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ARTICLE

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Resonance Raman investigation of lysine and N-acetylmethionine complexes of ferric and ferrous microperoxidase

Influences of the axial ligation on the heme c structure

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Abstract In order to evaluate the steric and electronic effects of mixed axial ligations on the heme c structure, lysine (Lys) and N-acetylmethionine (AcMet) complexes of ferric and ferrous microperoxidase-8 (MP8(III) and MP8(II), respectively) are characterized by absorption and resonance Raman (RR) spectroscopies. Spectrophotometric titrations establish that MP8(III) binds one molecule of exogenous ligand while MP8(II) forms mono(ligated) and bis(ligated) compounds. The Soret-excited RR spectra of the six-coordinated low-spin MP8(III) complexes show that the macrocycle can adopt different structures between planar and ruffled conformations. The ferriheme c conformation is primarily determined by the ionization state of the His side chain of MP8(III) and, secondarily, by the bonding and nonbonding heme-ligand interactions. As far as the RR spectra of the MP8(II) complexes are concerned, they permit us to conclude that the mixed His/Lys and His/AcMet coordinations induce a nonplanar heme conformation, the extent of deformation again depending on the ionization state of the endogenous His ligand. In contrast, the RR spectra of the bis(Lys) and bis(AcMet) compounds are associated with a planar heme structure. When the His of MP8 is bound to heme c, the stabilization of distorted heme conformations is thus associated with constraints exerted by the Cys-Ala-Gln-Cys-His-peptide on the porphyrin macrocycle. More generally, the spectroscopic data obtained in this study can be used to predict both the axial coordination and the structure of heme in c-type cytochromes.

Key words Heme-ligand interaction · Heme structure · Porphyrin modes

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Abbreviations *MP8* Microperoxidase-8 · *cyt* Cytochrome · *CTABr* Cetyltrimethylammonium bromide · *ImH* Imidazole · *Im* Imidazolate · *His* Histidine · *His*$^-$ Histidinate · *Lys* Lysine · *AcMet* N-acetylmethionine · *Met* Methionine · *5c* Five-coordinated · *6c* Six-coordinated · *LS* Low-spin · *HS* High-spin · *RR* Resonance Raman

Introduction

The axial ligation of heme constitutes one of the major factors determining the electron transfer properties of cytochromes (cyt). Considering the proteins carrying one or several six-coordinated heme(s), a survey of the available X-ray structures shows two main types of coordination. On one hand, N(His)/N(His) ligation was found in monoheme cyt b_2 and b_5 and in tetraheme cyt c_3 (Mathews et al. 1972; Pierrot et al. 1982; Xia and Mathews 1990). On the other hand, N(His)/S(Met) heme coordination was characterized in several mitochondrial and bacterial c-type cytochromes and in *E. coli* cyt b_{562} (Salemme et al. 1973; Takano and Dickerson 1981; Matsuura et al. 1982; Carter et al. 1985; Lederer et al. 1981). More recently, two new types of heme coordination were confirmed by X-ray crystallography for chloroplast cyt f with a N(His)/N(amine) coordination and for *E. coli* cyt b_1 with a bis-S(Met) coordination (Martinez et al. 1994; Frolow et al. 1994).

Studies using heme model compounds and cytochromes modified by either semisynthesis or site-directed mutagenesis showed that, in the absence of measurable changes in the heme environment, an imidazole/imidazole → imidazole/thioether substitution in an axial position provokes a positive shift of the redox potential by 110–220 mV (Harbury et al. 1965; Warme and Hager 1970; Mashiko et al. 1979; Raphael and Gray 1989; Dolla et al. 1994). In contrast, a negative shift of 190–330 mV is detected upon His/thioether → His/aliphatic amine change in microperoxidase-8 (MP8) and cyt c_{550} (Harbury and Loach 1960b; Harbury et al. 1965; Ubbink et al. 1994). These observations clearly illustrate how the His, Met or amine ligands

Table 1 Axial coordinations, formation constants and absorption maxima of lysine and N-acetylmethionine complexes of MP8(III)

Ligand	Solvent conditions[a]	Axial heme ligation	K_a or $1/c_{1/2}$ (M^{-1})	CT[b]	α	β (nm)	Soret
Lys	–CTABr, pH 10.2	N(His)/N(Lys)	$2 \cdot 10^3$		530	556	405
	–CTABr, pH 14	$N(His^-)$/N(Lys)	n.d.		533	560	408.5
	+CTABr, pH 12–14	$N(His^-)$/N(Lys)	$4 \cdot 10^1$		532	560	408
AcMet	–CTABr, pH 7.5	N(His)/S(AcMet)	1.6	690	526	558	409.5
	+CTABr, pH 7.5	N(His)/S(AcMet)	1	698	526	557	409
	+CTABr, pH 12.5	$N(His^-)$/S(AcMet)	n.d.	632	537	557	412

[a] Absence or presence of CTABr (– or +), pH; [b] Charge transfer band

can influence the redox potential of heme. Full understanding of the effects of axial heme ligands on the cytochrome redox site however necessitates a knowledge of the interdependent bonding and non-bonding interactions of the iron-porphyrin with its ligands. Whereas many studies concern complexes modeling the bis(His) coordination in heme proteins, few investigations have been devoted to heme compounds containing mixed His/Met or His/amine coordinations. The MP8 system offers the possibility of preparing heme complexes with different axial ligands. This heme c-octapeptide, obtained by enzymatic cleavages of horse cyt c, contains a His residue linked to the heme iron and is able to react with a variety of exogenous ligands (Harbury and Loach 1960a, b; Harbury et al. 1965; Byfield et al. 1993). As part of our studies on heme c model compounds, we previously investigated, by absorption and resonance Raman (RR) spectroscopies, the imidazole (ImH) and imidazolate (Im) complexes of ferric and ferrous MP8 (Othman et al. 1994). Characterization of lysine (Lys) and N-acetylmethionine (AcMet) complexes of oxidized and reduced MP8 is of interest in terms of biomimetic studies since they simulate the His/amine and His/Met heme coordinations of c-type cytochromes and permit us to measure the influence of mixed iron ligations on the heme c structure.

Materials and methods

Microperoxidase and exogenous ligands. Microperoxidase-8 (Sigma) was used without further purification. The coordination of Lys and of AcMet to oxidized and reduced MP8 (MP8(III) and MP8(II), respectively) has been studied as a function of pH. These ligands were purchased from Merck, Sigma, Lancaster or Aldrich and used without further purification. The equilibrium constants for the ligand binding to MP8(III) and MP8(II) were investigated at 20 ± 1 °C by spectrophotometric titrations according to methods previously described (Brault and Rougée 1974; Othman et al. 1994). The preparations of ligated MP8 derivatives in water have been previously reported (Harbury and Loach 1960a, b; Harbury et al. 1965). However, in order to limit the tendency of MP8 to form aggregates and to facilitate the ionization of the proximal His, a cationic detergent, cetyltrimethylammonium bromide (CTABr) (Sigma), was added to the aqueous solutions of MP8 at a micelle-forming concentration (1%) (Othman et al. 1993, 1994). For pH values from 7.0 to 12.0, the MP8 solutions were buffered with 50–100 mM potassium phosphate, Tris-HCl or sodium carbonate. At pH values higher than 12.0, concentrated KOH solutions (1–10 N) were added to unbuffered aqueous solutions of MP8. The reduced MP8 derivatives were obtained by addition of solid dithionite under vacuum (Othman et al. 1993, 1994).

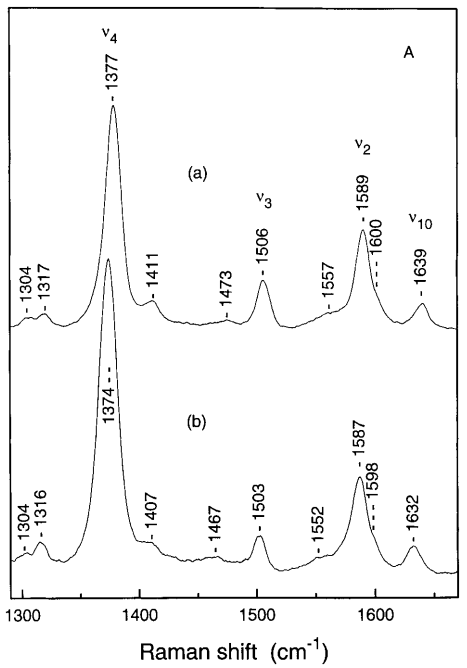
Spectroscopy. The spectrophotometric titrations and UV-visible absorption spectra were obtained with Beckman DU7 and Shimadzu UV-160 spectrophotometers. The Raman spectra were recorded at room temperature (20 ± 1 °C) on a Jobin-Yvon spectrometer (HG 2S) with Soret excitations, i.e. the 406.7- and 413.1-nm lines of a Kr^+ laser (Coherent Innova) and/or the 441.6-nm line from a He-Cd laser (Liconix). Fluorescent impurities in commercial MP8 do not allow a RR investigation with Q-band excitations. Radiant laser powers of 15–40 mW were used. The Raman spectrometer was calibrated at each excitation with the bands of a 1/1 mixture (v/v) of benzene and dichloromethane (Merck, Spectroscopic grade).

Under particular solvent (pH, ligand concentration, presence of detergent) and excitation conditions, the AcMet and Lys complexes of MP8(III) can exhibit slight heme photoreduction. This process was greatly limited by stirring the solutions of MP8(III) complexes (Desbois et al. 1989). The small reduced contributions observed in some RR spectra were removed by computer subtraction using the spectra of the dithionite-reduced MP8 derivatives, prepared under the same solvent conditions. Elimination of the photoreduced contributions was performed using Spectra Calc software (Galactic Industries) (Othman et al. 1994). The RR spectra shown were the unsmoothed sums of several scans (2–8). To ascertain the positions of overlapping bands in the 340–450 and 1450–1650 cm^{-1} regions of RR spectra, fittings with Lorentzian band shapes and linear backgrounds were made with a nonlinear least-squares program of the Grams/32 software (Galactic Industries).

Results

Lysine complexes of MP8(III)

Considering the weak acidity of the Lys ammonium groups [$pK_a(\alpha\text{-}NH_3^+) = 8.9$ and $pK_a(\varepsilon\text{-}NH_3^+) = 10.3$], the prepara-



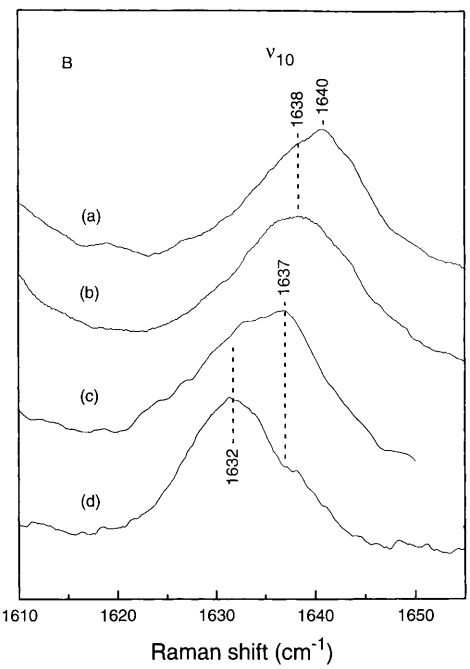


Fig. 1 **A** High-frequency regions (1300–1650 cm^{-1}) of RR spectra of lysine complexes of MP8(III): *a* 0.05 M Lys, pH 10.2, excitation: 406.7 nm; *b* 0.5 M Lys, CTABr 1%, pH 12.0, excitation: 413.1 nm. **B** ν_{10} regions (1610–1650 cm^{-1}) of RR spectra of lysine complexes of MP8(III): *a* 0.05 M Lys, pH 10.2, excitation: 406.7 nm; *b* 0.05 M Lys, pH 10.2, excitation: 413.1 nm; *c* 0.5 M Lys, CTABr 1%, pH 12.0, excitation: 406.7 nm; *d* 0.5 M Lys, CTABr 1%, pH 12.0, excitation: 413.1 nm

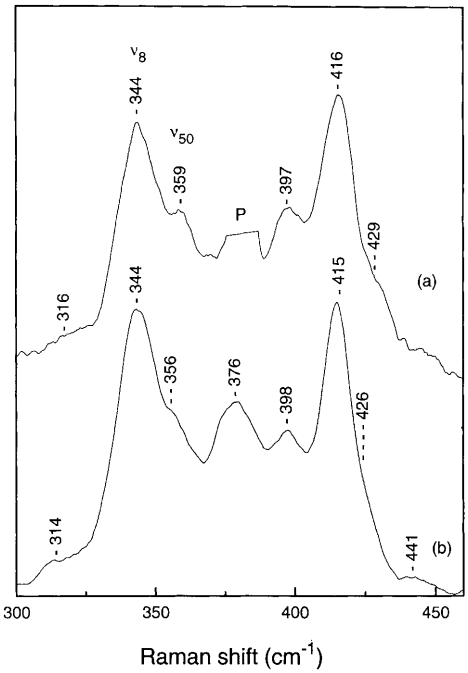


Fig. 2 Low-frequency regions (300–460 cm^{-1}) of RR spectra of lysine complexes of MP8(III): *a* 0.05 M Lys, pH 10.2, excitation: 406.7 nm; *b* 0.5 M Lys, CTABr 1%, pH 12.0, excitation: 413.1 nm. P in (*a*) indicates a truncated laser plasma line

tion of the MP8-(Lys) complexes has been performed at pH equal to or higher than 10.2 to form saturated complexes with reasonable Lys concentrations (0.01–1.7 M).

The optical titration of MP8(III) with Lys in aqueous CTABr solution at pH 12 is consistent with the association of one molecule of ligand ($K_a = 4 \cdot 10^1 \cdot M^{-1}$) (Table 1). The absorption spectrum of this MP8(III)-(Lys) complex exhibits band maxima at 408, 532 and 560 nm. These band positions are not significantly affected by an increase in hydroxide concentration up to 1 M (Table 1). In the absence of detergent, the absorption spectrum of MP8(III)-(Lys) at pH 10.2 displays peaks at 405, 530 and 556 nm (Table 1). Under these solvent conditions, spectral titrations give a binding constant of $2 \cdot 10^3$ M^{-1}. When the pH is raised, the α, β and Soret bands are gradually redshifted, approaching, at 1 M hydroxide, the spectral characteristics found for MP8(III)-(Lys) in aqueous CTABr solution at pH 12 (Table 1). A pK_a of 12.5 is determined from the apparent shift of the Soret band of MP8(III)-Lys in water (data not shown).

The RR spectra of the Lys complexes of MP8(III) are shown in Figs. 1 and 2. The ν_{10} regions exhibit two bands at 1638 and 1640 cm^{-1} with relative intensities depending on the laser excitation (Fig. 1B). Whatever the excitation used, the ν_4, ν_3 and ν_2 modes exhibit single bands at 1377, 1506 and 1589 cm^{-1} (Fig. 1 and spectra not shown). In the RR spectra of the Lys complex in aqueous CTABr at pH 12, the corresponding set of frequencies is detected at 1374, 1503 and 1587 cm^{-1}, respectively (Fig. 1A). Two ν_{10} bands of variable intensity with the excitation are again observed. However, the frequencies of the ν_{10} pairs are decreased with respect to those detected for the complex at pH 10.2, in the absence of detergent (1632 and 1637 cm^{-1} versus 1638 and 1640 cm^{-1}) (Fig. 1B).

In the low-frequency regions of RR spectra, the band corresponding to the ν_8 mode is broad and detected at 344 cm^{-1}

but the ν_{50} mode downshifts from 359 to 356 cm^{-1} when both pH is increased and CTABr added (Fig. 2). A band seen at 404–409 cm^{-1} in the RR spectra of the imidazole complexes of MP8(III) is downshifted to 397–398 cm^{-1} in those of the Lys complexes (Othman et al. 1994, and Fig. 2).

Lysine complexes of MP8(II)

The spectrophotometric titrations of MP8(II) by Lys carried out at pH 12 in aqueous CTABr solutions correspond to a sequential binding of Lys to ferroheme c with two association constants. The first K_a value is 10 M^{-1} while the second one, obtained by extrapolation, is 2.5 M^{-1}. The MP8(II)-(Lys) complex, formed either in water or in aqueous CTABr solutions, displays visible bands at 415, 519–520 and 548–549 nm (Table 3). Upon increasing the Lys concentration, a small redshift of these bands is observed (416, 521 and 550 nm) (Table 3).

Since the pK_a of an ImH group bound to a ferroheme is between 13 and 14.1 (Desbois and Lutz 1992; Othman et al. 1993, 1994), we have also investigated the possible formation of a MP8(II)-(Lys) complex in which the bound His side chain of MP8 is N_1-deprotonated. In aqueous CTABr solutions containing 2.5 M hydroxide, the titration curves of MP8(II) with Lys correspond to a ligand binding with a low affinity ($4 \cdot 10^{-1}$ M^{-1}). The absorption spectrum of this very alkaline MP8(II)-(Lys) complex exhibits redshifted Soret, β and α bands (421, 524 and 553 nm, respectively) when compared to the band positions observed for the MP8(II)-(Lys) complex in aqueous CTABr, at pH 10.2–12 (Table 3).

The RR spectra of the Lys complexes of MP8(II) are presented in Figs. 3 and 4. Using various Soret excitations and different solvent conditions, three Lys complexes of MP8(II) are characterized by the frequency changes of the ν_{10}, ν_{38}, ν_{11} and ν_4 modes. The RR spectra of a first complex exhibit these modes at 1620, 1559, 1535 and 1359 cm^{-1}, respectively (Fig. 3 A, B). It is mainly detected with the 413.1- and 441.6-nm excitations using low Lys concentrations at pH 10.2–12, in the presence or absence of CTABr (Fig. 3 and spectra not shown). A second complex is partially observable with the 441.6-nm excitation and when the ligand concentration is increased at 1.2–1.7 M. It exhibits the frequencies of its ν_{10}, ν_{38} and ν_{11} modes at 1625, 1566 and 1540 cm^{-1}, respectively (Fig. 3). The third complex is fully detected when the solution is very alkaline and contains CTABr. Its RR spectra show the ν_{10}, ν_{38}, ν_{11} and ν_4 modes at 1614, 1554, 1529 and 1356 cm^{-1}, respectively (Fig. 3). The low-frequency RR spectra of the three Lys complexes of MP8(II) are similar (Fig. 4). The ν_8 and ν_{50} modes are observed at 348–349 and 360–363 cm^{-1}, respectively.

N-acetylmethionine complexes of MP8(III)

The Hill plots of the titrations of MP8(III) with AcMet at pH 7.4, in the presence or absence of CTABr, exhibit

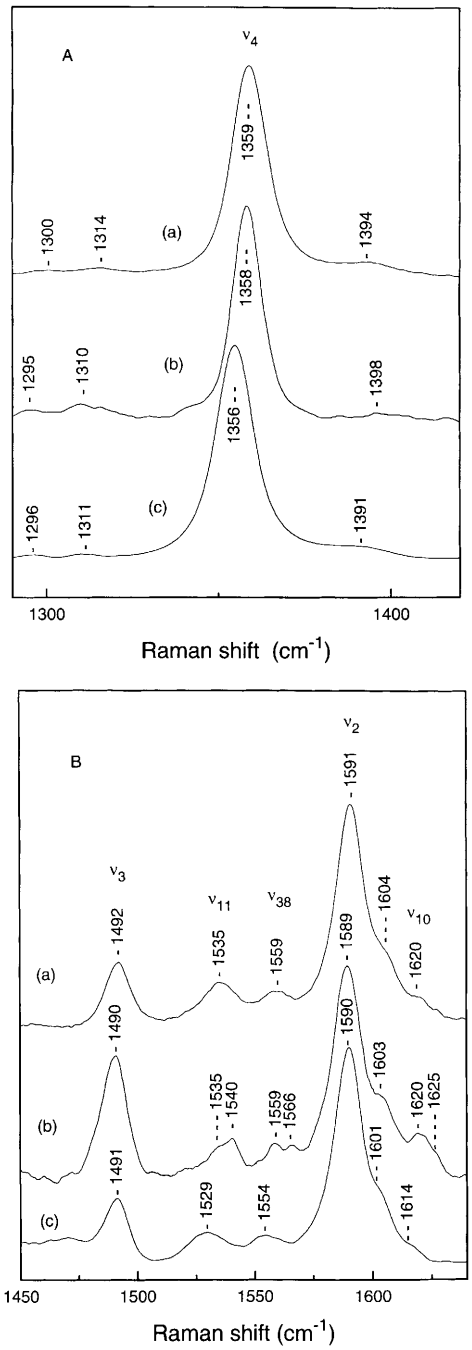


Fig. 3 1290–1420 **A** and 1450–1640 **B** cm^{-1} regions of RR spectra of lysine complexes of MP8(III). *a* 0.5 M Lys, 1% CTABr, pH 12.0, excitation: 413.1 nm; *b* 1.7 M Lys, 1% CTABr, pH 12.0, excitation: 441.6 nm.*c* 2 M Lys, 1% CTABr, 2 M KOH, excitation: 413.1 nm

curved lines. The apparent ligand affinity of MP8 is low with a $c_{1/2}$ value of 0.6 M in the absence of detergent and of 1 M in its presence. The absorption spectra of the AcMet complex of MP8(III) in neutral aqueous CTABr solution (409, 526 and 557 nm) are practically the same as those obtained in the absence of detergent (409.5, 526 and 558 nm). In the near IR region of these spectra, a weak broad band is observed at 690–698 nm (Table 1). An alka-

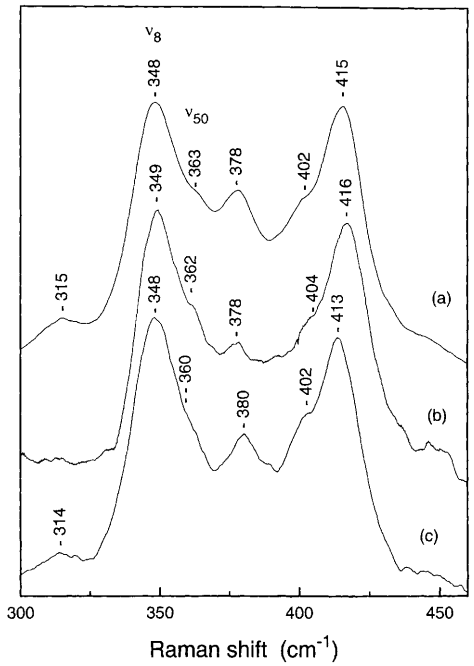
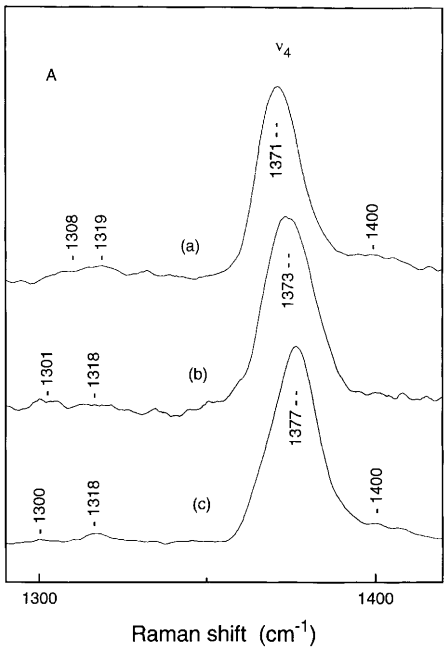


Fig. 4 Low-frequency regions (300–460 cm^{-1}) of RR spectra of lysine complexes of MP8(II): *a* 0.5 M Lys, 1% CTABr, pH 12.0, excitation: 413.1 nm; *b* 1.7 M Lys, 1% CTABr, pH 12.0, excitation: 441.6 nm; *c* 2 M Lys, 1% CTABr, 2 M KOH, excitation 413.1 nm



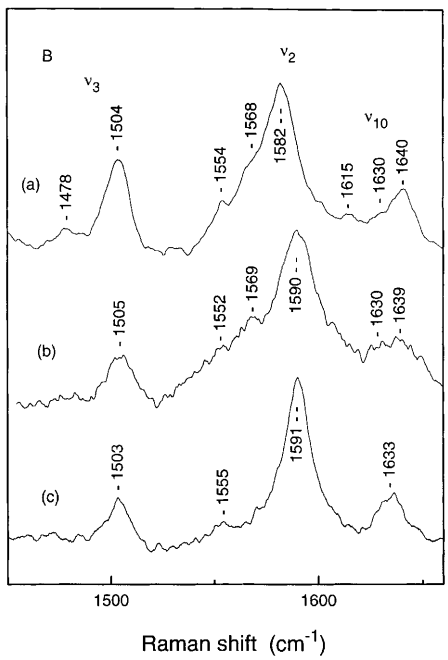


Fig. 5 1290–1420 **A** and 1450–1660 **B** cm^{-1} regions of RR spectra of N-acetylmethionine complexes of MP8(III): *a* 2 M AcMet, pH 7.5, excitation: 441.6 nm; *b* 2 M AcMet, pH 7.5, excitation: 413.1 nm; *c* 2.2 M AcMet, CTABr 1%, pH 12.2, excitation: 413.1 nm

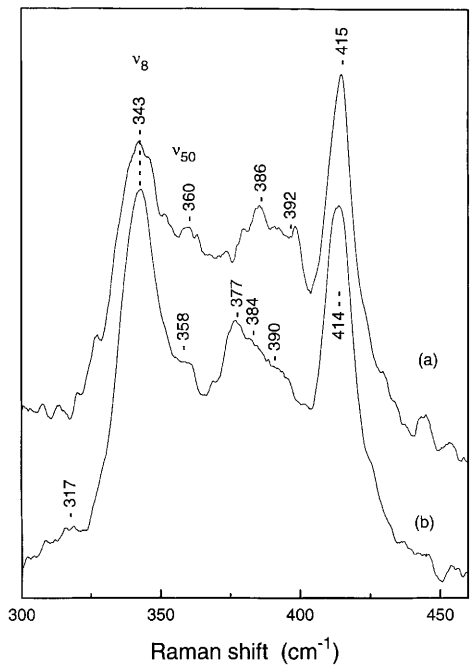
line form of the AcMet complex of MP8(III) is observable in the presence of CTABr. This form exhibits visible absorption bands at 412, 537 and 557 nm but no feature is seen in the 690–700 nm region (Table 1). A marked shoulder is however observed at 632 nm, on the red side of the α band.

The RR spectra of the AcMet complex of MP8(III) at pH 7.5 in the presence or absence of detergent are wavelength-dependent (Fig. 5 and spectra not shown). The 441.6-nm excited spectra exhibit the ν_4, ν_3, ν_{37} and ν_2 modes at 1371, 1504, 1554 and 1582 cm^{-1}, respectively (Fig. 5A, B). In the 1620–1650 cm^{-1} region, two ν_{10} modes are observed at 1630 and 1640 cm^{-1}. Bands corresponding to a high-spin (HS) form are also seen at 1478 (ν_3), 1568 (ν_2) and 1615 cm^{-1} (ν_{10}) (Fig. 5B). The RR spectra excited at 413.1 nm exhibit single bands for ν_4, ν_3, ν_{37} and ν_2 (1373, 1505, 1552 and 1590 cm^{-1}, respectively) (Fig. 5B, spectrum (b)). Two ν_{10} modes are detected at 1630 and 1639 cm^{-1} but no HS contribution is observable (Fig. 5).

The alkaline form of the AcMet complex of MP8(III) exhibits the ν_4, ν_3 and ν_{10} modes at 1377, 1503 and 1633 cm^{-1} (Fig. 5). No HS component is found in the RR spectra excited at either 441.6, or 413.1 or 406.7 nm. The low-frequency regions of RR spectra of the AcMet complexes of MP8(III) show the ν_8 and ν_{50} modes at 343 and 358–360 cm^{-1}, respectively. The band observed at 397–398 cm^{-1} in the spectra of the Lys complexes of MP8(III) is downshifted to 390–392 cm^{-1} in those of the AcMet complexes (Figs. 2 and 6).

N-acetylmethionine complexes of MP8(II)

The spectrophotometric titrations of MP8(II) with AcMet in aqueous CTABr solutions at pH 7.5 are consistent with a large overlap in the sequential binding of two AcMet molecules per MP8(II) molecule (Table 3). The absorption spectra of MP8(II)-(AcMet) obtained with low ligand concentrations (0.05–0.1 M) in water or in aqueous CTABr at pH 7.5 exhibit absorption maxima at 414.5, 521 and



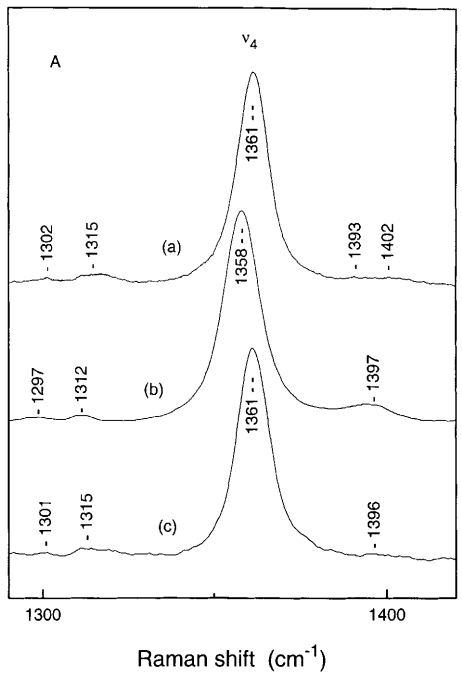


Fig. 6 Low-frequency regions (300–460 cm^{-1}) of RR spectra of N-acetylmethionine complexes of MP8(III): *a* 2 M AcMet, 1% CTABr, excitation: 413.1 nm; *b* 2.2 M AcMet, CTABr 1%, pH 12.2, excitation: 413.1 nm

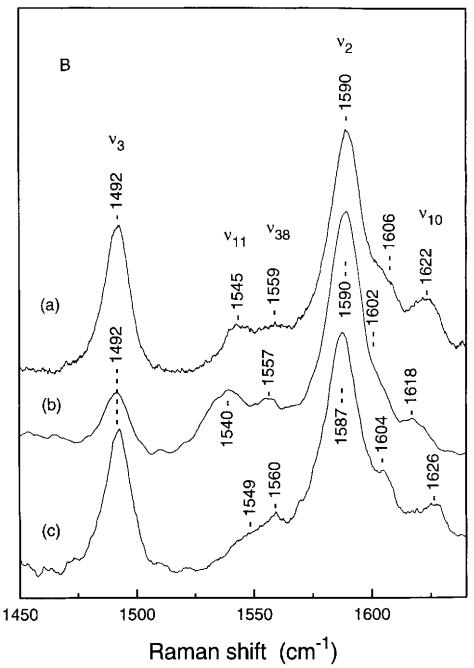


Fig. 7 1290–1420 **A** and 1450–1640 **B** cm^{-1} regions of RR spectra of N-acetylmethionine complexes of MP8(II): *a* 0.25 M AcMet, pH 7.5, excitation: 441.6 nm; *b* 0.2 M AcMet, pH 7.5, excitation: 413.1 nm; *c* 1.7 M AcMet, pH 4.5, excitation: 441.6 nm

550 nm (Table 3). A large increase in AcMet concentration (2–3 M) redshifts the absorption bands resulting in a spectrum that is very similar to that obtained for the bis(AcMet) complex of MP8(II) prepared at pH 4.5 (Harbury et al. 1965) (Table 3), i.e. at 420.5, 524 and 554 nm (Table 3). Aggregation due to elevated concentrations of both AcMet and KOH did not permit the characterization of a well-defined alkaline form of MP8(II)-(AcMet).

The RR spectra of the AcMet complex of MP8(II) at pH 7.5, in the presence of a low ligand concentration and excited at 441.6 nm, exhibit single bands at 1361, 1492 and 1590 cm^{-1} for the ν_4, ν_3 and ν_2 modes, respectively (Fig. 7 A, B, spectra (a)). In contrast, the RR bands observed at 1545 (ν_{11}), 1559 (ν_{37}) and 1622 (ν_{10}) cm^{-1} are significantly broader. In the RR spectra excited at 413.1 nm, the ν_3 and ν_2 modes have the same frequencies (1492 and 1590 cm^{-1}, respectively), but the ν_4, ν_{11}, ν_{37} and ν_{10} frequencies differ significantly (1358, 1540, 1557 and 1618 cm^{-1}, respectively) (Fig. 7 A, B, spectra (b)). In the low-frequency RR spectra, the bands corresponding to the ν_8 and ν_{50} modes are detected at 343–345 and 354–355 cm^{-1}, respectively (Fig. 8).

The RR spectra of the bis(AcMet) complex of MP8(II) are different from those of the mono(AcMet) complexes. With respect to the MP8(II)-(AcMet) spectra, clear frequency differences concern the ν_{11}, ν_2, ν_{10} and ν_{50} modes (Figs. 7 B and 8, spectra (c)). The ν_{10} and ν_{11} frequencies are upshifted from 1540–1545 to 1549 cm^{-1} and from 1618–1622 to 1626 cm^{-1}, respectively (Fig. 7 B). Those of the ν_2 and ν_{50} modes are downshifted from 1590 to 1587 cm^{-1} and from 354–355 to 350 cm^{-1}, respectively (Figs. 7 B and 8).

Discussion

Lysine complexes of MP8(III)

Equilibrium and absorption data. The titrations of MP8(III) by Lys under different solvent conditions are consistent with the binding of one molecule of Lys per

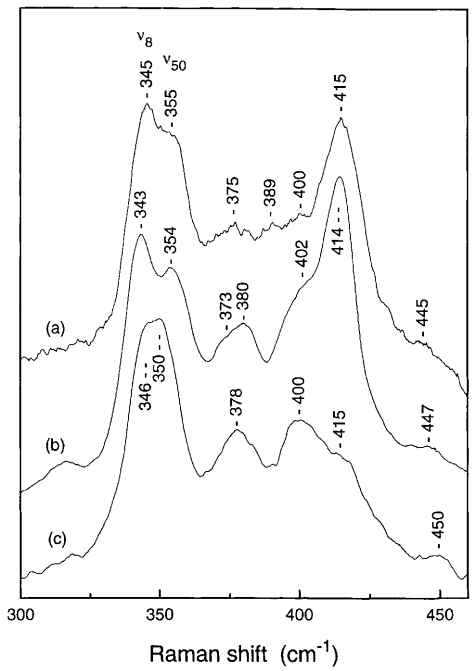


Fig. 8 Low-frequency regions (300–460 cm^{-1}) of RR spectra of N-acetylmethionine complexes of MP8(II). *a* 0.25 M AcMet, pH 7.5, excitation: 441.6 nm; *b* 0.2 M AcMet, pH 7.5, excitation: 413.1 nm; *c* 1.7 M AcMet, pH 4.5, excitation: 441.6 nm

MP8(III) monomer, forming a MP8(III)-(Lys) complex (Table 1). Among the α- and ε-amino groups of Lys, both the stronger basicity and the smaller steric hindrance of the ε group are expected to result in a better ligation to heme (Byfield et al. 1993). The absorption data allow the characterization of two mono(Lys) complexes differing by the ionization state of the bound His residue of MP8 (Table 1). On one hand, the His ligand of the MP8(III)-(Lys) complex obtained in aqueous CTABr solution at pH 12–14 is deprotonated considering both the invariance of the absorption spectrum between pH 12 and 14 and the fact that alkaline solutions of CTABr (pH > 12) favor the ionization of the N_1H group of imidazole rings bound to a ferriheme (Desbois and Lutz 1992; Othman et al. 1994). The similarity of the absorption spectrum of MP8(III)-(Lys) in aqueous CTABr at pH 12–14 with that of MP8(III)-(Lys) in water at pH 14 is also indicative that the His ligand in the latter complex is anionic. On the other hand, the His ligand of the MP8(III)-(Lys) complex formed in water at pH 10.2–11 is protonated since the Soret band maximum is blue-shifted from 408.5 to 405 when the pH is decreased from 14 to 10.2 (Table 1). A similar spectral shift (–2.5 nm) was found for the protonation of the bound His of the MP8(III)-(NH_3) complex in 20% aqueous methanol (Marques et al. 1993). Moreover, in the alkaline transition of the MP8(III)-(ImH) complex, an average 3 nm Soret red-shift is estimated for the ionization of each imidazole group (Othman et al. 1994).

RR spectra. The RR spectra of the protonated and deprotonated MP8(III)-(Lys) complexes show that the His ionization sizeably affects the frequencies of the ν_3, ν_4 and ν_{10} modes. A similar downshift of ν_{10} was previously observed in the RR spectra of the ImH and Im^- complexes of MP8(III) (Othman et al. 1994) (Table 2). An increase in heme ruffling was associated with the imidazole deprotonations of the ImH complex of MP8(III) (Othman et al. 1994).

Table 2 RR frequencies (cm^{-1}) of the structure-sensitive modes of the imidazole, imidazolate, lysine and N-acetylmethionine complexes of MP8(III)

Heme coordination	ν_{10}	ν_2	ν_3	ν_4	ν_{50}	
His/ImH[a]	1637/1641	1587	1504	1377	404	362
His/Lys	1638/1640	1589	1506	1377	397	359
His/AcMet (LS)	1630/1640	1590	1505	1373	392	360
(HS)	1615	1568	1478			
His^-/Im^-[a]	1631/1636	1588	1502	1375	409	360
His^-/Lys	1632/1637	1587	1503	1374	398	356
His^-/AcMet	1633	1591	1503	1377	390	358

[a] RR data from Othman et al. 1994

Two low-frequency bands in the 360–362 and 404–409 cm^{-1} regions of RR spectra of the ImH and Im^- complexes of MP8(III) were previously assigned to out-of-plane porphyrin modes coupled with Fe-ligand modes (Othman et al. 1994). The corresponding bands are observed at 356–359 and 397–398 cm^{-1} in the RR spectra of the Lys complexes (Table 2). These frequency variations detected upon ligand substitution support the proposed assignments of the two bands.

Lysine complexes of MP8(II)

Equilibrium and absorption data. The titrations of MP8(II) with Lys at pH 12 are consistent with the sequential formation of mono(Lys) and bis(Lys) complexes (MP8(II)-(Lys)) and MP8(II)-$(Lys)_2$, respectively) (Table 3). The absorption spectrum of MP8(II)-$(Lys)_2$ is slightly redshifted relative to that of MP8(II)-(Lys) (Table 3). Aqueous CTABr solutions containing 2.5 M hydroxide are generally required to deprotonate the imidazole ring(s) bound to a ferroheme (Desbois and Lutz 1992; Othman et al. 1994). Under these solvent conditions, the His ligand of the MP8(II)-(Lys) complex is also deprotonated given both the red-shifted positions of the absorption bands and the decreased affinity for Lys (Table 3) (Nappa et al. 1977; Swartz et al. 1979).

RR spectra. The preceding absorption and equilibrium data provide strong indications for the existence of three Lys complexes of MP8(II) (protonated and deprotonated MP8(II)-(Lys) and MP8(II)-$(Lys)_2$). The RR data confirm this interpretation. The RR spectra of the protonated MP8(II)-(Lys) complex is observed at low Lys concentrations and enhanced by the 413.1-nm excitation. Although this complex is not fully formed at 0.05 M Lys (Table 3), the resonance conditions at 413.1 nm allow the sole observation of the RR contribution of the 6cLS species even in

Table 3 Axial coordinations, formation constants and absorption maxima of lysine and N-acetylmethionine complexes of MP8(II)

Ligand	Solvent conditions[a]	Accessible heme ligation(s)	K_a or $1/c_{1/2}$	α	β (nm)	Soret
Lys	–CTABr, pH 12	N(His)/N(Lys)	$5 \cdot 10^2$ M^{-1}	548	519	415
		N(Lys)/N(Lys)	$2.5 \cdot 10^1$ M^{-1}	550	520	416
	+CTABr, pH 12	N(His)/N(Lys)	10 M^{-1}	549	520	415
		N(Lys)/N(Lys)	2.5 M^{-1}	550	521	416
	+CTABr, 2.5 M OH^-	$N(His^-)$/N(Lys)	$4 \cdot 10^{-1}$ M^{-1}	553	524	421
AcMet	–CTABr, pH 7.5	N(His)/S(AcMet)	10 M^{-1}	550	521	414.5
	+CTABr, pH 7.5	N(His)/S(AcMet)	1.2 M^{-1}	552	523	416.5
	–CTABr, pH 4.5	S(AcMet)/S(AcMet)	$2 \cdot 10^2$ M^{-2}	554	524	420.5

[a] Absence or presence of CTABr (– or +), pH or hydroxide concentration

the presence of unreacted 5cHS species (Othman et al. 1993). In this way, the ν_4, ν_3, ν_{11}, ν_{38}, ν_2 and ν_{10} modes of the protonated MP8(II)-(Lys) complex are observed at 1359, 1492, 1535, 1559, 1591 and 1620 cm^{-1}, respectively (Fig. 3, spectra (a), and spectra not shown). With respect to the preceding complex, the bis(Lys) complex is relatively more RR-enhanced by the 441.6-nm excitation. This effect is likely due to the small redshift of its absorption spectrum (Table 3). Its ν_{11}, ν_{38} and ν_{10} modes contribute as single bands at 1540, 1566 and 1625 cm^{-1}, respectively, in the RR spectra of the MP8(II)-(Lys) complex (Fig. 3B, spectrum (b), and spectra not shown). Finally, the deprotonated form of the MP8(II)-(Lys) complex show the ν_4, ν_3, ν_{11}, ν_{38}, ν_2 and ν_{10} modes at 1356, 1491, 1529, 1554, 1590 and 1614 cm^{-1}, respectively (Fig. 3, spectrum (c)). The 6 cm^{-1} downshift of ν_{11} from 1535 cm^{-1} for MP8(II)-(Lys) at pH 10.2–12 to 1529 cm^{-1} for MP8(II)-(Lys) in a CTABr/KOH mixture is consistent with the His deprotonation of the MP8(II)-(Lys) complex (Othman et al. 1994).

N-acetylmethionine complexes of MP8(III)

Equilibrium and absorption data. The weak basicity of the thioether sulfur group (pK_a ca. –6.8) does not favor its coordination with hard metal ions such as Fe(III). Consequently, the low affinity of S(AcMet) for ferriheme requires high concentrations of ligand to ensure the full complexation of MP8(III) (Harbury et al. 1965). However, high concentrations of free ligand induce an aggregation of the unligated MP8(III) species so that a competition between ligation and aggregation occurs even in the presence of detergent. For these reasons, the Hill plots of the titrations of MP8(III) with AcMet are not linear. Nevertheless, the absorption data show that the MP8(III)-(AcMet) complex is fully formed in the presence of excess ligand. In particular, the observation of a 690–698 nm band in the absorption spectra of the AcMet complexes of MP8(III) is a clear indication of S(AcMet) binding to MP8(III). This spectral feature is similar to that previously detected in the spectra of the AcMet complex of microperoxidase-11 and MP8 and of various c-type cytochromes (Shechter and Saludjian 1967; Smith and McLendon 1981; Moore and Pettigrew 1990). It is associated with the thioether ligation to ferriheme c and assigned to a charge transfer transition (Moore and Pettigrew 1990).

As far as the 632 nm band of the alkaline form of the MP8(III)-(AcMet) is concerned, it cannot be attributed to a residual HS form of MP8(III) since in this case other bands would be observed in the 490 and 400 nm regions of the absorption spectrum (Othman et al. 1993). Nevertheless, the spectra of *E. coli* cyt b_{562} which has His/Met residues as heme ligands offer interesting analogies. A band observed at 720 nm for the neutral form of cyt b_{562} is shifted at 680 nm in the spectrum of its alkaline form (Myer and Bullock 1978; Moore et al. 1985). These bands were assigned to charge transfer transitions specific to the S(Met) heme ligation and the 40 nm blue-shift was attributed to the ionization of the proximal His of cyt b_{562} (Myer and Bullock 1978; Moore et al. 1985). Thus, the 632 nm band of the alkaline form of MP8(III)-(AcMet) can be assigned to a charge transfer band corresponding to a $N(His^-)$/S(Met) heme coordination in MP8(III).

RR spectra. The RR spectra of the neutral MP8(III)-(AcMet) complex exhibit a HS form beside the expected 6cLS form. Observed with the 441.6 nm excitation, this HS species is characterized by three bands at 1478, 1568 and 1615 cm^{-1} (Fig. 5; Table 2) and can be assigned to a 6c form considering the RR spectra of the fluoride complex of N-acetyl-MP8(III) (1479 (ν_3), 1572 (ν_2) and 1609 (ν_{10}) cm^{-1}, respectively) (Wang and Van Wart 1989). This 6cHS species cannot be attributed to either an aquo complex (MP8(III)-(H_2O)) or a bis(AcMet) complex (MP8(III)-$(Met)_2$) of MP8(III) since its contribution relative to that of the normal 6cLS species is unchanged when the AcMet concentration is varied between 1 and 2.4 M (spectra not shown). These observations thus reveal that the MP8(III)-(AcMet) complex assumes two spin states: a major LS state and a minor HS state. A HS/LS equilibrium in a His/Met-coordinated ferriheme finds precedence with ferricyt b_{562} (Wu et al. 1991). The comparisons of the circular dichroism and nuclear magnetic resonance spectra of MP8(III)-(AcMet) and ferricyt c indicate that the histidylimidazole ring orientation is the same in the model system and the protein but also suggests large differences in the Met orientation (-Smith and McLendon 1981). In particular, a rotation about the Fe-S(AcMet) bond is predicted in the MP8(III) complex. Different N(His)-Fe-S(AcMet) bonding geometries, altering the ligand field of AcMet, could therefore account for the HS/LS states of the neutral MP8(III)-(AcMet) complex (Scheidt and Gouterman 1983).

The heme of the alkaline MP8(III)-(AcMet) form is 6cLS (Table 2). It exhibits a single ν_{10} mode at 1633 cm^{-1} while the neutral 6cLS MP8(III)-(AcMet) form has two bands at 1630 and 1640 cm^{-1} (Table 2). These changes in the ν_{10} region reflect differences in heme structure (vide infra).

The two low-frequency ligand-sensitive bands, observed in the 350–362 (ν_{50}) and 400–407 cm^{-1} regions of RR spectra of MP8(III)-(ImH) and MP8(III)-(Im^-) (Othman et al. 1994), are seen at 358–360 and 390–392 cm^{-1}, respectively, in the spectra of the AcMet complexes (Table 2). Observed at 397–398 cm^{-1} in those of the Lys complexes, the latter band is particularly sensitive to ligand substitution in the 6cLS MP8(III) complexes.

Table 4 RR frequencies (cm^{-1}) of the structure-sensitive modes of the imidazole, imidazolate, lysine and N-acetylmethionine complexes of MP8(II)

Heme coordination		ν_{10}	ν_{38}	ν_{11}	ν_4	ν_{50}
His/ImH[a]		1621	1561	1539	1360	359
His/Lys		1620	1559	1535	1359	363
His/AcMet	Form I	1622	1559	1545	1361	355
	Form II	1618	1557	1540	1358	354
His^-/Im^-[a]		1618	1558	1527	1357	357
His^-/Lys		1614	1554	1529	1356	360
Lys/Lys		1625	1566	1540		
AcMet/AcMet		1626	1560	1549	1361	350

[a] RR data from Othman et al. (1994)

N-acetylmethionine complexes of MP8(II)

Equilibrium and absorption data. The thioether ligands are known to bind more easily to ferroheme than ferriheme (Harbury et al. 1965; Warme and Hager 1970; Momenteau et al. 1976). The titrations of MP8(II) with AcMet confirm this trend since bis(AcMet) complexes are formed while bis(ligation) is not detected for MP8(III). In aqueous acidic conditions (pH 4–5), MP8(II) binds two molecules of AcMet. The bis(coordination) is facilitated by the low pH that protonates the His ligand of MP8 ($pK_a = 5.4$) and thus provokes the disruption of the Fe–His bond (Harbury et al. 1965). The absorption spectra of the bis(AcMet) complex of MP8(II) are red-shifted relative to those of the mono(AcMet) complex (Table 3). These spectra alterations, and particularly the shift of the Soret band, are in good agreement with published data on similar mesoheme and deuteroheme complexes (Warme and Hager 1970; Momenteau et al. 1976). An analogous trend is also observed when the absorption spectra of reduced cyt b_{562} (His/Met heme coordination) and its H102M mutant (Met/Met coordination) are compared (Barker et al. 1996).

RR spectra. Changing the excitation conditions permits the observation of two different His/Met coordinations in the neutral MP8(II)-(AcMet) complex (Fig. 7). The invariance of the ν_2 and ν_3 frequencies support the simultaneous presence of two 6cLS complexes. However, these two conformers exhibit clearly distinct ν_{10}, ν_{11} and ν_4 frequencies (Table 4). Considering that the ν_{10} frequency is sensitive to the porphyrin conformation and the ν_{11} and ν_4 frequencies to the heme electronic structure induced by the ligands (Spiro and Burke 1976; Alden et al. 1989; Czernuszewicz et al. 1989), different coordination modes of the axial ligands can account for the observation of two 6cLS forms for MP8(II)-(AcMet). The preceding RR data have shown the stabilization of two spin states for the neutral MP8(III)-(AcMet) complex. This effect has been at least in part related the free rotation of the S(Met) ligand. Indeed, the relative orientations of the CH_3–S–CH_2– thioether group of AcMet with, on one hand, the imidazole ring of His and, on the other hand, the FeN_4(pyrrole) bonds are expected to participate in the modulation of the bonding parameters of the axial ligands with the heme iron (Moore and Pettigrew 1990). This variable stereochemical interaction may be modulated by H-bonding effects on the ligands. H-bonding at the N_1H group of the coordinated ImH increases the ring electronegativity inducing a better σ and π donor ability of the ligating N_3(ImH) atom. A more direct H-bonding effect may be operative on the S(Met) heme ligand since the latter is a H-bond acceptor. For instance, a H-bond between S(AcMet) and a solvent water molecule is expected to decrease the electron density of the sulfur ligand. Therefore, it is highly probable that the spectral forms I and II of neutral MP8(II)-(AcMet) correspond to complexes in which the H-bonding states and/(or) the coordination geometries of the His/AcMet ligands are/(is) different.

Axial ligation and heme c structure in oxidized MP8 derivatives

In ferriheme complexes axially coordinated by two ImH rings, the absolute and relative ligand orientations largely determine both the axial bond lengths and the porphyrin structure (Scheidt and Gouterman 1983; Scheidt et al. 1987a, b; Safo et al. 1991). Two types of heme structure maintain the LS electronic state of the iron atom. A first one corresponds to a nearly planar heme with short axial bonds (Scheidt et al. 1987a; Safo et al. 1991). A second type of complex lengthens the axial bonds and shortens the Fe-N(pyrrole) bonds provoking a porphyrin ruffling (Scheidt et al. 1987b). The RR data obtained on the 6cLS complexes of MP8 can be interpreted in the context of similar alternative structures.

The RR spectra of the MP8(III)-(ImH) complex exhibited a ν_{10} doublet (1641 and 1637 cm^{-1}) reflecting the simultaneous presence of planar and ruffled heme conformations (Othman et al. 1994). The RR spectra of the Lys and AcMet complexes of MP8(III) show similar features with double bands at 1638/1640 and 1630/1640 cm^{-1}, respectively (Table 2). The ν_{10} frequency at 1640–1641 cm^{-1} was previously attributed to a planar heme c conformation while lowest ν_{10} frequencies were associated with more or less ruffled conformations (Othman et al. 1994, 1997). From the ν_{10} frequencies of the examined 6cLS MP8(III)

complexes, one can conclude that the extent of out-of-plane heme distortion is ligand-dependent. It can reach a higher degree in the MP8(III)-(AcMet) complex (1630 cm^{-1}) than in the MP8(III)-(ImH) and MP8(III)-(Lys) complexes (1637–1638 cm^{-1}).

When the His ligand of MP8(III) is deprotonated, no ν_{10} component at 1640–1641 cm^{-1} is detected (Table 2). This observation reveals that the planar heme structure is not stabilized. The strong σ bonding interaction between the Fe(III) ion and the negatively charged His^- ligand of MP8(III), as well as the steric constraints of its Im^- ring on the porphyrin core, are electronic and steric factors able to stabilize strongly ruffled porphyrins. The ν_{10} pairs of the deprotonated MP8(III)-(Im^-) and MP8(III)-(Lys) complexes have distinct frequencies corresponding to two clearly different degrees of nonplanar heme distortion (1631/1636 and 1632/1637 cm^{-1}, respectively) (Table 2). For the alkaline AcMet complex, a broad ν_{10} band at 1633 cm^{-1} is indicative of heme conformers having a more uniform nonplanar structure.

In conclusion, ferriheme c in MP8 can easily adopt ruffled structures. The extent of heme distortion is ligand-dependent, being particularly marked when the bound His of MP8(III) is deprotonated. The covalent thioether links, the strong σ interaction between Fe(III) and His and the steric constraints of the histidylimidazole ring on the porphyrin macrocycle are parameters that can contribute to the stabilization of nonplanar heme structures in MP8. Recent investigations on ferric cyt c and cyt c_2 suggest that the Cys–X–Y–Cys-His-protein segment could be the source of heme ruffling in c-type cytochromes (Othman et al. 1997; Jentzen et al. 1997). Our present data on derivatives of an octapeptide-ferriheme c system further support this suggestion.

Axial ligation and heme structure of reduced MP8

From the comparisons of the RR spectra of ImH, Lys and AcMet complexes of MP8(II), we can deduce that the ν_{11}, ν_{10}, ν_{38}, ν_4 and ν_{50} modes are sensitive to the nature of the axial ligands of ferroheme c (Table 4). The ν_{11} frequency was frequently used to characterize the axial ligation of ferrohemes (Desbois 1994). However, taking this frequency as a sole marker may introduce some uncertainties in the heme coordination. For instance, Table 4 shows an ambiguous ν_{11} frequency region at 1539–1540 cm^{-1} for the His/ImH, Lys/Lys and His/AcMet (form II) coordinations. Nevertheless, Table 4 also shows that other RR porphyrin modes can be used in a further determination of the ferroheme ligation. Close examination reveals that the frequencies of the ν_{10}, ν_{38}, ν_4 and ν_{50} modes are sensitive to the heme coordination and, more important, their frequency changes are not necessarily the same as those of the ν_{11} mode. Reconsidering the coordination states exhibiting a similar ν_{11} frequency, the ν_{50} mode is observed at a frequency significantly higher for the His/ImH coordination (359 cm^{-1}) than for the His/Met (354 cm^{-1}) coordination. The ν_{10} and ν_{38} frequencies of the Lys/Lys coordination (1625 and 1566 cm^{-1}, respectively) are higher than those of the His/ImH and His/AcMet coordinations (1618–1621 and 1557–1561 cm^{-1}) (Table 4). The different behaviors of the ν_{11}, ν_{10}, ν_{38}, ν_4 and ν_{50} frequencies upon ligand replacements in MP8(II) are clues about different sensitivities of these modes to electronic and/or steric interactions between ligands and heme. These differing sensitivities obviously have their origin in the different normal mode compositions of ν_{11}, ν_{10}, ν_{38}, ν_4 and ν_{50} (Li et al. 1989). The properties of these modes upon ligand change can be exploited to be used for diagnostic purposes for c-type ferrocytochromes of unknown structure.

Table 5 Frequencies (cm^{-1}) of the ν_{11} mode of 6cLS complexes of MP8(II) and of c-type ferrochromes as a function of the heme ligation

MP8(II) ligation	ν_{11}	cyt	(ligation)	ν_{11}
His^-/Im^-	1527			
His^-/Lys	1529			
His/Lys	1535	f	(His/NH_2)	1532–1538
His/ImH	1539	c_3	(His/His)	1539–1541
Lys/Lys	1540			
His/Met	1540	c_1	(His/Met)	1540–1543
	1545	c_{551}	(His/Met)	1543–1545
		c_2	(His/Met)	1544–1547
		c	(His/Met)	1544–1548
Met/Met	1549			

Ligand effects on the ν_{11} mode. Table 5 summarizes the sensitivity of the ν_{11} frequency to the ferroheme ligation in 6cLS complexes of MP8(II). This $\nu(C_b–C_b)$ mode is influenced by the electronic Fe(II)-ligands interactions (Spiro and Burke 1976; Li et al. 1989; Desbois and Lutz 1992). The ν_{11} frequency is ordered as follows: Im^- < Lys < ImH < AcMet, i.e. according to the decreased pK_a value of the axial ligand (14.2, 10.3, 7.1 and –6.8, respectively). The pK_a values giving an index of the donor power of the ligand, the ν_{11} frequency is inversely related to this parameter. A similar relative order has been characterized from the position of the near-IR charge transfer band of magnetic circular dichroism spectra of ferriheme compounds (Simpkin et al. 1989; Gadsby et al. 1990).

Shifts in redox potential have been related to ligand substitutions in model compounds and cytochromes (Harbury and Loach 1960b; Warme and Hager 1970; Mashiko et al. 1979; Quinn et al. 1983; Raphael and Gray 1989; Wallace and Clark-Lewis 1992; Dolla et al. 1994; Ubbink et al. 1994; Barker et al. 1996). Considering the differences in the heme environment for each system and taking the bis(ImH)-type ligation as a reference for the potential variations, the following qualitative scale between changes in heme redox potential and changes in heme coordination can be drawn:

Im^-/Im^- < Im^-/amine < ImH/amine < ImH/ImH < ImH/thioether < thioether/thioether

In this scale, the most negative potentials corresponds to the bis- and mono-(imidazolate) ligations while the most

positive potentials are associated with the mono- and bis-(thioether) ligations. The ν_{11} frequencies obtained for the 6cLS MP8(II) complexes rank with the preceding redox scale (Table 5). Further potentiometric studies on the investigated MP8 derivatives in aqueous detergent media would be very informative to quantitatively relate changes in redox potential with changes in ν_{11} frequency.

Ligand effects on the ν_{11} mode. Assigned to a $\nu(C_aC_m)$ stretch (Li et al. 1989), the ν_{10} mode is sensitive to the electronic properties of the axial ligands (Spiro and Burke 1976), but much more influenced by the out-of-plane deformations of the porphyrin macrocycle (Alden et al. 1989; Czernuszewicz et al. 1989). As for the oxidized compounds, it is expected to measure the ability of the axial ligands to bring about a heme distortion.

The MP8(II) complexes having both a mixed-type ligation and a protonated His exhibit a ν_{10} mode at 1618–1622 cm^{-1} (Table 4). This frequency is indicative of a significant heme c distortion (Othman et al. 1994, 1997). The comparison of the ν_{10} frequency of the His/ImH, and His/Lys coordinations (1621 and 1620 cm^{-1}, respectively) shows that, with a common His ligand, the ImH $\rightarrow$ Lys substitution in the trans position has no significant effect on the porphyrin structure. Thus, steric constraints induced by the proximal side of MP8 are determinants for porphyrin ruffling in the MP8(II)-(ImH) and MP8(II)-(Lys) complexes. As far as the mono(AcMet) complexes of MP8(II) are concerned, the heme-His bonding interaction and the relative orientation of the thioether and imidazole groups likely influence the degree of heme distortion. Form I of the His/AcMet coordination has a ν_{10} frequency (1622 cm^{-1}) close to that of the His/ImH and His/Lys coordinations (1620–1621 cm^{-1}). The corresponding moderate heme distortion appears to be again induced by proximal constraints in MP8. For form II, the ν_{10} frequency is significantly decreased at 1618 cm^{-1}, reflecting an increased nonplanar deformation. This additional distortion likely originates from the geometry of the S(AcMet) ligation since a perpendicular orientation between the CH_3–S–CH_2-axis of AcMet and the imidazole plane of His can produce a strong heme distortion in cyt c_4 (Gadsby et al. 1989).

Table 4 shows the lowest ν_{10} frequency for the His^-/Lys coordination (1614 cm^{-1}). A strong Fe(II)-His^- bonding interaction, an increased steric constraint of the Im^- ring of His and a minimal restraint of the amine ligand on the porphyrin core are three factors able to generate a more strongly distorted macrocycle.

A 1625–1626 cm^{-1} frequency is observed for the bis(Lys) and bis(AcMet) complexes of MP8(II) (Table 4). Therefore, when the His of MP8 is unbound, ferroheme c adopts a nearly planar conformation (Othman et al. 1994, 1997). The release of constraints exerted by the –Cys–Ala–Gln–Cys–His– peptide on heme c are certainly at the origin of the porphyrin flattening when the His of MP8 is deligated. Long axial bonds, similar to those observed in symmetric bis(amine) and bis(thioether) model compounds (Scheidt and Gouterman 1983), and the low steric demands of the amine and thioether groups are factors that likely participate in the stabilization of a planar structure.

In conclusion, the nonplanar structure of ferroheme c is essentially determined by the internal binding of the His side chain of MP8. The extent of heme deformation is then modulated by the electronegativity of the histidylimidazole ring. Moreover, the ν_{10} mode exhibits a single band for each MP8(II) complex. By contrast with the 6cLS derivatives of MP8(III), this observation indicates a single heme conformer. These comparisons point to a substantial difference in heme deformbility when the iron atom of MP8 is either oxidized or reduced.

Ligand effects on the ν_{10} mode. Like ν_{11}, the ν_{38} mode mainly corresponds to a $\nu(C_bC_b)$ stretching mode (Li et al. 1989). However, ν_{38} appears sensitive to both electronic and steric interactions between heme and its ligands. When Lys is the sixth ligand (His^-/Lys, His/Lys and Lys/Lys coordinations), a rough linear correlation is observed between the ν_{11} and ν_{38} frequencies (data not shown). For the ligations involving at least one AcMet (AcMet/AcMet and forms I and II of His/AcMet), another linearity is found between the two frequencies. The points corresponding to the His/ImH and His^-/Im^- coordinations are outside the two preceding lines and could constitute another correlation (data not shown). Therefore, the three preceding classes of heme coordination can be ranked according the electronic and/or steric nature(s) of the sixth ligand. In each class, the behavior of the ν_{38} mode appears to follow that of the ν_{11} mode.

Ligand effects on the ν_4 mode. The ν_4 mode consists of C_aN and C_aC_b bond stretches and is sensitive to the π electron density at the heme (Spiro and Burke 1976; Li et al. 1989). A small systematic shift of the ν_4 frequency was detected for ferrous cyt c having His/His, His/Met and His/amine ligations (Desbois 1994). This effect is not clearly observed for the same coordination schemes in MP8(II) (Table 4). In fact, the ν_4 frequency is mostly affected by the deprotonation of the proximal His producing downshifts of 3 cm^{-1}. This property may be used to determine the ionization state of His but complicates the ligand attributions.

Ligand effects on the ν_{50} mode. The ν_{50} mode is an out-of-plane Fe-N(pyrrole) vibration likely coupled with an Fe-ligand vibration (Othman et al. 1994; Li et al. 1989). Hence, its frequency is expected to be dependent on several factors, in particular the strength and the arrangement of the axial bonds, the masses of the axial ligands and the conformation of the FeN_4(pyrrole) grouping. For the investigated MP8(II) derivatives, the ν_{50} frequency is significantly influenced by the axial heme coordination (Table 4).

In conclusion, the ν_{11}, ν_{10}, ν_{38}, ν_4 and ν_{50} frequencies that correspond to different porphyrin modes discriminate electronic and/or steric effects of the ligands on the porphyrin macrocycle. All these modes are therefore good

markers in the determination of the heme ligation in c-type ferrocytochromes.

Heme coordination in c-type cytochromes

The ImH complexes of MP8 were previously characterized to simulate the bis(His) coordination of several c-type cytochromes (Othman et al. 1994). The Lys and AcMet complexes of MP8 have been investigated to model the two other most plausible heme coordinations in c-type cytochromes and to measure the influence of mixed axial coordinations on the heme structure.

Coordination geometry is an energetic compromise between the electronic and steric properties of the heme and its ligands (Scheidt and Gouterman 1983). In MP8, the internal His ligation is relatively well fixed (Smith and McLendon 1981). As far as the sixth coordination of MP8 is concerned, its free access and the absence of peptide atoms in its proximity allow the exogenous ligand to accomodate unconstrained ligation mode(s). In the cytochromes, the protein fold decreases the degree of freedom of the axial ligands and could induce some strains on the coordinating atoms. Therefore, the 6cLS complexes of MP8 are illustrative of relaxed coordination situations and can serve to evaluate the "entatic" or "rack" states of the heme-ligands complexes in c-type cytochromes (Williams 1995).

Ferric cytochromes. The ν_{11} mode was found to be sensitive to the axial ligation in ferricyt c (Kitagawa et al. 1977). However, its narrow frequency variation (1562–1567 cm^{-1}) makes its use as a safe marker difficult in the characterization of the heme ligation of ferricytochromes. The low-frequency bands observed in the 356–362 and 390–410 cm^{-1} regions of RR spectra of 6cLS MP8(III) complexes also exhibit frequency variations with the heme coordination (Table 2). They thus can be used for complementary probes to identify the axial heme ligands in 6cLS ferricytochromes.

Through the analysis of the ν_{10} region, the RR spectra of the 6cLS MP8(III) complexes have shown that the heme-peptide-ligand(s) interactions can induce different degrees of out-of-plane macrocycle deformation. This effect is due to the small core size of ferriheme and the constraints exerted by the peptide fold, the intramolecular binding of the proximal His, and the thioether linkages of heme c. These observations establish that the ν_{10} frequency is a good marker for the determination of heme distortions in the c-type ferricytochromes (Othman et al. 1996, 1997).

Ferrous cytochromes. The RR investigations on various complexes of MP8(II) show that the frequencies of the ν_{11}, ν_{10}, ν_{38}, ν_{50} and ν_4 modes behave differently with the axial heme ligations (Table 4). The ν_{11} frequency was generally used to characterize the axial ligation of ferrous cytochromes (Desbois 1994). However, to remove some ambiguities in the heme coordination, an additional characterization of the ν_{10}, ν_{38} and ν_{50} frequencies appears particularly useful.

From magnetic circular dichroism and RR spectra, it was proposed that the heme coordination of plant cyt f is either His/Lys or His/amine (Siedow et al. 1980; Desbois and Lutz 1984; Davis et al. 1988). The recent resolution of the crystal structure of spinach cyt f has revealed a His/amine heme ligation. However, the amine coordination is not provided by a Lys side chain but derives from the α-amino group of Thr-1 (Martinez et al. 1994). The ν_{11} frequency of various ferrous cyt f is detected at 1532–1538 cm^{-1} (Davis et al. 1988; Desbois 1994). The His/NH_3 and His/Lys coordinations in MP8(II) lead to a ν_{11} mode at 1535 cm^{-1}, the His deprotonation decreasing this frequency to 1529 cm^{-1} (Table 5 and data not shown). In proteins, H-bonding at the coordinated histidylimidazole decreases the ν_{11} frequency (Desbois and Lutz 1992). The interaction of one of the amine protons with a H-bond acceptor increases the electron density at the coordinated nitrogen and thus can also decrease the ν_{11} frequency. The ν_{11} frequency variation of ferrous cyt f could therefore be interpreted as corresponding to different degrees of H-bonding at the α-amino and/or histidylimidazole ligands. It seems also possible to attribute the slight difference in the ν_{11} frequency of cyt f and MP8(II)-(Lys) to the lower pK_a value and the higher steric hindrance of an α-amino acid group relatively to those of an aliphatic amine group.

The ν_{11} mode of ferrous cyt c containing a His/Met heme ligation was observed at 1540–1548 cm^{-1}. More precisely, it is detected at 1540–1543, 1544–1547, 1544–1548 and 1543–1545 cm^{-1} for cyt c_1, cyt c_2, cyt c and cyt c_{551}, respectively (Strekas and Spiro 1972; Adar and Erecinska 1974; Kitagawa et al. 1975; Shelnutt et al. 1981; Lewis et al. 1985; Lou et al. 1993; Desbois 1994; Othman et al. 1997) (Table 5). As previously discussed for the two His/AcMet ligations of the MP8(II)-(AcMet) complex, the different sets of ν_{11} frequency can be attributed to differences in H-bonding states and/or coordination geometry of the His/Met ligands. The fold of the Cys–X–Y–Cys–His–motif and its binding mode to heme c imposing a very similar orientation of the histidyl-imidazole ligand (Senn and Wüthrich 1985), the expected differences in the coordination geometry most likely concern the S(Met) ligand. A difference in Met configuration was exemplified by horse cyt c and *Pseudomonas aeruginosa* cyt c_{551} (Takano and Dickerson 1981; Matsuura et al. 1982; Senn and Wüthrich 1985). The ν_{11} mode of these two cytochromes is observed at 1547 (±1) and 1544 (±1) cm^{-1} (Strekas and Spiro 1972; Lewis et al. 1985; Lou et al. 1993; Desbois 1994). This ca. 3 cm^{-1} difference may indicate that the R and S configurations of the Met side chain have a weak effect on the ν_{11} frequency. However, the H-bonding state of the Met and/or His ligand(s) may compensate the effect of the Met configuration on the ν_{11} frequency. In this context, the crystallographic structures of reduced cyt c and c_{551} show different H-bonding interactions of the S(Met) ligand with the protein. In ferrocyt c, the hydroxyl group of a conserved Tyr residue is at a hydrogen-bonding distance from this ligand (Berghuis and Brayer 1992). In cyt c_{551}, the amine group of an Asn side chain constitutes the H-bond donor (Matsuura et al. 1982). It is thus probable that the H-bond-

ing state and the conformation of the thioether group inversely modulate the S(Met) electronegativity to produce similar ν_{11} frequencies for cyt c and cyt c_{551}.

Our investigations of the MP8 derivatives (Othman et al. 1993, 1994, and this work) have characterized different electronic and steric influences of the peptide and axial ligand(s) on the structures of ferric and ferrous heme c. Future RR investigations on heme compounds, in which rotational constraints on the ligand coordinations occur, will be helpful to measure its effects on the heme structure.

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