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Identification of heme macrocycle type by near-infrared magnetic circular dichroism spectroscopy at cryogenic temperatures

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The electron paramagnetic resonance (EPR) and near-infrared magnetic circular dichroism (MCD) spectra of the azide and cyanide adducts of nitrimyoglobin and hydroperoxidase II from *Escherichia coli* have been measured at cryogenic temperatures. For the first time, ligand-to-metal charge-transfer transitions in the near-infrared have been observed for an Fe(III)-chlorin system. It is shown that near-ultraviolet-to-visible region electronic spectra of 'green' hemes such as these are an unreliable indicator of macrocycle type. However, the combined application of EPR and near-infrared MCD spectroscopies clearly distinguishes between the porphyrin-containing nitrimyoglobin and the chlorin-containing hydroperoxidase II.

Electron paramagnetic resonance; Magnetic circular dichroism; Catalase; Hydroperoxidase; Nitromyoglobin; Chlorin; Nitriheme

1. INTRODUCTION

The majority of hemoproteins, sometimes in addition to other non-heme cofactors, contain iron-protoporphyrin IX (i.e. protoheme) as the only kind of macrocyclic iron complex. However, a significant number of examples are now known in which some alternate heme structure is found [1]. A noteworthy feature of these 'unusual' hemes, is that where they occur, they are always observed to be the substrate binding prosthetic group, even if protoheme is also present. There is currently not enough data available on the physiochemical properties of the unusual hemes to explain why they have evolved to fulfill certain enzymatic functions, seemingly in preference to other cofactors. For example, *Escherichia coli* produces two catalases, hydroperoxidase I and hydroperoxidase II, with the latter containing an unusual heme as the only prosthetic group [2]. The structure of this heme has quite recently been shown to be a chlorin type system, with the identical core structure as heme *d* found in a terminal oxidase complex from the same bacterium, but as a different isomer [3].

Near-infrared magnetic circular dichroism (MCD) spectroscopy, in conjunction with electron paramagnetic resonance (EPR) measurements, has been shown to be a powerful method for studying low-spin ferric hemoproteins, particularly in the area of axial ligand assignment [4]. Until now, all substantiated cases of near-infrared MCD signals observed for hemoproteins have involved a fully unsaturated tetrapyrrole (i.e. porphyrin) ring. The present study was undertaken in order to determine the feasibility of extending this ligand assignment methodology to include chlorins and we now report the detection by MCD spectroscopy of near-infrared ligand-to-metal charge-transfer transitions for low-spin derivatives of hydroperoxidase II. In addition, and for purposes of comparison, we have also measured the near-infrared MCD and EPR spectra of the analogous low-spin derivatives of metnitrimyoglobin. Despite having split Soret bands in the absorption spectra of all its reported derivatives, suggestive of the presence of a chlorin ring, this green hemoprotein contains a porphyrin macrocyde [5]. The results provide an instructive lesson concerning the unreliability of near-ultraviolet-to-visible region electron absorption spectra as a 'fingerprint' of heme type.

2. EXPERIMENTAL

Previously published procedures were used to prepare hydroperoxidase II [2] and nitromyoglobin [5]. Heme *d* concentrations were determined for the uncomplexed hydroperoxidase II using $\varepsilon_{590} = 19$ $mM^{-1} \cdot cm^{-1}$ [6]. Nitriheme concentrations were determined using $\varepsilon_{618} = 16$ $mM^{-1} \cdot cm^{-1}$ and $\varepsilon_{615} = 13$ $mM^{-1} \cdot cm^{-1}$ for the azide and cyanide adducts of metnitrimyoglobin, respectively [7].

EPR spectra were obtained using a hybrid instrument consisting of a Varian E109E console, used to provide the field modulation to a Bruker B-E 25 magnet, with an ER 082 power supply and B-H 15 field controller, plus a Varian E102 microwave bridge. The spectrometer

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); MCD, magnetic circular dichroism.

was fitted with an Oxford Instruments ESR 900 liquid helium flow cryostat.

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 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 substracted from the spectrum.

3. RESULTS

In Fig. 1 are shown the X-band EPR spectra at 11 K of the azide and cyanide complexes of hydroperoxidases II (HPII) and metnitrimyoglobin (NMb$^{\bullet}$). The broken lines in Fig. 1B and C indicate where it has been necessary to subtract a background signal (arising from cavity contaminants) from the data and this has led to some variability in the results obtained between samples. The spectrum of metnitromyoglobin-azide (g_{xyz} = 2.77, 2.20, 1.73; Fig. 1A) is very like that observed [4] for normal metmyoglobin-azide (g_{xyz} = 2.77, 2.20, 1.73; Fig. 1A) is very like that observed [4] for normal metmyoglobin-azide (g_{xyz} = 2.79, 2.21, 1.73). Similarly, as far as can be ascertained given that only one g-value is readily determinable, the spectrum of metnitrimyoglobin-cyanide (g_z = 3.57; Fig. 1C) is very like that observed [4] for normal metmyoglobin-cyanide (g_{xyz} = 3.45, 1.89, 0.93). The spectrum of hydroperoxidase II- azide shows a small rhombic signal around g = 6 (Fig. 1B) indicating a minority high-spin species. The low- spin component (g_{xyz} = 2.38, 2.16, 1.80) is noticeably less rhombic than the corresponding metnitrimyoglobin-azide spectrum (Fig. 1A). The low-spin ferric signal of hydroperoxidase II-cyanide (Fig. 1D, g_{xyz} = 2.34, 2.23, 1.79) is also clearly less rhombic than the analogous metnitrimyoglobin derivative (Fig. 1C). The spectra of Fig. 1B and D are quite in keeping with the results expected for low-spin FE(III)-chlorins [8].

In Fig. 2 are shown the near-infrared MCD spectra of the azide (1.27 μm low energy maximum) adducts of metnitrimyoglobin at 4.2 K and 5.0 T. These spectra are both qualitatively and quantitatively very similar to the previously reported spectra of the analogous normal metmyoglobin derivatives [9], but the present data sets appear blue shifted by some 120 and 140 cm^{-1}, respectively. The near-infrared MCD spectra of the corresponding hydroperoxidase II derivatives at 2.0 K and 7.0 T are presented in Fig. 3. The native hydroperoxidase II (high-spin ferric system) contained no detectable bands between 800 and 2,500 nm (data not shown). The azide adduct has a distinct low energy maximum at about 1.35 μm. The cyanide adduct consists of at least two poorly resolved transitions of similar intensity in this region, but 1.6 μm is clearly a reasonable estimate of the lower energy one and will certainly suffice for present purposes. Note that when the recording conditions are taken into account, the signals of Fig. 3 are

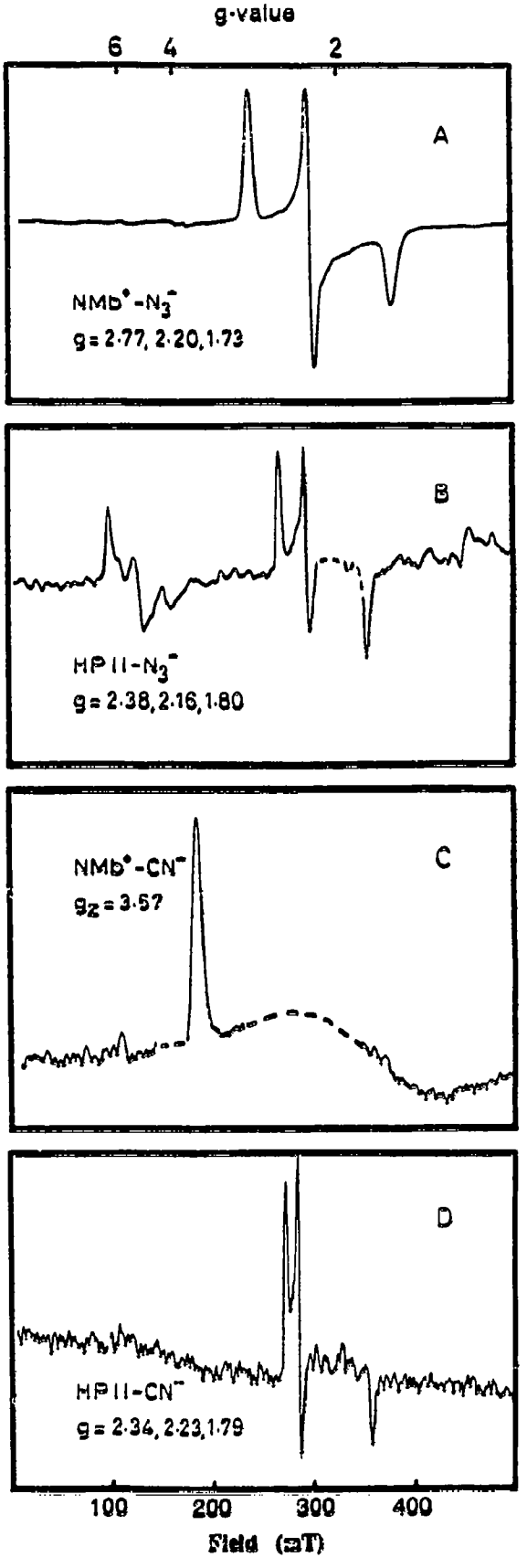

Fig. 1. X-band EPR spectra at 11 K. Recording conditions: 2 mW microwave power, 10 G modulation amplitude, 8 min scan time, 1 s time constant. (A) 1.5 mM metnitrimyoglobin-azide, pH 7.7, in 20 mM HEPES, 2 mM in EDTA; 9.21 GHz, 3.2×10^4 amplifier gain. (B) 0.2 mM hydroperoxidase II-azide, pD 8.4, in 9 mM HEPES, 1 mM in EDTA, 54% (v/v) d_6-ethanediol; 9.20 GHz, 2×10^5 amplifier gain. (C) 1.9 mM metnitrimyoglobin-cyanide, pH 7.7, in 20 mM HEPES, 2 mM in EDTA; 9.21 GHz, 2×10^3 amplifier gain. (D) 0.2 mM hydroperoxidase II-cyanide, pD 8.4, in 9 mM HEPES, 1 mM in EDTA, 54% (v/v) d_6-ethanediol; 9.22 GHz, 2.5×10^4 amplifier gain.

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about an order of magnitude weaker than those of a typical low-spin Fe(III)-porphyrin system like the metnitrimyoglobin-azide spectrum of Fig. 2. Furthermore, the sample concentration used to produce the spectra of Fig. 3 was 0.2 mM. Until now, it has commonly been the practice to use sample concentrations of 2–3 times this value in near-infrared MCD studies (e.g. [9]). Thus, the present hydroperoxidase II spectra represent signals that are at least twenty-times weaker than those arising from low-spin ferric hemoproteins routinely studied by near-infrared MCD spectroscopy in the past.

4. DISCUSSION

It has previously been quite-well documented that the energies of the porphyrin (π)-to-ferric (t_{2g}) ion charge-transfer transitions of low-spin ferric hemoproteins, measured by near-infrared MCD spectroscopy, are sensitive to changes in the ligand field experienced by the heme iron, and thereby provide a means of axial ligand assignment. More recently, Gadsby and Thomson have shown [4] that there is, in fact, a linear correlation between the low energy maximum in the near-infrared spectrum and the energy of the ferric d_{yz} hole relative

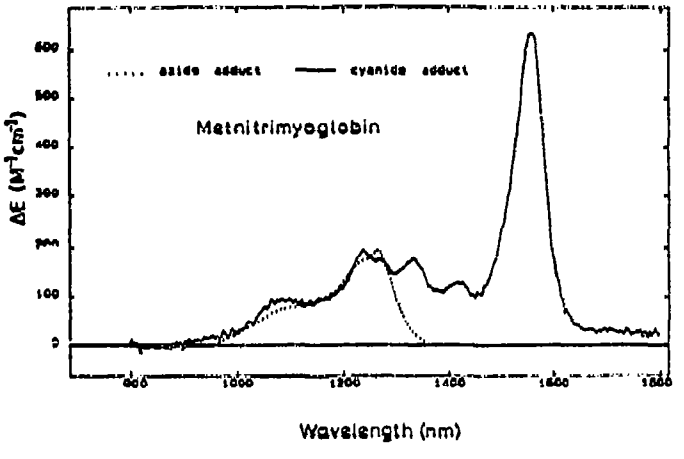

Fig. 2. Near-infrared MCD spectra of metnitrimyoglobin derivatives at 4.2 K and 5.0 T applied field. 0.18 mM protein, pD 8.4, in 9 mM HEPES, 1 mM in EDTA, 54% (v/v) d_6-ethanediol; azide adduct (....), cyanide adduct (—); 1.0 mm pathlength, 6 nm maximum spectral bandwidth, single spectral scans.

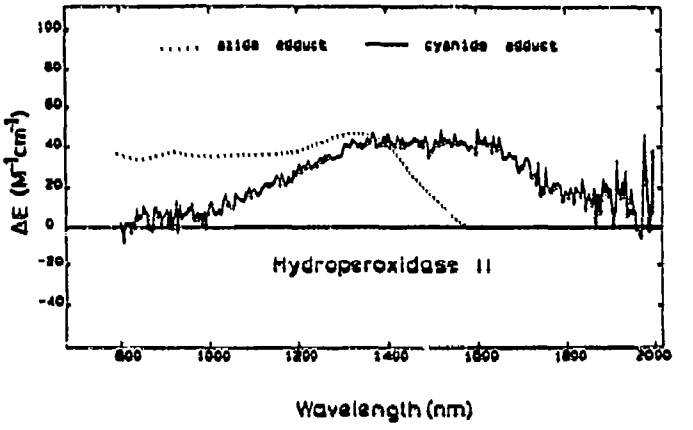

Fig. 3. Near-infrared MCD spectra of hydroperoxidase II derivatives at 2.0 K and 7.0 T applied field. 0.2 mM protein, pD 8.4, in 9 mM HEPES, 1 mM in EDTA, 54% (v/v) d_6-ethanediol; azide adduct (....), cyanide adduct (—); 1.0 mm pathlength, 12 nm maximum spectral bandwidth, data are the average of 2 spectra.

to the baricenter of the t_{2g} 3d subshell. These authors established this empirical relationship using more than thirty derivatives of metmyoglobin, metleghemoglobin and ferricytochromes. The present metnitrimyoglobin-azide data are fully consistent with these previous results. The metnitrimyoglobin-cyanide data are more difficult to compare with the earlier results using Gadsby and Thomson's relationship, because evaluation of the energy of the ferric d_{yz} hole requires knowledge of all three principal g-values for the system [10] and only one has been reliably determined (Fig. 1C).

More interestingly though, we note that the current results for low-spin ferric hydroperoxidase II derivatives are not entirely consistent with the previous observations for Fe(III)-porphyrins. That is, if following appropriate manipulation of the data, these hydroperoxidase II results are plotted on Fig. 5 of Gadsby and Thomson paper [4], where their parameterizations are summarized, it is immediately obvious that the present Fe(III)-chlorin parameters lie a little outside the range of values delineated by the earlier findings. At this juncture, further discussion is probably unwarranted, since it would be based on only two data points. Suffice it to say that the current hydroperoxidase II results of Fig. 3 are at least encouraging insofar as they show that the position of the low energy maximum is sensitive to the nature of the axial ligands. However, without many more well-characterized examples of Fe(III)-chlorins with various axial ligands, we cannot confirm the recent suggestion by Dawson et al. [11] that the proximal ligand to iron in hydroperoxidase II is tyrosinate. Efforts are presently underway to extend the near-infrared MCD study to some Fe(III)-chlorin model compounds.

The electronic absorption spectra of nitrimyoglobin derivatives exhibit a split Soret band and in addition, a relatively intense α band in some cases, more like

chlorin-containing systems than other Fe(III)-porphyrins [5]. The Soret band is a degenerate transition under the pseudo-tetragonal (D_{4h}) symmetry usually assumed for porphyrins [12]. It follows that anything causing a lifting of the degeneracy may produce a split Soret. It has now become clear that in hemoproteins at least the following three factors may produce this effect: (i) the presence of a reduced porphyrin ring, e.g. a chlorin; (ii) the combined effects of certain axial ligands, especially mercaptide [13]; (iii) extension of the macrocycle conjugation by some exocyclic substituents like the nitrovinyl group of nitriheme. Furthermore, of course, this leads to the conclusion that the electronic absorption spectra of hemes in the near-ultraviolet-to-visible region can be wholly unreliable indicators of macrocycle type, since they cannot easily distinguish between these possibilities.

On the other hand, based on the information documented herein, the combined application of EPR and near-infrared MCD spectroscopies to low-spin ferric derivatives appears to unambiguously distinguish between porphyrins and chlorins. In particular, near-infrared MCD signals of similar intensity to those of Figure 2, which are detectable in experiments at room temperature, are indicative of a porphyrin ring being present. So, for example, the intense near-infrared transitions reported by Eglinton et al. [14] for the cyanide adducts of myeloperoxidase do not support the presence of a chlorin ring, but are more in keeping with the suggestion of Sono et al. [15] that the green heme of this enzyme contains a porphyrin macrocycle.

Low energy charge-transfer transitions, like those of Figs. 2 and 3, have not yet been detected for hemes with more reduced macrocycles than chlorins. Consequently, it is at this time unclear if the Fe(III)-chlorin spectra of Fig. 3 are distinct from, say, those of low-spin Fe(III)-isobacteriochlorin derivatives. However, this *is* expected to be the case. It has recently been argued that the near-infrared MCD transitions observed for low-spin hemes are of maximum intensity when the hole in the t_{2g} 3d subshell of the ferric iron is equally divided between the d_{xz} and d_{yz} orbitals, but drops to zero if the hole becomes localized in only one [16]. Naively then, the more reduced the porphyrin ring, the greater are the chances that the ferric ion d_{xz} and d_{yz} orbitals will have significantly different energies, leading to decreased MCD intensity in the following anticipated order: Fe(III)-porphyrin > Fe(III)-chlorin > Fe(III)-isobacteriochlorin. These expectations remain to be experimentally verified.

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