Chlorophyll–Amino Acid Interactions in Synthetic Models

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Abstract. A number of amino acids and molecules of related functionality have been covalently connected to pyrochlorophyllide *a* (PChl*a*) and bacteriopyrochlorophyllide *a* (BPChl*a*) to determine their impact on the properties of the chromophore. PChl*a* and BPChl*a* esterified with side chains containing the thio-ether functionality (methyl-6-hydroxyhexylsulfide, a Met analogue) were studied in detail by NMR. Intramolecular coordination at the central metal is observed for the Zn-PChl*a* and Zn-BPChl*a* thio-ether derivatives, but not for the Mg-PChl*a* derivative. The NMR data for the Zn-PChl*a* derivative is interpreted as providing evidence for two, distinguishable five-coordinate complexes, with the ligand on either side of the macrocycle. In contrast, the Zn-BPChl*a* derivative appears to be coordinated exclusively on one side. It is proposed that stereoselective coordination may provide a simple mechanism for altering the spectroscopic properties of BChl*a* when it binds to a protein. PChl*a* was covalently attached to the methyl esters of L-Trp, L-Tyr, L-Phe and L-Val. Very large, similar chemical shift changes in the NMR spectra in the absence of a coordinating ligand indicate that all of these compounds form a novel dimer, in which the carbonyl group of the carbomethoxy group on the amino acid coordinates to the central metal, bridging between macrocycles. An average structure is proposed for this dimer in which the macrocycle planes are parallel, overlap in ring IV and have their *x*-axes antiparallel.

It is becoming increasingly evident that the majority of chlorophyll (Chl) in photosynthetic organisms is associated intimately with proteins.[1] We can divide the nature of the interaction between Chls and proteins into three broad classes. First, the fifth and/or sixth ligands on Chl are likely to be amino acid side chains. There are many possible candidates based on studies of Chl ligation in organic solvents: hydroxyl, such as Ser, Thr and Tyr; amines, such as Lys, Arg and His; and sulfur-containing, such as Cys and Met. Second, the amino acids could form π-complexes with the macrocycle. Possible examples are Phe, Tyr and Trp. Third, the tertiary structure of the protein may compress or twist the chromophore or restrict the free rotation of chromophore side chains.

The first and third of these classes of interactions can have significant effects on the spectroscopic and functional characteristics of the chromophore. For example, the ligand has been shown to affect the position of absorption bands in organic solvents[2] and the distribution of unpaired spin density in the radical ions.[3] The second class of interactions is much weaker and is not expected to affect the properties of the chromophore. Aromatic residues are likely to be found in nonpolar regions of the protein and may participate in solvating the nonpolar chromophore. Their presence can have a substantial effect on the circular dichroism (CD) of the chromophore, as they possess intense optical transitions which can couple to highly excited Chl transitions.[4]

This study was motivated by the preparation in our laboratory of a large number of well defined 1:1 Chl–protein complexes by combining chlorophyllides with apomyoglobin.[5,6] These protein complexes in solution generally exhibit very minor spectroscopic changes relative to the chromophore in organic solvents, but the chromophore absorption is changed in single crystals of the complexes.[7] We have undertaken a systematic examination of Chls which are covalently connected to a wide range of compounds, many of which are amino acids or contain closely related functionality. The advantage of covalent attachment is simply that intramolecular association is favored, so that well defined species are formed in solution, whose average structures and spectroscopic properties can be investigated. This principle was nicely demonstrated by Sanders et al.,[8] who covalently attached an imidazole side chain to chlorophyllide *a* and found that the complex was internally coordinated and monomeric regardless of solvent. A disadvantage is that the covalent linkage may restrict the interaction to certain possibilities and exclude others. In this paper we have selected certain amino acids which produce complexes with interesting properties.

We have found that high-resolution NMR spectroscopy provides the best method to characterize the interaction between ligands and the Chl macrocycle in solution. Extensive studies have shown that Chl aggregates with itself in the absence of a coordinating ligand.[9] The NMR spectra of these aggregates are characterized by very broad lines, and all resonances show an upfield shift relative to the monomer. The largest upfield shifts are observed for protons in rings III and V, suggesting that aggregates are formed by interaction between the central Mg (or Zn) atom and the keto-carbonyl group at position 9. The broad lines are presumably a consequence of slow exchange among many possible conformations of this dimeric aggregate. This observation must be borne in mind when interpreting the NMR data for our synthetic models. The observation of sharp NMR lines in noncoordinating solvents ($CDCl_3$, C_6D_6) can be taken as evidence that the covalently attached ligand competes favorably with the intermolecular Mg $\cdots$ O = C interaction.

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EXPERIMENTAL

L-Trp-methylester, tryptamine, L-Tyr-methylester, L-Phe-methylester, L-Val-methylester and ethyl-2-hydroxyethylsulfide were obtained commercially. Methyl-6-hydroxyethylsulfide was a generous gift from Professor Collman. Crystalline pyropheophorbide *a* (PP*a*) and bacteriopyropheophorbide *a* (Fig. 1) were prepared by well established methods.[6,10] All solvents were highly purified in our laboratory.

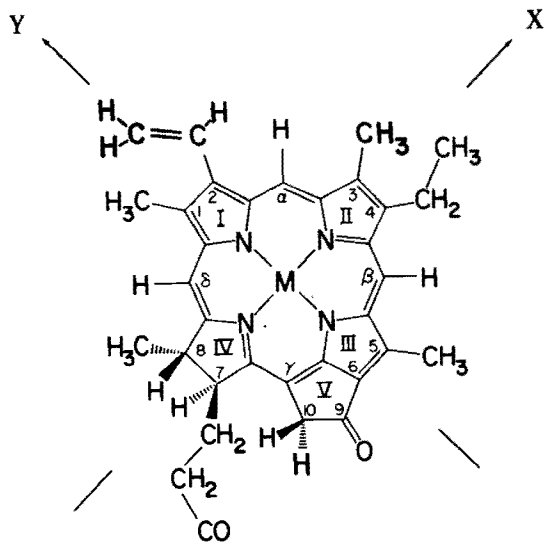

Fig. 1. Structure and numbering system for the pyrochlorophyllide *a* fragment. The metal-free compound, pyropheophorbide *a* is abbreviated PP*a*; metal (M) containing compounds are abbreviated M-PChl*a*. Bacteriopyrochlorophyllide *a* (BPChl*a*) has ring II trans-reduced and acetyl in place of vinyl at position 2.

The covalently linked compounds were prepared by the following procedure: the activated ester of PP*a* was prepared by reaction with a five-fold excess of 2-chloro-N-methylpyridinium iodide and triethylamine in dry methylene chloride.[11] A five-fold excess of the compound to be attached was added, and the reaction was stirred under argon at 50 °C for 1 h. The coupled product was purified by silica thin layer chromatography (TLC, typical solvent 1/3, acetone/CCl_4). NMR of the resulting compound at 360 MHz provides very adequate structure proof, as no side reactions occur with this very mild coupling reaction. Zn[12] or Mg[13] insertion followed well established methods, and the final product was purified on silica TLC plates, which were deactivated by prerunning with methanol.

Water was removed from the samples prepared for NMR by codistillation with dry CH_2Cl_2 prior to addition of the NMR solvent. NMR spectra were obtained at 360 MHz at room temperature and chemical shifts are referenced to TMS. Optical absorption and CD were measured on a Cary 17 and Durrum-Jasco spectrometers, respectively, using 10^{-3}-cm pathlength cells so that the same sample could be studied by NMR and optical methods.

RESULTS AND DISCUSSION

Methionine Analogues

Pyropheophorbide *a* was esterified with ethyl-2-hydroxyethylsulfide and methyl-6-hydroxyhexylsulfide, and both the Mg and Zn complexes were prepared. Both Mg complexes were found to have very broad NMR spectra in the absence of an added coordinating ligand. Thus it appears that the interaction between a thio-ether type sulfur and Mg does not compete effectively with the intermolecular interaction between Mg and the keto-carbonyl group. By constrast the Zn complexes exhibit sharp NMR spectra. This difference is not surprising because Zn is a "softer" atom than Mg and sulfide is a "soft" ligand. This result does not, of course, imply that methionine will never be found to be a ligand for chlorophyll in vivo, although we expect that the interaction will be quite weak.

The NMR spectrum of Zn-pyrochlorophyllide *a* 2-(ethylthio)ethylester has sharp lines and upfield shifts for all positions on the attached ligand and for all protons on the periphery of the macrocycle (largest for the 5, 10, and β-methine peaks). Furthermore, the methylene protons of the terminal ethyl are found to be diastereotopic. The upfield shifts were found to be strongly dependent on concentration, with only slight upfield shifts at high dilution, relative to the spectrum following addition of pyridine. The sulfur atom in this compound is six atoms removed from the edge of the macrocycle at position 7. Examination of space-filling models shows that this barely reaches the central metal. Taken together the data suggest the formation of a dimer (or larger aggregate) bridged by coordination of one or both sulfides. Many possible structures can be drawn. If both sulfides serve as bridging ligands, maximum ring overlap is expected for rings III and V, which could lead to the observed shifts.

The NMR spectrum of the longer chain sulfide is remarkable and is shown in Fig. 2. In this case *none* of the ring protons is found to shift, but each resonance is *doubled*. The attached sulfide terminal methyl is dramatically upfield shifted and doubled, and all methylene protons on the ligand are upfield shifted, diastereotopic and doubled (we have not attempted to assign them). The terminal methyl peaks are found at -0.69 and -0.74 ppm for the compound in $CDCl_3$, and shift to $+1.80$ ppm after addition of excess pyridine ($\Delta\delta = 2.51$ ppm). The spectrum is *not* affected by changes in concentration. In this case there are ten atoms between the sulfur and ring IV, and models show that this is more than sufficient to reach the central metal. In fact, it is sufficiently long that the metal can be reached from either side of the macrocycle; we believe this is the origin of the double set of peaks.

Zn-complexes of porphyrins have been shown to be five-coordinate in a number of studies,[14] and we expect the same to be true for Zn-chlorins. The NMR spectra of coordinated Zn-chlorins show a single set of resonances (e.g., Fig. 2B where pyridine is the fifth ligand), which implies either that the metal coordinates exclusively on one side of the chlorin, or that either one side or the other can be coordinated, but these species interchange faster than the inverse in the chemical shift difference. Although the central metal is found about 0.4 Å out of plane on the opposite side to the propionic acid side chain in crystals of ethyl chlorophyllides *a* and *b*, it is likely that this is a consequence of an unusual network of hydrogen bonds found in the crystal.[15] Several examples of synthetic covalently linked chlorophyll dimers provide strong evidence for coordination on the other side.[16] Our data suggests that ligation is possible on both sides, and the chemical shifts for these two species are found to be slightly different. Exchange between the two modes of coordination requires a very substantial motion of the chain from one side of the macrocycle to the other and inversion of the metal through the plane; thus it is not surprising that it is very slow. An alternate explanation for the doubling of peaks is that the side chain binds exclusively on one side, but assumes two distinguishable orientations. This hypothesis requires a substantial steric

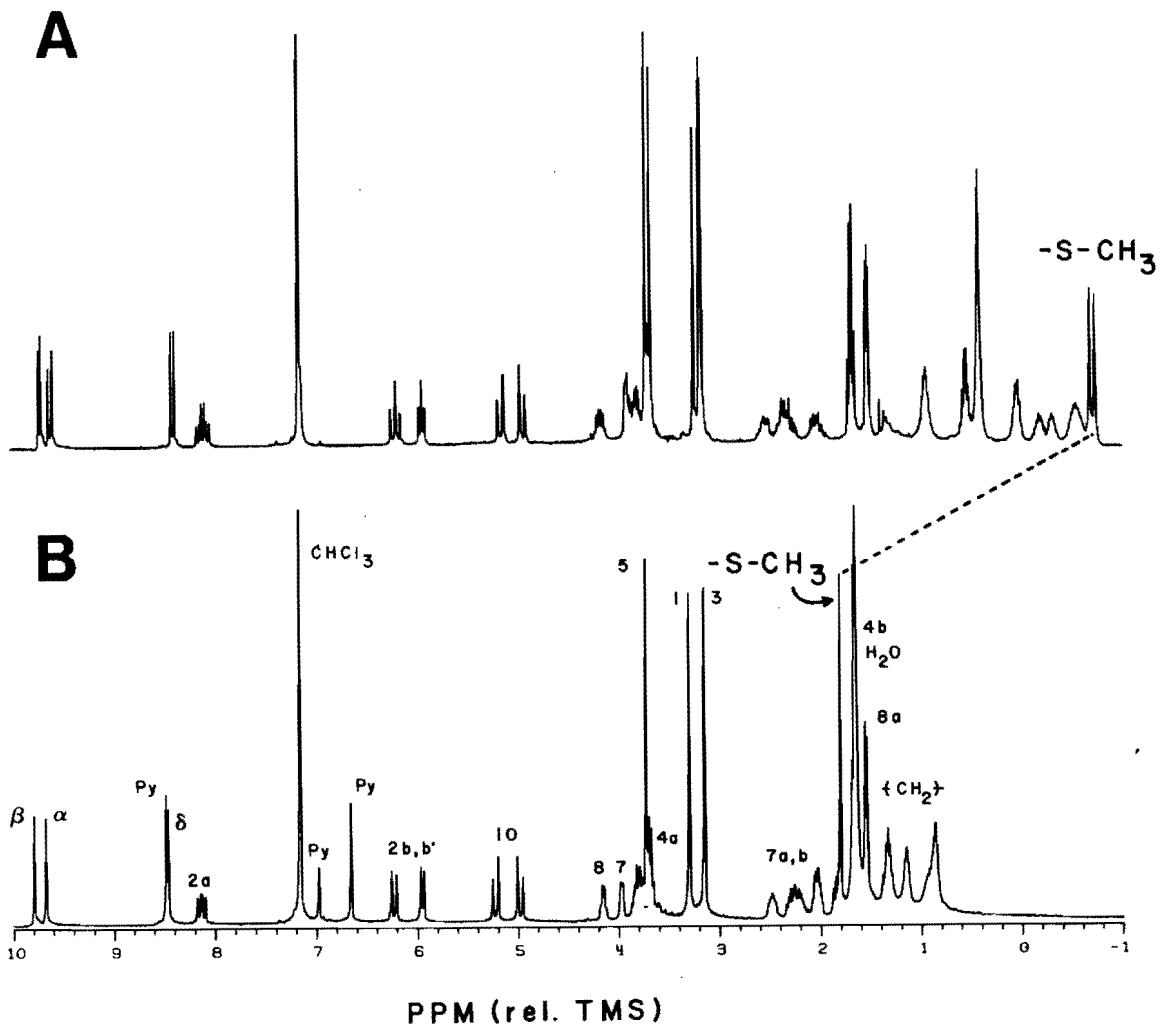

Fig. 2. 360 MHz ^{1}H-NMR spectra of ZnPChl*a*–$(CH_2)_6SCH_3$. (A) $CDCl_3$; note that all peaks are doubled (in several cases, e.g., the 10 protons, the doubling is not evident due to the reduction of the photograph, but it can be seen readily on an expanded plot). (B) The same sample with a 100-fold excess of pyridine-d_5 added.

or potential barrier at the surface, and we have no reason to expect it.

The analogous compound was prepared with Zn–bacteriopyrochlorophyllide *a*. Similiar changes are observed in the NMR spectrum for the thio-ether-containing side chain. The terminal methyl group is found at 0.00 ppm in $CDCl_3$ and shifts downfield to +2.03 ppm after addition of excess pyridine ($\Delta\delta = 2.03$ ppm); all resonances are sharp. Unlike the ZnPChl*a* complexes, neither the side chain peaks nor protons on the periphery of the macrocycle are doubled. The sharp peaks and large upfield shift for the terminal methyl protons indicate that the thio-ether side chain serves as a ligand for the central Zn-atom in this compound as well. The difference in total upfield shift (2.51 ppm for the chlorin vs. 2.03 ppm for the bacteriochlorin) is exactly what one would expect given the difference in ring current intensity associated with different levels of reduction of the macrocycle.[17,6]

The contrast between sharp doubled peaks for the chlorin (Fig. 2A) and the sharp single peaks for the bacteriochlorin is striking. Continuing the arguments advanced above to explain the doubled peaks, the single set of peaks in the latter case implies either that the Zn-atom coordinates exclusively on one side or that the exchange rate between coordination on one side or the other is faster than the inverse of the chemical shift difference. There is no reason to expect a difference in the rate of exchange of position of the side chain. It is possible that the metal-to-ligand dissociation rate is rate-limiting, however we have no evidence that there is a large difference between a chlorin and bacteriochlorin.[18]

The other hypothesis is that the metal has a great preference for one side, and only one species is present. If this hypothesis is correct, it could have significant consequences for the spectroscopic properties of bacteriochlorophyll (BChl) bound to proteins in vivo. The only structurally characterized natural chlorophyll–protein complex at this time is a water soluble BChl*a* protein, isolated from the green bacterium *P. aestuarii*.[19] This complex has been studied by X-ray crystallography and contains twenty-one BChls per protein (a trimer).[20] In all cases the BChl is found to be five-coordinate, and it is estimated that the metal is 0.4 Å out of plane. Of the seven distinguishable BChls, two are coordinated on the same side as the phytyl side chain and five are coordinated on the opposite side.

If our hypothesis is correct, and there is a strong preference for coordination on one side, it is possible that most spectral data obtained for BChls applies to this species (note that our experiment can not specify which side). The presence of two reduced rings and ring V causes considerable ruffling of the BChl macrocycle. Thus, a displacement of the metal from one side to the other may be associated with considerable distortion of the macrocycle, which could affect its electronic and redox properties. Some evidence is available suggesting that the central metal is coordinated by histidine on the same side as the propionic acid side chain in our synthetic Zn-bacteriopyrochlorophyllide *a*-apomyoglobin complexes.[6] The absorption maxima of this complex are red-shifted somewhat from those for the chromophore in diethyl ether, but they are not outside the range found in strongly coordinating solvents, such as pyridine. By contrast, the large change in the absorption spectrum observed for the *P. aestuarii* protein[21] and other in vivo complexes, which cannot be explained by exciton coupling, may be a simple consequence of ligand binding to the other side of the macrocycle. Although this argument is based on a plausible series of assumptions, proof for this concept awaits the isolation and structure elucidation of other simpler chlorophyll-protein complexes or a much larger series of Chls and BChls with fully defined ligation.

Tryptophan Analogues

L-Tryptophan methylester and tryptamine were covalently connected through an amide linkage to the propionic acid side chain, and the Mg and Zn complexes were studied by NMR. The NMR spectra of both the Mg and Zn complexes with L-Trp-methylester showed very unusual chemical shifts in the absence of a coordinating ligand, which converted to the usual chemical shifts when pyridine was added; the NMR spectra are shown in Fig. 3. The spectrum in the absence of pyridine was assigned by titration with pyridine to the assigned spectrum of Fig. 3B, accompanied by extensive homonuclear decoupling studies. The tryptophan NMR spectrum has recently been unambiguously assigned in our laboratory by the

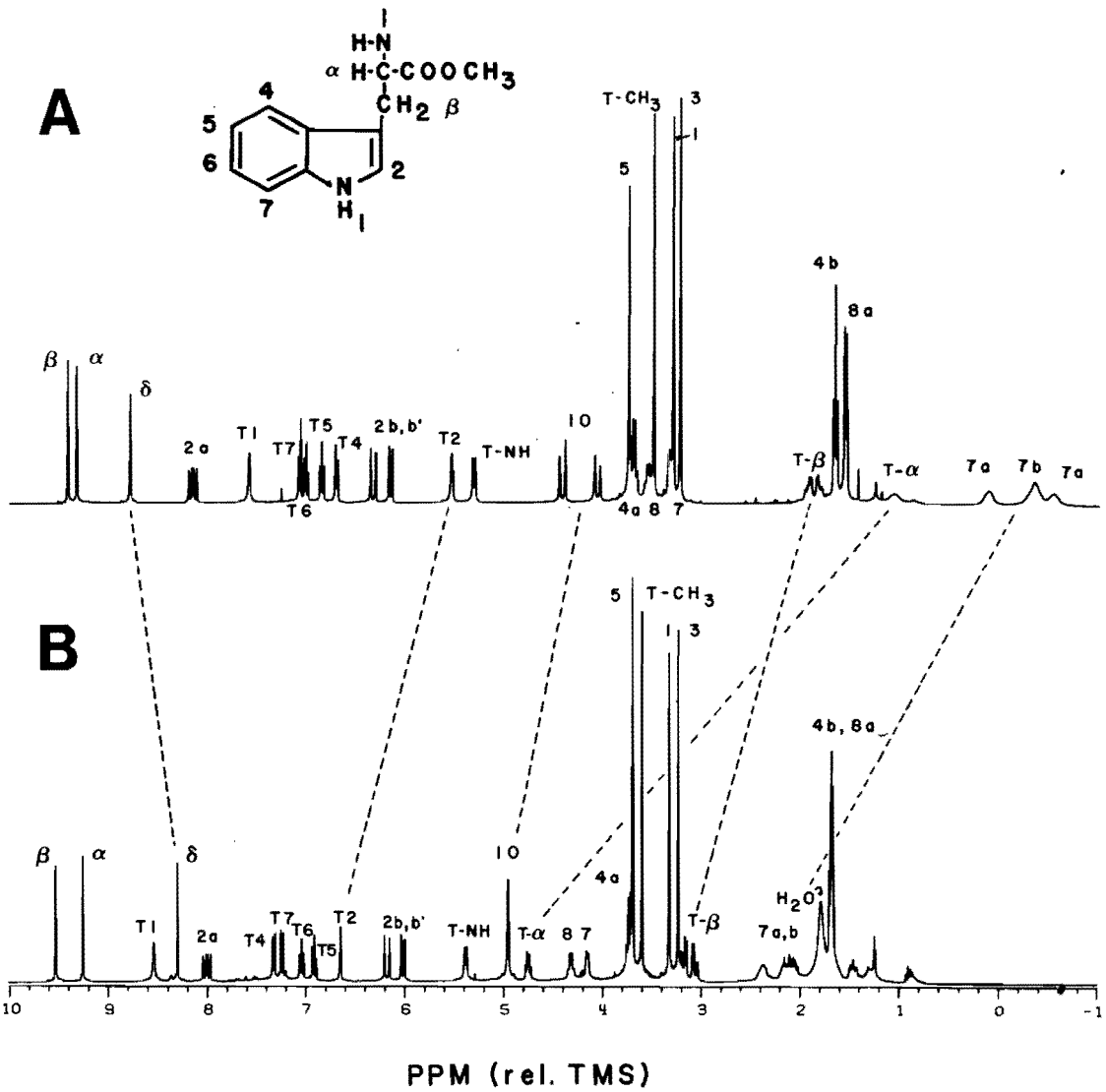

Fig. 3. 360 MHz ^{1}H-NMR spectra of ZnPChl*a*-L-Trp-methylester in (A) $CDCl_3$; (B) same sample with a 100-fold excess of pyridine-d_5 added. The Mg-complex shows the same pattern of chemical shifts. See Table 1 for quantitative data on the shift differences.

laser-CIDNP method,[22] and this method was used with the metal-free PP*a*-Trp-methylester to confirm this assignment.[22] The largest chemical shift changes depend on concentration and are summarized in Table 1. Great care was taken to insure the absence of water from these samples, but neither careful drying nor intentional addition of D_2O had any effect. No unusual shifts were observed for the metal-free compounds or the metal-complexes with tryptamine attached.

Table 1. Chemical Shift Differences for ZnPChl*a*-L-Trp-methylester and ZnPChl*a*-L-Val-methylester in $CDCl_3$ With and Without Added Pyridine-d_5

Proton[a]	Chemical Shift Difference ($\Delta\delta$)[b]	
	Zn-PChl*a*-L-Trp	Zn-PChl*a*-L-Val
Val-α		+4.40
Trp-α	+3.67	
7a[c]	+2.69, +2.26	+2.32, +2.30
7b[c]	+2.42	+2.54, +2.00
Val-CH_3		+1.43, +0.88
Trp-β	+1.24	
Trp-2	+1.11	
Val-β		+0.97
Trp-1	+0.95	
δ	−0.48	−0.57

a. See Figs. 1 and 3 for labelling of protons. b. $\Delta\delta \equiv \delta(CDCl_3) - \delta(CDCl_3 + \text{pyridine})$ in ppm. c. The absolute assignment of the 7a and b protons in the aggregate is not certain. However, a reversal of their assignments does not lead to a significant difference in the $\Delta\delta$ values.

The largest chemical shift difference for this complex ($\Delta\delta = 3.67$ ppm) is substantially larger than has been previously observed for other chlorophyll aggregates.[16,23] This observation and the concentration dependence of the chemical shift differences suggest the formation of an aggregate. The indole ring is not expected to be a ligand for the central metal, yet the central metal is required to produce the effect. Tryptamine and L-Trp-methylester differ only in the asymmetric α-carbon and carbomethoxy group. To further identify the origin of these large shifts, we covalently connected the methyl esters of L-Tyr, L-Phe and L-Val to PP*a* and prepared the Zn-complexes. Suprisingly, *all* of these complexes show the same pattern of strongly shifted resonances as the L-Trp compound. The L-Val complex is striking because the two terminal methyl groups shift upfield and differ by 0.6 ppm (the data for L-Val is presented in Table 1). There is no possibility for π-π interaction between the L-Val isopropyl group and the macrocycle, and this amino acid has no ring current to shift other protons. This result clearly implicates the carbonyl group of the carbomethoxy ester as the cause of aggregate formation, not a specific interaction with the amino acid.

Since a dimer (or larger aggregate) is formed for each of these amino acids with very similar structures, these compounds are not useful for determining the specific effect of individual amino acid side chains on chlorophyll properties. On the other hand, the data demonstrate the formation of a specific aggregate, and it is worthwhile to consider its structure. Only one NMR peak is associated with each type of proton, requiring at least a C_2 axis on the NMR time scale. The lines are very sharp, except for the most shifted 7a, b and amino acid α protons. These two observations and the analysis below, suggest that the predominant species in solution is a dimer. The NMR data show that dimer formation depends both on the presence of the central metal and a carbomethoxy group on the amino acid. The simplest suggestion is that the carbomethoxy carbonyl group from one macrocycle coordinates to the central metal of another, forming a bridged dimer, as illustrated schematically in Fig. 4.

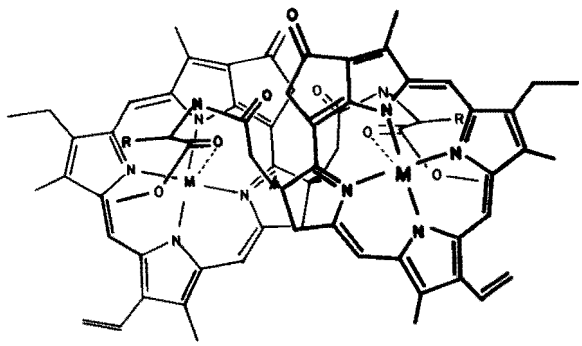

Fig. 4. A schematic illustration of the bridged dimer proposed in the text for ZnPChl*a* covalently connected to the methyl esters of L-Trp, L-Tyr, L-Phe or L-Val (the amino acid side chain is labeled R for simplicity).

Inspection of the structure presented in Fig. 4 with the aid of molecular models shows that the macrocycles overlap very slightly, in the region of ring IV. This positions the propionic acid side chain (methylene protons 7a and b) directly over the center of the other macrocycle. Likewise the amino acid α and β protons are positioned directly over the other macrocycle. The amide bond NH proton is found at the edge of the macrocycle in the isoshielding region and experiences no shift. The carbomethoxy protons are over the center of one ring and at the side of the other, leading to no shift. The 7, 8 and 10 protons are upfield shifted by the other ring. Though it is not obvious from the two-dimensional representation of Fig. 4, both the ring δ-methine and 5-methyl protons are in the deshielding region of the other macrocycle, if the rings are tilted slightly from plane parallel. This can explain the downfield shift of these protons. Thus, Fig. 4 offers a structural representation of a dimer which is consistent with all the NMR data. The exact positioning of the propionic acid side chain is only suggestive, because we expect considerable flexibility in its position (this also provides a simple explanation for the broadness of the most shifted 7a, b and amino acid α protons).

We have previously prepared a covalently linked pyrochlorophyllide *a* dimer.[16] This molecule and its Chl*a*[24] and BChl*a*[25] analogues form an intramolecular aggregate in the presence of hydroxylic ligands in which the macrocycle planes are parallel and overlap at rings III and V, so that the *y*-axes (see Fig. 1) are antiparallel. This leads to a substantial shift in the red absorption maximum from 663 to 697 nm and enchanced, not split, CD.[26] The aggregate structure proposed in Fig. 4 for the covalently connected amino acid methyl esters, is much less precisely known because the macrocycles are not covalently linked. The absorption spectrum of this complex is nearly indistinguishable from the dissociated monomer. This suggests that the *x*-axes are not antiparallel, because this would lead to parallel *y*-axes and a center-to-center separation roughly the same as the covalently linked dimer. This would lead to a blue-

shifted absorption maximum which is not observed. The CD spectrum of Zn–PChl*a*–L–Trp–methyl ester in $CDCl_3$ (the same sample whose NMR spectrum is shown in Fig. 3A) is intense and shows a conservative splitting which is characteristic of an exciton interaction.[27] In the absence of a measurable splitting in the absorption spectrum, the CD data serve primarily to confirm the presence of an aggregate, but can not provide a specific structure. Though this is a novel solution structure for Chl, this complex is only weakly bound by the bridging ligand, and its absorption spectrum is not red-shifted. Thus, it is not expected to provide a useful experimental model for comparison with in vivo reaction center dimers.

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