Optical Properties and Structure of Tetrapyrroles

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Introduction

The bacterial photosynthetic apparatus contains several bacteriochlorophyll (Bchl) proteins of well defined functions and spectroscopic properties (1,2). In all these chromoproteins, the electronic spectra are distinctly different from that of Bchl in solution. The near infrared (Qy) absorption maxima are shifted by up to 230nm ($3500cm^{-1}$) to the red and increased in intensity, the visible (Qx) and/or the near UV (Soret) band(s) are red shifted to a lesser degree and usually decreased in intensity. Even more pronounced changes are observed in the circular dichroism (cd) spectra. They are more complex in the Bchl proteins, and their anisotropies are increased by up to two orders of magnitude as compared to free Bchls.

Similar, although less dramatic changes are known for the chlorophylls of green plants, where they have mainly been attributed to aggregation (see 3 for leading references). In solution, coordinative unsaturation of the central magnesium and the presence of several carbonyl donor groups has been demonstrated as the main driving force for

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Abbrevitations: Bchl *a*gg = bacteriochlorophyll, type (*a* or *b*) and esterfying alkohols (p=Pphytol or gg = geranylgeranol) are given as suffix and subscript, respectively. cd = circular dichroism, nir = near infrared, vis = visible. uv = ultraviolet,

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aggregation from a series of detailed experiments. Similar aggregates have been proposed to be present in chlorophyll proteins. It is not yet clear, however, to which extent the mechanisms for aggregation identified in solution are also relevant for the interactions of chlorophyll in proteins.

Much less work has been carried out with Bchl (4-11) and it has already been pointed out by Katz et al (8), that its interactions are more complex to do the presence of an additional donor, e.g. the 3-acetyl group. Two laboratories have recently taken up the subject and studied the properties of Bchl and related pigments in detergent solution (9-11). The outset of these studies was the observation, that the most commonly used detergent for the isolation of Bchl proteins were capable by themselves to form complexes with Bchl, which had many of the characteristic spectral properties of the former. A more detailed investigation of these complex may be helpful in understanding the spectra of Bchl proteins. It has also a practical aspect, because detergent effects may obscure the interpretation of experiments with Bchl proteins (see e.g. 12).

Gottstein and Scheer (9) reported on three distinct complexes of Bchl in Triton X-100, which were suggested to contain a minimum of one, two and three strongly interacting pigment molecules per micelle, respectively. Scherz and Parson (10) investigated complexes with LDAO which are also believed to contain only a small number of strongly interacting molecules. They developed a theory which related the spectral changes to exciton interaction and hyperchromism (11). One of the most remarkable results of the latter authors was the observation, that the complexes are not only formed by Bchl, but also its Mg-free derivative bacteriopheophytin (Bphe). These and earlier similar observations of Krasnovskii et al. (5) with Bchl films strongly questioned the dominant role of the central Mg for aggregation in the detergent complexes and suggested a different type of interaction. Since the pigment enviroment in a micelle is probably more similar to that in the protein, than is a homogenous solution, these aggregation mechanisms should also be considered in Bchl proteins. Here, we wish to report further results on long-wavelength forms of Bchl, which indicate an important function of the long-chain

terpenoid alcohol in the aggregation of Bchl in micelles.

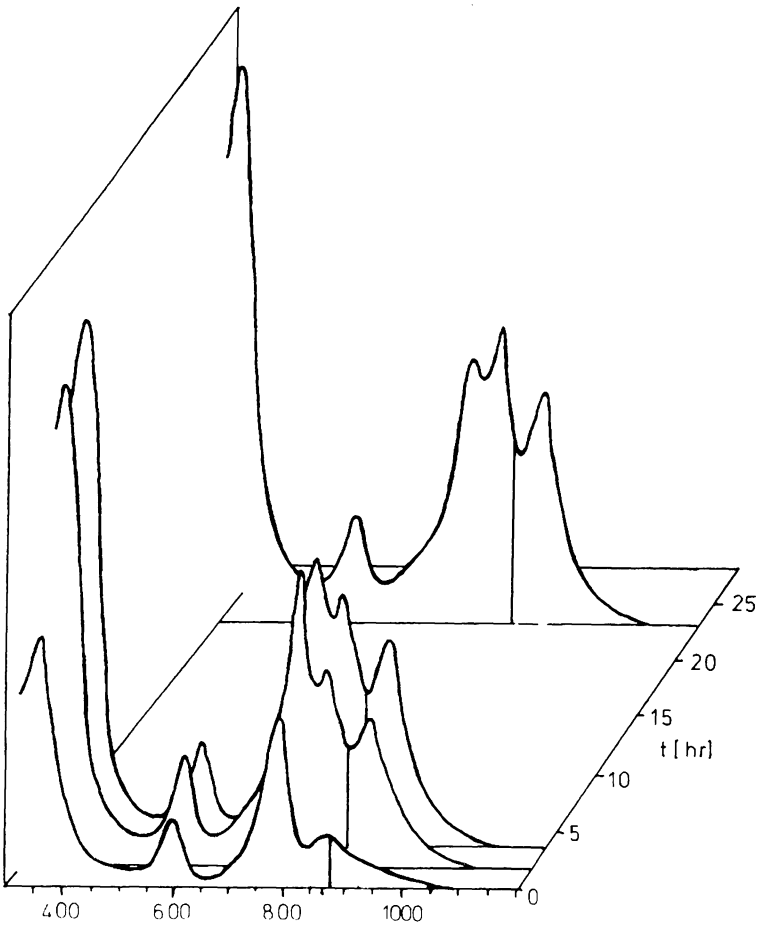
MATERIALS AND METHODS

Bchl esterfied with phytol and geranylgeranol was isolated from Rhodopseudomonas spheroides and Rhodospirillum rubrum, respectively, by the method of Svec (13, modified by dioxan precipitation of the crude extract) and chromatographed on DEAE-cellulose (14). Bphe was prepared by demetalation of Bchl with 6% HCl. Triton X-1oo was obtained from Serva, Heidelberg, and LDAO from Bayrol, München. All solvents were reagent grade. Uv - vis - nir absorption spectra were recorded with a model DMR 22 spectrophotometer (Zeiss, Oberkochen), cd spectra with a Dichrograph V equipped with a red sensitive photomultiplier (ISA - Yvon-Jobin, München). The spectra are not corrected for scattering. Controls in a scattering insensitive photometer gave shifts $\leq$ 1onm.

RESULTS AND DISCUSSION

Absorption Spectra of Triton X-1oo complexes

The Triton X-1oo complexes have been routinely prepared by dissolution of Bchl in a buffer (tris-HCl, 1omM, pH 8.o) containing o.1% (v/v) Triton X-1oo (fig. 1). Like in the previous study (9), the absorptions in the nir are composed of up to four peaks (λmax 775, 83o, 86o and 92o nm) (fig.1). Two of them (83o, 92o nm) are always present in a fairly constant ratio (2:1, corrected for absorption of B86o and B77o). This suggests, that the two absorption bands belong to a single species, and that there are two different long - wavelength absorbing complexes, B86o and B83o/93o, which absorb at 86o nm and 83o/92o nm, respectively. The Bchl/micelle ratio in these complexes has been estimated to approximately two and three, respectively, which agrees with the minimum number of strongly interacting pigment molecules derived from the cd spectra (fig.2). Whereas complexes absorbing around 86o nm are common to all



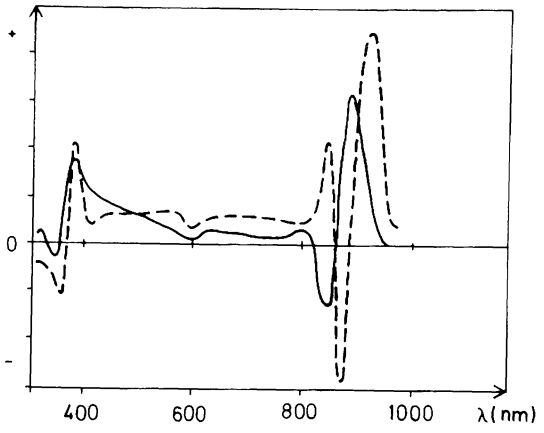


Fig. 1: Absorption spectra of Bchl agg during the dissolution in tris buffer (10 mM, pH 8.0) containing 1% Triton X-100

Fig. 2: Circular dichroism spectra of the sample shown in Fig. 1 at t=0 (——) and 3 hrs (----).

previous studies, the further development is different in different preparations. B830/ 930nm type complexes have been observed also by Komen (4), whereas forms absorbing mainly at 830 or 800 nm without any absorption maxima $\gtrsim$ 860 nm have been reported by others (8, 15). It is not yet clear, if the 830 nm absorption in B830/930 orginates from one of the latter, or is part of a more complex system as suggested by the constant band ratio.

In both complexes, but in particular the 83o/92o nm one, the Soret and the Qx absorption (fig.1) and cd bands are reduced in intensity relative to the nir bands. During the time - course of dissolution, the absorptions around 37o and 59o nm remain rather constant, even though the intensity of the bands $\gtrsim$ 8oo nm increase several fold during this process. Scherz and Parson (1o) have succeded in the preparation of a B85o type complex which is almost free of monomer, which clearly shows this hyperchromic effect without the necessity for curve resolution. This effect is one of the characteristics not only for the long - wavelength absorbing forms in vitro, but also for many Bchl proteins.

Concentration dependence of complex formation with Triton X-1oo.

Concentration effects have been studied varying the pigment/detergent ratios. Increasing pigment concentrations promoted the formation of the B830/930 nm complex, which is agreement with the assignment of a larger aggregation number to this complex. Increasing the detergent concentration, reduced vice versa the amounts of these complexes and promoted monomer formation (λmax $\approx$ 77o nm, fig.3). At Triton concentrations $\gtrsim$ 1%, the red shifted complexes are no longer observed. Complexes formed at o.1% Triton X-1oo are likewise gradually destroyed by the addition of increasing amounts of the detergent. These results can be explained by a random and rapid distribution of the Bchl molecules on the detergent micelles present, and support the idea (9, 11) that each aggregate comprises only a small number of Bchl molecules in a single micelle.

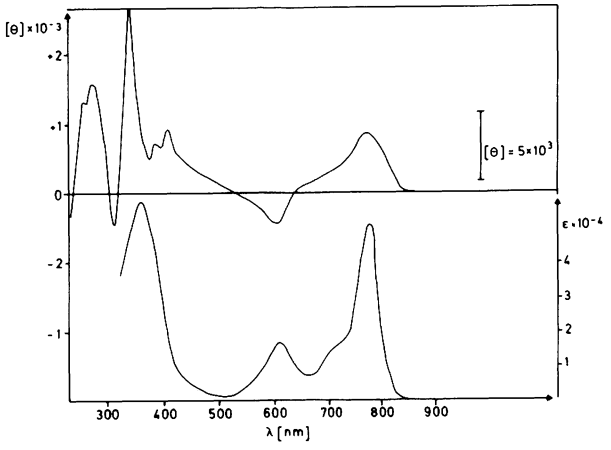


Fig. 3: Absorption (lower trace) and cd spectra of monomeric Bchl a*gg* in methanol, from ref. 9.

Comparison of detergent and aqueous organic solvent induced complexes

As observed first by Komen (4), Bchl complexes with strongly red - shifted absorptions are formed under two different conditions, e.g. in mixtures of organic solvents (methanol, acetone, and others) containing $\gtrsim$ 5o% water, and in solutions with detergents like sodium dodecyl sulfate. Long wavelength absorbing forms of Bchl in mixtures of organic solvents with water have also been obtained by others (5,6,7). We have obtained rather similar absorption and cd spectra for complexes of Bchl with the detergent, Triton X-1oo (figs. 1,2, see also ref.9), and in the mixtures of acetone or methanol with water (figs. 4,5). In the latter systems, the onset of complex formation with λmax $\gtrsim$8oo nm is at water concentrations of 45-5o%, and the absorption spectra are rather similar up to the highest water concentrations studied, e.g. 95%. These results reproduce the findings of Komen (4). It should be noted, that the kinetics of the complex formation depend on the preparation procedure. The results shown in figs. 4,5 have been obtained by injection of a Bchl stock solution in methanol or acetone into appropriate aqueous organic mixture. The complex formation is slowed down (but the same

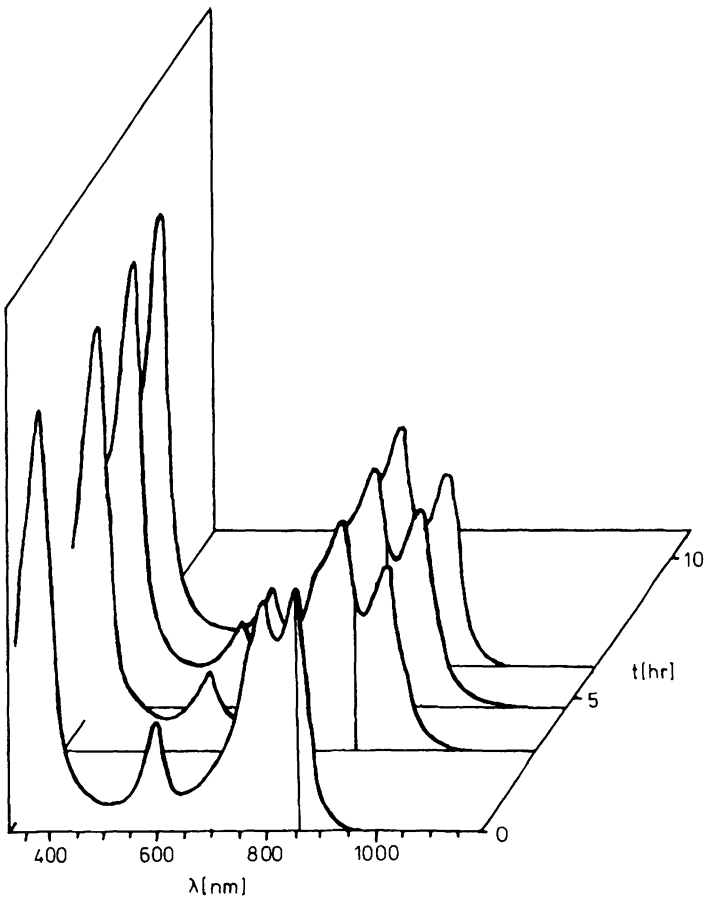


Fig. 4: Absorption spectra of Bchl agg after the dilution of an acetonic solution with water to a final concentration of 70% water.

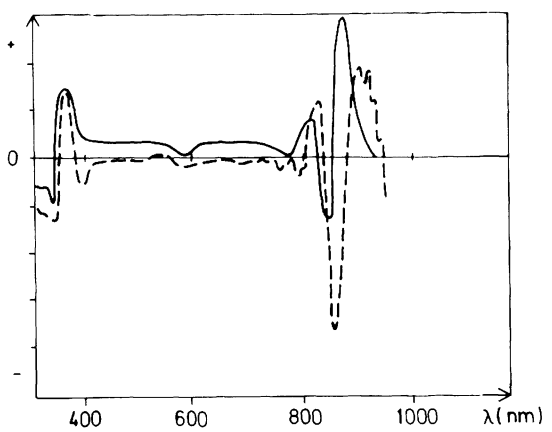


Fig. 5: Circular dichroism spectra of the sample shown in Fig.4 after 0 (——) and 4.25 hrs (----).

spectrum is eventually obtained), if the stock solution is first diluted with the respective pure solvent, and the resulting dilute solution is then further diluted with water.

The absorption and cd spectra of Bchl in these solvent systems (figs. 4, 5), are very similar to the ones observed in detergent micelles (fig. 6). Distinct differences are in the absorption ratios of the two nir bands of the B830/939 complex (4:3 vs. 2;1 in Triton X-100), and a more Gaussian appearance of the bands (less pronounced tails at the red wings, fig. 6). In spite of this similarity, the size of the aggregates seems to be rather different in the two systems. The following observations suggest the presence of large aggregates in the mixed aqueous/organic solvents. Solution of Bchl in the latter solvents are optically clear, but loose there color upon prolonged standing (hrs), due to the formation of a colored precipitate. Centrifugation in a small laboratory centrifuge is already sufficient to pellet all the pigment present, and the original spectrum is restored upon resuspension of the precipitate. The pigments thus seems to be present in dense aggregates of colloidal dimensions.

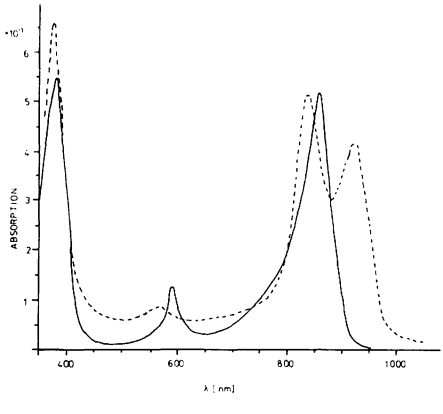


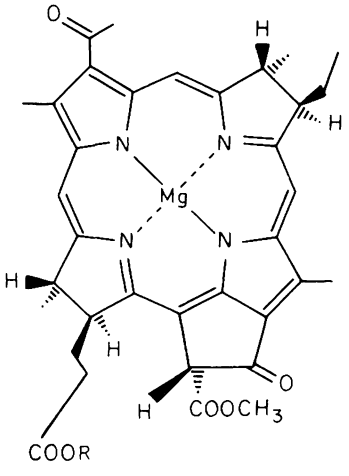
Fig. 6: Resolved spectra of the two long - wavelength absorbing forms, B860 and B830/930 of Bchl agg in aqueous methanol.

Katz et al.(8) and Scherz et al. (10) have earlier observed Bchl forms with similar spectra but rather varying aggregation number. Long-wavelength absorbing species of Bchl are also formed in hydrated films of the pigment (6-8). It then appears, that the spectrum depends mainly on a specific next -neighbor relationship, which is rather similar irrespective of the size of the aggregate.

Structure and complex formation of Bchl´s

The influence of the central Mg has been tested by a series of similar experiments with Bchl agg and Bphe agg. Both form complexes in the mixed aqueous-organic solvent system and with Triton X-100. The Bphe complexes have also strongly red-shifted absorptions (λmax $\approx$850 nm) and show the same hyperchroism of the Qy band, with the Qx band being almost reduced to zero (fig.7). There are noneless distinct spectroscopic differences among the complex of the two pigments. Bphe forms only one type of complex, with an absorption maximum close to 850 nm and a strong S-shaped cd signal in the nir (fig.8). This complex is spectrosopically similar to the B860 complex of Bchl. The bandwidth of the Bphe complexes are, however, considerably narrower than in either form of Bchl complexes (fig.9), and there is essentially no additional absorption in the nir correponding to either monomeric Bphe (λmax $\approx$770 nm) or a B830/930 type complex. Whereas the absorption spectra of the Bphe complexes are similar in different mixtures of acetone with $\gtrsim$ 50% water, the cd spectra are rather different (fig.7,8) and indicate a different arrangement in both forms.

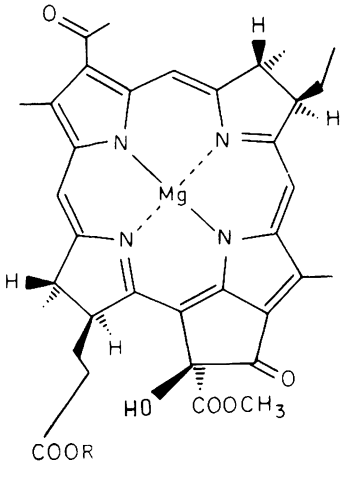
Most of the results are similar to those obtained by Scherz and Parson with Bphe in a solvent system containing LDAO in dilute acetic acid (10, the same authors have recently also obtained rather pure B860 complexes of Bchl, private communication). We assume, therefore, a similar arrangement as has been suggested from the theoretical analysis of these authors (11), with only a slight overlap of the non-parallel macrocycles. Krasnovskii et al. (5) have on the other hand suggested,



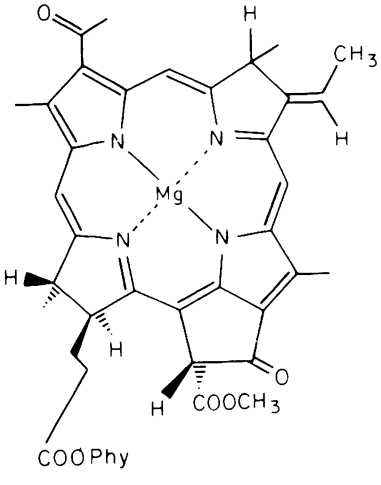
Bchl a_p R =

Bchl a_{gg} R =

Bphe = Bchl without central Mg



10-hydroxy-Bchl a



Bchl b

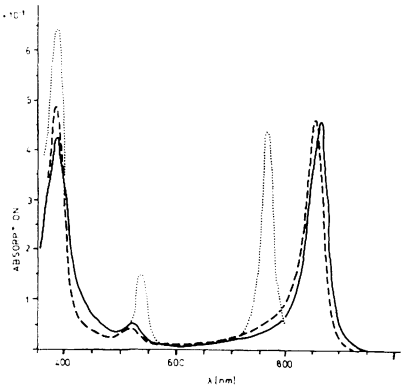


Fig. 7: Absorption spectra of Bphe agg in aqueous acetone (1:1, ——) and (9:1, ----), and in pure acetone (.....).

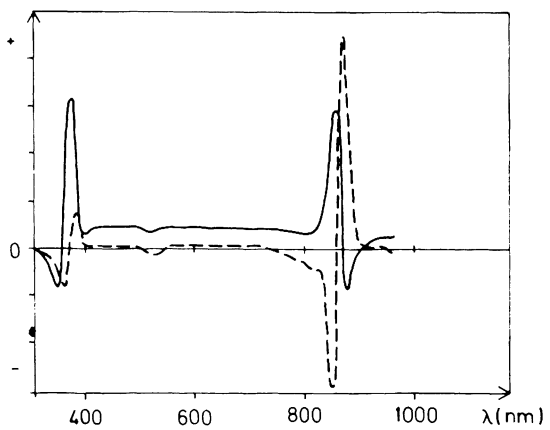


Fig. 8: Circular dichroism spectra of Bphe agg in aqueous methanol (1:1, ——) and (9:1, ----).

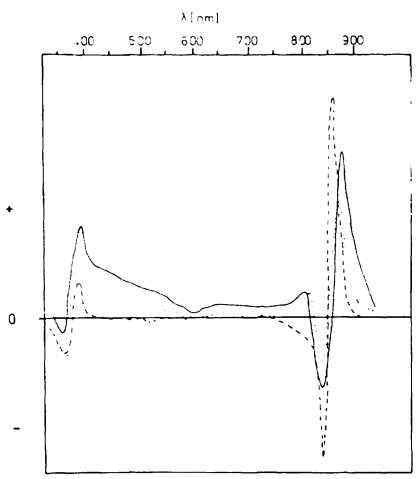


Fig. 9: Comparison of circular dichroism of Bchl agg in tris buffer containing Triton X-100 (0.1%, ——), and in aqueous acetone (8:2, ...), and of Bphe agg in aqueous (95:5, -------).

that π-π interactions are predominant in the aggregation of Bphe, which would rather favor a larger overlap of the two macrocycles. Aggregates of this type are also found in vivo. Angerhofer et al.(16) have identified Bphe species with rather similar spectra in whole bacteria by fluorescence techniques. In particular do they exhibit only a very small Qx band in the excitation spectra. The formation of complexes irrespective of the presence of the central Mg sets them apart from the aggregates investigated in detail by Katz et al., in which the Mg is essential for the coordination with other chlorophylls or bridging ligands. A possible benefit of an aggregation which does not involve the central Mg, is the fact that this would free the latter for coordination e.g. with amino acid side chains in the protein (Scherz, private communication). This situation prevails in the excitation transfer protein from Prostecochloris aestuarii (17).

To test the influence of the esterfying alcohol, a series of experiments was carried with Bchl's carrying different alcohols. Many former results (4,9,10) have been obtained with Bchl isolated from Rs. rubrum, which is esterfied with geranylgeranol (Bchl agg) (18),and no source for the pigment given in the other studies (5 -8). When Bchl ap (esterified with phytol, isolated from Rp. speroides) is used instead, the ability to form complexes is strongly reduced. In mixtures of water with methanol or acetone, the initial absorptions $\geq$ 800 nm are reduced by more than 50%, and they remain at a much lower level throughout the experiments, which involved generally the observation over 24 hrs. In particular is the formation of B830/930 strongly inhibited, and B860 somewhat stabilized. The results are again essentially independent of the solvent (water/methanol or water/acetone) and of the water concentration above a threshold value of 50% up to 95%. The difference between Bchl agg and Bchl ap is even more pronounced in the Triton X-100 micelles. With the pigment esterfied with phytol, we have been unable to prepare any solutions with significant absorptions - 800 nm by the procedure given above, and only 50% complex is formed by the procedure of Rosenbauch and Scherz (15) using aqueous formamide as the solvent.

The long chain terpenoid alcohols are a characteristic structural feature of almost all chlorophylls, but little is known about their function. To our knowledge, the complex formation with Triton X-100 is the first example of a non-enzymatic (19) reaction which is profoundly influence by these alcohols. It is noteworthy in this context, that the high-resolution x-ray structure of a Bchl a -protein shows a rather peculiar arrangement of the alcohol chains (which are phytol in this case), which indicates that it may be involved in the binding of the pigment (17). Scherz and Parson have recently observed a strong cooperativity in the formation of the long-wavelength absorbing forms of Bchl, with a seed formation preceeding the actual aggregation process (private communication). They suggested, that the alcohol could affect the first step of this sequence.

There is as yet only marginal data on the influence of other structural modifications on the complex formation. Bchl b esterfied with different alcohols (20) gives only shoulders on the long-wavelength side of the monomer absorption band. Since part of the pigment is oxidized during the incubation, the significance of this finding is not clear. Preparations containing allomerized Bchl a (= 10-hydroxy-Bchl a) do not form long-wavelength absorbing complexes. This is also a tentative explanation for the degradation of the Bchl complexes upon prolonged standing (see figs. 1.4), because the pigment becomes gradually oxidized. The allomerization is, however, slowed down in the Triton X-100 complexes as compared to Bchl in solution, which can be used to stabilize labile Bchl´s.

Taken together, the results indicate an important influence of the long -chain terpenoid alcohol and the enolizable ß-ketoester system at the isocyclic ring on the formation of long-wavelength absorbing complexes, and only a lesser influence of the central Mg and the substituents at ring 2. Further modifications are necessary to fully understand the role of the different functional groups present in chlorophylls on this type of aggregate formation.

CONCLUSIONS

Bchl agg and its demetalated derivative e.g. Bphe agg form strongly red-shifted and Qy hypochromic complexes in micelles with the detergent, Triton X-100 and in mixed organic-aqueous solvent systems containing $\gtrsim$ 50% water. Both types of complexes are aggregates of the pigments, if judged from the exciton splittings in the cd spectra. According to their similar absorption and cd spectra, they appear to have rather similar next-neighbor relationships, but the aggregation number is small (2-4) in the detergent micelles and probably much larger in the aqueous organic solvents. The aggregation does not require the presence of the central Mg, but is critically affected by the esterfying terpenoid alcohol present in Bchl's, and is inhibited by oxidation at C-10 of the pigments. The complexes formed in detergent micelles may be a useful model to analyze the influence of aggregation geometry on the spectra, and a help towards understanding the spectra of Bchl-proteins.

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Discussion

Woody: How many Bchl molecules are there in the reaction centers from the purple photosynthetic bacteria?

Scheer: There are four Bchl and two Bphe. Two Bchl constitute the "special pair" primary donor, another one serves as the primary acceptor, and the Bphe as the secondary acceptor. Only the spectrum of the special pair and the active pheophytin is known in some detail from several difference spectroscopy methods.