Characterization of Bacteriochlorophyll Interactions in Vitro by Resonance Raman Spectroscopy

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Abstract: The effect of several types of BChl interactions of its resonance Raman (RR) spectrum has been examined. In monomeric BChl species, ligation interactions at the central Mg atom produce significant changes in its spectrum and two structure-sensitive bands are identified, which are analogous to those in other porphyrins. Hydrogen-bonding interactions in monomeric BChl affect only the C=O modes, which are weakly in resonance at the excitation wavelength used here. In aggregated BChl species, large differences are observed in the RR spectra as compared to monomeric BChl for two of the three types of aggregates studied. These are the BChl pyrazine adduct (bifunctional ligand aggregate) and the BChl hydrate (hydrogen-bonded aggregate) absorbing at 845 nm. Both of these aggregates display RR spectra which are distinct from one another as well. The third type of BChl aggregate, that formed through coordination interactions of the acetyl or keto carbonyl group of one molecule with the Mg atom of another (self-aggregate), exhibits a spectrum which differs from monomeric BChl only in relative band intensities; no frequency shifts are observed. Additional results which are discussed include the identification of metal-sensitive and deuteration-sensitive bands. A comparison of the RR spectra of BChl a and its metal-free analogue, BPheo a, shows there are three bands which are sensitive to metalation in the region 1000–1800 cm^{-1}. The fully deuterated spectrum of BChl is substantially different from the fully protonated.

Researchers in the field of photosynthesis have long recognized the need for determining the molecular organization in the photosynthetic membrane in order to understand the processes of energy transfer among the light-harvesting components and photoinduced charge separation in the reaction center. Electronic absorption spectroscopy has been used extensively to study the chlorophylls, which play a major role in both of these processes.[1,2] The popularity of this technique results not only from its experimental simplicity but also because the chlorophylls have intense electronic transitions in the visible region of the electromagnetic spectrum which are significantly perturbed in the intact organism. Large shifts to lower energy are observed in the red transitions of chlorophyll (Chl) in green plants and bacteriochlorphyll (BChl) in photosynthetic bacteria relative to the extracted pigments in organic solvents.[3,4] The shifts are most pronounced in the case of BChl-containing organisms. For example, antenna BChl in *Chromatium vinosum* has an absorption maxima at 890 nm or approximately 120 nm to the red of that for BChl in diethyl ether.

Several theories have evolved concerning the nature of Chl and BChl interactions in vivo which purport to explain the red shift in their absorption maxima. The two most common include (1) noncovalent interactions of the pigment molecules with proteins[4] and (2) aggregation (Chl) or hydration (BChl) of the pigments.[1,2] These interactions are thought to result in a lowering of the electronic transition energy through medium effects, donor–acceptor interactions, exciton interactions, or some combination of the aforementioned. In the case of the first theory, there is strong experimental evidence to support chlorophyll–protein interactions from disk gel analysis of the photosynthetic membrane proteins.[4] In many cases almost 100% of the pigment can be accounted for in the various protein bands if mild detergent treatments are used to dissociate the membranes. Also, X-ray crystallographic analysis of the BChl–protein complex from *Prosthecochloris aestuarii* has shown that the seven bacteriochlorophyll a molecules of this complex are contained within a protein matrix. It appears that functional groups on the protein side chains are coordinated to the Mg atoms of the BChl molecules. With respect to the second theory, in vitro studies of model systems have shown that sizable red shifts in the chlorophyll absorption maxima are achieved only in cases where there are chlorophyll–chlorophyll interactions.[1a] In Chl a, self-aggregation causes shifts on the order of those observed in vivo.[1] In BChl a, various polymeric hydrates in nonpolar solvents exhibit absorption maxima analogous to those observed in bacterial antenna complexes.[1b] Thus far there have been no reports of in vivo or synthetic chlorophyll–protein complexes which clearly contain monomeric chlorophyll and exhibit sizable red shifts in the chlorophyll absorption spectrum.[6] Although such negative evidence is not conclusive, it does suggest that perhaps the absorption properties of the chlorophylls in vivo are a result of both chlorophyll–chlorophyll and chlorophyll–protein interactions.

There are obvious limitations to the use of absorption spectroscopy for the study of chlorophyll interactions. Broad absorption bands do not contain structural information and cannot, for that matter, be correlated with unique chlorophyll associations. Other spectroscopic techniques which have been used to define chlorophyll interactions in solution such as NMR and IR cannot be used in the traditional mode to study molecular interactions in complex biological membranes or large protein molecules. One of the most promising spectroscopic approaches for studying chromophore-containing systems is resonance Raman spectroscopy (RRS), which employs laser excitation frequencies in, or near, resonance with an electronic transition in the chromophore-bearing system. There are at least two main advantages to RR spectroscopy. First, the molecular vibrations of the chromophore are preferentially enhanced over those of its environment when the laser wavelength is in resonance only with the chromophore. Second, the vibrational spectrum of the chromophore which results is simpler than its normal Raman (NR) spectrum because only those modes which are coupled to the electronic transition are resonance enhanced. The RR spectra, therefore, can also be used to determine information about the excited state of the molecule by monitoring vibrational intensities as a function of excitation

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(6) Synthetic Chl a derivative complexes with myoglobin have been reported by two groups, but neither complex exhibits a substantial red shift in the complex relative to that of the isolated chromphore. See the following: (a) Boxer, S. G.; Wright, K. A. *J. Am. Chem. Soc.* **1979**, *101*, 6791–6794. (b) Davis, R. C.; Pearlstein, R. M. *Nature (London)* **1979**, *280*, 413–415.

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wavelength. In many instances the vibrational spectrum of the chromophore is sensitive to its interactions with the environment. Frequency shifts are observed in the bound vs. free chromophore spectrum which can be related to the mode of bonding.

There are numerous examples of the successful use of RR spectroscopy in the study of chromophore interactions in complex biomolecules. Two of the most extensively studied systems are the heme-containing proteins[7] and the visual pigments.[8] In both cases, a comparison of the spectral features observed in the intact complex with those observed in the isolated chromophore has indicated the nature of the bonding interactions present between the chromophore and its protein carrier.

In photosynthetic systems, Lutz and co-workers have studied the RR spectra of chlorophyll–protein complexes from a variety of alga and green plants as well as intact chloroplasts.[9,10] The spectra were compared with those obtained for Chl a in a polar solvent, acetone, and as a dry or hydrated solid. Several conclusions were reached concerning the nature of the pigment interactions in these complexes. From frequency shifts in the ketone C=O group of ring V, it was concluded that there are at least five distinct and universal forms of Chl a in green plants. Because the RR spectra of the protein complexes in the skeletal mode region (1600–400 cm^{-1}) resembled that of monomeric Chl a in acetone at low temperatures more than the self-aggregated solid or hydrate, it was concluded that Chl is bound directly to the protein side chains through its ketone carbonyl, probably through hydrogen-bonding interactions. An analysis of the low-frequency region (400–50 cm^{-1}) indicated that the Chl a was pentacoordinate in the protein complexes and the authors suggested that a protein functional group may provide a fifth ligand to the central Mg atom of Chl a. It was pointed out, however, that the low-frequency region of the protein complexes did not entirely resemble any of the in vitro Chl a preparations.

There have been fewer RR studies on photosynthetic bacterial preparations. In a preliminary report on reaction centers (RC), it was established that selective excitation can produce RR scattering from either bacteriochlorophyll or bacteriopheophytin in these preparations.[11] Furthermore, it was shown that BChl and its electrogenerated cation radical, $BChl^{+\cdot}$, in solution exhibit distinctive RR spectra[12] and subsequent studies on RC preparations showed bands which could be assigned to the cation radical of the special pair BChl donor (P870).[13] In each of the cited studies, laser excitation wavelengths used to excite RR scattering from the strong Soret transitions in BChl and $BChl^{+\cdot}$ were far from resonance. When stimulated Raman scattering from H_2 or D_2 was used as an excitation source in a later investigation,[14] it was possible to excite exactly in resonance with the $BChl^{+\cdot}$ transition near 420 nm. In this case, the spectral differences between BChl neutral and its cation radical were more dramatic, and a marker band unique to the latter species was identified at 1420 cm^{-1}. This result suggests that it should be possible to monitor BChl cation radical formation and decay in RC's during photoexcitation by using time resolved RR spectroscopy.

In the results reported here, the RR spectra of BChl and BPheo, in vitro are described as a function of sample conditions. The objective of this study has been to define spectral features that are characteristic of monomeric, self-aggregated, and hydrated BChl. In the monomeric systems, the RR spectrum of BChl is examined in solvents in which the central Mg atom is five- or six-coordinate and its ketone C=O is either free (unassociated) or hydrogen bonded. The effect of solvents on the RR spectrum of BPheo is also examined. Since BPheo lacks Mg, it does not undergo aggregation and coordination interactions that are characteristic of BChl. Hence, the changes that are observed in the RR spectrum as a function of solvent are due solely to solvent effects. Finally, a comparison of the spectra of protonated with that of fully deuterated BChl is given, and the modes sensitive to deuteration are defined.

Experimental Section

The BChl a used in these studies was purified by chromatography on powdered sucrose according to the published procedure.[15] BPheo a was prepared by the acidification of BChl a, followed by chromatography on powdered sucrose. Both pigments were dried by codistillation from CH_2Cl_2 and heating at 60 °C under vacuum (10^{-5} mm) for at least 30 min.

The solvents were purchased from Burdick and Jackson (distilled in glass) and were dried by repeated vacuum distillation onto activated molecular sieves.[16]

Solutions of dry BChl or BPheo in the various solvents were prepared in a nitrogen-purged glovebox. Aliquots were transferred to 5-mm Pyrex tubing, vacuum degassed by using three freeze–pump–thaw cycles, and sealed under vacuum for the RR experiments.

The BChl a pyrazine complex was prepared by adding a stoichiometric quantity of pyrazine dissolved in hexane to a solution of BChl in hexane.[17] A precipitate formed immediately and was collected by centrifugation. The precipitate was dispersed on the inner walls of a 5-mm Pyrex tube and the residual solvent evaporated under a stream of nitrogen gas. The tube containing the BChl–pyrazine film was sealed under vacuum.

The BChl a hydrate was prepared by the procedure of Ballschmiter and Katz.[18] After being dried, the BChl was dissolved in cyclohexane and a drop of deionized water added to the solution. The solution was then sonicated for approximately 10 min, at which point the hydrate had precipitated. It was collected by centrifugation and prepared for the RR experiments as described above for the pyrazine adduct.

The Raman instrumentation, optics, and data collection procedures used in these experiments were identical with those described previously.[12] A Coherent Radiation Model 52 AR^+ laser was used as an excitation source for all of the spectra.

Electronic absorption spectra were recorded on a Cary 17D spectrometer.

Results and Discussion

Raman excitation spectra of porphyrins have been interpreted in terms of Herzberg–Teller (coupling between excited states), pseudo-Jahn–Teller (coupling between components of the Q (visible) and B (Soret) states), and Franck–Condon mechanisms. The extent to which each of these mechanisms contribute to the observed vibrational intensities is dependent upon the excitation wavelength.[19] Thus, a consideration of the absorption properties of the chromophore under study is important. In the case of the chlorophylls, the electronic transitions overlap considerably and are extremely complex, as shown by the recent theoretical interpretations of the BChl absorption spectrum.[20] Consequently, it is not easy to discuss the RR spectra as a function of a single well-defined electronic state. For the present purposes, reference will be made to the qualitative aspects of the absorption spectra of BChl preparations, solely for the purpose of characterizing the BChl interactions.

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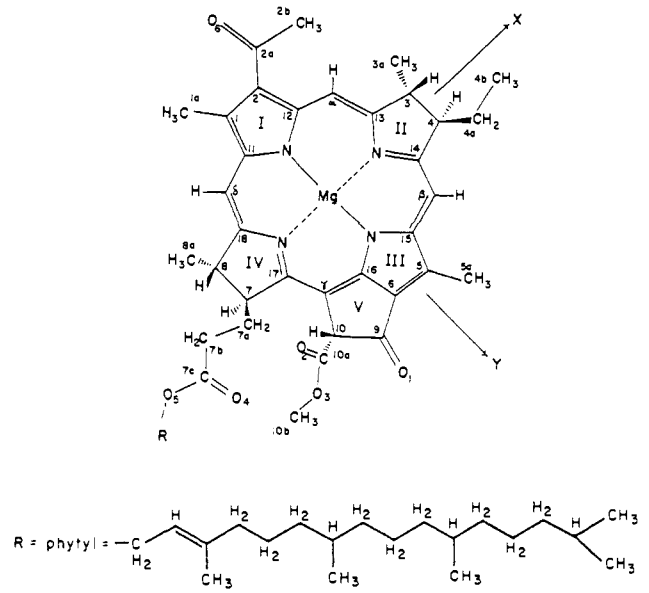

Figure 1. Molecular structure of BChl.

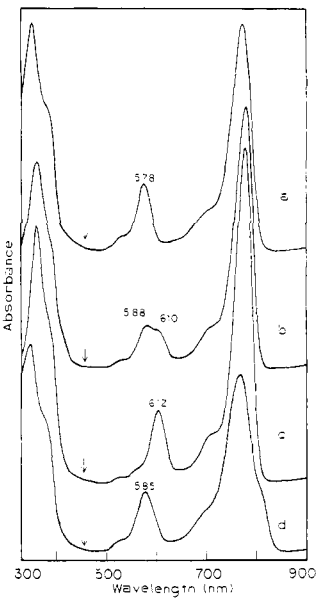

Figure 2. Electronic absorption spectrum of BChl as a function of solvent: (a) CH_2Cl_2 solution with 2 mol of pyridine added/mol of BChl; (b) CH_2Cl_2 solution with 50 mol of pyridine added/mol of BChl; (c) neat pyridine solution; (d) neat CH_2Cl_2 solution. BChl concentration equals 1×10^{-2} M in all solutions. Arrows indicate the position of the laser excitation wavelength used for RR spectroscopy.

The absorption spectrum of BChl a as a function of solvent or its interactions has been studied to some extent,[1b] albeit not as extensively as has been Chl a.[1a,17a] In addition to bulk solvent effects (dielectric constant), other gross changes occur with solvent and these have been related to coordination of axial ligand(s), self-aggregation, and aggregation via hydration interactions. Each of these types of interactions can be understood by examining the molecular structure of BChl as shown in Figure 1. The functional groups which regulate BChl interactions with its environment have been defined as the central Mg atom, the C-9 keto carbonyl, and the C-2 acetyl group, as a result of extensive studies by Katz and co-workers.[1,21] Briefly, the Mg atom apparently requires a coordination number of at least five, or one axial ligand in addition to the four pyrrole nitrogens. In dry, nonpolar solvents, which cannot provide an axial ligand for coordination, the requirement is satisfied through BChl self-aggregation. Either the C-2 acetyl or C-9 ketone group of one molecule coordinates to the Mg atom of another. In polar solvents, either one or two solvent molecules, depending upon the basicity of the solvent, coordinate to the Mg or BChl. In those instances where the axial ligand is bifunctional, or can form hydrogen bonds (e.g., water), colloidal aggregates are produced as a result of crosslinking of BChl molecules through the ligand.

Each of the above types of BChl interactions produces changes not only in its absorption spectrum but also in its IR spectrum. Therefore, it is reasonable to expect that the RR spectrum of BChl might also be sensitive to these interactions. If significant changes are observed as a function of ligation, hydration, and the various types of aggregation, the potential for using RR spectroscopy as a probe of BChl interactions in vivo is considerable. In presenting the results of our investigation of the effect of defined BChl interactions on its RR spectrum, the absorption properties of the BChl species will be presented first, followed by the RR data.

Electronic Absorption Spectrum of BChl as a Function of Its Interactions in Nonaggregating Solvents. BChl dissolved in nonpolar solvents (CH_2Cl_2) in the presence of stoichiometric quantities of a strong Lewis base (pyridine) exhibits an absorption spectrum typical of that displayed in Figure 2a. The band maxima and full width at half-height (fwhh) for this, as well as other spectra to be discussed, are listed in Table I. Under these conditions, BChl is five-coordinate and contains one molecule of pyridine coordinated as an axial ligand to the Mg atom. If additional ligand (pyridine) is added to the solution, several changes occur in the spectrum, as shown in Figure 2b, where the mole ratio of pyridine to BChl is 50:1. The Q_y band increases in intensity, narrows, and shifts to the red. The Q_x band shifts from 578 to 588 nm and develops a shoulder at 610 nm. These changes continue with the addition of more pyridine until the spectrum approaches that observed in neat pyridine (Figure 2c), where it may be noted that Q_x has shifted completely to 612 nm. This shift in Q_x with the addition of pyridine to a CCl_4 solution of BChl was previously observed by Evans and Katz[22] and attributed to the formation of six-coordinate BChl, with two molecules of pyridine functioning as axial ligands to Mg. Although there was no definitive structural data to support this assignment, it is a reasonable assumption in view of the fact that Mg is known to coordinate two ligands in other porphyrins.[23] The RR data to be presented support this interpretation and offer more substantial structural evidence.

Table I. Bacteriochlorophyll Electronic Absorption Bands in Various Solutions[a]

solvent	B_y	B_x[b]	Q_x	Q_y
CH_2Cl_2 + 2:1 (M/M) pyridine-BChl	358	385–390	578	773 (48)
CH_2Cl_2 + 50:1 (M/M) pyridine-BChl	366	385–390	588, 610	777 (40)
pyridine, neat	372	390–395	612	780 (38)
EtOH, absolute	363	~390	605, 580 sh	770 (52)
CH_2Cl_2, dry	358	385–390	585	770, 810 sh
BChl film, dry	362	385	590	790 (100)
BChl-pyrazine (1:1)	368	385	588	845 (40)
BChl hydrate	368	385	588 (br)	845 (90)

[a] Band positions are in nm; sh = shoulder; numbers in parentheses for Q_y are fwhh values. [b] B_x is not resolved from B_y. The position is approximate.

For purposes of comparison, Figure 2d shows the absorption spectrum of BChl in dry CH_2Cl_2 with no external ligand added. On the basis of vapor-phase osmometry experiments,[24] it is believed

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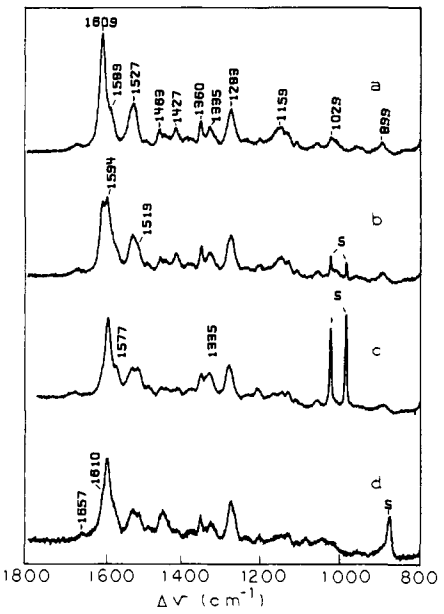

Figure 3. Resonance Raman spectrum of BChl as a function of solvent: (a) CH_2Cl_2 solution with 2 mol of pyridine added/mol of BChl; (b) CH_2Cl_2 solution with 30 mol of pyridine added/mol of BChl; (c) neat pyridine solution; (d) absolute ethanol solution. BChl concentration equals 1×10^{-2} M in all solutions. Laser excitation wavelength equals 457.9 nm; and power equals 40 mW. Scan parameters are as follows: rate = 0.1667 Å/s; counting interval = 1.0 s; slit width = 5 cm^{-1}.

that the average size of the BChl aggregate present in CCl_4, a comparable solvent, at this concentration is a trimer. The most significant difference between the absorption spectrum of the aggregate and that of monomeric, five-coordinate BChl (Figure 2a) is the shoulder on Q_y at approximately 810 nm. This shoulder is absent if the solvent is not dried carefully, indicating that water can function as a nucleophile and dissociate the BChl aggregates.

RR Spectrum of BChl as a Function of Its Interactions in Nonaggregating Solvents. The RR spectra of BChl which are discussed here and in subsequent sections were observed by using the 457.9-nm line of an Ar^+ laser. The relationship of this line with respect to the electronic transitions in BChl is shown in Figure 2. In all spectra, the excitation wavelength is near the foot of the intense Soret bands and in a region of very low absorptivity. There is an advantage to exciting RR scattering in this region, even though it is weaker than that resulting from excitation closer in resonance with an electronic transition. The BChl appears less susceptible to photodamage, which may result from sample heating. Moreover, the spectral features which are observed by using this wavelength are very similar to those resulting from higher energy excitation wavelengths such as 363.8 nm and, therefore, appear to result from coupling with the Soret transitions rather than with the lower energy Q_x transitions. If, on the other hand, lower energy excitation wavelengths are used (e.g., 488.0 and 514.5 nm) the overall intensity of the RR spectrum is considerably reduced, which provides some additional support for coupling of the 457.9-nm line with the Soret transitions. However, a complete understanding of the scattering process in this region would require detailed excitation studies, since interference effects at the longer wavelengths might also reduce the Raman intensities.

The effect of ligation and hydrogen-bonding interactions on the RR spectrum of BChl is shown in Figure 3. Vibrational frequencies for these spectra are listed in Table II. The spectra in parts a–c of Figure 3 correlate with the optical spectra in parts a–c of Figure 2, although significantly higher BChl concentrations were required for the RR experiments than were used in absorption spectroscopy. Only the 800–1800-cm^{-1} region of the RR spectra are shown, since the modes below 800 cm^{-1} are very weak in spectra obtained by using this laser line. The spectrum of five-coordinate BChl (Figure 3a) is characterized by an intense band near 1609 cm^{-1} (designated band A) and two somewhat less intense bands at 1530 cm^{-1} (designated band B) and 1285 cm^{-1},

Table II. Resonance Raman Vibrational Frequencies for Bacteriochlorophyll a in Monomeric Solvents[a]

BChl–pyridine (five-coordinate)	BChl-bis-(pyridine) (six-coordinate)	BChl·EtOH (five and six-coordinate)
1671 w	1675 w	1657 w
1609	1594	1597
1589 sh	1577 sh	1579 sh
1529	1531	1531
	1519	1516
1497 w	1490	1490
1463	1458 w	*b*
1451 w	1446 w	*b*
1427	1417	*b*
1392 w	1392 w, sh	1389 w
1382 w	1382 w	1380 w
1360	1358	1359
1335	1335	1332
1283	1288	1284
1247 w	1252 w	1245 w
1211 w	1216	1215 w
	1175 w	1167 w
1159	1153	1154 w
1142 sh	1139	1139
1117	1120 w	*b*
1064	1065	*b*
1029	*b*	1027 w, sh
1017 sh	*b*	
965	*b*	965
950	*b*	*b*
899	893	*b*
	792	794
	772	

[a] Band positions are within ±3 cm^{-1}. [b] Solvent interference in this region.

as well as numerous other weaker bands. In Figure 3b, a mixture of five- and six-coordinate BChl is present and the effect of an additional ligand on the RR spectrum is obvious. Band A splits into two bands at 1609 and 1594 cm^{-1}, and band B develops a shoulder at 1519 cm^{-1}. In the all six-coordinate form (Figure 3d), the RR spectrum of BChl shows a complete shift in band A to 1594 cm^{-1} and the shoulder on band B is increased in intensity to equal that of band B. Other smaller differences in the RR spectrum of all six vs. all five (Figure 3a) coordinate BChl include an increase in intensity of the 1335-cm^{-1} band and a decrease in intensity for bands in the 1100–1200-cm^{-1} region.

The effect of hydrogen bonding interactions on the RR spectrum of BChl can be seen from a comparison of its spectrum in EtOH (Figure 3d) with that in pyridine (Figure 3c). From both the absorption spectrum (not shown) as well as the RR spectrum, it appears that BChl is predominantly six-coordinate in absolute ethanol. Unlike the case in neat pyridine, there is some five-coordinate present as evidenced by the shoulder near 1610 cm^{-1} on band A. This is reasonable since EtOH is not as strong a ligand as is pyridine. The only significant effect of hydrogen bonding observed on comparing these spectra is a shift in the weak acetyl vibration from 1675 cm^{-1} in pyridine to 1657 cm^{-1} in ethanol. The other functional group capable of hydrogen bonding, the C-9 ketone group, is not observed in the 457.9-nm RR spectrum of BChl but requires excitation closer in resonance with the Soret bands. Spectra observed at 363.8 nm[11,14b] and 396.7 nm[14a] show greater C=O intensities for both the C-2 acetyl and the C-9 keto modes in BChl and its cation radical than are observed in the 457.9-nm spectrum. These results can be rationalized in terms of experimental polarization data as well as a recent theoretical analysis of the Soret region of the BChl absorption spectrum.[20] Excitation at 457.9 nm is closest to the strong *x*-polarized transition (see Figure 1) which would not be expected to produce distortion of the C=O bonds in the excited state. On the other hand, excitation at 363.8 nm is close to a strong y-polarized transition, which appears much more likely to produce distortion along the C=O bond in the excited state. These results are consistent with RR studies of Chlorophyll a as well. Excitation at 441.6 nm is

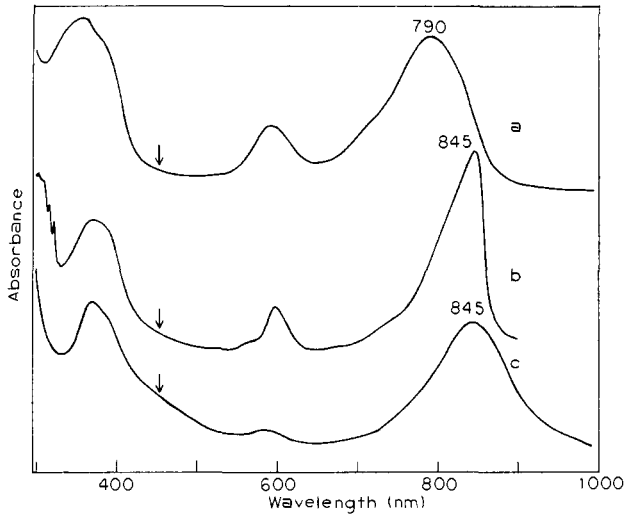

Figure 4. Electronic absorption spectrum of BChl aggregates cast as films on the absorption cell window: (a) anhydrous BChl film; (b) BChl–pyrazine (1:1, M/M) adduct; (c) BChl hydrate. Arrows indicate the position of the laser excitation wavelength used for RR experiments.

close to the *y*-polarized transition in Chl a, and the C-9 carbonyl mode is readily observed.[9,10]

Electronic Absorption Spectrum of BChl as a Function of Aggregation. Changes observed in the electronic absorption spectrum of BChl as a function of aggregation are much greater than those produced by solvent or ligation effects. As noted in the introduction, the shifts in Q_y with aggregation are very large and can equal those observed in antenna or reaction center BChl. The spectra of three different types of aggregates are shown in Figure 4. Figure 4a depicts the spectrum of self-aggregated BChl cast as a film onto the absorption cell window. Note that the Q_y band is shifted to 790 nm and is extremely broad. The fwhh and other peak positions for the aggregates are given in Table I. Figure 4b depicts the spectrum of a BChl–pyrazine adduct (1:1, M/M). In this species pyrazine acts as a bifunctional ligand and cross-links the BChl molecules through its coordination interactions at Mg. These adducts have been studied in Chl a,[17] but not previously in BChl. The Q_y transition experiences a large shift in this aggregate to 845 mm, a value close to that of antenna BChl in vivo. The absorption band is also much narrower than in the spectrum of the self-aggregate. There is some asymmetry at shorter wavelengths, which may be caused by a distribution of aggregate sizes. Figure 4c depicts a spectrum of the third type of aggregate, a BChl hydrate. BChl hydrates result when water simultaneously acts as a nucleophile, with its oxygen coordinated to the Mg of BChl, and a hydrogen-bond donor, with its hydrogen(s) coordinated to one of the acetyl or keto carbonyl groups of a second BChl. These aggregates are quite large and form precipitates in nonpolar solvents.[1b] The value of Q_y varies between 800 and 865 mm according to the procedure used to prepare the hydrate.[1b,18] The spectrum shown in part c of Figure 4 is of a form absorbing at 845 nm. The fwhh of Q_y is very broad, suggesting that a much greater distribution of sizes may be present, or more disorder, than in the pyrazine adduct. Alternatively, stronger exciton interactions in the hydrate could produce an increase in the breadth of Q_y. These two possibilities cannot be distinguished at the present time. In both the pyrazine adduct and the hydrate, light scattering is present due to the large size of particles; this is apparent from the rise in the base line in the blue region of the spectrum.

RR Spectrum of BChl as a Function of Aggregation. RR spectra for each of the three types of BChl aggregates discussed above are shown in Figure 5b–d. Figure 5a is that of five-coordinate, monomeric BChl and is shown for purposes of comparison. The effects of self-aggregation in an anhydrous BChl film, Figure 5b, can readily be seen by comparing this spectrum with Figure 5a. The greatest differences are observed in band A, which is considerably reduced in intensity, and the band near 1147 cm^{-1}, which is increased in intensity in the aggregate. No shifts greater than

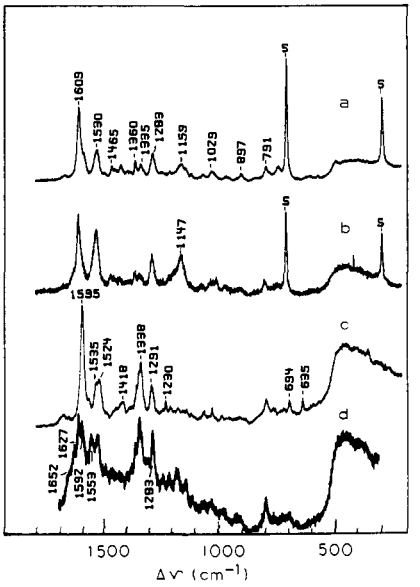

Figure 5. Resonance Raman spectrum of BChl as a function of aggregation: (a) 1×10^{-2} M BChl in CH_2Cl_2 with two mol of pyridine/mol of BChl added (shown for comparison purposes); (b) anhydrous BChl film; (c) BChl–pyrazine adduct (1:1, M/M); (d) BChl hydrate. Instrumental parameters are identical with those in Figure 3.

Table III. Resonance Raman Vibrational Frequencies for Aggregated BChl a[a]

$(BChl)_3$	$(BChl\text{-}pyrazine)_n$	BChl hydrate
	1679	
	1654	1652
		1637
		1627
		1608
1609	1595 (−14)	1592 (−17)
1589 sh	1569 sh	
1531	1535	1553 (+24)
	1524	1525
1427	1418 (−9)	1419 (−8)
		1376
	1365	
1360		1360
1335	1338	1332
1285	1291 (+6)	1283
	765	
	754	
	717	
689	694	
	635	
	589	
	565	
	351	

[a] Only bands which show differences (frequency shift or intensity changes) are listed. Band positions are within ±3 cm^{-1}. Numbers in parentheses are frequency shifts with respect to $(BChl)_3$.

±3 cm^{-1} are observed with aggregation, indicating that the ground electronic state of BChl is not appreciably perturbed by this interaction.

The BChl–pyrazine adduct (Figure 5c) exhibits a quite different RR spectrum from that of the self-aggregate. Frequencies are listed in Table III. There are large intensity increases in band A and a series of overlapping bands near 1338 cm^{-1}. New bands are observed at 1230, 694, 635, and 350 cm^{-1}. Frequency shifts are also observed: band A (−14), 1589 (−22), and 1291 cm^{-1} (+6) (where the numbers in parentheses indicate the direction and magnitude of the shifts). The band at 1147 cm^{-1} in the dry aggregate is decreased considerably in the pyrazine preparation. Finally, band B is split into two peaks of equal intensity at 1535 and 1524 cm^{-1}, indicating the BChl is six-coordinate, as would be expected in this type of aggregate.

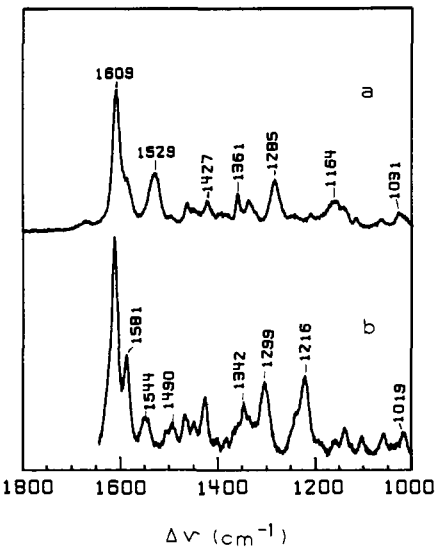

Figure 6. Resonance Raman Spectrum of BChl compared to that of BPheo: (a) BChl in CH_2Cl_2 with two mol or pyridine added/mol of BChl; (b) BPheo in CH_2Cl_2 (4×10^{-2} M). Instrumental parameters are identical with those in Figure 3.

When the RR spectrum of the BChl hydrate is observed, it is immediately obvious that it further differs from either of the other two types of aggregates. First, it may be noted that the quality of spectrum is not as good as that of the others. There is a broad underlying background signal, and the individual bands are not well resolved. Poor resolution may be caused by the diversity of species present in this system, as appeared from the absorption spectrum as well. It is also possible that this preparation is more susceptible to photodegradation, which could cause an increase in the background signal and decrease the overall RR intensities. In addition to decomposition of BChl, two other types of degradation are conceivable—water may be lost from the hydrate or BChl may lose Mg and form BPheo. It does not appear that BPheo is formed, however, since bands characteristic of BPheo (for example, 1216 cm^{-1} as discussed later) are not present.

Changes in the RR spectrum of the BChl hydrate as compared to the dry aggregate include splitting of both bands A and B and a shift in band B from 1531 to 1553 cm^{-1}. These splittings suggest that a portion of the BChl in the hydrate is six-coordinate, or at least that its ground-state geometry is more like that of six- than five-coordinate BChl. Another difference is the increase in intensity of the 1341-cm^{-1} mode, which has a structure similar to that observed in the BChl–pyrazine species. Note, however, that low-frequency modes below 700 cm^{-1} are not intensified as was the case in the pyrazine adduct. Finally, a number of poorly resolved shoulders are seen in the hydrate at 1652, 1637, and 1627 cm^{-1}. These are reminiscent of hydrogen-bonded keto and acetyl C=O vibrations as observed in the IR.[18]

A Comparison of the RR Spectra of BChl and BPheo. Identification of Metal Sensitive bands. BPheo lacks Mg and does not, therefore, undergo the aggregation and ligation interactions described for BChl. Its RR spectrum in a variety of solvents (CH_2Cl_2, pyridine, and EtOH) attest to this fact. No frequency shifts greater than ±3 cm^{-1} are observed with solvent, but only small differences in relative band intensities are noted. Therefore, only one spectrum is shown in Figure 6b, that of BPheo in CH_2Cl_2. The greatest change in the RR spectrum of BPheo is observed on comparing a film spectrum with that of a solution. In films, π–π overlap of the porphyrin macrocycles is maximal, and intensity differences are observed in the mode involving C=C stretching vibrations of the methine bridges (1607 cm^{-1}).

Identification of metal sensitive vibrations in BChl can be made by comparing its spectrum in Figure 6a with that of BPheo in Figure 6b. The vibrational frequencies are listed in Table IV. Only the band near 1216 cm^{-1} appears to be unique to BPheo (NH in-plane bending mode). This vibration, then, provides a means for distinguishing the spectra of the two closely related pigments.

Table IV. Resonance Raman Vibrational Frequencies in BChl a and BPheo a[a]

BChl	BPheo	BChl	BPheo
1609	1607	1285	1299 (+14)
1589 sh	1581 (−8)	1243	1240 sh
1529	1544 (+15)		1216
1497	1501 sh	1164	1153 (−11)
	1490	1142	1132 (−10)
1464	1461	1117	
1448 sh	1444		1099
1427	1427	1065	1054 (−11)
1361		1031	1019 (−12)
1337	1342 (+5)		

[a] Only bands which show differences (frequency shifts or intensity changes) are listed. Band positions are within ±3 cm^{-1}.

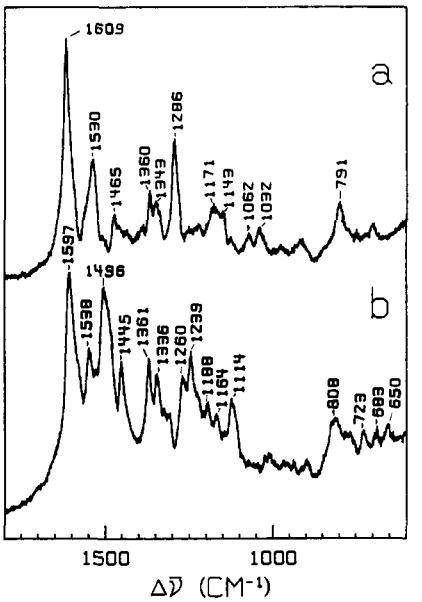

Figure 7. Resonance Raman spectrum of protonated BChl vs. that of fully deuterated BChl: (a) protonated BChl film; (b) deuterated BChl film. Both samples were dried according to the procedure given in the Experimental Section. Instrumental parameters are identical with those in Figure 3.

In addition, the following band shifts are observed in BPheo: 1581 (−7), 1544 (+15), and 1297 (+16) (where the numbers in parentheses indicate the magnitude (cm^{-1}) and direction of the shifts with respect to BChl.

The Effect of Deuteration on the RR Spectrum of BChl. An examination of the molecular structure of BChl indicates that the D_{4h} symmetry of the parent metalated porphin macrocycle is broken by its peripheral substituents, including ring V, and the reduction of the C_b–C_b double bonds of rings II and IV. If the peripheral substituents are ignored, the highest possible symmetry in BChl is D_{2h}. However, this space group possesses a center of symmetry, and, therefore, the IR and RR spectrum of BChl should have no common frequencies. This is not the case,[25] indicating the symmetry of BChl must be much lower than D_{2h}. Depolarization ratios for the various modes are in agreement with this interpretation; i.e. all values are less than 0.75—no B-type modes are observed. In spite of the fact that the symmetry of BChl is quite low, there are a number of correlations which can be made between Cu porphin, Cu chlorin, and BChl. Solovyov and coworkers[26] have noted that the reduction of Cu porphyrin symmetry by peripheral substituents and hydrogenation of ring IV to produce a Cu chlorin does not markedly affect the vibrational frequencies. Nor did a change in the central metal atom markedly affect frequencies above 800 cm^{-1}. Therefore, a determination of the

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deuteration sensitive vibrations in BChl would be helpful in assigning its vibrations to a first approximation.

The effect of deuteration on the RR spectrum of BChl may be seen by comparing the spectrum in Figure 7a (fully protonated) with that in Figure 7b (fully deuterated). Both spectra were obtained on dry films. As may be seen, large differences exist between the two spectra and it is difficult in some cases to correlate the bands. Caution must be exercised as discussed in the case of Cu porphin where deuteration was found to produce either a decrease in a particular vibrational frequency (due to the pure isotope effect) or an increase in its value (because of a change in composition or form of the normal mode). With this caveat in mind, bands most sensitive to deuteration are tentatively identified as follows: 1609 (-12), 1589 (-51), 1286 (-26 or -47) (where the number given is the value (cm^{-1}) for the fully protonated form and the number in parentheses is the direction and magnitude of the shift on deuteration). A more detailed analysis of the effects of deuteration is underway.

Conclusions

The resonance Raman results presented here indicate that it is possible to distinguish certain types of BChl interactions through changes in its spectrum. In monomeric BChl systems, the most easily distinguished are coordination interactions. Two structure sensitive bands have been identified, band A (1609 cm^{-1}) and band B (1529 cm^{-1}). (A shoulder on band A near 1589 cm^{-1} is also sensitive to coordination, but it is not well resolved.) The formation of six-coordinate BChl, through the interaction of two ligands at the central Mg atom, causes band A to shift 15-cm^{-1} to lower frequencies and band B to split into two bands of equal intensity (1530 and 1519 cm^{-1}). These changes are consistent with the core expansion correlation[27] which has been invoked to explain similar shifts in structure sensitive bands in a variety of porphyrins. In BChl the movement of its Mg atom into the plane of the macrocycle with an increase in coordination number should lead to an expansion of the porphyrin core. This expansion, in turn, results in an increase in bond length and a decrease in vibrational frequencies for those modes which are most affected (i.e., the methine C=C and pyrrole C=N stretching modes). In aggregated BChl systems, it has been observed that substantial differences are present in the RR spectra with respect to monomeric BChl, as well as between two of the three types of aggregates studied. In the pyrazine aggregate, formed through bifunctional coordination of pyrazine to Mg in BChl, both bands A and B indicate BChl is six-coordinate. Differences which are present between the RR spectrum of this aggregate and monomeric six-coordinate BChl include an enhancement of low-frequency modes between 600 and 700 cm^{-1}, which either are not observed or are extremely weak in monomeric BChl spectra, and an enhancement of the 1341-cm^{-1} mode. The RR spectrum of a second type of aggregate, the BChl hydrate, is distinctive from both that of monomeric BChl and the pyrazine adduct. Both bands A and B are split, and band B is shifted to higher frequencies as compared to those of monomeric BChl. Shoulders on band A at 1637 and 1627 cm^{-1} are characteristic of hydrogen-bonded C=O vibrations (the C-2 acetyl and C-9 keto). Hydrogen bonding of the C=O appears to enhance their RR scattering, perhaps through an increase in conjugation with the porphyrin π systems.[28]

BChl interactions which produced much smaller changes in its RR spectrum include hydrogen bonding in monomeric solutions and self-aggregation. Hydrogen bonding in EtOH caused only a shift in the weak 1675-cm^{-1} acetyl C=O band. Self-aggregation produced changes only in the relative intensities of the bands, especially that at 1609 cm^{-1}, resulting primarily from C=C stretching vibrations of the methines. It is possible, however, that greater effects in the RR spectrum of BChl as a result of hydrogen bonding or self-aggregation might be observed by using a different laser excitation wavelength. For example, excitation close in resonance with the Soret absorption bands produces stronger C=O scattering and would be more useful for studying hydrogen-bonding interactions.

In summary, the RR results discussed above are encouraging with respect to its potential application to the study of BChl interactions in vivo. Although reaction center and BChl protein complexes have already been examined by this technique,[29] the effect of defined BChl interactions in vitro on its RR spectrum has not been examined in detail.[30]

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Secondary Ion Mass Spectrometry of Small-Molecule Solids at Cryogenic Temperatures. 2.[1] Rare Gas Solids

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Abstract: Secondary ion mass spectra of neat solid argon, krypton, and xenon were measured as a function of the nature and energy of the primary ions, He^+-Xe^+. With primary ions of large momentum, considerable quantities of large cluster ions of the matrix material are produced.

In secondary ion mass spectrometry (SIMS), the surface of a solid is bombarded by a beam of primary ions and the positive or negative secondary ions emitted from the solid are analyzed by a mass spectrometer. SIMS has been a very useful tool for examining surfaces as well as the bulk of metallic and ionic solids.[2] More recently, its utility for molecular solids of low volatility has begun to be explored.[3,4] Recently, it was also used to obtain the

(1) Part 1: H. T. Jonkman and J. Michl, *J. Am. Chem .Soc.*, **103**, 733, (1981).

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