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# Spectroscopic Studies of Ferric Porphyrins with Quantum Mechanically Admixed Intermediate-Spin States: Models for Cytochrome *c′*

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Several five- and six-coordinate ferric octaalkylporphyrin (OEP) complexes [Fe(OEP)X, X = $ClO_4^-$, $SO_3CF_3^-$, $SbF_6^-$; [Fe(OEP)(3,5-$Cl_2$Py)$_2$]$ClO_4$, Py = pyridine] previously reported to display quantum mechanically admixed intermediate-spin behavior have been spectroscopically examined as possible models for cytochrome *c′*. The magnetic behavior of the complexes has been confirmed using NMR and EPR spectroscopy. The magnetic circular dichroism (MCD) spectra of these complexes have been investigated in the Soret/visible (300–700 nm) and near-IR (700–2000 nm) regions for the first time and compared to the spectra of high-spin Fe(OEP)Cl and low-spin [Fe(OEP)(HIm)$_2$]Cl and [Fe(OEP)(NMeIm)$_2$]Cl. The MCD spectral features in the Soret/visible region of the four admixed intermediate-spin model complexes are similar in band shape and intensity to the spectrum reported for ferric cytochrome *c′* from *Alcaligenes sp.*, NCIB 1105 (Yoshimura, T.; Suzuki, S.; Nakahara, A.; Iwasaki, H.; Masuko, M.; Matsubara, T. *Biochim. Biophys. Acta* **1985**, *831*, 267–274). The near-IR MCD spectra of these complexes are also very similar to those of cytochrome *c′* isolated from several bacterial sources (Rawlings, J.; Stephens, P. J.; Nafie, L. A.; Kamen, M. D. *Biochemistry* **1977**, *16*, 1725–1729) and are distinctly different from those of known high- and low-spin ferric heme systems or of complexes containing a thermal mixture of high- and low-spin states. These results provide new evidence for the utility of MCD spectroscopy, especially in the near-IR region, as a probe of spin state in ferric heme systems and support the use of the quantum mechanically admixed intermediate-spin ferric porphyrins examined herein as models of cytochrome *c′*.

## Introduction

The cytochromes *c′* are an anomalous group of heme proteins found in a wide variety of photosynthetic and denitrifying bacteria.[1] A single axial histidine bound to the *c* heme prosthetic group has been observed by X-ray crystallography.[2] The heme is five-coordinate, and there are no distal residues capable of hydrogen bonding to endogenous ligands. Unexpectedly, the empty coordination site faces the interior of the protein and hence only small ligands (CO, NO, cyanide) bind to the iron.[3] The general features of the electronic absorption spectrum of the ferric state are typical of high-spin heme proteins. At neutral pH, however, the magnetic susceptibility is unusually low and a broad derivative-shaped feature is observed near $g = 4$ in the electron paramagnetic resonance (EPR)[4] spectrum.[5] In addition, a ligation-sensitive high-frequency resonance Raman mode is seen that is intermediate in energy between those observed for high- and low-spin ferric porphyrins.[6] Mössbauer studies of the ferric protein clearly indicate that only one magnetic species is present.[7] Moss and Maltempo have proposed the existence of an intermediate-spin ($S = {}^3/_2$) state with a quantum mechanical admixture of the more common high-spin ($S = {}^5/_2$) state for the protein.[8]

Model complexes of ferric octaethylporphyrin (OEP) and tetraphenylporphyrin (TPP) having a single weakly coordinating anionic axial ligand (e.g. $ClO_4^-$,[9-11] $SO_3CF_3^-$,[12] $SbF_6^-$,[13] and $C(CN)_3^-$)[14] or two weakly basic axial pyridine ligands (e.g. 3-chloropyridine[15] and 3,5-dichloropyridine[16]) have been reported that display admixed intermediate-spin behavior similar to that of cytochrome *c′*. Structural,[17] spectroscopic (Mössbauer, EPR, and NMR), and magnetic susceptibility studies confirm the $S = {}^3/_2$, $S = {}^5/_2$ quantum mechanically admixed-spin behavior.[18] Teraoka and Kitagawa have identified resonance Raman vibrational modes sensitive to ligation environment for Fe(OEP)$ClO_4$ and several substituted derivatives and noted strong spectral similarities to those of the native protein.[19] Model studies such as these have provided a basis for understanding the anomalous spin behavior of cytochrome *c′*.

Many of the spectroscopic techniques that are used to characterize heme proteins and model complexes are sensitive to the spin state of the iron. EPR[20] and Mössbauer[21] spectroscopy have been routinely employed for the characterization of ferric hemes. The various spin states of ferric porphyrins can be distinguished by $^1H$ and $^{13}C$ NMR spectroscopy.[22] Magnetic circular dichroism (MCD) is particularly well suited to the study of heme proteins and porphyrin model systems.[23] All heme iron oxidation states (ferrous, ferric, and ferryl) exhibit MCD spectra; the technique is sensitive to the coordination structure of the heme complex while

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(1) Meyer, T. E.; Kamen, M. D. *Adv. Prot. Chem.* **1982**, *53*, 105–212.
(2) (a) Weber, P. C.; Howard, A.; Xuong, N. H.; Salemme, F. R. *J. Mol. Biol.* **1981**, *153*, 399–424. (b) Finzel, B. C.; Weber, P. C.; Hardman, K. D.; Salemme, F. R. *J. Mol. Biol.* **1985**, *186*, 627–743. (c) Weber, P. C.; Bartsch, R. G.; Cusanovich, M. A.; Hamlin, R. C.; Howard, A.; Jordan, S. R.; Kamen, M. D.; Meyer, T. E.; Weatherford, D. W.; Xuong, N.-H.; Salemme, F. R. *Nature (London)* **1980**, *286*, 302–304.
(3) Kassner, R. J.; Kykta, M. G.; Cusanovich, M. A. *Biochim. Biophys. Acta* **1985**, *831*, 155–158.
(4) Abbreviations used: EPR, electron paramagnetic resonance; OEP, octaethylporphyrin; TPP, tetraphenylporphyrin; MCD, magnetic circular dichroism; PPIXDME, protoporphyrin IX dimethyl ester; 3,5-$Cl_2$Py, 3,5-dichloropyridine; HIm, imidazole; NMeIm, *N*-methylimidazole.
(5) (a) Maltempo, M. M.; Moss, T. H.; Cusanovich, M. A. *Biochim. Biophys. Acta* **1974**, *342*, 290–305. (b) Maltempo, M. M. *Biochim. Biophys. Acta* **1975**, *379*, 95–102.
(6) Hobbs, J. D.; Larsen, R. W.; Meyer, T. E.; Hazzard, J. H.; Cusanovich, M. A.; Ondrias, M. R. *Biochemistry* **1990**, *29*, 4166–4174.
(7) Moss, T. H.; Bearden, A. J.; Bartsch, R. G.; Cusanovich, M. A. *Biochemistry* **1968**, *7*, 1583–1591.
(8) (a) Maltempo, M. M.; Moss, T. H. *Q. Rev. Biophys.* **1976**, *9*, 181–215. (b) Maltempo, M. M. *J. Chem. Phys.* **1974**, *61*, 2540–2547.
(9) (a) Dolphin, D. H.; Sams, J. R.; Tsin, T. B. *Inorg. Chem.* **1977**, *16*, 711–713. (b) Masuda, H.; Taga, T.; Osaki, K.; Sugimoto, H.; Yoshida, Z.-I.; Ogoshi, H. *Inorg. Chem.* **1980**, *19*, 950–955. (c) Ogoshi, H.; Sugimoto, H.; Watanabe, E.; Yoshida, Z.; Maeda, Y.; Sakai, H. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 3414–3419.
(10) (a) Kastner, M. E.; Scheidt, W. R.; Mashiko, T.; Reed, C. A. *J. Am. Chem. Soc.* **1978**, *100*, 666–667. (b) Reed, C. A.; Mashiko, T.; Bentley, S. D.; Kastner, M. E.; Scheidt, W. R.; Spartalian, K.; Lang, G. *J. Am. Chem. Soc.* **1979**, *101*, 2948–2958.
(11) Toney, G. E.; ter Haar, L. W.; Savrin, J. E.; Gold, A.; Hatfield, W. E.; Sangaiah, R. *Inorg. Chem.* **1984**, *23*, 2563–2564.
(12) Boersama, A. D.; Goff, H. M. *Inorg. Chem.* **1982**, *21*, 581–586.
(13) (a) Shelly, K.; Bartczak, T.; Scheidt, W. R.; Reed, C. A. *Inorg. Chem.* **1985**, *24*, 4325–4330. (b) Gupta, G. P.; Lang, G.; Reed, C. A.; Shelly, K.; Scheidt, W. R. *J. Chem. Phys.* **1987**, *86*, 5288–5293.
(14) Summerville, D. A.; Cohen, I. A.; Hatano, K.; Scheidt, W. R. *Inorg. Chem.* **1978**, *17*, 2906–2910.
(15) Scheidt, W. R.; Geiger, D. K.; Hayes, R. G.; Lang, G. *J. Am. Chem. Soc.* **1983**, *105*, 2625–2632.
(16) Scheidt, W. R.; Osvath, S. R.; Lee, Y. J.; Reed, C. A.; Shavez, B.; Gupta, G. P. *Inorg. Chem.* **1989**, *28*, 1591–1595.
(17) Scheidt, W. R.; Lee, Y. L. *Struct. Bonding* **1987**, *64*, 1–70.
(18) Goff, H. M.; Shimomura, E. *J. Am. Chem. Soc.* **1980**, *102*, 31–37.
(19) Teraoka, J.; Kitagawa, T. *J. Phys. Chem.* **1980**, *84*, 1928–1935.
(20) Palmer, G. In *Iron Porphyrins, Part II*; Lever, A. B. P., Gray, H. B., Eds.; Addison Wesley: Reading, MA, 1983; pp 43–88.
(21) Debrunner, P. G. In *Iron Porphyrins, Part III*; Lever, A. B. P., Gray, H. B., Eds.; VCH Publishers: New York, 1989; pp 137–234.
(22) Goff, H. M. In *Iron Porphyrins, Part I*; Lever, A. B. P., Gray, H. B., Eds.; Addison Wesley: Reading, MA, 1983; pp 238–281.
(23) Dawson, J. H.; Dooley, D. M. In *Iron Porphyrins, Part III*; Lever, A. B. P., Gray, H. B., Eds.; VCH Publishers: New York, 1989; pp 1–93, 93–136.

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being insensitive to the surrounding environment. Previous MCD studies of cytochrome *c′* in the UV–visible region have focused on the native ferric, ferric–cyanide, ferrous–NO, and ferrous–CO states.[24–28] With the native ferric protein,[28] it was observed that the MCD spectrum does not resemble those of typical high-spin ferric heme proteins. It was not possible to assign the spin state from the MCD data.

MCD spectroscopy of ferric heme systems in the near-IR region has been less extensively investigated. The bands in this region arise from charge–transfer transitions from the porphyrin macrocycle to the iron.[29] The electronic absorption spectra of ferric heme derivatives in the near-IR region are sensitive to both spin and axial ligation. In one of the first studies using MCD in the near-IR region, Stephens and co-workers examined ferric cytochrome *c′* from several sources.[30] Their results, however, were inconclusive regarding the spin state of the cytochrome due to the lack of reference data "for definitively characterized mixed-spin species."[30]

Stephens's results suggest that further investigation of the near-IR region of the MCD spectrum of model complexes of known spin state would lead to a clearer understanding of the anamolous spin state behavior of cytochrome *c′*. We have been interested in the utility of MCD as a probe of ferric heme spin state. To that end, we have examined the visible and near-IR MCD spectra of a series of five- and six-coordinate ferric porphyrin model complexes reported to display quantum mechanically admixed-spin behavior as possible models for cytochrome *c′*. Our results show that MCD is capable of distinguishing such complexes from the more commonly encountered high- and low-spin-state species. Comparison of the data reported herein with that previously published[30] for cytochrome *c′* provides further evidence for the existence of an admixed intermediate-spin ground state in the protein.

## Experimental Section

**Materials.** Solvents were washed with acid and distilled from $CaH_2$ ($CH_2Cl_2$) or sodium/benzophenone (benzene).[31] HPLC grade heptane was obtained from Fisher. Chloroform-$d_1$ (Aldrich 99.8 atom % D) was distilled from $CaCO_3$. Methylene-$d_2$ chloride (100 atom % D), Fe(OEP)Cl, $AgClO_4$, $AgSbF_6$, and $AgSO_3CF_3$ were used as received (Aldrich). Imidazole (Eastman) and 3,5-dichloropyridine (Aldrich) were recrystallized from ethanol and dried in vacuo overnight.

**Spectroscopic Methods.** NMR spectra were recorded at 300.16 MHz using a Bruker AM-300 spectrometer at ambient probe temperature unless otherwise noted. Spectra were obtained using a 60-kHz sweep width, 90° pulse with a 5-ms delay, and a 32 K word data set zero-filled to 64 K words before transforming. When necessary, the probe temperature was determined using published procedures and was accurate to ±0.5 °C.[32] EPR spectra were obtained at 77 K with a Varian E-109 Century Series spectrometer. IR spectra were measured on a Perkin-Elmer Model 1600 FTIR spectrophotometer using samples prepared as KBr pellets. Electronic absorption spectra were recorded on Varian Cary 210 (300–750 nm) and 2300 (280–2000 nm) spectrophotometers. MCD spectra were obtained with JASCO Model J-40C, J-500A (300–800 nm), and J-200D (750–2000 nm, indium antimony detector) spectropolarimeters equipped with 1.5-T electromagnets. A specially designed, glass-stoppered "Y" cuvette with both 0.10- and 1.00-cm path lengths was used. Electronic absorption spectra were recorded before and after each MCD scan; only when the two showed less than 5% change were the MCD spectra deemed valid. MCD scans have been normalized to path length, concentration, and magnetic field strength [$(M\cdot cm\cdot T)^{-1}$]. Data handling was carried out as previously described.[33]

**Table I.** Proton NMR Spectral Data for Ferric Octaethylporphyrin Complexes

| complex[a] | meso | methyl | methylene |
|---|---|---|---|
| Fe(OEP)Cl | −56.8 | 6.8 | 44.4, 40.9 |
| $Fe(OEP)ClO_4$ | −6.0 | 6.5 | 36.5 |
| $Fe(OEP)ClO_4$[b] | −5.5 | 6.4 | 35.5 |
| $Fe(OEP)SO_3CF_3$ | −24.6 | 7.5 | 49.9, 34.8 |
| $Fe(OEP)SO_3CF_3$[c] | −24.6 | 7.2 | 49.4, 34.6 |
| $Fe(OEP)SbF_6$ | −5.4 | 6.1 | 39.5, 35.5 |
| $[Fe(OEP)(3,5\text{-}Cl_2Py)_2]^+$ | −4.6 | 6.4 | 35.6 |

[a] 25 °C, 0.001 M, shifts in ppm relative to internal TMS. The chloride, perchlorate, and bis(dichloropyridine) complexes were examined in $CD_2Cl_2$, and the other adducts were studied in $CDCl_3$. [b] Reference 18. [c] Reference 12.

**Synthesis of Ferric Porphyrin Complexes.** $[Fe(OEP)(3,5\text{-}Cl_2Py)_2]ClO_4$,[16] $Fe(OEP)ClO_4$,[9] and $Fe(OEP)SO_3CF_3$[12] were prepared and their integrity confirmed by NMR and electronic absorption spectroscopy using published procedures. Extinction coefficients for the bis(dichloropyridine) adduct were determined in $CH_2Cl_2$ [$\lambda_{max}$, nm ($\epsilon$, $(M\cdot cm)^{-1}$): 387 ($9.8 \times 10^4$), 499 ($9.0 \times 10^3$), 624 ($1.6 \times 10^3$)]. Published extinction coefficients of the perchlorate [$\epsilon_{504} = 1.0 \times 10^4$ $(M\cdot cm)^{-1}$][9a] and triflate [$\epsilon_{380} = 1.1 \times 10^5$ $(M\cdot cm)^{-1}$][12] complexes were utilized. $Fe(OEP)SbF_6$ was synthesized by metathesis of Fe(OEP)Cl with $AgSbF_6$ as reported for the analogous TPP complex.[13a] The product was recrystallized from benzene–heptane and dried in vacuo at 60 °C.[34a] IR (KBr, $cm^{-1}$): $\nu(SbF_6)$ 656.0, 639.4. UV–vis ($CH_2Cl_2$) [$\lambda_{max}$, nm ($\epsilon$, $(M\cdot cm)^{-1}$)]: 387 ($1.8 \times 10^5$), 498 ($1.3 \times 10^4$), 620 ($2.0 \times 10^3$). The sample concentration was determined by conversion to the bis(imidazole) complex [$\epsilon_{527} = 1.1 \times 10^4$ $(M\cdot cm)^{-1}$][34b] by addition of excess solid imidazole. For spectral studies, $[Fe(OEP)(HIm)_2]Cl$ or $[Fe(OEP)(NMeIm)_2]Cl$ was prepared by dissolving Fe(OEP)Cl ($5.0 \times 10^{-3}$ M) and the imidazole in the appropriate solvent.[35] Due to the high moisture sensitivity, all intermediate-spin ferric porphyrins were prepared in an inert-atmosphere box (Vacuum Atmospheres) and handled in sealed cuvettes.

## Results and Discussion

**Quantum Mechanically Admixed Intermediate-Spin Ferric Porphyrins.** According to Maltempo's model for quantum mechanically admixed-spin systems, the heme iron electronic environment contains substantial contributions from two or more heme iron spin states.[5] The mechanism for the quantum mechanical mixing between the high-spin ($S = {}^5/_2$, ${}^6A$) and intermediate-spin ($S = {}^3/_2$, ${}^4A$) states is a spin–orbit interaction. Such mixing will occur when the energy difference between the pure sextet (${}^6A$) and quartet (${}^4A$) states is comparable to or less than the spin–orbit coupling constant (approximately 300 $cm^{-1}$). The relative contribution of each unperturbed spin state to the five resulting Kramer's doublets is determined by the ratio of a crystal field parameter and the spin–orbit coupling constant. If the unperturbed quartet state is lower in energy than the sextet, the two lowest energy Kramer's doublets exhibit relatively more $S = {}^3/_2$ character, and the two highest energy doublets relatively more $S = {}^5/_2$ character, while the intermediate doublet has total sextet character. This gives rise to a single species with magnetic properties distinct from either pure spin state. By contrast, in a thermal mixture, two magnetically distinguishable pure spin states exist in equilibrium. The difference between the two cases can be readily seen using spectroscopic techniques such as Mössbauer and EPR. Quantum mechanically admixed-spin species give rise to a spectral signature characteristic of a single

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(24) Yoshimura, T.; Suzuki, S.; Nakahara, A.; Iwaski, H.; Masuko, M.; Matsubara, T. *Biochemistry* **1986**, *25*, 2436–2442.

(25) Suzuki, S.; Yoshimura, T.; Nakahara, A.; Iwasaki, H.; Shidara, S.; Matsubara, T. *Inorg. Chem.* **1987**, *26*, 1006–1008.

(26) Yoshimura, T.; Suzuki, S.; Iwasaki, H.; Takakuwa, S. *Biochem. Biophys. Res. Commun.* **1987**, *144*, 224–231.

(27) Suzuki, S.; Nakahara, A.; Yoshimura, T.; Iwasaki, H.; Shidara, S.; Matsubara, T. *Inorg. Chim. Acta* **1988**, *153*, 227–233.

(28) Yoshimura, T.; Suzuki, S.; Nakahara, A.; Iwasaki, H.; Masuko, M.; Matsubara, T. *Biochim. Biophys. Acta* **1985**, *831*, 267–274.

(29) Smith, D. W.; Williams, R. J. P. *Struct. Bonding* **1970**, *7*, 1–45.

(30) Rawlings, J.; Stephens, P. J.; Nafie, L. A.; Kamen, M. D. *Biochemistry* **1977**, *16*, 1725–1729.

(31) Perrin, D. D.; Armarego, W. L. F.; Perrin, D. R. *Purification of Laboratory Chemicals*; Pergamon Press: Oxford, England, 1980.

(32) Van Geet, A. L. *Anal. Chem.* **1968**, *40*, 2227–2229.

(33) (a) Dawson, J. H.; Andersson, L. A.; Sono, M. *J. Biol. Chem.* **1982**, *257*, 3606–3617. (b) Dawson, J. H.; Andersson, L. A.; Sono, M. *J. Biol. Chem.* **1983**, *258*, 13637–13645. (c) Svastits, E. W.; Dawson, J. H. *Inorg. Chim. Acta* **1986**, *123*, 83–86. (d) Svastits, E. W.; Alberta, J. A.; Kim, I.-C.; Dawson, J. H. *Biochem. Biophys. Res. Commun.* **1989**, *165*, 1170–1176.

(34) (a) ${}^1H$ NMR and EPR data are compiled in Tables I and II, respectively. (b) Svastits, E. W. Ph.D. Thesis, University of South Carolina, 1986.

(35) Walker, F. A.; Lo, M.-W.; Reed, M. T. *J. Am. Chem. Soc.* **1976**, *98*, 5553–5560.

magnetic species. For the thermal mixture, the observed spectrum reflects the superposition of the two magnetically distinct species present.

Studies of quantum mechanically admixed intermediate-spin ferric porphyrin model complexes have employed both TPP and OEP. Only the latter have been examined herein. Except for Fe(OEP)SbF$_6$, the complexes have been previously prepared, have been structurally and magnetically characterized, and have been reported to exhibit admixed intermediate-spin behavior.[9,12,16] This is also true for Fe(TPP)SbF$_6$. The magnetic behavior of all the complexes examined herein has been characterized by NMR and EPR spectroscopy.

**NMR Studies.** The positions of the NMR hyperfine shifts of ferric porphyrins have been shown to be diagnostic of the iron spin state.[22] The chemical shifts of pyrrole protons have been correlated with single occupation of the $d_{x^2-y^2}$ orbital. A similar correlation is observed for the resonances of meso protons of octaalkylporphyrins, which are sensitive to $\pi$-spin density. Species exhibiting quantum mechanically admixed-spin behavior in the ground state display unique hyperfine spectra.[12,18] The resonance positions for the meso protons of such species reflect the net contribution of the quartet and the sextet and are characterized by a shift of approximately 10–25 ppm upfield relative to TMS. The observed meso proton resonances of the admixed-spin complexes examined herein are all shifted upfield (Table I). For the triflate adduct, this peak was observed at −24.6 ppm (CDCl$_3$), identical to the literature value.[12] This signal was observed between −4 and −6 ppm in the hexafluoroantimonate, perchlorate, and bis(dichloropyridine) adducts. Following Goff's interpretation,[12] we attribute the increased upfield shift in Fe(OEP)SO$_3$CF$_3$ to a greater contribution from the sextet ($^6$A, $S = {}^5/_2$) to the overall spin. On the contrary, the less upfield shifted peaks of the latter three species indicate a greater contribution from the quartet ($^4$A, $S = {}^3/_2$).

In addition, variable-temperature NMR spectroscopy can be used to distinguish mixed-spin ferric heme complexes from pure low- and high-spin species. For simple, magnetically ideal paramagnetic molecules, the absolute value of the isotropic shifts should increase in proportion to $1/T$. Previously examined admixed-spin species such as Fe(TPP)ClO$_4$, Fe(TPP)SO$_3$CF$_3$, and Fe(TPP)C(CN)$_3$ show reversed Curie law behavior.[12,18] In the present study, anomalous behavior was observed in studying the temperature dependence of the spectra of Fe(OEP)SO$_3$CF$_3$ and Fe(OEP)SbF$_6$. A plot of the proton resonance position vs temperature is linear but inverted from normal Curie behavior (data not shown) with the greatest deviation seen for the resonance of the meso proton. At 222 K, the methylene protons of Fe(OEP)SO$_3$CF$_3$ and Fe(OEP)SbF$_6$ display two sharp signals at 64.3 and 37.4 ppm and at 61.2 and 41.2 ppm, respectively. As the temperature is increased to 323 K, the methylene resonances of Fe(OEP)SbF$_6$ broaden and coalesce into one signal centered at 36.1 ppm. Similar behavior is observed for Fe(OEP)SO$_3$CF$_3$; however, two signals (46.8, 33.6 ppm) are still observed at 323 K. The ring methyl and methylene proton resonances of another quantum mechanically admixed-spin octaalkylporphyrin, iron(III) etioporphyrin perchlorate, also move in an upfield direction with increased temperature (208–325 K).[18] This inverted Curie behavior is characteristic of a mixed-spin species.[22] However, it is not possible to distinguish a thermal equilibrium of spin states from a quantum mechanically admixed-spin species resulting from thermal population of Kramer's doublets by NMR measurements alone.[18] The NMR time scale is likely not fast enough to resolve resonances for "pure" spin states.

**EPR Studies.** The EPR spectra of mixed-spin complexes are a function of the spin-mixing mechanism.[20] Samples displaying temperature-dependent spin-state behavior will give rise to two sets of EPR signals, one for each spin species present. In contrast, a quantum mechanically admixed-spin system will give rise to a single set of EPR signals which is the average of the two participating spins. Previous EPR studies of Fe(OEP)ClO$_4$,[9b] [Fe(OEP)(3,5-Cl$_2$Py)$_2$]$^+$,[16] and Fe(OEP)SO$_3$CF$_3$[12] have confirmed the admixed intermediate-spin behavior of these species. The *g* values of these complexes are listed in Table II along with those for typical high-spin,[36] Fe(OEP)Cl, and low-spin, [Fe(OEP)(HIm)$_2$]$^+$, complexes. The EPR signal obtained for Fe(OEP)SbF$_6$ (Figure 1) confirms the admixed intermediate-spin assignment. Therefore, SbF$_6$ is a sufficiently weakly coordinating anion to induce spin mixing in the ferric OEP system also. Trautwein, Weiss, and co-workers[37] have recently shown that the six-coordinate triflate/aquo-ligated complex of a tetraphenylporphyrin exhibits an EPR spectrum with *g* values at 5.75 and 2.00, distinctly different from those reported in Table II for the admixed intermediate-spin complexes. On that basis, we conclude that the admixed intermediate-spin complexes studied herein have not added a water ligand in solution. Trautwein, Weiss, and co-workers[37] also used Mössbauer spectroscopy to probe the properties of the triflate/aquo complex and, in combination with the EPR data, conclude that it is a weakly spin-mixed (85% $S = {}^5/_2$, 15% $S = {}^3/_2$) species.

**Table II.** EPR Spectral Data for Ferric Octaethylporphyrin Complexes

| complex[a] | obsd *g* values | | | assgnd spin state[b] |
|---|---|---|---|---|
| | $g_1$ | $g_2$ | $g_3$ | |
| Fe(OEP)ClO$_4$[c] | 3.83 | 1.97 | | QMAIS |
| Fe(OEP)SO$_3$CF$_3$[d] | 3.79 | 2.02 | | QMAIS |
| Fe(OEP)SbF$_6$ | 4.03 | 1.99 | | QMAIS |
| Fe(OEP)(3,5-Cl$_2$Py)$_2$ | 4.23[e] | 2.00[e] | | QMAIS |
| Fe(OEP)Cl | 6.14 | *f* | | high-spin |
| [Fe(OEP)(HIm)$_2$]$^+$[g] | 2.95 | 2.26 | 1.51 | low-spin |

[a] 77 K, 0.001 M solution in 1:1 methylene chloride/toluene glass. [b] QMAIS = quantum mechanically admixed intermediate spin. [c] Similar EPR values have been previously reported.[9b] [d] Similar EPR values have been previously reported.[12] [e] Taken from ref 16. [f] The expected signal at $g = 2.00$ is difficult to observe at 77 K but has been observed for the analogous high-spin ferric photoporphyrin chloride complex at lower temperatures.[36] [g] Similar EPR values have been previously reported.[46]

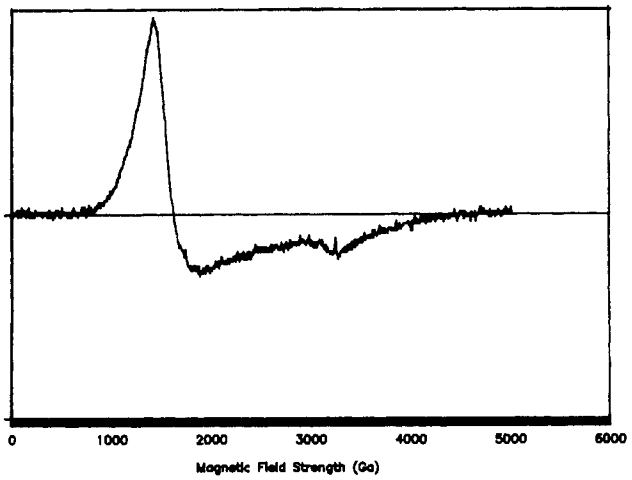

**Figure 1.** EPR spectrum of Fe(OEP)SbF$_6$ in a 1:1 methylene chloride/toluene glass at 77 K: frequency, 9.11 GHz; power, 40 mW; sweep width, 5000 G; center, 2500 G; modulation frequency, 100 kHz; modulation amplitude, 8 G; scan time, 4 min; time constant, 0.25 s; gain, 6.3 × 10$^3$.

**MCD Studies in the Soret and Visible Regions.** Having established that the selected "intermediate-spin" complexes exhibit quantum mechanically admixed-spin behavior, we turn our attention to the investigation of such complexes with MCD spectroscopy. MCD studies of heme iron systems have demonstrated the utility of the technique as a probe of spin state.[23] With ferric myoglobin, for example, high-spin ligand adducts display much

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(36) Uenoyama, H.; Sakai, K. *Spectrochim. Acta* **1975**, *31A*, 1517–1521.

(37) Gismelseed, A.; Bominaar, E. L.; Bill, E.; Trautwein, A. X.; Winkler, H.; Nasri, H.; Doppelt, P.; Mandon, D.; Fischer, J.; Weiss, R. *Inorg. Chem.* **1990**, *29*, 2741–2749.

**Table III.** UV-Visible Magnetic Circular Dichroism Spectral Characteristics of Ferric Octaethylporphyrin Complexes

| | Fe(OEP)X | | | | $[Fe(OEP)L_2]^+$ | | cytochrome *c'*[d] |
|---|---|---|---|---|---|---|---|
| features[a] | Cl[b,c] | $ClO_4$ | $SO_3CF_3$ | $SbF_6$ | 3,5-$Cl_2$Py | HIm[c] | native |
| Soret | | | | | | | |
| peak | 403 (10.3) | 378 (9.0) | 375 (10.2) | 382 (14.7) | 378 (11.7) | 398 (60.1) | 402 (5.9) |
| crossover | 412 | 387 | 384 | 388 | 389 | 404 | |
| trough | 420 (−6.8) | 396 (−11.8)<br>407 (−9.3) | 395 (−13.5) | 395 (−21.0) | 400 (−11.8) | 410 (−89.2) | 416 (−8.2) |
| β | | | | | | | |
| peak | 528 (7.3) | 525 (3.3) | 525 (4.2) | 518 (2.1) | 524 (4.9) | 548 (2.6) | 522 (2.1) |
| crossover | 537 | 536 | 536 | 529 | 538 | 554 | |
| trough | 548 (−12.1) | 548 (−4.7)<br>555 (−6.1) | 551 (−8.2) | 545 (−4.2) | 553 (−6.2) | 567 (−5.6) | 544 (−6.5) |
| α | | | | | | | |
| peak | 620 (2.2) | | 622 (0.6) | 610 (3.9) | | | 614 (1.1) |
| crossover | 637 | 630 | 631 | 629 | 634 | | |
| trough | 647 (−4.2) | 641 (−2.5)<br>658 (−1.6) | 640 (−1.1)<br>656 (−3.3) | 640 (−1.5) | 641 (−2.0) | | 638 (−2.4) |

[a] Values given are peak position, in nm, and peak intensities (in parentheses), in $(M{\cdot}cm{\cdot}T)^{-1}$. [b] Spectra obtained in benzene solution. All other spectra of model compounds obtained in methylene chloride solution; peak and trough positions are not sensitive to solvent. [c] See ref 40 for discussion about previously published work with these model compounds. [d] Cytochrome *c'* from *alcaligenes sp.*, NCIB 11 015.[28]

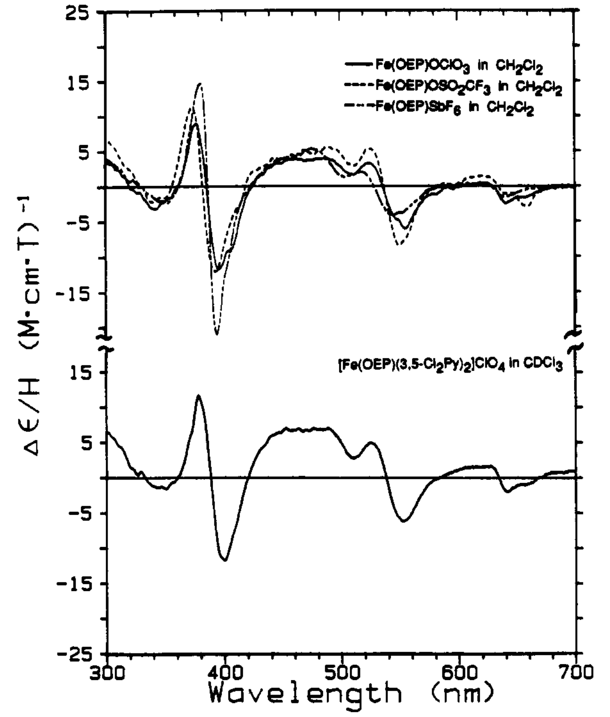

**Figure 2.** MCD spectra in the UV–visible region (300–700 nm) of $Fe(OEP)ClO_4$ (solid line), $Fe(OEP)SO_3CF_3$ (dashed line), and $Fe(OEP)SbF_6$ (dotted dashed line) (top) and of $[Fe(OEP)(3,5\text{-}Cl_2Py)_2]^+$ (bottom) obtained at 25 °C with sample concentrations between $7.5 \times 10^{-5}$ and $1.2 \times 10^{-4}$ M in methylene chloride.

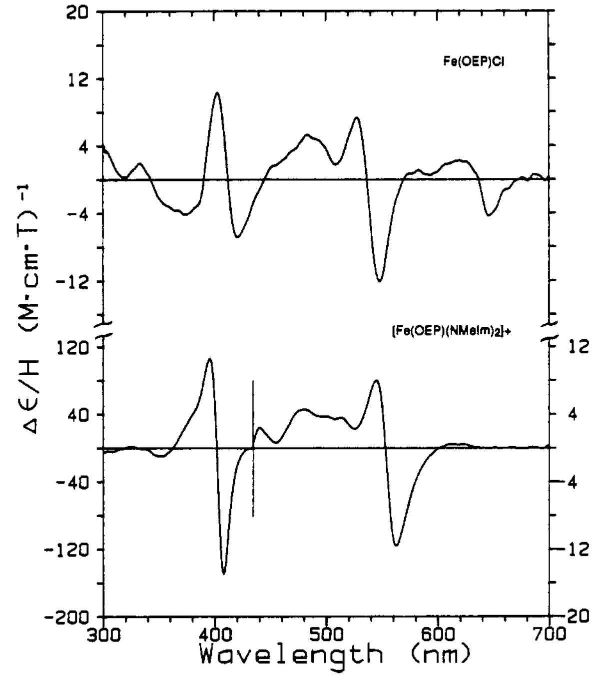

**Figure 3.** MCD spectra in the UV–visible region (300–700 nm) of Fe(OEP)Cl in benzene (top, $4 \times 10^{-4}$ M) and of $[Fe(OEP)(NMeIm)_2]^+$ (bottom, $8 \times 10^{-5}$ M) in methylene chloride obtained at 25 °C. The portion of the bottom spectrum above approximately 435 nm has been enlarged by a factor of 10.

weaker Soret MCD signals than low-spin ones.[38] The MCD signals in this region are dominated by *C* terms which are sensitive to both spin state and temperature. Stephens and co-workers described the characteristic dependence of the shape of the near-IR (700–2000 nm) MCD signal of the analogous ferric hemoglobin derivatives on the spin state.[39] MCD signals in this region are sensitive to the iron spin state because they originate from charge-transfer transitions of the π orbitals of the porphyrin to the $d_{xz}$ and $d_{yz}$ orbitals of the iron.[29]

MCD studies of ferric PPIXDME and OEP model complexes have shown that their spectra are nearly identical to those of analogously ligated protein samples.[23] For example, the MCD spectrum of $[Fe(PPIXDME)(HIm))_2]$ in the UV–visible region is identical to that of the bis(histidine)-ligated ferrous cytochrome $b_5$.[33c] The spectrum of the OEP analogue is similar in band shape and intensity but blue-shifted approximately 8 nm. For a study of *c* type cytochromes in which the porphyrin macrocycle has eight alkyl substituents, OEP is preferred over PPIXDME because of its octaalkyl substituent pattern.

The MCD spectra in the Soret/visible region (300–700 nm) of the admixed intermediate-spin and the exemplary high-spin (Fe(OEP)Cl) and low-spin ($[Fe(OEP)(NMeIm)_2]^+$) complexes[40] are plotted in Figures 2 and 3, respectively, and the relevant parameters are listed in Table III. A close resemblance is seen in the MCD spectra of the five-coordinate (Fe(OEP)X, X = $ClO_4$,

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(38) (a) Vickery, L.; Nozawa, K.; Sauer, K. *J. Am. Chem. Soc.* **1976**, *98*, 343–350. (b) Vickery, L. E. *Methods Enzymol.* **1978**, *54*, 284–302.

(39) Stephens, P. J.; Sutherland, J. C.; Cheng, J. C.; Eaton, W. A. in *The Excited States of Biological Molecules*; Birks, J. B., Ed.; Wiley: New York, 1976; pp 434–442.

(40) The spectra of Fe(OEP)Cl and of $Fe(OEP)(NMeIm)_2$ displayed in Figure 3 are similar to those previously reported for Fe(OEP)Cl and $Fe(OEP)(HIm)_2$, respectively: Kobayashi, H.; Higuichi, T.; Eguchi, K. *Bull. Chem. Soc. Jpn.* **1976**, *4*, 457–463.

**Table IV.** Near-IR Magnetic Circular Dichroism Spectral Characteristics of Ferric Octaethylporphyrin Complexes

| | Fe(OEP)X | | | | $[Fe(OEP)L_2]^+$ | | cytochrome *c'*[c] |
|---|---|---|---|---|---|---|---|
| features[a] | Cl | $ClO_4$ | $SO_3CF_3$ | $SbF_6$ | 3,5-$Cl_2$Py | HIm[b] | native |
| | 914 (0.45) | 942 (−0.25) | 907 (−0.05) | 967 (−0.35) | 942 (−0.20) | | 910 (−0.10 to −0.05) |
| crossover | 1007 | 1070 | 963 | 1097 | 1033 | | 944–972 |
| | 1093 (−0.25) | 1174 (0.15) | 1124 (0.25) | 1222 (0.25) | 1186 (0.35) | 1310 (sh) | 1100–1172 (0.50–0.70) |
| crossover | | 1343 | 1312 | 1399 | 1396 | | 1244–1283 |
| | | 1543 (−0.25) | 1451 (−0.20) | | 1517 (−0.20) | 1557 (1.05) | 1322–1390 (−0.40 to −0.60) |

[a] Values given are peak position, in nm, and peak intensities (in parentheses, rounded to nearest 0.05), in $(M{\cdot}cm{\cdot}T)^{-1}$. Spectra of model compounds obtained in chloroform-$d_1$ solution. [b] Similar MCD values have been previously reported; see text.[46] [c] Range of spectral parameters for cytochrome *c'* from several bacterial sources.[30]

$SO_3CF_3$, $SbF_6$) and six-coordinate ($[Fe(OEP)(3,5\text{-}Cl_2Py)_2]ClO_4$) intermediate-spin species in the Soret and visible regions (Figure 2). The Soret region (300–500 nm) of all four MCD spectra is dominated by a derivative-shaped band with a peak near 375 nm, a trough around 395 nm, and a crossover point at 386 nm. In contrast, low-spin complexes such as $[Fe(OEP)(NMeIm)_2]^+$ (Figure 3, bottom) display a much more intense derivative-shaped Soret band that is red-shifted by about 15 nm. The Soret MCD band of high-spin Fe(OEP)Cl (Figure 3, top) is also red-shifted relative to that of the intermediate-spin species but is somewhat less intense. In the visible region (500–700 nm), the spectrum of the low-spin complex is distinct from those of the high-spin and intermediate-spin cases. However, the MCD signals of the latter two spin types are not that readily distinguished in this region (Figures 2 and 3). All of the admixed intermediate-spin complexes examined herein exhibit a weak charge-transfer band at 640 nm. A similar band is seen at 638 nm in the spectrum of cytochrome *c'*.[28] The analogous band for Fe(OEP)Cl is at 647 nm and is entirely absent in low-spin cases.

The MCD spectral features of the admixed intermediate-spin model complexes are quite similar in band shape and intensity to the spectrum reported for cytochrome *c'* (Table III).[28] Taken as a whole, the MCD spectral features of the protein in the Soret/visible region bear a closer resemblance to those of the admixed intermediate-spin model complexes than to those of high-spin five-coordinate models,[28,41,42] but the case is not compelling.

**MCD Studies in the Near-IR Region.** The distinction between high-, low- and mixed-spin states of ferric hemes is especially clear using near-IR MCD. The bands in this region are charge transfer in origin arising from in-plane transitions from the highest occupied porphyrin $\pi$ orbitals ($a_{1u}$, $a_{2u}$) to the $e_g$ iron $d\pi$ orbitals ($d_{xz}$, $d_{yz}$).[43] Studies with ferric hemoglobin and myoglobin first demonstrated the sensitivity of near-IR MCD to spin state.[39,44,45] For example, the spectrum of a low-spin ferric complex such as $[Fe(OEP)(HIm)_2]^+$ (Figure 4) resembles its electronic absorption spectrum with a maximum at 1557 nm and broad shoulder at 1310 nm.[39,44,46] Thompson has examined several six-coordinate imidazole-ligated OEP complexes.[46] *In all cases*, the spectra are positive with maxima between 1540 and 1610 nm and intensities of about 0.2 $(M{\cdot}cm{\cdot}T)^{-1}$. The energy of the transitions correlate with the ligand field strength at the iron. Additional low-spin myoglobin, hemoglobin, and cytochrome *c* derivatives follow the same pattern.[44,47,48]

All pure high-spin complexes examined to date give rise to a *single derivative-shaped MCD feature* in the near-IR region. For example, Fe(OEP)Cl (Figure 4) has a trough at 1093 nm, a crossover at 1007 nm, and a peak at 914 nm. The MCD peak is close to the electronic absorption maximum at 925 nm. Hatano and co-workers have examined a series of five-coordinate, high-spin ferric protoporphyrin IX complexes with oxygen- and sulfur-donor ligands.[41] They showed that the energy of derivative-shaped bands observed between 746 and 885 nm is a function of the ligand field strength at the iron. The six-coordinate, high-spin fluoride adducts of ferric myoglobin[47] and hemoglobin[39] also display derivative-shaped curves between 750 and 1100 nm.

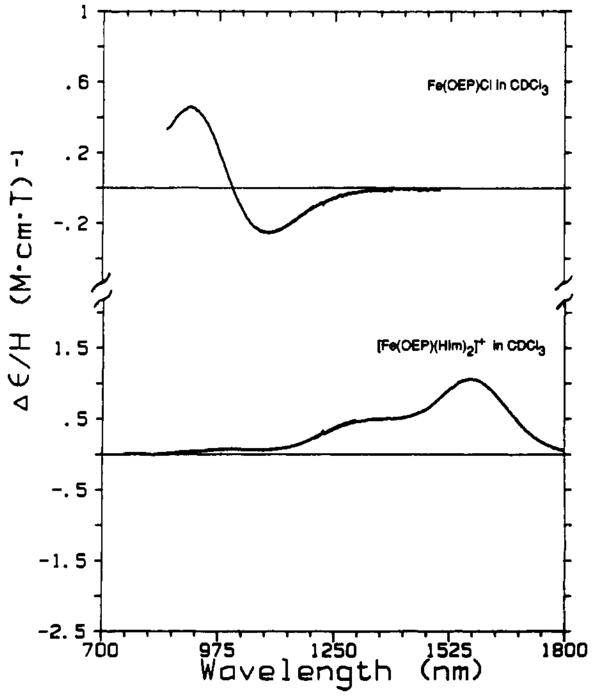

**Figure 4.** MCD spectra in the near-IR region (700–1800 nm) of Fe(OEP)Cl (top) and of $[Fe(OEP)(HIm)_2]^+$ (bottom) obtained at 25 °C with sample concentrations of $8 \times 10^{-4}$ M in chloroform-$d_1$.

The aquo and hydroxy forms of the ferric heme–octapeptide exist as a high-spin/low-spin thermal equilibrium.[49] The near-IR MCD spectrum has a derivative-shaped curve corresponding to an absorption peak around 1000 nm plus a positive Gaussian-shaped curve between 1100 and 1800 nm with a maximum around 1510 nm similar to the corresponding absorption spectrum.[50] This pattern is a composite of the near-IR MCD spectral features of high- and low-spin myoglobin derivatives.[47] As with EPR, the MCD spectral signature of complexes possessing a thermal equilibrium of high- and low-spin states is a combination of the spectra of the complexes with "pure" spin states.

The MCD spectra in the near-IR region of the quantum mechanically admixed-spin complexes examined (Figure 5) are all similar. The MCD spectra differ significantly from those of low- and high-spin complexes, of complexes that exist in a thermal equilibrium of high- and low-spin states, or of any previously

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(41) Nozawa, T.; Ookubo, S.; Hatano, M. *J. Inorg. Biochem.* **1980**, *12*, 253–267.
(42) Ookubo, S.; Nozawa, T.; Hatano, M. *J. Inorg. Biochem.* **1989**, *35*, 305–317.
(43) Makinen, M. W.; Churg, A. K. In *Iron Porphyrins, Part I*; Lever, A. B. P., Gray, H. B., Eds.; Addison Wesley: Reading, MA, 1983; pp 141–235.
(44) Cheng, J. C.; Osborne, G. A.; Stephens, P. J.; Eaton, W. A. *Nature* (*London*) **1973**, *241*, 193–194.
(45) Gadsby, P. M. A.; Thomson, A. J. *FEBS Lett.* **1982**, *150*, 59–63.
(46) Gadsby, P. M. A.; Thomson, A. J. *FEBS Lett.* **1986**, *197*, 253–257.
(47) Nozawa, T.; Yamamoto, T.; Hatano, M. *Biochim. Biophys. Acta* **1976**, *427*, 28–37.
(48) Kobayashi, N.; Nozawa, T.; Hatano, M. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 919–920.
(49) Huang, Y.-P.; Kassner, R. J. *J. Am. Chem. Soc.* **1981**, *103*, 4927–4932.
(50) Kobayashi, N.; Osa, T. *Chem. Pharm. Bull.* **1989**, *37*, 3105–3107.

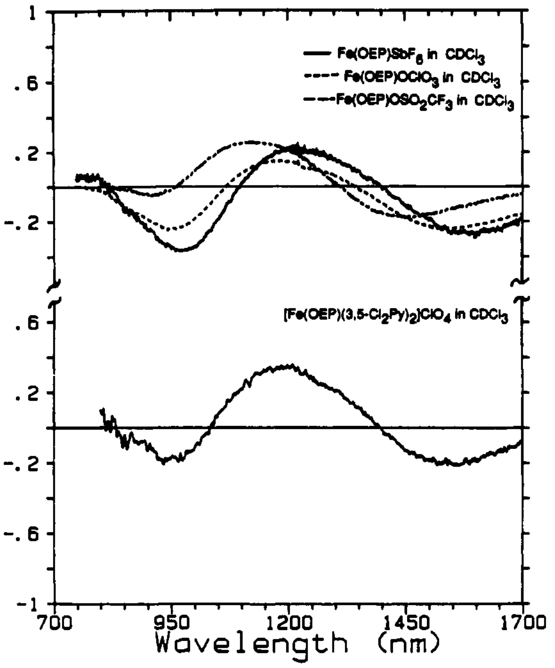

**Figure 5.** MCD spectra in the near-IR region (700–1800 nm) of Fe(OEP)SbF$_6$ (solid line), Fe(OEP)ClO$_4$ (dashed line), and Fe(OEP)SO$_3$CF$_3$ (dotted dashed line) (top) and of [Fe(OEP)(3,5-Cl$_2$Py)$_2$]$^+$ (bottom) obtained at 25 °C with sample concentrations of $2.5 \times 10^{-4}$ to $2.5 \times 10^{-3}$ M in chloroform-$d_1$.

reported model complexes of known spin state. The electronic absorption spectra of the quantum mechanically admixed-spin complexes display broad, weakly absorbing bands that appear to overlap. The high-energy maximum is centered around 975 nm for all four complexes. A second band between 1125 and 1235 nm is unique to each sample examined and corresponds to a peak in the MCD spectrum between 1120 and 1225 nm. The MCD spectrum also contains a high-energy trough between 907 and 967 nm (Table IV) corresponding to the electronic absorption maximum at 975 nm. On the basis of the near-IR electronic absorption studies of ferric heme systems, the bands appear to be charge transfer in origin and are probably composed predominantly of *C* terms.[43,50,51]

The near-IR MCD spectra of the admixed intermediate-spin complexes examined herein display the same negative–positive–negative band pattern (with increasing wavelength) seen for cytochrome *c'* by Stephens and co-workers[30] (Table IV). They examined the protein from several bacterial sources over a wide pH range (pD 1–13) and also identified a high-spin form and an alkaline low-spin form. They found, however, that "MCD cannot categorically distinguish between normal $S = {}^5/_2$ and mixed $S = {}^3/_2, {}^5/_2$ ground states" due to a lack of reference data for "definitively characterized mixed-spin species".[30] The present study provides those reference data. The near-IR MCD spectra of Fe(OEP)X (X = ClO$_4$, SO$_3$CF$_3$, SbF$_6$) and [Fe(OEP)(3,5-Cl$_2$Py)$_2$]$^+$, all of which contain an admixed $S = {}^3/_2, {}^5/_2$ ground state, are unique (Figure 5). Because MCD bands can be positive or negative, the near-IR MCD spectra are far more distinctive than near-IR electronic absorption spectra of the same species. The negative–positive–negative band pattern (with increasing wavelength) of the admixed intermediate-spin complexes are readily distinguished from the positive–negative derivative-shaped pattern (with increasing wavelength) seen in high-spin cases,[41,42] from the all positive features seen in low-spin cases[46] (Figure 4), or from the spectra of complexes containing a thermal mixture of high- and low-spin states.[49] The close similarity between the near-IR MCD spectra of the quantum mechanically admixed-spin models (Figure 5) and cytochrome *c'*[30] (Table IV) confirms the proposed admixed intermediate-spin ground state of the protein.[5]

**Concluding Remarks.** Our results further demonstrate the utility of MCD, particularly in the near-IR region, as a probe of spin state of ferric heme species. We have shown that MCD is capable of discriminating between low-, high-, and intermediate-spin-state ferric porphyrins at ambient temperatures. Our results for the quantum mechanically admixed-spin systems, when compared with data for a system in thermal equilibrium between low- and high-spin, suggest that the technique is also able to distinguish between mixed-spin systems in which the two spin states are in thermal equilibrium and a quantum mechanical admixture of high ($S = {}^5/_2$) and intermediate spin ($S = {}^3/_2$). An additional advantage to near-IR MCD is that the results are obtained at ambient temperatures in contrast to EPR and Mössbauer studies, which usually require temperatures below 100 K. Finally, a comparison of our data for the quantum mechanically admixed-spin complexes with MCD data for cytochrome *c'* found in the literature[28,30] provides further support for the use of these complexes as models for the mixed-spin state of cytochrome *c'*.

**Acknowledgment.** Support for this research was provided by NIH Grant GM-26730. The JASCO J-500 magnet was purchased through a grant from the Research Corp. The JASCO J-200 and J-500 spectrometers were purchased with NIH grant RR-03960. Additional support was provided through a NIH Biomedical Research Support Grant. The AM-300 NMR spectrometer was purchased with NSF Grant CHE 84-11172 and NIH Grant RR-02425. We thank Ann M. Huff for measuring the MCD spectrum of [Fe(OEP)(NMeIM)$_2$]$^+$ shown in Figure 3 and Drs. Edmund W. Svastits, Joseph Nardo, and John J. Rux for assembling the computer-based spectroscopic data system.

**Registry No.** Fe(OEP)Cl, 28755-93-3; Fe(OEP)ClO$_4$, 50540-30-2; Fe(OEP)SO$_3$CF$_3$, 79872-94-9; Fe(OEP)SbF$_6$, 137540-32-0; [Fe(OEP)(3,5-Cl$_2$Py)$_2$]$^+$, 119679-65-1; [Fe(OEP)(NMeIm)$_2$]$^+$, 60151-09-9; [Fe(OEP)(HIm)$_2$]$^+$, 48242-03-1; cytochrome *c'*, 9035-41-0.

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(51) Yamamoto, T.; Nozawa, T.; Kobayashi, N.; Hatano, M. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 3059–3063.