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OLIGOMERS OF BACTERIOCHLOROPHYLL AND BACTERIOPHEOPHYTIN WITH SPECTROSCOPIC PROPERTIES RESEMBLING THOSE FOUND IN PHOTOSYNTHETIC BACTERIA

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Oligomers of bacteriopheophytin (BPh) and bacteriochlorophyll (BChl) were formed in mixed aqueous-organic solvent systems, and in aqueous micelles of the detergent lauryldimethylamine oxide (LDAO). Conditions were found that gave relatively homogeneous samples of oligomers and that allowed quantitative comparisons of the spectroscopic properties of the monomeric and oligomeric pigments. The formation of certain types of oligomers is accompanied by a large bathochromic shift of the long-wavelength (Q_y) absorption band of the BChl or BPh, and by a substantial increase in its dipole strength (hyperchromism). The hyperchromism of the Q_y band occurs at the expense of the Soret band, which loses dipole strength. The Q_x band shifts slightly to shorter wavelengths and also loses dipole strength. The CD spectrum in the near-infra-red (Q_y) region becomes markedly nonconservative. (The net rotational strength in the Q_y region is positive.) This also occurs at the expense of the bands at shorter wavelengths, which gain a net negative rotational strength. The spectroscopic properties of the oligomers resemble those of some of the BChl-protein complexes found in photosynthetic bacteria. The oligomerization of BPh in LDAO micelles is linked to the formation of large, cylindrical micelles that contain on the order of 10^5 LDAO molecules. However, the spectral changes probably occur on the formation of small oligomers of BPh; they begin to be seen when the micelles contain about 10 molecules of BPh. The BPh oligomers formed in LDAO micelles fluoresce at 865 nm, but the fluorescence yield is decreased about 40-fold, relative to that of monomeric BPh. The fluorescence yield is insensitive to the BPh/LDAO molar ratio, suggesting that the oligomers formed under these conditions are predominantly dimers. When the oligomers are excited with a short flash of light, they are converted with a low quantum yield into a metastable form. This transformation probably involves alterations in the geometry of the oligomer, but not full dissociation.

Introduction

Chlorophylls and bacteriochlorophylls serve as the energy collectors and initial electron carriers in plants and photosynthetic bacteria. The pigments generally are bound to proteins as complexes that contain from two to seven molecules of chlorophyll or bacteriochlorophyll [1–3]. The absorption spectra of these complexes differ significantly from the spectra of the monomeric pigments in vitro. The lowest-lying (Q_y) absorption band is shifted to longer wavelengths, and in some cases has a substantially larger dipole strength and rotational strength. The circular dichroism (CD) spectrum in the Q_y region is frequently nonconservative (the

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Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; Chl, chlorophyll; LDAO, lauryldimethylamine oxide; CD, circular dichroism.

positive and negative rotational strengths do not sum to zero). The four Bchl molecules in the photosynthetic reaction centers of *Rhodopseudomonas sphaeroides*, for example, have absorption bands at 800 and 865 nm, compared to 775 nm for BChl in organic solvents. The combined dipole strength of the 800 and 865 nm bands is about 230 debye^2, or 55 debye^2 per BChl, compared to 41 debye^2 for monomeric BChl in vitro; the net rotational strength is approx. 0.8 debye-Bohr magneton, compared to 0.05. The 865 nm band, which probably is due to a special pair of BChls (P-870) [4], has a dipole strength of about 130 debye^2 and a rotational strength of about 2.92 debye-Bohr magnetons. A doubling of the Q_y dipole strength and a nonconservative CD spectrum also are seen in some of the BChl-protein complexes that act to transfer energy to the reaction center [5–7]. It is important to explore the origin of these spectral effects, because they probably reflect the interactions that enable the pigments to initiate light-induced electron transfer in the photosynthetic apparatus.

It has been known for some time that the formation of oligomers of Chl and BChl in vitro can cause a bathochromic shift (a movement to longer wavelengths) of the Q_y transition. In the absence of other ligands, Chl forms oligomers in which the C9 keto group of each molecule appears to bind to the magnesium of an adjacent molecule [8]. The Q_y band in these aggregates is shifted to lower energies by about 280 cm^{-1}. Dimers and higher oligomers also can be formed in the presence of bifunctional ligands such as H_2O [9]. In aprotic organic solvents containing traces of H_2O, Chl forms oligomers in which an H_2O molecule probably bridges the magnesium and C9 keto group of neighboring chlorophylls. Another H_2O molecule may be hydrogen-bonded to the first and to the C10 carbomethoxy group of a third chlorophyll [10]. Repeating structures of this sort are found in crystalline ethylchlorophyllide $\cdot$ $2H_2O$ or Chl $\cdot$ $2H_2O$, in which the energy of the Q_y transition is decreased by about 1400 cm^{-1} [11,12]. Dimers with a variety of structures also have been prepared by covalenty linking two residues of chlorophyllide, pyrochlorophyllide or bacteriochlorophyllide [13–17]. The spectral properties of these dimers have been related to the relative orientations of the two macrocycles [17,18].

BChl in CCl_4 forms a species absorbing at 812 nm that is probably a dimer [19]. Species with larger red-shifts are seen in solid films of BPh [20], and are generated when water is added to a dry film of BChl [21,22]. Suspensions of BChl in water, in acetone-water mixtures, or in micelles of Triton X-100 form heterogeneous aggregates with absorption bands extending from 770 to 920 nm [20,23,24].

The spectral shifts seen in oligomers of Chl and BChl have been ascribed to several factors, including (1) electron redistribution in the individual macrocyclic residues due to Lewis acid-base reactions with adjacent residues or other ligands [25], (2) exciton interactions between degenerate excited states of neighboring chromophores [11,18,19,26], (3) orbital overlap or charge-transfer interactions between neighbors [27,28], and (4) microscopic dielectric effects of the solvent [27]. However, these considerations do not account adequately for the hyperchromism and nonconservative circular dichroism that are observed in the Q_y region [4,29]. Alternative explanations suggested for the red shift of the Q_y transition in vivo have included specific interactions with proteins. These might involve the formation of a protonated Schiff's base of the C9 carbonyl group [30,31], enolization at C9–C10 [32], or coulombic interaction with nearby charged groups [33]. Again, it has not been shown that any of these types of interactions can account completely for the spectra found in vivo. Enolization shifts the Q_y transition to the blue and decreases the dipole strength [32]. The introduction of a positively charged ammonium group on C3a of Chl*a* also decreases the dipole strength slightly [30]. Molecular orbital calculations have shown that two oppositely charged groups in appropriate positions 3.5 Å above a BChl ring could lower the energy of the Q_y transition by 1400 cm^{-1} [33], but the dipole strength would be increased only slightly (Honig, B., personal communication). In addition, specific interactions of this kind seem unlikely to be responsible for the large spectral shifts that are found under a variety of conditions in vitro.

The present paper describes several new procedures for preparing comparatively well-defined oligomers of BChl or BPh. Because the preparations are relatively homogeneous, they allow a

detailed comparison of the spectral properties of monomers and oligomers. The Q_y band in the oligomers exhibits a strong bathochromic shift, hyperchromism, and a rotational strength comparable to that seen in vivo. In the accompanying paper [34] we show that many of these properties can be understood by extending the exciton-interaction theory to include interactions between non-degenerate states, i.e., by considering the mixing of the Q_y transition with higher-energy transitions in the neighboring molecules.

Materials and Methods

Bacteriochlorophyll *a* was purified from *Rhodospirillum rubum* as follows. Lyophilized cells (2–3 g) were ground to a powder and extracted with 10 ml of acetone. The extract was filtered through a Buchner funnel and discarded. The brown-green residue was washed twice with acetone, and reextracted by grinding with 5–6 ml methanol. After filtering, the pale grey solid was discarded and the solution was evaporated under a stream of N_2. The green residue was redissolved in 2 ml acetone, filtered, and chromatographed on a column (1 cm diamter, 5 cm length) of diethylaminoethylsepharose (Pharmacia Fine Chemicals DEAE-sepharose CL-6B) at 5 °C. The column was prepared as described by Omata and Murata [35] for chromatography of chlorophyll. The column was washed with approx. 50 ml acetone to remove yellow materials (carotenoids), and then with 7–8 ml methanol/acetone (1 : 3, v/v) to elute a dark blue solution of BChl. The total time required for the chromatography was 10–12 min. The BChl was checked spectrophotometrically for residual carotenoids and other impurities such as BPh or chlorins, and was rechromatographed if necessary. The methanol/acetone then was evaporated, and the BChl was redissolved in pyridine and stored in the dark at −5 °C. All of the operations described above were carried out in dim light or darkness, and were completed as rapidly as possible to minimize degradation. Spectral grade solvents were used without further purification.

To prepare BPh, 0.5 ml of a 0.1 mM solution of BChl in pyridine was evaporated under N_2. The addition of 50 μl of glacial acetic acid caused the color of the sample to change rapidly from blue to pink, indicating conversion of the BChl to BPh. A fresh sample of BPh was made for each experiment. BPh concentrations were measured spectrophotometrically in acetic acid, using a molar extinction coefficient $\varepsilon_{760} = 5.5 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [36]. BChl was assayed by conversion to BPh.

Micelles of lauryldimethylamine oxide (LDAO) containing BPh were prepared by adding 0.1 M aqueous KCl dropwise to a stirred solution of BPh and LDAO in acetic acid. Except where indicated otherwise, the final solution contained 20% acetic acid in water (v/v) and 0.08 M KCl. The concentrations of BPh and LDAO are indicated in the figures. The final solutions were sonicated for 2 min at 0 °C, using the 3 mm probe of a Bronwill Biosonik 3 sonicator at its maximum rated power. Suspensions of BChl in pyridine-formamide were sonicated similarly.

Absorption spectra were measured with a GCA-McPherson EU-700 spectrophotometer. Turbidities of LDAO micelle suspensions were measured from 360 to 420 nm with the same instrument. To study the concentration dependence of the absorption spectra, we used cuvettes with path lengths varying from 0.1 to 10 cm. A fresh solution was prepared for each measurement, and each measurement was repeated several times. To check for changes in the sample, the absorbance was measured at 760 and 850 nm before and after the spectrum was recorded. Changes in the absorbance were always less than 2%.

Flash-induced absorbance changes were measured as described previously [37]. The 857 nm excitation flashes were obtained from a dye laser (Kodak IR-144 dye) that was pumped by a ruby laser. They were about 30 ns in width and had an energy density of about 70 $mJ \cdot cm^{-2}$. The spectrometer light passed through monochromators before and after the sample; the combined band-pass was about 3.5 nm.

Fluorescence measurements were made as described previously [38]. The excitation light from a Xe flash lamp passed through a monochromator with a 7 nm band-pass. Emission was detected at 90 ° through a monochromator with a 15 nm band-pass.

CD spectra were measured on a Jasco J-500C spectrophotometer at Stanford University with the help of Dr. S. Boxer. Additional spectra were

measured by Dr. D Middendorf in the laboratory of Dr. R.K. Clayton at Cornell University.

Ultracentrifugation of BPh-LDAO micelles was done by Dr. D.C. Teller, using a Beckman Model E analytical ultracentrifuge equipped with absorption optics. Light-scattering autocorrelation spectroscopy was done with the help of Drs. J. Wilcoxin and J.M. Schurr, using methods described previously [39]. The measuring wavelength was 6328 Å. Teflon micropore filters were obtained from Gelman Instrument Co.

Results

Large aggregates of BPh

When distilled H_2O is added dropwise to a stirred solution of BPh in acetic acid or acetonitrile, the 760 nm Q_y absorption band of monomeric BPh is replaced by a new band at 850 nm. The extent of the transformation depends on the final concentrations of both the water and the BPh. Fig. 1a shows the optical absorption spectra of four solutions with the same BPh concentration but with different ratios of H_2O to acetic acid. Fig. 1b shows similar measurements with mixtures of H_2O and acetonitrile. The shift of the Q_y band to longer wavelengths with increasing water concentration is accompanied by a large increase in its dipole strength. The Q_x band (528 nm) experiences a decrease in dipole strength, and shifts to slightly shorter wavelengths. The absorption maximum in the Soret region shifts from 355 to 380 nm, and the total dipole strength in the Soret bands decreases. There are isosbestic points at several wavelengths, indicating that the solutions contain only two spectroscopically distinct species. Table I summarizes the optical properties of the two species.

Solutions of 10^{-6} M BPh in acetic acid/H_2O (1 : 9, v/v), in which the Q_y absorption is almost completely shifted to 850 nm, are stable for periods of hours at room temperature. The absorbance at 850 nm decreases by 1–2% per h. If the solution is centrifuged at $39000 \times g$, or filtered through a teflon filter with pores of 0.2 μm, a pink precipitate separates, leaving a colorless supernatant solution or filtrate. When solutions exhibiting both the 760 and 850 nm absorption bands are centrifuged, the component that absorbs at 850 nm sediments and the 760 nm component remains in solution. The 850 nm form evidently consists of a large aggregate of BPh.

Fig. 2. shows the results of experiments in which the composition of the solvent was held constant at 25, 29, 36, or 40% H_2O in acetic acid, and the

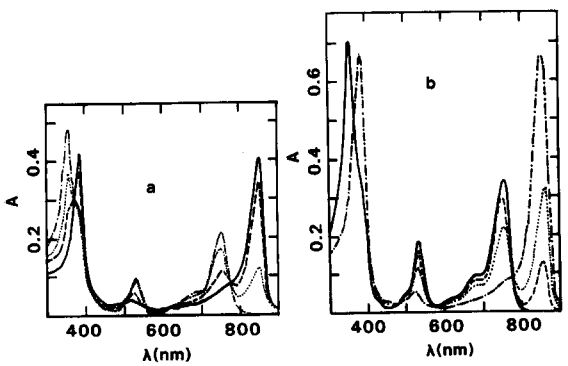

Fig. 1. Absorption spectra of BPh in organic solvents containing various amounts of H_2O. (a) 3.8 μM BPh in acetic acid (–·–·–); 30% H_2O in acetic acid (·····); 35% H_2O in acetic acid (- - - - -); 50% H_2O in acetic acid (———). (b) 5.5 μM BPh in CH_3CN (———); 30% H_2O in CH_3CN (- - - - -); 33% H_2O in CH_3CN (·····); 40% H_2O in CH_3CN (–·–·–).

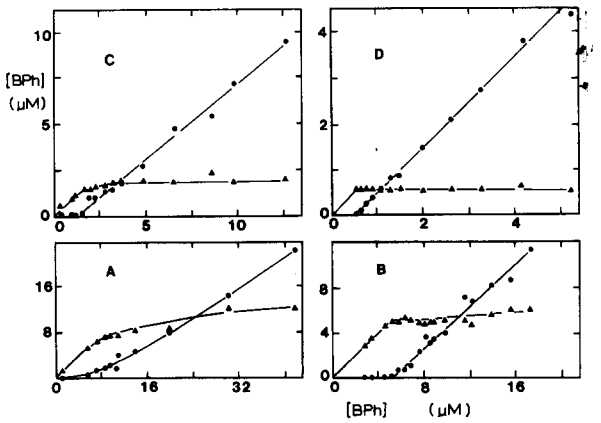

Fig. 2. Concentration dependence of BPh forms in mixed solutions of acetic acid and water. The concentration of BPh in the form of oligomers (●) was calculated from the absorbance at 850 nm using the extinction coefficient $\varepsilon_{850}^{olig} \approx 1.2 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$; the concentration of BPh monomers (▲) was calculated from the absorbance at 760 nm after subtraction of the contribution due to oligomeric BPh at this wavelength ($\varepsilon_{760}^{olig} \approx 1.2 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$, $\varepsilon_{760}^{mon} \approx 4.9 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$). In each experiment the water content was held constant: (A) 25% H_2O; (B) 29% H_2O; (C) 36% H_2O; (D) 40% H_2O. Note that the scales are different in the four panels.

TABLE I

SPECTROSCOPIC PROPERTIES OF MONOMERIC AND OLIGOMERIC BACTERIOCHLOROPHYLL AND BACTERIOPHEOPHYTIN

Compound	Q_y			Q_x			$B_{x,y}$ [c]		
	λ_{max} (nm)	Dipole Strength [a] (debye^2)	Rotational Strength [b] (debye·BM)	λ_{max} (nm)	Dipole Strength [a] (debye^2)	Rotational Strength [b] (debye·BM)	λ_{max} (nm)	Dipole Strength [a] (debye^2)	Rotational Strength [b] (debye·BM)
BPh *a*									
monomer in acetic acid/H_2O	758	39(0.24)	+0.01 [e]	528	13 (0.12)	0.00 [e]	355	117(1.52)	– [d]
oligomer in acetic acid/H_2O	849	74(0.41)	+1.18	525	9.2(0.08)	−0.06	380	105(1.30)	+0.46
	815	– [g]	−0.79				368	– [g]	−0.94
BChl									
monomer in acetone	770	41(0.25)	+0.05 [f]	581	14 (0.11)	−0.05 [f]	358	104(1.35)	– [d]
oligomer in pyridine/H_2O/ formamide	852	65(0.41)	– [d]	592	13 (0.09)	– [d]	390	101(1.21)	– [d]

[a] The dipole strength μ^2 is $9.18\cdot10^{-3}\cdot9n/(n^2+2)^2\cdot\int\varepsilon\nu^{-1}\,d\nu$ debye^2, where n is the refractive index, ν the frequency and ε the molar extinction coefficient (1 debye $=10^{-18}$ esu cm). Numbers in parentheses are oscillator strengths, $1.44\cdot10^{-19}\cdot9n/(n^2+2)^2\cdot\int\varepsilon\,d\nu$. Oscillator and dipole strengths for Q_y include both 0–0 and 0–1 vibrational bands; those for Q_x include only 0–0; see Ref. 34 for discussion of this point. The dipole and oscillator strengths of the oligomers are calculated on the basis of the molarity of monomeric BChl or BPh.

[b] The rotational strength is $0.248\cdot9n/(n^2+2)^2\cdot\int(\varepsilon_l-\varepsilon_r)\nu^{-1}\,d\nu$ debye Bohr-magnetons, where ε_l and ε_r are the molar extinction coefficients for left- and right-handed circularly polarized light. Rotational strengths for oligomeric BPh are calculated on the basis of dimeric BPh; i.e., ε was multiplied by 2.

[c] The B_x and B_y bands are not well resolved. The total dipole, oscillator and rotational strenghts for the Soret region are given.

[d] Not measured.

[e] Middendorf, D., personal communication.

[f] Calculated from Ref. 24.

[g] These bands are seen only in the CD spectrum.

concentration of BPh was changed. The ordinate scales give the concentrations of BPh in the monomeric and aggregated forms, as calculated from the optical densities at 760 and 850 nm. The concentration of monomeric BPh increases linearly with total BPh concentration until the latter reaches a critical value. Above this point, the 760 nm absorbance approaches an asymptote, and the 850 nm absorption associated with aggregates increases linearly. If the H_2O concentration is increased, the threshold for aggregation sharpens and the critical concentration decreases. An abrupt onset of aggregation at a critical concentration of monomers is typical of systems that form micelles or helical polymers.

The extent of the aggregation, and the absorption spectra of the monomeric and aggregated species were not greatly sensitive to the pH. Increasing the pH from 1 to 12 with NaOH accelerated the precipitation of the aggregates but had no significant effect on the absorption spectrum. Decreasing the pH below approx. 1 with HCl converted the BPh reversibly to a species with absorption maxima at 605 and 812 nm. A similar species was formed upon the addition of trifluoroactic acid to a solution of BPh in CCl_4.

BChl aggregates

When a solution of BChl in pyridine is added to stirred H_2O, the 780 nm absorption band is replaced by a broad band near 800 nm. The new spectrum (not shown) is similar to that obtained with the folded form of a covalently linked BChl dimer [14]. Although the Q_y band moves to slightly longer wavelengths on the formation of the aggregate (or on folding of the synthetic dimer), the

dipole strength of the band changes very little. We did not investigate the 800 nm species further.

If BChl in pyridine is added dropwise to stirred formamide to give about 10 μM BChl in pyridine/formamide (1 : 20, v/v), the 780 nm band is replaced by a strong, narrow band at 853 nm. The solution is stable for only a few min, before becoming turbid. A clear solution can be obtained by sonication at 0 °C. If one part of H_2O is then added per three parts of formamide, the BChl is converted almost completely into the form absorbing at 853 nm (Fig. 3a). This solution is stable at room temperature (and in darkness) for periods of hours. Over longer periods of time, a green precipitate forms, and the absorbance at 853 nm decreases relative to that at 780 nm. It thus seems clear that the 853 nm form is an oligomeric form of BChl.

The spectral properties of the 853 nm BChl aggregates are included in Table I. Like the BPh aggregate, the BChl preparation shows a large red-shift and hyperchromism of the Q_y band and compensatory hypochromism in the Soret and Q_x bands. Its spectrum is remarkably similar to that of a light-harvesting BChl-protein complex that has been isolated from *Rhodopseudomonas sphaeroides* [7,26,40] (Fig. 3b). The spectrum also has some of the main features of the spectrum of P-870, the photochemically reactive BChl dimer in bacterial reaction centers.

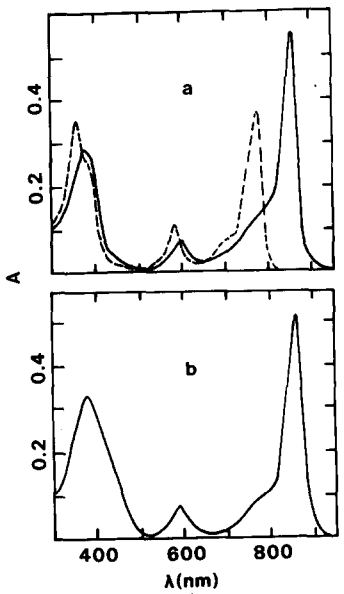

Fig. 3. Absorption spectra of monomeric and oligomeric BChl: (a) 3.9 μM BChl in acetone (- - - - -) and in 24% H_2O, 72% formamide, 3.3% pyridine (———); (b) 4 μM light-harvesting B850 BChl-protein complex from *Rps. sphaeroides* R-26 (replotted from Sauer and Austin [40] and scaled using the 850 nm extinction coefficient given by Clayton and Clayton [7]).

The similarity of the spectral changes that accompany the aggregation of BPh and BChl suggests that the changes result from basically similar interactions. As Krasnovskii et al. [20] have noted previously, the magnesium of BChl evidently is not necessary for these interactions. However, stable oligomers with a strongly shifted Q_y band can be obtained with BPh under a wider range of conditions, perhaps because the magnesium allows BChl to form other types of aggregates. We therefore have focused on aggregates formed from BPh rather than BChl.

BPh oligomers in LDAO micelles

The results presented above show that large aggregates of BChl or BPh can have spectral properties similar to those of the BChl oligomers found in vivo. They do not indicate how large an aggregate needs to be in order to have those properties. To approach this question, we tried to trap small oligomers of BPh in micelles of the detergent LDAO. In acetic acid containing only a small amount of H_2O, LDAO probably is monomeric.

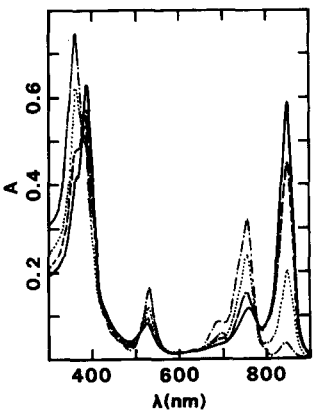

Fig. 4. Absorption spectra of BPh in acetic acid-water solutions containing various concentrations of LDAO. [BPh]/[LDAO] = $0.54 \cdot 10^{-4}$ (- · - · -); [BPh]/[LDAO] = $0.9 \cdot 10^{-4}$ (· · · · · ·); [BPh]/[LDAO] = $1.1 \cdot 10^{-4}$ (- - - - -); [BPh]/[LDAO] = $1.3 \cdot 10^{-4}$ (———). A 4 cm cell was used with 1.67 μM BPh in 80% H_2O/20% acetic acid with 0.08 M KCl for all measurements. The reference cuvettes contained the same concentrations of LDAO, acetic acid and KCl.

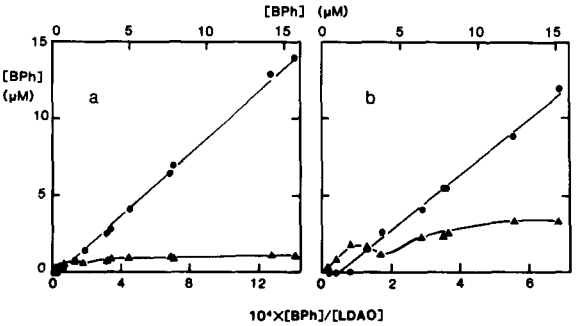

Fig. 5. Formation of BPh oligomers in solutions of H_2O containing 20% acetic acid, 0.08 M KCl, and two different concentrations of LDAO. The concentration of BPh in the form of oligomers (●) is calculated from A850, and that of monomeric BPh (▲) from A760 after filtration through a 0.2 μm filter. Extinction coefficients given in the legend to Fig. 2 were used. The final LDAO concentration was held constant at 11 mM (a) or 22 mM (b) and the BPh concentration varied.

When the amount of H_2O is increased dropwise above about 33%, the formation of micelles is seen by an increase in light scattering. If BPh is included in the acetic acid-LDAO solution, the addition of H_2O causes the Q_y absorption band to shift to 850 nm. The extent of the conversion depends on the BPh/LDAO concentration ratio (Fig. 4). As the molar ratio of BPh to LDAO increases, the absorption spectrum approaches that of the BPh aggregates obtained in the absence of LDAO (Fig. 1). Again, isosbestic points indicate that the solutions contain only two spectroscopically distinct species.

Fig. 5 shows the concentrations of the monomeric and oligomeric forms of BPh as functions of the total BPh concentration. The final composition of the solvent was 80% H_2O/20% acetic acid (v/v) with 0.08 M KCl in all cases. The final LDAO concentration was 11 mM (Fig. 5a) or 22 mM (Fig. 5b). The curves are qualitatively similar to those obtained in the absence of LDAO (Fig. 2), but differ in one important respect. The concentration of the monomeric species reaches an initial maximum or shoulder at approximately the point where oligomers begin to appear. It then drops or levels off, before rising again to an asymptote.

It seems clear that the monomeric form of BPh is associated with LDAO micelles, because its concentration can be considerably higher than the maximum concentration of monomers obtainable with the same mixture of H_2O and acetic acid in the absence of LDAO. The oligomeric species also must be associated with detergent micelles, because the oligomers do not precipitate on standing. In fact, the material absorbing at 850 nm floats on centrifugation, suggesting that it is associated with a micelle whose density is less than that of the solvent. In contrast, the micelles containing monomeric BPh sediment in the ultracentrifuge. Additional evidence that the monomeric and oligomeric forms of BPh are associated with different types of micelles was obtained by filtering the solutions. When BPh-LDAO solutions in 80% H_2O/20% acetic acid containing 0.08 M KCl were filtered through filters of 0.2 μm, essentially all of the 760 nm absorbing material passed through in the filtrate and the 850 nm material was retained on the filters.

LDAO can form several different types of micelles, depending on the conditions [41]. To characterize the two types of micelles formed here, we determined their translational diffusion coefficients by measuring the decay times of the autocorrelation functions of the scattered light intensity. The apparent diffusion coefficient (D) is related to the hydrodynamic radius (R_h) by the expression $D = kT/6\pi\eta R_h$, where k, T and η are the Boltzmann constant, temperature and solution viscosity, respectively [39]. After passage through a filter of 0.2 μm, LDAO solutions in 80% H_2O/20%acetic acid (with 0.08 M KCl, but no BPh) gave an autocorrelation function decay time indicative of $D = (1.0 \pm 0.1) \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1}. Taking $\eta \approx 0.01$ poise this gives $R_h \approx 21$ Å, which agrees with the hydrodynamic radius expected for a spherical micelle of LDAO. Such a sphere would contain 50–55 molecules of LDAO.

A similar value for the size of the micelles containing the monomeric form of BPh was obtained by measuring their sedimentation velocity in solutions containing varying ratios of D_2O to H_2O. The distribution of the micelles in the ultracentrifuge cell was monitored by measuring the absorbance of the BPh at 392 nm. The calculated molecular weight, including the contribution of the bound BPh, was $13\,800 \pm 500$. This is consistent with a micelle containing one molecule of BPh plus 56 molecules of LDAO alone, or 46 molecules of protonated LDAO and an equivalent amount of

bound acetate as a counterion.

The light-scattering properties of the large LDAO micelles were studied in solutions of 33% H_2O/67% acetic acid with 0.04 M KCl. This is approximately the composition of the solvent when the micelles form and the BPh begins to aggregate, during the dropwise addition of H_2O. The micelles formed in the absence of BPh under these conditions had scattering autocorrelation function decay times indicative of $D = 5.7 \times 10^{-9}$ cm$^2 \cdot$ s^{-1} or $R_h \approx 2100$ Å. Although the solutions presumably also contained the smaller spherical micelles, the light scattering evidently was dominated by the large micelles. If, as a first approximation, the large micelle is considered to be a rigid cylinder with a radius of 21 Å, its length can be estimated from D with the aid of expressions given by Tirado and García de la Torre [42]. The calculated length is $5.7 \cdot 10^4$ Å. Such a micelle would contain approx. 10^5 molecules of LDAO. The actual size is probably somewhat larger than this, because flexibility of the micelle would decrease the diffusion coefficient. However, the measurement of the diffusion coefficient is relatively inaccurate for such large micelles.

To estimate the critical micelle concentration for the formation of the large micelles, we measured the turbidity of the solution as a function of the LDAO concentration. These measurements also were made in 33% H_2O/67% acetic acid with 0.04 M KCl and no BPh. The turbidity increased linearly with the LDAO concentration between 8 and 50 mM, with the line extrapolating back to a critical micelle concentration of about 6 mM. The critical micelle concentration for the formation of the small, spherical micelles was lower than this, but could not be determined accurately because of the scattering due to the large micelles.

This information on the sizes and critical micelle concentrations of the two types of micelles allows a qualitative interpretation of the data shown in Fig. 5. In both experiments, the total LDAO concentration was sufficiently high, so that as the H_2O concentration was raised through 33% a substantial fraction of the LDAO would be incorporated into large, cylindrical micelles. The solutions also would contain spherical micelles, which are numerically more abundant but much smaller in size. BPh is incorporated into both types of micelles, but each spherical micelle evidently can hold no more than one molecule of BPh. We attribute the absorbance at 760 nm to the BPh that is isolated in spherical micelles, or in rod-shaped micelles that do not contain enough BPh molecules to support oligomerization. The amount of BPh in the latter pool is expected to go through a maximum at approximately the point when oligomers begin to form. The concentrations of spherical micelles can be estimated from the asymptote of the 760 nm absorbance at high BPh concentration, when each micelle of this type presumably contains 1 BPh, and essentially all of the BPh in the cylindrical micelles is oligomeric. The asymptotes are approx. 1 μM in Fig. 5a and 3 μM in Fig. 5b. Since the LDAO concentrations are 11 and 22 mM, most of the LDAO must be in cylindrical micelles. If the large micelles contain $1 \cdot 10^5$ LDAO molecules, their concentrations are 0.1 μM in Fig. 5a and 0.2 μM in Fig. 5b. Oligomers absorbing at 850 nm begin to form to a significant extent when the BPh concentrations are about 10-times these latter values. If the BPh is distributed between the two types of micelles in proportion to their shares of the LDAO, each cylindrical micelle would contain, on the average, about 10 molecules of BPh. The BPh oligomers are probably smaller than this, but their size cannot be determined reliably because the association constant for oligomerization in the micelles is unknown. On the alternative (and perhaps less plausible) assumption that the BPh is distributed on the basis of the concentrations of the two types of micelles, the large micelles would have an average of only about 1 molecule of BPh.

Gottstein and Scheer [24] recently have concluded that oligomers absorbing at 860 nm can form in micelles of Triton X-100 when each micelle contains only two molecules of BChl. It is likely that the oligomers that form at low concentrations of BPh in LDAO also are predominantly dimers (see below), but the very large size of the cylindrical micelles makes this point difficult to settle.

Fluorescence of BPh oligomers

When solutions containing BPh in LDAO micelles were excited at 392 nm, fluorescence emission bands peaking at 765 and 865 nm were observed. The band at 765 nm evidently is due to

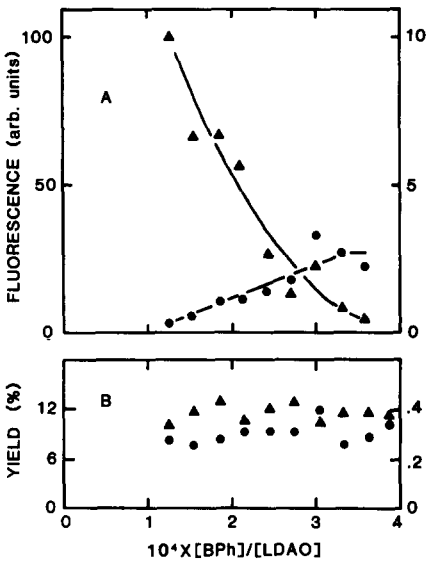

Fig. 6. (A) Fluorescence of BPh oligomers measured at 865 nm (●) and monomers measured at 765 nm (▲). The solutions contained 20% acetic acid, 0.08 M KCl, 4.84 μM BPh and variable LDAO concentration. Excitation was at 392 nm. (B) Fluorescence yields for oligomeric (●) and monomeric (▲) BPh, obtained as described in the text. The left-hand ordinate scales are for monomeric BPh; the right-hand scales, for oligomeric. The mean fluorescence yield of the monomers was, within experimental error, identical with the fluorescence yield of BPh in acetic acid. This yield is taken to be approx. 12%, on the basis of the fluorescence lifetime measured for BPh in organic solvents [36,43].

monomeric BPh, and that at 865 nm to oligomers. If the sample was excited at 530 nm, where the absorbance of monomeric BPh exceeds that of the oligomers (Fig. 4), only the 765 nm emission was detected. With 392 nm excitation, the ratio of the emission intensities at 865 and 765 nm increases with increasing [BPh]/[LDAO] molar ratio up to [BPh]/[LDAO] $\approx 4 \cdot 10^{-4}$ (Fig. 6A).

To calculate the relative fluorescence yield of the oligomeric BPh, we divided the emission intensity at 865 nm by the fraction of the 392 nm excitation light that was absorbed by the oligomers. This was compared with the emission from the BPh monomers in the same LDAO solutions, as measured at 765 nm with 530 nm excitation. Appropriate corrections were made for the relative intensities of the excitation light at 530 and 392 nm. The emission yield of the oligomers was about 1/40 that of the monomeric BPh. The yield was essentially independent of the [BPh]/[LDAO] molar ratio up to $4 \cdot 10^{-4}$ (Fig. 6B), but decreased as the [BPh]/[LDAO] ratio was increased above 10^{-3}. No fluorescence could be detected from the large aggregates of BPh that were formed in the absence of LDAO. The fact that the 865 nm fluorescence yield is relatively constant at small values of [BPh]/[LDAO], therefore supports the view that the BPh oligomers that are present under these conditions are predominantly dimers or other small oligomers of constant size.

A metastable state of oligomeric BPh

There are several possible explanations for the observation that fluorescence from the small oligomers of BPh is strongly quenched relative to that from monomeric BPh. Intermolecular electron transfer could convert the excited oligomer into a radical-pair that decays rapidly to the ground state. Radiationless decay also could occur by direct transitions to excited vibrational levels of ground states with altered nuclear geometries. Evidence for the occurrence of such a process with obtained by measuring changes in the absorption spectrum of the oligomers, following excitation with a short flash. Excitation of the small oligomers of BPh in LDAO micelles ([BPh]/[LDAO] $\approx 5 \cdot 10^{-4}$) with a 30 ns flash at 857 nm converted the oligomers partially into a metastable state. The data points in Fig. 7 show the spectrum of the absorbance changes that accompany this transformation. The most pronounced features are increases of the

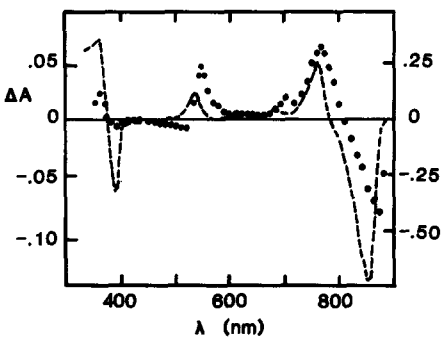

Fig. 7. Absorbance changes measured approx. 1 μs after excitation at 857 nm (ordinate scale on left). The solution contained 20% acetic acid, 13 mM LDAO and 6 μM BPh, which was almost entirely in the form of oligomers. The dashed curve is the expected difference spectrum for complete dissociation of the oligomers (ordinate scale on right).

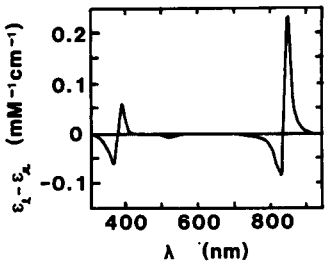

Fig. 8. CD spectrum of oligomeric BPh (measured with 16 μM BPh in 20% acetic acid, 35 mM LDAO). $\Delta\varepsilon$ is expressed on the basis of the molarity of dimers.

absorbance at 775 and 540 nm and a decrease at 870 nm.

The transient absorbance changes decay exponentially with a lifetime of approx. 21 ms at 295 K. The decay is not accelerated by O_2. The slow decay kinetics and insensitivity to O_2 indicate that the metastable state is unlikely to be an excited triplet state, or a photooxidized or reduced form of BPh. In agreement with this conclusion, the difference spectrum does not show the broad absorption band at 400–500 nm that would be expected for triplet BPh [43], nor the band at 425 nm expected for the cationic radical of BPh [44], nor that at 650 nm, where the anionic radical absorbs [45]. The spectral changes are not consistent with full dissociation of the oligomer to monomeric BPh, as has been suggested to occur in the triplet states of chlorophyllide dimers [46–48]. As shown by the dashed curve in Fig. 7, dissociation of the BPh oligomer should cause larger absorbance changes in the Soret bands and in the 830 nm region, relative to the change at 870 nm, and smaller absorbance changes in the 550 and 770 nm regions. The absorbance changes near 520 nm should be opposite in sign. We conclude that the absorbance changes probably reflect the reversible conversion of the oligomer into a ground state with an altered geometry.

The amplitude of the absorbance changes associated with the metastable state increased linearly with the intensity of the excitation flash, up to an incident energy of at least 70 mJ · cm^{-2}. At this point each BPh molecule in the central region of the cuvette could be excited on the order of 20 times. From the fluorescence yield, we estimate the lifetime of the excited singlet state is about 50 ps, which is much less than the width of the excitation flash. Because the lifetime of the metastable state is much longer than the width of the flash, these observations imply that the quantum yield of the state is less than 5%. Judging from the magnitude of absorbance changes near 860 nm, the quantum yield is probably greater than 0.5%. The metastable state evidently does not form efficiently enough to account for the 40-fold quenching of the fluorescence in the oligomers. However, the state that we detect may be only one, particularly long-lived representative of a family of products with altered geometries.

Absorbance changes indicative of the formation of a triplet state [43] were seen when monomeric BPh in micellar LDAO (or in organic solvents) was excited at 694 nm. The triplet state had a lifetime of about 50 μs in the absence of O_2, and decayed much more rapidly in the presence of O_2.

Circular dichroism of BPh oligomers

Fig. 8 shows the CD spectrum of the small BPh oligomers in LDAO micelles. The rotational strengths of the major CD bands are given in Table I. The long-wavelength transition at 850 nm has a large, positive rotational strength. There also is a negative CD band near 815 nm, but the sum of the rotational strengths in the near infrared region is significantly greater than zero. The sum of the rotational strengths in the 300–550 nm region is negative, so that the sum over the entire spectrum is close to zero.

The CD spectra of large BPh aggregates prepared in the absence of LDAO were similar to that shown in Fig. 8, but the relative amplitude of the negative band at 815 nm was variable and tended to increase with time. An additional negative band also developed near 860 nm. These changes correlated with precipitation of the aggregates. It therefore seems likely that the negative bands are due at least in part to light scattering, which has the effect of mixing the optical rotatory dispersion and CD spectra [49]. Light scattering could contribute to the CD spectrum of the small oligomers of BPh also, because the oligomers are formed in large micelles of LDAO.

Discussion

When BChl or BPh are dissolved in certain mixed solvent systems, they form oligomers whose spectroscopic properties resemble those of the BChl-protein complexes found in photosynthetic bacteria. The most obvious spectral change that occurs on formation of the oligomer is a shift of the Q_y absorption band to longer wavelengths by about 90 nm, coupled with an increase in the band's rotational and dipole strengths. The same phenomena can be obtained with BPh in micelles of LDAO under conditions such that most of the micelles probably contain a relatively small number of molecules of BPh. The fluorescence measurments are consistent with the view that the spectroscopic changes require only the formation of dimers. Although the results presented here do not allow us to deduce the structure of the oligomers unambiguously, the availability of comparatively well-defined preparations should be helpful in future efforts toward this goal. However, LDAO clearly is not an ideal detergent for studies of this type, because of the very large size of its micelles. Detergents such as Triton X-100 may be more satisfactory.

It seems significant that very similar spectroscopic features can be obtained with oligomers of BPh as well as with BChl, because ligands attached to the magnesium have played a key role in many of the structures that have been suggested for BChl oligomers previously [25,50]. Such ligands cannot be invoked in the case of BPh. One of the observations suggesting a role for the magnesium is that an infrared absorption band near 1695 cm^{-1}, which is assigned to the C9 keto group, shifts to the 1640 cm^{-1} region when BChl (or Chl) aggregates [21]. There is little or no shift of the corresponding band on the aggregation of BPh [20]. The shift to 1640 cm^{-1} has been attributed to ligation of the C9 carbonyl of one BChl to the magnesium of its neighbor, either directly or via an intervening molecule of H_2O [21]. However, the same shift of the C9 keto band is associated with the formation of an aggregate that absorbs near 800 nm [21]. The shift also occurs in BChl films that absorb at longer wavelengths [21], but it seems not well correlated with the spectroscopic properties of the oligomers.

BChl and BPh also have an infrared band near 1735 cm^{-1}, which is assigned to the C7c and C10a ester carbonyls. This band is unaffected in BChl aggregates that absorb at 800 nm, but splits into two bands (1715 and 1738 cm^{-1}) in BChl aggregates that absorb near 850 nm [21]. The infra red spectra that have been published for BPh aggregates in films that absorb near 850 nm have not had sufficient spectral resolution to show the behavior of the ester band in detail [20]. However, the spectra are consistent with the view that a similar splitting of the ester band occurs in these aggregates also, since a broadening and reduction of the 1735 cm^{-1} band with respect to the 1695 cm^{-1} band is apparent. We suggest, therefore, that oligomers of BPh and BChl that absorb in the 850–860 nm region are structurally similar, and that one or both of the ester carbonyls may play a role in stabilizing their structures. An X-ray structure obtained recently for crystalline methylbacteriopheophorbide places the C10a ester group close to the C9 keto group of a neighboring molecule [51]. In solution, these groups could be linked by H-bonds to a bridging H_2O.

With regard to the oligomers of BChl that occur in vivo, it seems likely that the magnesium participates mainly in linking the BChl to the protein, and not in the interactions of neighboring BChls [52,53]. In the water-soluble BChl-protein complex isolated from *Prosthecochloris aestuarii*, the magnesium atoms are liganded by histidines, by another unidentified amino acid, and by a carbonyl group of the polypeptide chain [52]. Other interactions that could help to stabilize oligomers of either BChl or BPh are hydrophobic interactions between the phytyl chains and π–π interactions between the bacteriochlorin rings. Kratky and Dunitz [12] have pointed out that stacks of overlapping chlorin rings are a common structural feature in crystals of chlorophyll derivatives.

The suspensions of oligomers or micelles that we studied had some turbidity due to light scattering, but the scattering by dilute solutions was weak enough to allow spectroscopic measurements in the near ultra violet region, as well as in the visible and near infrared. It was possible to make quantitative comparisons of the spectra of the monomeric and oligomeric pigments. The comparisons indicate that the increase in the dipole

strength of the Q_y band that occurs in the oligomers is obtained at the expense of the bands at shorter wavelengths. The sum of the oscillator strengths of the B_x, B_y, Q_x and Q_y bands changes very little on aggregation (Table I). The sum of the rotational strengths of the aggregate's CD bands is close to zero, so the overall CD spectrum also is conservative. These observations suggest that the spectroscopic changes result simply from exciton interactions of neighboring pigments. There is no indication that the valence structure of the pigments is modified significantly. It is clear, however, that the spectrum cannot be rationalized by considering only the exciton interactions between the degenerate excited states of the molecules. To account for the transfer of oscillator strength from the Soret and Q_x bands into the Q_y bands, one must consider the interactions between nondegenerate states of the neighboring pigments. In the accompanying paper [34], we discuss the theory of hyperchromism in further detail, and consider its implications for the structure of the oligomers.

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