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EFFECTS OF DENATURANTS ON THE ABSORPTION SPECTRUM OF THE BACTERIOCHLOROPHYLL–PROTEIN FROM THE PHOTOSYNTHETIC BACTERIUM *CHLOROPSEUDOMONAS ETHYLICUM*

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SUMMARY

1. The absorption spectrum of the water-soluble bacteriochlorophyll–protein complex in the 230- to 900-nm range is chiefly due to 20 bacteriochlorophylls per macromolecule and remains essentially unchanged in 5% Triton X-100, 6 M urea, 0.1 M $AlCl_3$, 1 M NaCl or 1 M $MgCl_2$. However, the bacteriochlorophyll is rapidly bleached by 0.1 M $FeCl_3$.

2. The spectrum of the complex is stable in the pH range 3–12. Below pH 3, at room temperature, the bacteriochlorophyll is converted into bacteriopheophytin at a rate proportional to the fifth power of $[H^+]$. The rate increases with increasing ionic strength. Below pH 1.5 a blue intermediate accumulates prior to the formation of pheophytin. Similar blue intermediates are formed above pH 12 and upon treatment of the complex with sodium lauryl sulfate at pH 8. This suggests a conformational change in the native complex as a common initial step in these transformations.

3. The absorption spectrum of the complex changes relatively little upon addition of methanol, until in 90% methanol bacteriochlorophyll is extracted from the complex.

4. Spectrophotometric study of heat denaturation of the complex is complicated by thermal degradation of the chlorophyll. Thirty sec at 100° causes a solution of the complex to coagulate. Concomitantly, the bacteriochlorophyll absorption band at 809 nm is split into 2 bands at 785 nm and 830 nm.

INTRODUCTION

The biological role of a bacteriochlorophyll-protein complex[1–4] (mol. wt. = $1.5 \cdot 10^5$) isolated from the green bacterium *Chloropseudomonas ethylicum* has been derived from a study of the transfer of the excitation energy in the intact bacterium[5,6]. Light absorbed by chlorobium chlorophyll, the chief pigment constituent in the bacterium, is transferred to the bacteriochlorophyll present in the complex, and the fluorescence emission from bacteriochlorophyll competes with photosynthesis. From this observation, the photosynthetic reaction center (P 840) studied by SYBESMA AND VREDENBERG[7,8] appears to be close to the bacteriochlorophyll–protein *in vivo*. The

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possibility exists that the complex contains the molecule or the group of molecules which performs the primary photochemical reaction. However, the spectrum of the bacteriochlorophyll–protein as obtained after isolation and purification shows no significant absorption at 840 nm (Fig. 1). Moreover, the high solubility of the complex in water and aqueous salt solutions suggests that the protein surface is hydrophilic and that the hydrophobic chlorophyll molecules are probably embedded in the non-polar interior of the protein[1,4]; thus the chlorophyll would be inaccessible for reaction. VERNON's observations that the bacteriochlorophyll–protein is unable to sensitize the photoreduction of methyl red[9] or the photooxidation of reduced phenazine methosulfate[10] support this model.

The present study was undertaken to shed more light on the question of the location of the 20 chromophores in the protein. The absorption spectrum of bacteriochlorophyll–protein is changed by various protein denaturants. Some of the agents chemically react with bacteriochlorophyll. If the bacteriochlorophyll molecules are embedded in the protein and therefore inaccessible to the solvent, a change in the conformation of the protein would be required before the agent could react with bacteriochlorophyll. The kinetics of such a transformation are expected to be different from those of a straightforward chemical reaction of bacteriochlorophyll. Kinetic measurements were undertaken in attempts to determine the mechanisms of the reactions. During these experiments denatured bacteriochlorophyll–proteins were observed as reaction intermediates. It has been suggested that the complex *in vivo* may have an absorption band at 840 nm due to a conformational state which is different from that of the isolated macromolecule in solution[11]. The absorption spectra of the intermediates were determined in a search for an absorption maximum at 840 nm in the altered complex.

MATERIALS AND METHODS

The isolation and purification of the bacteriochlorophyll–protein has been described previously[1,2]. Samples were stored in the dark at −5° as concentrates in 1 M NaCl–0.01 M phosphate (pH 7.8). Solutions were prepared from these stock solutions by dilution.

NaCl, $MgCl_2$, $FeCl_3$, $AlCl_3$, urea and methanol were Baker "analyzed" reagent grade. Sodium lauryl sulfate was U.S.P. reagent grade. Standardized solutions (Bio-Rad) of KOH (CO_2 free) and HCl were diluted to obtain the molarities desired.

Absorption measurements were made in a Cary 14R spectrophotometer with 1-cm path quartz cells. Absorption characteristics of the stable products reported were obtained from a slow scan (5 Å/sec) of the spectra, followed by static measurements at the band maxima. For transient species, spectra were obtained by a fast scan (25 to 50 Å/sec).

For the kinetic experiments, 5 ml of bacteriochlorophyll–protein solution were added to a solution (5 ml or less) of the denaturant and mixed before the volume was made up to 10 ml. The decrease in the absorbance at 809 nm (band maximum of bacteriochlorophyll–protein) was recorded *versus* time. The first measurement (A_t) could be recorded 1.5 to 2.0 min after the mixing of the solutions; A_0 was determined on a control solution without the denaturant. The temperature in the cell compartment of the spectrophotometer was controlled by circulating water through the cell housing

from an outside thermostat. The temperatures of the reacting solutions were brought to that of the thermostat before they were mixed.

For the denaturation studies at 70° to 100°, an oil bath was used. Semi-micro volumetric apparatus was used with an accuracy of ± 2 % in dilution.

RESULTS

Effect of hydrochloric acid

The absorption spectrum of the bacteriochlorophyll–protein complex remains relatively constant in the pH interval 3 to 12, although the absorptivity ratio,

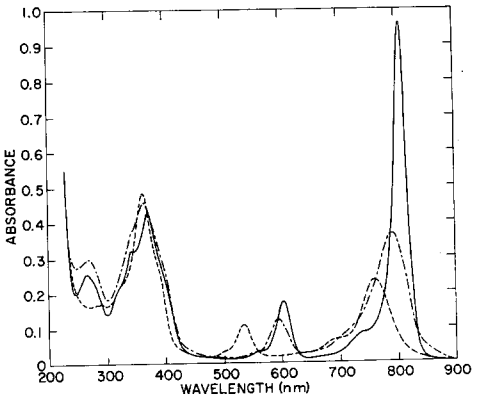


Fig. 1. Absorption spectra of bacteriochlorophyll–protein and its derivatives. ———, bacteriochlorophyll–protein in 10 mM phosphate (pH 7.2); –·—·–, I 790 in 0.2 M NaCl and 10 mM phosphate (pH 5–6); – – – –, bacteriopheophytin–protein in 20 mM HCl. [Bacteriochlorophyll] ≃ 6.2 μM.

TABLE I

SPECTRAL CHARACTERISTICS OF BACTERIOCHLOROPHYLL–PROTEIN AND ITS DERIVATIVES

λ in nm, ε in mM^{-1} cm^{-1} based on bacteriochlorophyll. Limit of error, ± 4 % for ε.

	Red band		*Orange band*		*Soret band*		*Ultraviolet band*	
	λ_1	ε_1	λ_2	ε_2	λ_3	ε_3	λ_4	ε_4
Bacteriochlorophyll–protein (0.01 M Tris, pH 7.8)	809	154	603	28.4	371	67	267	39
I 790, acid (0.02 M NaCl)	792	58	593	20	364	74	268	48
I 780, alkali (0.05 M KOH)	780	56.5	596	19	358	65		
I 780, detergent (0.04 M sodium lauryl sulfate)	780	48	591	21	361	68	270	48
I 770, alkali (0.05 M KOH)	770	58	580	15	355	65		
Bacteriopheophytin–protein, acid (0.02 M HCl)	760	36.4	534.5	18.5	363	78	none	none
Bacteriopheophytin–protein, detergent (0.04 M sodium lauryl sulfate)	756	36.0	534	19	361	84	none	none
Oxidation product (0.1 M $FeCl_3$)	690	12.5						

$\varepsilon_{809\ nm}/\varepsilon_{371\ nm}$, drops approx. 10 % as the pH is lowered from 7.8 to 4.0. Below pH 3, however, the blue-green complex is gradually converted to the pink bacteriopheophytin–protein. The absorption spectra of bacteriochlorophyll–protein and bacteriopheophytin–protein are shown in Fig. 1, and the absorption characteristics are listed in Table I.

The conversion of bacteriochlorophyll–protein into bacteriopheophytin–protein at room temperature was studied in the pH interval 1.5 to 2.1. Fig. 2 shows absorption spectra of the reaction mixture in 14 mM HCl at different stages of the reaction. The absorption spectra of bacteriochlorophyll–protein and bacteriopheophytin–protein intersect at 775, 635, 560, 450, 388, 344, 320, and 290 nm; the spectra of all intermediate reaction mixtures go through these same isosbestic points. This indicates

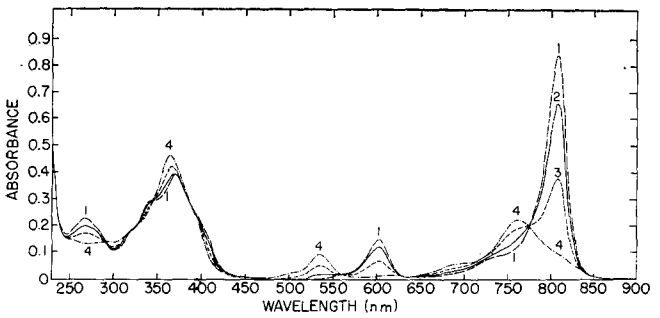


Fig. 2. Pheophytinization of bacteriochlorophyll–protein at 21°. [Bacteriochlorophyll] = 5.5 μM, [HCl] = 14 mM, [NaCl] = 25 mM. Curve 1 shows the absorption spectrum of bacteriochlorophyll–protein in 10 mM phosphate buffer (pH 7.3). Curve 2, 22 min; curve 3, 83 min; and curve 4, 176 min after the reaction in acid was initiated.

that the concentration of any intermediate is negligible compared with [bacteriochlorophyll–protein] and [bacteriopheophytin–protein] during the course of the reaction, and the concentrations of the two species can be readily obtained from the absorbance of the reaction mixture.

The reaction kinetics have been followed in this limited pH range by measuring the change in absorbance at 809 nm with time. In Fig. 3 the data for two acid concentrations have been plotted using the equation for a first order reaction. The solutions are in 25 mM NaCl. A linear relationship between log $(A_0-A_\infty)/(A_t-A_\infty)$ and time is maintained for a considerable part of the reaction, as would be expected for a first order reaction with respect to bacteriochlorophyll–protein. The extrapolated plots have positive intercepts at $t = 0$, instead of going through the origin. This presumably is due to an initial drop in absorbance at 809 nm not connected with the slower first order pheophytinization reaction. The presence of NaCl is important in determining the course of the pheophytinization reaction, as shown in Figs. 3 and 4. When the NaCl concentration is increased to 0.1 M (Fig. 4) the first order plots bend toward the horizontal axis, showing a sensitivity of the initial phase of the reaction to the increase in salt concentration. The reaction rate for the initial phase in 0.1 M NaCl is nearly twice the rate in 25 mM NaCl for the same hydrogen ion concentration (20 mM).

The dependence of the reaction rate on the acid concentration is more revealing. Apparent rate constants k_a at 21° in 25 mM NaCl were determined from linear plots

like those in Fig. 3. As shown in Fig. 5, pk_a is linear with respect to pH, and the slopes of the plots at both 5° and 21° indicate a direct proportionality of k_a to the fifth power of acid concentration. Approximately similar relations are obtained at other salt concentrations. To demonstrate this, there are shown two pk_a values (0.1 M NaCl) obtained from the slopes of the initial phases of the plots in Fig. 4. From the temperature dependence of k_a, the apparent activation energy (Arrhenius equation) is approx. 40 kcal/mole of reacting species.

The rate of the formation of pheophytin from chlorophyll,

chlorophyll + $2H^+$ → pheophytin + Mg^{2+}

could be proportional to either the first or the second power of $[H^+]$, depending on the reaction mechanism. MACKINNEY AND JOSLYN[12] reported a first power dependence

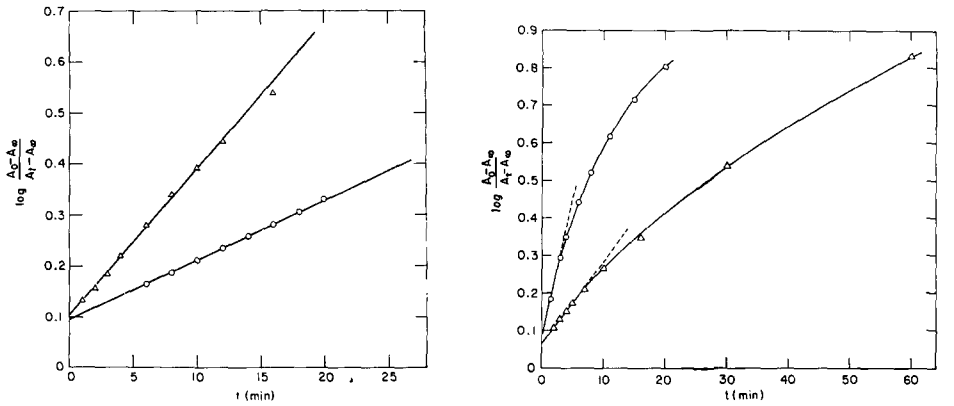


Fig. 3. First order kinetic plots for the conversion of bacteriochlorophyll–protein into bacteriopheophytin–protein at 21°. [NaCl] = 25 mM, [bacteriochlorophyll] = 5.5 μM, [HCl] = 17.5 mM (O—O), or 20 mM (△—△).

Fig. 4. First order kinetic plots for the conversion of bacteriochlorophyll–protein into bacteriopheophytin–protein at 21°. [NaCl] = 0.1 M, [bacteriochlorophyll] = 5.5 μM, [HCl] = 15 mM (△—△), or 20 mM (O—O).

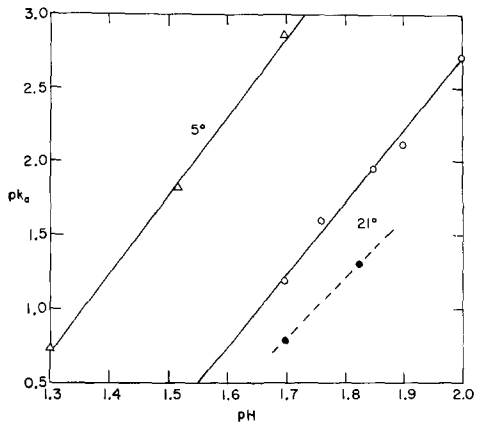


Fig. 5. Dependence on pH, temperature, and ionic strength of the apparent first order rate constant for pheophytinization of bacteriochlorophyll–protein. △—△, [NaCl] = 25 mM, 5°; O—O, [NaCl] = 25 mM, 21°; ●---●, [NaCl] = 0.10 M, 21°. The slopes of the solid lines (5° and 21°) are 5.2 and 4.9 respectively; that of the dashed line (21°) is 4.1.

for chlorophylls *a* and *b* in 90% acetone. On the other hand PAKSHINA AND KRASNOVSKII[13] indicated that in acetone–water mixtures, the rates of pheophytinization of chlorophylls *a* and *b* and their chlorophyllides show $[H^+]$ power dependence between 1 and 2. In the conversion of bacteriochlorophyll–protein to bacteriopheophytin–protein the actual pheophytinization step is proportional to the first power of acid concentration[14]. In protein denaturation, on the other hand, the rate of the reaction limiting step is sometimes proportional to the fourth or fifth power of $[H^+]$ (see ref. 15, p. 218 and ref. 16).

These considerations lead to the following scheme for the pheophytinization of each bacteriochlorophyll in the complex:

$$(\text{bacteriochlorophyll–protein})_{\text{native}} \xrightarrow{k_a} (\text{bacteriochlorophyll})_{\text{denatured}} \xrightarrow{k_b} \text{bacteriopheophytin–protein} \qquad (1)$$

where $k_a \sim [H^+]^5$ and $k_b \sim [H^+]$. The denatured bacteriochlorophyll–protein is presumed to be a reaction intermediate. In the pH range studied here, k_b is much larger than k_a so that the intermediate is not observed in the reaction mixture (see Fig. 2).

A corollary to the above reaction scheme would be the possibility of increasing the steady-state concentration of denatured bacteriochlorophyll–protein at sufficiently low pH, since $k_a/k_b \sim [H^+]^5$. A blue intermediate is indeed observed below pH 1.5. At these low pH values, the reactions are fast, and the following procedure was used to obtain the spectrum of the blue intermediate shown in Fig. 1: bacteriochlorophyll–protein was added to a solution of HCl (final concn. 0.2 M), and after 10 sec the solution was adjusted to approx. pH 5 with 1 M KOH. The absorption spectrum of this solution was determined 5 to 10 min after neutralization with corrections for the small amount of pheophytin formed during the reaction. Even though the blue intermediate so formed tended to coagulate in 15 to 30 min, the uncertainty in the absorption spectrum was estimated to be less than 7%. The absorption characteristics are given in Table I. The intermediate is designated I 790 on the basis of the average wavelength of the far-red maximum. (Minor variations ($\pm$ 3 nm) were observed in different preparations.)

The kinetics of the appearance and disappearance of I 790 in 0.05 M HCl (pH $\simeq$ 1.3) are shown in Fig. 6 along with kinetic curves for the disappearance of native bacteriochlorophyll–protein and the appearance of bacteriopheophytin–protein. Values for [bacteriopheophytin–protein] were obtained from the absorbance at 535 nm, and values for [bacteriochlorophyll–protein] were obtained at 809 nm (*cf.* Fig. 1). The curve for I 790 is the difference between the initial value of [bacteriochlorophyll–protein] and the sum of [bacteriochlorophyll–protein] + [bacteriopheophytin–protein] at any time. Evidence for the existence of a second intermediate has been found from a detailed study of the conversion of I 790 to bacteriopheophytin–protein[14].

Effect of potassium hydroxide

On addition of KOH to a final concentration of 0.5 M, the bacteriochlorophyll–protein is converted within 15 min at 22° to a blue product which is slowly bleached. The course of the reaction, as observed spectrophotometrically, is shown in Fig. 7. The blue intermediate initially formed (I_{alk} 780) has absorption maxima at 780, 596 and 358 nm; it is converted to a second intermediate (I_{alk} 770) in the course of the

next 2 h. (In Fig. 7, only the absorption changes in the 650- to 850-nm region are shown.) The new intermediate has absorption maxima at 770, 580 and 355 nm. The spectroscopic properties of the intermediates are given in Table I. The conversion of I_{alk} 780 to I_{alk} 770 is followed by a much slower reaction in which the bands in the orange and the red region of the spectrum disappear. The absorption spectrum of the straw-colored final solution (48 h) is shown by curve 6 of Fig. 7. Similar transformations are observed at lower KOH concentrations.

The kinetics of the alkaline denaturation of bacteriochlorophyll–protein were studied spectrophotometrically by recording the decrease in absorbance at 809 nm with time. The data were plotted assuming a first order reaction with respect to

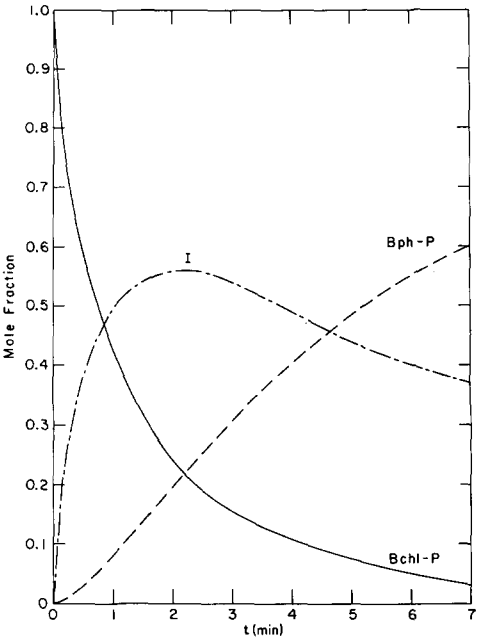


Fig. 6. Time course of the reactions in 50 mM HCl at 25°. ———, indicates the disappearance of native bacteriochlorophyll–protein observed at 809 nm. – – – –, indicates the appearance of bacteriopheophytin–protein observed at 535 nm. –·—·–, indicates the formation and disappearance of the denatured intermediate I 790. (Data of T. R. BROKER.)

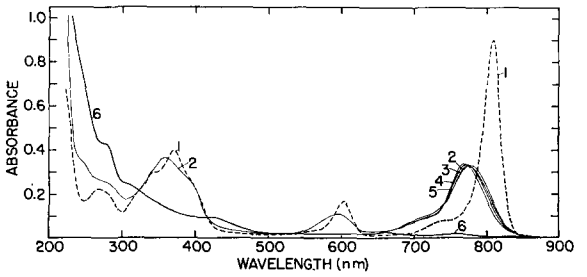
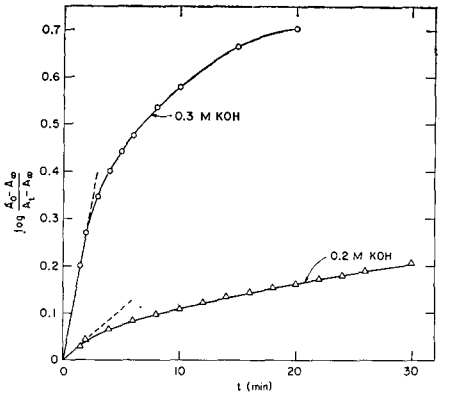


Fig. 7. Alkaline denaturation of bacteriochlorophyll–protein at 22°. [KOH] = 0.5 M, [bacteriochlorophyll] = 6 μM. Curve 1 shows the absorption spectrum of bacteriochlorophyll–protein in 10 mM Tris buffer (pH = 7.8). Curve 2, 15 min; curve 3, 35 min; curve 4, 60 min; curve 5, 120 min; curve 6, 48 h after addition of alkali.



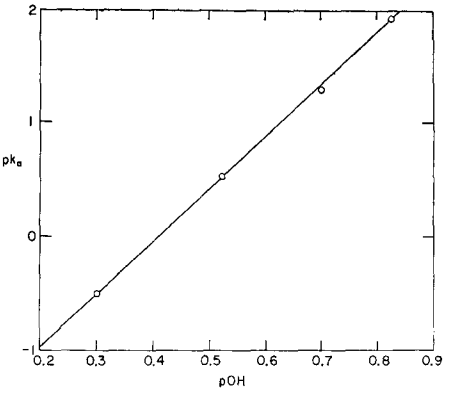


Fig. 8. First order kinetic plots for the alkaline denaturation of bacteriochlorophyll–protein at 22°. [Bacteriochlorophyll] = 5.5 μM; [KOH] = 0.2 M (△—△), or 0.3 M (○—○).

Fig. 9. Changes in the rate of alkaline denaturation of bacteriochlorophyll–protein with the concentration of alkali.

bacteriochlorophyll–protein, and A_∞ was taken as the absorbance of I_{alk} 780 at 809 nm. Two representative plots are shown in Fig. 8. The plots show marked bending towards the horizontal axis, which indicates that the denaturation of bacteriochlorophyll–protein to form I_{alk} 780 may consist of more than one step.

The dependence of the reaction rate on the concentration of alkali is shown in Fig. 9. The apparent first order rate constants k_a were obtained from the slopes of the first order plots (Fig. 8) at the initial stages of the denaturation. In Fig. 9 pk_a has been plotted against pOH; showing a great increase in reaction rate as the alkali concentration is increased. The slope of the curve is 4.7. The initial step in the alkaline denaturation of the bacteriochlorophyll–protein complex is probably proportional to the fifth power of $[OH^-]$.

Effect of sodium lauryl sulfate

The effect of sodium lauryl sulfate on the absorption spectrum of the bacteriochlorophyll–protein complex was studied as part of the search for new conformational states of the complex with different spectroscopic properties. The changes in the absorption spectrum of the complex in buffered 0.04 M sodium lauryl sulfate (Tris 0.01 M, pH 7.2) at 25° are shown in Fig. 10. A slow conversion of the bacteriochlorophyll–protein to a blue product (absorption maxima at 780, 591 and 361 nm), similar to those formed at high and low pH values, takes place. This blue intermediate is eventually pheophytinized, and the solution turns pink after 24 h. As shown in Table I the absorption spectrum of this pheophytinized product is somewhat different from that of the bacteriopheophytin–protein produced in acidic media. This might be due to partial removal of bacteriopheophytin from the protein by detergent. The rates of the two steps are of similar magnitude and considerable pheophytinization occurs before the conversion of bacteriochlorophyll–protein into the blue intermediate is complete. The absorption properties of the blue intermediate and the pheophytin are listed in Table I. A correction for the absorption of the pheophytin was made to obtain the spectroscopic properties of the blue intermediate.

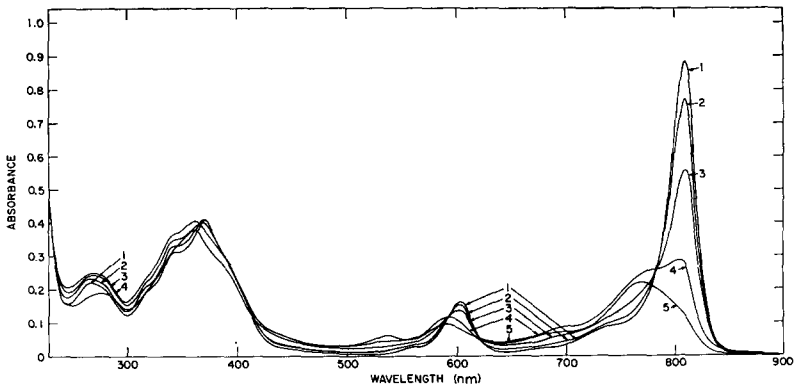


Fig. 10. Denaturation of bacteriochlorophyll–protein by sodium lauryl sulfate at 25°. [Bacteriochlorophyll] = 6 μM, [sodium lauryl sulfate] = 40 mM, [Tris] = 6 mM, pH = 7.95. Curve 1 shows the absorption spectrum of bacteriochlorophyll–protein in 10 mM Tris buffer (pH = 7.8). Curve 2, 1 h; curve 3, 3 h; curve 4, 6 h; curve 5, 7 h after addition of sodium lauryl sulfate.

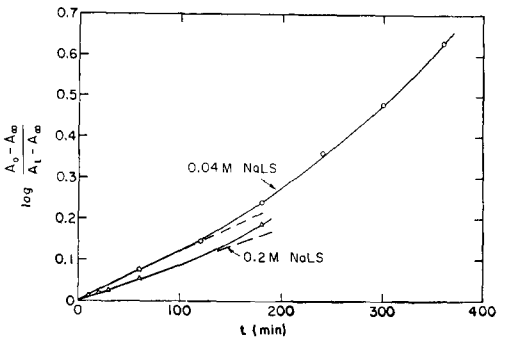


Fig. 11. First order kinetic plots for the denaturation of bacteriochlorophyll–protein in sodium lauryl sulfate (NaLS) at 25°. [Bacteriochlorophyll] = 6 μM, [sodium lauryl sulfate] = 40 mM (O—O), or 0.2 M (△—△).

The kinetics of the transformation of the bacteriochlorophyll–protein into the blue intermediate were followed by the usual method. First order plots of such transformations for two sodium lauryl sulfate concentrations are shown in Fig. 11. The plots are linear up to about 10 % conversion and then bend towards the vertical axis. Also the rate is greater in 0.04 M sodium lauryl sulfate than that in 0.2 M sodium lauryl sulfate. The rate of denaturation in sodium lauryl sulfate appears to be maximal at some value of sodium lauryl sulfate concn. less than 0.2 M, but further experiments are necessary to determine the optimum concentration.

Treatment of kinetic data

For the denaturation of bacteriochlorophyll–protein at extremes of pH and in sodium lauryl sulfate, simple first order plots were used to obtain apparent rate constants for the initial phases of the reactions (see Figs. 4, 8, and 11). The non-linearity of these plots is consistent with the probability that these denaturations involve several sequential reactions[17]. The rate of denaturation should therefore be described by more than one rate constant. We have calculated the apparent rate

constant for the initial phase of each reaction with the assumption that the contribution of the first step to the initial phase would be much greater than that of any subsequent step. Thus Figs. 5 and 9 presumably show the dependence of one initial reaction on denaturant concentration. At extremes of pH the first steps in the denaturation are faster than the later steps, while in sodium lauryl sulfate the later steps are faster than the primary ones.

Effect of Triton X-100

Solutions of bacteriochlorophyll–protein (50 mg/l) in 0.01 M Tris (pH 7.8) were prepared in the presence of the non-ionic detergent Triton X-100 in concentrations ranging from 0.001 % to 5%. No significant effect on the absorption spectrum was evident at any concentration after 24 h at room temperature in contact with this detergent.

Effect of inorganic salts

Both the solubility and the stability of the bacteriochlorophyll–protein complex are increased in the presence of NaCl. As shown in Table II the spectral characteristics are maintained in the presence of 0.75 M NaCl for several days at room temperature, whereas a steady reduction in the ratio $A_{809\ nm}/A_{371\ nm}$ and in the concentration of bacteriochlorophyll–protein in solution occurs at low ionic strength.

TABLE II

EFFECT OF SALT AND METHANOL ON THE ABSORPTION SPECTRUM OF BACTERIOCHLOROPHYLL–PROTEIN

Solvent	*Red band*		
	λ_{max}	$\varepsilon_{red}/\varepsilon_{soret}$	$\varepsilon_{red}/\varepsilon_{orange}$
0.01 M Tris (pH 7.9)*	809	2.0	—
0.01 M Tris (pH 7.9)* + 0.75 M NaCl	809	2.4	—
0.01 M Tris (pH 7.7)**	809.8	2.4	5.4
50% methanol–50% buffer**	811.4	1.0	4.7
70% methanol–30% buffer**	811.3	1.7	4.4
90% methanol–10% buffer**	773	0.7	approx. 2.7

* Spectra recorded at 21–24° after 5 days at 21–24°.
** Spectra recorded at 4–5° after 24 h at 4–5°.

Also the effects of methanol (50 to 70%) shown in Table II can be fully reversed by addition of only 0.1 M NaCl in 0.01 M Tris (pH approx. 8) and removal of the methanol.

Multivalent metallic ions have been known to produce aggregation of globular proteins (ref. 15, p. 23; and ref. 18). The effects of $AlCl_3$ and $FeCl_3$ were studied for a possible aggregation of the bacteriochlorophyll–protein. After 24 h in 0.1 M $AlCl_3$ the spectrum of the bacteriochlorophyll–protein is unchanged. $FeCl_3$ (0.1 M, pH approx. 1.6) on the other hand, rapidly bleaches the bacteriochlorophyll in the complex. The resultant solution has a small absorption band at 690 nm (Table I). This is presumably due to an oxidation of bacteriochlorophyll[19], similar to the reaction studied by RABINOWITCH AND WEISS[20]. No aggregation was observed.

Effect of urea

In 24 h at 4 to 5°, 6 M urea produces no significant change in the absorption spectrum of the complex.

Effect of methanol

Although the bacteriochlorophyll can be extracted quantitatively from the protein by 90% methanol at 40°, the complex appears to be stable in the presence of alcohol if the temperature is sufficiently low. At −44° the native complex is soluble in a mixture of 60% methanol, 30% ethanol, and 10% H_2O (vol. %).

Experiments were carried out at 0 to 5° to determine methanol effects more subtle than complete removal of the chlorophyll from the protein. At 0° the short term effects of increasing methanol concentration on the absorption spectrum of the bacteriochlorophyll (approx. 6 μM) in the complex were studied. In 50% methanol–50% buffer (0.01 M Tris, pH 7.7), the far-red peak shifted about 1 nm to the red, and the ratio of the red peak to the 371-nm peak dropped from 2.3 to 1.9. When the methanol concentration reached 90%, the ratio dropped to 1.4. In 99% methanol, the chlorophyll was rapidly removed from the protein, and the far-red band shifted down to 770 nm. After 24 h in the dark at 4 to 5°, the sample in 50% methanol was essentially unchanged; a sample in 70% methanol was cloudy due to partial precipitation of the complex, and in 90% methanol most of the chlorophyll was extracted from the protein into the methanol–water mixture. The results at 24 h are summarized in Table II.

Effect of temperature

At 40° no change occurs in the absorption spectrum of bacteriochlorophyll–protein (1 mM Tris, pH 7.7) in 24 h. Alteration of the pH produces a small reversible change. At 40° in 0.05 M phthalate buffer (pH 4), the 809-mμ band drops so that the ratio $\varepsilon_{red}/\varepsilon_{orange}$ decreases from 5.4 to 4.7 in 20 h.

More drastic irreversible changes are noticed at still higher temperatures. The changes produced at 72° are shown in Fig. 12. During the first hour a relatively rapid decrease in the absorption of bacteriochlorophyll–protein (1 mM Tris, pH 7.7) at 809 nm takes place, while the blue and the orange bands show lesser changes in intensity with band positions remaining unchanged. A slower transformation is noted during the next few hours; in 5.5 h a diffuse band has replaced the red band, the orange band has shifted to 595 nm, and the solution shows considerable light scattering. After 22 h, the bacteriochlorophyll has been completely bleached, and a straw-

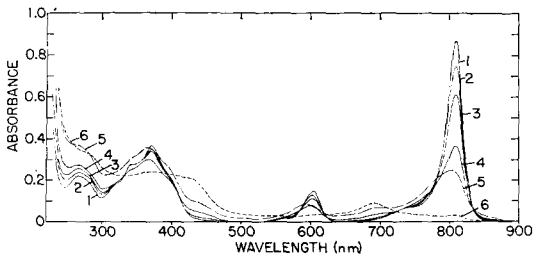


Fig. 12. Heat denaturation of bacteriochlorophyll–protein at 72°. [Bacteriochlorophyll] = 5.7 μM, [Tris] = 1 mM, pH = 7.7. Curve 1, 0 min; curve 2, 15 min; curve 3, 35 min; curve 4, 1 h; curve 5, 5.5 h; curve 6, 22 h.

colored solution is produced with a small absorption band at 690 nm and a broad band in the violet end of the spectrum.

No unique interpretation is possible in view of the complexity of the changes. The bacteriochlorophyll molecule clearly undergoes thermal degradation when heated for a long period of time. Yet, the relatively rapid changes in the spectrum in the first 60 min (accompanied by an increase in the light scattering) may be due to changes in the orientation of the bacteriochlorophyll molecules as a result of thermal denaturation of the protein.

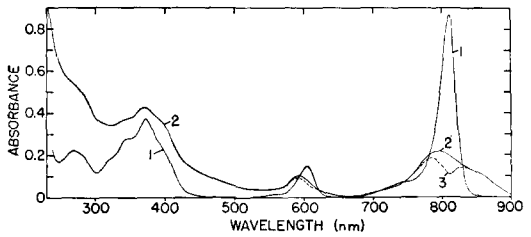


Fig. 13. Heat denaturation of bacteriochlorophyll–protein at 100° (30 sec). [Bacteriochlorophyll] = 6.3 μM, [phosphate] = 10 mM, pH 7.2. Curve 1, spectrum before heating; curve 2, spectrum after heating; curve 3, corrected for 10% unchanged bacteriochlorophyll–protein.

This view is supported by the changes produced in the absorption spectrum of bacteriochlorophyll–protein by heating a solution for 30 sec at 100°. The resulting opalescent mixture has a broad band in the red region of the spectrum in which a shoulder at approx. 840 nm can be observed (Fig. 13). If it is assumed that approx. 10% of the bacteriochlorophyll–protein remained unchanged, two bands at 785 and 830 nm and a band and shoulder at 590 and 620 nm appear in the corrected absorption spectrum. The thermal degradation of the bacteriochlorophyll molecule is less likely here, and the alternative hypothesis of a conformational alteration of the protein to produce new alignments of the chromophores is more attractive. Positive evidence for a conformational change in the protein upon heating is the fact that heat-treated bacteriochlorophyll–protein is readily digested by proteolytic enzymes, while native material is not (J. P. THORNBER, personal communication). The splitting of the 809- and 603-nm bands has been observed previously in a difference spectrum between modified complex and the native complex and may be due to the close spacing of two chlorophyll molecules in proper alignment to form a dimer[1,11]. Further evidence that the appearance of two far-red bands indicates chlorophyll aggregation is given by the absorption spectrum of colloidal bacteriochlorophyll[21].

DISCUSSION

The effects of denaturing agents on the absorption spectrum of bacteriochlorophyll–protein are complex and reveal some unanticipated results. Urea, which is one of the more common agents used for the denaturation of proteins, has no effect on the spectrum. The observed changes fall into different groups according to the degree of alteration. The minor, reversible changes in the absorption spectrum observed at pH 4 and in methanol–water mixtures, where the location of the band maxima remain substantially the same but the ratio $\varepsilon_{red}/\varepsilon_{soret}$ decreases are probably due to a minor

change in the shape of the bacteriochlorophyll–protein complex with slightly altered geometry of the pigment molecules in the protein. The blue intermediates formed with acid, alkali, and detergent are substantially different from the native bacteriochlorophyll–protein complex. Though the bacteriochlorophyll molecules are still chemically unaltered, the blue-shift of the absorption maxima, a considerable decrease in the absorptivities, and the reactivity of the bacteriochlorophyll molecules indicate increased accessibility to the solvent.

A possible explanation for the apparent fifth power dependence of pheophytinization on $[H^+]$ between pH values of 1.3 and 2.0 would be the existence of 5 key groups on the protein responsible for maintaining the tertiary structure of the macromolecule. According to this hypothesis, the tertiary structure remains essentially intact so long as at least one of these key groups remains unprotonated. For simplification we assume that the 5 groups are similar or identical, so that the binding constant K_1 for the first added proton is independent of the binding group. Likewise the binding constants for the other added protons depend only on the order of addition. The denaturation can then be represented in terms of the following series of reaction:

$$\begin{aligned}
&(\text{bacteriochlorophyll–protein}) + H^+ \overset{K_1}{\rightleftharpoons} (\text{bacteriochlorophyll–protein})^+ \\
&(\text{bacteriochlorophyll–protein})^+ + H^+ \overset{K_2}{\rightleftharpoons} (\text{bacteriochlorophyll–protein})^{2+} \qquad (2) \\
&(\text{bacteriochlorophyll–protein})^{4+} + H^+ \overset{K_5}{\rightleftharpoons} (\text{bacteriochlorophyll–protein})^{5+} \xrightarrow{K_6} I_{790}
\end{aligned}$$

The apparent rate constant for the rate-limiting denaturation step would be given by

$$k_a \cong k_6 K_1 K_2 K_3 K_4 K_5 [H^+]^5 \qquad (3)$$

as long as $-pK_i \leqslant pH - 1$.

Changes in the optical rotatory dispersion spectrum during pheophytinization indicate that the interaction between chromophores is lost, but that the secondary structure of the protein is maintained. The ORD spectrum of bacteriochlorophyll–protein has a large trough at 233 nm due to the protein and a large trough near 800 nm due to bacteriochlorophyll[22]. At pH 2.0 the disappearance of the Cotton effect near 800 nm is parallel to the disappearance of the absorption band at 809 nm, while the 233-nm Cotton effect remains essentially unchanged (B. KE, personal communication).

The dependence of the initial phase of the alkaline denaturation on the approximately fifth power of $[OH^-]$ may indicate that the 5 key groups postulated for the pH dependence of acid denaturation are also involved in the initial step of alkaline denaturation. The subsequent course of the reaction at high pH is, however, quite different from the kinetic behavior at low pH (*cf.* Figs. 3 and 8). Recent evidence that the bacteriochlorophyll–protein consists of 4 subunits[2], each containing 5 chlorophylls, suggests that the proposed 5 key groups involved in denaturation by either acid or alkali may also be involved in the binding of the 5 bacteriochlorophyll groups in each subunit.

The chemical reactions of bacteriochlorophyll reported in this paper require a conformation change of the protein prior to any chemical modification of the pigment. This indicates that the bacteriochlorophyll molecules are inside the protein and are inaccessible to the solvent or a chemical reactant. The blue intermediates produced in acid, alkali, or detergent have less compact conformations, and the reagents can

penetrate the protein and react with chlorophyll. From the experiments it appears that none of the 20 bacteriochlorophyll molecules can be located on the surface of the native complex. From the data of PAKSHINA AND KRASNOVSKII[13] for the pheophytinization of bacteriochlorophyll in acetone–water mixtures, the half-time of pheophytinization at 25° can be calculated to be 13 h at pH 3.1. If a few of the bacteriochlorophyll molecules in the bacteriochlorophyll–protein were on the surface of the macromolecule they would have reacted at pH 3.1 in 13 h. We find, however, that at 25° the complex is very stable at pH 3, and at lower pH, once pheophytinization begins it usually goes to completion. This supports the view that all the pigment molecules are located inside the protein.

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