show significant changes in the composition of bacteriochlorophyll and carotenoid. As long as the subchromatophores remain unaggregated or only slightly aggregated, Bchl 880 is readily pheophytinized. As Triton X-100 is removed from the subchromatophores by thorough dialysis, they undergo considerable reaggregation and the pheophytinization of Bchl 880 becomes more difficult. This indicates that when subchromatophore particles reaggregate, Bchl 880 is again buried within a lipoprotein matrix.

The decrease of carotenoid visible absorption bands and the separation of the carotenoid complex could be due to an acid-induced denaturation of lipoproteins, enhanced by the action of Triton X-100. It is interesting to note that this carotenoid complex is obtained at a lower pH in the absence of any enzyme. As suggested by KE *et al.*[9] for their carotenoid complex, this carotenoid complex could also be aggregates of carotenoids released by the action of Triton X-100 from the deformed lipoproteins. Further investigations on the nature of this complex are in progress.

Spectral characteristics of two different forms of bacteriopheophytin suggest that Bph 755 and Bph 850 are a monomeric and an aggregated form of bacteriopheophytin, respectively. When Bchl 800 or Bchl 880 (in the presence of Triton X-100) is pheophytinized, the ability for bacteriochlorophyll to interact is lost during pheophytinization leading to the formation of Bph 755. Intermediates absorbing between 795 and 770 nm (probably several forms of bacteriochlorophyll-proteins modified by acidification[3]) are formed in this conversion to Bph 755. In subsequent reactions, the interaction between bacteriopheophytin becomes possible forming Bph 850. These processes are summarized as follows:

$$\begin{matrix}\text{Bchl 800}\\ \text{Bchl 880 (Triton X-100)}\end{matrix} \longrightarrow \text{Bchl 795–770} \longrightarrow \text{Bph 755} \longrightarrow \text{Bph 850}$$

The fact that the rearrangement of Bph 755 to Bph 850 in the case of Bchl 880 is dependent upon the concentration of Triton X-100 supports the contention that Bph 755 is monomeric and Bph 850 is polymeric. The higher molar extinction coefficient of Bph 850 and the direct formation of Bph 850 from Bchl 880 in low concentrations of Triton X-100 suggest a unique structural arrangement of bacteriopheophytin, reminiscent of that of Bchl 880.

This rearrangement of bacteriopheophytin appears to be related to the association of lipoprotein, since there is also a relationship between the concentration of Triton X-100 and the degree of disintegration of chromatophores. The pH-dependence of the Bph 755 → Bph 850 rearrangement seems to imply some role for the pH-dependent protein conformation in this lipoprotein association. There exists the possibility, however, that bacteriopheophytin molecules released from the lipoprotein

matrix may reaggregate among themselves. The nature of the Bph 850 complex separated from the carotenoid complex is also at present under investigation.

Other forms of bacteriopheophytin have been reported. A bacteriopheophytin-containing particle was obtained by KIHARA AND FRENKEL[10] from young *R. rubrum* cells. CLAYTON[11] obtained "pheophytinized" cells from an old culture of a blue-green mutant of *Rhodopseudomonas spheroides* kept under anaerobic conditions in the light for several weeks. These forms of bacteriopheophytin showed a major absorption band at about 800 nm and are bound forms of bacteriopheophytin differing from Bph 850 obtained by direct acidification.

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