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PIGMENT–PROTEIN COMPLEXES DERIVED FROM *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES BY ENZYMATIC DIGESTION*

LEO P. VERNON AND AUGUSTO F. GARCIA

Charles F. Kettering Research Laboratory, Yellow Springs, Ohio (U.S.A.)

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SUMMARY

Rhodospirillum rubrum chromatophores are acted upon by pancreatin (a crude mixture of pancreatic digestive enzymes) or α-chymotrypsin in the presence of 0.5 % Triton X-100 to produce three clearly defined pigment–protein complexes which are separable by sucrose density-gradient centrifugation. The first to appear temporally is a brown band which contains carotenoids in an unusual combination, since the complex does not show the absorption peaks in the 400 to 500 nm range normally shown by carotenoids in the chromatophore. The complex does show a large absorption band with a maximum at 368 nm. The addition of acetone and methanol to this complex allows the usual absorption properties of the carotenoids to appear. Two bacteriochlorophyll (Bchl)–protein complexes are formed. A blue Bchl–protein complex has absorption maxima at 922, 835, 580 and 372 nm. The 922 nm band is partially bleached by addition of ferricyanide, indicating there are two Bchl types in the complex. Addition of ascorbate largely restores the 922 nm peak. The band at 372 nm is due to some carotenoids which are present. A green Bchl–protein complex has absorption maxima at 780, 587 and 360 nm. The green complex (but not the blue) is active in simple photochemical electron transfer reactions such as reduced phenazine methosulfate oxidation in the presence of ubiquinone and the photoreduction of 5,5′-dithiobis-(2-nitrobenzoic acid) in the presence of ascorbate and 2,6-dichlorophenolindophenol. The green complex is more labile than the blue complex once they are formed. The Bchl–protein complexes could represent modified Bchl–protein complexes which exist *in situ* or could be formed from Bchl and proteins which are liberated by the digestive enzymes. Treatment with α-chymotrypsin or pancreatin produces similar complexes from *R. rubrum* chromatophores. Treatment of a blue-green mutant of *R. rubrum* which lacks carotenoids yields the blue and the green bands, but shows no brown band following centrifugation. Treatment of *Chromatium* chromatophores with either pancreatin or α-chymotrypsin in the presence of Triton X-100 results in complete digestion of the chromatophore with no discernible pigment complexes being formed.

Abbreviations: Bchl, bacteriochlorophyll; PMS, phenazine methosulfate (*N*-methylphenazinium methyl sulfate); DCIP, 2,6-dichorophenolindophenol; UQ-6, ubiquinone with six isoprene units in the side chain; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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INTRODUCTION

Although many studies on the effects of detergents upon the photosynthetic apparatus of bacteria have been made (see refs. 1 and 2 for a brief review), only a few cases of enzymatic digestion have been reported. NEWTON AND LEVINE[3] treated *Chromatium* chromatophores with trypsin to obtain smaller pigmented particles which were less reactive toward antiserum prepared from the intact chromatophores. Treatment with chymotrypsin caused a partial destruction of the 850 and 880 nm forms of bacteriochlorophyll (Bchl) (see ref. 1). A subsequent investigation by BRIL[4], however, failed to show any effect of tryptic digestion upon *Chromatium* chromatophores. Treatment of *Rhodospirillum rubrum* chromatophores with lipase produced particles enriched in Bchl, but such particles were devoid of enzymatic activity[5]. In view of the reported alteration of physical and chemical properties of bacterial chromatophores through enzymatic digestion, we have conducted an investigation into the effect of combined detergent action and enzymatic digestion, which shows that *R. rubrum* chromatophores in the presence of the detergent Triton X-100 are susceptible to the action of pancreatin and α-chymotrypsin. Such treatment causes extensive breakdown of the chromatophore and produces two separate Bchl–protein complexes and one carotenoid–protein complex whose properties are described herein.

METHODS

R. rubrum cells, strain S-1, used for this study were grown on malate medium according to the directions of NEWTON[6]. Chromatophores were released from the cell by sonication and separated from other cellular material by centrifugation[2]. The experimental procedure used for density-gradient centrifugation was that previously reported[1]. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) photoreduction was followed by the method reported by NEWTON[7], and the photooxidation of reduced phenazine methosulfate ($PMSH_2$) coupled to ubiquinone (UQ) photoreduction was determined as previously reported[8]. For protein determination a small aliquot was treated according to the directions given by LOWRY *et al.*[9] with no prior extraction of the pigments. Absorption spectra of the various fractions were obtained with a Cary 15 spectrophotometer. The experimental conditions relating to the various enzymatic digestions are reported for the appropriate figure. When anaerobic conditions were employed, the system was evacuated twice with intermediate flushing with argon gas, which was continuously flushed through the system during the incubation period with the digestive enzymes. Bchl was determined by the method of CLAYTON[10]. The pancreatin and α-chymotrypsin and phenazine methosulfate (PMS) were commercial preparations purchased from Sigma Chemical Co. of St. Louis, Mo. Ubiquinone with six isoprene units in the side chain (UQ-6) was obtained from Mann Research Laboratories. The Triton X-100 was obtained from Rohm and Haas Co., Philadelphia, Pa.

RESULTS

Preliminary experiments showed that pancreatin treatment of chromatophores of *R. rubrum*, as well as the particles derived by the action of Triton X-100 (see refs. 1, 2), produced no significant change in the particles as evidenced by a lack of change

in appearance of sucrose density-gradient centrifuge tubes containing the treated material. When the incubation with pancreatin was carried out in the presence of 0.5 % Triton X-100, however, there was a marked breakdown of the chromatophore structure with the appearance of three new bands. Fig. 1 shows the appearance of the centrifuge

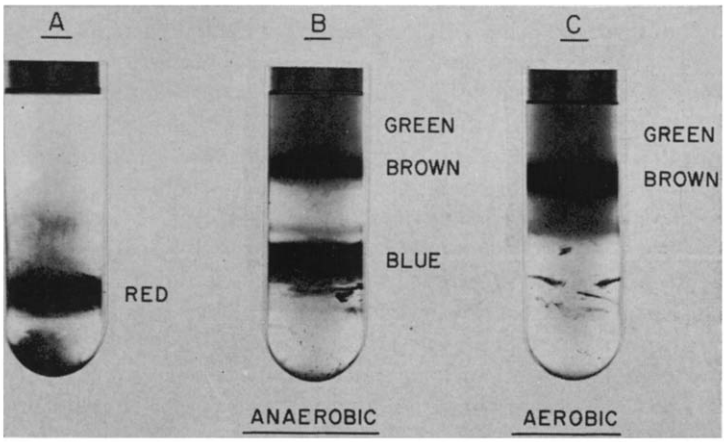


Fig. 1. Distribution of pigmented material following centrifugation of *R. rubrum* chromatophores and digestion mixtures for 2 h at 110000 × *g* in a discontinuous sucrose density gradient composed of equal volumes of 57, 28 and 15 % sucrose. (A) untreated chromatophores. (B) chromatophores digested with pancreatin (4 mg/ml of incubation mixture also containing 0.5 % Triton X-100 and 0.25 mg Bchl/ml) at 36° for 24 h. The incubation mixture was then layered directly on the sucrose gradient. Anaerobic conditions were employed during the digestion by means of two initial evacuations and flushing with argon, which was bubbled through the mixture during the incubation period. A small white band was located between the blue and brown bands, and probably was derived from pancreatin added originally. (C) chromatophores digested with pancreatin under aerobic conditions. Other conditions were the same as in (B). Some of the brown material extended below the narrow, intense brown band.

tubes after density-gradient centrifugation. The chromatophores treated under anaerobic conditions yielded a narrow, blue band, a narrow brown band of lesser density and a more diffuse green band located near the top of the tube. The blue band was absent in the digestion mixture which was open to the air, but once formed under anaerobic conditions it was stable to further treatments in the air. The data presented below show that both the blue and green bands are chlorophyll–protein complexes and the brown band is a carotenoid–protein complex.

The three bands produced by the action of pancreatin do not appear at the same time during the digestion. After 1 h digestion with pancreatin (4 mg/ml in a suspension containing 0.5 % Triton X-100 and *R. rubrum* chromatophores equivalent to 0.2 mg Bchl/ml) only the brown band was visible after centrifugation. Within 3 h of digestion the green band was also visible along with a faint indication of the blue band. After 8 h all three bands were clearly visible, along with some remaining material which sedimented in a manner resembling the original chromatophores. After 24 h of digestion there was no residual material relating to the original chromatophores, and the distribution shown in Fig. 1 was obtained. If Triton concentrations lower than 0.5 % were used, more blue band was formed at the expense of the green band. In the presence of 4 % Triton, the pancreatin digestion for 24 h failed to produce any of the blue band, and the green band was more prominent. Therefore, the

Bchl–protein complex found in the blue band is formed preferentially under anaerobic conditions and at low concentrations of Triton X-100, and is not obtained either under aerobic conditions or in the presence of 4 % Triton. The presence of protein in all three bands (brown, blue and green) was shown by reaction of the isolated material with the Folin–Ciocalteu reagent according to the directions of LOWRY *et al.*[9].

A similar digestion pattern was obtained by using α-chymotrypsin in place of pancreatin. Using similar digestion conditions of Triton concentration, the same three bands were observed following centrifugation. Similar procedures were tried on *Chromatium* chromatophores, but in this case the digestion was complete and there appeared to be complete destruction of the chromatophores with no appearance of the various bands noted for *R. rubrum* chromatophores.

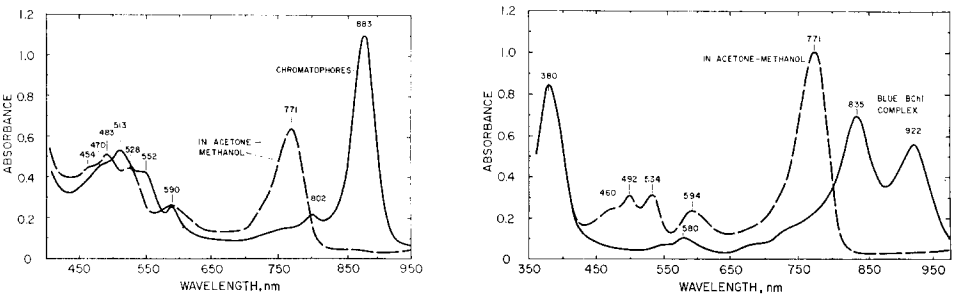


Fig. 2. Absorption spectra of *R. rubrum* chromatophores suspended in 0.01 M Tris buffer (pH 7.8) or extracted by an acetone–methanol solution. For the latter, an aqueous buffered suspension of chromatophores was diluted with 60 vol. of an acetone–methanol solution (7:2,v/v) at room temperature and centrifuged to remove precipitated protein. For the spectrum of chromatophores, the initial suspension was diluted with 60 vol. of Tris buffer to give a final Bchl concentration of 0.024 μmole/ml.

Fig. 3. Absorption spectra of the blue Bchl–protein complex obtained by anaerobic incubation with pancreatin in the presence of 0.5 % Triton X-100 as given in Fig. 1. For the acetone–methanol treatment the procedure outlined in Fig. 2 was used including the equal dilution with buffer or organic solvent. In the present case no protein precipitated upon addition of the organic solvent. The Bchl concentration in the Bchl complex after dilution was 0.04 μmole/ml.

The absorption spectra of *R. rubrum* chromatophores suspended in aqueous buffer and after treatment with acetone–methanol (7:2, v/v) are shown in Fig. 2, with the location of the absorption peaks also indicated. Treatment with a mixture of acetone and methanol (7:2, v/v) removes the pigments from the body of the chromatophore and shifts the absorption peaks to those found in organic solvents. Fig. 3 shows similar spectra for the blue band, which show that the main pigment in this band is Bchl. Two absorption bands are seen in the near infrared, which indicate the Bchl on the complex is in two forms (see data below on oxidation of the Bchl–protein complex). Treatment with acetone–methanol (7:2, v/v) shifts the absorption spectrum to that of Bchl in solution, showing that Bchl is the pigment responsible for the absorption in the near infrared region on the complex. The solution treated with acetone–methanol also shows the presence of some carotenoids, which absorb in the 400 to 500 nm region. The large Bchl peak observed at 380 nm in the complex apparently covers a carotenoid peak at 372 nm which is shown by the brown band which is a carotenoid–protein complex. In that band also the regular absorption peaks of the carotenoids between 400 and 500 nm appear only after treatment with acetone–methanol.

Fig. 4 shows the spectra for the diffuse green band, and shows that the pigment contained in this complex also in Bchl, since it shows the usual Bchl spectrum after treatment with acetone–methanol. The 685 nm band represents an oxidized form of Bchl[11]. There is a difference in the spectra of the complexes obtained by digestion under aerobic and anaerobic conditions, with the red maximum occurring at 771 nm in the former and 780 nm in the latter case. This indicates some difference in the protein part of the complex under these differing conditions. In no case is there any evidence for the presence of carotenoids on this complex.

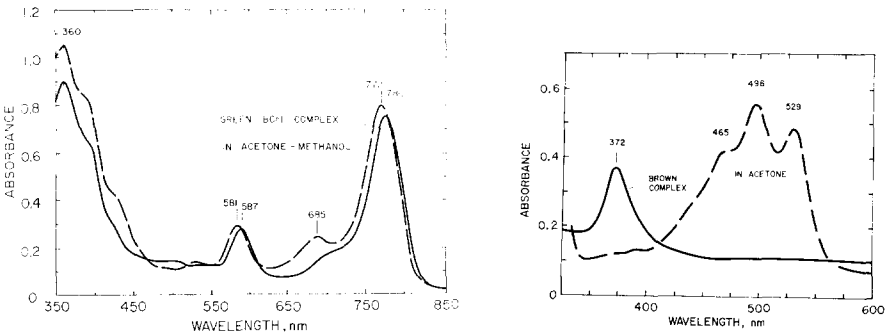


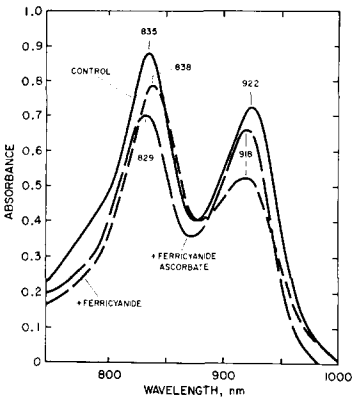
Fig. 4. Absorption spectra of the green Bchl–protein complex obtained by anaerobic incubation with pancreatin in the presence of 0.5% Triton X-100. Other conditions as in Fig. 3, except that the solution of the Bchl–protein complex was diluted with only 5 vol. of acetone–methanol (7:2, v/v) or 0.01 M Tris buffer (pH 7.8) prior to obtaining the spectra.

Fig. 5. Absorption spectra of the brown carotenoid–protein complex purified by chromatography on DEAE-cellulose (see text for details) following the initial separation from the pancreatin incubation mixture *via* sucrose density-gradient centrifugation. For these spectra, 0.02 ml of the purified complex was diluted to 3.0 ml with 0.01 M Tris buffer (pH 7.8) or acetone. The protein content of the material after dilution with buffer was 0.015 mg/ml.

The absorption spectra for the brown band are shown in Fig. 5, which shows that the material in this band is a carotenoid–protein complex. The material used for these spectra was purified by chromatography on a DEAE-cellulose column following the initial sucrose density-gradient centrifugation. Some contaminating material from the blue and green bands was strongly adsorbed at the top of the column and was not eluted with the 0.25 M NaCl in 0.01 M Tris buffer (pH 8.0) which was used to elute the brown band. The absorption spectra of the purified material are unique, since the usual absorption peaks of the carotenoids in the region of 400 to 500 nm are absent in the pigment–protein complex. Only when the organic solvent acetone is added does the usual carotenoid spectrum become apparent. The absorption peaks of the carotenoids from the brown band are shifted about 10 nm towards the blue when compared to the spectra obtained with chromatophores. The band at 372 nm for the carotenoid–protein complex acts in an inverse manner, being prominent in the protein complex and absent in the organic solvents. The transition between the two states for the carotenoids is freely reversible, and addition of more water to the acetone suspension will cause a reversal to the original spectrum of the carotenoid–protein complex. Of the many solvents tried, only acetone is capable of transforming the spectrum of the carotenoid–protein complex to the more usual type observed for the carotenoids.

Treatment with ferricyanide ion causes a significant change in the spectrum

of the blue band as shown in Fig. 6. The 922 nm absorption markedly decreases while the 835 nm absorption is less affected. Subsequent treatment with ascorbate largely restores the 922 absorption showing that the oxidation of this Bchl band is reversible. Since the 922 band behaves independently of the 835 nm band, we can conclude that these two absorption bands represent Bchl in two different environments on the complex, and are not two different absorption bands of the same Bchl molecule.



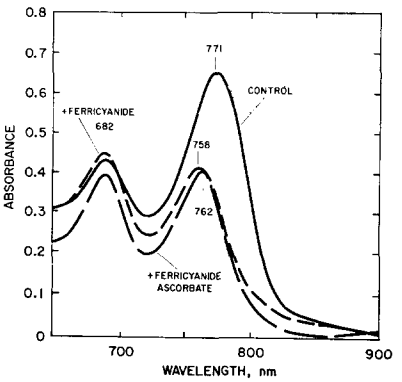


Fig. 6. Oxidation of the blue Bchl–protein complex by ferricyanide. To 3.5 ml of the complex in 0.01 M Tris buffer (pH 7.8) was added sequentially 0.05 ml of 0.5 M $K_3Fe(CN)_6$ and 0.05 ml of 1.5 M sodium ascorbate to obtain the lower two spectra. The system was allowed to react for 3 min after each addition before the spectra were obtained.

Fig. 7. Oxidation of the green Bchl–protein complex by ferricyanide. The sequential addition of $K_3Fe(CN)_6$ and sodium ascorbate was performed as given for Fig. 6.

The response of the green band to ferricyanide oxidation is shown in Fig. 7. In this case the green band was isolated from a digestion carried out under aerobic conditions, which gave a preparation with a different absorption maximum than observed under anaerobic conditions, *i.e.* 771 nm as compared to 780 nm for the anaerobic treatment. The salient features are the bleaching of the 771 nm band and a corresponding shift to 758 nm. The 758 band could be related to bacteriopheophytin formation instead of an oxidation of the Bchl. The band at 682 nm also increases slightly, however, which can be related to an oxidized form of Bchl which has recently been described[11]. These changes (oxidation) caused by ferricyanide are not reversed by the addition of ascorbate.

The material in the green band, once isolated, was quite labile. Upon standing at room temperature, either in darkness or light, there was a steady disappearance of the 771 absorption band with a corresponding increase in the 682 nm band. This indicated an oxidative destruction of the complex.

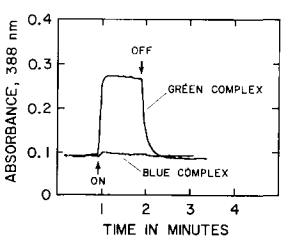
A blue-green mutant of *R. rubrum*, kindly supplied by Dr. R. CLAYTON, was subjected to the pancreatin digestion procedure. Except for the absence of the brown band which contains the carotenoid–protein complex, the same results were obtained as with the wild-type *R. rubrum*. Blue and green bands were obtained which were similar in all respects to those obtained from the wild-type bacterium.

The distribution of Bchl between the blue and green Bchl–protein complexes was in the ratio of approx. 3 (blue) / 1 (green), averaged over a number of preparations.

This ratio was obtained only for treatments under anaerobic conditions, since the presence of air allowed only the formation of the green band. In one experiment, the ratio of Bchl/protein for purified blue complex was one (Bchl) to four (protein) on a weight basis. Similar measurements have not been made for a green fraction.

The photochemical activities of the two Bchl–protein complexes have been tested. Fig. 8 shows that the green band is very active in catalyzing the photooxidation of $PMSH_2$ coupled to the reduction of UQ-6. This reaction, described previously by ZAUGG, VERNON AND TIRPACK[8], is readily catalyzed by *R. rubrum* chromatophores and by Bchl suspended in aqueous media through the use of detergents. It is not carried out, however, by chromatophores of a mutant of *Rhodopseudomonas spheroides* which has the normal complement of bulk Bchl but lacks the reaction center Bchl[12]. It appears that the main requirement for this reaction is for Bchl which is readily available to the reagents in an aqueous environment. This would be supplied by the reaction center Bchl in the intact chromatophore (and further indicates that the bulk of the Bchl on the chromatophore is not available to aqueous reactants), by 'solubilized' Bchl in the presence of detergents, and by the green Bchl–protein complex described in this investigation. The green Bchl–protein complex differs from the blue one, which is inactive in this reaction. In this regard the blue Bchl–protein complex resembles the Bchl–protein complex isolated by OLSON[13,14]. Experiments performed in this laboratory on a sample of that Bchl–protein complex supplied by Dr. OLSON showed that it also is inactive in the $PMSH_2$–UQ reaction system. The rate of $PMSH_2$ photooxidation for the reaction shown in Fig. 8 is 4300 μmoles $PMSH_2$ oxidized per h per mg Bchl, which should be compared to the rate of 6000–8000 μmoles observed for intact *R. rubrum* chromatophores[8].

Another simple photochemical electron transfer reaction catalyzed by *R. rubrum* chromatophores is the photoreduction of DTNB in the presence of the electron donor



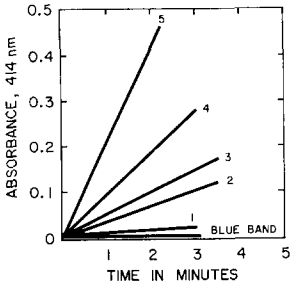


Fig. 8. Photooxidation of $PMSH_2$ coupled to UQ-6 by the green Bchl–protein complex. The reaction mixture contained initially in 2.8 ml the following components (in μmoles): reduced UQ-6, 0.56; PMS, 0.3; Tris buffer (pH 7.5), 200. Also present were the green Bchl–protein complex, 0.074 mg Bchl, or blue Bchl–protein complex, 0.15 mg Bchl. The reduced UQ-6 reduced the PMS chemically in the dark, to poise the system for the subsequent photooxidation of the $PMSH_2$.

Fig. 9. Photoreduction of DTNB by the green Bchl–protein complex in the presence of viologen dyes. The reaction mixture contained in 2.8 ml final volume the following (in μmoles): Tris buffer (pH 7.5), 200; DCIP, 2.0; DTNB, 0.5; sodium ascorbate, 10; and viologen dyes, 0.6. Also present were the green Bchl–protein complex, 0.14 mg Bchl, or the blue Bchl–protein complex, 0.15 mg Bchl (lower curve). The curves represent reaction mixtures containing the green complex and viologen dyes of decreasing oxidation potential: (1) diquat (1,1′-ethylene-2,2′-dipyridylium dibromide), —362 mV; (2) methyl viologen (1,1′-dimethyl-4,4′-dipyridylium dichloride), —426 mV; (3) 1,1′-trimethylene-2,2′-dipyridylium dibromide, —521 mV; (4) 1,1′-trimethylene-2,2′-dipyridylium-5,5′-dimethyl dibromide, —636 mV; and (5) 1,1′-trimethylene-2,2′-dipyridylium-4,4′-dimethyl dibromide, —656 mV.

system of ascorbate–2,6-dichlorophenolindophenol (DCIP). Again the blue Bchl–protein complex is inactive, while the green complex catalyzes the reaction. It is interesting to note that whereas the complex alone catalyzes the reaction, it is increased in rate by the addition of the low-potential viologen dye 1,1′-trimethylene-2,2′-dipyridylium-4,4′-dimethyl dibromide. The potential of this viologen dye is listed as −656 mV (see ref. 15) and its activity in this reaction shows that the Bchl–protein complex is capable of reducing compounds of such low potential. Methyl viologen and other viologens also activate this reaction although the rates in these cases are slower than with diquat. The mode of action of the viologen dyes in photosynthetic systems is generally thought to be that of an electron acceptor from the Bchl of bacteria or the chlorophyll of plants. The reduced form of the viologen could then transfer the electron either to oxygen (to catalyze an aerobic oxidation of the substrate) or to some other electron acceptor such as DTNB. Methyl viologen couples very readily with the plant chloroplast system[15,16].

The green Bchl–protein complex is also active in the aerobic photooxidation of reduced mammalian cytochrome *c*. The complex also catalyzes an active oxidation of the cytochrome in the dark, however, which makes it difficult to study the photooxidation reaction.

DISCUSSION

Pancreatin (a crude preparation of pancreatic digestive enzymes) readily breaks down *R. rubrum* chromatophores, but only in the presence of Triton X-100. It is also inactive on the small particles derived from the chromatophore through the action of Triton X-100 followed by centrifugation[1,2] unless there is Triton present in the digestion medium. It appears that Triton X-100 alters the structure of the chromatophore in a significant way so that the digestive enzymes are then able to interact with their substrate molecules within the organized chromatophore membrane, allowing them to partially digest the chromatophore with the production of a series of complexes which contain the pigment molecules Bchl and carotenoid. Since similar results are obtained with pancreatin and α-chymotrypsin, we conclude the changes are due primarily to alteration of the protein structure of the chromatophore.

The carotenoids are bound in an unusual manner to the protein with which they complex. The usual absorption properties of the carotenoids in the 400–500 mμ ranges are lost, and a new absorption band appears at 368 mμ. Addition of acetone brings out the usual carotenoid spectrum, but further addition of water causes a reversion back to the original spectrum. We have not yet determined if the acetone removes the carotenoids from the complex, or if it merely alters the binding (which is apparently very tight) of the carotenoids to the protein. Little is known concerning the nature of the protein, but it does behave in a usual manner on a DEAE-cellulose column.

The two Bchl–protein complexes formed differ quite markedly. The blue complex is apparently more sensitive to conditions during the digestion, since either the presence of oxygen or high Triton concentrations prevent its formation. Because of the inactivity of the blue complex in the photochemical electron transfer reaction, and since these reactions seem to require primarily adequate contact between the reagents

in the aqueous medium and the Bchl, it seems reasonable to say that the Bchl in this complex is in some fashion unavailable to the aqueous medium. Perhaps the Bchl molecules are contained within the interior of the complex. However, ferricyanide is able to react with the 922 mμ form of Bchl in this complex, indicating that some contact with the aqueous environment is possible.

The green Bchl–protein complex is a more interesting complex, since it will carry out some photochemical reactions. Furthermore, once formed it is much more labile than is the blue complex. The absorption maxima of the green complex at 780 nm (anaerobic conditions) or 771 nm (aerobic conditions) show that the environment of the Bchl on this complex is not much different from that in organic solvents, which produce an absorption peak of Bchl at 770 mμ. This represents a marked shift in the absorption peak of the Bchl on this complex, since the majority of the Bchl originally absorbed light at 880 nm on the chromatophore. For this reason it appears that the green Bchl–protein complex isolated in this investigation is significantly altered from the Bchl–protein complex which exists *in situ*. This could represent either a breakdown of the original structure of the chromatophore so that the Bchl remains attached to the original protein (or fragments of it) which are separated from other components which were tightly coupled to it in the original structure, or it could indicate a separation of the Bchl from its original protein during the digestion period followed by a recondensation on some other protein liberated by the proteolytic enzymes. We are not presently in a position to say which of these possibilities obtains.

By virtue of the absorption peaks at 835 and 922 mμ, the blue Bchl–protein complex represent a state of Bchl more nearly representative to that found in the chromatophore. Again, however, the location of these absorption bands does not coincide with any of the bands found *in vivo*, which indicates a rearrangement of the Bchl during the digestion procedure to form the blue complex. The inactivity of the blue complex in the simple photochemical reactions indicates the Bchl is removed from the aqueous environment and may be sequestered within the protein molecule. This would be similar to the bulk of the Bchl in the chromatophore which has long-wavelength absorption maxima and is not available to catalyze photochemical electron transfer reactions with reagents in the aqueous phase of the reaction mixture[12].

The fate of the other protein components of the chromatophore during the digestion procedure is not known. It has not been possible to detect any cytochromes or other enzymatic activities on the various complexes isolated following the pancreatin digestion. The green band will catalyze the oxidation of reduced mammalian cytochrome *c* in the dark, which proceeds even faster in the light.

The availability of the Bchl–protein complexes, especially the green one, will be of value since it will allow physical and photochemical studies to be performed on Bchl in aqueous media in the absence of high concentrations of detergents to keep the Bchl in 'solution'.

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