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Solution Properties of Synthetic Chlorophyllide- and Bacteriochlorophyllide–Apomyoglobin Complexes†

Karen A. Wright and Steven G. Boxer*

ABSTRACT: Well-defined 1:1 complexes have been formed between apomyoglobin (apoMb) and a number of chlorophyllide derivatives. The chlorophyllides substitute for heme in the pocket of myoglobin. These include magnesium chlorophyllide *a*, magnesium and zinc pyrochlorophyllide *a*, zinc pyrochlorophyllide *b*, zinc pyrochlorophyllide *d*, zinc pyromesochlorophyllide *a*, zinc 2-acetyl-2-devinylpyrochlorophyllide *a*, zinc protopyrochlorophyllide *a*, and zinc bacteriopyrochlorophyllide *a*. The effects of the protein on the electronic absorption, circular dichroism (CD), magnetic circular dichroism, and triplet state electron spin resonance spectra and fluorescence lifetimes in solution are compared with appropriate models in organic solvents. With the exception of the CD spectra, the protein causes shifts and intensity changes which are within the range observed for solvent effects. The CD spectra change substantially: the signs of several transitions are entirely reversed in the chlorins, and 3–6-fold intensity increases are observed with zinc bacteriochlorophyllide *a*. High-field ^{1}H NMR spectra of ring current shifted Val-E11 methyl protons for the series porphyrin–, chlorin–, and bacteriochlorin–apoMb are used to establish the probable absolute orientation of the chromophore in the heme pocket. Doubled peaks in the NMR spectra of certain complexes are shown to arise from interconvertible species. The temperature dependence of the peak intensities and saturation transfer studies show that the species giving rise to the doubled peaks exchange on the time scale of about 1–60 s. Arguments are presented against inversion of the macrocycle in the heme pocket by either an inter- or an intramolecular mechanism as the origin of doubled peaks, and simple two-site exchange is ruled out by the NMR data. We suggest that the data are consistent with the idea that at least two slowly interconverting conformational substates of the protein are populated, depending sensitively on small changes in rings I and II of the macrocycle and temperature.

Although it is widely believed that most chlorophyll in photosynthetic organisms is associated with proteins, little information is available on the consequences of this association. The spectroscopic and photochemical properties of chlorophyll in a large number of different reaction center and antenna complexes have been described (Thornber et al., 1979). Obvious features, such as the electronic absorption maxima and band shapes, and more subtle properties, such as oxidation and reduction potentials, excited state lifetimes, circular dichroism (CD)[1] intensities, and ESR properties of π radicals and excited triplet states, have been shown to differ substantially between various natural chlorophyll–protein complexes and chlorophylls in organic solvents (Sauer, 1978; Thornber et al., 1978; Norris & Katz, 1978). "Chlorophyll–protein interactions" of an undefined nature are commonly invoked to explain the variability of chlorophyll properties in vivo, yet we have little insight into the mechanism by which the protein exerts its influence. By contrast, a substantial literature has developed which suggests the basis for the diversity of properties of other important prosthetic groups in proteins, such as hemes (Collman et al., 1980), iron–sulfur clusters (Holm, 1977), retinal (Nakanishi et al., 1980), and flavins (McCormick, 1977).

An alternative hypothesis is that spectroscopic and functional variability are primarily a consequence of chlorophyll–chlorophyll interactions. Extensive studies of chlorophyll aggregates in organic solvents (Katz et al., 1976), and singly (Boxer & Closs, 1976) and doubly covalently linked chlorophylls (Wasielewski et al., 1978; Bucks & Boxer, 1981), do indeed show a considerable diversity of absorption and chemical properties. It is quite likely that both chlorophyll–protein and chlorophyll–chlorophyll interactions play significant roles in determining the properties of photosynthetic pigments in vivo, and extensive research is directed toward a deeper understanding of both types of interactions [see, for example, Rafferty et al. (1979)].

Our approach to understanding the role of chlorophyll–protein interactions is to prepare *synthetic chlorophyll–protein complexes of defined structure*. An example of this type of

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[1] Abbreviations: 2-Ac-PChl*a*, 2-acetyl-2-devinylpyrochlorophyllide *a*; ANS, 8-anilino-1-naphthalenesulfonate; BPChl*a*, bacteriopyrochlorophyllide *a*; CD, circular dichroism; DMF, dimethylformamide; DSS, 4,4-dimethyl-4-silapentanesulfonate; ESR, electron spin resonance; Hb, hemoglobin; Mb, myoglobin; MCD, magnetic circular dichroism; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PChl*a*, pyrochlorophyllide *a*; PChl*b*, pyrochlorophyllide *b*; PChl*d*, pyrochlorophyllide *d*; PMesoChl*a*, pyromesochlorophyllide *a*; ProtoPChl*a*, protopyrochlorophyllide *a*; ZFS, zero-field splitting parameters.

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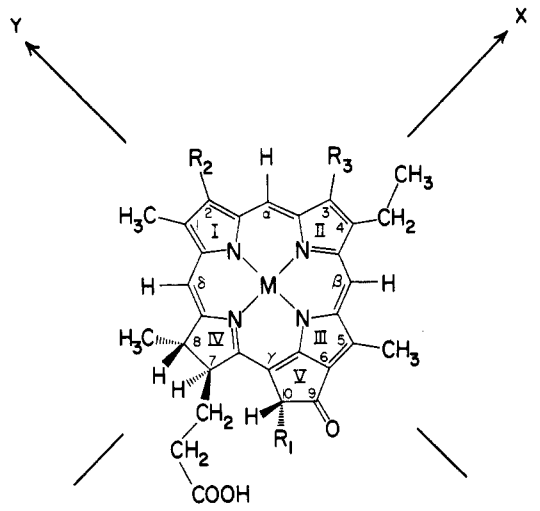

ABBREVIATION	R_1	R_2	R_3
Chl *a*	-COOMe	vinyl	methyl
PChl *a*	-H	vinyl	methyl
PChl *b*	-H	vinyl	aldehyde
PChl *d*	-H	aldehyde	methyl
PMesoChl *a*	-H	ethyl	methyl
2-Ac-PChl *a*	-H	acetyl	methyl

FIGURE 1: Structure, numbering system, and abbreviations for chlorophyllides discussed in the text.

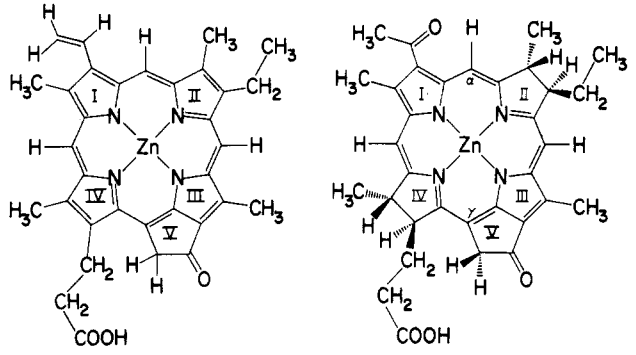

FIGURE 2: Structures, numbering systems, and abbreviations for Zn protopyrochlorophyllide *a* (left) (a porphyrin) and Zn bacteriopyrochlorophyllide *a* (right).

complex was prepared by substituting chlorophyllide *a* for heme in the extensively characterized protein myoglobin (Mb) (Boxer & Wright, 1979). The chlorophyll macrocycle (Figures 1 and 2) is very similar to heme, especially in the region of rings I and II, which is most intimately involved in hydrophobic contacts with the protein in native Mb (Perutz, 1975; Antonini & Brunori, 1971). The heme pocket in apoMb is notoriously promiscuous, and such diverse chromophores as 8-anilino-1-naphthalenesulfonate (ANS) (Stryer, 1965; Dochink et al., 1979), numerous modified hemes (Brunori et al., 1969; Gottfried et al., 1980), and now chlorophyllides are tightly bound.

Davis & Pearlstein (1979) have presented data on a complex between "chlorophyllin" and apoMb. "Chlorophyllin" is generally taken to denote magnesium chlorin e_6: chlorophyllide *a* in which ring V is opened to give a propionic acid side chain at position 6 and an acetic acid side chain at the γ position. Davis and Pearlstein demonstrated that the red absorption maximum of this complex undergoes a red shift when the complex is irradiated with red light. It is unlikely that this red shift is due to specific "chlorophyllin"–protein interactions, because similar spectral changes accompany photolysis of "chlorophyllin" in methanol, free from the protein. By contrast, *none* of the chlorophyllide–apoMb complexes discussed in the present work is photochemically altered under comparable conditions.

Most natural chlorophyll-containing proteins are rather hydrophobic, particularly in the best characterized reaction center complexes (Okamura et al., 1974; Steiner et al., 1974). None of the chromophores described in the present work has the naturally occurring hydrophobic phytyl ester at the propionic acid side chain. Although we doubt that this side chain is the source of any of the unique spectral or photochemical properties of the chlorophylls in vivo, it undoubtedly plays a major role in stabilizing the interactions between the chromophore and its natural hydrophobic protein and lipid hosts. The only structurally characterized natural chlorophyll–protein complex at this time is a water-soluble bacteriochlorophyll *a* protein, isolated from the green bacterium *Prosthecochloris aestuarii* (Olson & Romano, 1962). This has been studied in detail by X-ray crystallography (Matthews & Fenna, 1980) and optical methods (Matthews et al., 1979; Pearlstein & Hemenger, 1978). Unfortunately the protein contains seven distinct bacteriochlorophylls in each monomeric unit of a trimer; thus, the nature of individual chromophore–protein contacts is not well understood. This structure does provide considerable insight into chlorophyll–protein binding, including the result that most bacteriochlorophylls are ligated by histidine, a property shared with the Mb complexes.

Our original purpose in preparing these synthetic complexes concerned the basic spectroscopic properties of the chromophore. Large single crystals of a number of these synthetic complexes have been prepared and studied by X-ray diffraction and optical methods (Boxer et al., 1981).[2] A protein crystal offers many desirable features as a host for spectroscopic studies of chromophores of this type (Eaton & Hochstrasser, 1968). The present paper focuses on the properties of the complexes in solution.

Materials and Methods

Preparation of Chromophores. The structures and abbreviations for all macrocycles which were combined with apoMb are shown in Figures 1 and 2. Crystalline pheophorbide *a* and pyropheophorbides *a* and *b* were prepared by well-established methods from spinach (Strain & Svec, 1966). Zn (Furhop & Smith, 1975) or Mg (Isenring et al., 1975) was inserted, and the metalated chlorophyllide was chromatographed on thin-layer silica plates (deactivated by prerunning in methanol; 9:1 CH_2Cl_2:methanol, $R_f \approx 0.3$, typically). Zn was frequently used due to its ease of insertion and the well-established similarity between Zn and Mg chlorophyllides.

Zinc and magnesium protoporphyrin IX were prepared by standard methods (Furhop & Smith, 1975) from commercial protoporphyrin IX. Pyromesopheophorbide *a* methyl ester and pyropheophorbide *d* methyl ester were prepared from pyropheophorbide *a* methyl ester by the methods of Fischer et al. (1934) and Holt & Morley (1959), respectively.

Selective deuteration of the formyl hydrogen for both pyropheophorbides *a* and *d* was accomplished by reduction of the aldehyde with $NaCNBD_3$ to -CHDOH (Borch et al.,

[2] Well-formed orthorhombic single crystals of ZnPChl*a*Mb and MgPChl*a*Mb have been prepared. The space group of the latter is $P2_12_12_1$, and the unit cell parameters are within 0.5% of those for orthorhombic native Mb (Kendrew & Parrish, 1956). A detailed analysis of the optical properties of these crystals is the subject of a separate paper (Boxer et al., 1981).

1971; Boxer & Bucks, 1979), followed by reoxidation with activated MnO_2 (Fieser & Fieser, 1967). Deuteration is typically 70–80% (greater than 50% due to the isotope effect on oxidation) as judged by ^1H NMR. Protopyropheophorbide *a* methyl ester (2-vinylphylloerythrin methyl ester) was isolated in low yield (10–20%) from the oxidation of pyropheophorbide *a* methyl ester with dichlorodicyanobenzoquinone. All methyl esters were hydrolyzed in HCl, and Zn or Mg was inserted as described above.

Bacteriopyropheophytin *a* was prepared by pyrolysis of bacteriopheophytin *a* (ex. *Rhodopseudomonas sphaeroides*) in rigorously anaerobic collidine at 150 °C for 1 h (Kenner et al., 1973). Hydrolysis for 2 h at 30 °C in oxygen-free 5:1 trifluoroacetic acid:HCl gave the free acid. 2-Acetyl-2-devinylpheophytin *a* was prepared according to the procedure of Lindsay Smith & Calvin (1966).

As a model for histidine binding in the Mb heme pocket, an analogue of imidazole, 3-(1-imidazolyl)propylamine (Chang & Traylor, 1973; Denniss & Sanders, 1978), was coupled to the free carboxylic acid on the propionic acid side chain of a number of pheophorbides. Metal insertion gave the desired model, which has been shown to be internally coordinated and monomeric even in nonpolar solvents (Denniss & Sanders, 1978). These compounds are abbreviated PimChl.

Preparation of Chlorophyllide–apoMb Complexes. The apoproteins of sperm whale and horse heart metmyoglobins (Sigma Corp.) were prepared according to a procedure adapted from Teale (1959), Breslow (1964), and Alston & Storm (1979). Hemin was extracted by the acid–butanone procedure (Teale, 1959), followed by dialysis against deionized water, then 0.6 mM $NaHCO_3$, and finally 10 mM Tris-HCl (pH 8.3). The solution was passed through a column of DEAE-cellulose equilibrated with the same buffer to remove residual heme-containing protein, concentrated, dialyzed against deionized water, and lyophilized.

Chlorophyllide–apoMb complexes were prepared by one of the following two procedures. Method A: Solutions (1–2% w/v) of apoMb in 0.1 M sodium phosphate buffer (pH 6.3) were centrifuged to remove undissolved protein. A 1.5–3-fold molar excess of the chlorophyllide was dissolved in sufficient 1:1 pyridine:water such that the total pyridine concentration was 5% or less when combined with the apoMb solution. The chlorophyllide solution was added slowly to the apoMb solution with rapid stirring at 4 °C. The mixture was stirred for 10–15 min and immediately applied to a column of Sephadex G-25, equilibrated with 10 mM sodium phosphate (pH 6.3), to remove the bulk of the pyridine, excess chlorophyllide which precipitates at the top of the column, and larger aggregates. The intensely pigmented band which eluted was dialyzed against 10 mM sodium phosphate (pH 6.3) to remove traces of pyridine and bound to a column of CM32 cellulose equilibrated with the same buffer. The chlorophyllide–apoMb complex was eluted as a clean band with 20 mM sodium phosphate (pH 7.6), was dialyzed exhaustively against deionized water, and was lyophilized.

Method B: Some chlorophyllides are insoluble under the conditions described above, so an alternate method was developed. The chlorophyllide was dissolved in a minimum volume of 1:1 DMF:buffer mixture and was applied to a Sephacryl S-200 column, which had been equilibrated in 10 mM sodium phosphate (pH 6.3). Typically the entire column became colored and was washed with several column volumes of buffer. A 1% (w/v) solution of apoMb in the same buffer was passed through the column, stripping off the chromophore as the complex was formed. The chlorophyllide–apoMb complex was then further purified as described above for method A.

Chlorophyllide–apoMb complexes are abbreviated with the name of the chromophore (Figures 1 and 2) followed by the name of the protein; e.g., ZnPChl*a*Mb is zinc pyrochlorophyllide *a*–apoMb complex (sperm whale Mb is implied unless otherwise indicated).

Measurements. ^1H NMR spectra were obtained at 360 MHz (298 ± 1 K, unless otherwise noted). The NMR samples were approximately 0.6 mM protein in D_2O and were centrifuged before use to remove undissolved protein. The chemical shifts at 298 K are referenced to the residual HDO peak at 4.76 ppm relative to DSS, and typically 1000–4000 scans were collected for each spectrum. Absorption spectra were obtained on a Cary 14 or 17 spectrophotometer and CD and MCD (applied field strength 15 kG, corrected by subtracting CD) spectra on a Durrum-Jasco instrument. Steady-state fluorescence emission was measured at right angles for samples whose optical density at the excitation wavelength (420 nm) was less than 0.2, and whose optical density at the red absorption maximum was less than 0.1. Time-resolved fluorescence lifetimes were measured by using synchrotron radiation (excitation pulse typically 500 ps, full width at half-maximum, at 420 nm) and single-photon correlation methods (Monro et al., 1979; Monro & Sabersky, 1980), and lifetimes were obtained by the curve-fitting method of Shrager (1970) and Grinveld & Steinberg (1974). Triplet state ESR spectra were obtained at 80–90 K or 10 K by using either broad-band irradiation in the wavelength range 400–700 nm or a Kr ion laser (647 or 752 nm) and standard light modulation and data reduction methods to obtain the zero-field splitting parameters (Wasserman et al., 1964; Levanon & Norris, 1978). All samples were degassed either by several freeze–pump–thaw cycles or by purging with a stream of Ar.

Results

General Properties of the Protein Complexes. The combination of extinction coefficient measurements, ANS binding studies, and elemental analysis of the Zn complexes (Zn/C ratio) is used to prove that the chlorophyllide is substituted for hemin in all of our complexes [see Boxer & Wright (1979) for details]. We have been unsuccessful in combining metal-free pyropheophorbide *a* with the protein by either methods A or B; only superficially complexed species were formed which would not pass through a CM32 ion-exchange column. This is disappointing in view of the fact that metal-free protoporphyrin IX binds fairly well in the pocket of apoMb (Breslow et al., 1967).

^1H NMR Spectroscopy. The ^1H NMR spectra of the chlorophyllide–apoMb complexes are very similar to that of diamagnetic CO-Fe^{II}Mb (Shulman et al., 1970). One of the most useful features of the spectra of the reconstituted chlorophyllide– and bacteriochlorophyllide–Mb complexes is the appearance of several ring current shifted resonances in the region upfield (0 to −4 ppm) from the protein envelope, as seen in Figure 3. Four resonances are evident in all cases, each with an area of 3 protons. It is seen that the most upfield shifted of these, peaks I and II, are also readily identified in the spectra of CO-Fe^{II}Mb and MgMb and are absent in the apoprotein. For CO-Fe^{II}Mb, these peaks have been assigned to the methyl group protons of Val-E11, lying on the distal side of ring I of the heme (Shulman et al., 1970). Figure 4 shows the reduction in the upfield shift of peak I with increasing chemical reduction of the complexed tetrapyrrole ring system. The chemical shifts of both peaks also show a minor dependence on the nature of the substituent at ring positions

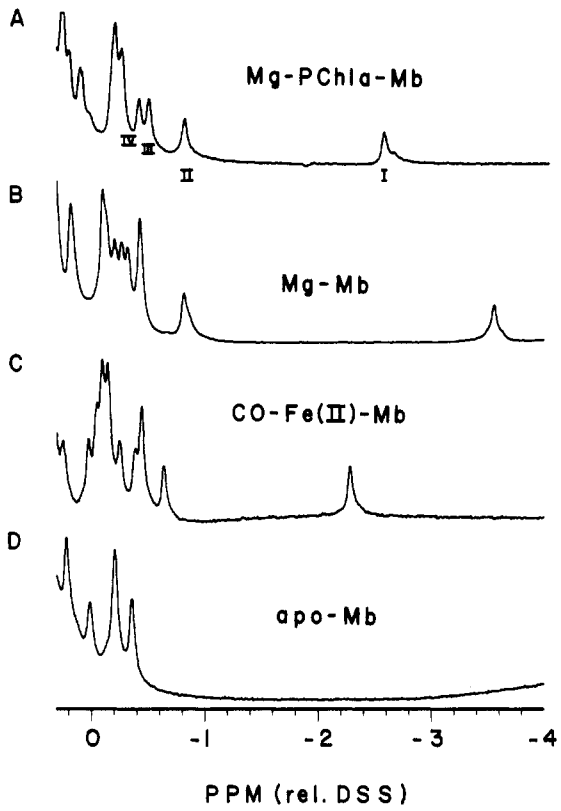

FIGURE 3: ^{1}H NMR spectra (360 MHz) in the upfield region of (A) MgPChl*a*Mb, (B) MgMb (Mg replaces Fe in reconstituted Mb), (C) diamagnetic CO-Fe^{II}Mb, and (D) apoMb. All samples are ca. 10^{-3} M in D_2O at 25 °C.

Table I: ^{1}H NMR Chemical Shifts for the Upfield Resonances of Various Chlorophyllide-apoMb Complexes (in ppm from DSS)

chromophore	peak I[a]	peak II	peak III	peak IV
ZnProtoIX[b]	−3.62	−0.85	−0.47	−0.15[d]
ZnProtoPChl*a*	−3.36	−0.76	−0.42[d]	−0.34[d]
MgPChl*a*	−2.50, −2.59[c]	−0.73	−0.42	−0.31
ZnPChl*a*	−2.59, −2.45[c]	−0.70	−0.41	−0.31
ZnPChl*b*	−2.48, −2.32[c]	−0.68	−0.40	−0.32
ZnPChl*d*	−2.73	−1.08	−0.45	−0.36
ZnPMesoChl*a*	−2.40	−1.04	−0.44	−0.34
Zn-2-Ac-PChl*a*	−2.60	−0.81	−0.47	−0.40
ZnBPChl*a*	−2.16	−0.85	−0.45	−0.36

[a] See Figure 5 for labeling of peaks. Peaks I and II are Val-E11 methyl protons. [b] ZnProtoIX is zinc protoporphyrin IX. [c] Doubled peaks; see Figure 5 and text. [d] Not well resolved from the protein envelope.

2 and 3. All shifts are presented in Table I.

The upfield regions for a number of different zinc pyrochlorophyllide–Mb complexes are compared in Figure 5. The spectra of both ZnPChl*a*Mb and MgPChl*a*Mb consistently exhibit a small shoulder or distinct peak on the upfield side of peak I with a similar line width. The result for ZnPChl*b*Mb is more dramatic: both peaks I and II are split into two components with intensity ratios of nearly 1:1 at room temperature. In all other cases, only single sets of resonances are observed. Figure 5 also shows the downfield region (11.6–8.9 ppm), which is particularly informative for the complexes of ZnPChl*b* and ZnPChl*d*, due to the formyl substituent at ring positions 3 or 2, respectively. Each of the three downfield peaks for ZnPChl*d*Mb has an area of one proton. In contrast, ZnPChl*b*Mb exhibits *six* clearly resolved peaks in this region, each of area no greater than ca. 0.5 proton.

Reconstitution with ZnPChl*b* or ZnPChl*d* selectively deuterated at the aldehyde position unambiguously assigns the

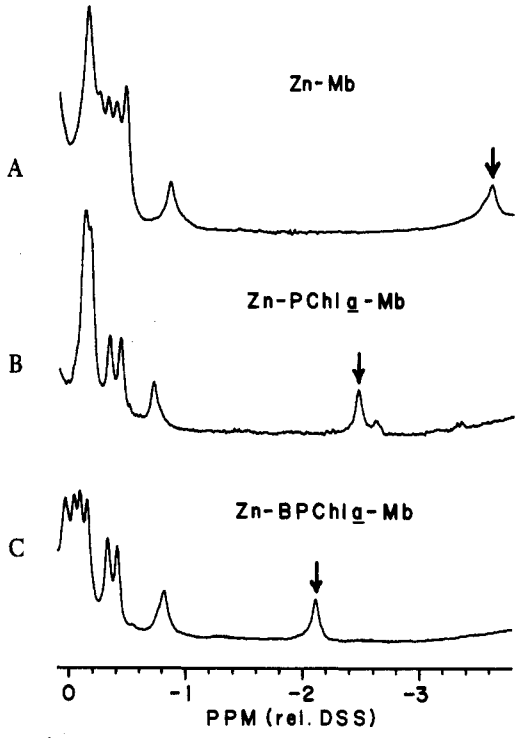

FIGURE 4: ^{1}H NMR spectra (360 MHz) in the upfield region of (A) ZnMb (Zn replaces Fe in reconstituted Mb), (B) ZnPChl*a*Mb, and (C) ZnBPChl*a*Mb, showing the effect of reduction of the macrocycle ring current on Val-E11 shifts (indicated with an arrow). All samples are ca. 10^{-3} M in D_2O at 25 °C.

single most downfield-shifted peak in ZnPChl*d*Mb and the *two* most downfield-shifted peaks in ZnPChl*b*Mb to the formyl proton in each complex. Reconstitution with ZnPChl*b* deuterated at the δ-methine position does not noticeably affect resolved peaks. The intensity ratios of split peaks for ZnPChl*a*Mb and ZnPChl*b*Mb are reproducible and independent of whether method A or B is used for reconstitution. Samples stored in solution for 6 months at 4 °C showed no change in peak intensities. Sanders et al. (1978) have unambiguously confirmed the original assignment of the lowest field methine resonance (Closs et al., 1963) to the α proton in chlorophyll *b* by measuring the nuclear Overhauser effect (NOE) enhancement of this peak following radio frequency irradiation at the aldehyde peak position. Using this approach, we have similarly assigned the lowest field methine resonance to the α proton for ZnPChl*d* in acetone solution and to this peak in the protein [a weak negative NOE is observed; see Wüthrich et al. (1978)]. Unfortunately NOE measurements were unsuccessful for ZnPChl*b*Mb, so the assignment of the α proton is less certain for this complex.

The NMR spectra of both ZnPChl*a*Mb and ZnPChl*b*Mb were studied as a function of temperature from 5 to 60 °C (the protein suffers irreversible denaturation at higher temperatures). Representative results for ZnPChl*b*Mb are shown in Figure 6. All resonances exhibit significant changes of position and a pronounced *narrowing* of the peaks with increasing temperature. A most surprising result is the observation of a large and completely *reversible* increase in the ratio of upfield to downfield components of peak I for ZnPChl*b*Mb. Parallel changes in the relative intensities are observed for peak II, the α and the aldehyde resonances, leading to the pairing of assigned resonances labeled A and B in Figure 6. For peak I, the ratio of peak A to B varies from slightly greater than 1.0 at 5 °C to 0.5 at 60 °C. Saturation transfer was not detected for the well-separated aldehyde protons whose spin–lattice relaxation times were measured to be 0.4 ± 0.25 s. A

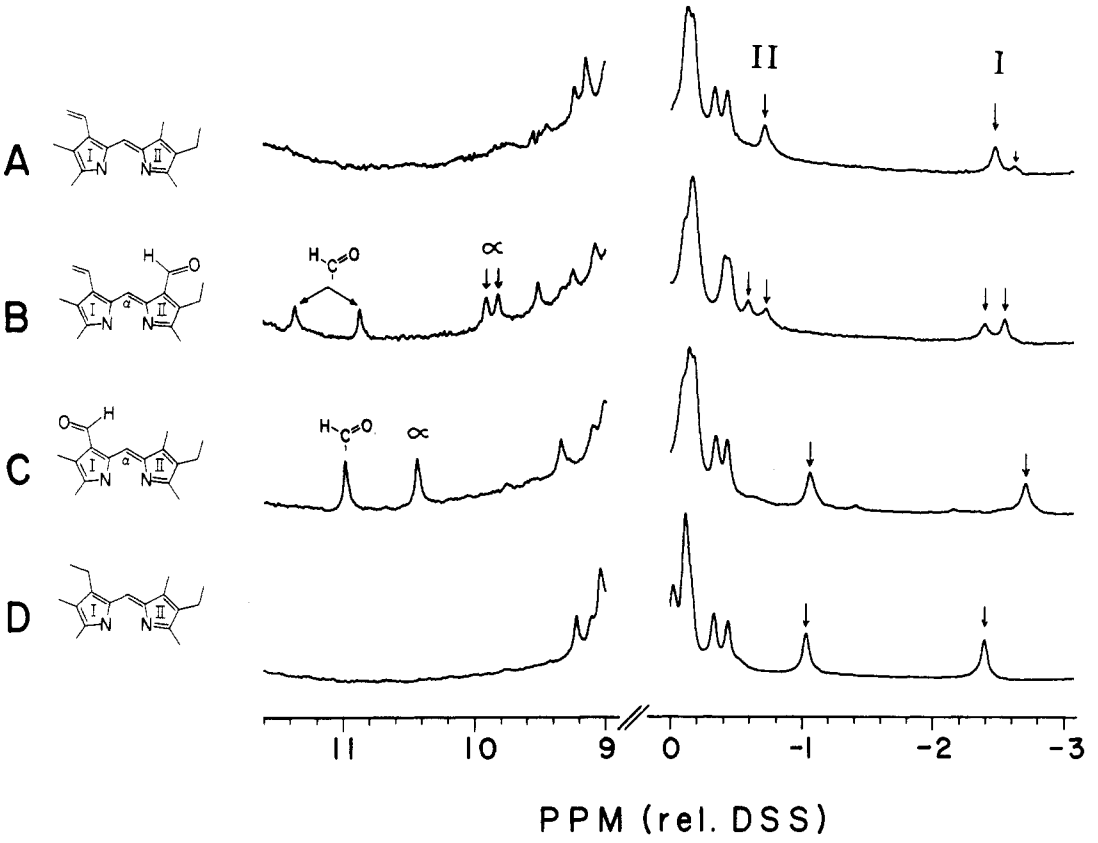

FIGURE 5: High- and low-field regions of the 360-MHz ^1H NMR spectra of (A) ZnPChl*a*Mb, (B) ZnPChl*b*Mb, (C) ZnPChl*d*Mb, and (D) ZnPMesoChl*a*Mb. All samples are ca. 10^{-3} M in D_2O at 25 °C. Peaks I and II are ring current shifted valine E-11 protons. See text for assignment of low-field resonances.

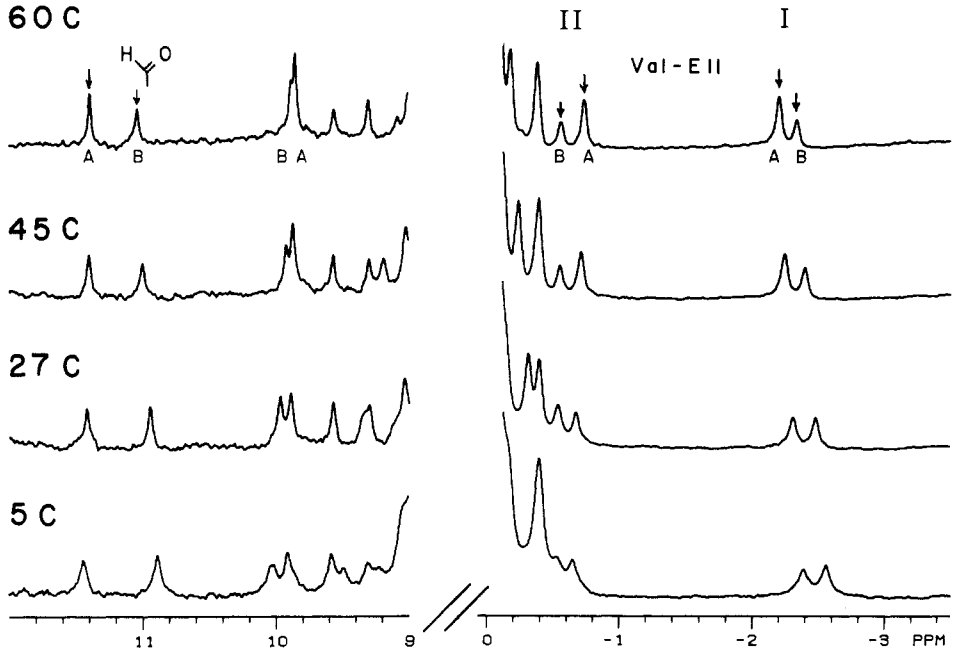

FIGURE 6: Temperature dependence of aldehyde, α-methine, and Val-E11 resonances for ZnPChl*b*Mb. Sample was ca. 10^{-3} M in D_2O. All changes are reversible. The chemical shifts are relative to internal DSS; the shifts are not corrected for the small temperature dependence of the DSS chemical shift.

crude temperature jump experiment was attempted by dropping the sample, equilibrated at room temperature, into the probe at 65 °C. As quickly as data of sufficient quality could be obtained (1–2 min), the relative peak intensities of peaks IA and IB resembled those seen at the higher temperature. ZnPChl*a* and ZnPChl*b* were reconstituted with apoMb from horse heart. For ZnPChl*a*, a single peak was observed for peaks I and II. For ZnPChl*b*, peak doubling was again observed; however, the ratio of A to B type peaks was much greater at room temperature, resembling most closely the high-temperature ratio for the sperm whale Mb complex (Figure 6). The classification of A and B type peaks is the same as for the complexes with sperm whale apoMb.

Electronic Absorption, CD, and MCD Spectroscopy. The electronic absorption spectra of the chromophores in organic solvents and complexed in the protein, along with band as-

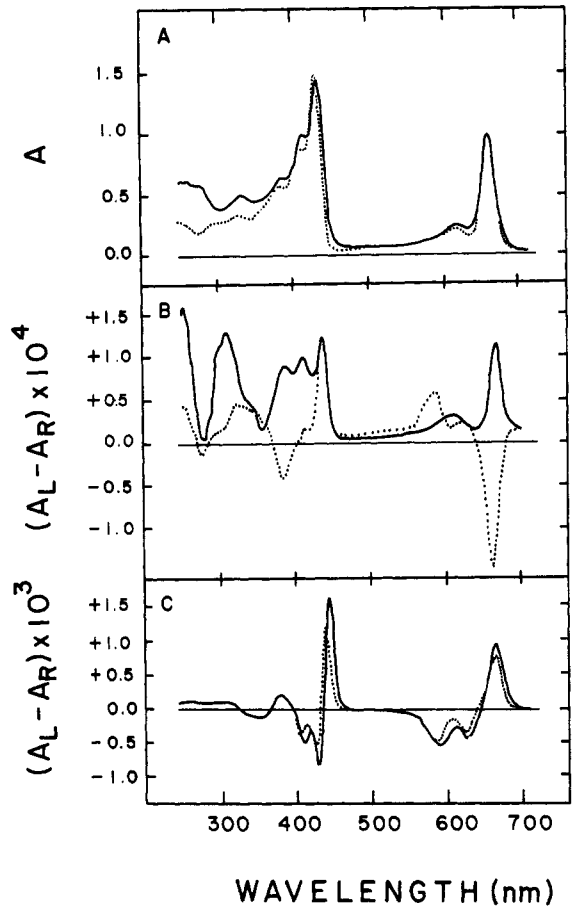

FIGURE 7: Comparison of (A) electronic absorption, (B) CD, and (C) MCD, 1-cm path length at 20 °C, spectra for MgPChl*a*Mb (—), 10^{-5} M in H_2O, and MgPimChl*a* (···), 10^{-5} M in CH_2Cl_2.

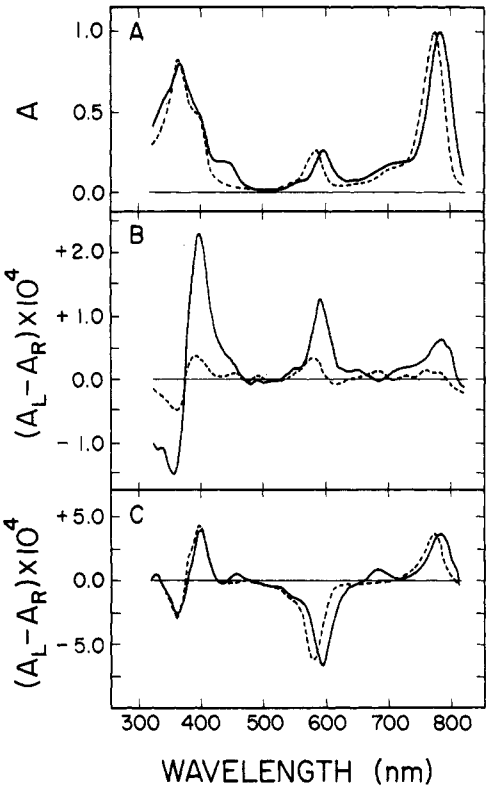

FIGURE 8: Comparison of (A) electronic absorption, (B) CD, and (C) MCD, 1-cm path length at 20 °C, spectra for ZnBPChl*a*Mb (—), 10^{-5} M in H_2O, and ZnBPChl*a* (---), 10^{-5} M in 1% pyridine in CH_2Cl_2. The small intensity long-wavelength shoulders observed at 435 and 675 nm arise from a trace of contaminating Zn-2-Ac-PChl*a*, formed by oxidation.

signments, are summarized in Table II. In general, the electronic absorption spectrum of the chromophore in the protein in the visible and near-ultraviolet regions (>300 nm) is very similar to that of the corresponding model in an organic solvent with the appropriate ligand. Examples of the smallest changes (MgPChl*a*) and largest changes (ZnBPChl*a*) are shown in Figures 7A and 8A, respectively.

The CD spectra are substantially altered when all chromophores are inserted into apoMb. MgPimChl*a* and MgPChl*a*Mb are compared in Figure 7B, and it is observed that the strong $Q_y(0–0)$ band changes *sign* from negative to positive. The long-wavelength components of the Soret transitions retain both their sign and their intensity. The $Q_x(0–0)$ transition retains the same sign but is reduced in intensity. Qualitatively similar changes are found for all other chlorins. The CD spectrum of ZnProtoPChl*a*Mb is very similar to that of ZnPChl*a*Mb in the Q and B bands (note that ZnProtoChl*a* itself has no asymmetric centers). Unlike the chlorins, the signs of the CD bands are unaffected when ZnBPChl*a* is inserted into apoMb, but very large enhancements of the intensities of the visible and Soret transitions are found (3–6-fold, see Figure 8B).

The MCD spectra of all these chromophores are relatively insensitive to the presence of the protein as seen for ZnPChl*a*Mb and ZnBPChl*a*Mb in Figures 7C and 8C, respectively. This observation is consistent with the notion that the protein does not appreciably alter the excited singlet electronic states of the chromophores.

Fluorescence and Fluorescence Lifetimes. Changes in fluorescence maxima parallel changes in the absorption maxima of the $Q_y(0–0)$ transitions. The fluorescence lifetimes are slightly perturbed by the protein in some cases, and the results are presented in Table III.

Triplet ESR Data. The zero-field splitting parameters (ZFS) for the lowest triplet excited states of MgPChl*a*Mb, ZnPChl*b*Mb, and ZnBPChl*a*Mb are compared with their appropriate model in organic glasses in Table IV. We find that the ZFS fall within the range observed for the corresponding chlorophylls in organic glasses (Levanon & Norris, 1978).

Discussion

NMR and Orientation. ^1H NMR provides a valuable probe of the structure and integrity of the chlorophyllide–Mb complexes due to the influence of the macrocycle ring current on the chemical shifts of protein side chains. The original assignment (Shulman et al., 1970) for the most upfield-shifted or shielded peak in the spectra of diamagnetic native Mb's was based on considerations of the environment in the vicinity of the heme, as depicted in Figure 9. One of the methyl groups of Val-E11 is positioned directly over ring I at a distance of about 4.4 Å and should be subject to the greatest shielding of any aliphatic protein side chain. In native Mb, this peak is found between −2.2 and −2.8 ppm, depending on the sixth ligand on Fe.

The data in Table I show that the position of peak I for our complexes spans the range from −2.16 to −3.62 ppm. There are three reasonable contributions to the range of positions observed. First, the range could arise from differing degrees of ring current associated with each macrocycle. Second, the position of the Val-E11 methyl group may change in relation to the macrocycle, due to different packing of the side chains of the pocket when different chromophores are introduced. Third, different chromophores may insert with either ring I or ring II in close contact with Val-E11. This amounts to

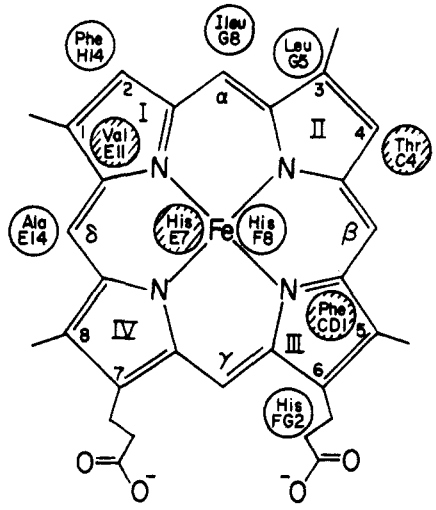

FIGURE 9: Illustration of the positions of Mb residues in the vicinity of the heme pocket from the crystal structure of metaquoMb[adapted from Kendrew (1962)]. Shaded residues are on the distal side of the heme group.

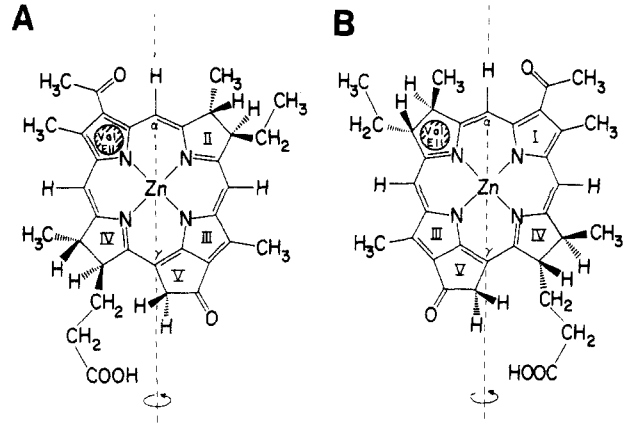

FIGURE 10: Illustration of the effect of rotation of the ZnBPChl*a* macrocycle around its α–γ axis in the heme pocket on the relationship between the Val-E11 methyl groups and rings I and II.

rotation about the α–γ axis, as illustrated in Figure 10.

We believe that the first of these is most significant. It is well-known that the magnitude of the macrocycle ring current decreases in the order porphyrin > chlorin > bacteriochlorin. For example, the central N–H protons are found at −3.74, −2.46, and −1.88 ppm in the series octaethylporphyrin, chlorin, and bacteriochlorin (Scheer & Katz, 1975).[3] The chemical shifts for peak I in our complexes (see Table I and Figure 4) cluster around −3.5 ppm for porphyrins, around −2.5 ppm for chlorophyllides, and −2.2 ppm for bacteriochlorophyllide, precisely paralleling the expected trend. Minor variations due to substituent effects are noted within each class. Cases have been studied in which the substituents overlap between classes as closely as possible, for example, ZnProtoPChl*a* vs. ZnChl*a* and Zn-2-Ac-PChl*a* vs. ZnBPChl*a*, and in each case, the chemical shift of Val-E11 is characteristic of the class (e.g., porphyrin vs. chlorin) rather than the substituent.

It is conceivable that the orientation around the α–γ axis (see Figure 1) is different for some chlorophyllides. An analogous possibility has been raised for a number of Mb's reconstituted with modified hemes from an analysis of doubling of hyperfine shifted resonances in their NMR spectra (LaMar et al., 1978). This result in solution contrasts with crystallographic data on native Mb in which hemin has the unique orientation illustrated in Figure 9 (Perutz, 1975). Hemin is very nearly symmetric for rotation about the pseudo-C_2 α–γ axis. The chlorophyllides are far less symmetrical, particularly considering ring V and the two asymmetric centers in ring IV. For this reason, we favor the idea that chlorophyllides have a specific orientation in the heme pocket (see the discussion of the CD data below for further evidence on this point).

If correct, this analysis has an important consequence. If ZnBPChl*a* were oriented with its *reduced* ring II close to Val-E11 (see Figure 10B), we would expect a much smaller ring-current shift. Thus, the orientation of ZnBPChl*a* is almost certainly as shown in Figure 10A, that is, identical with the orientation of hemes. Furthermore, if the orientation of chlorophyllides is the same in all complexes due to steric constraints, as argued above, then the chlorophyllides are also oriented such that ring I, rather than ring II, is in close proximity to Val-E11. Knowledge of the absolute orientation of the macrocycle in the heme pocket is central to further studies of the spectra of these complexes in single crystals,[2] because rotation about the α–γ axis exchanges the directions commonly associated with the transition dipole moments for the lowest excited states (labeled X and Y in Figure 1). Though the NMR analysis supports the orientation discussed above, final resolution of this question awaits the solution of the X-ray crystal structure.[2]

Electronic Structure of Chlorophyllides in Mb. The electronic absorption, MCD, triplet state ESR, and fluorescence lifetime data attest to the fact that the protein matrix in apoMb imposes no severe perturbation on the electronic structure of any of the chlorophyll derivatives which we have studied. The absorption maxima for the $Q_y(0$–$0)$ transition of Chl*a* (Seely & Jensen, 1965) and BChl*a* (Sauer, 1975) have been measured in a very wide range of solvents, and none of the cases studied in this work lies outside this range, with the exception of ZnPChl*b*Mb. The latter has an absorption maximum at 653 nm. This is an interesting case because an absorption band at 650 nm has been observed in the spectrum of the light-harvesting *a*/*b* protein isolated from green plants (Thornber, 1975) and from various Chl*b*-containing organisms (French et al., 1972). It has been suggested that this spectral shift can be explained by exciton coupling in a hydrated Chl*b* dimer (Serlin et al., 1975). Our data show that a rigorously monomeric Chl*b* derivative can also absorb this far to the red.

The CD spectra of these complexes show the greatest impact of the protein on the properties of the chromophore. It is well-known that chromophores containing no asymmetric centers, such as hemes or flavins, have strong CD spectra when the chromophore is associated with a protein (Hsu & Woody, 1971). The chlorophylls have asymmetric centers and well-characterized intrinsic CD spectra (Sauer, 1978), so we were surprised that the *CD spectra of the protein complexes are dominated by the interaction with the protein.*

Philipson et al. (1971) have shown that the signs of the CD spectra for chlorophyll monomers can be accounted for by the coupled oscillator interaction between chiral centers and the transition dipoles of the macrocycle. A possible explanation for the effect of the protein on the CD is that the chromophore is twisted or buckeled by the protein, changing this interaction. Since no other characteristic of the excited state is drastically altered, we consider this to be a very unlikely explanation.

[3] We have covalently attached the coordinating methionine analogue, methyl 6-hydroxyhexyl sulfide, to ZnPChl*a* and ZnBPChl*a* by esterification at the propionic acid side chain. Coordination between Zn and the thioether sulfur positions a methyl group over the macrocycle. The change in chemical shift on coordination for the chlorin is +2.5 ppm and +2.03 ppm for the bacteriochlorin (Boxer & Bucks, 1981).

Table II: Absorption, CD, and MCD Data for Various Chlorophyllide–apoMb Complexes and Corresponding Free Chromophores

	absorption[a]		CD		MCD		
compd	λ_{max}	A	λ_{max}	$\Delta A \times 10^5$	λ_{max}	$\Delta A \times 10^5$	assignment[f]
ZnPimChl*a*[b]	660	0.600	660	−10.9	660	+50.8	$Q_y(0-0)$
	618	0.110	618		619	−23.7	$Q_y(1-0)$
	585	0.050	578	+3.8	583	−33.0	$Q_x(0-0)$
	535	0.025					$Q_x(1-0)$
	433	1.000	436	+10.9	438	+89.3	B_x
					426	−44.2	B_y
ZnPChl*a*Mb	661	0.58	661	+7.8	664	+59.5	$Q_y(0-0)$
	621	0.15	612	+2.0	626	−30.5	$Q_y(1-0)$
					589	−31.2	$Q_x(0-0)$
	438	1.00	440	+8.3	444	+10.7	B_x
					432	−56.5	B_y
MgPimChl*a*[b]	665	0.660	660	−15.0	664	+8.0	$Q_y(0-0)$
	627	0.125	622	+2.5	622	−3.5	$Q_y(1-0)$
	588	0.070	583	+5.8	584	−5.0	$Q_x(0-0)$
	537	0.025					$Q_x(1-0)$
	433	1.000	435	+12.0	438	+11.5	B_x
					425	−5.5	B_y
MgPChl*a*Mb	664	0.655	665	+10.1	664	+9.0	$Q_y(0-0)$
	622	0.145	610	+3.0	626	−4.8	$Q_y(1-0)$
					590	−5.8	$Q_x(0-0)$
	438	1.000	437	+12.0	441	+16.0	B_x
					429	−8.8	B_y
ZnPimChl*b*[b]	643	0.290	642	−8.9	643	+21.6	
			617	+3.3	617	−30.8	
	592	0.085					
	545	0.045					
	462	1.00	464	+6.2	473	+63.3	
					457	−62.0	
ZnPChl*b*Mb	652	0.250	645	<−1	655	+22	
					626	−32.6	
	599	0.080					
	470	1.000	467	+7.1	479	+71.4	
					462	−73.6	
ZnPChl*d*[c]	686	0.90	686	−16.0	686	+79.6	$Q_y(0-0)$
	640	0.27	628	−3.5	642	−20.6	
	599	0.10			601	−52.6	
	552	0.04					
	449	1.00	448	+8.5	458	+89.7	B_x
					440	−39.3	B_y
ZnPChl*d*Mb	690	0.750	696	+6.4	692	+79.4	$Q_y(0-0)$
	655	0.270	634	+3.3			
					615	−52.0	
	461	1.000	457	+5.3	468	+85.6	B_x
					452	−36.6	B_y
ZnProtoPChl*a*[d]	620	0.145	*e*	*e*	623	+34.3	$Q_y(0-0)$
					599	−41	$Q_x(0-0)$
	568	0.050	*e*	*e*			$Q_y(1-0)$
	529	0.030	*e*	*e*			
	431	1.000	*e*	*e*	443	+12.8	B_x
					422	−9.2	B_y
ZnProtoPChl*a*Mb	622	0.125	625	+4.2	627	+38.8	$Q_y(0-0)$
					610	−46.6	$Q_x(0-0)$
	571	0.055					$Q_y(1-0)$
	535	0.025					
	441	1.000	445	+24	491	<+5	B_x, B_y
ZnBPChl*a*[c]	772	1.000	772	<+1	772	+39	$Q_y(0-0)$
	580	0.260	580	+3.8	579	−60	$Q_x(0-0)$
			394	+3.8	394	+49	B
	360	0.830	364	−4.8	360	−30	N
ZnBPChl*a*Mb	780	1.000	780	+6	785	+39	$Q_y(0-0)$
	592	0.265	591	+12	593	−63	$Q_x(0-0)$
			396	+23	399	+39	B
	365	0.803	357	−17	361	−24	N

[a] All samples approximately 10^{-5} M. Absorbance data normalized to 1.00 relative to most intense transition. [b] Solvent, CH_2Cl_2. [c] Solvent, 1% pyridine in CH_2Cl_2. [d] Solvent, MeOH. [e] No asymmetric centers. [f] The notation Q_y, Q_x, B, and N is a convention in the literature [see, e.g., Weiss (1972)] and is not meant to imply that the orientation of the associated transition dipole moment has been related experimentally to the molecular geometry.

It is much more likely that the protein environment itself is responsible for the effects we observe. The specific case of native Mb has been dealt with in detail by Hsu & Woody (1971), who were able to account for most CD features in the Soret region by calculating the coupled oscillator interaction of the heme with the π–π^* transitions of aromatic residues in the heme pocket within 12 Å. These authors have also shown that the magnitudes of the rotation in the Soret region are sensitive to the orientation of the transition moments in the heme plane. We find that the CD spectra of chlorin

Table III: Fluorescence Lifetimes for Various Chlorophyllide–apoMb Complexes and Corresponding Free Chromophores

compd	fluorescence lifetime (ns)
MgPimChl*a*[a,d]	5.73 ± 0.02
MgPChl*a*Mb[b,d]	6.8 ± 0.1
ZnPimChl*a*[a,d]	3.54 ± 0.03
ZnPChl*a*Mb[b,d]	4.0 ± 0.1
ZnPimChl*b*[a,e]	1.40 ± 0.02
ZnPChl*b*Mb[b,e]	1.72 ± 0.02
ZnBPChl*a*[c,f]	2.7 ± 0.1
ZnBPChl*a*Mb[b,f]	2.4 ± 0.1

[a] Solvent, CH_2Cl_2. [b] Solvent, water. [c] Solvent, 1% pyridine in CH_2Cl_2. [d] Excitation, 438 nm. [e] Excitation, 468 nm. [f] Excitation, 380 nm.

Table IV: Zero-Field Splitting Parameters for the Lowest Photoexcited Triplet State of Various Chlorophyllide–apoMb Complexes and Corresponding Free Chromophores[a]

compd	$\lvert D\rvert/(hc)$ (cm^{-1})	$\lvert E\rvert/(hc)$ (cm^{-1})
MgPimChl*a*[b]	0.0273 ± 0.0003	0.0039 ± 0.0003
MgPChl*a*Mb[c]	0.0297 ± 0.0003	0.0035 ± 0.0003
ZnPimChl*b*[b]	0.0309 ± 0.0002	0.0055 ± 0.0001
ZnPChl*b*Mb[c]	0.0308 ± 0.0016	0.0036 ± 0.0009
ZnBPChl*a*[d]	0.0228 ± 0.0003	0.0063 ± 0.0002
ZnBPChl*a*Mb[c]	0.0224 ± 0.0002	0.0057 ± 0.0002

[a] 80–90 K, 100-Hz light modulation. [b] Solvent, 2-methyltetrahydrofuran. [c] Solvent, 50/50 (v/v) glycerol/water. [d] Solvent, 10% pyridine in toluene.

chromophores change in the same direction when they are incorporated in the protein (the magnitudes of the change vary among the chromophores). This indicates that those features of the interaction which influence the CD spectra are not significantly affected by small structural changes. ZnProtoPChl*a* gives very similar results, indicating that the altered CD spectrum does not originate with changes in the coupling to the chiral centers of the chromophore. The similarity of the relative magnitudes of the CD spectra for the Q and B transitions suggests that the macrocycles (at least the transition moments) possess comparable orientations with respect to the protein, although we cannot specify the absolute orientation from the CD data alone. It is clear from this result that one cannot ignore the effects of the protein when analyzing the CD of natural Chl–protein complexes.

Doubling of NMR Peaks. As discussed earlier, the upfield-shifted Val-E11 methyl resonances provide a very sensitive test for the integrity of chlorophyllide–apoMb complexes. The observation of peak doubling for ZnPChl*a*Mb and ZnPChl*b*Mb, but not for other complexes, is the subject of further and continuing investigation. The discussion which follows is a qualitative analysis of this intriguing observation.

Our original hypothesis to explain double peaks was that solutions of these particular complexes were noninterconvertible mixtures containing chromophores in both orientations depicted in Figure 10, that is, *static disorder* in the heme pocket (Boxer & Wright, 1979). The chlorin ring current is sufficiently unsymmetrical when inverted about the α–γ axis that different shielding would be expected. The temperature-dependent interconversion of peak intensities shown in Figure 6, however, demonstrates that the cause of the peak doubling is *dynamic*. We present below three possible hypotheses to explain this phenomenon.

(i) Inversion in the Pocket. It is possible that the macrocycle can rotate about its α–γ axis in the heme pocket, either by inter- or by intramolecular exchange. We may safely assume that an intramolecular mechanism does not involve rotation of the 12-Å diameter chromophore in place, but it could occur by chlorophyllide–apoMb dissociation, inversion, and recombination. Chlorophyllides are quite insoluble in water so this is less likely in our complexes than in the natural heme complex.

The absence of saturation transfer for the unambiguously assigned, doubled aldehyde proton in ZnPChl*b*Mb places a firm upper limit of about 1 s^{-1} on the exchange rate. The crude temperature-jump experiment suggests that the change in peak intensities follows the temperature rise, so the lower limit is approximately 1 min^{-1}. If hemin is added to a solution of ZnPChl*a*Mb in H_2O (both 1×10^{-5} M), the characteristic metMb absorbance grows in with a first half-life of about 24 h. This experiment does not definitively rule out rapid dissociation of the chlorophyllide–apoMb complex, because the rates of dissociation and recombination could greatly exceed that of dissociation followed by displacement. However, the rate constant for the reaction hemin + apoMb → Mb is approximatley 10^5 M^{-1} s^{-1} (Adams, 1977); thus, at these concentrations, hemin would complex with apoMb in less than 1 s. Since this mechanism requires full dissociation, inversion, and recombination, displacement by heme would compete, leading to rapid exchange. The observation that displacement occurs so slowly suggests that this mechanism is not operative.

(ii) Restricted Rotation of Macrocycle Side Chains. Because NMR peak doubling is only observed for the apoMb complexes of certain macrocycles, it is natural to consider structural differences among the cases in Figure 5. All variations are at positions 2 and 3, a region of the macrocycle periphery known to have important functional consequences in Mb and Hb (Antonini & Brunori, 1971; Asakura & Sono, 1974; Seybert et al., 1976). Restricted rotation of vinyl or aldehyde groups could lead to the doubling of peaks, as these groups are conjugated to the macrocycle and are quite close to Val-E11 (see Figures 9 and 10). The 2-vinyl group is about 90° out of the plane in Mb single crystals (Perutz, 1975). Little definitive work is available on the orientations of these groups in solutions of the native proteins (LaMar et al., 1980). The barriers to rotation in simple molecules such as styrene or benzaldehyde are typically 8–12 kcal/mol (Drakenburg, 1980); hence slow exchange in the NMR spectrum is only observed at very low temperatures (we have been unable to detect the effects of restricted rotation for pyropheophorbide *b* methyl ester as low as −80 °C in CD_2Cl_2).

Even if rotation around the single bond were severely restricted by the protein, this alone would not lead to the *interchange of peak intensity* observed in Figure 6. A simple two-site exchange mechanism leads to NMR line broadening and coalescence as the temperature increases, and the two sites are sampled more rapidly. The data in Figure 6 show line narrowing and interchange of peak intensity, so simple two-site exchange is not consistent with the data. This argument also applies to hypothesis i.

(iii) Macrocycle-Dependent Conformational Changes in the Protein. In order to explain the NMR data, we postulate that the protein is present in at least two slowly interconverting conformational states, whose relative stabilities depend sensitively on substituents on rings I and II of the macrocycle and on temperature. The NMR spectrum of a protein in solution ordinarily exhibits single peaks for each type of proton, the exceptions being in the vicinity of the denaturation temperature, when signal intensity from native and denatured forms coexists, or free enzyme vs. enzyme–substrate complexes. The fact that single peaks are the rule for proteins in solution implies that the barriers to conformational fluctuations are

sufficiently low that each conformation is sampled on a time scale faster than the inverse of the largest chemical shift differences. Our observations suggest that for very specific macrocycle side chains in chlorophyllide–apoMb complexes, there is a substantial barrier between at least two "sites" which leads to nonaveraged chemical shifts. The observed line narrowing with increasing temperature is partially the consequence of more effective averaging within each site and partially the decrease in solvent viscosity, with attendant faster overall tumbling.

For the reasons outlined above, it is necessary to postulate that the relative stabilities of the two sites depend on temperature. If this were not the case, we would observe coalescence behavior. Unlike thermal denaturation, which occurs over a narrow temperature range, ZnPChl*b*Mb shows peak doubling over the entire range we have examined at this time. This analysis does not specify the molecular structures which lead to the doubled peaks. Although the chlorophyllide influences the populations of protein conformations, it is quite possible that the effect is detectable because these conformations differently restrict rotation of the aldehyde or vinyl groups or the lateral position of the macrocycle itself. We only have the aldehyde and Val-E11 peaks unambiguously assigned at this time, and these are insufficient to map out the shifts in each conformation in greater detail. The observation that the Val-E11 peaks are not doubled in the ZnPChl*a* horse heart apoMb complex and are doubled, but in very different ratio, for the ZnPChl*b* complex supports our interpretation. Phenomenologically, it is as if the room temperature horse heart Mb complexes are very similar to the high-temperature sperm whale Mb complexes. Apparently the small differences in primary sequence are sufficient to cause this change in relative conformational stability.

LaMar et al. (1978) have observed double sets of hyperfine shifted resonances in the NMR spectra of reconstituted Mb's. The patterns of these sets of peaks are similar to what is observed when the heme is strongly electronically perturbed by substitution of acetyl groups at positions 2 or 4 in model compounds. LaMar and co-workers argue that the protein could exert a similar unsymmetrical effect. Then, if one imagines the protein frozen in place and rotates the heme about the α–γ axis, the perturbation is placed on the other side of the ring, and this is postulated to account for the other set of resonances. It is not at all clear that the peak doubling in reconstituted Mb's is related to the phenomenon reported in this paper. Nonetheless, we can suggest the alternate possibility that the protein is present in two (or more) conformations, which exert a markedly different perturbation on the macrocycle. Our work shows that the ratio of populations at any temperature strongly depends on minor structural modifications. Regardless of interpretation, we conclude that modifications in the "prosthetic" group modify the protein conformation.

In summary, we find that the NMR spectra of some of the synthetic chlorophyllide–apoMb complexes provide evidence for very slowly interconverting conformations, whose relative populations are very sensitive to temperature and structural changes in the prosthetic group. Much more extensive studies of chlorophyllide–apoHb complexes, complexes with protein fragments, and the effects of solvent viscosity and pH on conformational changes will be presented in a separate paper.

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