Models of Cytochrome b: Mössbauer Studies on Bis-ligated Complexes of (Protoporphyrinato IX)iron(III) with Histidine and its Substituted Derivatives

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Mössbauer spectroscopic studies have been carried out on a series of complexes of the type $[FeL(L')_2]^+$, where H_2L = 3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropionic acid, L' = histidine, N^α-acetylhistidine, histamine, or pilocarpate. Measurements were made at various pH values in the range 7.5—12.0 in 20% (v/v) ethanol–water solution frozen at 80 K. When the pH is 8.0—8.5, the major species are low-spin bis complexes [δ = 0.25(2) mm s^{-1} and ΔE_Q = 2.0—2.2 mm s^{-1}]. These are rapidly converted into a molecular complex of histidine and $[(FeL)_2O]$ at high pH (10.1—12.0). The results for the bis(histidine) and related complexes show that histidine binds as a sterically hindered imidazole and that the iron–imidazole bonds are weak. The ΔE_Q value of 2.14 mm s^{-1} and large linewidths (0.48—1.22 mm s^{-1}) of the complexes indicate that the two imidazole planes in the bis(histidine) complex are non-parallel with a large angle between the planes. The observation of asymmetric quadrupole doublets and broad lines are typical of slow spin–lattice relaxation of iron similar to that observed for cytochrome c. The present Mössbauer spectroscopic results for the bis(histidine) complexes are similar to those found for low-spin iron(III)-cytochromes and cytochrome b_5. Steric strain due to the histidine side-chains and electrostatic interactions between the charged groups and the porphyrin propionate carboxylates are found to influence the iron electronic structure and the imidazole plane orientations.

Studies on the question of the identity of axial ligands of cytochromes b from various mitochondrial and chloroplast sources have revealed that the haem in these proteins is co-ordinated to two histidine residues.[1–8] For example, cytochrome b_5 from liver[2] and erythrocytes of animals,[3] chloroplast,[4,5] cytochrome b_6 and b_{559}, yeast[6] flavocytochrome b_2, mitochondrial[4] b_{562} (b_K) and b_{566} (b_T), and cytochrome a of cytochrome oxidase[7,8] have all been shown to have these axial ligands. Steric and/or electronic influences of these ubiquitous histidine ligands constitute the primary mechanism of fine control of haem iron reactivity in haemoproteins.[9] The wide variety of physical properties[5,10–13] of the cytochrome b, having apparently little change in their iron co-ordination environments, have been ascribed to differences in the orientation of the two imidazole planes of histidine,[5,12,14–16] steric strain of bis(histidine) ligation,[11,12] or hydrogen bonding of axial histidines.[13,14] A powerful secondary control mechanism also exists which involves perturbation of the porphyrin ring involving π donor–acceptor[17] interactions between the haem and an aromatic amino acid residue such as histidine, phenylalanine, or tyrosine.

Detailed studies on a bis(histidine) complex of an iron porphyrin as a protein-free model system of a cytochrome have not been carried out, presumably due to the fact that haems undergo extensive aggregation in aqueous solutions.[18–20] Recently it was shown that in an aqueous ethanolic solution the monomeric low-spin six-co-ordinated bis(histidine) complexes can be prepared.[21–23] It was shown that histidine co-ordinates to iron(III) as the anion, $NH_2CHRCO_2^-$ (R = imidazolyl), in the pH range 8.5—12.0 and that the visible spectra of the bis(histidine) complexes of iron-(III) and -(II) protoporphyrin IX (3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropionic acid, H_2L) [FeL] are similar[21–23] to that of cytochrome b_5. Coulombic interaction between the residual positive charge of the iron in the porphyrin and ionizable functional groups in the side-chains of histidine have been shown be quite significant in these complexes.[21–23]

Mössbauer spectroscopy provides unambiguous characterization of iron electronic structure and may provide information on the influence on the iron centres due to any possible interaction by the neighbouring charged groups. We have recently found,[24] based on a Mössbauer study on $[FeL(L')_2]^+$ (L' = imidazole or substituted imidazole) complexes in various solvents, that a definite relationship exists between the axial imidazole plane orientation and the observed quadrupole splittings (ΔE_Q) and lineshapes. Large ΔE_Q values of *ca.* 2.34—2.43 mm s^{-1} have been assigned to structures where the two imidazoles are oriented more or less in parallel alignment, whereas values of *ca.* 1.9 mm s^{-1} were assigned to perpendicular orientation of imidazole planes.

We have also studied the formation of a molecular complex between histidine and $[(FeL)_2O]$ and reported its Mössbauer results.[25] We have suggested that a role of the amino group of histidine is to form a $NH \cdots {}^-O_2CR$ type of hydrogen bond with the propionic acid group of L so that this interaction can be envisaged as a model to study the influence of the non-co-ordinated amino acid residues neighbouring a haem in a protein pocket.

In this paper, we extend our earlier Mössbauer spectroscopic study[24] to the bis(histidine) complexes of $Fe^{III}L$ where the histidine side-chains are modified by using histamine (1*H*-imidazole-4-ethanamine), N^α-acetylhistidine, and pilocarpate (α-ethyl-β-hydroxymethyl-1-methyl-1*H*-imidazole-5-butanoate) (Figure 1). We also report the pH dependence of the low-spin bis(histidine) complex as well as of the histidine–$[(FeL)_2O]$ donor–acceptor complex.

Experimental

The frozen-solution samples for Mössbauer spectroscopic measurements were prepared by dissolving ^{57}Fe-enriched $[FeL]^+$, prepared and purified according to the method of Caughey *et al.*,[26] in 20% (v/v) ethanol–water. The choice of this solvent system is based on the solubility and stability of the

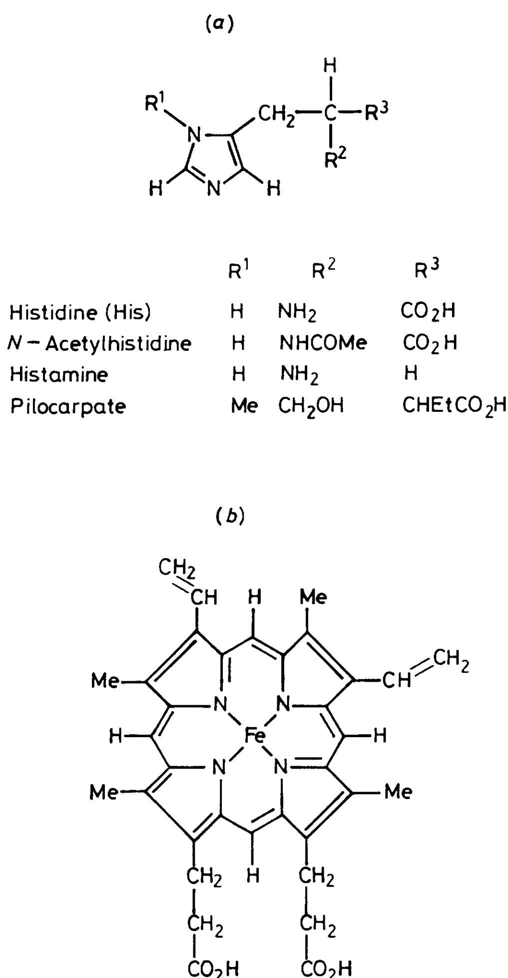

Figure 1. Structures of (*a*) histidine and its derivatives relevant to this work, (*b*) (protoporphyrinate IX) iron

monomeric bis(histidine) complexes.[21–23] The formation of the bis-ligated species was verified by visible spectroscopy.[21–23]

The Mössbauer spectra were recorded using an instrument and techniques previously described.[27] The pH values of the solutions were measured using a Philips PW 9420 digital pH meter and the visible spectra were recorded on a Perkin-Elmer Lambda 5 spectrophotometer.

Results and Discussion

pH *Dependence of Mössbauer Spectroscopic Data.*—The bis(histidine) complexes of (protoporphyrinato IX)iron(III), $[FeL]^+$, exist in the pH range 7.5—10.1.[21–23] Though the bis(histidine) complex is reported to be stable in this pH range according to visible spectroscopy (at micromolar concentrations) at the higher concentrations (millimolar) used for this Mössbauer study, it is susceptible to attack by OH^- ions or H_2O molecules depending on the pH, as seen from the formation of a histidine–$[(FeL)_2O]$ complex (see below).[25]

At pH 8.0, the $[FeL(His)_2]^+$ (His = histidine) complex is the major species in frozen solution (Table 1 and Figure 2). The isomer shift of *ca.* 0.25(2) mm s^{-1} and a quadrupole splitting range of 1.99—2.28 mm s^{-1} for the bis(histidine) complexes in Table 1 are typical of low-spin iron(III) porphyrin complexes.[25,28–31] As the pH is increased to 8.5 a new species appears in the frozen-solution Mössbauer spectrum with δ = 0.39 mm s^{-1} and ΔE_Q = 0.46 mm s^{-1}, which is identified as the donor–acceptor complex of histidine with $[(FeL)_2O]$[25] (Figures 2 and 3).

On lowering the pH of the solution to 7.5, the frozen-solution Mössbauer spectrum shows the presence of two sets of asymmetrically split quadrupole doublets similar to the ones found for dehydrated metmyoglobin.[26] The asymmetric doublet of the inner pair of lines is typical of high-spin monomeric iron(III) porphyrin complexes with slow electronic relaxation,[26,28,32] however the visible spectrum of the solution at room temperature provides evidence for only the low-spin $[FeL(His)_2]^+$ species ($\lambda_{max.}$ = 411, 535, and 556 nm). The outer pair of lines with a ΔE_Q value of 2.09(5) mm s^{-1} is indicative of the presence of small amounts (*ca.* 25%) of a low-spin iron(III) porphyrin complex similar to that which was found in dehydrated metmyoglobin[26] and in this work at pH 8.0.

As the pH is increased to 10.1 the molecular complex of histidine with $[(FeL)_2O]$ is formed[25] as the major species in solution (Table 1). The histidine ligands of the low-spin bis complexes are probably first displaced by OH^- ions, then as the pH increases the $[(FeL)_2O]$ forms. Though we expected a deprotonation of the NH of imidazoles[23] at this pH, at the high (millimolar) concentrations used for Mössbauer spectroscopy the driving force for the OH^- attack would be the stabilization of the $[(FeL)_2O]$ complexes before molecular complex formation with histidine. The fact that OH^- ions compete with imidazole at high pH for (porphyrinato)iron(III), particularly in aqueous solutions, is well known.[33,34]

It is seen from the Mössbauer spectroscopic data in Table 1 that the molecular complex formation between histidine, N^{α}-acetylhistidine, or histamine with (protoporphyrin IX)iron(III) is favoured at high pH.[25] If the reason for this was due to any preference for $[(FeL)_2O]$ (over the monomeric high-spin haemin) by a donor such as histidine, we would have expected other similar donors such as 1,10-phenanthroline or 1-methylimidazole (1-mim) to form such molecular complexes with $[(FeL)_2O]$ in similar pH ranges. However, our previous Mössbauer studies[24] show that these donors do not perturb the spectrum of $[(FeL)_2O]$ under identical conditions to those for the histidine adducts. This suggests that $[(FeL)_2O]$ is more stable than bis(histidine)-type complexes of $[FeL]^+$ at high pH. It is therefore probable that the presence of a side-chain is necessary to stabilize the molecular complex between histidine and $[(FeL)_2O]$.[25] The hydrogen bonding of the NH_2 group of histidine with the propionic acid CO_2^- is probably involved in aligning the imidazole ring of histidine parallel to a pyrrole ring of $[(FeL)_2O]$ so that sufficient π–π interactions can take place. The hydrogen-bonding interaction is likely to be more important at high pH since at lower pH the amino group would be present as NH_3^+ (pK_a = 8.5)[21,23] and would be separated from CO_2^- groups by solvation. Thus the pH dependence of formation of the molecular complex is attributed to the interaction of the side groups of histidine with the propionic carboxylates. These will be favoured at high pH. On lowering the pH, protonation of the NH_2 will break the hydrogen bonds, and concomitantly the $[(FeL)_2O]$ complex itself will break up.

Pilocarpate is said to form a molecular complex with haematin in aqueous alkaline solution.[22] However, the present Mössbauer spectroscopic results show that the electron density at the iron centres of $[(FeL)_2O]$ is essentially the same as that for normal uncomplexed $[(FeL)_2O]$ and obviously not perturbed by any possible adduct formation with pilocarpate. An increase in the pH of the solution above 10.1 did not significantly change the spectrum of $[(FeL)_2O]$ in the presence of pilocarpate. The difference between pilocarpate and histidine toward the formation of a donor–acceptor complex must reflect the difference in their structures (Figure 1). The replacement of the NH_2 group of histidine by CH_2OH and the presence of a bulky group (R^3 in Figure 1) decreases the possibility of hydrogen-bonded interactions with the propionate, thereby not favouring molecular complex formation. Thus the molecular

Table 1. Mössbauer data (80 K) for bis(histidine)(protoporphyrinato IX)iron(III) complexes $[FeL(L')_2]^+$ in solutions frozen at the indicated pH[a]

L	pH[b]	Site[c]	Isomer shift (with respect to Fe)/ mm s^{-1}	Quadrupole splitting/mm s^{-1}	Linewidth (half-width at half-height)/ mm s^{-1}	Relative area (%)
Histidine	7.5	(1)	0.21(5)	2.09(5)	0.41(7), 0.25(9)	15.9 (3.2), 8.5 (3.4)
		(2)	0.42(2)	0.40(3)	0.36(4)	75.6 (8.7)
	8.0	(1)	0.24(2)	2.15(2)	0.27(2), 0.35(4)	43.8 (4.0), 41.1 (5.1)
		(2)	0.38(5)	0.50(7)	0.20(6)	15.1 (1.9)
	8.5	(1)	0.25(2)	2.14(2)	0.24(2), 0.33(3)	24.3 (2.3), 25.3 (3.0)
		(2)	0.39(1)	0.46(1)	0.14(1)	50.4 (2.8)
	10.1	(1)	0.26(6)	1.99(6)	0.19(8), 0.30(9)	9.8 (4.7), 14.4 (5.4)
		(2)	0.38(1)	0.43(1)	0.14(1)	75.8 (6.2)
N^{α}-Acetylhistidine	8.4	(1)	0.21(3)	2.09(3)	0.40(3), 0.61(6)	46.8 (5.4), 53.2 (6.6)
	12.0	(2)	0.42(1)	0.46(1)	0.19(1)	100.0
Histamine	8.3	(1)	0.21(6)	2.17(6)	0.21(5), 0.37(9)	17.8 (4.4), 21.4 (6.4)
		(2)	0.40(1)	0.47(2)	0.18(2)	60.8 (7.5)
	11.0	(1)	0.28(5)	2.28(5)	0.32(4), 0.42(8)	25.4 (3.9), 22.0 (4.9)
		(2)	0.42(1)	0.47(2)	0.20(2)	52.6 (5.7)
Pilocarpate	8.3		0.24(3)	2.17(3)	0.37(2), 0.56(4)	49.4 (3.7), 50.6 (4.5)
	10.1	(1)	0.26(2)	2.22(2)	0.36(2), 0.44(4)	43.7 (4.2), 50.6 (3.5)
		(2)	0.42(3)	0.60(4)	0.08(3)	5.7 (2.8)

[a] Solvent: 20% (v/v) ethanol–water. [b] At 300 K in the mixed solvent. [c] Site 1 corresponds to low-spin iron(III) bis complexes, site 2 to either a donor–acceptor adduct or a high-spin complex (see text).

Table 2. Mössbauer data for low-spin iron(III) bis(imidazole) models and proteins

Compound[a]	Experimental conditions	δ(Fe)/ mm s^{-1}	ΔE_Q/ mm s^{-1}	Ref.
Cytochrome b_5 (calf liver)	195 K	0.23(3)	2.27(3)	43
Cytochrome a	4.2 K	0.20	2.0	*b*
Cytochrome c_1aa_3 (*Thermus. Thermophilus*)	4.2 K	0.25	1.95—2.1	*c*
Metmyoglobin (dehydrated)	77 K	0.18(2)	2.04(2)	26
[FeL(Him)(im)]	dmso, 80 K	0.24(3)	2.43(3)	24
$[FeL(Him)_2]^+$	dmso, 80 K	0.22(2)	2.38(2)	24
$[FeL(1\text{-mim})_2]^+$	Ethanol–water (1:1), 80 K	0.26(1)	2.34(1)	24
	dmso, 80 K	0.23(1)	2.24(1)	24
$[FeL(2\text{-mim})_2]^+$	Ethanol–water (1:1) 80 K	0.16(2)	1.87(2)	24

[a] Him = Imidazole. [b] T. A. Kent, L. J. Young, G. Palmer, J. A. Fee, and E. Münck, *J. Biol. Chem.*, 1983, **258**, 8543. [c] T. A. Kent, E. Münck, W. R. Dunham, W. F. Filter, K. L. Findling, T. Yoshida, and J. A. Fee, *J. Biol. Chem.*, 1982, **257**, 12489.

structure of histidine appears specially suited for forming a stable donor–acceptor complex with L and any modification of the amino group in the side chain changes the extent of π–π interactions.

Comparison between Bis(histidine) and Bis(imidazole) Complexes of $[FeL]^+$.—The isomer shift and the quadrupole splitting of the bis(histidine) complexes in Table 1 are similar to those reported for the bis complexes of substituted imidazoles[24] such as 1- or 2-mim (Table 2) and are clearly different from those of the analogous unsubstituted imidazole complexes.[24,28,29] Co-ordination of 2-mim to an iron(III) porphyrin imposes considerable steric restriction[12] so that the two imidazoles in a bis(2-mim) complex are oriented in nearly perpendicular planes.[14] This accounts[24] for small ΔE_Q values of *ca.* 1.9 mm s^{-1}. In the case of the bis(1-mim) complex in dimethylsulphoxide (dmso), the iron–imidazole bonds are weak when compared to those of unsubstituted imidazole complexes.[35] This allows some degree of freedom for rotation of axial ligands, the two 1-mim planes are thus non-parallel with a large angle between them (ΔE_Q = 2.24 mm s^{-1}). A strong iron–imidazole bond in non-hindered imidazole complexes stabilizes parallel orientation of axial ligand planes[24] and the expected value of ΔE_Q is 2.34—2.43 mm s^{-1}. We have previously reported[24] the correlation of ΔE_Q values of bis(imidazole) complexes (Table 2) with such angular alignment of axial ligand planes. Thus the quadrupole splittings in the range 2.0—2.2 mm s^{-1} observed for the bis(histidine) complexes reported here indicate that histidine binds similarly to a sterically hindered imidazole and that the iron–imidazole bonds are weak, allowing some freedom of rotation of the axial imidazole planes.

Though the side-chains of histidine should not impose as severe a steric restriction as the Me Group in 2-mim, the interaction of the side-chains with the propionic acid groups or with solvent molecules will lead to some steric strain in the histidine binding to (protoporphyrinate IX)iron(III). The resulting strain in bis(histidine) ligation is responsible for aligning the two imidazole planes in a non-parallel orientation with a large angle between the axial ligand planes.

The relatively large values of Mössbauer linewidths observed (0.48—1.22 mm s^{-1}) for the bis(histidine) complexes (Table 1, site 1) are comparable to those of the bis(imidazole) complexes where we found evidence for large angles between the axial imidazole planes.[24] As we have reported previously,[24] the large linewidths of such complexes are due to the presence of many similar iron(III) complexes that vary in the angular alignment of the axial imidazole planes.

The two lines of the quadrupole doublet in the Mössbauer spectra (Figure 2) of the bis(histidine) complexes are of unequal intensity; the higher-velocity line is unusually broad though the areas of both lines are similar (Table 1). Such spectra are attributed to slow spin–lattice relaxation.[24,26,36–40] This indicates that magnetic hyperfine interactions are responsible for the line broadening.[26,41,42] Similar spectra are also obtained when the imidazole planes are non-parallel.

Relevance to Cytochrome b.—The bis(histidine) complexes of (protoporphyrinato IX)iron(III) have Mössbauer parameters similar to that which was observed for cytochrome b_5 as well as to those found for the bis(substituted imidazole) complexes[24] of (protoporphyrinato IX)iron(III) (Table 2). Münck[43] has

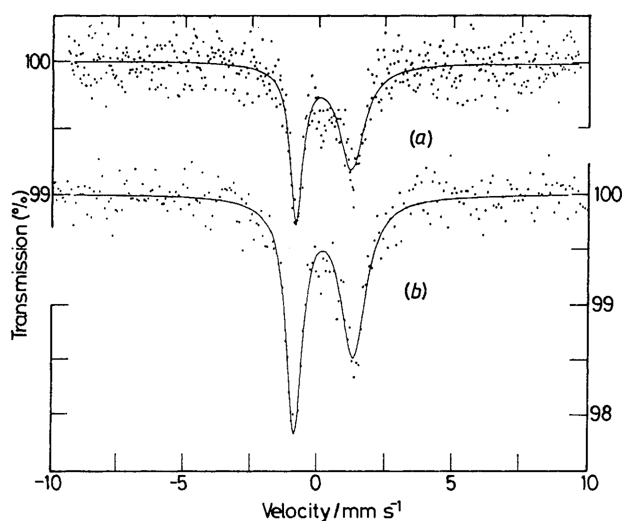

Figure 2. Frozen-solution Mössbauer spectra (80 K) of the bis(histidine) (pH 8.0) (*a*) and bis(pilocarpate) (pH 8.3) (*b*) adducts of (protoporphyrinato IX)iron(III)

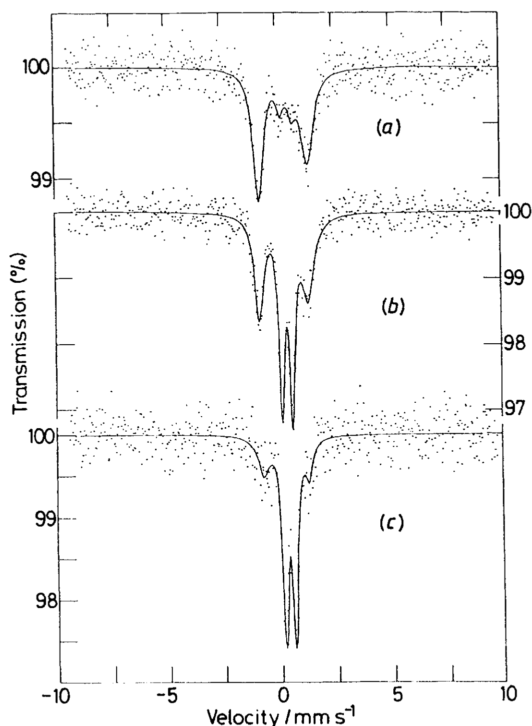

Figure 3. Frozen-solution Mössbauer spectra (80 K) of (protoporphyrinato IX)iron(III) chloride in the presence of excess of histidine at pH 8.0 (*a*), 8.5 (*b*), and 10.1 (*c*)

reported that the Mössbauer spectrum of cytochrome b_5 at low temperature is broad and that its slow relaxation is similar to that of cytochrome c. Similar broad spectra and slow relaxation are observed for the bis(histidine) complexes discussed above and also for the bis complexes of haemin with substituted imidazoles.[24] The above results, along with those reported previously,[24] are in keeping with the suggestion that steric strain imposed by the side-chains of the histidine ligands in cytochrome b, *can* align* the two imidazole planes in a non-parallel orientation with a large angle between the axial ligand planes. It is reported that the physiologically relevant form of chloroplast cytochrome b_{559} contains a near-perpendicular orientation of axial imidazole planes.[5]

The e.s.r. data for cytochrome b_5 show that the protein exists in two different forms[33] in neutral (g_z = 3.03, g_y = 2.23, g_x = 1.43) and alkaline (g_z = 2.76, g_y = 2.28, g_x = 1.68) media. These data are similar to those reported[5] for $[FeL(1\text{-mim})_2]^+$ in neutral (g_z = 2.95, g_y = 2.26, g_x = 1.52) and alkaline (g_z = 2.74, g_y = 2.27, g_x = 1.72) solutions and for chloroplast cytochrome b_{559} (high-potential form, g_z = 3.08, g_y = 2.16, g_x = 1.36; low-potential form, g_z = 2.94, g_y = 2.26, g_x = 1.50). Based on a comparison of Mössbauer spectroscopic and e.s.r. data for the bis(imidazole) models,[24] we attributed this to be due to two different orientations of the axial imidazole planes. In neutral solution the two imidazole planes are in non-parallel orientation with a large angle between the planes, whereas in alkaline solution the two ligand planes are in parallel orientation. The results for the bis(histidine) complexes indicate that the two imidazole planes are non-parallel due to steric reasons as well as a weak iron–imidazole bond and that the parallel form is non-accessible by increasing the solution pH for the model complex.

If the parallel orientation of imidazole planes is not accessible in a simple bis(histidine) model compound, then the question to be asked is: what causes the parallel orientation of imidazole planes in cytochrome b_5, c_3, and in chloroplast cytochrome b_{559}? Deprotonation of the axial imidazole NH is a possibility.[24] However, this is not always necessary since the results for the *N*-methylimidazole complex show that electrostatic interactions of the propionate carboxylate with solvent water molecules may also stabilize the parallel orientation of the axial imidazole planes.[24] The effect of negatively charged groups (such as the propionates) near the haem stabilizing the charge on the iron centre[44] may be quite important. It may be pointed out that the crystal structure of a tetrahaem protein cytochrome c_3 [a protein having bis(histidine) co-ordination to haem] shows that three haems have nearly parallel imidazole planes, while the fourth haem has the axial imidazoles perpendicular to each other.[45] These findings that are the opposite to those for other model bis(histidine) complexes are obviously imposed by the protein structure. Thus in a bis(histidine) co-ordinated haem of a haemiprotein the solvent effects, the steric influence of histidine side-chains, and the charge effects of carboxylate and other nearby charged groups can be modulated by the apoprotein conformation so as to align the two imidazole planes in a perpendicular or a parallel orientation.

Conclusion

Mössbauer spectroscopic studies on the bis(histidine) and related complexes of (protoporphyrinato IX)iron(III) indicate that histidine binds as a sterically hindered imidazole. The steric hindrance of the 4-substituted imidazole is due to the electrostatic and hydrogen-bonding interactions of the histidine side-chain and the propionic acid carboxylate group. The iron–imidazole bonds in the bis(histidine) complexes are weak so that OH^- ion can replace the co-ordinated histidines at high pH. The combined consequence of the long iron–imidazole bond, steric strain of the histidine co-ordination, and the electrostatic interaction between charged groups in histidine and the propionic carboxylate is to align the two imidazole planes in a

* The experimental conditions under which the Mössbauer data for cytochrome b_5 (ref. 43) were obtained are not known to us. The protein preparation used in the Mössbauer experiment, according to the discussions in the text, should contain a haem with two imidazoles of histidine in a non-parallel orientation. However, other available information on the protein[2,33] indicate that parallel orientation is more favoured. However, the e.s.r. data[33] do indicate two forms of the protein as in chloroplast[5] b_{559} (see text and also ref. 24). Further work on the protein is needed to clarify this point.

non-parallel orientation with a large angle between the planes in bis(histidine) haem complexes. The results for the bis(histidine) complexes, taken together with those for the bis(imidazole) complexes reported previously,[24] indicate that steric strain in the histidine co-ordination, solvent effects, and the influence of charged groups near the haem in cytochromes b may play a combined role in orienting the planes of the two imidazole ligands to the haem.

The results from this study, taken together with those of related work,[4,5,10–16,24,25] may lead to an understanding of the various possible roles of the side-chains attached to the imidazole ring of the histidine residues in the electronic structure of iron in the cytochromes.

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