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Identification of the imidazolate anion as a ligand in *met*-myoglobin by near-infrared magnetic circular dichroism spectroscopy

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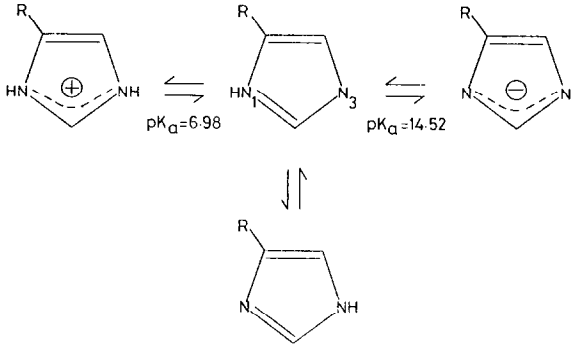
The near-infrared (700–1900 nm) magnetic circular dichroism (MCD) spectra of a horse-heart metmyoglobin–imidazole complex have been measured as a function of pD between 9.1 and 12.2 at room temperature. Two low-spin ferric haem complexes with MCD peaks at 1600 and 1350 nm, respectively, interconvert with an apparent pK_a of just above 11.0. Since this process has been identified with the deprotonation of the added imidazole ligand at N-1 we identify the species showing its main peak at 1600 nm as the histidine–imidazole complex and that at 1350 nm as the histidine–imidazolate form of the haem. This study points to the value of near infrared MCD in discriminating clearly between these two species.

Haemoprotein *Metmyoglobin* *Magnetic circular dichroism* *Imidazolate anion* *Ligand-binding*

1. INTRODUCTION

Imidazole (R=H) (imid) is a di-basic ligand with pK_a values, in water at 25°C, of 6.98 and 14.52 [1]. The neutral form exists in aqueous solution as a tautomer with an equilibrium constant equal to 1 [2].



For histidine ($R=CH_2 \cdot CH(NH_2) \cdot COOH$) in aqueous solution the true form of the tautomeric pair is distinguishable [3]. The tautomer which dominates depends entirely upon the conditions, that is, whether in solution or the solid state and the pH value [2]. The pK_a-values are shifted by complexing the N-3 position in the case of histidine to the ferric ion in haemoproteins [1]. The histidyl (hist) residue is the proximal ligand for haem iron in a wide variety of haemoproteins but is also often an invariant residue of functional significance within the haem pocket (known as the distal histidine). In some proteins this latter group can coordinate the haem iron to generate a hist–hist complex. There has been considerable interest in being able to determine the state of protonation of coordinated imidazole groups, that is, whether under a given set of pH conditions the ligand is in the neutral ($imid^0$) or the mono-anionic ($imid^-$) form [4–6].

Optical spectra of the imidazole complex of sperm-whale metmyoglobin (met.Mb) have shown that the complex titrates with pK_a 10.45 at 25°C [1]. This process was ascribed to the loss of a proton from the N-1 group of the added imidazole ligand. Apparently complexation of imidazole via N-3 to ferric haem lowers the pK_a^2 of the N-1 position by ~4 units. The process observed was not a simple one since other species such as the hydroxyl ligand are present at high pH values. A similar

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study was subsequently carried out of the imidazole (and other triazole) complex of haemoglobin from *Chironomus plumosus* which has a high affinity for added imidazole ligand [7]. A similar lowering by ~4 units of the pK_a^2-value of the coordinated base was observed [7].

Because there are a number of titrable residues present in a complex protein the measurement of a pK_a-value does not furnish proof of the nature of the group being titrated, especially if the pK_a determined is at a previously unrecognised value. A spectroscopic probe is required which is capable of distinguishing between $imid^0$ and $imid^-$ and, hence, $hist^0$ and $hist^-$ as ligands of haem iron. It has been proposed that the electron paramagnetic resonance (EPR) spectra are distinctive [6]. Peisach and co-workers studied the EPR of a bis-imidazole–hemin complex in solution and in a melt of NaOH in order to generate species representative of a bis–$imid^0$ and an $imid^0$–$imid^-$ haem complex[4] The *g*-factors reported were 3.02, 2.22, 1.65 and 2.8, 2.22, 1.81, respectively. In [8] imidazole and imidazolate complexes of Fe(III) TPP (where TPP = tetraphenylporphinato) were identified and EPR spectra recorded. It is suggested that the species can be distinguished by classifying the complexes by their locations on a plot of rhombicity *vs* tetragonality, both parameters being derived from the EPR spectra. This procedure (originally from [4]) groups complexes with ligands in common into clustered regions of the plot. However, protein-bound haem complexes of imidazole have *g*-factors which vary over a range. There is some uncertainty in assignment therefore unless a titratable proton can be observed. Clearly it is desirable to have available a second spectroscopic probe, especially one which can make a diagnosis at room temperature.

We show here that the near infrared magnetic circular dichroism (MCD) spectra of the hist–$imid^0$ and hist–$imid^-$ complexes of protohaem IX are quite distinctive and provide a useful means of identifying these species. Low-spin ferric haems have charge-transfer transitions, of the type porphyrin-to-Fe(III), whose energies lie in the wavelength range 800–2000 nm and with energies dependent upon the nature of the axial ligand pair which bind the ferric ion [9,10]. We show here that hist–$imid^0$ and hist–$imid^-$ generate sufficiently different field strengths at the Fe(III) ion to give charge-transfer bands peaking at ~1600 nm and ~1350 nm, respectively. We have used this observation to diagnose the presence of the species hist–$imid^-$ as a ligand set in oxidised leghaemoglobin *a* imidazole complex and hist–$hist^-$ in oxidised lactoperoxidase [11,12].

2. MATERIALS AND METHODS

Horse-heart metmyoglobin (type III, Sigma) and imidazole (grade 1, Sigma) were dissolved in buffers made from AnalaR glycine (BDH), NaOD (Aldrich) in D_2O (Aldrich). MCD spectra were recorded on a home-built instrument, previously described, fitted with a superconducting solenoid, maximum field 4.7 Tesla (T) [13]. EPR spectra were on an ER-200D (Bruker) spectrometer using a flow cryostat (ESR-9, Oxford Instr.).

pD-values quoted are as measured and are not corrected for the K_W of D_2O. MCD spectra are expressed in units of $\Delta\epsilon = \epsilon_L - \epsilon_R$, where ϵ_L and ϵ_R are the molar extinction coefficients for left and right circularly polarised light, respectively, and $\Delta\epsilon$ values are normalised to unit Tesla (T).

3. RESULTS

The near infrared MCD spectra of horse-heart met.Mb in the presence of added imidazole over pD 9.1–12.2 are shown in fig. 1. Three distinct species, which vary in proportion as the pD is raised, can be observed in these spectra. Assignment of these species is assisted by the MCD spectra of met.Mb in the absence of added imidazole at pD 10.4 and in the presence of 300-fold molar excess of imidazole ligand at pH 8.6 (fig. 2). In the absence of added imidazole the hydroxyl ion is the ligand of the haem at pD 10.4. The MCD spectrum of this species has positive peaks at ~780 nm and 1050 nm and a sharp negative trough at 850 nm [10,14]. This results from a mixture of high- and low-spin species of the hydroxy-complex at room temperature. The positive peak at 1050 nm which dominates the low-temperature (4.2 K) MCD spectrum [15] arises from the low-spin ferric haem species whereas the sigmoidal-shaped curve with a crossing point at ~820 nm is due to the high-spin component [10,14]. In the absence of added imidazole these hydroxy species are the only ones evident in the MCD spectrum of met.Mb above

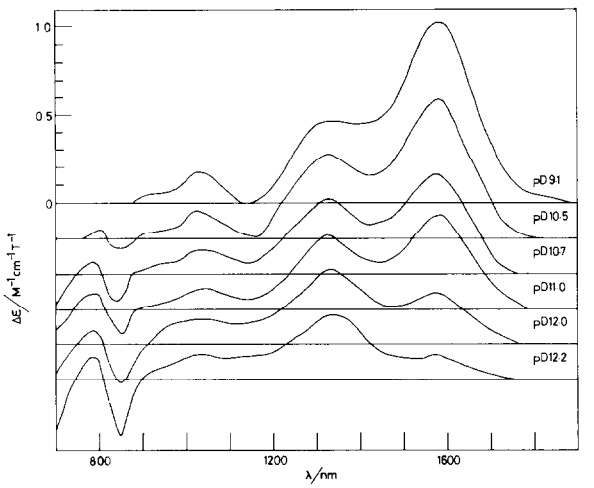


Fig. 1. Near-infrared MCD spectra of metmyoglobin–imidazole compound at various pD values. Buffer–glycine–NaOD (0.05 M); pathlength 5 mm; magnetic field 4.7 T, pD 9.1, 10.5, 11.0, 12.0; 0.6 mM met.Mb; met.Mb–imid = 300. pD 10.7, 12.2; 0.52 mM met.Mb; met.Mb–imid = 400. Room temperature.

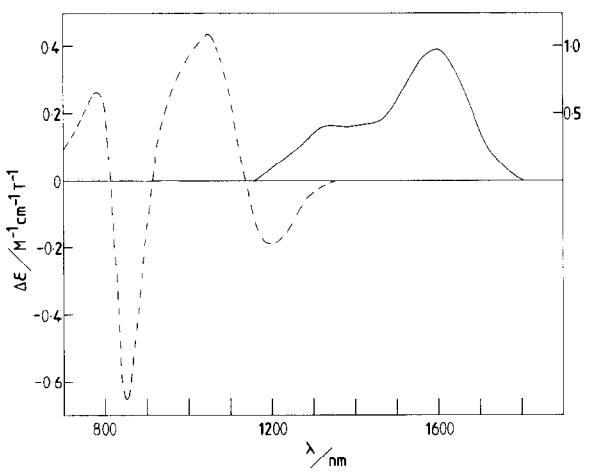


Fig. 2. Near-infrared MCD spectra of: metmyoglobin (pD 10.4) in glycine–NaOD buffer, plus deuterated ethane-diol (50%, v/v), protein 1.3 mM, pathlength 1 mm (———) (left scale); and metmyoglobin–imidazole (pD 8.4) in phosphate buffer, protein 0.5 mM, imidazole 150 mM, pathlength 5 mm (——) (right scale); field 4.7 Tesla; room temperature.

pD 10. The concentration of these species is reduced as the imidazole:haem ratio is increased from 300 to 900:1.

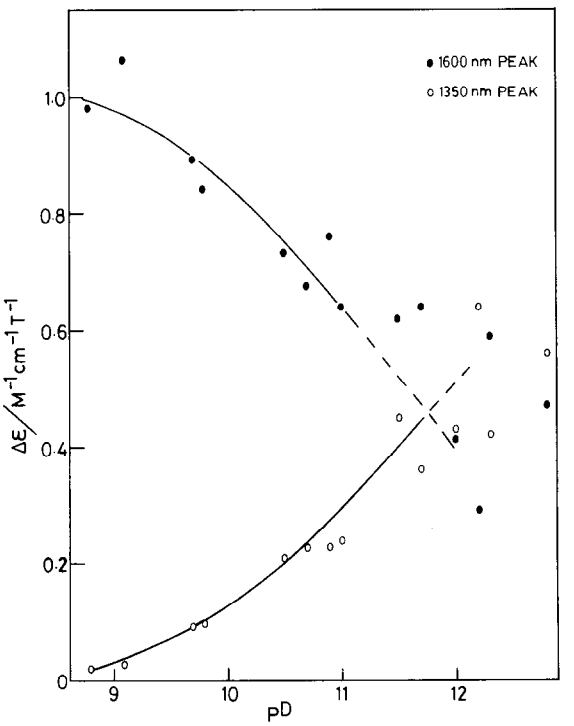


Fig. 3. A plot of the intensities of the MCD peaks at 1350 nm (○) and 1600 nm (●) against pD value for the species in fig. 1. The values have been corrected for the presence of hydroxy-met.Mb determined from the intensity of the MCD trough at 850 nm.

The second species which is readily identified in the set of spectra in fig. 1 is due to the imidazole complex of haem. The MCD spectrum consists of positive peaks at ~1350 and 1600 nm and arises from the low-spin ferric haem hist–imid^0 complex [10]. As the pD-value is raised the intensity of the peak at 1600 nm is steadily reduced. This is due both to the formation of hydroxy-haem but also to the presence of a new species with a positive MCD peak at 1350 nm and a poorly resolved shoulder at ~1150 nm. Fig. 3 gives a plot of the disappearance of the species characterised by the 1600 nm MCD peak. The points are corrected for the loss of available haemoprotein caused by the competition from the hydroxy species. The negative trough at 850 nm was used to estimate the amount of hydroxy-species present given the intensity of the fully-formed species, fig. 2.

The third species apparent in fig. 1 gives rise to a positive MCD peak at ~1350 nm. The species

with a positive peak at 1600 nm also has a second peak at 1350 nm. However, at pD 12.2, the concentration of this species, as indicated by the intensity of the peak at 1600 nm is low and it clearly contributes little to the MCD spectrum in the region of 1350 nm. Fig. 3 shows the appearance of the third species at 1350 nm. The MCD intensity at 1350 nm is plotted against pD value, and has been corrected for the contribution from the hist–imid^0 complex at this wavelength. The interconversion of the two species as a function of pD is demonstrated by fig. 3. The pK_a value cannot be accurately estimated but is just above 11.0. The data quality is poor above pD ~ 11.0 because the concentration of the hydroxy species rises rapidly with pD. If the imidazole:haem ratio is raised too high then protein denaturation takes place. A pK_a value of 10.45 was determined for met.Mb–imidazole complex in H_2O [1]. Applying the correction for the K_W of D_2O we take our estimate of the pK_a to be in reasonable agreement with that in [1]. We therefore assign the species with a positive MCD peak at 1350 nm to the imidazolate (imid$^-$) complex of met.Mb; that is, to protohaem coordinated imid$^-$–hist^0. This species is never fully formed under our experimental conditions, but we estimate from fig. 3 that the $\Delta\epsilon$ value at 1350 nm of this species is close to $1.0\,M^{-1}.cm^{-1}.T^{-1}$.

Table 1

EPR data for low-spin ferric haem imidazole complexes

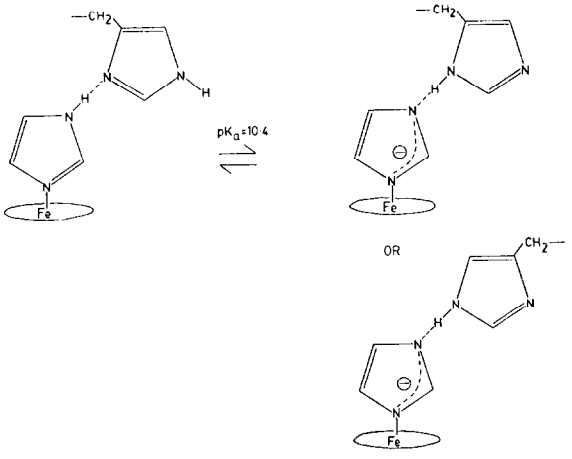
Complex	EPR g-values	[Ref.]
Hist–imid^0		
met.Mb–imidazole pD 9.6	2.93, 2.22, 1.52	[Here]
Fe TPP (imidazole)$_2$Cl	2.92, 2.30, 1.56	[8]
Fe protoporphyrin IX –bis-imidazole	3.02, 2.24, 1.51	[4]
Cytochrome b_5	3.03, 2.23, 1.43	[17]
Histi–imid$^-$		
met.Mb–imidazole pD 12.1	2.80, 2.25, 1.67	[Here]
Fe TPP (imidazole) (imidazolate)	2.73, 2.28, 1.74	[8]
Fe protoporphyrin IX–bis-imidazole + NaOH	2.78, 2.26, 1.72	[4]

The EPR spectra have also been determined over a similar range of pD-values (table 1). At pD 9.6 low-spin rhombic haem g-values of 2.93, 2.22 and 1.52 are obtained which are converted at pD 12.1 to values of 2.80, 2.25 and 1.67. Some hydroxy species can be seen at 2.59, 2.17 and 1.85. The decrease in rhombicity of the g-values upon deprotonation of imidazole coordinated to Fe(III)-porphyrins was observed in [4] and re-investigated in [8]. The values are compared in table 1 with those obtained by us for met.Mb–imidazole complex at two pD values.

Hence, we conclude that the hist–imid$^-$ complex of low-spin ferric haem has a positive peak in the MCD spectrum at 1350 nm, whereas that of a bis-imidazole (or hist–imid^0) complex is at ~1600 nm. This provides a useful additional diagnostic criterion for the presence of a deprotonated form of imidazole coordinated to haem. We have used this assignment to show the presence of hist–imid$^-$ coordination of the ferric haem in soybean leghaemoglobin a at pH $\geq$ 7.0 [11]. This attests to a remarkable drop in the pK_a^2 of the coordinated imidazole and may be related to the ability of leghaemoglobin a to bind anionic ligands such as nicotinate. We have also observed a positive MCD band at ~1350 nm at 4.2 K in the spectrum of lacto-peroxidase [12]. The spectrum is independent of pH over 5–8. This is postulated to arise from bis-histidine coordination of the haem at low temperature in which the distal histidine has become deprotonated at N-1. These reports appear to be the first unambiguous evidence for the presence of imidazolate as a ligand to ferric haem in proteins. The proximal histidyl ligand in horseradish peroxidase may become deprotonated when the haem is in the five-coordinate state [5].

In a report of the X-ray structure of the imidazole complex of methaemoglobin (met.Hb) it is shown that imidazole is bound only partially but appears to become hydrogen-bonded to the distal histidine (E7) [16]. The hydrogen-bonded interaction can be maintained in the case of imidazolate ligand either by rotation of 180° about the Cβ–Cγ bond of the distal histidine sidechain or by tautomerism of the proton on the distal histidine.

Clearly considerable stabilisation of the imidazolate ligand will result if the N atom of the distal histidine *not* H-bonded to imidazolate can become H-bonded to yet another protein sidechain



such as, for example, a carboxylic acid residue. It is possibly the presence of this extended network of H-bonding in addition to the charge of the Fe(III) ion which contributes to the lowering of the imidazole pK_a^2.

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