

Giant circular dichroism of high molecular weight chlorophyllide–apomyoglobin complexes

(myoglobin/photosynthesis/protein complexes)

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ABSTRACT Chlorophyllide *a* and the apoprotein of myoglobin (Mb) spontaneously form three types of complex. The M ($M_r \approx 3 \times 10^5$) and H ($M_r \geq 4 \times 10^6$) complexes, but not the L ($M_r \approx 1.7 \times 10^4$), display a circular dichroism (CD) spectrum that is highly red-shifted, nonconservative, and very intense—characteristics shared by the CD spectra of reaction center complexes from purple photosynthetic bacteria. At its 710-nm peak, the H complex CD spectrum has a larger magnitude, 0.06 differential absorbance per unit total absorbance, than has been reported for chlorophyll in any medium.

Complexes in which a chlorophyll (Chl) derivative, such as chlorophyllide (Chlide), is substituted for heme in Mb have been reported previously (1, 2). In both of these earlier studies, the complexes were monomeric in apoMb (molecular weight $\approx 17,000$), and the pigment-to-protein ratio was 1:1. In one of them (1), the Chl derivative Mg chlorin e_6 , as a monomeric pigment bound to apoMb, was shown to undergo an irreversible photoconversion reaction to a long-wavelength-absorbing form. The other (2) used Mg pyrochlorophyllide *a*, which shows little change in absorbance on binding to apoMb (and no analogous photoconversion). Both types of 1:1 complex displayed CD spectra whose peak absolute magnitude per unit absorbance in the red were no more than 3 times that of monomeric Chl in a solvent (ref. 2 and unpublished data)—i.e., $\Delta A/A < \approx 3 \times 10^{-4}$. (ΔA is the differential absorption of circularly polarized light, $A_{\text{left}} - A_{\text{right}}$.)

More recently, we have shown (unpublished data) that Chlide *a* and apoMb spontaneously form not only 1:1 monomeric complexes but also complexes of much greater molecular weight. These high molecular weight complexes have some exceptional optical properties, which we report and discuss in this paper.

MATERIALS AND METHODS

The preparation of high molecular weight complexes will be described in greater detail elsewhere. Briefly, using 0.1 M sodium borate buffer (pH 9.2), a pyridine solution of Chlide *a*, prepared by the chlorophyllase digestion of Chl *a* (3), is diluted with a solution of apoMb, prepared from Mb by acid/butanone heme extraction (4). The final concentrations were typically 0.3 mM apoMb, 0.6 mM Chlide *a*, and 6% (vol/vol) pyridine. This solution was chromatographed on Sepharose CL-6B and gave two high molecular weight complexes and one monomeric complex. Boxer and Wright (2) reported monomeric apoMb complexes of Chl derivatives. However, their use of Sephadex G-25 probably precluded resolution of the high molecular weight complexes from the monomeric complex.

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Absorption and CD spectra were recorded on a Cary 118C spectrophotometer and Durrum–Jasco J-10 spectropolarimeter, respectively. These optical measurements were made on samples at room temperature.

RESULTS

Gel-filtration chromatography of the Chlide *a*–apoMb reconstitution mixture on Sepharose CL-6B resolved three bands containing both Chlide and protein and enabled us to estimate their molecular weights. These components are L (light), which consists of the expected 1:1 monomeric Chlide *a*–apoMb; M (medium), which has a molecular weight of $\approx 3 \times 10^5$; and H (heavy), with molecular weight $\geq 4 \times 10^6$.

Of the total protein recovered after gel filtration, approximately a third occurs in the M and H bands together. (Proportions vary somewhat between the latter two from preparation to preparation.) This large fraction, by itself, establishes that the protein of the M and H bands is indeed apoMb and not an impurity. The molar ratios of pigment to protein are approximately 2:1 in the M band and 4:1 in the H band. (Pigment and protein concentrations were determined as in ref. 1.) The M band therefore consists of aggregates of perhaps 16 apoMb molecules and 32 Chlide *a* molecules. H band aggregates are obviously much larger, with twice as much Chlide per unit protein.

Absorption and CD spectra of Chlide *a*–apoMb L, M, and H complexes are shown in Figs. 1 and 2. The red absorption maxima occur at 666, 677, and 681 nm, respectively. The ratio of Soret (blue) to red absorption maximum is noticeably greater for the H complex. The CD spectrum of the L complex is similar to that of Chl *a* in diethyl ether (5). In both cases, there is a single CD peak in the red near the absorption maximum (red-shifted 6 nm in the L complex, blue-shifted 4 nm in ether), with $\Delta A/A \approx 2 \times 10^{-4}$ but positive in the L complex and negative in ether. Boxer and Wright's CD spectrum of 1:1 Mg pyrochlorophyllide *a*–apoMb monomers (2) is quite similar to that of our Chlide *a*–apoMb L complex.

The CD spectra of M and H complexes contrast sharply with those of the L complex and Chl *a* in ether. The red CD bands of M and H complexes consist of two or more features, of both signs. For both complexes the longest-wavelength CD peak is positive and red-shifted (to 710 nm) ≈ 30 nm from the longest-wavelength absorption peak (itself red-shifted 16 or 20 nm compared to the red absorption peak of Chl *a* in ether) (5). The red CD peaks are also quite narrow (8–12 nm), only about a third the width of the absorption peaks. Most striking are the magnitudes of the 710-nm CD peaks for the M and H complexes. The M complexes have a maximum $\Delta A/A = 0.015$ –0.04, with the spectral shape varying somewhat from preparation to preparation. The H complexes have a maximum $\Delta A/A = 0.06$ with

Abbreviations: Chl, chlorophyll; Chlide, chlorophyllide, the acid of which chlorophyll is the phytol ester.

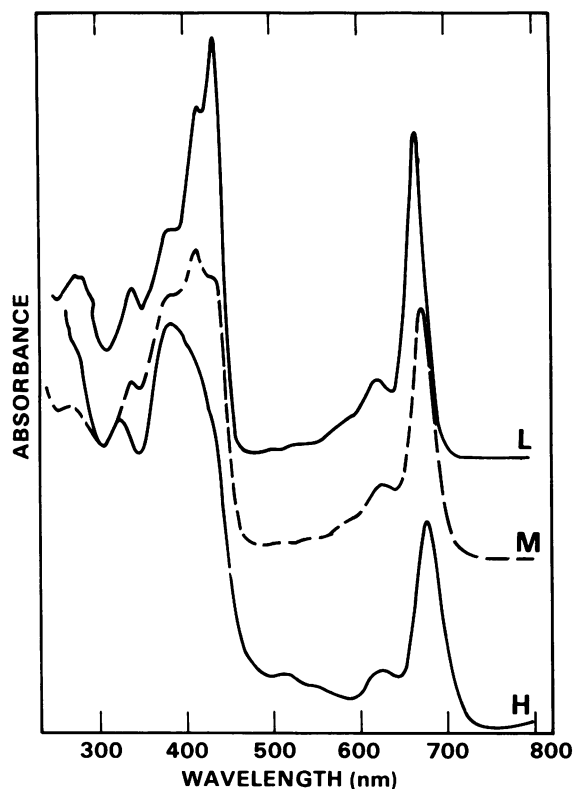


FIG. 1. Absorption spectra of Chlide-apoMb complexes in borate buffer (pH 9.2): L (top); M (middle); H (bottom).

the spectral shape being quantitatively reproducible among preparations.

DISCUSSION

Giant Magnitude of CD. The CD of the H complex is impressive when compared with natural or artificial complexes of pigments with biopolymers. The value of $\Delta A/A$ for the H complex 710-nm CD peak is ≈ 500 times larger than that for Mb or the L complexes of Chlide and apoMb, ≈ 50 times larger than that of the red CD peaks typical of Chl-protein complexes purified from green plants (6, 7), and 7 times larger than that of the negative CD peak at 821 nm in the Bchl *a*-protein from *Prosthecochloris aestuarii* (8), the largest reported value of $\Delta A/A$ for a Chl or Bchl red or near-infrared CD peak of which we are aware. Comparisons to induced CD in biologically related artificial systems also demonstrate how large $\Delta A/A$ is for the 710-nm CD of the H complex. Complexes of dyes such as Cibacron blue or congo red with enzymes have $\Delta A/A$ ratios 1/50th–1/2000th of this value (9). Complexes of polynuclear dyes such as acridine orange, proflavin, or ethidium bromide with DNA or poly(L-glutamate) have $\Delta A/A$ ratios 1/500th–1/5000th times as large (10–13). The values of $\Delta A/A$ for the 710-nm CD bands of the M and H Chlide-apoMb complexes are not as large as $\Delta A/A$ for some weak electric-dipole-forbidden transitions (14, 15). The only CD bands of electric-dipole-allowed transitions (e.g., the red transitions of chlorins) of which we are aware, that have $\Delta A/A$ as large as 0.05 occur in molecules (such as anthracene) in ordered liquid crystal matrices (16).

Other Distinguishing Features of CD. Two other characteristics of M and H complex CD spectra are noteworthy. With some minor exceptions only for the long-wavelength CD bands of some preparations of M complexes, the CD spectra of complexes in both the red and Soret regions are remarkably constant in shape; i.e., positions, widths, signs, and relative magnitudes

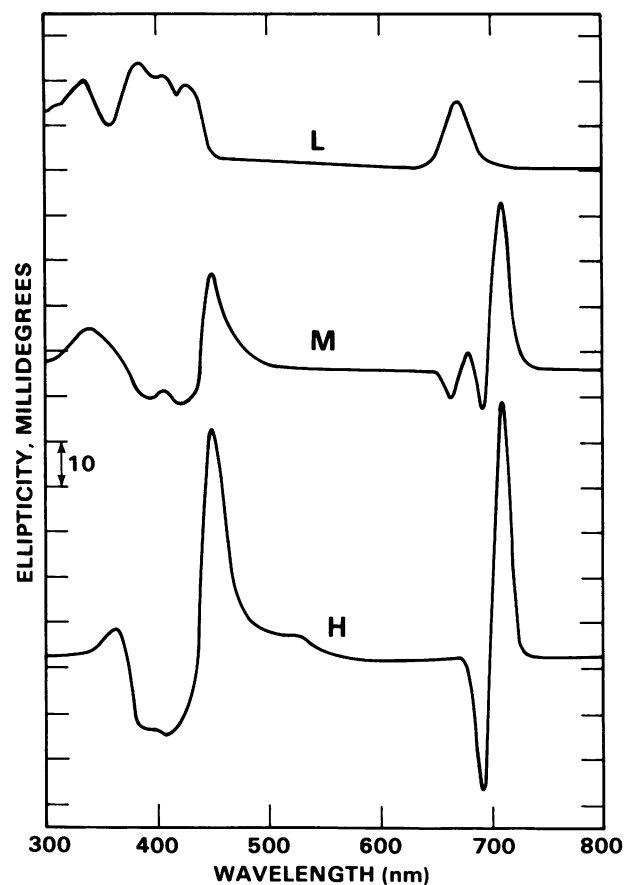


FIG. 2. Circular dichroism spectra of Chlide-apoMb complexes in borate buffer (pH 9.2): L (top), Chlide = 33 μM ; M (middle), Chlide = 9.7 μM ; H (bottom), Chlide = 0.93 μM . Optical path = 1 cm in all cases.

of bands are invariant over the M and H band elution profiles within each run and from preparation to preparation in spite of some variability in the Sepharose CL-6B elution profiles. This suggests the existence of a well-defined structural "core" of some sort that gives rise to the giant chirality (see below). Also, the red CD bands are distinctly nonconservative (do not integrate to zero area), the positive lobe(s) having at least twice the area of the negative.

Resemblance to Reaction Center CD. M and H complex CD spectra resemble, in three ways, those of Bchl-containing reaction center complexes from purple photosynthetic bacteria. Both are highly red-shifted ($\geq 10^3 \text{ cm}^{-1}$) relative to the corresponding pigment spectra in ether, both have peak magnitudes (per unit absorbance) $\geq 10^2$ times that of the corresponding pigment in ether, and both are nonconservative with the longest-wavelength band positive and having twice the area of the negative band(s) (17, 18). The reaction centers differ, among other ways, in having only six pigment molecules per complex and much broader CD bands.

Structural Considerations. We can propose no detailed model of the structure of M or H complexes based on current data. However, we can suggest a specific aggregation mechanism. It is based in part on the observation (unpublished) that, when Chlide *a* is mixed with Mb rather than apoMb, under otherwise identical conditions, $< 5\%$ of protein subsequently eluted from Sephadex G-75 is complexed with Chlide (and cochromatographs with Mb). Moreover, this Chlide is not spectrally red-shifted. This leads us to conclude that Chlide *a* binds

to the surface of the Mb protein only with difficulty* and without engaging in the red-shifting interactions prevalent in M and H complexes.

A mechanism that can account for the binding of the extra Chlide per protein in M and H complexes, explain the red shift, and provide a basis for interprotein aggregation is the axial liganding interaction of the Mg in one Chl molecule with the oxygen atom of the C-9 carbonyl group (not present in hemes) of another Chl. It is well established (19) that Chl *a* in which the C-9 keto oxygen is thus coordinated absorbs maximally near 680 nm, as do the M and H complexes. In this view, a Chlide bound in an apoMb heme cleft would have its C-9 keto oxygen [which, if in a binding mode analogous to heme in Mb (20), very likely extends outside the pocket] coordinated in one axial position of a Mg belonging to an external Chlide. This implies that those apoMb molecules whose clefts are occupied by Chlides can interact with additional Chlides to form M or H complexes. To build up aggregates of the observed size by this mechanism, some of the external Chlides must have their Mg atoms coordinated in both axial positions. We have no explanation why 300-kilodalton aggregates are favored, but this specific size would seem to be related to the regular structure, which also gives rise to the remarkably large and almost invariant CD spectrum.

CD Mechanism. The nonconservative nature of the individual M and H complex CD bands rules out a chiral mechanism based exclusively or even largely on resonance (exciton) interactions among Chlide electronic transition moments, which would necessarily give rise to a conservative CD band (18). In view of the large number of Chlides in each M or H complex, this is a surprising result. Another mechanism that apparently can be ruled out as an explanation of the giant CD is selective scattering (21, 22). This is a phenomenon in which light of one circular polarization is scattered to a greater extent (depending on direction) than light of the other polarization. It requires structures that are large enough to scatter considerable total light. The M and H complexes, particularly the ≈ 300 -kilodalton M complex, are too small to be expected to satisfy this requirement. Additionally, preferential circularly polarized scattering depends on the dispersive (i.e., optical rotatory dispersion) as well as absorptive (i.e., CD) properties (21). Because there is optical rotation outside absorption (and CD) bands one expects CD artifacts caused by scattering to be broadened. This is the case for circularly polarized scattering from chloroplasts and similar structures (22). The ≈ 700 -nm CD features of the M and H complexes are remarkably narrow, which argues against scattering as the origin of the giant CD.

Without a detailed model of M or H complexes, we can only speculate that the giant CD arises from a small subset of the Chlide *a* whose absorbance is centered near 700 nm. This chiral Chlide could be in-pocket but skewed relative to "normal" in-pocket Chlide so as to substantially change its interaction with the protein. Alternatively, the chiral Chlide could be external with the chirality arising either from (nonresonant) Chlide-Chlide interactions or from Chlide interactions with external apoMb

amino acids. In either case, we cannot altogether rule out the possibility that it is interaction of Chlide with some altered (i.e., partially denatured) form of apoMb in the heavy complexes that gives rise to the giant CD. If the chirality arises from amino acid interactions it is unlikely to be of the Kirkwood-Tinoco dynamic-coupling type as applied by Hsu and Woody (23) to successfully calculate the visible CD of Mb. Their model considers the dynamic coupling of the ultraviolet transitions of aromatic amino acids with visible transitions of the heme. The Hsu-Woody model might be able to explain the CD of Chlide-apoMb L complexes because the rotational strengths of the latter's visible bands are comparable to the strength of the Soret band of heme in Mb. We believe it unlikely, however, that this model can explain our observations with Chlide-apoMb heavy complexes, because the giant CD is two orders of magnitude greater than that of the heme in Mb.

The possibility that there can be other types of substantial chiral interactions of Chl with charged amino acids, which may include shifts of Chl absorption spectra (24), should not be overlooked. It is our hope that further studies of the M and H complexes will yield the mechanism of the giant CD, and perhaps suggest an explanation for similar phenomena observed in photosynthetic reaction centers.

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* Greater ($\approx 20\%$) surface binding of Zn pyrochlorophyllide (rather than Chlide *a*, which we use) to Mb is claimed in ref. 2. This conclusion is based on observations with a mixture of the Zn pigment and Mb eluted from Sephadex G-25.