Imidazole- and Alkylamine-Ligated Iron(II, III) Chlorin Complexes as Models for Histidine and Lysine Coordination to Iron in Dihydroporphyrin-Containing Proteins: Characterization with Magnetic Circular Dichroism Spectroscopy

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An extensive series of five- and six-coordinate (1-methylimidazole)-, (1-butylamine)-, and (pyridine)iron(II, III) chlorin complexes are characterized for the first time with magnetic circular dichroism (MCD) spectroscopy. The chlorins (dihydroporphyrins) employed are octaethylchlorin, mesochlorin, and "methyl"chlorin (2,2,4-trimethyldeuterochlorin, featuring a *gem*-dimethyl-substituted peripheral carbon). The species studied include the bis(1-methylimidazole)ferric, bis(1-methylimidazole)ferrous, (1,2-dimethylimidazole)ferrous, and 1-methylimidazole/CO or NO complexes together with analogous bis(pyridine)- and bis(1-butylamine)ferrous adducts and their CO-ligated derivatives. The results presented establish MCD spectral signatures for use in determining whether an iron chlorin-containing protein bears either a histidine or a lysine axial ligand. Analysis of data obtained with different ring-reduced mesochlorin isomers shows that the method is insensitive to the site of pyrrole ring reduction. This latter observation will facilitate the use of mesochlorins in reconstitution experiments with structurally-defined heme proteins in order to investigate mixed-ligand complexes which are difficult to generate synthetically. In general, the MCD spectra of iron chlorin complexes are most sensitive to the identity, number, and type of *axial* ligand, along with the oxidation and spin state, and are relatively insensitive to changes in the *equatorial* plane such as the site of pyrrole ring reduction. Therefore, it appears that MCD spectroscopy will be of particular use in the identification of proximal and distal axial ligands in chlorin-containing proteins, as has repeatedly been shown to be the case with protoheme-based iron porphyrins.

Introduction

Iron chlorins (dihydroporphyrins) have been found as the prosthetic groups of a number of heme proteins in recent years. The most well documented cases are the green catalases from *Escherichia coli* (HPII catalase) and *Neurospora crassa*[1] as well as the heme *d*-containing terminal oxidase from *E. coli*.[2] Sulfmyoglobin, formed by addition of H_2O_2 to the protein in the presence of H_2S, contains a unique chlorin macrocycle resulting from addition of a sulfur atom to the porphyrin.[3] The green hemeprotein from spleen[4] (now known to be spleen myeloperoxidase[5]) and myeloperoxidases from other sources[6] have been proposed to contain an iron chlorin prosthetic group,[7] but recent results suggest that the green color may be due to the presence of a formyl-substituted heme group such as is found in heme *a*.[8] In an effort to explore the potential of magnetic circular dichroism (MCD) spectroscopy as a probe of the coordination structures of iron chlorin-containing proteins, we have initiated a three stage effort involving the study of synthetic iron chlorin model complexes described herein, the investigation of structurally defined proteins such as myoglobin reconstituted with iron chlorin prosthetic groups[9] and the examination of naturally occurring iron chlorin proteins such as those mentioned above.[10]

MCD spectroscopy[11-13] has been repeatedly shown to be of great utility in the identification of axial ligands in protoporphyrin IX (protoheme)-containing proteins. All heme iron oxidation states (ferrous, ferric and ferryl) display MCD spectra and the method is most sensitive to factors that affect the electronic structure such as the nature of the axial ligands. Examination of the MCD spectra of "new" protoheme-containing proteins in different oxidation and ligand-bound states can often lead to the establishment of the axial ligand structure in a single, simple study.[11,14-17] In order to establish whether MCD spectroscopy can be similarly used to probe axial ligand identity in iron chlorin

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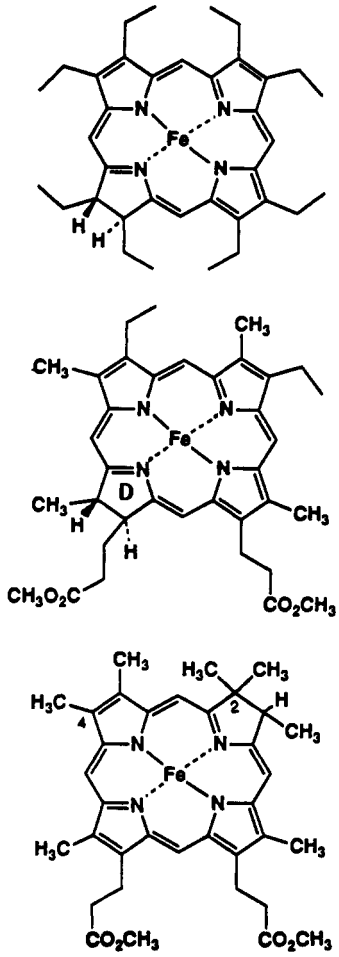

Figure 1. Chlorins used in the study: structures of the iron complexes of *trans*-octaethylchlorin (top), of the ring D-reduced isomer of *trans*-mesochlorin dimethyl ester (center), and of methylchlorin dimethyl ester (bottom). For simplicity, axial ligands are omitted and only the ring D-reduced isomer of mesochlorin is shown.

proteins, we have studied the MCD properties of high- and low-spin iron(II, III) complexes of three related chlorins. There has been a relative lack of MCD data on well characterized iron chlorin systems. The only major previous study of the MCD properties of iron chlorins is that of Stolzenberg et al.[18] which emphasized high-spin ferric complexes with non-nitrogenous anionic biomimetic ligands. The present study is complementary in that we focus on low-spin ferric as well as low- and high-spin ferrous complexes with biomimetic neutral nitrogenous ligands. The results described herein represent a substantial increase in the MCD database for use in probing the active site coordination structures of iron chlorin-containing proteins.

Because reduction of a peripheral double bond of a naturally occurring porphyrin to form a dihydroporphyrin (chlorin) product can yield four different pyrrole ring-reduced isomers, we have also investigated the extent to which MCD spectroscopy is sensitive to the site of ring reduction by examination of a pure isomer as well as mixtures of isomers of several different iron *meso*chlorin complexes. The chlorins used in this study are displayed in Figure 1. By addressing the effect of changes in the nature of both axial as well as equatorial ligands and by substantially expanding the available MCD data on biomimetic iron chlorin complexes to include low-spin ferric as well as high- and low-spin ferrous cases, the present findings lay a solid foundation for the use of the technique in the study of iron chlorin-containing proteins.

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Experimental Section

Materials. Reagents and chemicals were obtained from Aldrich or Fisher unless otherwise indicated. Benzene and CH_2Cl_2 were carefully purified using literature procedures.[19] Ethanethiol, triethylamine, 1-methylimidazole (1-MeIm)[20] and pyridine were distilled under nitrogen, the latter two from KOH. 1-Butylamine (1-$BuNH_2$) was dried over KOH then distilled from P_2O_5 under nitrogen. 1,2-Dimethylimidazole (1,2-Me_2Im) was recrystallized from benzene. Nitric oxide (Matheson, 99.0% min.) was purified by passage through a column of KOH pellets. $CDCl_3$ (100% D) was used for NMR studies. Sodium dithionite (Virginia Chemicals), CO (Matheson, 99.5% min.), and phenol (99+%) were used without further purification. Hexane and $CHCl_3$ used for chromatography were reagent grade and were used without additional purification. Preparative silica gel thin layer chromatography plates (1000 μm, preconcentrating zone) were obtained from Curtin Matheson Scientific.

Preparation of Complexes.[21] Because of possible light sensitivity, complexes were prepared and handled in subdued light. All complexes except the ferric bis(imidazole) and chloride adducts were prepared and handled under nitrogen in a Vacuum Atmospheres inert atmosphere box. For spectral analysis, O_2-sensitive samples were studied in sealed cuvettes. All complexes other than the ferric chloride adducts were generated in situ from solutions of the corresponding chloride complex of defined concentration. Except for the butylamine species, complex formation was confirmed by comparison of the absorption spectrum to those of previously reported octaethylchlorin or related chlorin adducts.[18,22-24]

A. Fe(chlorin)Cl. The ferric chloride complexes of *trans*-octaethylchlorin (octaethylchlorin) and *trans*-mesochlorin dimethyl ester (mesochlorin), both containing small amounts of the corresponding iron porphyrin (see below), as well as of methylchlorin dimethyl ester (methylchlorin) were prepared as described elsewhere.[21,25-27] The structures of the chlorins used herein are shown in Figure 1. Metal-free *trans*-octaethylchlorin was synthesized by the method of Whitlock[25a] and purified by preparative thin layer chromatography. A $CHCl_3$ solution of the crude product was applied to 1 mm silica gel plates that were saturated with ammonia vapor and then developed with hexanes/$CHCl_3$ [3:2 (v/v)]. The porphyrin-free, green free base chlorin was metalated to produce Fe(octaethylchlorin)Cl using a modification[18] of the direct iron insertion procedure of Collman.[25b] Metallation typically led to generation of a small amount (1–8%) of Fe(octaethylporphyrin)Cl.[21] Three forms of ferric mesochlorin chloride were used: (a) a mixture of ring A- and ring B-reduced isomers (~1:1 ratio), (b) a mixture of ring C- and ring D-reduced isomers (~1:1 ratio), and (c) the separate ring D-reduced isomer. Extinction coefficients, determined by weight (octaethylchlorin and mesochlorin) or by back-calculation from the ferrous pyridine hemochrome (methylchlorin), were used to establish the concentration of the ferric chloride complexes[28] and of products subsequently generated from the ferric chloride species.

B. [Fe(chlorin)(1-MeIm)$_2$]Cl, Fe(chlorin)(1-MeIm)$_2$, and Fe(chlorin)(CO)(1-MeIm). The ferric bis(imidazole) complexes were generated by addition of neat 1-MeIm to the Fe(chlorin)Cl in benzene or CH_2Cl_2 until no further spectral change occurred. The ferrous bis(imidazole) octaethylchlorin and mesochlorin adducts were prepared by addition of a large excess of neat 1-MeIm to the appropriate ferric chloride complex

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in benzene followed by reduction with freshly prepared aqueous $Na_2S_2O_4$.[29] Additional 1-MeIm was added to the organic layer until changes in the absorption spectrum were no longer observed. Fe(methylchlorin)(1-MeIm)$_2$ was formed by treatment of the corresponding ferric chloride complex in benzene with 1-MeIm followed by reduction with ethanethiol in the presence of triethylamine.[18,30-32] The CO adducts were prepared by briefly bubbling CO through the ferrous bis(imidazole) solution until the color changed from blue to bright green.

C. Fe(chlorin)(1,2-Me$_2$Im). Benzene solutions of 1,2-Me$_2$Im and of Fe(chlorin)Cl were mixed, reduced with aqueous $Na_2S_2O_4$ and the organic layer treated with excess ligand as above. No chlorin bis(imidazole) formation was observed, as previously reported.[33]

D. Fe(chlorin)(pyridine)$_2$ and Fe(chlorin)(CO)(pyridine). Concentrated stock solutions of Fe(methylchlorin)Cl or Fe(octaethylchlorin)Cl in benzene were diluted with pyridine and reduced with solid $Na_2S_2O_4$ to produce the pyridine hemochromes. The ferrous pyridine CO complexes were prepared as described above for the imidazole CO adducts.

E. Fe(chlorin)(1-BuNH$_2$)$_2$. A concentrated stock solution of Fe(chlorin)Cl in benzene was diluted with 1-BuNH$_2$ to produce Fe(chlorin)(1-BuNH$_2$)$_2$. As with ferric porphyrins,[34] the iron(III) chlorins were reduced in the presence of large excess of aliphatic amine. Several amine concentrations were used to assure that the complexes were fully formed.

F. Fe(octaethylchlorin)(1-BuNH$_2$)(CO). A benzene solution of Fe(octaethylchlorin)Cl was shaken with aqueous $Na_2S_2O_4$ in the presence of CO[35] to give Fe(octaethylchlorin)(CO)$_n$ (n = 1 or 2)[32,36] which was titrated with neat 1-BuNH$_2$ until no further spectral changes were noted. Overtitration resulted in the appearance of some of the ferrous *porphyrin* bis(amine) species, readily detected by examination of the visible region of the MCD spectrum.

G. Fe(octaethylchlorin)(1-MeIm)(NO). Fe(octaethylchlorin)(NO) was prepared from Fe(octaethylchlorin)Cl as previously described.[32,37] A solution of Fe(octaethylchlorin)Cl and triethylamine in benzene was degassed (repetitive freeze–pump–thaw cycles) and a small amount of degassed ethanethiol was added by vacuum distillation. NO was briefly introduced into the resulting ferrous chlorin solution followed by evaporation to dryness in vacuo. A portion of the solid Fe(octaethylchlorin)(NO) was dissolved in benzene for spectral analysis. The remaining solid was dissolved in 5 M 1-MeIm in benzene. MCD spectral examination showed this solution to contain a mixture of the Fe(octaethylchlorin)(1-MeIm)(NO), Fe(octaethylporphyrin)(1-MeIm)(NO), and Fe(octaethylporphyrin)(1-MeIm)$_2$.[38] Upon treatment with additional NO, the MCD features due to the ferrous bis(imidazole) species disappeared.

Spectral Analysis. With low-spin six-coordinate ferrous octaethylchlorin complexes, the small amount (1–8%) of the corresponding ferrous octaethylporphyrin derivatives present significantly affected the MCD spectra. The MCD signals of many ferrous six-coordinate low-spin porphyrin complexes are very intense, up to 20 times more intense than the chlorin counterparts in the region near 550 nm.[39] The MCD signals due to contaminating octaethylporphyrin complexes were eliminated from the spectra of these octaethylchlorin complexes by computer subtraction. The quantity (percentage) of the porphyrin spectrum subtracted was determined by MCD spectroscopy and confirmed by ^{1}H NMR spectroscopy (see below). The octaethylporphyrin complexes were prepared using the same general procedures as for the chlorin complexes. In each case, iterative subtractions were carried out using increasing percentages of the octaethylporphyrin spectra until the predominant porphyrin signals were eliminated. For the low-spin six-coordinate ferrous mesochlorin complexes, the corresponding octaethylporphyrin complexes were again used for the computer subtraction because the mesoporphyrin data were unavailable.

The ^{1}H NMR spectrum of [Fe(octaethylchlorin)(1-MeIm)$_2$]Cl has previously been used to quantitate the amount of octaethylporphyrin contamination in octaethylchlorin complexes.[40] We examined the adduct at −41 °C in $CDCl_3$.[41] The resonance used for integration was that of the singlet at 22.2 ppm for the *N*-methyl group on the coordinated 1-MeIm.[40]

Spectral Measurements. Electronic absorption spectra were collected on Varian/Cary 210 or 219 spectrophotometers interfaced to an IBM PC. MCD measurements were obtained with a JASCO J-500A spectropolarimeter interfaced to an IBM PS/2 Model 50 computer via a JASCO IF-500II interface box and equipped with a JASCO MCD-1B electromagnet (1.5 T). The magnetic field direction was set to be parallel to the direction of light propagation. Magnetic field strength was determined using a freshly prepared solution of potassium ferricyanide.[12] The instrument was calibrated for CD intensity with ammonium d-10-camphorsulfonate ($[\theta]_{290.5nm}$ = 7910 deg cm^2 dmol^{-1}).[43] The integrity of the CD modulator was insured by a two-point calibration and the wavelength drive was calibrated using a neodymium glass filter.[44] The baseline-subtracted MCD spectra have been normalized to pathlength, concentration and magnetic field strength [(M·cm·T)$^{-1}$]. Where appropriate, the data were smoothed using Savitzky-Golay routines.[45] Absorption and MCD data were obtained using a customized spectra database designed and written primarily by Dr. Edmund W. Svastits. Absorption spectra were acquired before and after each MCD spectrum; only those cases where the pre- and post-MCD absorption spectra differ by less than 5% are reported herein. All optical spectral measurements were acquired at ambient temperature.

Proton NMR spectra were recorded on a Bruker AM-300 spectrometer operating at 300.16 MHz. The temperature of the probe was determined using published procedures and was accurate to ±0.5 °C.[46] Spectra were obtained using a 60 kHz sweep width, 90° pulse with a 5 ms delay, and a 32K-word data set zero-filled to 64K words before transforming.

Results

High-Spin Ferric Chlorin Complexes. The MCD spectra of the five-coordinate high-spin[47] ferric chlorin chloride complexes of octaethylchlorin and methylchlorin are compared in Figure 2A while the spectra of the corresponding adducts of two different ferric mesochlorin isomer mixtures (ring A- and ring B-reduced isomer mixture vs ring C- and ring D-reduced isomer mixture) are shown in Figure 2B. All four MCD spectra are quite similar to each other as well as to the MCD spectrum of the same complex of the separate ring D-reduced mesochlorin isomer (data not shown). The spectra feature broad troughs centered near 380 and 575 nm, each clearly composed of two partially resolved bands, and three very broad peaks between about 420 and 535 nm. The UV–visible absorption spectra of these complexes (data not shown[21]) closely match that of the octaethylchlorin complex.[18]

Low-Spin Ferric Chlorin Complexes. The MCD spectra of the six-coordinate bis(1-methylimidazole) chlorin low-spin[18] adducts of octaethylchlorin and methylchlorin are compared in Figure 3A. In Figure 3B, the MCD spectrum of the same adduct of the

(28) The following values were used for λ and ε for Fe(chlorin)Cl in methylene chloride: Fe(octaethylchlorin)Cl, 378 nm (88 mM^{-1} cm^{-1}), 472 nm (7.8 mM^{-1} cm^{-1}), 604 nm (20 mM^{-1} cm^{-1}); Fe(mesochlorin)Cl, the ring A- and ring B-reduced isomer mixture, 377 nm (91 mM^{-1} cm^{-1}), 472 nm (7.6 mM^{-1} cm^{-1}), 602 nm (20 mM^{-1} cm^{-1}). For Fe(methylchlorin)(pyridine)$_2$ in pyridine: 416 nm (90 mM^{-1} cm^{-1}).

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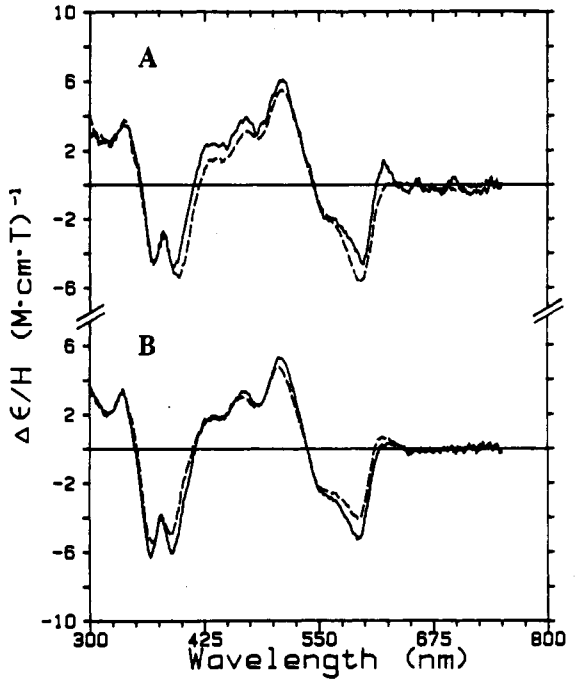

Figure 2. MCD spectra of Fe(chlorin)Cl complexes in methylene chloride: (A) octaethylchlorin (—) vs methylchlorin (- - -); (B) the ring A- and ring B-reduced mesochlorin isomer mixture (—) vs the ring C- and ring D-reduced mesochlorin isomer mixture (- - -).

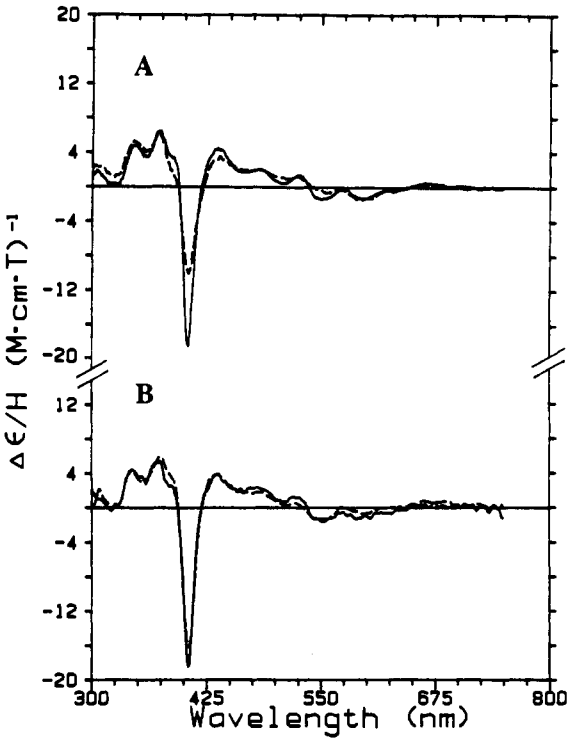

Figure 3. MCD spectra of $[Fe(chlorin)(1\text{-}MeIm)_2]^+$ complexes in methylene chloride: (A) octaethylchlorin (—) vs methylchlorin (- - -); (B) the ring A- and ring B-reduced mesochlorin isomer mixture (—) vs the ring C- and ring D-reduced mesochlorin isomer mixture (- - -).

ring A- and ring B-reduced ferric mesochlorin isomer mixture is overplotted with that of the corresponding complex of the ring C- and ring D-reduced mesochlorin isomer mixture. Once again, the four MCD spectra are nearly identical to each other and, as before, also match the spectrum of the same complex of the separate ring D-reduced mesochlorin isomer (data not shown). The spectra are dominated by a sharp trough at about 405 nm with two or three weak peaks on the low wavelength side, and with a series of increasingly weaker peaks on the higher wavelength side leading to weak trough centered at about 550 nm. The UV–visible spectra of these complexes (data not shown) are very similar to that reported by Ozaki et al.[22] for ferric bis(imidazole) octaethylchlorin. The data presented in Figure 3 are the first examples of the MCD spectra of low-spin ferric chlorin model complexes.

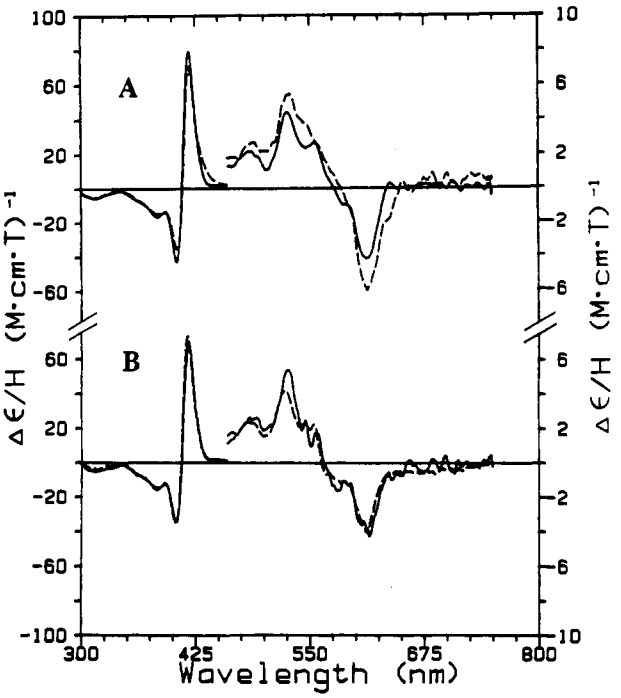

Figure 4. MCD spectra of $Fe(chlorin)(1,2\text{-}Me_2Im)$ complexes in benzene: (A) octaethylchlorin (—) vs methylchlorin (- - -); (B) the ring C- and ring D-reduced mesochlorin isomer mixture (—) vs the ring D-reduced mesochlorin isomer (- - -).

High-Spin Ferrous Chlorin Complexes. In Figure 4A, the MCD spectra of the five-coordinate, high-spin[33] 1,2-dimethylimidazole adducts of ferrous octaethylchlorin and methylchlorin are displayed. The same adducts of the ring C- and ring D-reduced isomer mixture and of the separate ring D-reduced isomer of ferrous mesochlorin are compared in Figure 4B. As with the high- and low-spin ferric complexes just described, the four MCD spectra in Figure 4 are nearly identical to each other; the ring A- and ring B-reduced isomer mixture of the corresponding ferrous mesochlorin complex was not examined. The spectra feature a very intense (for iron chlorins) negatively-signed derivative-shaped curve in the Soret region centered near 410 nm that is asymmetric (peak intensity 2-fold greater than trough intensity) followed by a lower intensity trough at shorter wavelength. The remaining spectral features are considerably lower in intensity and contain a complex species of peaks between 425 and 560 nm and a broad trough at about 615 nm. The UV–visible absorption spectra of the complexes shown in Figure 4 (data not shown[21]) are very similar to that of a mono(imidazole)ferrous chlorin adduct in which the imidazole is covalently linked to the chlorin.[23] No MCD spectra have previously been reported for high-spin ferrous chlorins.

Low-Spin Ferrous Chlorin Complexes. Figures 5–7 display the MCD spectra of several six-coordinate low-spin[33,48,49] ferrous chlorin complexes with nitrogenous ligands (imidazole, alkylamine or pyridine) and, in some cases, with CO or NO as a ligand. No MCD spectra of low-spin ferrous chlorin complexes have been

(48) Chatfield, M. J.; La Mar, G. N.; Kauten, R. J. *Biochemistry* **1987**, *26*, 6939.

(49) The ferrous CO/pyridine, CO/1-$BuNH_2$, and NO/1-MeIm complexes are assumed to be low-spin by analogy to the known spin state of the corresponding porphyrin complexes: (a) Scheidt, W. R.; Reed, C. A. *Chem. Rev.* **1981**, *81*, 543. (b) Svastits, E. W. Ph.D. Dissertation, University of South Carolina, 1986. (c) The MCD spectrum of Fe(octaethylchlorin)(CO)(1-MeIm) in Figure 6A has been recently reported by our laboratory.[Nb]

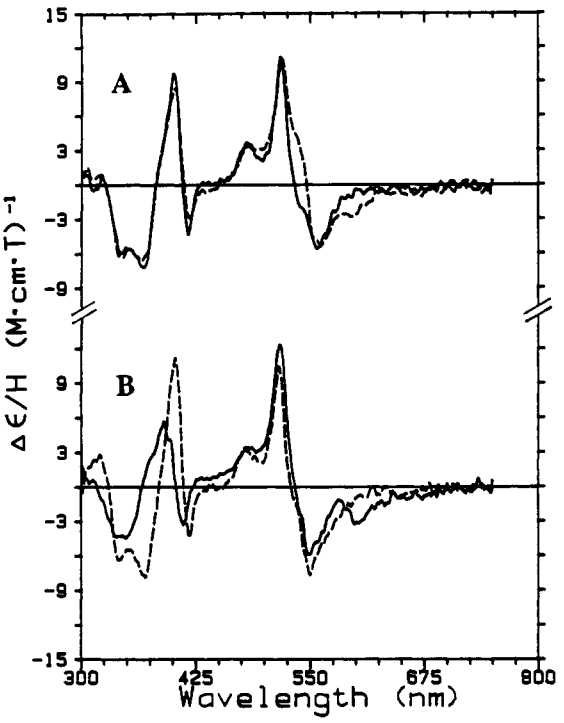

Figure 5. MCD spectra of ferrous chlorin bis(nitrogen donor) complexes: (A) Fe(octaethylchlorin)(1-MeIm)$_2$ (—) vs Fe(methylchlorin)-(1-MeIm)$_2$ (- - -) in benzene; (B) Fe(octaethylchlorin)(pyridine)$_2$ in ~20:1 pyridine/benzene (—) vs Fe(octaethylchlorin)(1-BuNH$_2$)$_2$ in ~20:1 1-butylamine/benzene (- - -).

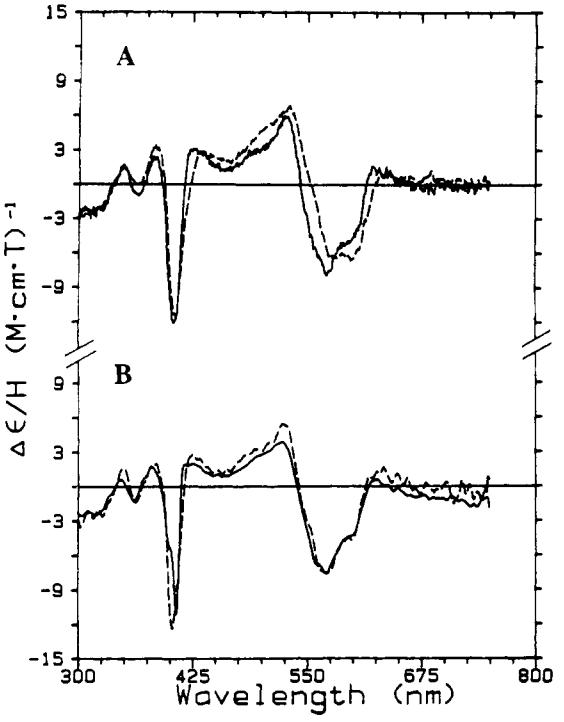

Figure 6. MCD spectra of Fe(chlorin)(CO)(1-MeIm) complexes in benzene: (A) octaethylchlorin (—) vs methylchlorin (- - -); (B) the ring C- and ring D-reduced mesochlorin isomer mixture (—) vs the ring D-reduced mesochlorin isomer (- - -).

previously reported.[49c] The MCD spectra of the bis(1-methylimidazole) adducts of ferrous octaethylchlorin and methylchlorin and of the bis(1-butylamine) and bis(pyridine) adducts of ferrous octaethylchlorin are shown in Figure 5. Except for the lower intensity of the features in the Soret region for the bis(pyridine) adduct, the four spectra are quite similar to each other. As noted in the Experimental Section, the spectra of the octaethylchlorin complexes have been corrected for the presence of a small percentage of porphyrin contamination. The MCD

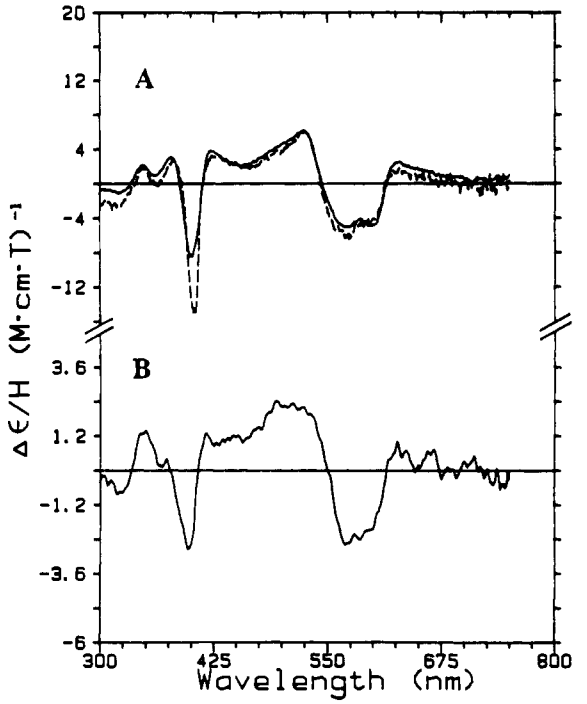

Figure 7. (A) MCD spectra of Fe(octaethylchlorin)(CO)(pyridine) in ~20:1 pyridine/benzene (—) vs Fe(octaethylchlorin)(CO)(1-BuNH$_2$) in benzene (- - -). (B) MCD spectrum of Fe(octaethylchlorin)(NO)-(1-MeIm) in 5 M 1-MeIm/benzene.

spectra of the bis(1-butylamine) and bis(pyridine) adducts of ferrous methylchlorin (data not shown[21]) are essentially identical to the corresponding octaethylchlorin adducts shown in Figure 5B. The MCD spectra contain a complex trough–peak–trough pattern with increasing wavelength in the 325–450 nm region followed by a peak–peak–trough pattern with increasing wavelength between 475 and 625 nm. The UV–visible absorption spectra of the bis(1-methylimidazole) and bis(pyridine) complexes (data not shown) closely match those previously published by Stolzenberg et al.[18] for the respective octaethylchlorin adducts. The UV–visible absorption spectrum of the ferrous octaethylchlorin bis(1-butylamine) complex[21] compares well with that of the bis(imidazole) complex.[18] The bis(1-butylamine) complex has been investigated previously by resonance Raman spectroscopy.[33]

The MCD spectra of the 1-methylimidazole/CO adducts of ferrous octaethylchlorin and methylchlorin are overplotted in Figure 6A, and the spectrum of the ring C- and ring D-reduced mixture of ferrous mesochlorin is compared to the spectrum of same adduct of the separate ring D-reduced isomer in Figure 6B. The ring A- and ring B-reduced isomer mixture of the ferrous mesochlorin species was not examined. As described in the Experimental Section, the spectra of the octaethylchlorin and mesochlorin complexes have been corrected for the presence of a small amount of porphyrin impurity. All four MCD spectra shown in Figure 6 are quite similar to each other. The most intense band in the spectra is a sharp trough centered near 405 nm with weaker flanking peaks on either side and a very broad peak–trough pattern with increasing wavelength between 450 and 625 nm. The UV–visible absorption spectra of these complexes (data not shown) compare well with that reported for the corresponding octaethylchlorin derivative.[18]

In Figure 7A, the MCD spectra of the pyridine/CO and 1-butylamine/CO adducts of ferrous octaethylchlorin are compared. The spectrum of the imidazole/NO complex of ferrous octaethylchlorin is displayed in Figure 7B. The spectra have all been corrected for the presence of a small amount of the corresponding porphyrin (see Experimental Section). As will be discussed below, except for the lower intensity of the spectrum of the ferrous–NO species, the three spectra shown in Figure 7

are quite similar to the four spectra shown in Figure 6. This is also true of the MCD spectra of the pyridine/CO and 1-butylamine/CO adducts of ferrous methylchlorin (data not shown) which closely match the spectra of the corresponding octaethylchlorin complexes shown in Figure 7A and are generally similar to the other spectra of ferrous octaethylchlorin with a nitrogenous ligand trans to CO or NO displayed in Figures 6 and 7B. The UV–visible absorption spectrum of the pyridine/CO ferrous octaethylchlorin adduct compares well to that reported previously.[18] The UV–visible absorption spectrum of the five-coordinate NO adduct matches the published data[37] and the MCD spectrum of the subsequent six-coordinate 1-methylimidazole/NO is nearly identical to that of the ferrous–NO complex of chlorin-reconstituted myoglobin.[9]

Discussion

MCD spectroscopy has been employed with considerable success over the past 15 years in the study of heme proteins to establish the identity of axial ligands in structurally undefined heme proteins.[11] Such applications of the method have been based on comparisons of the MCD spectra of various ligand-bound derivatives of the protein in different oxidation states to the spectra of ligation and oxidation state derivatives of structurally established heme proteins and model complexes. This approach has been successful because of the demonstrated sensitivity of MCD spectroscopy to changes in axial ligation (for a given porphyrin macrocycle) and insensitivity to changes in heme environment (either the solvent or the protein matrix). Many examples of the success of this approach are described in two recent review articles and in other recent publications.[11,14-17]

Iron chlorins are porphyrin-derived iron-containing prosthetic groups in which one of the peripheral double bonds of the porphyrin macrocycle has been reduced to yield an iron dihydroporphyrin. Only a limited number of iron chlorin complexes, all high-spin ferric species with anionic non-nitrogenous ligands, have been investigated with MCD spectroscopy;[18] the purpose of this study is to expand the coverage of iron chlorin adducts with MCD spectroscopy to include low-spin ferric as well as high- and low-spin ferrous model complexes with biomimetic nitrogenous ligands including imidazole and alkylamines as well as pyridine. In so doing, we will address the question of whether MCD spectroscopy can be used as profitably to establish the coordination structures of iron chlorin complexes, as has been possible with "regular" iron porphyrins. In pursuing this work, we quickly became aware of two "problems" which needed to be addressed. The first concerns the noticeably lower intensity of the MCD signals for iron chlorins, relative to that of iron porphyrins, especially in the low-spin ferrous state. A number of solutions to this problem will be described below. The second problem, really a limitation of the model system approach itself, is the difficulty in making very many mixed-ligand ferric and ferrous complexes. A simple solution to that problem is to study the MCD spectra of iron chlorins reconstituted into structurally defined heme proteins such as myoglobin or horseradish peroxidase which readily bind exogenous ligands. However, to do such reconstitutions, a suitable iron chlorin prosthetic group needed to be identified for insertion into the apoproteins just mentioned.

MCD Spectroscopy as a Structural Probe of N-Donor-Ligated Iron Chlorin Complexes. As can be readily seen in the figures, within a given type of complex, the MCD spectra of analogous derivatives of octaethylchlorin, methylchlorin and mesochlorin are essentially identical (the spectral comparison of the different mesochlorin isomers will be discussed later). It therefore appears that changes in the nature and position of peripheral alkyl groups on the macrocycle, even the presence of the *gem*-dimethyl substituent in methylchlorin, do not affect the MCD spectrum in a significant way. The naturally occurring iron chlorin prosthetic groups, however, likely contain vinyl substituents as has been proposed in the case of HPII catalase.[50] In the porphyrin series, we have examined the effect on the MCD spectrum resulting from the two vinyl substituents in protoporphyrin IX, the hexalkyl-, divinyl-substituted heme that is most prevalent in nature, relative to mesoheme, the octaalkyl analogue.[51] We reconstituted mesoheme into myoglobin and horseradish peroxidase and compared the resulting MCD spectra of numerous ferric and ferrous derivatives having different spin states and ligands bound to analogous forms of the native proteins. We consistently observed that the spectra of the mesoheme-reconstituted species were about 8–10 nm blue-shifted throughout the 300–700 nm region with about 2–3-fold greater signal intensity in the Soret region (300–500 nm) and with smaller intensity differences in the visible region (500–700 nm). The trends were reproducible and predictable. It seems likely that similarly predictable wavelength shifts and relatively small intensity differences will occur between the chlorins studied herein with alkyl substituents at all peripheral positions and the naturally occurring chlorins that likely have one or two vinyl groups in conjugation with the π-electron system of the macrocycle. For these reasons, we conclude that the chlorins employed in this study are appropriate ones for use with MCD spectroscopy.

In the high-spin ferric state, the MCD spectra of the chloride-ligated chlorins displayed in Figure 2 are actually rather similar to the spectra of other high-spin ferric chlorin complexes such as with oxyanion ligands (acetate and ethoxide)[18] as well as the high-spin ferric state of chlorin-reconstituted horseradish peroxidase[9] featuring a histidine fifth ligand and a presumably vacant sixth coordination site. As shown by Stolzenberg et al.,[18] the MCD spectrum of the high-spin ferric octaethylchlorin thiolate adduct is sufficiently different from the spectra of the oxyanion complexes (and therefore of the spectra in Figure 2) to use MCD as a method to distinguish the presence of a thiolate ligand from other ligand types in the high-spin ferric state. Thus, the situation with the MCD spectra of high-spin ferric chlorins appears to be reminiscent of that with regular porphyrins in the low-spin ferrous-CO state where the spectra are distinctly different when the ligand trans to CO is thiolate but otherwise independent of ligand type.[49b,52] A six-coordinate high-spin ferric chlorin such as in aquo-met myoglobin reconstituted with a chlorin prosthetic group also has a distinctly different MCD spectrum.[9]

The MCD spectra of low-spin ferric bis(imidazole)-ligated chlorins seen in Figure 3 are clearly different from those observed for the high-spin complexes just discussed. In this case, the MCD spectra also differ when one of the imidazole ligands is replaced with typical anionic heme ligands such as cyanide in the chlorin-reconstituted myoglobin system.[9] Likewise, the MCD spectra of low-spin ligand adducts of *E. coli* HPII catalase which appears to contain a tyrosinate proximal ligand[10] are also considerably different from those seen for bis-imidazole ligated complexes in Figure 3. Thus, to the extent of the currently available data, it appears that the MCD spectra of low-spin ferric chlorin complexes are quite sensitive to the combination of axial ligands and therefore will be useful as "fingerprints" for assigning endogenous ligands in chlorin proteins.

In contrast to the ferric chlorin complexes already discussed whose MCD spectra are completely different from those of corresponding ferric porphyrins, the MCD spectral band shape of five-coordinate high-spin imidazole-ligated ferrous chlorins (Figure 4) resemble those of the corresponding porphyrin derivatives in the Soret region to a large extent.[11,39b,53] The Soret features in the high-spin ferrous case are red-shifted and less intense than the corresponding mesoporphyrin-reconstituted high-spin deoxymyoglobin[51] but share the same characteristic asymmetric derivative-shape of inverted sign.[11] In the visible region,

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(51) Dawson, J. H.; Kadkhodayan, S.; Zhuang, C.; Sono, M. *J. Inorg. Biochem.* **1992**, *45*, 179.
(52) Collman, J. P.; Sorrell, T. J.; Dawson, J. H.; Trudell, J. R.; Bunnenberg, E.; Djerassi, C. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 6.
(53) Carter, K.; Palmer, G. *J. Biol. Chem.* **1982**, *257*, 13507.

the MCD spectral features for the chlorin adducts are very different from the corresponding porphyrins[11,53] as well as from the low-spin nitrogen donor ligated complexes shown in Figures 5–7. Unfortunately, the only other MCD spectrum of a high-spin ferrous chlorin of which we are aware is that of chlorin-reconstituted deoxyferrous myoglobin and horseradish peroxidase.[9] As would be expected since they possess the same five-coordinate imidazole-ligated structure, the spectra of those two protein derivatives are essentially identical to those reported in Figure 4. Therefore, it is not yet possible to say how distinctive the MCD spectra of high-spin ferrous chlorins will be as a function of proximal ligand. It is clear, however, that the MCD spectra of high-spin imidazole-ligated ferrous chlorins differ significantly from those of the low-spin ferrous chlorins investigated herein throughout the spectral region examined.

The low-spin ferrous chlorins whose MCD spectra have been reported in this study fall into two categories, those having two nitrogenous axial ligands (Figure 5) and those with a nitrogenous ligand trans to CO or NO (Figures 6 and 7). The MCD spectra of the complexes in the first category are quite similar to each other in shape with differences seen only in the relative intensities of the features in the visible region compared to the Soret region. With 1-methylimidazole and 1-butylamine, the intensities in the two regions are comparable while the bis(pyridine) adduct has 2-fold lower intensity in the Soret region. The Soret features of the bis(pyridine) complex are also blue-shifted by 8–12 nm compared to the other two spectra (Figure 5). As would be expected, the MCD spectra of bis(histidine)-ligated cytochrome b_5 reconstituted with ferrous methylchlorin[9] closely resembles that of the bis(imidazole)-ligated complex reported herein. Once again, the MCD spectra of the bis(nitrogen donor)-ligated species shown in Figure 5 are distinctly different from those of any of the other complexes reported in this study showing the responsiveness of the technique to the oxidation, spin and ligation state of the iron chlorin complex. On the other hand, the method clearly is not especially sensitive to the nature of the nitrogenous ligand in the bisligated state. No MCD spectra of non-nitrogenous ligand-bound low-spin ferrous complexes have yet been reported so it is not possible to make conclusions of a more general nature.

For the complexes having a nitrogenous ligand trans to CO or NO (Figures 6 and 7), the MCD spectra are again distinctly different from the spectra of the other oxidation and spin state nitrogen donor chlorin complexes reported in this study. As just discussed for the adducts with two nitrogenous ligands, the spectra of the CO complexes with imidazole, alkylamine and pyridine ligands are essentially indistinguishable. The ferrous–CO states of the chlorin-reconstituted myoglobin and horseradish peroxidase also have MCD spectra that closely resemble those displayed in Figure 6. The spectrum of the imidazole/NO-ligated ferrous chlorin reported in Figure 7 is similar to those of the CO-ligated complexes in band shape but is about 3–5-fold less intense. The spectrum is very similar to that of the ferrous–NO state of chlorin-reconstituted myoglobin.[9]

As mentioned above, one of the problems we have had to face in the present study is the presence of small amounts of low-spin ferrous porphyrin impurities in the octaethylchlorin and mesochlorin samples of the same oxidation and spin state. One solution to that problem has been to employ complexes of methylchlorin, which can be purified rigorously free of porphyrin contamination. This has provided benchmark spectra for both the bis(nitrogen donor) and nitrogen donor/CO or NO low-spin ferrous chlorin state. Next, we subtracted small amounts of the appropriate octaethylporphyrin spectrum from the spectra of the low-spin ferrous octaethylchlorin and mesochlorin derivatives. The amount subtracted was based on the match to the corresponding methylchlorin spectrum with the quantitation confirmed by NMR (see Experimental Section). As can be seen in the spectra of the octaethylchlorin and mesochlorin adducts shown in Figures 5–7, this procedure works very well.

MCD Properties of the Ring-Reduced Isomers of Mesochlorin. Of the chlorins examined in this study, the substituents of mesochlorin dimethyl ester most closely match those thought to be present in naturally occurring chlorin systems.[50,54] Reconstitution of mesochlorin as the diacid into well studied heme proteins such as myoglobin and horseradish peroxidase having a proximal histidine ligand should provide a method to generate mixed-ligand adducts featuring a variety of ligand types trans to histidine. In the present study, we have assessed the sensitivity of MCD spectroscopy to the site of pyrrole ring reduction in several mesochlorin ring-reduced isomers or mixtures of isomers. In the bottom panels of Figures 2–4 and 6, we have made such comparisons for the high-spin ferric, low-spin ferric, high-spin ferrous and low-spin ferrous states, respectively. In each of these four cases, the MCD spectra of different mesochlorin isomer mixtures and of the separate ring D-reduced isomer have indistinguishable MCD spectra. These data demonstrate that MCD spectroscopy is insensitive to the site of pyrrole ring reduction and that this variable is not one which needs to be taken into account in future attempts to use the MCD method to establish the identity of axial ligands as has been successfully done repeatedly with iron porphyrins. Needless to say, if saturation of a pyrrole double bond in a naturally occurring chlorin were to also take a vinyl substituent out of conjugation, then additional wavelength shifts and small intensity changes would also be seen. Given the difficulty in obtaining pure ring-reduced isomers of mesochlorin, the results described herein also justify the use of mixtures of ring-reduced isomers in such reconstitution experiments.

Conclusions

The potential of magnetic circular dichroism spectroscopy as a probe of the coordination structure of iron chlorin-containing proteins has been explored through examination of the spectra of five- and six-coordinate model complexes containing nitrogenous axial ligands to both ferric and ferrous iron. The MCD spectra of iron chlorin complexes are most sensitive to the identity, number and type of axial ligand together with the oxidation and spin state. Analysis of the spectra of different ring-reduced isomers of mesochlorin reveals that the method is relatively insensitive to the site of pyrrole ring reduction. The results described herein indicate that MCD spectroscopy will be of comparable use in the identification of axial ligands in iron chlorin-containing proteins as has been shown to be the case in the study of iron protoheme systems.

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