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^{13}C- and ^{15}N-NMR Studies on the Intact Bacteriochlorophyll *c* Dimers in Solutions

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Abstract: ^{13}C and ^{15}N chemical shifts of the intact farnesyl (3^1R)-bacteriochlorophyll (BChl) *c* have been measured in methanol and carbon tetrachloride solutions. Two sets of resonances have been observed in carbon tetrachloride for all carbon and nitrogen atoms, indicating a formation of highly stable dimeric species with asymmetric configurations. Complete assignments have been made based on a combination of homonuclear and heteronuclear correlation experiments using the ^{13}C- and ^{15}N-labeled BChl *c* samples. Changes of the ^{13}C chemical shift in the two solvents can be interpreted in terms of mixed effects arising from (a) ring current due to the overlap of the macrocycles, (b) coordination state of the central magnesium, (c) excitation state of the π-electron system, (d) polarity of the solvents used, and (e) hydrogen bonding. Substantial ring current effect is observed on the ^{13}C chemical shifts for the carbon atoms around pyrrolic ring I upon the dimer formation. Remarkable differences in the line widths observed for all propionic carbons and some carbons of the farnesyl group suggest that the propionic-farnesyl side chains may adopt a “return” structure over the region from 17^1 to f2 carbons with much different conformation and mobility in the dimer. No clear evidence is obtained for a hydrogen bond formed with the C13^1 carbonyl group in CCl_4 solution, nor for ring overlap over the ring V. Comparison between the ^{15}N chemical shifts in both solvents indicates that the paramagnetic shielding effect is predominant and N_{IV} nitrogen is most sensitive to the dimer formation, followed by N_{II}, N_I, and N_{III}, respectively. The result reveals a high sensitivity of ^{15}N chemical shift to the electronic state and N−Mg bond length for each nitrogen atom in the dimer.

Introduction

Bacteriochlorophyll (BChl) *c* is found in a special antenna complex, known as chlorosome, of green photosynthetic bacteria as a major light-harvesting pigment. Since a large ratio of pigment to protein (2:1 ∼ 0.6:1, w/w) has been found in the multicomponent complex,[1,2] the BChl *c* molecules are considered to exist in an oligomeric form in the chlorosome. The oligomer has recently been estimated to be composed of 16 and 6 BChl *c* molecules for green filamentous species *Chloroflexus aurantiacus* and green sulfur species *Chlorobium tepidum*, respectively.[3] The absorption maximum of the native chlorosome at long wavelength (Q_y) is around 740 nm, about 70 nm red-shifted from that of its monomeric form as observed in methanol. On the other hand, isolated and purified BChl *c* is capable of self-association to form high aggregates in hexane with an absorption spectrum closely resembling that of native chlorosome.[4−6] Many spectroscopic measurements have been made to elucidate the in vitro structure of 740-nm aggregate in relation with the organization of BChl *c* in chlorosomes.[6−11] Two functional groups, 3^1-hydroxyl and 13^1-keto groups, were identified to play key roles in ligation and hydrogen bonding in the BChl *c* aggregates. However, due to large molecular weight and structural heterogeneity, the structure of the 740-nm aggregates has not been solved.

Beside the 740-nm high aggregate, there are several smaller aggregates formed in CH_2Cl_2, $CHCl_3$, CCl_4, and benzene with the Q_y absorption maxima at 680 and 710 nm for the BChl *c* of the *R*-configuration at the C3^1 chiral center.[12,13] The 710-nm species has been demonstrated as being predominated by BChl *c* dimers in a small-angle neutron scattering experiment.[14] Although structural connection between the small and high aggregates remains unclear and needs to be further investigated, the highly stable and structurally homogeneous small aggregates enable one to obtain precise information on the individual atom. This is believed to provide useful knowledge on the nature of self-association at a level as small as dimer, and to give insight into the higher-order structure of the high aggregate as well as arrangement of BChl *c* in chlorosome. In a previous study,[15] we reported on the complete assignment of ^{1}H-NMR for the

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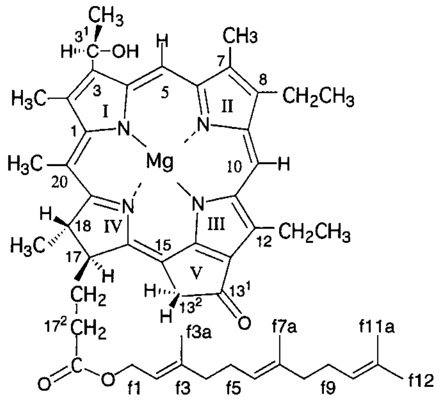

Figure 1. Structure and nomenclature of the intact (3[1]*R*)-[*E*,*E*]-BChl c_F.

710-nm aggregate of BChl *c* formed in carbon tetrachloride. The ^{1}H-NMR spectrum is characterized by two asymmetric resonances resolved for each individual proton, indicating that the two molecules in dimer experience slow exchange between two nonequivalent configurations. Results from a detailed structural analysis support an antiparallel "piggy-back" conformation for the BChl *c* dimers (see Figures 9 and 10 in ref 15).

In this study, we present detailed assignments of ^{13}C- and ^{15}N-NMR spectra of intact BChl *c* dimer in pure CCl_4 solution and compare with those in methanol. The sample used is (3[1]*R*)-[*E*,*E*]BChl c_F (8,12-diethyl-BChl *c* esterified with farnesol, Figure 1), the most abundant component constituting more than 50% of whole BChl *c* homologues in *Chlorobium tepidum*.[10,16] This pigment has a 710-nm absorption maximum in CCl_4 solution.[15] The ^{13}C NMR spectra in CCl_4 reveal two sets of resonances with highly asymmetric features in both chemical shift and spectral shape for many carbon atoms. Carbon−carbon connectivity is made using a fully ^{13}C-labeled sample on the basis of the results from two-dimensional ^{13}C−^{13}C double quantum INADEQUATE experiments with the aid of the ^{1}H−^{13}C long-range heteronuclear multibond correlation (HMBC) technique. Authentic farnesyl acetate was used to assist identification of the farnesyl carbon resonances. For the first time, we have directly observed solution ^{15}N-NMR signals for the Mg-chlorin macrocycles with all resonances assigned by the HMBC experiment. Again, two resonances are observed for each nitrogen atom of the BChl *c* dimer. The results of this study provide not only the crucial basis for the assignment of corresponding ^{1}H-spectra through H−C correlation but also alternative probes for detecting chemical and electromagnetic environments in the BChl *c* dimer. Such information is also relevant to other photosynthetic pigments in terms of their in vivo self-assembly property, e.g., two BChl *a* molecules form an approximately symmetric dimer, the so-called special pair, in the bacterial reaction center. Both experimental and computational approaches have been made to the ^{15}N and ^{13}C NMR behavior of the BChl *a*.[17−19]

In an earlier study,[7] we first applied solid-state CP/MAS ^{13}C NMR to the powder samples of both 740-nm high-aggregate and native chlorosome. It is suggested that structural arrangement of in vitro BChl *c* aggregates is very similar to that in the chlorosomes. The results have been confirmed and improved subsequently by 2D radio frequency-driven dipolar recoupling experiments.[10] Owing to large line width inherent in the spectra of the solid samples, it was difficult to explicitly determine the chemical shifts for all atoms including the farnesyl side chain. We will show that this can be achieved with the solution sample of the BChl *c* dimer.

Materials and Methods

Isotope Enrichment and Sample Preparation. (3[1]*R*)-[*E*,*E*]BChl c_F was extracted by methanol from *Chlorobium tepidum* dry cells and purified by a reverse-phase HPLC column as described previously.[20] For natural-abundance sample, the cells were grown in the medium of Wahlund[21] containing sodium bicarbonate, ammonium acetate, and ammonium chloride as carbon and nitrogen sources. For isotope-enriched samples, the ammonium acetate was replaced by sodium acetate with adjustment of the concentration of ammonium chloride to balance the ammonium concentration. Partially ^{13}C-enriched sample was obtained with a medium containing sodium acetate-*1*-^{13}*C* (^{13}C >99.9% atom, Isotec Inc. USA) and $NaHCO_3$ of natural abundance. This medium resulted in a highly randomly ^{13}C-labeled (3[1]*R*)-[*E*,*E*]-BChl c_F (thereafter referred to as fractionally ^{13}C-enriched sample). The average enrichment factor was estimated to be less than 40% based on ^{1}H-NMR integration of 3[1]-H signals. Fully ^{13}C-labeled sample was grown in a medium containing $NaH^{13}CO_3$(^{13}C >98% atom, Isotec Inc.) as the sole carbon source (thereafter referred to as fully ^{13}C-labeled sample). ^{15}N-labeled (3[1]*R*)-[*E*,*E*]BChl c_F was obtained with $^{15}NH_4Cl$ (^{15}N >99% atom, Isotec Inc.). Farnesyl acetate (*trans,trans*, purity >95%) was purchased from Aldrich Chem. Co. (Milwaukee, WI). Deuterated methanol (CD_3OD, D >99.95%) was from Merck (Darmstadt, Germany). Carbon tetrachloride (purity >99.8%, Infinity pure grade) was purchased from Wako Pure Chemical Industries, Ltd., Japan, and dried with Na_2CO_3 during storage. BChl *c* solutions were prepared under N_2 atmosphere by dissolving the dried pigments in solvents with a monomer concentration of 2 mM. The solutions were then moved into NMR tubes and further purged with N_2 gas before the NMR tubes were sealed.

NMR Measurements. NMR spectra were collected on Bruker DRX-400 and DRX-500 spectrometers at a temperature of 22 °C. Field-frequency lock for the sample in pure CCl_4 solution was achieved by inserting a 1-mm D_2O-filled inner tube in the 5-mm NMR tubes. One-dimensional proton-decoupled ^{13}C spectra were recorded with a 30° pulse, 32 K data points, and a repetition time of 1.8 s. Distortionless enhancement by polarization transfer (DEPT)[22] spectra were recorded using both natural abundance and fractionally ^{13}C-enriched samples with a 135° read pulse to give CH, CH_3 positive, and CH_2 negative followed by proton decoupling during acquisition. Two-dimensional ^{13}C−^{13}C double quantum INADEQUATE spectra were acquired using the fully ^{13}C-labeled BChl *c* sample with 512 t_1 points, 2 K data points in t_2, and 256 transients for each t_1 point using a pulse sequence described by Turner[23] in which the split t_1 domain is used to give a symmetric, COSY-like spectrum. ^{13}C chemical shifts were referenced to tetramethylsilane. One-dimensional proton-decoupled ^{15}N spectra were recorded with 32 K data points and a repetition time of 5 s. Two-dimensional ^{1}H−^{13}C and ^{1}H−^{15}N long-range HMBC spectra were acquired with a pulse sequence reported by Bax and Summers[24] using gradient pulses for selection and with low-pass *J*-filter to suppress one-bond correlation. A total of 512 (256 for ^{15}N) t_1 and 2K t_2 data points were recorded with a delay time of 100 ms for evolution of long-range

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Table 1. ^{13}C-NMR Assignment of *trans,trans*-Farnesyl Acetate in $CDCl_3$

position	chemical shift (ppm)	position	chemical shift (ppm)
CH_3**C**OO−	170.18	f4	39.00
f3	141.22	f9	26.20
f7	134.70	f5	25.66
f11	130.40	f12	25.06
f10	123.86	**CH**$_3$−COO	20.18
f6	123.15	f11a	17.02
f2	118.12	f3a	15.74
f1	60.67	f7a	15.35
f8	39.21		

couplings. ^{15}N chemical shifts were referenced to 3 M NH_4Cl in 1 M HCl (24.9 ppm).

Results and Discussion

^{13}C NMR Assignment of Farnesyl Acetate. To simplify analysis of the spectra of the intact (3^1R)-[*E*,*E*]BChl c_F, we first measured ^{13}C NMR for the authentic farnesyl acetate. Due to the structurally high similarities, it was difficult to clearly assign the resonances of f6 and f10, f4 and f8, and f5 and f9 pairs even from a well-resolved 1D spectrum. For this reason, we conducted two-dimensional ^{13}C−^{13}C correlation experiments with the natural-abundance *trans,trans*-farnesyl acetate in $CDCl_3$. From the INADEQUATE spectrum (Supporting Information), carbon−carbon connectivity can be unambiguously established, and the assignments for each carbon atom are given in Table 1. Chemical shifts of f6 and f10 were determined based on their correlations with f7 and f11, respectively, and these further led to the assignments for f5 and f9. The nearly identical resonances of f4 and f8 can be distinguished by their cross signals correlating with f3 and f7, respectively. Three side methyl groups at upfield, f3a, f7a, and f11a, were readily assigned by their correlations with corresponding quaternary carbons at downfield. The overall assignment can be compared with an early ^{13}C NMR study by Jautelat et al.[25] on farnesol, in which assignments for f3a and f7a methyls, f4 and f8 methylenes, and f2, f6, and f10 methines were only provisional. The chemical shifts for f2 and f6, f4 and f8 pairs were found to be in reverse order.

^{13}C NMR Assignments of the Intact [*E*,*E*]BChl c_F Monomer in Methanol-d_4. Before we show ^{13}C NMR assignments for the [*E*,*E*]BChl c_F dimer, it is necessary to review the assignment of monomer as, despite much effort, discrepancies still exist in the literature and they could largely affect the results of the dimer assignment. A number of the resonances, including our own reported previously, were found to need reassignment. The results are shown in Table 2. Chemical shifts of the farnesyl side chain carbons remained the same order as for farnesyl acetate in $CDCl_3$ described above. The most downfield resonance was confirmed to be C13^1 carbonyl from its long-range correlation with H13^2. Another carbonyl carbon, C17^3, can be assigned from its correlations with C17^2, H17^2, H17^1, and f1 protons. Due to the close resonances between meso carbons, C15 and C20, it was difficult to determine the order between C14 and C19 from ^{13}C−^{13}C correlation. However, these carbons can be easily assigned from HMBC experiment with C19 correlating to H18, H18^1, H20^1, and C14 to H13^2. Other quaternary carbons were assigned in a similar way. The assignments in the low-field region were in principle consistent with those reported for chlorophyll *a* in acetone by Lötjönen and Hynninen[26] and BChl *c* in methanol by this laboratory[7] except for a reverse order between C3 and C4, but were different from those for methyl pheophorbides in chloroform.[27−29] In contrast, more variations were found in the high-field region (15∼40 ppm). The order of f8 and f4 resonances was confirmed by the INADEQUATE experiment using the fully ^{13}C-labeled BChl *c* sample. The same experiment allowed unambiguous assignment for C17^2 and C17^1, as these signals can be readily distinguished from their correlations with C17^3 carbonyl and C17 carbons, respectively. This is contrary to previous assignments which relied on protonation shifts[30] or upon comparisons with chlorophyll *a* T_1 measurements,[31] but is in agreement with the conclusion from a single frequency off-resonance proton-decoupling experiment.[27] Resonances of C3^2 and f12 methyls appeared at higher field than those of f9 and f5 methylenes, as confirmed from the DEPT spectrum, with C3^2 assigned based on its correlation with C3^1. The order of C12^1 methylene and C20^1 methyl resonances was definitely determined from the DEPT spectrum, which is the reversal of that reported for BChl *c* in chloroform/methanol (9:1).[10] Resonances of C12^2 and f11a were coincident and appeared at higher field than C8^2 (correlating to C8^1) but at lower field than C2^1. The most upfield signal was assigned to C7^1 from its correlation with the downfield C7 resonance. These discrepancies mentioned above may be partly attributed to the differences in substituent groups and the solvents used; however, it is noted that for those well-assigned signals the values of chemical shifts of this study were essentially the same as those of BChl *c* in $CDCl_3/CD_3OD$ (9:1).[10]

Table 2. Assignments of ^{13}C NMR Spectra of Intact [*E*,*E*]BChl c_F

position	CD_3OD	CCl_4[a]	position	CD_3OD	CCl_4[a]
13^1	197.49	195.9, 193.6	5	99.66	100.7, 95.3
17^3	173.52	174.2, 171.7	3^1	64.71	64.5, 63.3
19	167.77	168.0, 166.9	f1	60.43	61.7, 60.3
14	160.88	162.7, 161.1	17	50.03	51.0, 50.7
16	153.70	154.6, 153.8	13^2	48.31	48.7, 48.2
1	153.66	152.6, 151.9	18	48.03	49.4, 49.1
6	150.77	150.1, 150.0	f8	39.02	39.5, 39.3
11	146.19	147.5, 147.1	f4	38.65	39.3, 39.2
9	145.91	146.5, 145.7	17^2	30.06	31.5, 30.6
3	145.60	144.2, 144.0	17^1	29.48	30.6, 28.8
4	145.23	143.7, 143.5	f9	25.93	26.6, 26.3
8	143.06	142.6, 142.7	f5	25.31	26.0, 25.9
f3	141.49	142.4, 140.6	3^2	24.98	24.3, 22.6
12	139.62	140.2, 139.8	f12	24.14	25.8, 25.6
2	134.91	135.5, 133.7	12^1	20.31	(19.9, 19.8)
f7	134.53	134.8, 134.6	20^1	20.24	21.3, 21.2
7	133.09	132.7, 131.3	18^1	19.68	21.4, 20.4
f11	130.29	130.3, 130.3	8^1	18.70	(19.9, 19.8)
13	129.67	128.5, 128.8	8^2	16.36	17.9, 17.6
f10	123.63	124.4, 124.1	12^2	16.00	16.9, 16.4
f6	123.13	123.6, 123.4	f11a	16.00	17.5, 17.4
f2	117.89	118.7, 117.2	2^1	15.93	15.3, 14.3
10	104.97	106.4, 106.2	f3a	14.64	16.3, 16.1
15	104.67	105.7, 104.9	f7a	14.30	15.9, 15.7
20	104.55	103.9, 103.7	7^1	9.41	11.4, 10.9

[a] Parentheses indicate provisional assignment, see text.

^{13}C NMR Assignments of the Intact [*E*,*E*]BChl c_F Dimer in CCl_4. With the reliable assignment of the monomeric form,

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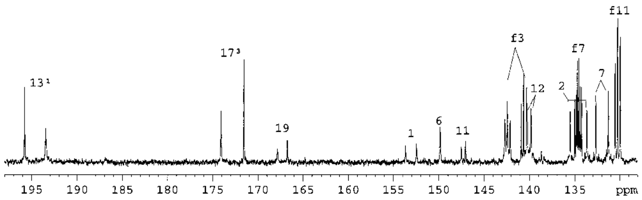

Figure 2. Low-field proton-decoupled ^{13}C NMR spectrum of the fractionally ^{13}C-enriched intact (3^1R)-[*E*,*E*]BChl c_F in pure CCl_4. Note that some quaternary carbons are not or only slightly labeled. For the full assignment, see Table 2 and text.

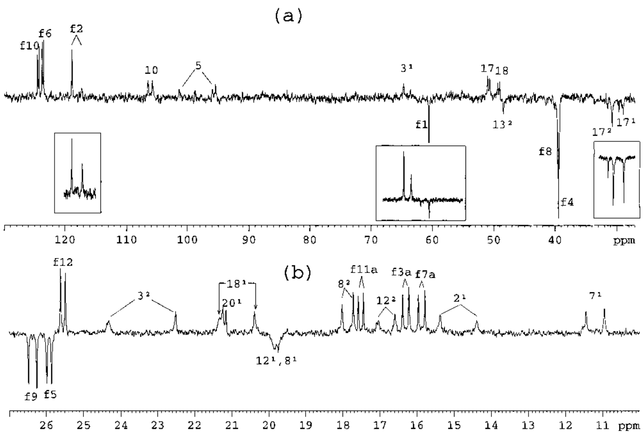

Figure 3. ^{13}C DEPT spectrum and assignments of natural abundance (3^1R)-[*E*,*E*]BChl c_F in pure CCl_4 for all proton-attached carbons. Negative peaks represent methylene carbons. Several resonances with low S/N ratio were confirmed using the fractionally ^{13}C-enriched sample, as shown in the insets.

we were able to specifically assign all carbon resonances of the intact BChl *c* dimer formed in pure CCl_4. The full assignments were listed in Table 2. Most assignments were made using natural-abundance sample, while the resonances of low S/N ratio were determined with the ^{13}C-enriched sample. A typical low-field region spectrum obtained with the fractionally ^{13}C-labeled sample is shown in Figure 2. The labeling pattern with 1-^{13}C-acetate as one of the substrates revealed that macrocyclic carbons C9, C14, C16, as well as all meso carbons were specifically unlabeled, and C3, C4, C8, and C13 were only slightly enriched. The former is in agreement with the biosynthetic pathway of tetrapyrrole compounds via δ-aminolevulinic acid. The carbon−carbon coupling pattern was found to be very useful in identifying the resonances of farnesyl quaternary carbon (f3, f7, and f11) in the overcrowded low-field region (130−150 ppm). Because each of these carbons gave to a triplet signal in the ^{13}C spectrum in methanol due to the coupling with its neighboring methine carbon (f2, f6, and f10), each of these signals further split into one triplet pair characteristic of the sharp line shape in CCl_4 (Figure 2). Closely spaced signals of C3 and C4, which were almost unlabeled with the 1-^{13}C-acetate medium, were distinguished by 2D-INADEQUATE experiment using the fully ^{13}C-labeled sample. The C4 pair was determined from its correlation with the C5 pair.

Figure 3 shows the DEPT-135 spectrum of natural-abundance [*E*,*E*]BChl c_F in CCl_4 for all proton-attached carbons. Negative peaks represent methylene carbons. The fractionally ^{13}C-enriched sample was used for the assignments of several resonances with low S/N ratio, as indicated in the insets in Figure 3a. Positions of meso carbon C5 were confirmed by a H−C correlation experiment.[15] Only one signal of C13^2 was observed from the DEPT spectrum, the other one that was almost coincident with that of the C18 but with opposite phase can be judged as a clear shoulder on the signal of the C18 peaks from a normal 1D spectrum (see Figure 5 in ref 15). The order of C17^2 and C17^1 methylenes remained unchanged upon dimer formation as was confirmed by a correlation observed between C17^2 and carbonyl C17^3. Three methyls C3^2, C18^1, and C20^1 can be easily assigned on the basis of the H−C and C−C correlation spectra despite large shifts of C3^2 signal to a higher field and the large separation of C18^1 peaks with respect to the chemical shifts of their corresponding monomeric form. Two methylene carbons C8^1 and C12^1 appeared as a total of two peaks in the dimer spectra, which could not be distinguished from each other even for the ^{13}C-enriched sample. Therefore, these signals can only be considered as being accidentally coincident. Resonances for C8^2, C12^2, and C2^1 methyl carbons that were closely spaced in the monomer spectrum were well resolved in the dimer spectra, and were assigned by the 2D-INADEQUATE spectrum.

Similar to ^{1}H-NMR, the most characteristic feature of the ^{13}C NMR spectra for [*E*,*E*]BChl c_F in CCl_4 is that each individual carbon gave rise to two resonances, indicating different environments for the corresponding carbon atoms in different molecules of the dimer. The wide spectral range of ^{13}C NMR allows one to evaluate not only the chemical shift change but also the spectral shape. There is a striking feature in the dimer spectra where several carbon pairs had significantly different line widths. The asymmetric resonance pairs were mainly found for the propionic side chain carbons (C17^1, C17^2, and C17^3) and the first two carbons of the farnesyl chain (f1 and f2). Such information was deficient in the corresponding ^{1}H spectra, because H17^1, H17^2, and f2 proton signals were heavily overlapped with other signals and only one resonance was observed for the f1 protons in the dimer spectrum.[15] The asymmetric behavior may reflect very different conformations adopted for this portion of the long side chain in the two molecules in dimer, leading to a substantial difference in the molecular motion. It was suggested, based on ^{1}H-NMR results, that the farnesyl side chain in dimer may take a folding-back conformation with the f1 methylene being positioned at the folding point and with most of the tail part fluctuating around the periphery of the macrocycle.[15] The ^{13}C NMR result of this study further suggested that the propionic-farnesyl side chain forms a “return” conformation with one of the “returns” experiencing a highly restricted motion compared with another. It was of interest to note that a similar phenomenon was reported for methyl bacteriochlorophyllide *d*,[32] in which two ester methyl signals in the ^{1}H spectra of dimer showed very different complexation shifts and line widths. These very contrasting signals were interpreted in terms of possible conformations of the propionic side chain.

The changes in ^{13}C chemical shift upon dimer formation were relatively small compared with those in ^{1}H spectra.[15] Very careful inspection was required as interchanges of the order in chemical shift occurred for a number of closely spaced, or overlapping signals, and the situation was further complicated by peak splittings in the dimer spectra. This was observed for the resonances of C2, C13^2, C3^2, C12^1, C18^1, C12^2, and C2^1 (see Table 2). For most of these carbons, one or both of the two signals shifted across their neighboring peaks to higher fields; whereas for C18^1 one resonance shifted across C20^1 to a lower field. Resonances of C12^2 and f11a, coincident in the spectrum with methanol, resolved in the dimer spectrum with that of C12^2 shifted to a higher field. The order of C2^1 and f3a,

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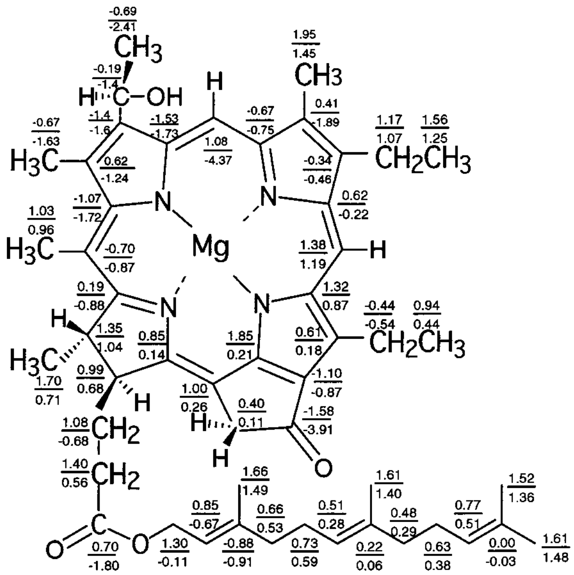

Figure 4. Schematic map showing the ^{13}C complexation shifts, $\Delta\delta = \delta_{dimer} - \delta_{monomer}$, of (3^1*R*)-[*E*,*E*]BChl c_F dimer. All values were obtained using Table 2.

f7a was exchanged. Figure 4 illustrates a schematic map of the complexation shifts, defined by $\Delta\delta = \delta_{dimer} - \delta_{monomer}$. Large chemical shift changes were found for C1, C3, C3^2, C4, C5, C7, C13^1, and C14. All carbons attached to pyrrole ring I exhibited large negative complexation shifts, while most other carbons including the farnesyl chain showed a general trend of positive complexation shifts. The farnesyl backbone carbons had small shift changes, whereas the side methyl carbons had relatively large downfield shifts.

The ^{13}C chemical shift changes upon dimer formation can be considered as a result of combined effects including (a) ring current due to the partial overlap of the macrocycle, (b) coordinating state of the central Mg atom, (c) changes in excitation states, as was observed from absorption spectrum, (d) polarity of the solvents used, and (e) hydrogen bonding. Of these factors, only ring current and hydrogen bonding could bring about an upfield shift under the conditions of this study in going from methanol to carbon tetrachloride as described below, while all other effects would result in downfield shift. The ring-current effect was the most remarkable, overriding other contributions to give substantial upfield shifts for the ring I and ring I-attached carbons (Figure 4). This is in accordance with our ^{1}H-NMR results,[15] showing significant upfield shifts for all resonances of H2^1, H3^1, H3^2, and H5, and supports that the [*E*,*E*]BChl c_F forms an antiparallel dimer mutually overlapped around the pyrrole ring I with an asymmetric configuration. The asymmetric feature was also characterized by the complexation shifts, for example, one of the resonances of C5 shifted more than 4 ppm to higher field while another shifted about 1 ppm to lower field with respect to the monomer form in methanol. Most macrocyclic carbons, except for those around ring I, experienced downfield shifts in CCl_4. A similar phenomenon was reported by Lötjönen and Hynninen for all macrocyclic carbons of chlorophyll (Chl) *a* in going from THF to acetone.[33] Since Chl *a* exists in monomeric form in both solvents, the result was explained in terms of a change in ligation of the solvent molecules to the axial positions of central Mg. Electronic absorption and resonance Raman spectroscopy suggested that the central Mg of Chl *a* is predominantly hexacoordinated in THF, whereas it is pentacoordinated in acetone.[34−36] The different interactions of THF and acetone with the Mg atom of Chl *a* may cause different conformations of the flexible macrocycle and redistribution of charge density within the molecule.[33] This conclusion can also be applied to [*E*,*E*]BChl c_F of this study, as we have shown that BChl *c* forms pentacoordinated aggregates in vivo as well as in acetone, ether, and other nonpolar solvents in vitro, whereas the Mg atom is hexacoordinated in methanol and pyridine from the results of resonance Raman,[37] absorption, CD, and magnetic CD.[38] Therefore, the downfield shifts of the macrocyclic carbons may partly be attributed to a change of coordination number from six in methanol to five in CCl_4. Changes in excitation state could also alter the ^{13}C chemical shift because the magnitude of the paramagnetic term can be approximated to be inversely proportional to an average excitation energy,[39] and such effect was first used by Boxer et al.[40] to interpret the large downfield shifts upon insertion of Mg into methyl pheophorbide. The strong Q_y absorption band of [*E*,*E*]BChl c_F shifted from 668 nm in methanol to 710 nm in CCl_4, corresponding to a decrease of 2.5 kcal/mol in the transition energy. Assuming that the average excitation energy in the dimer is dominated by the $\pi \rightarrow \pi^*$ transition, then about 6% increase to the maximum in the paramagnetic term (leading to downfield shifts) is expected for the macrocyclic carbons. Intrinsic solvent effect may be estimated from the chemical shifts of the farnesyl carbons provided that the side chains are in completely free motion in the solutions. A comparison of ^{13}C chemical shifts of farnesyl acetate in methanol-d_6 and $CDCl_3$ revealed that backbone carbons only had shift differences less than 0.18 ppm while all methyl carbons had about 0.9 ppm downfield shifts in chloroform with respect to those in methanol (data not shown). However, a somewhat different situation may be expected for the chlorin-attached farnesyl side chain. As mentioned above, all ^{13}C- and ^{1}H-NMR results indicate that large portions of the farnesyl groups are entangled around the periphery of the dimer macrocycles in CCl_4 with a restricted motion. Consequently, the ^{13}C chemical shifts for the farnesyl chain in CCl_4 might contain an effect of steric strain resulting from interactions between farnesyl and macrocyclic carbons or peripheral groups. The downfield-shift effect in CCl_4 was also demonstrated by a solvent-dependent experiment, in which the ^{13}C resonance of tetramethylsilane shifted about 1.7 ppm to higher field when CCl_4 was substituted for methanol as a solvent.[41] The large upfield shift observed for carbonyl carbon C13^1 is interpreted in terms of the effect of hydrogen bonding rather than ring current. This conclusion is based on our observation[7] that although [*E*,*E*]BChl c_F exists as monomer in both acetone and methanol an upfield shift of 3.6 ppm was measured when the solvent was changed to acetone from methanol which forms the hydrogen bond with C13^1 oxygen. The result is in good agreement with the conclusion from ^{1}H-NMR study[15] showing no evidence for a macrocycle overlap on the ring V, since any interaction between C═O and Mg or C═O and −OH would

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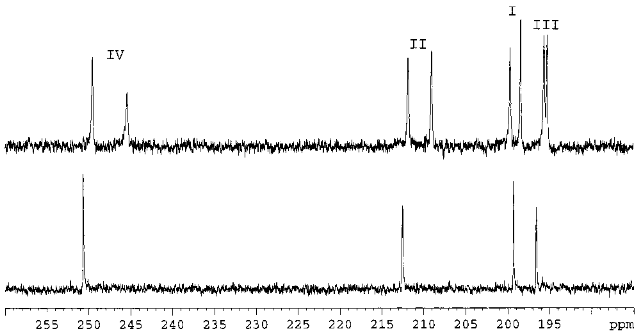

Figure 5. Proton-decoupled ^{15}N spectra for the fully ^{15}N-labeled (3^1R)-[*E*,*E*]BChl c_F in methanol-d_4 (bottom) and in CCl_4 (top). Both concentrations were 2 mM. Chemical shifts were referenced to 3 M NH_4Cl in 1 M HCl (24.9 ppm).

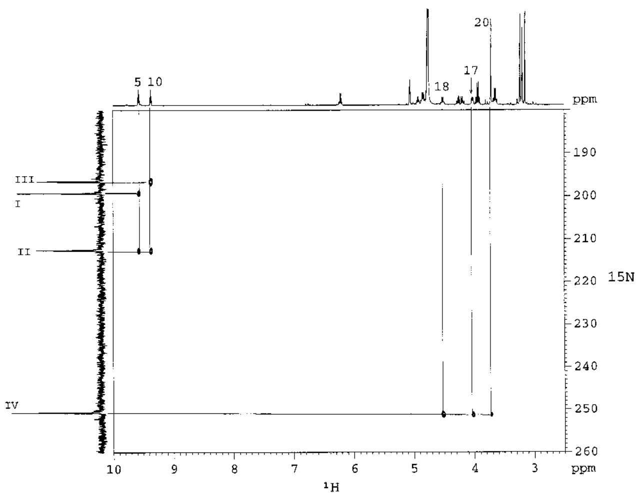

Figure 6. $^1H-^{15}N$ HMBC spectrum obtained with the fully ^{15}N-labeled (3^1R)-[*E*,*E*]BChl c_F in methanol-d_4 at a concentration of 2 mM, along with the assignment. Note that triplets can be seen on the signals of H5 and H10 with a coupling constant of 4.6 Hz.

result in a downfield shift. An example of the former has been given in a disaggregating process of chlorophyll *a* dimer by THF.[42]

^{15}N-NMR of the Intact [*E*,*E*]BChl c_F in Methanol and CCl_4. Figure 5 shows proton-decoupled ^{15}N-NMR spectra of the fully ^{15}N-labeled intact [*E*,*E*]BChl c_F in methanol and CCl_4. Assignment was made by long-range $^1H-^{15}N$ correlation experiment, which is shown in Figure 6 for the [*E*,*E*]BChl c_F in methanol. A coupling constant of $^3J_{NH} = 4.6$ Hz was obtained from the ^1H-NMR spectrum where clear triplets were observed on H5 and H10 methine signals. Table 3 shows the assignment. In methanol, resonance of N_{IV} appeared most downfield, followed by N_{II}, N_I, and N_{III} toward higher field. In CCl_4, two signals were observed for each type of nitrogen atom, confirming the existence of a stable and asymmetric [*E*,*E*]BChl c_F dimer. N_I resonances separated with one at a lower field and another to a higher field with respect to that in methanol, whereas all other resonances shifted to higher fields. Apparently different line widths were found for the N_{IV} and N_I pairs. Separations of the chemical shift between the two signals in each pair increased with the increase of chemical shift.

To our knowledge, this is the first observation of solution ^{15}N-NMR signals directly detected from Mg-chlorin compounds, although similar results have been reported for Mg-bacteriochlorins with a higher symmetry.[18,43] Boxer et al.[40] measured ^{15}N spectra of pheophytin *a*, but failed to detect the signals from 95% ^{15}N-enriched Chl *a* in acetone even at a maximum pulse interval for 2 days. The ^{15}N chemical shifts of the four BChl *c* nitrogen atoms in this study are close to those determined by an indirect method;[40] however, the order is reversed between N_I and N_{III}. The difference may be attributed to the different solvents used, as a strong solvent effect was observed on the relative ordering between N_I and N_{III}, as well as N_{II} and N_{IV}, in the case of BChl *a*.[18] Unlike ^{13}C and ^1H spectra, no obvious ring-current effect was measured on ^{15}N chemical shift of the dimer spectrum in CCl_4, implying that the ^{15}N chemical shift of BChl *c* is predominated by contributions from paramagnetic shielding and other factors. N_{IV} nitrogen atoms were most sensitive to the dimer formation, followed in the order of N_{II}, N_I, and N_{III}, as can be seen from the changes and separations of chemical shifts for each pair of resonances in the dimer spectrum. The differences in the ^{15}N chemical shifts of the same type nitrogens reflect different electronic environments involved with the respective molecules in the dimer, and may be interpreted in terms of their electronic states and N−Mg bond lengths. Spangler et al.[44] found a good quadratic correlation between ^{15}N chemical shifts and p-orbital populations for the magnesium-free and magnesium-containing porphyrin derivatives, which was not applicable to the ^{13}C chemical shifts. It is of interest to note that their calculations of ab initio quantum mechanics revealed significant changes in the populations of $N_{IV}-Mg$ σ bond, N_{IV} π-orbital, and $N_{II}-(Mg-N_{II})$ $\sigma-\pi$-type bond order upon coordination of H_2O to the magnesium of ethyl chlorophyllide *a*. This result may mean that the electronic states of N_{IV} and N_{II} nitrogens are more sensitive to environmental changes than those of N_I and N_{III}. Another ab initio calculation and solid state ^{15}N-NMR study by Strohmeier et al.[45] on highly symmetric metal−tetraphenylporphyrin compounds suggested that the ^{15}N chemical shifts tend to be determined by the metal−nitrogen separation. Considering the close proximity of about 3.3 Å separation between the overlapping macrocyclic planes of [*E*,*E*]BChl c_F dimer,[15] it is reasonable to explain the ^{15}N chemical shift differences between similar nitrogens in the dimer spectrum as a consequence of strong intermolecular interaction, which leads to a slightly asymmetric electronic state and bond lengths for corresponding nitrogen atoms in the dimer.

Table 3. Assignments of ^{15}N-NMR Spectra of Intact [*E*,*E*]BChl c_F

position	CD_3OD	CCl_4	complexation shift
N_I	199.32	199.72	0.41
		198.44	−0.87
N_{II}	212.48	211.87	−0.61
		209.04	−3.44
N_{III}	196.59	195.69	−0.90
		195.30	−1.29
N_{IV}	250.66	249.56	−1.10
		245.44	−5.23

Conclusions

In this study, complete assignments of ^{13}C and ^{15}N solution NMR spectra are reported for intact [*E*,*E*]BChl c_F in pure methanol and CCl_4. Two sets of resonances for each carbon and nitrogen atom were observed in CCl_4 solution, supporting a dimeric structure derived from ^1H-NMR study. The ring-

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current effect was large enough to override other effects in the ^{13}C spectrum as indicated by the large upfield shifts of the carbons around pyrrole ring I, where the two macrocycles apparently overlap, with respect to the chemical shifts of its monomeric form in methanol. Most other macrocyclic (including substituent groups) and farnesyl carbons exhibited downfield shifts. Three effects under the conditions of this study are considered to bring about a downfield shift, i.e., coordinating state of the central magnesium atom, transition energy of the π electron system, and polarity of the solvent. However, we are unable to explicitly evaluate each of these effects from the current data. Propionic-farnesyl side chains in the dimer seem to form a "return" structure over a region from 17^1 to f2 carbons with different conformation and molecular motion. There is no apparent hydrogen bond formed in the C13^1 carbonyl group, as well as no ring overlap over the ring V in the dimer. ^{15}N chemical shift appears to be predominated by the paramagnetic shielding term rather than the ring current. Resonances of N_{IV} and N_{II} nitrogens were most sensitive to the dimer formation. Sensitivity of the electronic state of the nitrogen atom suggests that the macrocyclic interactions could cause slight differences in the electron population and in the N−Mg bond length for each corresponding nitrogen atom in the dimer.

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Supporting Information Available: ^{13}C NMR spectral collection with assignment for the authentic *trans*,*trans*-farnesyl acetate and for the intact (3^1*R*)-[*E*,*E*]BChl c_F (natural abundance, fractionally ^{13}C-enriched and fully ^{13}C-labeled samples) in methanol-d_4 and CCl_4 including 1D proton-decoupled spectra, DEPT spectra, 2D-HMBC, and 2D-INADEQUATE spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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