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Author
Vickery, Larry E.

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Larry E. Vickery

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DETERMINATION OF SPIN STATE EQUILIBRIA IN
FE(III)-HEMEPROTEINS BY MAGNETIC CIRCULAR DICHOISM

Larry E. Vickery
Department of Chemistry and Laboratory of Chemical Biodynamics
Lawrence Berkeley Laboratory, University of California
Berkeley, California 94720

Summary. The intensity of the magnetic circular dichroism (MCD) associated with the near-UV Soret transition of ferric hemeproteins is paramagnetic in origin and is proportional to the amount of low spin ($S = 1/2$) component present. For characterization of low spin $\leftrightarrow$ high spin ($S = 5/2$) mixtures the MCD technique can offer advantages over other methods: (i) samples need not be pure if other components present exhibit weaker magneto-optical activity than the intense hemoprotein signals, (ii) high sensitivity allows for low (μm) concentrations to be used, (iii) measurements can be obtained at physiological as well as cryogenic temperatures and thermodynamic parameters can be determined, and (iv) rapid kinetic measurements should be possible. Results obtained with a variety of hemeproteins (myoglobin, hemoglobin, cyt. c, cyt. f, cyt. b$_5$, cyt. P-450, cyt. oxidase, and hemopexin) are described to illustrate the utility of MCD as a spectral probe for monitoring spin state equilibria both in vitro and in situ.

Hemeproteins serve catalytic roles in a wide variety of biological processes, including oxygen transport and storage, electron and proton transfer, and substrate oxidation, reduction and hydroxylation reactions. The ability of hemeproteins to participate in so many diverse reactions is due in a large part to the fact that the electronic properties of the heme iron can be modulated drastically by specific interactions with the protein. Thus, the iron atom can exist stably in several oxidation-reduction states and in several configurations of d-orbital electron pairing. Moreover, within a given redox and spin state, the chemical identity and the stereochemical positioning of the protein axial ligand(s) to the iron can affect kinetic and thermodynamic properties such as substrate interaction rates and electron affinity midpoint potentials.
This report will describe the use of a relatively new technique, magnetic circular dichroism (MCD), to determine a single type of change in electronic structure of the heme group, viz. the high spin \(S = 5/2\) \(\Leftrightarrow\) low spin \(S = 1/2\) equilibrium of Fe\(^{3+}\) complexes. This spin state change has been observed to occur in a number of ferric hemeprotein systems, and examples will be given for each of the classes of hemeproteins mentioned above. It will be shown that, for each type of hemeprotein investigated, the MCD spectra in the near ultraviolet, or Soret region, of ferric heme complexes is paramagnetic in origin and that the intensity can provide a useful measure of the amount of low spin component present. The general origins of magneto-optical activity, or the Faraday effect, has been presented in the preceding paper by Nozawa (1) and have been extensively reviewed (2,3), and these will not be covered here. Details of the experimental technique and descriptions of instruments can be found in the recent literature (4-6). Magnetic optical rotatory dispersion (MORD) measurements have also been carried out on a number of hemeproteins. This technique yields the same information as MCD but suffers from a more complex band shape, and because it is a refractive phenomenon gives rise to effects outside of regions of absorption causing overlapping of bands. References to MORD studies relevant to this report can be found in Shashoua’s article (7) and cited in references (2), (9) and (15).

Several other experimental approaches are also available for determining the spin state of Fe(III)-hemeproteins, but each of these has certain limitations. Absorption spectroscopy is perhaps the easiest method to use but requires a purified sample and an accurate knowledge of extinction coefficients of high and low spin forms. If other absorbing chromophores are present, however, difference spectroscopy can be used to monitor band shifts. These shifts can be interpreted in terms of changes in spin ratios but determinations of absolute spin states are not possible. Electron spin resonance (ESR) can often be used to overcome the contamination problems encountered in absorption spectroscopy.
since impurities present may not be paramagnetic or may exhibit microwave absorption at different magnetic field values. ESR suffers, however, from the limitations that relatively high concentrations, on the order of 100's μm, and low, non-physiological temperatures, which may alter spin equilibria, are required. Magnetic susceptibility and Mössbauer techniques can also be applied, but these methods also require high concentrations of heme and are sensitive to the presence of impurities. MCD will be shown to compliment these approaches since results can be obtained over a wide temperature range, on dilute solutions (sub-micromolar), both on purified hemeproteins in vitro and on crude preparations in situ.

MCD spectra have been reported for many hemeproteins (see ref. 2), but until recently it was assumed that the intense "S-shaped" MCD associated with the Soret band of low spin ferric forms was a Faraday A type term. The MCD observed for this band resembles the first derivative of the absorption spectrum as expected for a transition to an orbitally degenerate excited state. Briat et al. (8), however, found that the MCD spectrum of ferricytochrome b\textsubscript{2} core exhibits a temperature dependence expected for Faraday C terms, indicating a paramagnetic origin for the magnetically-induced optical activity.

We observed a similar dependence on temperature for the MCD of the low spin cyanide complex of met-myoglobin (9). Fig. 1 shows the results obtained at room and liquid nitrogen temperatures. Both curves show a similar derivative shape with a zero crossing near the absorption maximum of the complex. Lowering the temperature from 22° to -196°C causes about a four-fold increase in the intensity of the MCD, as would be expected on the basis of a Boltzmann redistribution of ground states. Thus this MCD band is composed predominantly of C terms and must arise from the removal of a ground state spin degeneracy by the applied magnetic field.

The paramagnetic origin of the MCD in this spectral region suggests that the intensity should be very sensitive to the spin state of the iron. A
comparison of the absorption and MCD near 22°C of the low spin cyanide derivative and the essentially completely high spin fluoride form of met-myoglobin is presented in Fig. 2. The absorption of the high spin form is simply blue shifted, but the MCD is practically abolished. The temperature dependence of the MCD of fluoride myoglobin provided evidence for the presence of weak C terms (not shown).

The MCD spectra of other complexes of ferric myoglobin having various mixtures of low and high spin states were also measured at room temperature (Fig. 3). Each of the derivatives possessing appreciable low spin form exhibits a similar shape, and noting the difference in the left and right hand ordinates, it is clear that the intensity decreases as the fraction of the low spin form decreases in the order cyanide > imidazole > azide > hydroxide (pH 11) > thiocyanate > aquo (pH 6) > fluoride. An attempt to quantitate this relationship is given in Fig. 4. Here the absolute value of the MCD intensity of the troughs near 430 nm is plotted against the percentage of the low spin form as determined by absorption spectral (10) and magnetic susceptibility (11,12) measurements. Those derivatives possessing less than 40% low spin form and the cyanide derivative fall in a straight line indicating an approximately linear relationship between the amount of $S = 1/2$ ferric heme and the MCD intensity. The azide and imidazole complexes deviate considerably from the plot, however. The reason for this discrepancy is not understood, and plots of the MCD peak intensity, integration of the area under the MCD curve or corrections for differences in extinction coefficients of the derivatives did not improve the fit. The MCD intensity of several other completely low spin ferri-hemeproteins are also included in the figure. Cytochrome c (cyt. c), cytochrome f (cyt. f) and cytochrome b$_5$ (cyt. b$_5$) exhibit similar Soret trough magnetic ellipticities demonstrating that the myoglobin results can be extended to other iron-protoporphyrin IX systems, including covalently bound c-type heme. Fe(III)-deuteroporphyrin and
Fe(III)-protoporphyrin complexes of rabbit hemopexin (dRHx and pRHx, respectively) are also low spin (13) and exhibit a similar intense S-shaped MCD; the somewhat stronger MCD for dRHx is due to the characteristically narrower band width (14).

Further studies were carried out on the cytochromes to confirm the paramagnetic origin of the MCD and to establish the sensitivity of the MCD to spin state for this functional group of heme proteins (15). The results of a narrow temperature range study of horse heart ferri-cytochrome c are presented in Fig. 5. The MCD curves obtained near room temperature and about 100° lower have similar shapes and positions and the increase in intensity observed is expected for a Boltzmann effect. The inset shows a plot of the peak and trough ellipticity values at these and intermediate temperatures vs. reciprocal temperature. Both curves are approximately linear and show that MCD C terms can be detected for temperature changes as small as 10-20°.

The results obtained in a wider temperature range study on beef liver ferri-cytochrome b₅ serve to illustrate some additional points. The complete Soret region MCD curves obtained at room temperature and liquid nitrogen temperatures are given in Fig. 6 and the inset shows a plot of the peak and trough ellipticities vs. inverse temperature. There is no readily discernible change in shape of the MCD upon lowering the temperature, but the increase in intensity of the trough is somewhat greater than that of the peak. This effect is most clearly seen in the inset by extrapolation of the 419 and 406 nm curves to 1/T = 0. At infinite temperature the C terms will disappear and only Faraday A and B terms will remain. The fact that the data plotted for 419 nm do not extrapolate to zero intensity while the 406 nm results do, suggests the presence of positive A and B terms near 419 nm which are not present at 406 nm. A careful study of the temperature dependence of the MCD as a function of wavelength could be useful for extracting these parameters.

Cytochrome b₅ can be reversibly denatured to a high spin form at alkaline pH values (16,17) providing an opportunity for assaying the sensitivity of MCD
to this spin state equilibrium in another system in addition to myoglobin.

Figure 7 shows the Soret MCD curves for ferri-cytochrome b₅ at various pH values. The main effect is a large decrease in the MCD intensity as the pH is raised, but a small red shift of the zero crossing which appears to be complete by pH 12 is also evident. This may reflect the production of a different low spin form with a pK around pH 11.5; such a species would be difficult to observe by absorption spectroscopy due to the simultaneous appearance of the blue-shifted high spin form, but has been seen in ESR spectra in this pH region (16-18).

These data have been replotted as a pH titration in Fig. 8. The upper portion of the figure shows the decrease in the peak (---) and trough (—) magnetic ellipticity as the pH is raised. Both curves exhibit a midpoint for the transition near pH 12 under these conditions, and the MCD intensity has essentially completely disappeared by pH 13. Assuming that the neutral pH species is 100% low spin and that the alkaline form is completely high spin, the ratios of the two spin state forms have been calculated from the peak and trough ellipticity values and are plotted as a function of pH in the lower half of Fig. 8. The change in the logarithm of the low/high spin ratio is linear over almost two decades, and the slope indicates that more than a single proton is linked to the transition.

The preceding studies were all carried out with highly purified hemeproteins in vitro. In favorable cases, measurements can also be obtained for unpurified hemeproteins in situ. This is desirable since it might enable one to monitor the physiological behavior of hemeproteins coupled to other components, and since possible effects of harsh isolation procedures on hemeprotein structure can be avoided. The utility of MCD as a spectral probe for crude preparations of hemeproteins will be illustrated while discussing the MCD of a third class of hemeproteins, the oxidases.

Several laboratories have reported MCD spectra of cytochromes P-450,
enzymes involved in the hydroxylation of various hydrocarbon substrates (19-22). Of these proteins only P-450$_{cam}$, the camphor-hydroxylating enzyme of Pseudomonas putida, is water soluble, and hence this hemeprotein has been used as a model for other membrane-bound P-450 enzymes. The Soret region MCD spectra of the oxidized bacterial enzyme are shown in Fig. 9. In the absence of camphor a derivative-shaped MCD typical of a low spin, ferric hemichrome is observed. The intensity of the MCD, however, is much less than was found for the completely low spin myoglobin derivatives and b- and c-type cytochromes described above, indicating the presence of a considerable fraction of high spin component at room temperature. Further evidence for the existence of a high spin component was seen in the visible MCD and absorption spectra (21). In the presence of camphor the low spin signal disappears, but new MCD bands unlike those seen for high spin forms of myoglobin or cytochrome b$_5$ appear. The origin of the magneto-optical activity of the P-450-substrate complex is not yet known, but the properties are clearly different from other high spin ferric complexes.

MCD spectra of rat liver microsomal preparation containing P-450 and cytochrome b$_5$ are shown in Fig. 10. The intense hemeprotein A and C terms dominate the spectra as the flavoproteins, non-heme iron proteins and other chromophores present exhibit a much weaker magneto-optical activity. In the absence of any additions both hemeproteins are in the oxidized state, and the intense S-shaped band in the near UV contains C term contributions from each heme. Upon the addition of reduced pyridine nucleotide, cytochrome b$_5$ is reduced. This change is accompanied by the loss of the C terms in the Soret region and the appearance of a sharp A term in the visible. The MCD remaining in the Soret region is due primarily to low spin, ferric P-450. This band can then be used to monitor further redox changes upon sequential addition of reducing equivalents (21) or to follow spin state changes of the membrane bound enzyme upon substrate or effector binding. It is perhaps also noteworthy that a comparison of the MCD intensities of various extrema in the membrane-bound
mammalian enzyme with those of the soluble bacterial enzyme reveals subtle differences between the electronic structures of the two heme groups (21). The significance of this result is not known, but the difference was found to persist in the solubilized mammalian enzyme (22).

We have also investigated some of the properties of cytochrome c oxidase (23). The spectroscopic properties of this protein differ from those of the preceding hemeproteins primarily because of the substitution of heme a as the prosthetic group, but preliminary studies on isolated ferric heme a complexes have indicated that the Soret region MCD spectra of this porphyrin derivative are also very sensitive to the spin state of the central iron atom. The temperature dependence of the MCD of the oxidized form of beef heart cytochrome oxidase is shown in Fig. 11. These results clearly indicate the presence of C terms. The MCD increases linearly with the reciprocal of temperature without appreciable change in band shape, as expected for a paramagnetic origin of the magneto-optical activity, but the fact that the peak and trough intensities do not extrapolate to zero at infinite temperature suggests the presence of A and B terms in this region as well. The magnetic ellipticity values observed for cytochrome oxidase are less than would be expected if all of the heme a were in the low spin form. This could result from the presence of a low spin \( \Rightarrow \) high spin equilibrium or from the presence of two different populations of heme a, one low spin and the other high spin. Two types of heme a are, of course, known to exist in cytochrome oxidase, and the linearity of the temperature dependence of the MCD (Fig. 11, inset) as well as the Curie behavior of susceptibility measurements (24) argue against any thermal spin equilibrium.

The presence of a spin state equilibrium exhibiting a significant enthalpic change would, however, be expected to manifest itself in the temperature dependence of the MCD. Figure 12 presents some hypothetical cases
in which \( S = 5/2 \) \( \rightleftharpoons \) \( S = 1/2 \) spin equilibria poised at equal ratios near room temperature are altered upon lowering the temperature. Curves (1) and (2) correspond to low spin ground states or a negative \( \Delta H \) for the reaction, while curves (3) and (4) approach a high spin ground state. The broken lines represent typical MCD intensities for pure low spin (upper curve) and high spin (lower curve) ferric heme complexes; a linear temperature dependence which extrapolates to zero at infinite temperature (no \( A \) or \( B \) terms) has been assumed. Thermal spin equilibria having an \( S = 1/2 \) ground state would approach the low spin limit asymptotically, the curvature being most easily recognized in instances with a large \( -\Delta H \); spin mixtures with an \( S = 5/2 \) ground state would actually exhibit an initial decrease in MCD intensity as the temperature is lowered. Thermodynamic parameters could be extracted by curve analysis as has been done with magnetic susceptibility measurements (25).

This article has dealt solely with the MCD results obtained for the Soret spectral region and has been restricted to Fe(III) complexes. The visible and near infrared regions are, of course, also affected by the unpaired iron electrons, but the correlation between the spin state and the MCD intensity is not often as clear. Other MCD spectral regions do provide additional information, though, relating to the heme electronic structure. Fe(II) or ferrous heme also commonly exists in a low spin \( (S = 0) \) and \( (S = 2) \) state. Only the latter, paramagnetic state will give rise to \( C \) terms, and MCD is useful for characterizing these reduced heme complexes as well. Further discussions of these aspects are given in some of the references.
Acknowledgements. A large part of the work summarized here was carried out in collaboration with others and many of the ideas presented arose from discussions with these colleagues whom I gratefully acknowledge: Drs. Gerald Babcock, Mel Klein, William Morgan, Tsunenori Nozawa, Kenneth Sauer, Todd Schuster and John Sutherland. I also thank the National Institute of General Medical Sciences, USPHS, for a postdoctoral fellowship. Work performed under the auspices of the U. S. Energy Research and Development Administration.

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1. T. Nozawa, these proceedings, preceding paper.
18. L. E. Vickery and A. J. Bearden, unpublished observations. The formation of two different low spin forms, one with a low field $g$ value of 2.86 followed by a second with $g = 2.74$, were actually observed as the pH was raised above 11.
Figure 1. The effect of temperature on the MCD of sperm whale ferrimyoglobin cyanide. Solvent: potassium glycerophosphate, glycerol, 0.1 M sodium phosphate in equal volumes at pH 6.8. (Reproduced from Ref. 9 by permission.)
FERRI-MYOGLOBIN COMPLEXES
Comparison of Low and High Spin Forms

Figure 2. Comparison of absorption and MCD spectra of low and high spin complexes of ferrimyoglobin. Samples in 0.1 M sodium phosphate, pH 6.8, 22°.
Figure 3. MCD spectra of the Soret band of sperm whale ferrimyoglobin complexes. All samples in 0.1 M sodium phosphate, pH 6.8, except hydroxide at pH 11.5; room temperature (near 22°). Note that the right-hand scale is expanded. (Reproduced from Ref. 9 with permission.)
Figure 4. Correlation of the Soret region MCD intensity of ferrimyoglobin complexes with low spin ($S = 1/2$) content. (Reproduced from Ref. 9 by permission.)
Figure 5. Temperature dependence of the Soret MCD of horse heart ferri-cytochrome c. The solvent was glycerol and 0.5 M KCl (6:4 v:v) at pH 7.3. (Reproduced from Ref. 15 by permission.)
Figure 6. Temperature dependence of the Soret MCD of calf liver ferricytochrome \( b_F \). Solvent as Fig. 1. (Reproduced from Ref. 15 by permission.)
Figure 7. The effect of pH on the Soret MCD of ferricytochrome b$_5$. The solvent was 0.1 M KCl, temperature 22°. (Reproduced from Ref. 15 by permission.)
Figure 8. MCD spectrophotometric titration of ferricytochrome b₅. Data from Fig. 7. (Reproduced from Ref. 15 by permission.)
Figure 9. MCD spectra of *P. putida* cytochrome P-450<sub>cam</sub> in 0.1 M sodium phosphate, pH 7.0, near 22°. (Reproduced from Ref. 21 by permission.)
Figure 10. MCD spectra of rat liver microsomal preparation. Microsomes were suspended at a concentration of 0.8 mg/ml in 0.05 M Tris HCl, 0.25 M sucrose, pH 7.5 and spectra recorded at ambient temperature (near 22°). (Reproduced from Ref. 21 by permission.)
Fe(III) CYTOCHROME OXIDASE

Figure 11. Temperature dependence of the MCD of purified beef heart cytochrome c oxidase. The results are presented on the basis of the total heme a concentration. The sample was dissolved in glycerol and 0.5 M KCl, pH 7.4 (3:1 :: v/v) (23).
MCD for Fe(III) heme: $S = \frac{5}{2} \rightleftharpoons S = \frac{1}{2}$ Equilibria

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<thead>
<tr>
<th>$\Delta H$ (Kcal/mole)</th>
<th>$\Delta S$ (e.u.)</th>
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<tr>
<td>1) -10</td>
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<tr>
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Figure 12. Temperature dependence of MCD intensity for hypothetical cases of Fe(III) low spin$\rightleftharpoons$ high spin equilibria.
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