Resonance Raman Investigation of Imidazole and Imidazolate Complexes of Microperoxidase: Characterization of the Bis(histidine) Axial Ligation in *c*-Type Cytochromes

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ABSTRACT: In order to evaluate the steric and electronic influences of the heme axial ligands on the vibrational modes of heme *c*, various ferric and ferrous six-coordinate heme *c* compounds have been prepared from microperoxidase-8 (MP8) and different extrinsic ligands. In this paper, the absorption and Soret-excited resonance Raman (RR) spectra of imidazole, imidazolate, 1-methylimidazole, and histidine complexes of MP8 are presented. The absorption characteristics of the unligated forms, either aggregated or monomeric, as well as of the ligated forms of MP8(III) and MP8(II) have been determined as a function of pH, the presence of a cationic detergent, and the ligand concentration. Spectrophotometric titrations have shown that MP8(III) and MP8(II) can bind one or two molecules of exogenous ligand, forming monoligated or bisligated complexes. The latter form, observed with large excesses of ligand, results from the displacement of the intrinsic proximal His of MP8 by an exogenous ligand. Several structural marker bands have been detected in the high- and low-frequency regions of RR spectra. The high-frequency RR spectra of the ImH compounds of MP8(III) exhibit a ν_{10} mode sensitive to ligand deprotonation(s). Moreover, the replacement of His by an exogenous ImH in MP8(III) complexes induces the upshift of the ν_{10} mode frequency (1637−1641 cm^{-1}), indicating that the porphyrin skeleton is less distorted when the internal coordination of proximal His to heme is broken. A similar dependence of the out-of-plane porphyrin distortion is suggested for the low-frequency mode ν_8 (343−347 cm^{-1}). As far as the ferrous compounds are concerned, the mode most sensitive to the ImH deprotonation is ν_{11}, which is downshifted from 1539 to 1527 cm^{-1}. Comparisons of the low-frequency regions of the RR spectra of imidazole-type ligated MP8(III) and MP8(II) complexes, as well as observations based on isotopic substitutions of the corresponding 1-methylimidazole complexes (MeIm → MeIm-d_6), allow the assignment of two bands in the 184−197 and 400−409 cm^{-1} regions to modes involving the symmetric and asymmetric stretches of the axial ligands, respectively. Two other bands in the 343−347 and 359−362 cm^{-1} regions, sensitive to the mass and/or deprotonation states of the axial ligands, have been tentatively assigned to ν(Fe−N(pyrrole)) modes coupled to either a deformation mode of axial bonds or an internal mode of the bound imidazole(s). All of the preceding spectroscopic data are used to extract information on the ionization states of axial imidazoles, as well as on the heme structure in bis(histidine)-ligated hemes of cytochromes *c*. They will also provide good references in a study to follow on the *N*-acetylmethionine and lysine complexes of MP8.

The *c*-type heme, formed from the condensation of heme *b* with two cysteinyl protein residues, has been found to be the prosthetic group of a large number of cytochromes defined as cytochromes *c* (cyt *c*)[1] (Moore & Pettigrew, 1990; Yamanaka, 1992). These heme proteins fulfill essential electron transfer roles in the vital processes of living organisms, such as respiration, photosynthesis, or fermentation, and exhibit large variations in their biological functions and measured redox potentials at pH 7 (E_{m7}). Indeed, this parameter can change by ca. 0.8 V from −400 to +400 mV (Meyer & Kamen, 1982; Moore & Pettigrew, 1990; Yamanaka, 1992). Among the factors governing the redox properties of *c*-type cyt, the number, nature, conformation, and H-bonding interaction of axial heme ligands appear to be of primary importance (Harbury et al., 1965; Dickerson & Timkovich, 1975; Valentine et al., 1979; Mashiko et al., 1979; Quinn et al., 1984; Senn & Wüthrich, 1985). X-ray structures of various cyt *c* have indeed revealed that all have a heme bound to a histidine residue at the fifth coordination position of the iron atom (Takano & Dickerson, 1981; Weber et al., 1981; Matsuura et al., 1982; Pierrot et al., 1982; Higuchi et al., 1984; Carter et al., 1985; Bushnell et al., 1990; Benning et al., 1991).

On the other hand, the sixth coordination of heme(s) in the crystallized proteins exhibits more variability, with this site being either vacant in cyt *c'* (Weber et al., 1981), occupied by a methionine sulfur in cyt *c*, c_2, c_5, and c_{551}

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[1] Abbreviations: MP8, microperoxidase-8; cyt, cytochrome; RR, resonance Raman; CTABr, cetyltrimethylammonium bromide; OEP, octaethylporphyrin; PP, protoporphyrin; ImH, imidazole; Im^-, imidazolate; MeIm, 1-methylimidazole; His, histidine; His^-, histidinate; AcHis, *N*-acetylhistidine; LS, low-spin; HS, high-spin.

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(Takano & Dickerson, 1981; Matsuura et al., 1982; Carter et al., 1985; Bushnell et al., 1990; Benning et al., 1991), or occupied by another histidine nitrogen in cyt c_3 (Pierrot et al., 1982; Higuchi et al., 1984). Moreover, the number of combinations for plausible axial heme ligations could be relatively high if we take into account that (i) an overwhelming majority of cyt *c* has an unresolved structure; (ii) protein ligands, such as the amino group provided from a lysyl residue or the polypeptide chain, the guanidine function from an arginyl residue, the alcohol group from a threonyl residue, the phenol group from a tyrosyl residue, and the thiol group from a cysteinyl residue, are potentially able to coordinate heme *c*; and (iii) H-bonding and/or ionic interactions inside the proteins can increase the electronegative character of Tyr, Cys, and His side chains, forming tyrosinate, cysteinate, and histidine residues. Considering on one hand the large number of purified cytochromes *c*, and on the other hand the putative variability in heme ligation, which is one of the main factors controlling the redox potential, the determination of the axial heme ligand(s) is a first stage in the characterization of cytochromes for which the structure is unknown.

Resonance Raman (RR) spectroscopy is an increasingly useful technique for the characterization of a number of structural features of hemes in hemoproteins (Spiro, 1983, 1985; Kitagawa & Ozaki, 1987; Desbois et al., 1984b; Desbois, 1994). Taking into account the need for well-characterized compounds to deduce vibrational information that is transposable to the *c*-type cytochromes, microperoxidase-8 (MP8) has attracted a renewed interest from different points of view. Indeed, this heme-peptide system retains a heme *c* covalently linked to the characteristic proximal site of all mammalian cyt *c* and a great majority of cyt *c* from vegetal and bacterial sources (Cys-X-Y-Cys-His) (Meyer & Kamen, 1982; Moore & Pettigrew, 1990; Yamanaka, 1992). Considering that the RR spectra of hemoproteins are highly sensitive to the nature of peripheral heme substituents (Choi et al., 1982, 1983; Desbois et al., 1984a), the heme identity in the MP8 derivatives and *c*-type cytochromes ensures a direct transposability of RR data obtained for the two sets of heme compounds. On the other hand, a preceding study has described the absorption and resonance Raman spectra of five-coordinate ferrous derivatives of MP8, modeling the coordination site of ferrous cytochromes *c'* (Othman et al., 1993a). However, most *c*-type cytochromes have six-coordinate hemes in both oxidation states. Since MP8 offers the possibility of preparing six-coordinate compounds with different and mixed axial coordinations (Harbury & Loach, 1960a,b; Harbury et al., 1965), it is the compound of choice to study the effects of axial ligation on the spectroscopic properties of heme *c*.

We thus have studied the absorption and resonance Raman spectra of imidazole, imidazolate, histidine, histidinate, lysine, and *N*-acetylmethionine complexes of oxidized as well as reduced MP8, i.e., complexes representing a biomimetic approach to the heme ligation of most *c*-type cytochromes. We report here the data obtained on imidazole, imidazolate, 1-methylimidazole, and histidine complexes, simulating the coordination of low-potential hemes *c* associated with various proteins (Pierrot et al., 1982; Higuchi et al., 1984; Deisenhofer & Michel, 1989; Cohn et al., 1989; Morimoto et al., 1991). A preliminary account of this work has been published by Othman et al. (1993b).

EXPERIMENTAL PROCEDURES

Microperoxidase and Extrinsic Ligands. Microperoxidase-8 was purchased from Sigma and used without further purification. The samples were prepared by dissolving lyophilized MP8 at a final concentration of 0.1−1 mM. The coordination of different exogenous ligands, i.e., imidazole (ImH), imidazolate (Im^-), 1-methylimidazole (MeIm), histidine (His), histidinate (His^-), or *N*-acetylhistidine (AcHis), to oxidized and reduced MP8 (MP8(III) and MP8(II), respectively) was studied as a function of pH. These ligands were purchased from Merck, Sigma, Lancaster, or Aldrich and used without further purification. Perdeuteriated 1-methylimidazole (MeIm-d_6) (isotopic enrichment, 98%) was from the Bureau des Isotopes Stables at Saclay (France). The equilibrium constants for the ligand binding to MP8(III) and MP8(II) were investigated at 20 ± 1 °C by spectrophotometric titrations according methods previously described (Brault & Rougée, 1974). The preparation of liganded MP8 derivatives in water has been previously reported (Harbury et al., 1965; Harbury & Loach, 1960a,b; Myer & Harbury, 1966). However, in order to limit the tendency of MP8 to form aggregates (Harbury et al., 1965; Othman et al., 1993a), a cationic detergent, cetyltrimethylammonium bromide (CTABr) (Sigma), was added to the solutions of MP8 at a micelle-forming concentration (1−2%). In the 7.5−12 pH range, the MP8 solutions were buffered with 50−100 mM potassium phosphate, Tris-HCl, or sodium carbonate. At pH values higher than 12.0, concentrated NaOH or KOH solutions (1−10 N) were added to unbuffered aqueous solutions of MP8. The reduced derivatives of MP8 were obtained by addition of solid dithionite under anaerobic conditions as previously described (Othman et al., 1993a).

Spectroscopy. The spectrophotometric titrations and UV−visible absorption spectra were obtained with Beckman DU7 and Shimadzu UV-160 spectrophotometers. The Raman spectra were recorded at room temperature (20 ± 1 °C) on a Jobin-Yvon spectrometer (HG 2S) with the 406.7, 413.1, and/or 441.6 nm excitations of a Kr^+ laser (Coherent Innova) and a He−Cd laser (Liconix). Using radiant laser powers of 15−40 mW, the RR spectra (2−8 scans) were collected and averaged using a multichannel analyzer (Tracor Northern TN 1710). Under these conditions, the frequency precision is 0.5−3 cm^{-1}, depending on both band intensity and signal-to-noise ratio. The Raman shift frequencies were calibrated at each excitation with the bands of benzene and dichloromethane. Moreover, in the 300−450 cm^{-1} regions where overlapping bands occur, the band positions, shifts, and intensities were checked by difference spectra (Rousseau, 1981) and the use of band-fitting programs (Spectra Calc, Galactic Industries). The fits were performed with Gaussian or Lorentzian band shapes and bandwidths of 14−18 cm^{-1}. The band shape exhibited no influence on the band positions and bandshifts within 0.5−1 cm^{-1}.

RESULTS AND DISCUSSION

Visible Absorption and Resonance Raman Spectroscopy of Ferric MP8 Derivatives

Absorption of MP8(III) without Added Ligand. The absorption spectrum of MP8(III) in water at pH 7.5−13 is concentration-dependent, indicating that aggregation occurs (Harbury & Loach, 1960a; Urry & Pettegrew, 1967; Aron

Table 1: High-Frequency Modes (cm^{-1}) of MP8(III) in Aqueous Solutions with or without CTABr[a,b]

	−CTABr					+CTABr				
	pH 7.5			pH 13.0		pH 7.5			pH 13.0	
	442 nm[c]	413 nm	407 nm	442 nm	413 nm	442 nm	413 nm	407 nm	442 nm	413 nm
ν_{10}	1642	1638	1639	1640	1641	1642	1638	1639	1640	1638
	1633		1630		1631					1631
				1628	1625	1629	1625	1627	1628	
			1619			1621			1620	1623
ν_{37}			1603				1603	1600	1600	
ν_2	1586	1587	1587	1587	1588		1586	1586		1587
						1583			1584	
	1577			1578	1578	1577	1578	1578	1577	1578
ν_{11}		1571								1569
ν_{38}	1556	1554	1552	1557	1554	1555	1554		1557	1554
ν_3	1505	1505	1502	1503	1503	1504	1505	1502	1503	1505
	1490		1493	1490	1490	1490	1492	1491	1490	1491
	1484	1488								
ν_{29}	1410	1410	1409							1408
		1401	1402		1405	1404	1402	1403	1403	1400
ν_4	1372	1374	1374	1372	1374	1372	1374	1373	1373	1375
	1320			1323					1326	
ν_{21}		1318	1314	1316	1317	1318	1319	1318	1316	1314
	1311		1310			1308		1310		
	1303	1305	1301		1302	1300	1301	1300	1301	1298

[a] Mode numbering according to Li et al. (1989). [b] The underlined frequencies correspond to the major components of ν_2, ν_3, and ν_{10} modes at the given excitation. [c] Excitation wavelengths.

et al., 1986). MP8(III) is high-spin (HS) monomeric at ca. 10^{-6} M, but becomes dimeric, maintaining HS hemes, when the concentration is increased by a factor of 10 (Urry & Pettegrew, 1967; Aron et al., 1986). Further aggregates with low-spin (LS) hemes are detected at MP8(III) concentrations higher than 10^{-4} M. The addition of alcohol to the aqueous solution of MP8(III) (50% (v/v)) decreases these intermolecular interactions (Aron et al., 1986). A similar dispersing effect is observed when CTABr is present at a micelle-forming concentration in aqueous solutions of MP8 (Othman et al., 1993a). The Soret band maximum is observed at 399 and 403 nm for MP8(III) in aqueous CTABr at pH 7.5 and 13.0, respectively, while that of the LS form of aggregated MP8(III) is detected at 405 nm (spectra not shown).

Resonance Raman Spectra of MP8(III) without Added Ligand. The observed RR frequencies of MP8(III) in water or in aqueous CTABr solutions, at pH 7.5 or 13, excited at 441.6, 413.1, and 406.7 nm, are listed in Table 1. Taking into account that ν_{10} is the mode most sensitive to the spin and coordination states of heme in ferric octaethylporphyrin (OEP) derivatives (Teraoka & Kitagawa, 1980), four forms can be significantly detected in the RR spectra of MP8(III) (Figure 1, Table 1). The 1638−1642 cm^{-1} band (ν_{10}), as well as the ~1504 and 1587 cm^{-1} bands (ν_3 and ν_2, respectively), appears predominant in the absence of detergent (Figure 1, spectrum a). This set of frequencies is attributed to six-coordinate LS hemes (Teraoka & Kitagawa, 1980) corresponding to large aggregates of MP8(III) (Harbury & Loach, 1960a,b; Aron et al., 1986; Wang & Van Wart, 1989). Intermolecular interactions likely form heme coordinates with the His_5 residue of a MP8 molecule and the terminal amino group of Cys_1 or the His_5 residue of another MP8 molecule.

The addition of CTABr converts, in large part, the LS aggregates into a five-coordinate HS form of MP8(III), since

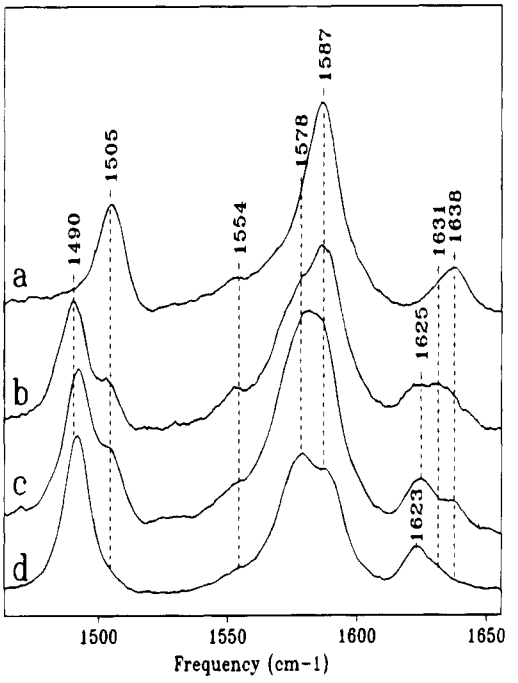

FIGURE 1: High-frequency regions (1450−1650 cm^{-1}) of RR spectra of MP8(III) in aqueous solutions: (a) 100 mM phosphate, pH 7.5; (b) 0.1 M KOH; (c) 1% CTABr, 100 mM phosphate, pH 7.5; (d) 1% CTABr, KOH 0.1 M. Excitation: 413.1 nm.

major bands are seen at 1490 (ν_3), 1578 (ν_2), and 1625−1629 (ν_{10}) cm^{-1} (Figure 1, spectra c and d, Table 1). The LS aggregates, however, are not totally dispersed at pH 7.5. This effect is related to the large difference in the MP8 concentrations used in the absorption and RR experiments. The 1619−1623 and 1631 cm^{-1} components of ν_{10} originate from a six-coordinate HS heme and a six-coordinate intermediate-spin heme, respectively (Teraoka & Kitagawa, 1980). All of these monomeric forms likely correspond to

Table 2: Low-Frequency Raman Modes (cm^{-1}) of MP8(III) in Various Aqueous Solutions[a,b]

	−CTABr					+CTABr				
	pH 7.5			pH 13.0		pH 7.5			pH 13.0	
	442 nm[c]	413 nm	407 nm	442 nm	413 nm	442 nm	413 nm	407 nm	442 nm	413 nm
γ_{22}	458	457	459		454	454				
		450	446		448					
	420	413	412	418	413	418	415	413	415	415
		403	400							
		388								
			(P)	381	380	384	381	(P)	383	386
		374				375	375		370	
		359	359		359					360
ν_8		348	353	350		351	347	347	351	
	344	340	342		344					345
ν_{51}							315		322	315
			309			302			300	
		293				285				
ν_9		272	275				272	275		271
				268	268	268			267	
										235
γ_{24}		223	226							
			217				217			217
		208					203			205
		199	203							
			188				191			

[a] Mode numbering according to Hu et al. (1993). [b] P represents bands hidden by a plasma line. [c] Excitation wavelengths.

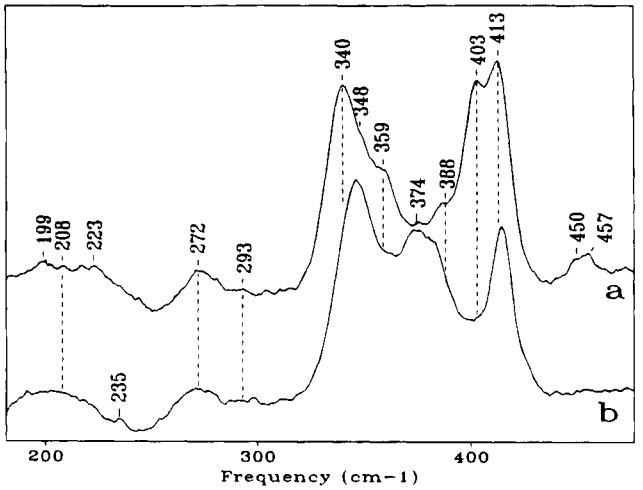

FIGURE 2: Low-frequency regions (180−480 cm^{-1}) of RR spectra of MP8(III) in aqueous solutions at pH 7.5: (a) 100 mM phosphate; (b) 1% CTABr, 100 mM phosphate. Excitation: 413.1 nm.

MP8 ligating a solvent molecule (H_2O or OH^-) (Huang & Kassner, 1981). As in the case of the ferrous form of MP8 (Othman et al., 1993a), the MP8(III) peptide likely exhibits a conformational flexibility that is sensitive to both the pH value and the presence of detergent and consequently can modulate the structure of the internal Fe−His bond, the ionization state of the His, and thus the affinity for the solvent molecules (Wang et al., 1992; Othman et al., 1993a).

The low-frequency RR spectra also indicate changes in the heme ligation of MP8(III) as a function of pH and the presence of detergent (Table 2, Figure 2). The ν_8 mode, which is a porphyrin core expansion mode involving the four pyrrole rings (Li et al., 1989; Hu et al., 1993), is observed at 340−341 cm^{-1} in the spectra LS aggregates. The presence of CTABr, which essentially stabilizes five- and six-coordinate HS complexes at pH 7.5 (Table 1), induces a shift in ν_8 to 347−351 cm^{-1} (Figure 2, spectrum b). These observations demonstrate that the frequency of ν_8 is spin state sensitive. Similarly, bands at 272−275 (ν_9) and 386−388 cm^{-1} are associated with LS aggregated forms, while bands in the 268−272 and 374−384 cm^{-1} regions correspond to the HS forms of MP8(III). The 359−360 and 400−403 cm^{-1} bands, as well as two overlapping bands in the 440−460 cm^{-1} region, are particularly active in the RR spectra of aggregated MP8(III) and do not exhibit any apparent homologous band in the spectra of HS forms (Figure 2, spectrum a).

Absorption of Imidazole and Imidazolate Complexes of MP8(III). In order to monitor the coordination state of the MP8(III) heme in the presence of an exogenous ligand, spectrophotometric titrations have been performed. They show that ImH is easily bound to MP8(III) in aqueous CTABr solution at pH 7.5. The binding constant ($K_a = 1.5 \times 10^4$ M^{-1}) is intermediate between those determined in water and those in methanolic aqueous solution ((0.8−2.8) $\times 10^4$ M^{-1}) (Harbury & Loach, 1960b; Baldwin et al., 1986). This high imidazole affinity warrants that the different possible initial forms of MP8(III), i.e., the aggregated forms, the monomeric form without the sixth ligand, and the aquo and hydroxo monomeric forms, are all converted to an imidazole complex of MP8(III), forming a bis(imidazole)-type heme coordination. Moreover, our results show the binding of a second imidazole in water ($K_a = 8 \times 10^{-1}$ M^{-1}). This additional binding likely corresponds to the displacement of the endogenous His of MP8(III) by an exogenous ImH molecule (MP8(III)−$(ImH)_2$). However, it maintains a bis(imidazole)-type heme coordination. The visible absorption spectra of the ImH complexes of MP8(III) are characteristic of LS six-coordinate derivatives, having the Soret, β, and α band maxima at 406−407.5, 526−527, and 555−556 nm, respectively (Table 3). Similar absorption spectra were published for these complexes in different solvent conditions (Harbury & Loach, 1960b; Urry & Pettegrew, 1967; Balwin et al. 1986; Owens et al., 1988).

Table 3: Absorption Maxima of Liganded MP8(III) Derivatives

ligand	α/β	Soret	solvent conditions[a]
ImH	526/556	406	0.1 M, −CTABr, pH 7.5
ImH	524/554	407.5	8 M, −CTABr, pH 7.5
ImH	526/555	407.5	0.1 M, +CTABr, pH 7.5
AcHis	526/555	407.5	0.1 M, +CTABr, pH 7.5
Im^-	540/555	414	0.2 M, +CTABr, pH 13
His^-	538/556	412.5	0.1 M, +CTABr, pH 13
His^-	540/556	414	0.5 M, +CTABr, pH 13

[a] Ligand concentration, absence or presence of CTABr (− or +), pH.

Despite the expected major overlap of the absorption bands of MP8(III)−ImH and MP8(III)−$(ImH)_2$, a small systematic red shift of the Soret maximum by 1.5 ± 0.5 nm is observed when the complexes are formed at low and high ligand concentrations in water (Table 3).

The presence of CTABr in aqueous solutions of mono- and bis(imidazole) complexes of ferric and ferrous hemes is known to facilitate the deprotonation of the N_1H group of coordinated imidazole group(s) (Mincey & Traylor, 1979; Desbois & Lutz, 1992; Othman et al., 1993a). This property has been used to obtain heme compounds in which at least the imidazole group of His_5 of MP8 is ionized. Thus, spectrophotometric titrations were performed in the 12−13 pH range, where the imidazole rings that are bound to ferrihemes are expected to be deprotonated (Harbury & Loach, 1960b; Sundberg & Martin, 1974; Desbois & Lutz, 1992). Two association constants (7×10^3 and 2×10^2 M^{-1}) were also determined for the apparent binding of imidazole to MP8(III) at pH 13. The former is attributable to the formation of the imidazolate complex of MP8(III) (MP8(III)−Im^-), in which the imidazole group of His_5 is deprotonated. The latter constant indicates that the histidine residue of MP8 normally bound to heme is displaceable by an exogenous ligand at alkaline pH, thus forming a bis(Im^-) complex (MP8(III)−$(Im^-)_2$). The absorption spectrum of the latter form, obtained using a ligand concentration of 1 M, exhibits visible bands at 414, 540, and 555 nm (Table 3). An apparent pK_a value of 12.2 is found for the MP8-(III)−ImH → MP8(III)−$(Im^-)_2$ transition. The 6 nm red shift of the Soret band (Table 3) is similar to that detected in the absorption spectra of bis(ImH) and bis(Im^-) complexes of ferric protoporphyrin (Desbois & Lutz, 1992). This behavior confirms that the axial ionizations of ImH or His take place in the ImH complexes of MP8(III), forming bis-(imidazolate)-type heme ligations.

High-Frequency Regions of Resonance Raman Spectra of Imidazole- and Imidazolate-Type Complexes of MP8(III). The high-frequency regions of the RR spectra (1250−1700 cm^{-1}) of the ImH, Im^-, MeIm, AcHis, or His^- complexes of MP8-(III) exhibit a LS six-coordinate pattern, with the ν_4, ν_3, ν_2, and ν_{10} modes occurring in the 1373−1376, 1502−1505, 1586−1588, and 1631−1641 cm^{-1} frequency regions, respectively (Figures 3A,B, Table 4). The comparison of RR spectra of imidazole- and imidazolate-type complexes of MP8(III) excited at 441.6, 413.1, and 406.7 nm, however, shows that the frequencies of several of these modes are dependent on the excitation wavelength. The ν_{10} mode is indeed detected at 1641 cm^{-1} when the ImH and His complexes of MP8(III), formed at pH 7.5 with ligand concentrations of 0.1 M, are excited at 441.6 nm (Figure 3B, spectrum a). Using the 413.1 nm excitation, a major

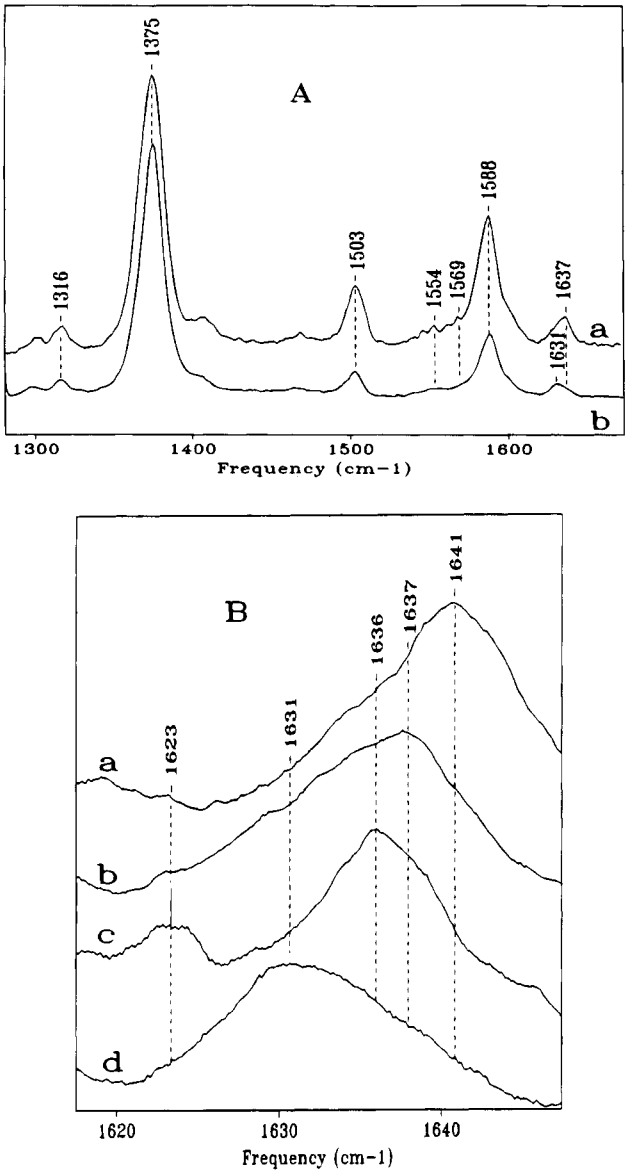

FIGURE 3: (A) High-frequency regions (1300−1650 cm^{-1}) of RR spectra of MP8(III) in 1% aqueous CTABr solutions containing 0.1 M ImH: (a) 100 mM phosphate, pH 7.5; (b) 0.1 M KOH. Excitation: 413.1 nm. (B) Extended scale of the 1620−1650 cm^{-1} regions of RR spectra of MP8(III) in 1% aqueous CTABr solutions containing 0.1 M ImH: (a) pH 7.5, 441.6 nm excitation; (b) pH 7.5, 413.1 nm excitation; (c) 0.1 M KOH, 441.6 nm excitation; (d) 0.1 M KOH, 413.1 nm excitation.

band is detected at 1637 cm^{-1}, along with a shoulder at 1629 cm^{-1} (Table 4, Figure 3B). The latter feature represents the persistence of an HS heme that is resonance-enhanced by the blue-shifted excitation. However, when the ligand concentration is increased to 5 M in water, the 1629 cm^{-1} shoulder disappears and is replaced by another shoulder at 1640 cm^{-1} on the 1637 cm^{-1} band (not shown). One may suggest that the 1640−1641 cm^{-1} band could correspond to the formation of a significant amount of MP8 aggregates (Table 1). However, this band exhibits no change in relative intensity with the addition of CTABr. Moreover, the aggregates of MP8(III) show a Soret maximum at 405 nm that is slightly blue-shifted with respect to the Soret position of ImH complexes of MP8(III) (406.5−407.5 nm). Thus, the 406.1 nm excitation, close to the Soret peak of MP8 aggregates, is expected to be more effective than the 441.6

Table 4: High-Frequency Raman Modes (cm^{-1}) of Imidazole (ImH), Imidazolate (Im^-), 1-Methylimidazole (MeIm), *N*-Acetylhistidine (AcHis), and Histidinate (His^-) Complexes of MP8(III) in Various Aqueous Solutions[a,b,c]

	ligand								
	ImH			AcHis	MeIm	Im^-			His^-
	442 nm[d]	413 nm	407 nm	442 nm	413 nm	442 nm	413 nm	407 nm	442 nm
ν_{10}	1641			1641	1640	1636	1637	1636	1637
		1637	1637		<u>1636</u>		<u>1631</u>		
							1627		
ν_{37}	1603	1603	1604	1604	1603	1603	1600		1602
ν_2	1586	1588	1586	1588	1587	1587	1588	1588	1588
ν_{11}	1564	1569		(L)		1568	1573		(L)
ν_{38}	1554	1554	1557	1557	1554	1557	1558	1556	1558
ν_3	1505	1503	1502	1505	1504	1503	1502	1501	1503
ν_{29}		1407	1406		1408		1403	1406	
ν_{20}	1398			1398					
ν_4	1373	1375	1376	1374	1375	1374	1375	1375	1374
ν_{21}	1318	1316	1316	(L)	1316	1318	1316	1316	(L)
	1300	1302	1300	(L)	1301	1295	1299	1298	(L)

[a] Mode numbering according to Li et al. (1989). [b] The underlined frequencies correspond to the major component of ν_{10} at the given excitation. [c] L represents bands hidden by a free ligand band. [d] Excitation wavelengths.

nm excitation in enhancing the ν_{10} mode of aggregates. Inasmuch as the 406.1 nm excited RR spectra of MP8(III)−ImH complexes show no trace of a 1641 cm^{-1} component (Table 4), we can exclude the possibility that the band observed with the 441.6 and 413.1 nm excitations corresponds to LS aggregates of MP8(III). Using the data obtained from the absorption and Raman titration experiments, the 1637 and 1641 cm^{-1} bands therefore are assigned to the MP8(III)−ImH and MP8(III)−$(ImH)_2$ complexes, respectively.

The ν_{10} Frequency and the Out-of-Plane Distortions of Oxidized Heme c. The preceding assignments are in good agreement with published RR data for a ferriheme *c*−peptide system containing 65 amino acids (H65) and a modified MP8(III) in which the proximal His is blocked and cannot bind heme (Myer, 1985). The addition of ImH to the latter MP8 compound forms a bis(ImH) complex that exhibits ν_{10} at 1641 cm^{-1}. On the other hand, the long peptide chain of H65 protects the proximal Fe−His bond, so that only the mono(ImH) is formed upon ImH addition. This complex exhibits a ν_{10} mode at 1637 cm^{-1} (Myer, 1985). Although heme *c* of MP8(III)−ImH and MP8(III)−$(ImH)_2$ is complexed in both cases by two imidazole rings (His/ImH versus ImH/ImH), the 4 cm^{-1} frequency difference in ν_{10} may be attributed to different porphyrin structures. Indeed, the crystal structures of several cyt *c* show that the porphyrin macrocycle is saddle-shaped (Takano & Dickerson, 1981; Matsuura et al., 1982; Louie & Bayer, 1990; Bushnell et al., 1990). This conformation seems essentially due to the three-points binding between heme and protein, i.e., the two thioether bridges and the Fe−His bond. Considering the chemical structure of MP8, its heme thus could adopt a saddled conformation if the internal His−heme link is preserved. On the contrary, when the Fe−His coordination is broken, one may expect that the out-of-plane distortion of heme is decreased significantly. Taking into account that out-of-plane distortions of the porphyrin skeleton of NiOEP can decrease the frequency of ν_{10} to a large extent (Alden et al., 1989; Czernuszewicz et al., 1989; Shelnutt et al., 1991, 1992; Sparks et al., 1993), the lowest frequency of ν_{10} (1637 cm^{-1}), assigned to the MP8(III)−ImH species, thus should be consistent with a porphyrin structure more distorted than that corresponding to the 1641 cm^{-1} frequency, which is assigned to the MP8(III)−$(ImH)_2$. This interpretation is reinforced by the fact that the six-coordinate LS ferriheme compounds can easily adopt a ruffled structure, with the extent of this out-of-plane porphyrin deformation in bis(imidazole) complexes of Fe(III)-porphyrins seeming to be related to the orientation of axial rings (Scheidt & Gouterman, 1983). Moreover, the wavelength dependence of the ν_{10} frequency of ImH complexes of MP8(III) is analogous to that detected for the same mode of flat and ruffled NiOEP (Alden et al., 1989).

Similarly, the RR spectra of Im^- complexes of MP8(III) show variable frequencies for ν_{10}: a single band at 1636 cm^{-1} is observed when using the 441.6 nm excitation, whereas with the 413.1 nm excitation, a major band at 1631 cm^{-1} is observed along with a shoulder at 1637 cm^{-1} (Figure 3B, spectra c and d, Table 4). By analogy with the behavior observed for the ImH complexes, the 1631 and 1636 cm^{-1} bands are associated with the MP8(III)−Im^- and MP8(III)−$(Im^-)_2$ complexes, respectively. In spite of the increase in porphyrin planarity induced by the displacement of His or His^- by ImH or Im^-, the ImH and His ionizations lead to an apparent decrease in the frequency of ν_{10}. This effect may also be interpreted in terms of a conformational change of the heme. Relative to the neutral ImH and His ligands, the negative charge associated with Im^- and His^- is indeed expected to increase the Fe(III)−ligand bonding interactions and, thus, to increase the out-of-plane deformation (saddling or ruffling) of the porphyrin macrocycle in order to minimize the nonbonding interactions between the heme pyrroles and the Im^- rings (Quinn et al., 1983; Desbois & Lutz, 1992). Therefore, the ν_{10} mode is sensitive to two opposite, related, and overlapping effects: it decreases its frequency with ligand ionization while it increases its frequency when porphyrin deformation is diminished.

The ν_{10} frequency therefore is expected to measure the protein-induced nonplanarity of heme *c* in cytochromes.

Low-Frequency Modes of Imidazole- and Imidazolate-Type Complexes of MP8(III). The 413.1 nm excitation is known to particularly enhance the low-frequency modes of oxidized and reduced cyt *c* (Cartling, 1983, 1988; Hu et al., 1993). Table 5 lists the frequencies observed in the 190−480 cm^{-1} region of the RR spectra of MP8(III) in aqueous CTABr solutions in the presence of ImH and MeIm. At neutral pH,

Table 5: Low-Frequency Raman Modes (cm^{-1}) of Imidazole (ImH), 1-Methylimidazole (MeIm), and Imidazolate (Im^-) Complexes of MP8(III) in Aqueous Solutions[a]

	ligand				
	ImH (pH 8)		MeIm (MeIm-d_6)	Im^- (pH 13)	
	442 nm[b]	413 nm	(pH 8) 413 nm	442 nm	413 nm
γ_{22}		445	447	448	445
	416	416	416 (416)	414	414
		404	409 (406)		409
	381	385		384	381
	377	380	378 (379)	375	376
	362	362	359 (362)	359	360
ν_8	347	344	344 (345)	347	343
ν_{51}					325
	315	318	317		316
	298				
ν_9	267	271	271	268	268
γ_{24}		225			225
		211	217 (216)		214
		199	203 (204)		199

[a] Mode numbering according to Hu et al. (1993). [b] Excitation wavelengths.

these complexes exhibit eight common bands at ca. 200, 215, 269, 345, 360, 377, 400, and 415 cm^{-1}; their relative band intensities depend on the excitation wavelength. Moreover, one may point out that the frequency of ν_8 (ca. 345 cm^{-1}) is wavelength sensitive (Table 5). This mode is observed at 347 and at 343−345 cm^{-1} using 441.6 and 413.1 nm excitation, respectively. This difference in the ν_8 frequency is analogous to the frequency dispersion of ν_{10} and, thus, is apparently sensitive to out-of-plane porphyrin distortions. This sensitivity is plausible for a mode involving the stretching of metal−N(pyrrole) bonds (Li et al., 1989; Hu et al., 1993). On the other hand, bands observed in the 215, 344, 360, and 400 cm^{-1} regions are sensitive to the mass of the bound imidazole and, at least in part, to the ionization state of the axial ligand(s) (Figure 4A,B). The bands at 211, 362, and 404 cm^{-1} in the spectrum of MP8(III)−ImH are observed at 214, 360, and 409 cm^{-1} at alkaline pH, respectively (Figure 4A). When MP8(III) is complexed to MeIm, these latter bands are detected at 217, 359, and 409 cm^{-1} (Table 5). The MeIm → MeIm-d_6 isotopic substitution in MP8(III)−MeIm reveals that the 344 (ν_8) and 359 cm^{-1} bands are upshifted by 1 (±0.5) and 3 (±1) cm^{-1}, respectively, while the 409 cm^{-1} band is downshifted by 3 (±1) cm^{-1} (Figure 4B). These different sensitivities therefore indicate that the 211−214, 359−362, and 403−409 cm^{-1} bands correspond to modes involving the axial bonds and/or the ligand(s) of heme *c* (vide infra).

Visible Absorption and Resonance Raman Spectroscopy of Ferrous MP8 Derivatives

Absorption of MP8(II) without Added Ligand. The absorption spectra of MP8(II) in aqueous solutions containing neither detergent nor ligand are dependent on the hemopeptide concentration and pH, like those of MP8(III) in the same solvent conditions. At pH 7.5−12, LS bands at 412−416, 548−549, and 520−522 nm are indeed more or less clearly superimposed on an HS spectrum with Soret and broad α/β bands at ca. 430 and 550 nm, respectively (spectra not shown). On the other hand, we previously showed that MP8-(II) in aqueous CTABr solutions is essentially HS, with a

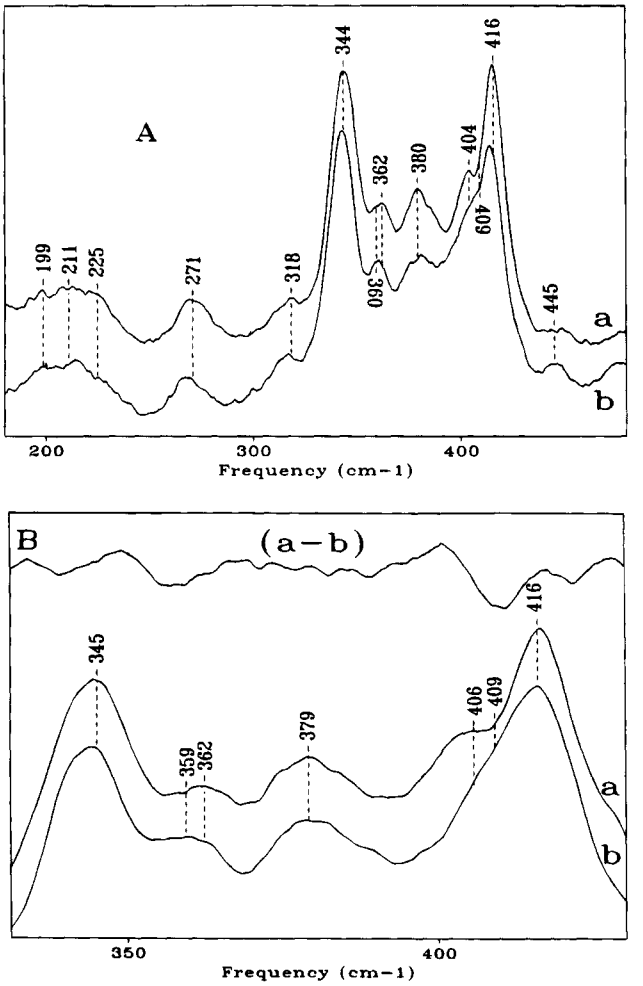

FIGURE 4: (A) Low-frequency regions (180−480 cm^{-1}) of RR spectra of MP8(III) in 1% aqueous CTABr solutions containing 0.1 M ImH: (a) 100 mM phosphate, pH 7.5; (b) 0.1 M KOH. Excitation: 413.1 nm. (B) 330−430 cm^{-1} regions of RR spectra of MP8(III) in 1% aqueous CTABr solutions containing 10^{-2} M MeIm (pH 7.5): (a) MeIm-d_6; (b) MeIm; (a − b) difference spectrum. Excitation: 413.1 nm.

Soret maximum dependent on the hydroxide concentration and seen in the 428.5−434 nm region (Othman et al., 1993a).

Absorption of Imidazole and Imidazolate Complexes of MP8(II). Spectrophotometric titrations of MP8(II) with ImH in neutral aqueous CTABr solution document two ligand binding reactions with apparent association constants of 5 × 10^1 and 2 × 10^{-1} M^{-1}. These data indicate both a lower affinity of ImH for MP8(II) than for MP8(III) and the possible displacement of the proximal His of MP8(II) by ImH at high ligand concentration. The absorption spectra of ImH and AcHis complexes of MP8(II) in aqueous CTABr both exhibit band maxima at 417.5, 522, and 551 nm (Table 6). The titration of these complexes in aqueous CTABr by hydroxide gives new LS spectra with red-shifted Soret, β, and α bands (Table 6). This general red shift is similar to that detected upon alkaline titration of Fe(II)PP(ImH)$_2$ in aqueous CTABr solution (Desbois & Lutz, 1992), but as in the latter case, the detergent precipitation at high hydroxide concentrations did not allow the full deprotonation of the two axial rings of MP8(II)−ImH or MP8(II)−(ImH)$_2$. The titration curves of MP8(II) with ImH in a CTABr aqueous solution containing 2.5 M KOH fit with two apparent binding constants of 2 × 10^1 and 8 × 10^{-1} M^{-1} (correcting for the total concentrations of free ImH and Im^-).

Table 6: Absorption Maxima of MP8(II) Derivatives

ligand	α/β	Soret	solvent conditions[a]
ImH	521/550	415.5	1 M, −CTABr, pH 8
ImH	521/550	416.5	7 M, −CTABr, pH 8
ImH	522/551	417.5	1 M, +CTABr, pH 7.5
AcHis	522/551	417.5	1 M, +CTABr, pH 8
ImH + Im^-	525/553	422.5	1 M, +CTABr, 2.5 M OH^-

[a] Ligand concentration, absence or presence of CTABr (− or +), pH or hydroxide concentration.

High-Frequency Regions of Resonance Raman Spectra of Imidazole- and Imidazolate-Type Complexes of MP8(II). The high-frequency RR spectra (1280−1680 cm^{-1}) of ImH and Im^- complexes of MP8(II) show marker bands specific to LS hemes (Table 7). As in the ferric series, the RR spectra of ImH and Im^- complexes of MP8(II) exhibit a sensitivity to the exciting wavelength, an effect particularly marked for the ν_{11} mode and to a lesser extent for the ν_{10} mode (Figure 5A,B, Table 7). The titration of ImH complexes of MP8-(II) with hydroxide leads to an apparent downshift of the ν_4, ν_{10}, and ν_{11} modes, as was qualitatively observed for the sequential ligand deprotonations of Fe(II)PP(ImH)$_2$ in aqueous CTABr solutions (Desbois & Lutz, 1992) (Table 7, Figure 5B). In the case of the ν_{11} mode, for example, its frequency is decreased from 1539 to 1527 cm^{-1} when the hydroxide concentration is gradually increased from 10^{-6} to 2.5 M (Figure 5B).

Given the values of association constants obtained for the binding of ImH to MP8(II), along with the ligand concentration used (1 M), the ImH complexes of MP8(II) in the experiments corresponding to Figure 5A,B are, in fact, mixtures of MP8(II)−ImH (83%) with MP8(II)−(ImH)$_2$ (17%). The ν_{10} and ν_{11} frequencies of the ImH complexes of MP8(II) are both dependent on the excitation wavelength: ν_{10} is detected at 1621 and 1617 cm^{-1} with 441.6 and 413.1 nm excitation, respectively, while ν_{11} is composed of two overlapping bands of variable relative intensity at 1539 and 1533 cm^{-1} (Figure 5A,B, Table 7). Considering the 4 and 6 cm^{-1} frequency differences detected for ν_{10} and ν_{11}, respectively, one may suggest that, as for ImH complexes of ferric MP8, these frequency variations correspond to porphyrin structure heterogeneities due to the displacement of the axial His by ImH. However, in NiOEP complexes, the frequency shift of ν_{10} is roughly twice as sensitive to porphyrin distortions as that of ν_{11} (Czernuszewicz et al., 1989; Shelnutt et al., 1992; Sparks et al., 1993). Thus, the frequency variations of the ν_{10} and ν_{11} modes of ImH complexes of MP8(II) cannot be totally attributed to a difference in the porphyrin saddling.

On the other hand, taking into account the high ligand concentrations used in our experiments, MP8(II) aggregation may come into play by canceling the detergent effect and, thus, may compete with the ImH ligation. Since the Soret maxima of LS MP8(II) aggregates are in the 412−416 nm region, 413.1 nm is expected to be the most selective wavelength to enhance their RR contributions. Using this excitation wavelength, the RR spectra of MP8(II) aggregates generated in 1 M phosphate buffer at pH 7.5 or in 3 M KOH exhibit bands at 1615 and 1532−1533 cm^{-1} corresponding to ν_{10} and ν_{11} (Figure 5A,B, Table 7). Therefore, we cannot exclude the possibility that the 1533 cm^{-1} component observed in ImH complexes of MP8(II) could correspond, at least partially, to contributions arising from LS MP8(II) aggregates (Figure 5b). Thus, the 1539 and 1621 cm^{-1} bands, respectively, are assigned to the ν_{11} and ν_{10} modes of a bis(imidazole)-type heme coordination in MP8 (MP8-(II)−ImH or MP8(II)−(ImH)$_2$). If we assume that the ν_{10} frequency of the ImH complexes of MP8(II) is influenced by a significant contribution from aggregates (Table 7), its sensitivity to the expected change in the porphyrin structure, induced by the replacement of His by an extrinsic ImH, cannot be precisely evaluated. However, inspection of Table 7 indicates that the corresponding ν_{10} shift cannot exceed 4 cm^{-1}. Considering again that the sensitivity to the heme conformation is weaker for the ν_{11} mode than for ν_{10} by a factor of 2 (Czernuszewicz et al., 1989; Shelnutt et al., 1992; Sparks et al., 1993), the frequency difference of the ν_{11} modes of MP8(II)−ImH and MP8(II)−(ImH)$_2$ should be, moreover, experimentally unattainable since it is close or equal to the frequency uncertainty of the corresponding bands (1 cm^{-1}).

On the other hand, although the 12 cm^{-1} maximal frequency variation observed for the ν_{11} modes of ImH and Im^- complexes of MP8(II) is significantly lower than the 16 cm^{-1} observed for the same modes of bis(ImH) and bis(Im^-) complexes of Fe(II)PP (Desbois & Lutz, 1992), the 1527 cm^{-1} frequency is attributed to the ν_{11} mode of a bis-(imidazolate) heme coordination, i.e., MP8(II)−Im^- or/and MP8(II)−(Im^-)$_2$. As far as the intermediate frequencies (1533−1536 cm^{-1}) are concerned, they could be assigned to the ν_{11} mode of an imidazole/imidazolate heme coordination: first, MP8(II)−ImH with the proximal His deprotonated, second, MP8(II)−Im^- with a protonated His, or finally, MP8(II)−(ImH,Im^-). They are, however, too close to the ν_{11} frequency of MP8(II) aggregates (1532−1533 cm^{-1}) to warrant complete confidence in these assignments.

Therefore, as in the case of *b*-type cytochromes, the frequency of the ν_{11} mode is expected to give information on the H-bonding and ionization states of His bound to the heme of *c*-type cytochromes (Desbois & Lutz, 1992).

Low-Frequency Modes of Imidazole- and Imidazolate-Type Complexes of MP8(II). The low-frequency regions of RR spectra of ImH, Im^-, and MeIm complexes of MP8(II) show strong analogies to those of the corresponding ferric complexes (Figures 6A−C). They also exhibit close similarities to those of various reduced cyt *c* (Desbois, 1994), supporting the validity of spectroscopic studies concerning the MP8 derivatives as coordination models of heme *c* in cytochromes.

As in the RR spectra of ImH, Im^-, and MeIm complexes of MP8(III), RR bands sensitive to the nature, the mass, and/or the protonation state of the imidazole ring are located in the ca. 190, 345, 360, and 400 cm^{-1} regions of the spectra of the corresponding reduced compounds (Table 8). In the lowest frequency region, a broad band is indeed shifted from 197 to 189 and 185 cm^{-1} in the spectra of the ImH, MeIm, and MeIm-d_6 complexes, respectively (Table 8, Figure 6A). The bands observed at 344 and 358 cm^{-1} for MP8(II)−MeIm are upshifted by 1 (±0.5) and 3 (±1) cm^{-1}, respectively, upon ligand perdeuteriation (Figure 6B). A homologue of the latter band observed at 361 cm^{-1} in the MP8(II)−ImH spectrum is downshifted to 359 cm^{-1} in the MP8(II)−Im^- spectrum (Figure 6C). As far as the ca. 400 cm^{-1} band is concerned, it is observed at 403, 401, and 403 cm^{-1} for the MeIm, ImH, and Im^- complexes, respectively, and is downshifted by 3 (±1) cm^{-1} from 403 to 400 cm^{-1} when the axial MeIm is perdeuteriated (Figure 6B,C).

Table 7: High-Frequency Raman Modes (cm^{-1}) of Imidazole (ImH), Acetylhistidine (AcHis), and Imidazolate (Im^-) Complexes of MP8(II) in Aqueous Solutions and of Aggregated MP8(II) in water[a,b]

	ligand (L)								
	ImH (pH 8)		AcHis	ImH + Im^- (1 M OH^-)		ImH + Im^- (2.5 M OH^-)		no L, 413 nm	
	442 nm[c]	413 nm	442 nm	442 nm	413 nm	442 nm	413 nm	(pH 7.5)	(2.5 M OH^-)
ν_{10}	1621	1617	1620	1619	1617	1618		1615	
ν_{37}	1603	1603	1604	1604	1603	1604	1603	1604	1602
ν_2	1590	1592	1590	1590	1590	1590	1590	1590	1591
ν_{38}	1561	1561	1561	1559	1556	1558	1556	1556	1556
ν_{11}	1539	1539	1540						
	1534	1533		1536	1534	1533	1533	1534	1532
						1527	1527		
ν_3	1491	1494	1490	1491	1493	1491	1494	1493	1493
ν_{29}	1396	1396	1396	1394	1395	1393	1391	1392	1393
ν_4	1360	1358	1359	1359	1357	1357	1356	1358	1356
ν_{21}	1312	1313	1315		1314	1310	1310	1313	1312
	1298	1297	1295	1296	1298	1296	1298	1297	1294

[a] Mode numbering according to Li et al. (1989). [b] The underlined frequencies correspond to the major component of ν_{11}. [c] Excitation wavelengths.

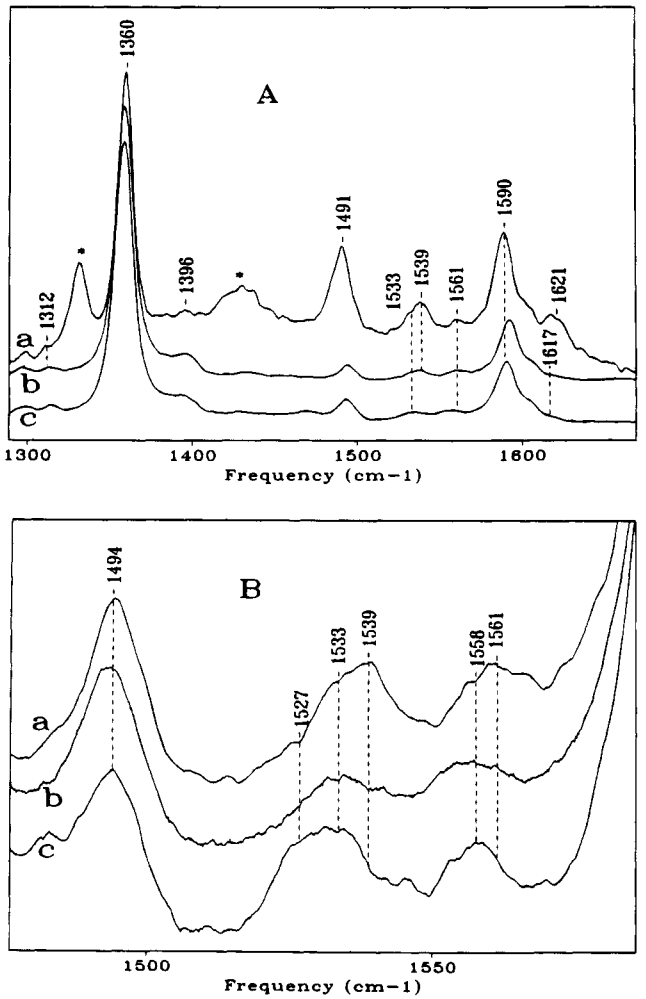

FIGURE 5: (A) High-frequency regions (1300−1650 cm^{-1}) of RR spectra of MP8(II) in aqueous solutions: (a) 1% CTABr, 1 M ImH, 100 mM phosphate, pH 7.5, 441.6 nm excitation; (b) 1% CTABr, 1 M ImH, pH 7.5, 413.1 nm excitation; (c) 1 M phosphate, pH 7.5, 413.1-nm excitation. (B) Extended scale of the 1480−1580 cm^{-1} regions of the RR spectra of MP8(II) in aqueous solutions: (a) 1% CTABr, 1 M ImH, 100 mM phosphate, pH 7.5; (b) 1 M phosphate, pH 7.5; (c) 1% CTABr, 1 M ImH, 2.5 M KOH. Excitation: 413.1 nm.

Modes Involving the Axial Ligands

The low-frequency bands observed at 185−217, 343−347, 357−362, and 400−409 cm^{-1} in the RR spectra of various imidazole complexes of MP8(II) and MP8(III) are sensitive to the mass and the ionization state of the bound imidazole(s) (Tables 5 and 8). The frequencies of two of them (the ca. 200 and 400 cm^{-1} bands) are significantly dependent on the oxidation state of MP8 (Figures 4B and 6B, Tables 5 and 8). All of these observations suggest that these four bands have, at least in part, origins in modes involving the axial bond(s) and/or the axial ligand(s) of heme.

For equivalent imidazoles (L) bound to Fe−porphyrins, the symmetric stretching mode of axial ligands [ν_s(Fe−L_2)] has been detected at 194−226 cm^{-1} with Soret excitations (Desbois & Lutz, 1981, 1992; Choi & Spiro, 1983; Mitchell et al., 1987; Desbois et al., 1989). Axial ligand perdeuteriation (MeIm → MeIm-d_6) on MP8(II)−MeIm produces a 4 ± 1 cm^{-1} downshift of the 189 cm^{-1} band (Figure 6A); this band is thus assigned to the ν_s(His−Fe−MeIm) mode. A comparison with the 194 cm^{-1} frequency of the ν_s(MeIm−Fe−MeIm) of Fe(II)PP−$(MeIm)_2$, which exhibits an 8 cm^{-1} downshift upon MeIm → MeIm-d_6 substitution (Desbois & Lutz, 1981; Desbois et al., 1989), further supports the assignment of the 189 cm^{-1} band of MP8(II)−MeIm. The 4 cm^{-1} difference in the isotopic shifts is indeed in agreement with the binding of only one MeIm molecule to MP8(II) in the experiments of Figure 6A.

The asymmetric stretching mode [ν_{as}(Fe−L_2)] is infrared-active for symmetrical ligations of two identical imidazoles to Fe−porphyrins and is located in the 390 cm^{-1} region (Ogoshi et al., 1973). It is predicted to be Raman-active if the ligands are inequivalent (L−Fe−L′) (Spiro & Li, 1988), as in the monoligated complexes of MP8. On the basis of the 2−3 cm^{-1} downshift of the 409 and 403 cm^{-1} bands of MP8(III)−MeIm and MP8(II)−MeIm, respectively, these bands therefore are assigned to a mode strongly involving ν_{as}(His−Fe−MeIm). Although the asymmetric stretching mode of axial bonds could be mixed with a pyrrole tilting mode (Mitchell et al., 1987), a calculation using a linear oscillator for the His−Fe(II)−MeIm bonds (Herzberg, 1945; Desbois & Lutz, 1992) satisfactorily reproduces the experimental frequencies, as well as the experimental isotopic shifts of the ν_s and ν_{as} modes of the axial bonds, if masses of 82 (His, MeIm), 56 (Fe), and 88 (MeIm-d_6) amu and force constants of 1.37 (Fe−His) and 2.51 (Fe−MeIm) mdyn $Å^{-1}$ are used. The low-frequency RR spectra of lysine and

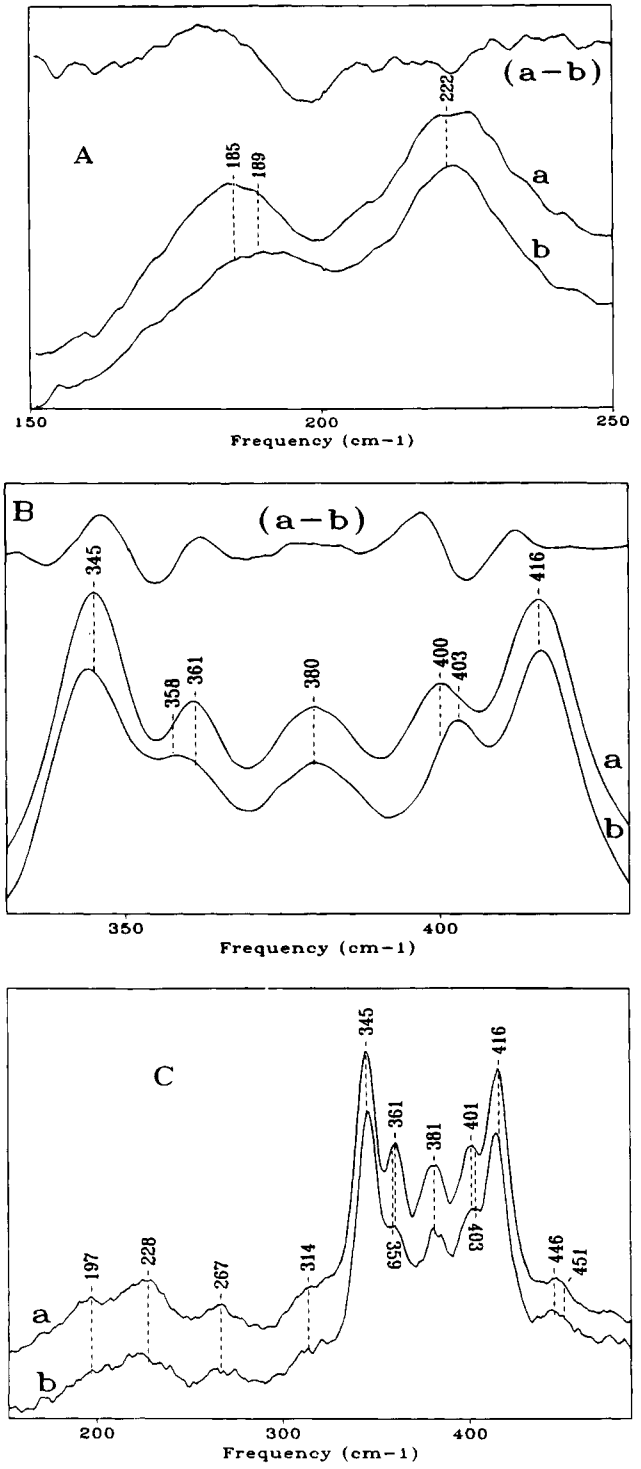

FIGURE 6: 150−250 cm^{-1} (A) and 330−430 cm^{-1} (B) regions of RR spectra of MP8(II) in 1% aqueous CTABr solutions containing 0.28 M MeIm (pH 7.5): (a) MeIm-d_6; (b) 1MeIm; (a − b) difference spectrum. The S-shaped band of the difference spectrum in A corresponds to a 4.1 (±1) cm^{-1} shift of the 189 cm^{-1} band (Rousseau, 1981). Excitation: 431.1 nm. (C) 180−480 cm^{-1} region of RR spectra of MP8(II) in 1% aqueous CTABr solutions containing 1 M ImH: (a) pH 7.5; (b) 2.5 M KOH. Excitation: 413.1 nm.

acetylmethionine complexes of MP8(II) and MP8(III), however, show that a shoulder at ca. 400 cm^{-1} remains present on the 414−416 cm^{-1} band, suggesting that the ν_{as}-(His−Fe−MeIm) mode largely overlaps a weak unspecific band (Othman et al., 1993b), presumably a bending mode of peripheral heme groups (Hu et al., 1993).

Table 8: Low-Frequency Raman Modes (cm^{-1}) of Imidazole (ImH), Acetylhistidine (AcHis), 1-Methylimidazole (MeIm), and Imidazolate (Im^-) Complexes of MP8(II) in Aqueous Solutions[a]

	ligand (L)						
	ImH (pH 8)		AcHis (pH 8)	MeIm (MeIm-d_6) (pH 8)	ImH + Im^- (2.5 M OH^-)		no L (pH 7.5)
	442 nm[b]	413 nm	442 nm	413 nm	442 nm	413 nm	413 nm
γ_{22}		451		451	449	451	448
		446		446		445	
	416	416	415	416 (416)	416	414	412
	398	401	398	403 (400)		403	402
					398	395	
	379	381	382	380 (380)		381	381
	375	376	376		377		
	359	361	361	358 (361)	357	359	360
							352
ν_8	347	345	347	344 (345)	347	346	347
ν_{51}		324		319	323	321	316
	308	314	309		307	311	
		303				299	
ν_9	266	267	265	264	266	267	269
γ_{24}	234	228	230		230	235	
				222 (222)		223	222
			215		210	206	205
		197	192	189 (185)	194	197	196

[a] Mode numbering according to Hu et al. (1993). [b] Excitation wavelengths.

Finally, the assignment of the ca. 345 and 361 cm^{-1} bands poses a problem since the MeIm → MeIm-d_6 substitutions in the oxidized as well as the reduced MP8−MeIm complexes induce upshifts of 1 and 3 cm^{-1}, respectively (Figures 4B and 6B, Tables 5 and 8). A positive shift is generally indicative of a change in the force constant of coupled modes, as was found for heterocycles containing nitrogen atoms (Susi & Ard, 1971; Abe & Kyogoku, 1987). Moreover, contrary to the 185−221 and 400−409 cm^{-1} bands, which show significant upshifts upon heme oxidation, the 345 and 361 cm^{-1} bands present no clear sensitivity to the oxidation state of heme. In the low-frequency regions of RR spectra of yeast cyt *c*, Hu et al. (1993) assigned bands at 347 and 360 cm^{-1} to stretching modes involving the metal−N(pyrrole) bonds (ν_8 and ν_{50}, respectively) from a normal mode calculation on NiOEP (Li et al., 1989), i.e., on a metalloporphyrin without any axial ligand. A deformation mode of the axial bonds and/or a internal mode of the imidazole ring(s) coupled with an Fe−N(pyrrole) stretching mode therefore could be plausible candidates for the assignment of these bands.

No RR band candidate for an Fe−ligand mode has been identified in the spectra of yeast cyt *c*, which has imidazole and thioether as heme ligands (Hu et al., 1993). Nevertheless, the MeIm → MeIm-d_6 substitution in the MP8−MeIm complexes produces a change in the relative mass of the ligand that is more significant (7.3%) than that corresponding to the CH_3 → CD_3 substitution performed on the thioether heme ligand of yeast cyt *c* (4.9%) (Hu et al., 1993). Moreover, the substitution of a thioether by an imidazole in the sixth axial coordination of heme *c* slightly changes the electronic properties of heme, which in turn may permit an RR enhancement sufficient for the observation of axial modes in the RR spectra of imidazole and imidazolate complexes of MP8.

Bis(histidyl) Coordination in c-Type Cytochromes

The RR data obtained here for the ImH, MeIm, and Im^- complexes of MP8(II) and MP8(III) demonstrate that the extraction of structural information concerning the porphyrin and its Fe−His bonds in *c*-type cyt is possible. They can be used in the determination of the heme coordination of cytochromes of unknown structure, thus constituting a predictive aspect of our investigation. The number of known cyt *c* structures that possess a bis(histidyl) coordination for heme is still relatively limited. This rarity may be related to the fact that the bis(His) ligation imposes low redox potentials for the heme (−400 to +20 mV) (Harbury & Loach, 1960b; Mashiko et al., 1979; Moore & Pettigrew, 1990). The classical example of bis(His) heme coordination in *c*-type cytochromes is that of the tetraheme and multiheme cyt c_3 groups from sulfate- and sulfur-reducing bacteria (Moore & Pettigrew, 1990; Yamanaka, 1992). The structure of the tetraheme cyt *c* associated with the photosynthetic reaction center reveals that one of the four hemes (c_{552}) is bis(His)-coordinated (Deisenhofer & Michel, 1989). The low-potential heme of some cyt c_{550} from cyanobacteria and algae also appears to be bis(His)-ligated (Cohn et al., 1989).

Among these cyt, only two tetraheme cyt c_3, in which all of the hemes are each coordinated by two His residues, have been investigated by RR spectroscopy (Kitagawa et al., 1975, 1977; Desbois & Lutz, 1984; Verma et al., 1988; Desbois, 1994). The electronic spectra of these cyt c_3 exhibit Soret maxima that are at ca. 410 and 419 nm for the oxidized and reduced states, respectively (Yamanaka, 1992). With respect to the absorption data obtained here for the ImH complexes of MP8(II) and MP8(III) (Tables 3 and 6), the 2.5−4 nm red shifts of the Soret bands of cyt c_3 may suggest that the His ligands of the four hemes *c* could possess markedly electronegative character. A similar conclusion may be reached by considering the redox potentials of the MP8−ImH complex and of cyt c_3: −210 mV versus −290 to −205 mV (Harbury & Loach, 1960a; Valentine et al., 1979; Yamanaka, 1992). However, the RR frequency of the ν_{11} mode has been located at 1539−1541 cm^{-1} for the fully reduced cyt c_3 from *Desulfovibrio vulgaris* Miyazaki and *Desulfovibrio vulgaris* Hildenborough at pH 7.5 (Kitagawa et al., 1977; Verma et al., 1988; Desbois, 1994). Table 7 shows that this frequency corresponds to imidazole-type ligands in the MP8(II) complexes. Thus, the absorption and redox properties of cyt c_3 appear to be determined primarily by environmental effects (Churg & Warshel, 1986) and not by the partial anionic character of the heme ligands.

We have shown, from the low-frequency RR spectra of ImH and Im^- complexes of MP8(II) and MP8(III), that the modes involving the axial bonds are obviously expected to provide the most information about the axial bonds and, thus, about the ionization states of the heme ligands in cyt *c*. Unfortunately, data on the low-frequency modes of c_3-type cyt are still scarce. The low-frequency RR spectra of reduced cyt c_3 from *Desulfovibrio vulgaris* Miyazaki excited at 514.5 nm and from *Desulfovibrio vulgaris* Hildenborough excited at 441.6 nm have been obtained (Kitagawa et al., 1975; Desbois & Lutz, 1984; Desbois, 1994). On the basis of comparison with several mitochondrial cyt *c*-containing His/Met heme ligations, a specific band at 209 cm^{-1} has been proposed to correspond to a ν_s(His−Fe−His) mode of cyt c_3 from *Desulfovibrio vulgaris* Hildenborough (Desbois & Lutz, 1984). On the other hand, strong bands have been observed at 396 and 400 cm^{-1} in the RR spectra of cyt c_3 from *Desulfovibrio vulgaris* Miyazaki and *Desulfovibrio vulgaris* Hildenborough, respectively (Kitagawa et al., 1975; Desbois & Lutz, 1984). Taking into account the RR data obtained for the ImH and MeIm complexes of MP8(II), they are good candidates for a ν_{as}(His−Fe−His) mode. In the frame of this assignment, the 4 cm^{-1} difference suggests slight changes in the His/heme interactions for the two cyt c_3.

CONCLUSION

The RR data obtained here for different imidazole complexes of MP8(III) and MP8(II) are expected to characterize the ionization and H-bonding states of heme ligands, as well as the porphyrin structure in *c*-type cytochromes containing two histidine residues as heme axial ligands. Although the low-frequency regions of the RR spectra of cyt *c* are very congested, this study has further demonstrated the possibility of direct observation of modes involving the axial bonds. All of these marker bands doubtless will yield more precise and detailed information concerning heme−protein interactions in cytochromes. Finally, this study on bis(imidazole) and bis(imidazolate)-type heme *c* ligations will be suitable for future comparison with absorption and RR data obtained on *N*-acetylmethionine and lysine complexes of MP8(II) and MP8(III) in which mixed heme coordinations occur.

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