Iron Chlorin-Reconstituted Histidine-Ligated Heme Proteins as Models for Naturally Occurring Iron Chlorin Proteins: Magnetic Circular Dichroism Spectroscopy as a Probe of Iron Chlorin Coordination Structure

Alma M. Bracete,[‡] Saloumeh Kadkhodayan,[‡] Masanori Sono,[‡] Ann M. Huff,[‡] Chengfeng Zhuang,[‡] David K. Cooper,[§] Kevin M. Smith,[§] Chi K. Chang,[∥] and John H. Dawson*[,†,‡]

Department of Chemistry and Biochemistry and the School of Medicine, University of South Carolina, Columbia, South Carolina 29208, and Departments of Chemistry, University of California, Davis, California 95616, and Michigan State University, East Lansing, Michigan 48824

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Apomyoglobin, horseradish peroxidase, and cytochrome b_5 have been successfully reconstituted with several iron chlorin prosthetic groups. The resulting green iron chlorin-bound derivatives have a histidine proximal ligand and therefore serve as models for naturally occurring histidine-ligated iron chlorin proteins. Characterization of these systems with electronic absorption and magnetic circular dichroism (MCD) spectroscopy has established definitive and diagnostic spectral signatures for both ferric and ferrous iron chlorin states. As with regular iron heme systems, MCD spectroscopy is sensitive to changes in the coordination structure, spin state and oxidation state of iron chlorin systems, while the nature of the protein environment or solvent does not contribute significantly to the band pattern. The MCD signals of the iron chlorin-reconstituted proteins were generally found to be broad and reduced in intensity and to have different line shapes when compared to the intense and often symmetric bands seen for iron porphyrin systems. This reflects the reduction in symmetry of the chlorin macrocycle. The MCD spectra of the iron chlorin systems are also distinct from those previously reported for the other type of green iron heme, namely iron formyl-substituted porphyrins. A significant change in the band pattern of the MCD spectra is associated with changes from high-spin to low-spin in the ferric iron chlorin complexes examined and from ferric to ferrous oxidation states in low-spin complexes such as iron chlorin-reconstituted cytochrome b_5, a bis(imidazole)-ligated species. The spectra of low-spin ferrous chlorin complexes with π-acceptor ligands such as CO or NO trans to histidine are also distinguishable from those of complexes with imidazole, a σ-donor, in the trans position. These results provide spectroscopic models for naturally-occurring iron chlorin proteins bearing a proximal histidine ligand and demonstrate the utility of MCD spectroscopy in the determination of iron chlorin coordination structure. In addition, MCD spectroscopy can readily distinguish the two green heme systems, iron chlorins and iron formyl-substituted porphyrins.

Introduction

Iron protoporphyrin IX is the prosthetic group for a wide variety of proteins and enzymes involved in oxygen transport (myoglobin, hemoglobin), oxygen and peroxide activation (cytochrome P-450, horseradish peroxidase), and electron transfer (cytochrome b_5). The fully unsaturated porphyrin macrocycle contains 11 conjugated double bonds; iron porphyrin complexes are generally red. If one or more of these double bonds is saturated, the compounds are called hydroporphyrins. A chlorin has one reduced pyrrole double bond. Nature utilizes metallochlorins, which are usually green, to perform diverse biological activities. Chlorophylls, for example, are magnesium chlorins.[1] Heme *d*, an iron protoporphyrin IX-derived chlorin, is the active site chromophore of a terminal oxidase and a catalase in *Escherichia coli*.[2-4] A catalase from *Neurospora crassa* contains a heme *d*-related prosthetic group.[5] Ferryl (Fe^{IV} = O) and oxygenated (Fe—O_2) intermediates of the cytochrome *d* terminal oxidase have recently been characterized.[6,7] Oxoiron(IV) chlorin π-cation radical and peroxoiron(III) chlorin complexes have been prepared as models for intermediates in the cytochrome *d* catalytic cycle.[8] The proximal heme iron ligand for the cytochrome *d* terminal oxidase may be histidine[9] although more recent evidence using ENDOR spectroscopy is inconsistent with that proposal.[10] Tyrosine has been proposed to be the proximal ligand to the chlorin-containing catalase from *E. coli*.[11] In sulfmyoglobin, a nonfunctional form of myoglobin formed by treatment with hydrogen peroxide and inorganic

* To whom correspondence should be addressed.
† School of Medicine, University of South Carolina.
‡ Department of Chemistry and Biochemistry, University of South Carolina.
§ University of California, Davis.
∥ Michigan State University.
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sulfide, the porphyrin macrocycle has been reduced to a chlorin by addition of a sulfur atom to a pyrrole ring.[12-14] Myeloperoxidase has been suggested to contain an iron chlorin prosthetic group.[15-18] However, more recently, evidence in favor of an iron porphyrin[19] bearing a formyl substituent[20,21] has been presented. Like iron chlorins, iron formyl-substituted porphyrins are generally green.

Heme proteins have been extensively studied with magnetic circular dichroism (MCD)[22] spectroscopy.[23,24] The technique has proven to be a powerful probe of spin state, oxidation state, and axial ligand identity in heme (iron porphyrin) systems while being insensitive to protein environment. The MCD properties of synthetic model complexes of known macrocycle and axial ligation structure are compared with those of naturally-occurring heme (iron porphyrin) proteins in order to delineate the structural properties of the latter.

The applications of MCD spectroscopy to characterize iron chlorin systems, on the other hand, have been very limited. MCD spectra have been reported only for a small number of high-spin ferric octaethylchlorin complexes with ligands such as phenolate, acetate, and thiolate.[25] Other studies have used metal-free chlorins and zinc or magnesium chlorin complexes.[26,27] Previous MCD studies of the active site chromophore of myeloperoxidase,[15,18] which was thought to possibly contain an iron chlorin, were limited by the lack of MCD spectral data for well-characterized iron chlorins.

The need for MCD spectroscopic models for iron chlorins to aid in identifying axial ligands in iron chlorin-containing proteins has prompted us to systematically study the MCD properties

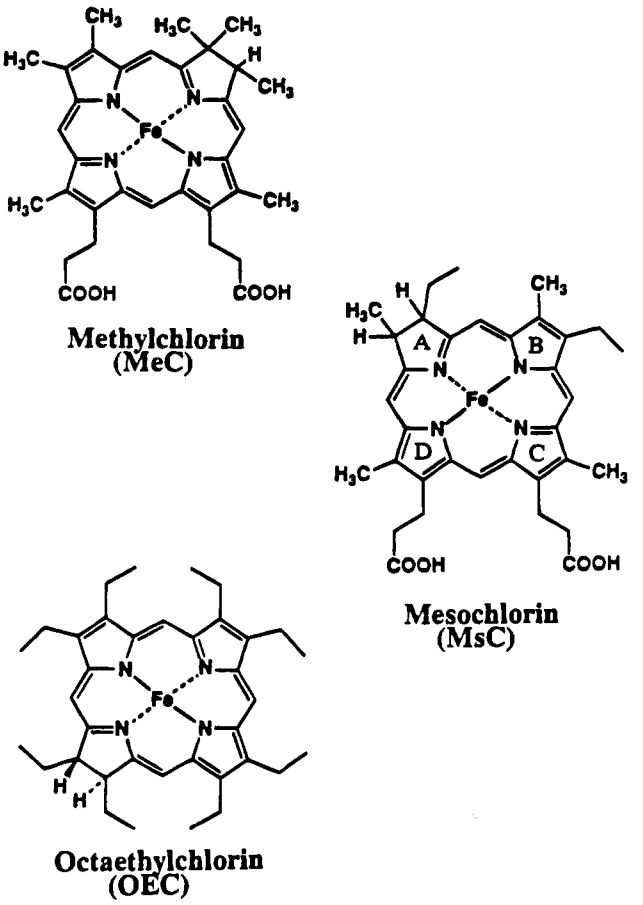

Figure 1. Structures of the three iron-bound alkylchlorin macrocyles used in this study. Iron methylchlorin (MeC) (2,2,4-trimethyldeuterochlorin) and mesochlorin (MsC) (only the ring A-reduced isomer is shown here) were incorporated in apomyoglobin, horseradish peroxidase, or cytochrome b_5. Iron octaethylchlorin (OEC) was used only in protein-free ligand complexes.

of synthetic iron chlorins.[28] We have recently shown that octaalkylporphyrins can replace protoporphyrin IX for MCD studies of normal heme systems.[29] This provides a valid basis for using octaalkylchlorins as models for protoheme-derived iron chlorins. In the present study, MCD spectra have been measured for a variety of ferric and ferrous derivatives of myoglobin reconstituted with iron methylchlorin and iron mesochlorin (Figure 1) (MeC−Mb and MsC−Mb, respectively) in the presence and absence of common heme ligands such as fluoride, cyanide, azide, imidazole, CO, and NO. Analogous states of iron methylchlorin-reconstituted horseradish peroxidase (MeC−HRP) have also been examined. In addition, iron methylchlorin has been incorporated[30] into apo-cytochrome b_5 (MeC−Cyt b_5) and has been characterized in its oxidized and reduced forms with MCD spectroscopy. The MCD data presented herein provide spectral signatures for histidine-ligated iron chlorin-containing proteins and demonstrate the utility of MCD spectroscopy as a probe of the spin state, oxidation state, and axial ligand identity in iron chlorin chromophores. It will also be shown that MCD spectroscopy provides a simple way of distinguishing the two different types of green hemes, iron chlorins and iron formyl-substituted porphyrins.

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(22) Abbreviations: MCD, magnetic circular dichroism; methylchlorin, 2,2,4-trimethyldeuterochlorin; octaethylchlorin, *trans*-octaethylchlorin; mesochlorin, *trans*-mesochlorin; Mb, myoglobin; HRP, horseradish peroxidase; Cyt b_5, cytochrome b_5; MeC−Mb, iron methylchlorin−reconstituted myoglobin; MsC−Mb, iron mesochlorin−reconstituted myoglobin; MeC−HRP, iron methylchlorin−reconstituted horseradish peroxidase; MeC−Cyt b_5, iron methylchlorin−reconstituted cytochrome b_5; EPR, electron paramagnetic resonance; 1-MeIm, 1-methylimidazole; 1,2-Me_2Im, 1,2-dimethylimidazole.
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Experimental Section

Materials. Horse heart Mb (Sigma) was purified as described by Dawson et al.[29] HRP (Sigma, Type VI) was used as received. Recombinant Cyt b_5 was a generous gift from Professor A. Grant Mauk (University of British Columbia). Iron *trans*-octaethylchlorin (octaethylchlorin) was synthesized[28] according to Whitlock et al.[31] Iron *trans*-mesochlorin (mesochlorin) was prepared as described.[32,33] Huff et al.[28] have shown that the MCD spectra of different ring-reduced mesochlorin isomers are identical; the mesochlorin isomer composition of each sample is indicated in the figure captions. The synthesis of iron 2,2,4-trimethyldeuterochlorin (methylchlorin) has been reported.[34] All other chemicals (Aldrich or Sigma, reagent grade) were used as received except 1-methylimidazole (1-MeIm), benzene, and methylene chloride which were distilled.

Preparation of Iron Chlorin-Reconstituted Proteins and Their Ligand Complexes. Apoheme proteins were prepared by the method of Teale.[35] The diacid forms of the iron chlorins were dissolved in minimal 0.1 N NaOH, diluted 10-fold with deionized water (Continental) to ~1 mM and added dropwise, with gentle stirring, to apo-Mb (0.1 M Tris, pH 8.0) or apo-HRP (0.1 M Tris, pH 7.4) at 4 °C. The reconstitution was followed by monitoring the increase in the Soret peak (390 nm) absorption. The mixture was then allowed to stand for an hour. The reconstituted proteins were dialyzed against water and against 5 mM Tris, pH 8.4 (Mb), or 5 mM acetate buffer, pH 4.4 (HRP), and then purified by DEAE− or CM−cellulose (Whatman) (Mb or HRP, respectively) column chromatrography using the same buffers. Elution occurred in the presence of 50 mM NaCl. Excess iron chlorin was tightly bound at the top of the column. MsC−Mb was separated from a small amount (≤ 7%) of iron mesoporphyrin−Mb by repetitive DEAE−cellulose chromatography using 5 mM Tris, pH 8.4 (without 50 mM NaCl). The MsC−Mb band (greenish brown) moved slightly faster than iron mesoporphyrin−Mb (brown). Iron methylchlorin was reconstituted into cytochrome b_5 as previously described by Martinis et al.[30,36]

Protein concentrations were determined from extinction coefficients[37] obtained using the pyridine hemochromogen assay by diluting concentrated protein stock solutions into a pyridine (25−30 % V/V)-NaOH (0.1 N) solution.[38a] A value of 90 mM^{-1} cm^{-1} was used for the Soret peak (415 nm) of the pyridine hemochromogen (ferrous bis(pyridine)) methylchlorin complex, prepared either in neat pyridine or in pyridine−NaOH[39] and for the iron mesochlorin pyridine hemochromogen. The ϵ values determined for iron octaethylchlorin complexes,[37] based on the weight of ferric octaethylchlorin chloride,[28] are in close agreement with those reported by Stolzenberg et al.[25] for the ferrous 1-MeIm/CO and bis-1-MeIm adducts, but are considerably smaller for the ferrous bis(pyridine) and pyridine/CO complexes.

Ligand complexes of ferric chlorin-reconstituted Mb and HRP were generated in 0.1 M potassium phosphate buffer, pH 6.0, by addition of ligand stock solution until no further spectral change was detected. [Fe(MsC)(1-MeIm)$_2$]Cl was obtained by adding neat 1-MeIm to Fe(MsC)Cl in benzene under nitrogen until no further spectral change occurred. Deoxyferrous iron chlorin proteins and ferrous MeC-Cyt b_5 were prepared under nitrogen by addition of minimal solid sodium dithionite to the ferric proteins. CO and NO complexes were formed by bubbling the gaseous ligand through the deoxyferrous and ferric proteins, respectively, followed by addition of solid sodium dithionite in the latter case. The synthesis of other iron chlorin model complexes has been described.[28]

Spectroscopic Measurements. Electronic absorption spectra were measured using a Varian/Cary 210 or 219 spectrophotometer interfaced to an IBM PC. MCD/CD spectra were recorded with a JASCO J-500A spectropolarimeter equipped with a JASCO MCD-1B electromagnet operated at 1.41 T. The J-500A was interfaced to an IBM PS/2 Model 50 by a JASCO IF-500-2 interface unit. Data acquisition and handling were performed as described elsewhere.[28,40] Spectral measurements were carried out at 4 and 25 °C for proteins and model complexes, respectively. Optical absorption spectra recorded before and after each MCD scan were essentially identical.

Results

Electronic Absorption Spectroscopy. Although iron mesochlorin-reconstituted Mb (MsC−Mb)[41] and iron methylchlorin-reconstituted cytochrome b_5 (MeC−Cyt b_5)[30] have been prepared previously, electronic absorption spectra were only reported from 500 to 750 nm for MsC−Mb and extinction coefficients were not provided for MeC−Cyt b_5. Therefore, in conjunction with our efforts to demonstrate the utility of magnetic circular dichroism (MCD) spectroscopy as a probe of iron chlorin coordination structure, absorption spectral data for iron alkylchlorin-reconstituted Mb, HRP, and Cyt b_5 and for selected model complexes are summarized in Tables S1 and S2[37] for the ferric and ferrous derivatives, respectively. The absorption peak positions are similar to those previously reported for analogous states of iron MsC−Mb and MeC−Cyt b_5. However, the ϵ values obtained for MsC−Mb derivatives[37] are about 2-fold greater than those reported by Chang et al.,[41] but are comparable to those of corresponding forms of iron MeC−Mb and of protein-free models.[25,28] The MCD and electronic absorption properties of various derivatives of iron alkylchlorin-reconstituted Mb, HRP, and Cyt b_5 and of selected model complexes are systematically described below.

High-Spin Ferric Derivatives.[42] The MCD spectra of the ferric states of MeC− and MsC−Mb in the absence of exogenous ligands are shown in Figure 2A. These species are likely six-coordinated high-spin aquo-ligated at pH 6-7 as in the ferric chlorin site of sulfmyoglobin.[12,14,43] The MCD spectra of these two iron alkylchlorin-containing Mbs are essentially identical, reflecting the structural similarity between the two chlorins. The Soret region (300−500 nm) of the MCD spectra consists of a derivative-like band of inverted sign (i.e., trough-crossover-peak with increasing wavelength). This band pattern is opposite to the normal pattern observed with native (iron porphyrin) ferric Mb in the same region.[24a] The MCD crossover points (~386 nm) for the iron chlorin-Mb correspond to the absorption maxima (~389 nm) (Figure 2C, solid line). The most prominent band in the visible region (500−800 nm) of the MCD spectra is a broad and unsymmetric trough at 550 nm. MCD signals between 700 and 800 nm were too weak to be detected under the experimental conditions used. Although

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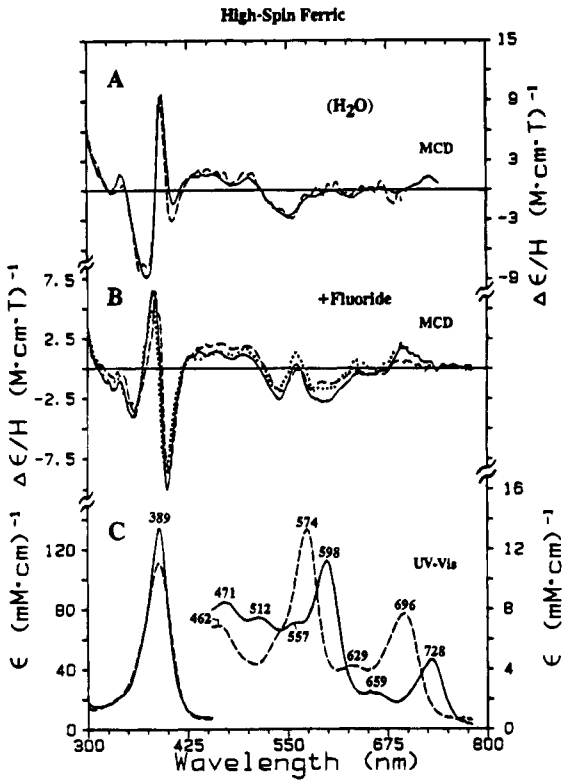

Figure 2. MCD spectra of the exogenous ligand-free ferric state of MeC−Mb (solid) and MsC−Mb (dashed) (A), MCD spectra of the ferric fluoride (200 mM KF) derivatives of MeC−Mb (solid), MeC−HRP (dashed) and MsC−Mb (dotted) (B), and electronic absorption (UV−vis) spectra of the corresponding ferric state (solid) and the ferric fluoride derivative (dashed) of MeC−Mb (C). All spectra were obtained in 0.1 M potassium phosphate buffer, pH 6.0 at 4 °C, using 25−40 μM protein concentrations.

the MCD band pattern in the Soret region for these iron chlorin-reconstituted Mbs is different from that of native (iron porphyrin) aquo-ligated Mb, the peak-to-trough intensities are similar. The electronic absorption peak positions for ferric MeC−[37] and MsC−Mb (Figure 2C) agree well with those reported for the latter.[41]

The binding of fluoride to ferric MeC−Mb, MsC−Mb, and MeC−HRP is associated with substantial MCD and electronic absorption spectral changes (Figure 2B,C). By analogy to ferric fluoride sulfmyoglobin,[12] the fluoride complexes reported herein are assumed to be high-spin. In the Soret region, normal sign derivative-shaped MCD bands are seen that are somewhat less intense than those of the aquo-ligated chlorin-reconstituted Mbs (Figure 2A). The MCD Soret crossover points (~391 nm) for the fluoride adducts closely match their absorption maxima (~389 nm). The visible region of the MCD spectra contains two broad troughs between 526−625 nm. Similar MCD and electronic absorption spectra are seen for other six-coordinate high-spin ferric chlorins such as ferric octaethylchlorin bis(dimethyl sulfoxide)[28] and the initially isolated form of sulfmyoglobin.[14,44]

As isolated, ferric MeC−HRP exhibits a distinctly different MCD spectrum (Figure 3A; solid line) from those of ferric MeC−Mb and MsC−Mb (Figure 2). The Soret MCD signal (Figure 3A) consists of two asymmetric troughs (~377 and 403 nm), one on each side of the absorption band (390 nm). The visible region contains two peaks (425−530 nm) and two troughs (530−625 nm) that are poorly resolved. Similar MCD

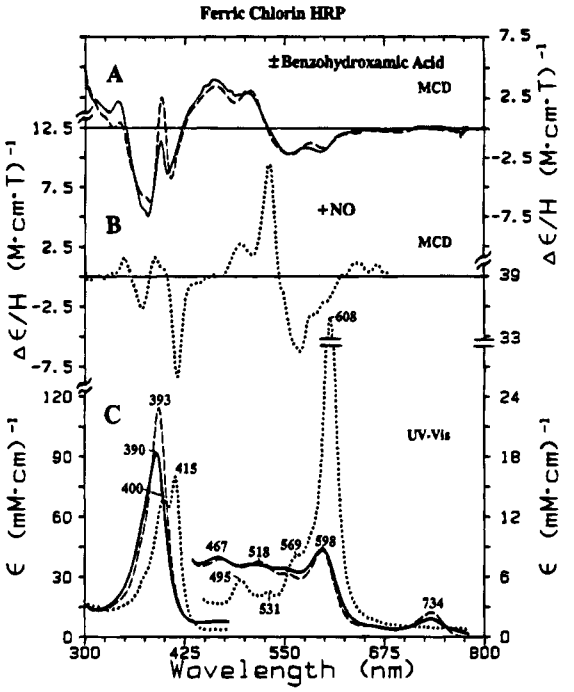

Figure 3. MCD spectra of ligand-free ferric MeC−HRP (solid) (A), ferric MeC−HRP + 1 mM benzohydroxamic acid (dashed) (A), and ferric MeC−HRP + NO (dotted) (B). The corresponding electronic absorption (UV−vis) spectra of these derivatives are overplotted in C. See the legend to Figure 2 for other conditions and the "Experimental Section" section for further details.

spectra are seen for five-coordinate ferric chlorin models.[28] Thus, the differences between the MCD spectra of MeC−HRP (Figure 3A) and of MeC− or MsC−Mb (Figure 2A) are likely due to the absence of water coordination in ferric MeC−HRP as in native (iron porphyrin) HRP.[45] For the latter, addition of benzohydroxamic acid leads to substantial changes in the electronic absorption and MCD spectra[46] arising from water ligation to the heme iron.[45] Benzohydroxamic acid addition to ferric MeC−HRP causes the Soret absorption band to shift from 390 to 393 nm and to become more intense (by ~24%) (Figure 3C). A new peak is seen at 394 nm in the MCD spectrum (Figure 3A, dashed line) with a line shape similar to that seen for aquo-ligated chlorin-reconstituted ferric Mbs (Figure 2A). By analogy to the case of iron porphyrin HRP, we suggest that the small but easily detected spectral changes for ferric MeC−HRP upon benzohydroxamic acid addition arise from water ligation.[47]

Low-Spin Ferric Complexes. Ferric chlorin−Mb and −HRP bind NO to form a complex presumed to be low-spin as in the case of their porphyrin counterparts.[38b] The Mb adducts were only examined by electronic absorption spectroscopy because they were readily autoreducible at 4 °C. The Soret absorption bands of the ferric-NO complexes are split into two peaks, and the longer wavelength peak in the visible region is unusually intense (see Figure 3C, dotted line for HRP). Interestingly, the spectral line shapes of these ferric−NO species closely resemble those of low-spin ferrous chlorins.[20,28] With iron porphyrins, a similar relationship has been seen between ferric imidazole−NO and low-spin ferrous bis(imidazole) ad-

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(47) Additional work will be necessary to determine whether benzohydroxamic acid addition leads to complete conversion to a six-coordinate aquo-ligated iron chlorin complex.

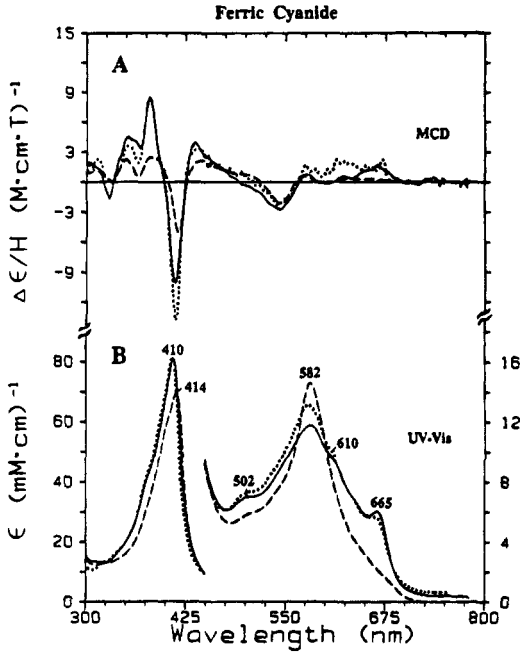

Figure 4. MCD (A) and electronic absorption (UV−vis) spectra (B) of the ferric cyanide (40 mM KCN) derivatives of MeC−Mb (solid), MeC−HRP (dashed), and MsC−Mb (dotted). See the legend to Figure 2 for other conditions.

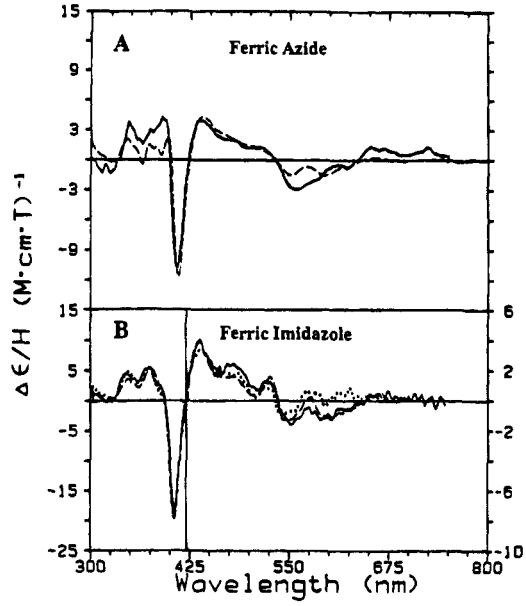

Figure 5. MCD spectra of the ferric azide derivatives of MeC−Mb (50 mM NaN_3) (solid) and MeC−HRP (100 mM NaN_3) (dashed) (A) and of the ferric bis(1-methylimidazole) complex of Fe(MsC) (prepared in methylene chloride using an equimolar mixture of the ring C- and ring D-reduced mesochlorin isomers in the presence of ~2 M ligand) (solid line), the 1-methylimidazole (~1 M) adduct of MsC−Mb (dotted line), and ferric MeC−Cyt b_5 (dashed line) (B). See the legend to Figure 2 for other conditions for the protein samples.

ducts.[48] The MCD spectrum of ferric−NO MeC−HRP in the Soret region consists of an asymmetric trough-crossover-peak pattern with decreasing wavelength. In the visible region, the spectrum has peaks at 495 and 531 nm and a trough at 570 nm with the latter peak being the most intense feature of the entire spectrum (Figure 3B). The line shape and intensity of the MCD spectrum of ferric−NO MsC−HRP in the visible region are almost indistinguishable from the spectra of the ferrous bis(pyridine) and bis(imidazole) chlorin adducts (*vide infra*).[20,28]

Figure 4A compares the MCD spectra of the ferric cyanide derivatives of MeC−Mb, MsC−Mb, and MeC−HRP.[49] Except for intensity differences in the Soret region, the spectra are very similar. The most prominent features are a trough at about 412 nm that is centered near the absorption maximum (~410 nm) and a peak at 380 nm corresponding to a shoulder on the shorter wavelength side of the Soret absorption band (Figure 4B). The visible region MCD spectra contain only one broad trough at 545 nm. The MCD band shapes of the cyanide derivatives (Figure 4), especially in the Soret region, are distinct from those of the high-spin systems (Figures 2 and 3). However, the peak-to-trough intensities are similar to the values observed for the aquo- and fluoride-bound iron chlorin complexes. Addition of cyanide to the ferric chlorin-reconstituted proteins shifts the Soret absorption band from 389 to 410 nm. In the visible region, the absorption spectra of the cyanide complexes of MsC− and MeC−Mb contain a broad band at 581 nm with a shoulder at 665 nm. Similar changes in the absorption spectrum of sulfmyoglobin upon cyanide binding have been reported.[12,14] With MeC−HRP, the absorption band at 582 nm is sharper and more intense and no shoulder is seen at 665 nm. The ferric iron chlorin complexes with cyanide (Figure 4), azide, and 1-MeIm (Figure 5) are assumed to be predominantly low-spin based on the similarity of the electronic absorption properties of these complexes with analogous iron chlorin systems known to be low-spin [sulfmyoglobin[12,14] and MeC−Cyt b_5[30]].

Figure 5A illustrates the MCD spectra of the ferric azide complexes of MeC−Mb and −HRP. The spectra of the 1-MeIm adduct of ferric MsC−Mb, of ferric MeC−Cyt b_5, and of ferric octaethylchlorin [$(1\text{-MeIm})_2$] are displayed in Figure 5B.[36] The latter spectra (Figure 5B) are characterized by a prominent trough in the Soret region such as was seen for the ferric−cyanide complexes (Figure 4A). The peak-to-trough intensities of the Soret MCD band for the azide adducts (Figure 5A) are very similar to that of the cyanide complex (Figure 4A), but are 2-fold less intense than for the bis(imidazole) species (Figure 5B). The visible region MCD spectra of the ferric imidazole/azide and bis(imidazole) derivatives, in comparison with the cyanide complexes, appear to exhibit additional bands which are better resolved in the bis(imidazole) cases. The electronic absorption spectra of the ferric−azide adducts of MeC− and MsC−Mb[37] are very similar to that of the ferric sulfmyoglobin−azide complex[12] except that the latter exhibits red-shifted (~15 nm) band positions due to the conjugated peripheral vinyl group. The absorption spectrum of MeC−Cyt b_5[37] agrees well with that reported by Martinis et al.[30]

High-Spin Ferrous Complexes. The MCD and electronic absorption spectra of deoxyferrous MeC−HRP and ferrous (MsC)(1,2-Me_2Im) are shown in Figure 6.[36] The MCD band shape in the Soret region for these iron chlorin derivatives is surprisingly similar to that observed for high-spin ferrous imidazole-ligated porphyrins[24a,50] although the intensity of the latter is twice as great. In the visible region, the MCD bands for these chlorin derivatives are broad and once again resemble those of high-spin ferrous iron porphyrins. The absorption spectra (Figure 6B) consists of an intense major band at the longest wavelength in the visible region and a single Soret peak, in agreement with spectra previously reported for other high-

(48) Sono, M.; Dawson, J. H. *Biochim. Biophys. Acta* **1984**, *789*, 170−187.

(49) Similar MCD spectra have also been obtained for the ferric cyanide, deoxyferrous and ferrous−CO derivatives of octaethylchlorin-reconstituted myoglobin (Patch, M. G.; Dawson, J. H. Unpublished results).

(50) Nozawa, T.; Kobayashi, N.; Hatano, M. *Biochim. Biophys. Acta* **1976**, *427*, 652−662.

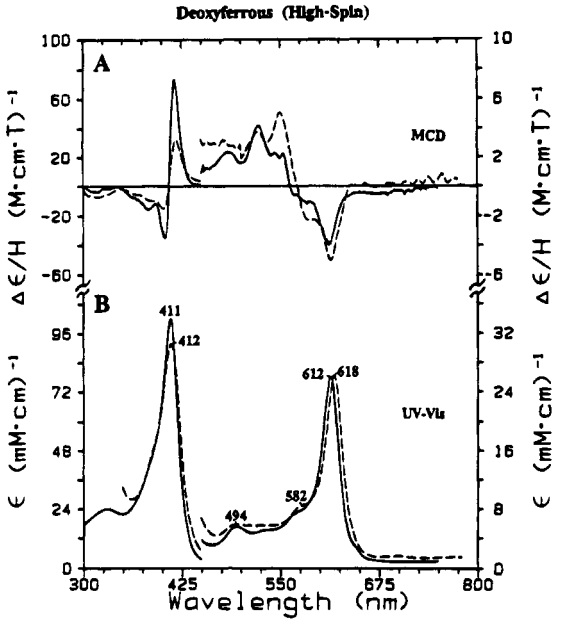

Figure 6. MCD (A) and electronic absorption (UV−vis) spectra (B) of the ferrous 1,2-dimethylimidazole complex of Fe(MsC) (prepared in benzene using the ring D-reduced mesochlorin isomer (solid line) and deoxyferrous MeC−HRP (dashed line). The Fe(MsC) sample contained approximately 7% iron mesoporphyrin which has not been subtracted. This contamination does not significantly change either MCD or electronic absorption spectra of the Fe(MsC) derivative (see text). See the legend to Figure 2 for other conditions for the protein samples and the "Experimental Section" section for further details.

spin ferrous imidazole-ligated chlorin systems such as the deoxyferrous sulfmyoglobin.[12,14]

Low-Spin Ferrous Complexes. The MCD spectra of low-spin ferrous (MeC)[(1-MeIm)$_2$] and of ferrous MeC−Cyt b_5 are displayed in Figure 7A. Except for small intensity differences in the Soret region, the band patterns for these two complexes are very similar and consist of a major peak at ~400 nm with relatively intense two-band troughs to shorter wavelength and a small trough to longer wavelength. The asymmetric and broad visible region MCD signals featuring a series of peaks between 450−550 nm and multiple troughs between 550−625 nm contrast with the intense derivative-shaped A term seen with bis(imidazole)ferrous porphyrins.[24b] The absorption spectra of the ferrous bis(imidazole) chlorins[37] feature a relatively intense major peak at the longest wavelength (~612 nm) and three smaller bands on the shorter wavelength side in the visible region and split Soret peaks.[30,51] The bis(pyridine and bis-(butylamine) complexes of ferrous methylchlorin and mesochlorin exhibit MCD and absorption spectra[20,28] that are similar to those of the bis(imidazole) adduct.

The MCD spectra of the ferrous imidazole−CO and imidazole−NO complexes are displayed in parts B and C of Figure 7, respectively.[49] The MCD band patterns for these two types of complexes are very similar in that two broad troughs are seen, one in the Soret region and one is the visible region. Note that the troughs seen in the spectra of the CO complexes are twice as intense as those for the NO complexes. The electronic absorption spectra of both the CO- and NO-bound ferrous chlorin complexes[20,28] are similar to each other.[37] The spectra consist of a prominent visible region peak at the longest wavelength with several additional smaller bands and split Soret bands although the shorter wavelength side Soret peak often appears as a shoulder to the other peak.

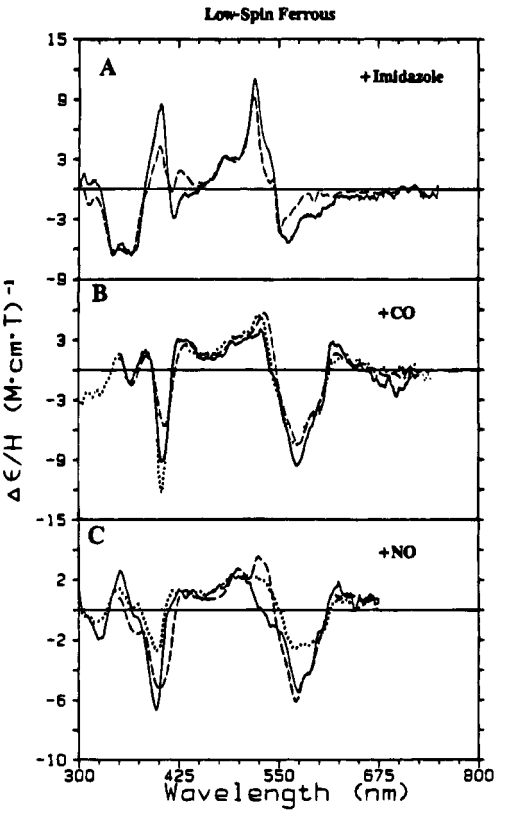

Figure 7. MCD spectra of ferrous low-spin iron chlorin derivative. (A) Ferrous bis(1-methylimidazole) complex of Fe(MeC) (solid line) and reduced MeC-Cyt b_5 (dashed line). (B) Ferrous−CO complexes of MeC−Mb (solid line), MeC−HRP (dashed line), and Fe(MsC) (1-methylimidazole) (prepared in benzene using the ring D-reduced mesochlorin isomer) (dotted line). A correction for 7% contamination of iron mesoporphyrin in the iron mesochlorin sample has been made. (C) Ferrous−NO complexes of MeC−Mb (solid line), MeC−HRP (dashed line), and octaethylchlorin−1-methylimidazole (dotted line). A correction for 3.5% contamination of iron octaethylporphyrin in the iron octaethychlorin sample has been made. See the legend to Figure 2 for other conditions for the protein samples and the "Experimental Section" section for further details.

Discussion

For iron porphyrin systems, it is well documented that MCD spectroscopy can detect changes in oxidation state, spin state, and the nature of the axial ligands.[23,24] Consequently, the technique has proven to be a powerful probe of coordination structure in heme proteins. The determination of axial ligand identity using MCD spectroscopy has been actively pursued in our laboratory. This effort has yielded evidence for an axial cysteine for cytochrome P-450, chloroperoxidase, and hemoprotein H-450, and for an axial histidine for indoleamine, 2,3-dioxygenase and secondary amine monooxygenase.[23a,40,48,52] However, the success of this approach is strongly dependent on the availability of iron porphyrin models of known coordination structure, spin state, and oxidation state. Indeed, only after the development of suitable models can MCD spectroscopy be used as a "fingerprint" technique in structural studies. The incorporation of iron chlorins into apo-Mb, apo-HRP, and apocytochrome b_5 described herein has generated "artificial" iron chlorin proteins with proximal histidine imidazole ligands. The resultant chlorin-reconstituted proteins have been used to test whether the established utility of MCD spectroscopy for iron porphyrin systems can be extended to probe the coordination structure of iron chlorin systems.

(51) Chang, C. K. In *Biological Chemistry of Iron*; Dunford, H. B., Ed.; Reidel Publishing Co.: Norwell, England, 1982; pp 313−334.

(52) Alberta, J. A.; Andersson, L. A.; Dawson, J. H. *J. Biol. Chem.* **1989**, *264*, 20467−20473.

MCD Spectroscopy as a Probe of the Prosthetic Group Identity: Iron Chlorin vs Iron Porphyrin. A chlorin is defined as a porphyrin macrocycle that has a saturated $\beta-\beta$ pyrrole bond. This disrupts the ring π conjugation[53] of the iron porphyrin and reduces the symmetry from D_{4h} to $C_{2(x)}$ for iron chlorins.[54] The two Q bands in the visible region of the electronic absorption spectrum of a metalloporphyrin are split into Q_x and Q_y components as a result of the electronic inequivalence in the x and y directions in the chlorin plane. The Soret band is also split, but the two components are poorly resolved in room temperature absorption spectra.[53] Due to this reduction in symmetry, derivative-shaped MCD A terms, which occur when the porphyrin π^* excited state degeneracy is lifted by an applied magnetic field, are not expected for iron chlorins.[55] MCD *B* terms, which are observed for systems with less than a 4-fold symmetry, are therefore expected to dominate the MCD spectra of iron chlorins; *C* terms are observed in cases where a paramagnetic complex is examined.[55,56]

The results presented herein show that the MCD properties of histidine-ligated iron alkylchlorin-containing proteins are significantly different, both in intensity and band pattern, from those of the iron porphyrin counterparts in most oxidation and ligation states. The diminished intensity and broadness of the MCD bands of iron chlorins reflects the reduction in the symmetry of the chlorin macrocycle. For example, the relatively intense derivative-shaped Soret MCD band seen for low-spin ferric Mb ligand complexes[24a] and other heme proteins [24b,50] is replaced by a broad and less intense trough that dominates the MCD spectra of the analogous complexes for the iron chlorin-substituted proteins (Figures 4 and 5). The five-coordinate high-spin ferrous complex of the iron alkylchlorin proteins, on the other hand, exhibit Soret region MCD spectra (Figure 6) which resemble that of the analogous iron porphyrin complex in band shape, but with reduced intensity. In this state, the MCD spectrum appears not to be significantly sensitive to the saturation of a pyrrole ring and the resultant reduction in symmetry. Heme *a*, which also has a symmetry-reducing formyl substituent, displays a similar MCD spectrum for the five-coordinate high-spin ferrous 1,2-Me_2Im model.[57] In the low-spin ferrous state, however, the iron chlorin protein complexes are readily distinguishable by MCD from analogous iron porphyrins. The broad MCD bands for the six-coordinate low-spin ferrous chlorins (Figure 7) are most likely *B* terms. In contrast, for the low-spin ferrous porphyrin systems,[24,50] relatively intense, derivative-shaped MCD *A* terms are observed in the Soret and visible regions.

MCD Spectroscopy as a Method for Distinguishing the Two Types of Green Hemes. Although the most common heme iron chromophores are red, two types of green heme iron systems have been described, iron chlorins such as heme *d* and iron formyl-substituted porphyrins such as heme *a*. Distinguishing these two types of green heme systems has often been difficult. The results described herein, however, provide a simple ambient temperature method for differentiating the two green heme chromophores. In an earlier study, we reported the MCD spectra of several derivatives of myoglobin reconstituted with iron-bound heme *s*, a formyl-substituted porphyrin macrocycle.[20] Comparison of the MCD data reported for the high-spin ferric (Figure 2A), low-spin ferric−cyanide (Figure 4A), low-spin ferrous−CO (Figure 7B) and low-spin ferrous bis(pyridine)[44] derivatives of MsC−Mb to parallel derivatives of heme *s*-reconstituted myoglobin[20] reveals substantial differences in band patterns and intensities especially in the low-spin ferrous cases.

Sensitivity of MCD Spectroscopy of the Spin, Oxidation, and Axial Ligation States of Histidine-Ligated Iron Chlorin Systems. Recently, we have characterized the MCD spectra of an extensive set of iron alkylchlorin models with imidazole, amine, and pyridine axial ligands in order to establish MCD spectral signatures for histidine- and lysine-ligated iron chlorin-containing proteins.[28] In the present study, the sixth coordination sites of chlorin-reconstituted Mb and HRP are available to bind exogenous ligands. This allows for the preparation of complexes with a variable ligand trans to histidine so as to further assess the sensitivity of MCD spectroscopy as a probe of coordination structure in iron chlorins.

The earlier work of Stolzenberg et al.[24] revealed that the five-coordinate high-spin ferric octaethylchlorin acetate and phenolate complexes can be distinguished from the analogous thiolate-ligated adduct based on the MCD band shape in the Soret region. These observations are substantially expanded in the present study by examination of additional ferric and ferrous chlorin ligand adducts. In the six-coordinate high-spin ferric state, the MCD spectra of the fluoride-ligated forms of the chlorin-reconstituted proteins (Figure 2B) are clearly different from those of the aquo-ligated states (Figure 1A), especially in the Soret region. The MCD spectra of the latter are, in turn, distinguishable from the spectrum of the ferric MeC−HRP as isolated (Figure 3A) likely reflecting the difference in coordination number of the iron chlorin complex of the two proteins (*vide supra*).

In general, the MCD spectra of the six-coordinate low-spin iron chlorin complexes with cyanide, azide and imidazole all exhibit a trough in the Soret region and generally similar visible region band patterns (Figures 4 and 5). Relative to iron porphyrins,[23b,24] the MCD spectra of iron chlorins are somewhat less dependent on the nature of the axial ligands. On the other hand, the change from high- to low-spin iron leads to dramatic changes in the overall MCD line shape, especially in the Soret region from a derivative-shaped pattern to a trough (Figure 2A,B vs Figures 4A and 5). The Soret MCD signal intensity for all of these ferric chlorins are nearly comparable regardless of spin state. This contrasts with the sensitivity of the MCD Soret signal intensity to spin state in ferric porphyrins.[24]

The marked MCD band shape changes that are seen between the spectra of ferric (Figure 5B) and ferrous (Figure 7A) MeC−Cyt b_5 illustrate the effect of oxidation state change on spectral properties in six-coordinate low-spin iron chlorins. The MCD spectra of ferrous bis(imidazole)iron chlorin complexes (Figure 7A) are also different in line shape and intensity from those of the imidazole−CO (Figure 7B) or −NO adducts (Figure 7C). As with ferrous porphyrins,[24] therefore, the MCD spectra of histidine-ligated ferrous chlorins with π-acceptor trans ligands (e.g., CO or NO) can be distinguished from those with trans σ-donors.

Conclusions

The successful reconstitution of several iron chlorins into well-defined histidine-ligated heme proteins described herein provides models to be used in establishing the relationship between spectroscopic properties and coordination structure in

(53) Weiss, C. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1978; Vol. 3, pp 211−223.
(54) Andersson, L. A.; Loehr, T. M.; Chang, C. K.; Mauk, A. G. *J. Am. Chem. Soc.* **1985**, *107*, 182−191.
(55) Sutherland, J. C. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1978; Vol. 3, pp 225−248.
(56) Assignment of the MCD spectra through variable temperature studies would further our knowledge of iron chlorin electronic structure but would be peripheral to the present goal of establishing spectral fingerprints to use in assigning iron chlorin coordination structure.
(57) Carter, K.; Palmer, G. *J. Biol. Chem.* **1982**, *257*, 13507−13514.

iron chlorin proteins. The present results demonstrate that MCD spectroscopy can be productively employed to probe the active site structures of iron chlorin proteins. As with heme iron systems, the MCD spectra of iron chlorins differing in spin state, oxidation state, and the type and number of axial ligands are distinctive. The MCD properties of iron chlorins and porphyrins are distinguishable and the method can also differentiate the two classes of green heme chromophores, iron chlorins and iron formyl-substituted porphyrins.[20] These results establish the utility of MCD spectroscoy in the assignment of the chromophore identity and coordination structure of naturally-occurring histidine-ligated iron chlorin proteins and demonstrate that the protein models described herein are appropriate MCD spectroscopic models for the heme *d* prosthetic group. Such models have already been used to exclude the possibility of a histidine proximal ligand in *E. coli* HPII catalase.[11]

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Supplementary Material Available: Tables S1 and S2 containing detailed electronic absorption spectra data (wavelengths and extinction coefficients) for the ferric and ferrous chlorin-reconstituted proteins, respectively (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.