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BACTERIOCHLOROPHYLL *a*-PROTEIN INTERACTIONS IN A COMPLEX FROM *PROSTHECOCHLORIS AESTUARII*

A RESONANCE RAMAN STUDY

MARC LUTZ [a], ARNOLD J. HOFF [b] and LIONEL BREHAMET [a]

[a] *Service de Biophysique, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, 91191 Gif-sur Yvette Cédex (France) and* [b] *Department of Biophysics, Huygens Laboratorium of the State University, Leiden (The Netherlands)*

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Raman spectra of bacteriochlorophyll *a* (BChl) bound to the soluble protein complex from *Prosthecochloris aestuarii* have been obtained at low temperature, using the resonance effect on their Q_x for Soret electronic bands. These spectra show that the acetyl carbonyls of at least four of the seven molecules bound to the monomer subunit of the complex and the ketone carbonyls of at least five of them are oriented close to the mean plane of the conjugated part of the dihydrophorbin macrocycle. Up to three bacteriochlorophyll molecules may have their ketone carbonyls free from hydrogen-bonding and up to two may have their acetyl carbonyls similarly free. Several of the binding sites of the remaining conjugated carbonyls are probably the same as those binding the conjugated carbonyls of bacteriochlorophyll (and of bacteriopheophytin) in reaction centers and in antenna structures of purple bacteria and as those binding chlorophyll in the antenna of higher plants and algae. The present resonance Raman spectra confirm that the magnesium atoms of most of the seven bacteriochlorophylls are pentacoordinated. They also show that polarisation effects from their local environments induce changes in the ground-state structures of the dihydrophorbin skeletons of the complexed molecules with respect to those of isolated, monomeric bacteriochlorophyll. These changes are quasi-identical for the seven molecules. These environmental effects predominate over any structural change brought about by intermolecular bonding of the conjugated carbonyls or of the magnesium atoms. The dihydrophorbin rings of the seven molecules thus appear to be immersed in a nearly homogeneous medium of low permittivity, although specific van der Waals interactions may polarise the free carbonyls to quite different extents. The possible implications of these observations on the interpretation of the electronic spectrum of the set of complexed bacteriochlorophylls are discussed.

Introduction

A steadily increasing number of experimental data indicates that, in the photosynthetic membrane, the chlorophylls most generally occur as complexes with polypeptide chains, whatever their functions and the organism considered. Much of the available evidence has resulted from the development of mild procedures for disaggregating the photosynthetic membrane [1]. Another piece of evidence, as far as antenna of higher plants and algae is concerned, came from resonance Raman data, which showed that all the binding sites for the conjugated carbonyls observed in the intact membrane are not other chloro-

Abbreviations: BChl, bacteriochlorophyll *a*; $(BChl_7\text{-}P)_3$, the soluble bacteriochlorophyll *a*-protein complex from *Prosthecochloris aestuarii*.

phyll molecules and are also present in the chlorophyll-protein complexes isolated from the membrane [2].

Yet, the structures of most chlorophyll-protein complexes are still unknown, because of both their general insolubility in water and their lability. The structure of a only one complex has been satisfactorily described, namely that of the soluble, crystallisable bacteriochlorophyll *a* (BChl)-protein complex from *Prosthecochloris aestuarii* [3], which has been determined with great detail from X-ray diffraction data by Fenna, Matthews and co-workers [4,5].

Raman spectroscopy yields structural information at the molecular and submolecular levels. Its applicability does not depend on the physical state of the sample and, through a resonance process, it permits one to observe vibrational spectra of chromophores, e.g., of chlorophylls, in complex media, such as chlorophyll-protein complexes, with a high selectivity [2,6–9]. Resonance Raman spectra of chlorophyll may even be obtained from intact membranes, chloroplasts and cells [10], thus allowing assay of the degree to which these molecules retain their native networks of bonding in the extracted protein complexes [2]. However, the obvious counterpart of such a selectivity is that only indirect information is obtained about the binding sites of chlorophyll.

For the reason just mentioned, it appeared useful to study the resonance Raman spectra of bacteriochlorophyll in the soluble complex from *P. aestuarii,* the structure of which is largely known, and to use them in interpreting the spectra from other complexes. In addition to permitting a calibration of the resonance Raman spectra of BChl in terms of their structural information content, these spectra proved useful in three other ways: firstly, they yielded additional data on the structure of the $(BChl_7\text{-}P)_3$ complex, particularly concerning the interactions assumed by the acetyl and ketone carbonyls and on their orientations with respect to the dihydrophorbin plane. Secondly, they gave some information on the effects of the environment on the molecular structures of the complexed BChl molecules. Thirdly, they confirmed that the $(BChl_7\text{-}P)_3$ complex is representative of the binding states of antenna (bacterio)chlorophylls [4,11] and of bacteriochlorophylls and -pheophytins in reaction centers of purple bacteria [8].

Materials and Methods

Samples

Two preparations of the $(BChl_7\text{-}P)_3$ complex from *P. aestuarii,* strain 2K, were studied. One was prepared in Leiden following the procedure outlined in Ref. 12 and was examined as a lyophilisate from a $(NH_4)_2SO_4$ solution. The second sample was a gift from Dr. J.M. Olson and was examined as a fresh centrifugation pellet from a 10 mg/ml solution in 30% $(NH_4)_2SO_4$. These two samples were characterised by their electronic absorption spectra in the 250–850 nm region, both when prepared and when the Raman experiments were run. The four sets of spectra agreed in both peak wavelengths and absorbances with the published reference values [3] [Fig. 1].

Bacteriochlorophyll *a* was extracted from *Rhodopseudomonas sphaeroides* cells, strain Y and was purified as described previously [7], then dried as described in Ref. 13. The monomer was obtained by dissolution in dry tetrahydrofuran to a concentration of approx. 10^{-3} M. Oligomers were formed by evaporating a dry BChl solution in CCl_4. All of these samples were handled in a dry nitrogen atmosphere.

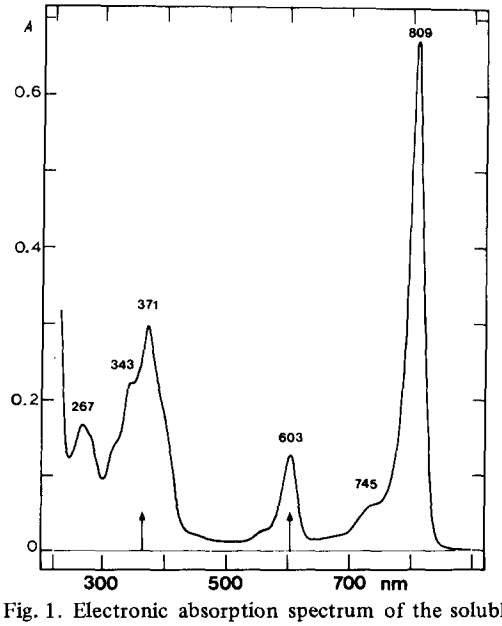


Fig. 1. Electronic absorption spectrum of the soluble BChl *a*-protein complex from *P. aestuarii.* Arrows at 363.8 and 604 nm indicate the wavelengths used to produce spectra of Figs. 2 and 3, respectively.

Droplets of the fluid samples were laid down on the sample holder under dry nitrogen then frozen in liquid nitrogen and introduced into a precooled cryostat. All samples were immersed in a flow of cold helium gas ensuring efficient cooling of the irradiation site and were studied at between 20 and 35 K. In these conditions no illumination damage was observed on any sample during the recording of the Raman spectra.

Spectroscopy

Grazing excitation on optically dense samples was used to produce the resonance Raman spectra. Radiant powers reaching the samples were less than 1 mW. Resonance on the Soret transitions of BChl was achieved using the 363.8 nm emission from an argon laser (Spectra Physics, model 170). The 351.1 nm emission as well as the plasma lines were eliminated by filtering through a three-prism monochromator (Anaspec). The Raman spectrometer (Jobin-Yvon HG2S-UV) was equipped with d.c. detection. Typical spectral resolution was 8 cm^{-1} at 1000 cm^{-1}. The S/N ratios in the 100–500 and in the 1600–1800 cm^{-1} regions were improved by summation of individual spectra in a multichannel analyser (Didac 4000, Intertechnique).

Resonance conditions on the Q_x transition were achieved using Rhodamine 6G emission in the 596–605 nm range from a dye laser (Spectra Physics, model 375). The radiant power of the dye laser was stabilised by a feedback system acting on the pump argon laser. The dye fluorescence was removed from the laser beam by filtering through the three-prism monochromator. The Raman spectrometer was a Coderg PHO equipped with d.c. detection. Typical spectral resolution at 1000 cm^{-1} was 4 cm^{-1}.

Results and Discussion

Resonance Raman spectra of the BChl molecules bound to the soluble complex from *P. aestuarii* were obtained at 20–35 K using resonance enhancement near the top of the Soret band (363.8 nm excitation, Fig. 2) and in the Q_x band (596–605 nm excitation, Fig. 3). Samples of both origins yielded identical spectra. The frequencies of most of the approx. 60 bands or shoulders observed in these spectra are noted on Figs. 2–5.

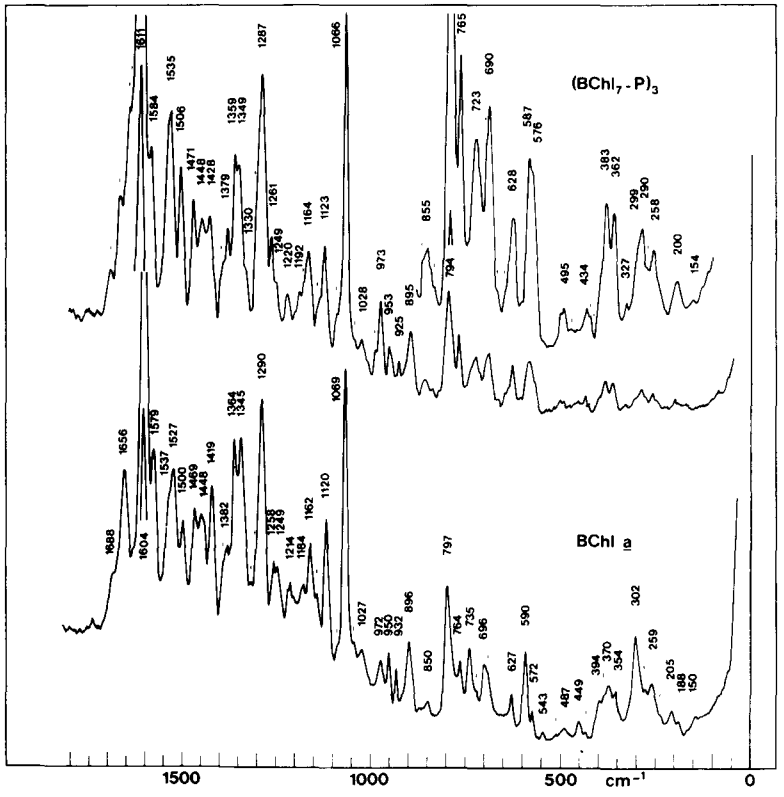
Resonance Raman spectra of BChl *a* contain direct information upon intermolecular binding of its 9-ketone and 2-acetyl carbonyl groups, as well as of its magnesium atom [7–9]. These three sites should play prominent roles in the binding of BChl *a* in vivo as well as in vitro, although ester carbonyls may also take part [8,14–16].

Conformations of the carbonyl groups

The stretching modes of the acetyl and ketone carbonyls of monomeric BChl *a* occur at 1693 and 1665 cm^{-1} in tetrahydrofuran at room temperature [17] and at 1688 and 1656 cm^{-1} at 30 K, respectively (Fig. 2). They are significantly resonance-enhanced in Raman spectra excited at 363.8 nm but not in those excited near 600 nm (Fig. 3). These two bands have the highest frequencies observed in resonance Raman spectra of BChl *a*. The next, lower frequency band occurs at 1605 cm^{-1}, predominantly arising from stretching of the methine bridges [7].

In the resonance Raman spectra of the $(BChl_7\text{-}P)_3$ complex a cluster of shoulders is observed on the high frequency side of the very strong 1612 cm^{-1} band (Fig. 4). The structure of this cluster is the same for the samples of both origins and up to 12 components may be present in both cases. With the exception of the approx. 1620 cm^{-1} feature (Fig. 4C), we attribute these components to the stretching modes of 2- and 9-C=O groups of the seven BChl molecules of the monomer unit of the complex. The 1620 cm^{-1} shoulder probably represents a skeletal component of the 1612 cm^{-1} band, also present in certain preparations of monomeric BChl *a*, e.g., in acetone at 30 K [18]. 9–11 distinct components may thus be assigned to stretching of the carbonyl groups, a number inferior to the 14 crystallographically unequivalent ketonic and acetyl carbonyls of the complex, although the seven BChl molecules should contribute equally to the resonance spectrum as a whole.

This discrepancy may have two origins. The most obvious one is that accidental degeneracies may occur among certain components. The second one is that certain carbonyl vibrators might be inactive in the resonance Raman spectra of the complex. The only possible reason for 9- or 2-C=O groups of certain BChls of the complex being resonance Raman active and those of others not, given the same experimental conditions, is that these groups assume different



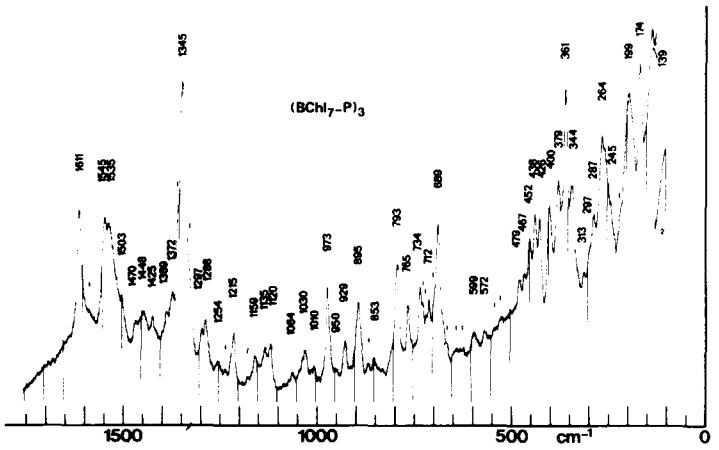


Fig. 2. Resonance Raman spectra of: $(BChl_7\text{-}P)_3$ the soluble antenna complex from *P. aestuarii* (uppermost spectrum, sensitivity ×4); BChl *a*, monomeric BChl *a*, 10^{-3} M in dry tetrahydrofuran. 30 K. Excitation 363.8 nm. Resolution at 1000 cm^{-1}, 8 cm^{-1}. Single scans. Frequencies are in cm^{-1}. Vertical bars point to minor features, the frequencies of which are not indicated here.

Fig. 3. Resonance Raman spectrum of the soluble antenna complex from *P. aestuarii* at 22 K, excitation 604 nm, resolution at 1000 cm^{-1}, 5 cm^{-1}. Single scan. Vertical bars point to minor features, the frequencies of which are not indicated here.

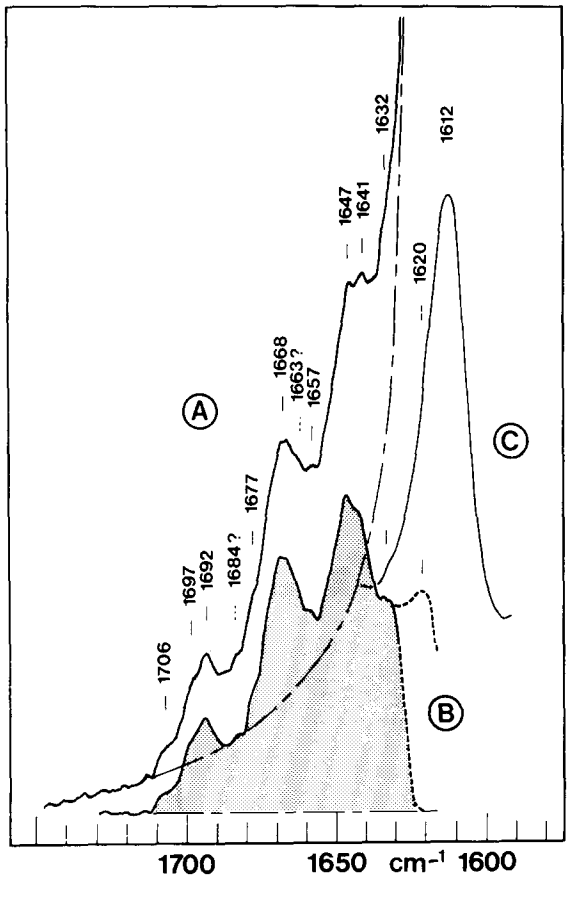


Fig. 4. A: Resonance Raman spectrum of the $(BChl_7\text{-}P)_3$ complex in the carbonyl stretching region, 30 K, excitation 363.8 nm, summation of ten scans. Spectral resolution at 1700 cm^{-1}, 6.5 cm^{-1}. B: Same spectrum after subtraction of the high-frequency end of the 1612 cm^{-1} band. C: Same spectrum, νC_a ··· C_m band, sensitivity ×0.2.

orientations with the dihydrophorbin ring. An out-of-plane orientation of any of these groups indeed results in a decrease of its conjugation with the main π-electron system of the molecule, involved in the Soret and visible transitions, and in an unfavorable coupling of the $\nu C{=}O$ mode with these in-plane transitions [19].

We showed that the vinyl C=C groups of chlorophyll *a* and *b* are never active in any of their observed resonance Raman spectra and concluded that these groups are out of the phorbin plane [19]. X-ray crystallography confirmed this point in the particular cases of hydrated crystals of ethylchlorophyllides *a* and *b*, by showing that the angles of the vinyl C=C groups with the pyrrolic nitrogens' plane were 22 and 18° for the *a* and *b* derivatives, respectively. On the other hand, the ketone and formyl carbonyls, active in the resonance spectra, indeed lay close to the nitrogens' plane [20,21]. Similarly, since at least one of the vinyls of heme appear to be active in resonance Raman spectra of hemoproteins [22,23], their inactivity in resonance Raman spectra of protoporphyrin IX [24] should be due to out-of-the-heme-plane conformations. X-ray crystallographic data again support this conclusion: in bis(1-methylimidazole)ferriprotoporphyrin IX, for instance, the vinyls are oriented at 21 and 41°C from the mean porphyrin plane [25]. Hence, angles as low as 20° with the plane of the conjugated frame appear to prevent any significant resonance Raman activity of the $\nu C{=}C$ mode of the vinyls of chlorophylls and heme. We may speculate that the resonance Raman scattering efficiencies of the stretching modes of the conjugated carbonyls of BChl may assume the same dependence on their angle with the molecular plane.

We calculated the angles of the 2- and 9-C=O bonds of the seven bacteriochlorophylls of the monomer subunit of the complex with respect to the (1N-3N, 2N-4N) plane, from the atomic coordinates published by Fenna and co-workers [26]. Angles higher than 18° were found for eight out of the 14 groups. These values may however be of limited significance, as they were derived from coordinates determined with rather high uncertainties of up to 1 Å [26]; Actually, these calculated angles raise two difficulties. First, the values found for certain groups appear surprisingly large if compared to those observed for the ethylchlorophyllides [20,21] and for methylpheophorbide [27]: 20 and 22° for the ketone carbonyls of BChl No. 3 of BChl No. 6, respectively; 59 and 60° for the acetyl carbonyls of BChl No. 6 and of BChl No. 7, respectively. Second, they would allow only two acetyl carbonyls (of BChls No. 3 and 4) to be active in the present resonance Raman spectra, assuming, on the basis of the above discussion for the vinyl groups, that angles higher than 18° drastically decrease this activity. Actually, Fig. 4B shows that at least four acetyl carbonyls must contribute to the resonance Raman spectrum: the frequency of the 1632 cm^{-1} component is too low, and the relative intensities of the 1641, 1647 and 1668 cm^{-1} compo-

TABLE I

FREQUENCIES (cm^{-1}) OF THE STRETCHING MODES OF KETONE AND ACETYL CARBONYLS OF BACTERIOCHLOROPHYLL *a* IN THE $(BChl_7\text{-}P)_3$ COMPLEX FROM *P. AESTUARII*, COMPARED TO FREQUENCIES OBSERVED IN OTHER STRUCTURES

Column 1: from resonance Raman spectra of two samples of different origins, obtained with 363.8 nm excitation at 30 K and averaged by summation of multiple scans (Fig. 3). Column 2: tentative assignment, assuming that the 14 crystallographically unequivalent carbonyl vibrators of the complex are active in these spectra: Ac, acetyl carbonyl vibrator; K, ketone carbonyl vibrator. Columns 5 and 6: from spectra excited at 363.8 nm and including contributions from the four BChls and the two bacteriophosphytins. Column 7: from spectra excited in the Q_x bands of the bacteriopheophytins and including contributions from these molecules only. Ox.: untreated reaction centers, *P*-870$^+$ state. Red.: reaction centers treated with dithiothreitol, *P*-870 state. (535), (545) tag frequencies assigned to the bacteriopheophytin molecules absorbing at 535 or at 545 nm at 30 K, respectively. Column 8: average frequencies of stretching modes of the formyl and ketone carbonyls of antenna Chl *a* and Chl *b* observed in RR spectra of chloroplasts. Mean values from seven (Chl *a*) and four different species (Chl *b*). From Ref. 2. (F), formyl carbonyls of Chl *b*; (Kb), ketone carbonyls of Chl *b*.

$(BChl_7\text{-}P)_3$		*Rhodopseudomonas spheroides*					Chloroplast
		Chromatophores		Reaction centers			Antenna Chl*a* + Chl*b*
	Assignment			Soret		Q_x (BPheo)	
		R26	2.4.1.	Ox.	Red.		
1632	Ac			1632	1632	1627? (535)	1630 (F)
			1637	1637	1638	1635 (545)	
1641	Ac, Ac	1643	1643	1643	1643		1640 (F)
1647	Ac, Ac			1652	1653		1653
1657	K			1659	1661	1660 (535)	1661
1663?							
1668	K, Ac } Ac	1668	1665	1666			1670
1677	K	1676?	1675?		1679	1678 (545)	1681
1684?	K	1684	1683	1684	1685		
1692	K			1690	1691?		1689
1697	K				1699?	1700 (535)	1694 (Kb)
1706	K	1705 [a]	1703 [a]	1708			1702

[a] Bands possibly not genuine to the structures studied.

nents are too high for ketone carbonyls alone.

Hence, there is no compelling reason to consider that any of the acetyl and ketone carbonyls of the seven BChl molecules should not contribute to the resonance Raman spectra of the complex. Indeed, planimetry of the carbonyl cluster – following subtraction of a background profile accounting for the high frequency end of the 1612 cm^{-1} band (Fig. 4B – yields a 0.25 value for its integrated intensity, normalised to that of the 1612 cm^{-1} band. This value falls inside the variability range of the normalised, integrated intensities of the ν(2-C=O) plus ν(9-C=O) bands in various in vitro preparations, for which extreme values are 0.22 and 0.50. This is thus consistent with the 14 vibrators being active in the resonance Raman spectrum of the complex, but does not demonstrate this activity, in view of the arbitrariness of the subtracted profile and of the large dispersion of the in vitro values. Table I shows a tentative assignment of the cluster components, using 14 carbonyl vibrators. This assignment is based on the respective frequency spans of acetyl and ketone carbonyls stretching modes in vitro [7,17,18] and on the relative intensities of the components, the intensities of the acetyl C=O bands being generally higher than those of the ketone carbonyl bands in resonance Raman spectra of bacteriochlorophyll [7,18].

Intermolecular binding of the carbonyl groups

Partial data on the binding states of the carbonyl groups have been obtained by Fenna and co-workers. The 9-C=O group of BChl No. 1 should be free from bonding, that of BChl No. 2 may be hydrogen-bonded to the peptidic chain of an adjacent subunit and that of BChl No. 6 may be linked to an arginine residue [5]. Raman frequencies at 1692, 1697 and 1706 cm^{-1} must arise from ketone carbonyls, probably free from hydrogen-bonding [18], in different local environments (see below). The 1677 and 1684 (?) cm^{-1} components of the cluster also necessarily arise from stretching of ketone carbonyls, as is also probably the case for the weak 1657 cm^{-1} component. These frequencies should correspond to intermolecularly bound groups. Similarly, the 2-C=O group(s) vibrating at 1668 cm^{-1} should be free, while those vibrating at 1647, 1641 and 1632 cm^{-1} are bonded. The differences in frequencies observed among the bonded ketone or acetyl carbonyls most probably originate in differences in the chemical natures of their binding sites. However, it cannot be excluded that the same carbonyls bound to chemically identical proteic sites might have somewhat different stretching frequencies depending on their local environments.

Some interesting comparisons can be made between the present set of νC=O frequencies of BChl in the soluble complex and those we observed for antenna BChl *a* in chromatophores of *R. sphaeroides* and for the six bacteriochlorophyll and -pheophytin molecules bound to reaction centers of the same bacteria [8,9,18]. Very close correlations are observed between the three sets of frequencies, except for a 1637 cm^{-1} component observed in the antenna of wild-type *R. sphaeroides* and in the reaction centers (Table I). This strongly suggests that the binding sites of the conjugated carbonyls of the pigments are the same (proteic) chemical groups in these three cases, except for one site that binds (acetyl) carbonyls in *R. sphaeroides* structures.

More surprisingly, an excellent agreement is also apparent between the sets of ketone νC=O frequencies observed for the soluble complex and for antenna chlorophyll *a* and *b* in higher plants and algae (Table I). It may even be noted that the average stretching frequencies of the formyl carbonyls of chlorophyll *b* in plant antenna, at 1630 and 1640 cm^{-1}, closely match with two of the frequencies we assign to stretching of acetyl carbonyls of BChl in the soluble complex; this correlation may well be not fortuitous, since the stretching frequencies of free 3-C=O groups of chlorophyll *b* in vitro and of free 2-C=O groups of BChl in vitro are the same within a very few wavenumbers [17,18]. It thus appears that the conjugated carbonyls of antenna chlorophyll *a* and *b* also bind to the same chemical sites as does BChl *a* in the $(BChl_7\text{-}P)_3$ complex. This confirms our previous conclusion that these sites are not other chlorophyll molecules, most of them probably being protein groups [2,11], and that the *P. aestuarii* complex constitutes a valuable model for the state of antenna chlorophylls in vivo [11].

These observations further indicate that the possible hydrogen-bond donor sites of the proteins are not randomly involved in anchoring the pigments, but that only certain specific ones actually assume this function in any of the antenna or reaction-center complexes we have studied.

Liganding of the magnesium atoms

Isotopic substitution of the ^{24}Mg atom of bacteriochlorophyll *a* by ^{26}Mg results in shifts of a complex band present around 290–305 cm^{-1} in the Soret-excited resonance Raman spectra of this molecule, either in vitro or in chromatophores or reaction centers of *R. sphaeroides* (Lutz, M., Reiss-Husson, F. and Kléo, J., unpublished data). This band is absent from resonance Raman spectra of bacteriopheophytin [18]. It is also sensitive to $^{14}N \rightarrow {}^{15}N$ (pyrrole) substitution and hence most likely corresponds to an in plane mode predominantly involving stretching of the Mg-N(pyrrole) bonds (Lutz, M., Reiss-Husson, F. and Kléo, J., unpublished data).

In tetrahydrofuran (Fig. 5) and in pyridine solutions [8] at 30 K, this 'Mg-N band' occurs at 302 and 303 cm^{-1}, respectively, and its halfbandwidth (measured from an arbitrary baseline joining the 270 and 320 cm^{-1} points) is 17 cm^{-1}. In both of these cases, the Mg atom binds two solvent molecules, that is, is hexacoordinated [18,28]. The Mg-N band occurs at 292–294 cm^{-1} in resonance Raman spectra of $(BChl)_n$ oligomers (Fig. 5), for which the Mg atoms of most molecules should be pentacoordinated, but the structure(s) of which is largely unknown [16]. Its halfwidth, 24 cm^{-1}, is larger than in polar solvents.

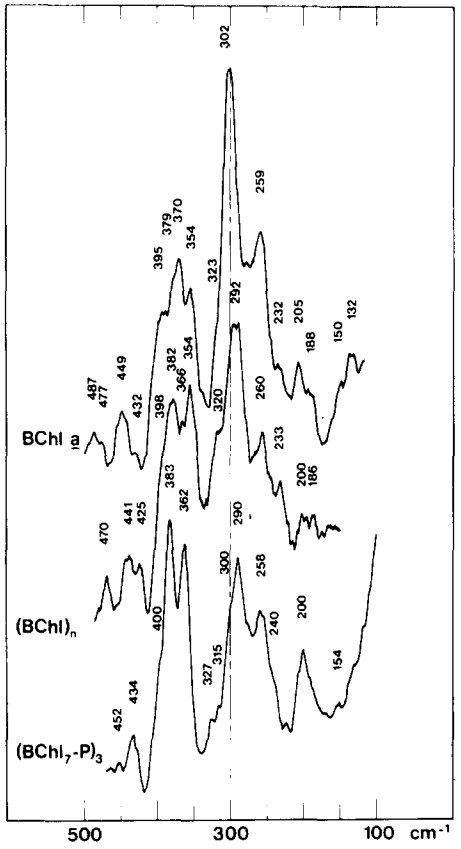


Fig. 5. Resonance Raman spectra (lower frequency region) of BChl *a*; monomeric BChl *a*, 10^{-3} M in tetrahydrofuran; $(BChl)_n$: dry BChl *a* oligomers in the solid state; $(BChl_7\text{-}P)_3$: the soluble antenna complex from *P aestuarii*. 30 K. Excitation 363.8 nm. Spectral resolution at 300 cm^{-1}, 8 cm^{-1}. Averaged by summation.

In the resonance Raman spectra of the $(BChl_7\text{-}P)_3$ complex excited at 363.8 nm, the 'Mg-N band' is observed at 290 cm^{-1} and its halfwidth is 22 cm^{-1}. Matthews and co-workers have shown that the seven BChl molecules of the monomer unit of the complex should have pentacoordinated Mg atoms [5]: hence, the frequency of the Mg-N band, together with its half-bandwidth, can safely be considered as indicators for the coordination number of the Mg atom: a frequency of 300 cm^{-1} or higher corresponds to an hexacoordinated magnesium and a frequency lower than 295 cm^{-1} to a pentacoordinated magnesium. The half-bandwidth of the Mg-N band appears to be lower than 20 cm^{-1} in the former case and higher than 20 cm^{-1} in the latter.

The presence of a 299 cm^{-1} shoulder in the resonance Raman spectra of the $(BChl_7\text{-}P)_3$ complex would then indicate that at least one of the BChl molecules should have an hexacoordinated Mg atom, in disagreement with the current interpretation of X-ray data. However, this shoulder may more plainly result from a complex structure of the Mg-N band, also observed for other samples, e.g., for $(BChl)_n$ oligomers (Fig. 5). This band indeed appears to contain a minor component less sensitive than the major one to the coordination number of the Mg atom. The frequency of the Mg-N band may also possibly be slightly sensitive to the chemical nature of the molecules bound to the Mg atom and its complexity might be increased for this reason in the case of the $(BChl_7\text{-}P)_3$ complex, since three types of ligand are probably involved, namely histidine side chains, a peptidic oxygen and a water molecule [5].

Environmental interactions on the dihydrophorbin skeleton

Most of the resonance Raman bands of BChl observed in the Soret and Q_x resonances arise from complex modes of the conjugated, dihydrophorbin skeleton. Isotopic substitution of the pyrrolic nitrogens permits one to distinguish modes predominantly involving C-N bonds in the inner part of the molecule (e.g. at 1364, 1290, 1162, 1120 and 797 cm^{-1}) and modes which involve only motions of carbon atoms, primarily concerning the outer part of the molecules (e.g. 1604, 1469, 1448, 1214 and 896 cm^{-1}) (Lutz, M., Reiss-Husson, F. and Kléo, J., unpublished data). In the spectra of the $(BChl_7\text{-}P)_3$ complex, most of these bands are found at frequencies differing slightly from those observed in spectra of monomeric BChl, and/or with different relative intensities (Fig. 2).

As far as the quantum yield for resonance Raman scattering depends on both the structural and electronic properties of the molecule in its ground and electronic excited states, the intensity variations observed when passing from monomeric BChl to BChl complexed to the protein should largely result from the observed changes in the excited electronic states of BChl molecules when complexed, due to their proteic and chromophoric environments [3]. On the other hand, the vibrational modes active in the resonance Raman spectra specifically belong to molecules in their ground electronic states only and any band-

TABLE II

COMPARED BEHAVIORS OF ENVIRONMENT-SENSITIVE BANDS OF BACTERIOCHLOROPHYLL *a* IN THE $(BChl_7\text{-}P)_3$ COMPLEX AND IN TETRAHYDROFURAN (THF) AT 25 K (363.8 nm EXCITATION)

Frequency in $(BChl_7\text{-}P)_3$ (cm^{-1})	1611	1506	1123	1066	925
Relative shift, $^{14}N \rightarrow {}^{15}N$ ($\times 10^{-3}$)	0	0	9	5	0
Shift, (THF) $\rightarrow$ $(BChl_7\text{-}P)_3$ (cm^{-1})	7	6	3	−3	−7
Half-bandwidth in THF (cm^{-1})	16.5	12.5	13.5	13	7.5
Half-bandwidth in $(BChl_7\text{-}P)_3$ (cm^{-1})	17	12.5	14	13	8

shift reflects a change in ground-state properties of certain bonds of the molecule.

Fig. 2 and Table II show that several bands involving vibrations of the dihydrophorbin skeleton are shifted by up to 9 cm^{-1} from their positions in monomer spectra. However, none of the shifted bands which are fully resolved assumes any increase in half-bandwidth higher than 0.5 cm^{-1}. The same observation is valid for spectra excited in the Q_x band (compare Fig. 3 to Fig. 1 in Ref. 7). This shows that except for their conjugated carbonyl bonds, the ground-state structures of the seven BChl molecules in the complex are altered almost exactly to the same extent by their local environments. The possible origins for the observed shifts may be discussed on this basis.

Frequency differences between skeletal bands of BChl in the complex and of monomeric BChl in vitro must result either, indirectly, from the differences occurring in the intermolecular bonding of the carbonyl groups and of the Mg atom in these two types of environment or from differences in distribution of bonding electrons on the skeleton more directly resulting from a polarisation effect by neighboring molecules in the complex, that is, other bacteriochlorophylls and/or the protein.

The interactions assumed by the 14 ketone and acetyl carbonyls cover a wide range of energies and induce frequency shifts of the ν(C=O) modes ranging from 0 to 40 cm^{-1} at least. If these interactions were the primary cause for the shifts of the skeletal bands, the latter should be different for the different BChl molecules.

Interchromophoric interactions in the ground state assumed by each of the seven BChls taken separately should also be largely different, as may be inferred from the unequivalent orientations and distances of any of the molecules with respect to the other six. Moreover, the overall effect of the formation of oligomers from monomeric BChl on the resonance Raman spectra is much smaller than that which would correspond to inserting a BChl monomer into the $(BChl_7\text{-}P)_3$ complex. These effects may be compared semi-quantitatively by calculating the partial variations in the zero-point energy of BChl corresponding to the skeletal bandshifts observed in the resonance Raman spectra, assuming that none of the modes involved is degenerate – a legitimate assumption considering the low molecular symmetry of BChl – and neglecting anharmonicities. The value corresponding to the insertion of a BChl molecule into the complex is $6 \cdot 10^{-15}$ erg, one order of magnitude higher that that found for the formation of oligomers in the solid state, $5 \cdot 10^{-16}$ erg. Considering further that the Mg–Mg distances in the complex subunit range from 11.4 (BChl No. 3–BChl No. 4) to 31.3 Å (BChl 1–BChl 4) [26], that is, they are equal to or higher than those involved in the oligomers, approx. 9–12 Å whatever their precise structure [14,29], allows the conclusion that the shifts cannot primarily arise from nearest neighbor interchromophoric interactions. This also excludes that they might originate from the difference in the co-ordination numbers of the Mg atom of BChl in tetrahydrofuran at 30 K – six – and in the complex – five – as far as oligomers of BChl in the solid state also involve pentacoordinated Mg atoms (Fig. 5).

We thus are led to conclude that the environmental effect on most of the skeletal modes of any BChl molecule of the $(BChl_7\text{-}P)_3$ complex, and hence on their ground-state structures, primarily consists of an averaged polarisation effect from the protein and from the six other pigments taken as a whole.

The influence of the surrounding medium on the vibrational frequencies of a molecule, even in the absence of specific intermolecular bonding, should be

best described in terms of microscopic factors characterising the local (van der Waals) interactions between the molecule and neighbouring groups, especially when the considered medium is heterogenous. However, for obvious reasons, macroscopic parameters characterising the 'solvent' medium as a whole have been most generally used.

Extensive work has shown that the stretching frequencies ν_s of a number of vibrators depend markedly on the dielectric permittivity of the surrounding medium and more-or-less obey Kirkwood-Bauer-Magat (KBM)-type laws (review, Ref. 30):

$$\frac{\nu_g - \nu_s}{\nu_g} = K \frac{\epsilon - 1}{2\epsilon + 1} \quad (1)$$

where ν_g is the frequency of the vibrator in vacuo. In many cases, including the stretching of carbonyls, the constant K is positive, that is, an increase in permittivity results in a decrease of ν_s. Relation 1 is often better obeyed when limiting high frequency values ϵ_∞ are considered rather than the static field values ϵ_0 [31].

The inner space of a globular protein is expected to be hydrophobic and weakly polar. Permittivity (ϵ_∞) values around 2, at room temperature, have been considered as reasonable in many cases [32]. The ϵ_∞ of tetrahydrofuran at room temperature, 1.98, is close to this value. However, the stretching frequencies of the ketone and acetyl carbonyls of monomeric BChl in tetrahydrofuran decrease by 5 and 9 cm^{-1}, respectively, upon cooling to 30 K. Similarly, the νC=O mode of 1% (v/v) acetone in tetrahydrofuran decreases by no less than 20 cm^{-1} between 300 and 77 K. These observations indicate that the permittivity of tetrahydrofuran increases markedly upon cooling. Indeed, assuming that the νC=O mode of acetone still obeys a KBM law, with a K of 0.052 [31], the ϵ_∞ of tetrahydrofuran at 77 K is found to be 7. On the other hand, the permittivity of the inner space of the apoprotein of the $(BChl_7\text{-}P)_3$ complex must remain low, even at 30 K, because at least one of the BChl molecules keeps its ketone carbonyl vibrating at a high frequency, 1706 cm^{-1} (Table I).

Hence, the frequency differences observed on skeletal bands of BChl in the complex and in tetrahydrofuran, at 30 K, may well primarily result from a difference in permittivities of the two types of environment. The fact that these differences have various values and different signs for the various bands of the spectrum is not at odds with such an interpretation [33].

In this interpretation, the fact that, in the complex, any skeletal band occurs at the same frequency for all of the seven BChl molecules shows that the local permittivities at the sites of the seven molecules are nearly the same. Assuming a moderate sensitivity to permittivity, as observed for ring modes of pyrrole [33], corresponding to a K value of $1 \cdot 10^{-2}$ in Eqn. 1, a frequency dispersion not higher than 0.5 cm^{-1}, at 1000 cm^{-1}, in a medium of average permittivity of 2, would correspond to differences in local permittivities at the sites of the seven BChl not higher than 0.3.

Such a small dispersion in local permittivities most probably cannot account for the differences between the three uppermost frequencies of the ketone carbonyls observed in the resonance Raman spectra of the complex at 1692, 1697 and 1706 cm^{-1}, which should be free from hydrogen bonding. It appears more likely that the downshifts of the components at 1697 and 1692 cm^{-1} from the 1706 cm^{-1} value are due to specific, local van der Waals interactions, in a medium of low average permittivity. On the other hand, the effect of local interactions on the vibrational modes of the phorbin rings appear to be largely averaged, so that the seven macrorings actually appear to be immersed in an homogenous medium of low permittivity.

Electronic spectra of the complexed bacteriochlorophylls

Recent calculations based on an excitonic interpretation of the electronic absorption and CD spectra of BChl in the $(BChl_7\text{-}P)_3$ complex have met with difficulties in accounting for the experimental spectra [34,35]. Various hypotheses have been suggested concerning the origin of this problem [35], including the possibility that the directions of the 'Q_y' and 'Q_x' electronic dipole moments relative to the molecular frame are rotated by some 90° in the protein with respect to their positions in the free molecule. Such a drastic effect of the protein environment actually is ruled out by the close overall similarity in band intensities of resonance Raman spectra of BChl in the complex and in any polar solvent (Fig. 3 and Ref. 18).

Most of the other hypotheses either are inconsistent with other data or result in too small shifts (Ref.

35 and Bréhamet, L. and Paillotin, G., unpublished results). A possible way which may ultimately lead to a correct description of the electronic spectra is to consider that the (proteic) van der Waals interactions (or the local permittivities) are different on each BChl molecule in the complex [35]. Such a heterogeneity should introduce non-zero and different values of the diagonal elements of the resonance matrix. The present Raman data obviously set an upper limit to the dispersion of these parameters among the seven BChl sites in the protein and hence may help to define a reasonable set of values which would account for the observed electronic spectra.

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