Low-temperature EPR and near-infrared MCD studies of highly anisotropic low-spin ferrihaem complexes

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Received 7 January 1986

The low-temperature EPR spectra and near-infrared magnetic circular dichroism (MCD) spectra are reported of the bis complexes of imidazole, 1-methylimidazole, 4-methylimidazole, 1,2-dimethylimidazole and 2-methylimidazole with Fe(III) octaethylporphyrin (OEP) in the mixed organic solvent dichloromethane/diethyl ether. It is shown that the latter two have highly anisotropic EPR spectra characteristic of the low-spin ferric state. The optical charge-transfer bands have an unusually high MCD intensity, with a narrow linewidth. It is proposed that this feature is typical of bis-histidine-ligated haem in cytochromes with sterically strained coordination and may be used as diagnostic of such a conformation. The EPR and near-infrared MCD spectra of the bis-butylamine complex of Fe(III(OEP are also reported. It is shown that bis-amine-ligated haem may be clearly distinguished from sterically hindered bis-imidazole (bis-histidine) by near-infrared MCD spectroscopy whereas the assignment is uncertain using EPR spectroscopy alone.

EPR MCD Methylimidazole Fe(III) octaethylporphyrin

1. INTRODUCTION

EPR spectroscopy has been widely used for the characterisation of low-spin ferric haem complexes at low temperature [1,2]. These studies have allowed the identification of the axial ligation in a range of haem proteins by comparison between haem model complexes and proteins of known axial ligation [2,3]. However, the EPR spectra reported for a number of haem proteins such as cytochromes *b* of the inner mitochondrial membrane are unusual in appearance [3]. Typical low-spin ferric haem EPR spectra consist of 3 anisotropic resonances, $g_z = 3.3–2.8$, $g_y = 2.3–1.8$ and $g_x = 1.8–1.0$. The spectra of the *b* cytochromes exhibit very large *g*-value anisotropy. Thus g_z ranges between 3.7 and 3.5 and g_y and g_x are generally unobservable. The peak observed has an asymmetric line shape often with a folded appearance at low field [5,6]. These observations originally led to the suggestion of ligation of the haem group by two lysine amine side chains in these proteins [4]. However, recently a number of reports have shown that this type of highly anisotropic EPR spectrum can be reproduced by model haem compounds ligated by imidazoles substituted at the 2-position. Consequently it has been proposed that the axial coordination of the mitochondrial cytochromes *b* involves bis-histidine subjected to a steric strain [7,8]. Since highly anisotropic EPR spectra have also been produced from bis-amine derivatives of protohaem [4], ligand assignment using EPR spectroscopy as a criterion alone remains uncertain.

Near-infrared magnetic circular dichroism (MCD) provides a complementary means for the identification of the axial ligands and the spin state of the Fe^{3+} of haem complexes [9]. In the wavelength range 700–2000 nm low-spin ferric haems display porphyrin-to-Fe(III) charge-transfer transitions with energies which are strongly dependent upon the nature of the axial ligation [10]. These transitions can be observed by absorption spectroscopy but are more readily detected by MCD spectroscopy [9–11].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

We show here that the ligation of haem by sterically hindered imidazoles has a marked effect upon the near-infrared MCD spectrum and that ligation of haem by bis-imidazole and bis-amine can be readily distinguished by MCD spectroscopy in the near-infrared region.

2. MATERIALS AND METHODS

Octaethylporphyrinato iron(III) [Fe(III) OEP(Cl)] was purchased from Strem Chemicals. Imidazole, 1-methylimidazole, 2-methylimidazole, 4-methylimidazole, 1,2-dimethylimidazole and *n*-butylamine were purchased from Aldrich. Solutions of the complexes were prepared by dissolving the porphyrin in dichloromethane/diethyl ether (2:1, v/v) and then adding the required base until no further change was observed as judged by absorption spectroscopy at room temperature. EPR spectra were recorded at 10 K with an ER-200D (Bruker) spectrometer using a flow cryostat (ESR-9, Oxford Instruments). MCD spectra were recorded at 4.2 K on a previously described home-built instrument fitted with a superconducting solenoid (maximum field 5.0 T) [11]. Intensities of MCD spectra are reported in units of $\Delta\epsilon$ where $\Delta\epsilon = \epsilon_L - \epsilon_R$, the molar absorption coefficients for left- and right-circularly polarized light, respectively. The concentrations of the complexes used to calculate $\Delta\epsilon$ values have been corrected for the contraction in the volume of the solvent on cooling from room temperature to 4.2 K. The correction factor has been estimated at 0.67. The MCD spectra are not normalized to unit magnetic field.

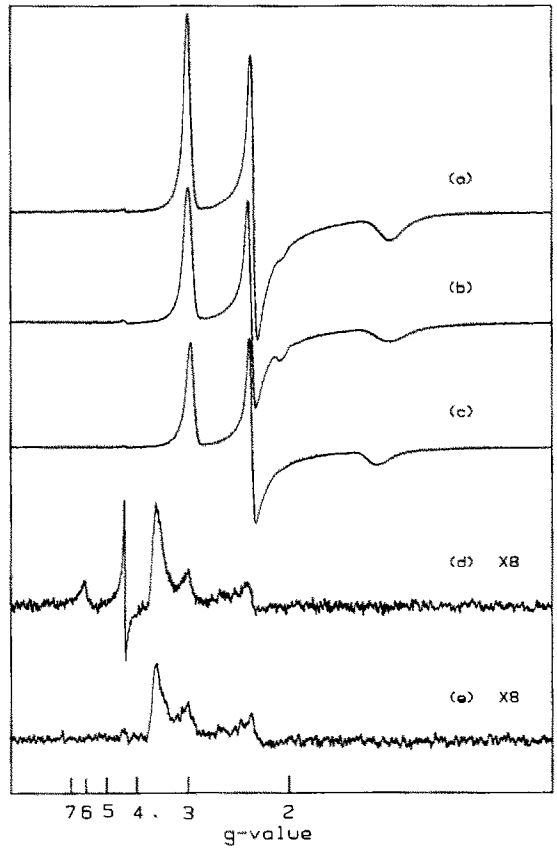

Fig.1. EPR spectra of ferric octaethylporphyrin-imidazole complexes in dichloromethane/diethyl ether (2:1, v/v) at 10 K, frequency 9.4 GHz and 6.3 G modulation amplitude. Complex: (a) imidazole, 2.58 mM, 2 mW; (b) 1-methylimidazole, 2.58 mM, 2 mW; (c) 4-methylimidazole, 2.58 mM, 2 mW; (d) 1,2-dimethylimidazole, 5.01 mM, 160 mW; (e) 2-methylimidazole, 2.58 mM, 160 mW.

3. RESULTS AND DISCUSSION

The EPR spectra of Fe(III) recorded in the presence of a series of imidazoles are shown in fig.1. The spectra presented for the haem coordinated with 1-methyl- and 4-methylimidazole, as well as imidazole itself, are typical for bis-imidazole haem models, and also for proteins with bis-histidine ligation such as cytochrome b_5 [12]. In the presence of 2-methylimidazole and 1,2-dimethylimidazole the haem exhibits much weaker EPR signals which are detected only at higher microwave powers. The bis(2-methylimidazole) complex is completely low-spin, the highly anisotropic majority species exhibiting an asymmetric g_z resonance at 3.53. g_y and g_x are not observed. A minority species with g values of 2.98 and 2.23 is also present. The bis(1,2-dimethylimidazole) complex also exhibits a highly anisotropic asymmetric majority species with $g_z = 3.52$ and a minority species at $g = 3.0$ and 2.26. Also present in this spectrum is a small amount of high-spin haem at $g = 6$, and adventitious iron at $g = 4.3$. The highly anisotropic EPR signals are much more difficult to saturate with increase in microwave power than those of typical low-spin ferric haems. The EPR spectra shown here are very similar to those reported for imidazole complexes of haemin chloride in dimethyl sulphoxide [7] except for the 2-meth-

ylimidazole complexes which have slightly larger values of g_z. This dramatic change in the appearance of the EPR spectra upon methyl substitution at the 2-position of imidazole, and the close similarity of these spectra to those of the mitochondrial *b*-type cytochromes, have led to the suggestion that these complexes may be good structural models for cytochrome *b*. The spectral properties may result from an unusual rotational orientation about the normal to the haem plane of the axial ligands under the influence of the non-bonded steric interaction between the 2-methyl group of the imidazole ring and a nitrogen atom of the porphyrin ring [7,8,13]. Since binding of imidazole to ferric haem is via the N-3 position, it might have been expected that the 4-methylimidazole complex would also exhibit a highly anisotropic EPR spectrum. However, 4-methylimidazole is tautomeric and exists as a mixture of 4- and 5-methylimidazole in solution [14]. The observation of a normally anisotropic EPR for this derivative suggests that the complex formed is bis(5-methylimidazole).

These reports and the remaining difficulty in distinguishing between bis-imidazole- and bis-amine-liganded haem by EPR led us to study a number of these haem models by near-infrared MCD spectroscopy. Presented in fig.2 are the near-infrared MCD of 5 imidazole complexes of

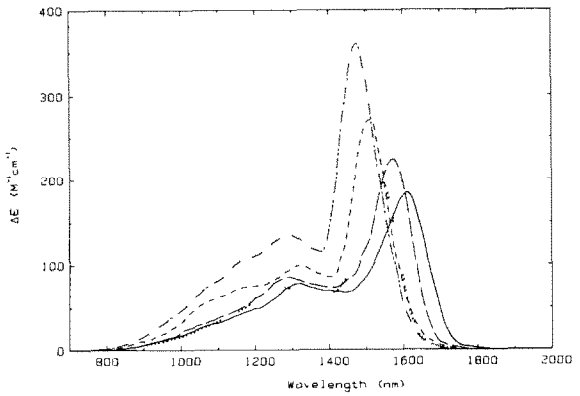

Fig.2. Near-infrared MCD spectra of ferric octaethylporphyrin-imidazole complexes in dichloromethane/diethyl ether (2:1, v/v) at 4.2 K and 5 T. Complex: (-----) imidazole, 600 μM; (——) 1-methylimidazole, 600 μM; (···) 4-methylimidazole, 600 μM; (– – –) 1,2-dimethylimidazole, 400 μM; (–·–·–) 2-methylimidazole, 600 μM.

Fe(III) OEP. The MCD spectra of the imidazole, 1-methylimidazole and 4-methylimidazole complexes are typical for bis-imidazole-ligated haem, consisting of a positive peak in the region 1540–1610 nm with an intensity of approx. 200 $mM^{-1} \cdot cm^{-1}$, at 4.2 K and 5 T and a shoulder at ≈1300 nm [15]. The MCD spectral features of the 2-methylimidazole complexes are similar. However, the peak positions are shifted slightly to higher energy. Also, the signals are considerably more intense and sharper in appearance. In the case of the 2-methylimidazole complex the peak is almost twice as intense as for the unhindered imidazole complexes. Although the 1,2-dimethylimidazole complex is apparently not quite as intense this is due to the presence of approx. 10–20% high-spin haem in this sample.

The shift in peak position points to a slight decrease in ligand field strength at the iron, possibly reflecting a lengthening of the iron-to-imidazole bond caused by the steric effect of the 2-methyl group. The intensification of the MCD spectrum appears to be correlated with the degree of anisotropy of the EPR signals. The optical transitions giving rise to the MCD spectrum are porphyrin orbitals (a_{1u}^{π},a_{2u}^{π}) to Fe(III) orbital (t_{2g}^{q}) charge-transfer (CT) transitions [15]. Only the a_{1u},$a_{2u} \longrightarrow d_{xz,yz}$ transitions carry intensity. Hence the intensity of CT transition will be reduced if the hole orbital in the Fe(III) ground state contains some d_{xy} character. It is noteworthy that in the case of bis-histidine complexes with highly anisotropic EPR spectra the hole orbital is thought to be almost purely axial, with little contribution from d_{xy} [8]. Theoretical work is in progress to make this conclusion quantitative (Gadsby, P.M.A. and Thomson, A.J., unpublished).

Whatever the reasons underlying this striking correlation the high MCD intensity for the infrared CT bands has proved useful in the detection and identification of the cytochrome *b* of formate dehydrogenase (*Pseudomonas aeruginosa*) particularly as the increased MCD intensity parallels a substantial decrease in the EPR intensity giving some difficulty in detection (Godfrey, C. et al., unpublished).

The EPR spectrum of the bis(*n*-butylamine) complex of Fe(III) OEP is shown in fig.3a. This exhibits a majority species showing a highly anisotropic spectrum with $g_z = 3.69$, $g_y = 1.69$ and

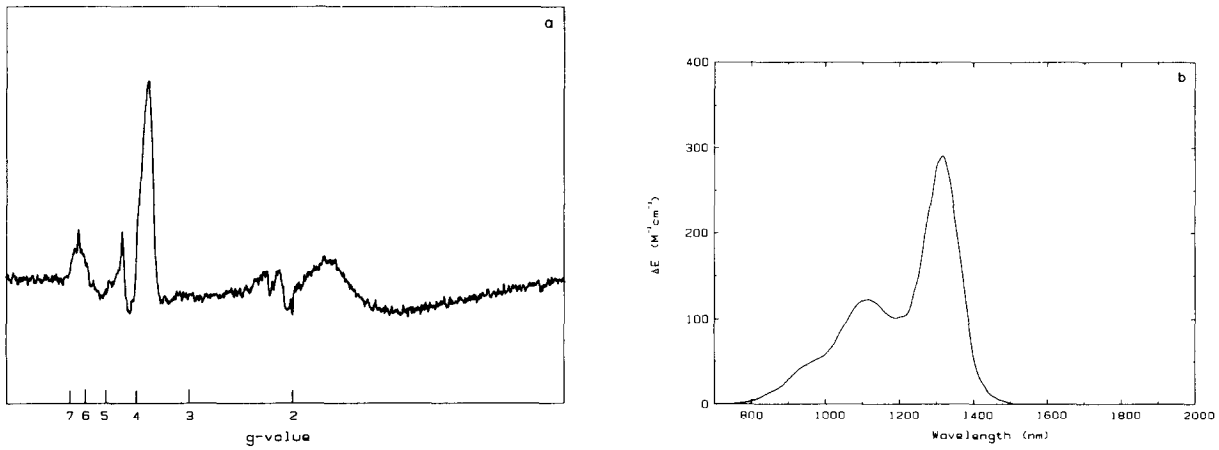

Fig.3. Ferric octaethylporphyrin–*n*-butylamine complex in dichloromethane/diethyl ether (2:1, v/v, 1.78 mM). (a) EPR spectrum, 10 K, 2 mW, 9.4 GHz, 6.3 G modulation amplitude; (b) MCD spectrum, 4.2 K, 5 T.

g_x not being observed. (A referee has pointed out that this EPR spectrum is not well-described by the t_{2g} hole model of Griffith [16] since $g_z^2 + g_y^2 \approx 16.5$. The sum of the squares of all the g values of a pure t_{2g} hole cannot exceed 16. This is equivalent to saying that the complex is not well-described as a pure low-spin ferrihaem.) Although this spectrum is highly anisotropic the intensity is greater than that of the 2-methylimidazole complexes. The corresponding near-infrared MCD spectrum is shown in fig.3b. As expected, from the form of the EPR spectrum the MCD is intense with $\Delta\epsilon$ 300 $M^{-1} \cdot cm^{-1}$ at 4.2 K and 5 T. However, the peak is located at 1320 nm. The blue shift of the peak, compared to the bis-imidazole complexes, reflects a decrease in the axial ligand field strength in the bis-amine complex. This is the first report of the MCD of a bis-amine haem complex and should thus provide a sensitive means of detecting this type of ligation in ferric haemoproteins. Only one other type of haemoprotein ligation has been observed with an MCD peak in the region of 1320 nm, namely, the histidine-histidinate ligate of lactoperoxidase [17]. However, this type of ligation is readily distinguished from that of bis-amine both by the EPR spectrum, which is not highly anisotropic, and also by the near-infrared MCD peak, which is of lower intensity.

In conclusion, the near-infrared MCD of haem complexes showing highly anisotropic EPR spectra have been shown to be considerably more intense than commonly observed for low-spin ferric haems. Thus, near-infrared MCD appears to provide a sensitive and useful means of characterising this type of haem coordination. Finally, although bis-(hindered imidazole) and bis-amine haem complexes can both produce highly anisotropic EPR spectra, the ligand field strength at the ferric iron is sufficiently different for these two types of axial ligation to be distinguished by near-infrared MCD.

ACKNOWLEDGEMENTS

Thanks are due to the SERC and the Royal Society for grants in support of this work.

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