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# The use of near-infrared charge-transfer transitions of low-spin ferric chlorins in axial ligand assignment

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**Abstract** The near-infrared magnetic circular dichroism spectra of some low-spin derivatives of ferric-octaethylchlorin substituted myoglobin have been recorded at cryogenic temperatures. The spectra, which include some of the lowest energy charge-transfer transitions ever observed for hemes, are clearly dependent upon the nature of the axial ligands present. While the results indicate that such spectra may have some practical utility in axial ligand assignment, as is now quite common practice for iron-porphyrin systems, there are some severe practical limitations to this protocol documented in the case of iron-chlorins.

*Key words:* Axial ligand; Chlorin; MCD; Near-infrared

## 1. Introduction

The use of near-infrared magnetic circular dichroism (MCD) spectroscopy for the assignment of the axial ligands in low-spin derivatives of ferric hemoproteins containing fully unsaturated tetrapyrrole rings (i.e. porphyrins) is now quite well established [1,2]. Recently, an effort has been started in our laboratory to extend this methodology to hemoproteins containing hydroporphyrins, such as chlorins [3]. This is potentially of some importance, since where they occur, iron-hydroporphyrins are invariably observed to be substrate binding hemes, even if iron-porphyrins are also present.

While the results of the earlier study were encouraging in that they clearly showed the near-infrared MCD spectra of *Escherichia coli* hydroperoxidase II (an iron-chlorin system) to depend on the identity of the exogenous ligands present [3], it was surprising that the positions of the low energy maxima observed were similar to those exhibited by the analogous adducts of metmyoglobin. Suspecting the proximal ligands in these two proteins to be different and consequently, the close correspondence between the low energy charge-transfer transitions of their ligand adducts to be fortuitous, we have now undertaken a study of the near-infrared MCD spectra of some metmyoglobin derivatives where the native iron-porphyrin macrocycle has been replaced with a synthetic iron-chlorin.

## 2. Experimental

The method of Whitlock et al. [4] was used to prepare *trans*-1,2,3,4,5,6,7,8-octaethylchlorin (OEC). Formation of the required product was confirmed by electronic absorption spectroscopy and liquid secondary ion mass spectrometry. Incorporation of OEC into apomyoglobin was accomplished following 'method B' for the insertion of chlorophyllides into apomyoglobin described by Wright and Boxer [5]. The previously reported preparation of iron-octaethylporphyrin substituted myoglobin by Dawson and colleagues [6] had already established that the very similar iron-OEC would almost certainly fit the heme pocket. Location of the alternate heme inside the heme pocket of myoglobin was confirmed by the fluorescence method of Stryer [7]. Concentrations of samples in OEC were determined using $\varepsilon_{416} = 90$ $mM^{-1} \cdot cm^{-1}$ for the pyridine hemochrome form of the iron-chlorin [8].

Electronic absorption spectra were recorded on Perkin-Elmer $\lambda$ 5 and Varian DMS 100 spectrophotometers. MCD spectra were recorded using an Aviv Associates 41DS circular dichroism spectrometer in conjunction with a Cryomagnetics Incorporated cryomagnet. A 'single spectrum' consists of data recorded in with the applied field in the forward direction minus the reverse field data, the difference being divided by two. In this manner, contributions arising from natural circular dichroism are subtracted from the spectrum.

## 3. Results

The near-ultraviolet to visible region electronic absorption characteristics of the ferric-OEC reconstituted myoglobin ($Mb^+\{OEC\}$) derivatives used in this study are given in Table 1. The spectrum of the imidazole adduct correlates rather well with that previously reported for ferric-oxochlorin reconstituted cytochrome $b_5$ ($\lambda_{max}$ = 404 nm and 586 nm) by Martinis et al. [9]. The spectrum of the cyanide adduct, with only two distinct bands, is clearly similar and representative of the characteristic low-spin spectrum for $Mb^+\{OEC\}$ derivatives. The presence of additional bands to longer wavelength than 600 nm in the azide and hydroxide adducts (Table 1) is undoubtedly indicative of some residual high-spin sites in samples at ambient temperatures. However, below 20 K, the EPR spectra of these samples (not shown) indicate residual high-spin ($g$ = 6) components to be minority species. The possible existence of high-spin components in samples is of no consequence to the MCD analysis, since such systems do not exhibit transitions in the near-infrared region of interest. The EPR spectral characteristics of the low-spin derivatives used in this study are given in Table 1, where they are compared to the data for the analogous adducts of ferric-sulfmyoglobin previously reported by Berzofsky et al. [10].

In Fig. 1 is shown the near-infrared spectrum of the imidazole adduct of $Mb^+\{OEC\}$ at 4.2 K and 5.0 T. The maximum at 2,340 nm represents the lowest energy charge-transfer transition yet reported for a low-spin ferric heme. Furthermore, with $\Delta\varepsilon = 43$ $M^{-1} \cdot cm^{-1}$ at 4.2 K and 5.0 T, the magnitude of the signal is similar to that previously reported for the azide and

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*Abbreviations:* CAPS, 3-(cyclohexylamino)propanesulfonic acid; EPR, electron paramagnetic resonance; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MCD, magnetic circular dichroism; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, *trans*-1,2,3,4,5,6,7,8-octaethylchlorin.


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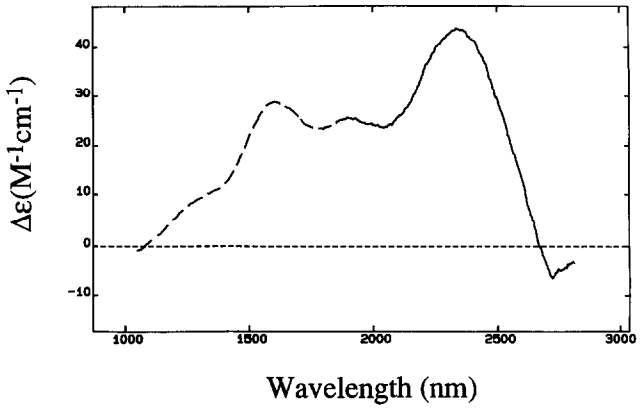


Fig. 1. Near-infrared MCD spectrum of the $Mb^+\{OEC\}$-imidazole complex, pD 6.3 in 50 mM MES buffer, 50% (v/v) $d_6$-ethanediol, 9.8 mM imidazole. Heme concentration was 0.64 mM, 0.5 mm pathlength, 12 nm maximum bandwith, average of 8 accumulated spectra at 4.2 K and 5.0 T.

cyanide adducts of hydroperoxidase II [3], significantly weaker than the analogous spectra of ferric-porphyrin derivatives. The portion of the spectrum shown by the dashed line was variable between samples and almost certainly represents contamination by bis(imidazole) coordinated ferric-porphyrin species. These contaminants arise from two potential sources. First, it does not seem possible to prepare apomyoglobin entirely free of protoheme, which is then residual in the finally obtained $Mb^+\{OEC\}$. Second, OEC preparations tend to reoxidize to the starting porphyrin upon manipulation and furthermore, this process appears to be promoted by certain combinations of axial ligands, e.g. bis(imidazole). The near-infrared MCD spectra of the bis(imidazole) adducts of ferric-octaethylporphyrin and protoheme have low energy maxima at 1,550 nm to 1,600 nm, with $\Delta\varepsilon = 200\ M^{-1} \cdot cm^{-1}$, or more [2]. The dashed part of the data of Figure 1 is consistent with contamination of the sample at the level of ca. 7% ferric-porphyrins relative to $Mb^+\{OEC\}$.

In Fig. 2 are shown the low temperature near-infrared MCD spectra of the hydroxide, azide and cyanide adducts of $Mb^+\{OEC\}$. In the case of the hydroxide adduct, the dashed portion of the spectrum was variable and probably represents some degree of coordination by other ligands in the vicinity of the heme pocket. The stronger ligands azide and cyanide gave very reproducible adducts. The spectra of Figure 2 clearly indicate that the position of the charge-transfer transition depends upon the strength of the axial ligands, as is the case for ferric-porphyrins. However, the present data set is significantly red shifted compared to the comparable spectra of the analogous derivatives of native metmyoglobin [1]. This comparison is presented in Table 2.

## 4. Discussion

It is evident from inspection of the data summarized in Table 2 that the position of the near-infrared charge-transfer transition provides a basis for assignment of the axial ligands in low-spin ferric-chlorin as well as low-spin ferric-porphyrin systems. In general, the spectra of ferric-chlorin derivatives are about 2,000 $cm^{-1}$ red-shifted with respect to those of ferric-porphyrins having the same axial ligands. This can readily be understood as follows. The near-infrared transitions of low-spin ferric hemes are considered to arise from porphyrin ($\pi$)-to-ferric ($d_{yz}$) charge-transfer processes [1,2]. For the same sets of axial ligands, the energy of the $d_{yz}$ orbital is not expected to vary much between chlorin and porphyrin derivatives. However, the energy of the highest occupied molecular ($\pi$) orbital of the chlorin is predicted to be greater than that of the corresponding porphyrin orbital [11]. Therefore, the energy of the charge-transfer transition (i.e. the difference between the $\pi$ and d orbital energies) should be less in the case of the ferric-chlorin derivative.

The near-infrared MCD spectra of the azide and cyanide adducts of ferric hydroperoxidase II, an iron-chlorin containing hemoprotein, have been reported to be at 1,350 nm and 1,600 nm respectively [3]. The results presented here clearly demonstrate that, as expected, these earlier observations are

Table 1
Electronic absorption and EPR characteristics of low-spin $Mb^+\{OEC\}$ derivatives

| Exogenous ligand | Absorption spectrum $\lambda_{max}$ (nm) [$\varepsilon(mM^{-1} \cdot cm^{-1})$] | EPR parameters ($g_z$, $g_y$, $g_x$) | |
|---|---|---|---|
| | | $Mb^+\{OEC\}$ | Sulfmyoglobin[a] |
| Hydroxide | 389 [120], 583<br>653, 704 | 2.46<br>2.27<br>—* | 2.44<br>2.20<br>1.88 |
| Azide | 402 [95], 589<br>626 | 2.60<br>2.26<br>1.77 | 2.61<br>2.23<br>1.80 |
| Cyanide | 409 [99], 578 | 2.63<br>2.44<br>1.71 | 2.65<br>2.43<br>1.65 |
| Imidazole | 403 [106], 586 | 2.58<br>2.41<br>—* | 2.57[b]<br>2.38<br>1.76 |

*Not reliably observed (broad and/or weak).
[a] Data taken from [10].
[b] Data for ferric-oxochlorin reconstituted cytochrome $b_5$, taken from [9].

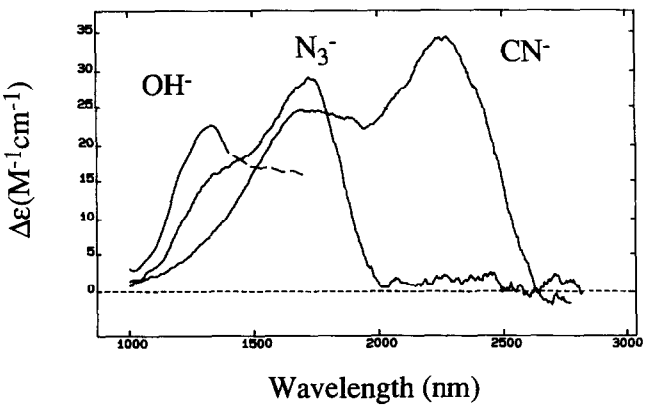


Fig. 2. Near-infrared MCD spectra of $Mb^+\{OEC\}$-adducts at 4.2 K and 5.0 T, 0.3 mm pathlengths and 12 nm maximum bandwidth. $OH^-$ complex: 0.27 mM heme, pD 10.5 in 50 mM CAPS, 50% (v/v) $d_6$-ethanediol, average of 9 spectra. $N_3^-$ complex: 0.88 mM heme, 156 mM sodium azide, pD 6.3 in 50 mM MES, 50% (v/v) $d_6$-ethanediol, average of 5 spectra. $CN^-$ complex: 0.74 mM heme, 13.2 mM sodium cyanide, pD 7.8 in 50 mM HEPES, 50% (v/v) $d_6$-ethanediol, average of 9 spectra.

inconsistent with histidine being the proximal ligand in hydroperoxidase II. However, the present near-infrared MCD data set for iron-chlorins is still too limited to allow the unambiguous identification of tyrosine as the proximal ligand species in this enzyme without access to other information.

It is also evident from the data of Table 2 that the differential extinction coefficients for the near-infrared transitions of the ferric-chlorin derivatives are at least four times less than those of the corresponding ferric-porphyrin transitions. This is in keeping with the intensities of low-spin hydroperoxidase II derivatives reported previously [3] and explained on the basis of the greater deviation from $D_{4h}$ symmetry exhibited by chlorin systems compared to porphyrins [12].

The present findings indicate that there are some important practical limitations to the application of near-infrared MCD spectroscopy in the assignment of axial ligands to iron-chlorins (and presumably other iron-hydroporphyrins). Since the unusual hemes of interest are normally found in hemoproteins which also contain iron-porphyrins [13], signals arising from the latter will tend to dominate the spectra because of their greater intensity. The substantial red shifts reported here for the $Mb^+\{OEC\}$ adducts compared to the metmyoglobin adducts (Table 2) suggest that the spectral characteristics of *some* ferric-chlorin derivatives will be resolved from the interfering signals of ferric-porphyrins and therefore, be diagnostically useful. However, in these cases, one is dealing with signals of such low intensity that they require accumulation of spectra at cryogenic temperatures in order that acceptable signal-to-noise be achieved. Furthermore, most of the useful signals appear to be located to longer wavelength than 2,000 nm, beyond the range of the vast majority of currently operating spectrometers [14].

Table 2
Comparison of the MCD characteristics of $Mb^+\{OEC\}$ and metmyoglobin derivatives

| | Low energy band (nm) [$\Delta\varepsilon$ $M^{-1} \cdot cm^{-1}$][a] | | |
|---|---|---|---|
| Exogenous ligand | *i* $Mb^+\{OEC\}$ | *ii* Metmyoglobin[b] | $\lambda_{ii}-\lambda_i$ ($cm^{-1}$) |
| Hydroxide | 1,330 [22] | 1,020 [100] | 2,280 |
| Azide | 1,730 [29] | 1,290 [130] | 1,970 |
| Cyanide | 2,270 [34] | 1,600 [590] | 1,840 |
| Imidazole | 2,340 [43] | 1,630 [220][c] | 1,860 |

[a] At 4.2 K and 5.0 T applied field.
[b] Data for horse metmyoglobin taken from [1].
[c] Data for soybean leghemoglobin, also from [1].

As the instrumentation and expertise required to perform the kind of MCD measurement described here is not widely available, Dawson and colleagues have suggested an alternate method to the assignment of axial ligands to iron-chlorins, using fingerprint comparisons of visible region MCD spectra recorded at ambient temperatures [15]. While interpretation of visible region electronic spectral data is subject to more ambiguity than is the case with near-infrared spectra [3], the alternate approach seems an entirely reasonable effort in view of the difficulties we now report.

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