N-Hydroxyguanidines as New Heme Ligands: UV−Visible, EPR, and Resonance Raman Studies of the Interaction of Various Compounds Bearing a C═NOH Function with Microperoxidase-8[†]

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ABSTRACT: Interaction between microperoxidase-8 (MP8), a water-soluble hemeprotein model, and a wide range of *N*-aryl and *N*-alkyl *N*′-hydroxyguanidines and related compounds has been investigated using UV−visible, EPR, and resonance Raman spectroscopies. All the *N*-hydroxyguanidines studied bind to the ferric form of MP8 with formation of stable low-spin iron(III) complexes characterized by absorption maxima at 405, 535, and 560 nm. The complex obtained with *N*-(4-methoxyphenyl) *N*′-hydroxyguanidine exhibits EPR *g*-values at 2.55, 2.26, and 1.86. The resonance Raman (RR) spectrum of this complex is also in agreement with an hexacoordinated low-spin iron(III) structure. The dissociation constants (K_s) of the MP8 complexes with mono- and disubstituted *N*-hydroxyguanidines vary between 15 and 160 μM at pH 7.4. Amidoximes also form low-spin iron(III) complexes of MP8, although with much larger dissociation constants. Under the same conditions, ketoximes, aldoximes, methoxyguanidines, and guanidines completely fail to form such complexes with MP8. The K_s values of the MP8−*N*-hydroxyguanidine complexes decrease as the pH of the solution is increased, and the affinity of the *N*-hydroxyguanidines toward MP8 increases with the pK_a of these ligands. Altogether these results show that compounds involving a -C(NHR)═NOH moiety act as good ligands of MP8−Fe(III) with an affinity that depends on the electron-richness of this moiety. The analysis of the EPR spectrum of the MP8−*N*-hydroxyguanidine complexes according to Taylor's equations shows a strong axial distortion of the iron, typical of those observed for hexacoordinated heme−Fe(III) complexes with at least one π donor axial ligand (HO^-, RO^-, or RS^-). These data strongly suggest that *N*-hydroxyguanidines bind to MP8 iron via their oxygen atom after deprotonation or weakening of their O−H bond. It thus seems that *N*-hydroxyguanidines could constitute a new class of strong ligands for hemeproteins and iron(III)-porphyrins.

Growing interest has recently emerged in *N*-hydroxyguanidines since it was proven that N^{ω}-hydroxy-L-arginine (NOHA)[1] is a key intermediate in the biosynthesis of nitric oxide (NO) from L-arginine (*1*). NO, an ubiquitous biological messenger, is involved in neurotransmission, vascular tone regulation, and immune responses (*2*). It is produced by hemeproteins called NO synthases (NOSs) through oxidation of the guanidino group of L-arginine with intermediate formation of NOHA (*3*). L-Arginine and NOHA positionings in the active site of NOSs have been recently solved by crystallographic studies (*4*, *5*). Moreover, it has been recently shown that NOSs can also produce NO from oxidation of exogenous *N*-aryl *N*′-hydroxyguanidines such as *N*-(4-chlorophenyl) *N*′-hydroxyguanidine (*6*). Preliminary results concerning the interaction between *N*-aryl *N*′-hydroxyguanidines and NOSs or cytochromes P450 (D. Lefevre-Groboillot, J.-L. Boucher, and D. Mansuy, to be published) led us to examine the interactions between various *N*-hydroxyguanidines and a simple hemeprotein model, microperoxidase-8 (MP8).

MP8 is a product of enzymatic cleavages of horse heart cytochrome *c* (*7*). It contains the type *c* heme and residues 14 to 21 from the protein. Cys 14 and Cys 17 are covalently linked to the porphyrin moiety by thioether bridges, and His 18 acts as an iron axial ligand, as in cytochrome *c* itself. At

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[1] Abbreviations: CTABr, cetyltrimethylammonium bromide; DMSO, dimethyl sulfoxide; EPR, electron paramagnetic resonance; MP8, microperoxidase-8; NOHA, N^{ω}-hydroxy-L-arginine; NOS, NO-synthase; PBS, phosphate buffer saline; RR, resonance Raman.

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pH between 5 and 9, in 20% methanol aqueous solution, and at concentrations below 10 μM, MP8 is monomeric with His 18 as an iron(III) fifth ligand, the sixth position being occupied by a water molecule (*8*, *9*). MP8 is able to catalyze both peroxidase- and cytochrome P450-type reactions (*10*, *11*), which makes it an attractive water-soluble hemeprotein model, suitable for structural and mechanistic studies (*12*−*15*).

As far as coordination chemistry is concerned, MP8 has been used to characterize factors that determine the affinity of heme ligands. Comparison of their binding affinities toward MP8 with those observed with full hemeproteins give insights into the distal environment of the heme in the hemeproteins (*12*, *16*). Equilibrium constants for the coordination of several members of different families of heme ligands to MP8−Fe(III) have also been determined, giving insights into the relative roles of basicity, steric hindrance, π stacking, and other factors in the heme affinity for ligands (*14*, *17*, *18*).

Little information is available about the coordination chemistry of *N*-hydroxyguanidines (*19*) and particularly about the possible roles of these compounds as iron ligands in hemeproteins. It has been recently shown that *N*-hydroxyguanidine itself binds to a cytochrome *c* peroxidase mutant bearing a cavity above its distal heme side (*20*). This binding results in changes of the visible spectrum that suggest conversion of the high spin ferric heme to a low spin state (*20*). Here we report the study of the interaction of MP8 with a wide range of *N*-alkyl and *N*-aryl *N*′-hydroxyguanidines and related compounds bearing a C═NOH function, using UV−visible, EPR, and resonance Raman spectroscopies. This study shows that, generally speaking, *N*-aryl (or *N*-alkyl) *N*′-hydroxyguanidines act as good ligands of MP8−Fe(III). Amidoximes also bind to MP8 but with a much lower affinity, whereas ketoximes, aldoximes, *N*-methoxyguanidines, and guanidines do not. These results demonstrate the existence of a new class of heme ligands, the compounds bearing a -C(NHR)═NOH moiety, and give insights into the effects governing the affinity of these molecules toward the heme of MP8.

MATERIALS AND METHODS

Microperoxidase-8 Preparation. MP8 was prepared by sequential peptic and tryptic digestions of horse heart cytochrome *c* (Sigma) as described by Aron et al. (*7*). The purity of the sample was more than 97%, based on matrix-assisted desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry analysis. MP8 concentration was determined according to the absorption of its Soret band [$\epsilon_{397} = 1.57 \times 10^5$ M^{-1} cm^{-1} (*7*)]. All experiments were carried out in 0.1 M phosphate buffer saline (PBS), pH 7.4, containing 20% methanol, unless indicated.

Chemistry-General. Chemicals and reagents of the highest grade commercially available were obtained from Aldrich, Fluka, or Janssen and used without further purification. Aminoguanidine bicarbonate came from Aldrich. Chemical reactions were monitored by TLC using Merck precoated silica gel $60F_{254}$ (0.25 mm thickness) plates with analysis of the product mixtures at 254 nm. Merck Kieselgel 60 (70−230 mesh ASTM) was used for flash chromatography. ^{1}H NMR spectra were recorded on a Bruker ARX 250 MHz spectrometer. Mass spectra were recorded at Ecole Normale Superieure, Paris, on a RiberMag system with fast atom bombardment, electron impact, chemical ionization, or high-resolution techniques. Elemental analyses were performed at the Service de Microanalyze, Université P. et M. Curie, Paris.

*Synthesis of Compounds **1**−**26**.* These compounds were prepared by classical methods that are briefly indicated in the following. All of them were completely characterized by ^{1}H NMR and mass spectroscopy and gave satisfactory elemental analyses. Their detailed synthesis and characteristics will be published elsewhere.

*Compounds **1**−**17**.* *N*-Aryl *N*′-hydroxyguanidines **1**−**10**, *N*-alkyl *N*′-hydroxyguanidines **11**−**13**, and *N*,*N*-disubstituted *N*′-hydroxyguanidines **14**−**15** were obtained, as well as small amounts of the corresponding ureas, by the addition of hydroxylamine hydrochloride to cyanamides RR′NHCN in anhydrous ethanol, following a classical method (*21*).

General Protocol for Synthesis of Cyanamides RR′NHCN (*22*). A solution of 10 mmol of the aryl or alkylamine in 15 mL of methanol was added dropwise at 0 °C to a solution of 1.2 mmol of BrCN in 15 mL of methanol containing 30 mmol of anhydrous sodium acetate. The reaction mixture was stirred 2 h at 0 °C and 2−20 h at room temperature. The solvent was removed under vacuum, and the crude product was extracted with CH_2Cl_2. The organic phase was washed with brine, dried, and evaporated. Crude cyanamides were purified by flash chromatography over SiO_2 using CH_2Cl_2 as solvent and/or recrystallized from CH_2Cl_2/cyclohexane mixtures.

*General Protocol for Synthesis of N-Hydroxyguanidines **1**−**15*** (*22*−*25*). A 0.2 M solution of the cyanamide in anhydrous ethanol containing 1.1 equiv of hydroxylamine hydrochloride was heated under reflux for 1−15 h. The solvent was evaporated, and the residue was dissolved in water. The byproduct urea was filtered off or extracted with ethyl acetate. The aqueous phase was brought to pH 8−9 by the addition of a saturated solution of $NaHCO_3$ and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and evaporated. The crude *N*-hydroxyguanidines were either recrystallized in diethyl ether/pentane mixtures or redissolved in minimal amounts of methanol and precipitated after the addition of a solution of anhydrous HCl (or acetic acid) in diethyl ether.

Symmetrically, *N*,*N*′-disubstituted *N*′′-hydroxyguanidines **16**−**17** were obtained upon reaction of hydroxylamine hydrochloride with the corresponding carbodiimides according to a previously described protocol (*26*, *27*).

*Compounds **19**−**26**.* Benzamidoximes **19**−**20** were prepared by refluxing anhydrous methanolic solutions of hydroxylamine hydrochloride with the corresponding nitrile in the presence of sodium carbonate following a classical protocol (*28*).

Acetamidoximes **21**−**22** were prepared by heating under reflux the corresponding anilines in anhydrous ethanol for 3 days in the presence of excess ethyl *N*-hydroxyacetimidate (Aldrich) according to a previously described protocol (*29*).

Ketoxime **23** and aldoxime **24** were prepared following conventional methods by reacting hydroxylamine hydrochloride with 4-chlorobenzaldehyde or 4-chloroacetophenone in aqueous ethanol as described previously (*30*).

N-(4-Chlorophenyl) *N*′-methoxyguanidine **25**: 45% yield from (4-chlorophenyl) cyanamide (*23*) and methoxylamine hydrochloride (Fluka) following the general method used to prepare *N*-hydroxyguanidines (*21*).

N-(4-Chlorophenyl) guanidine hydrochloride **26** (*31*): *N*,*N*′-Bis (*tert*-butyloxycarbonyl) *N*′′-(4-chlorophenyl) guanidine was obtained in 90% yield by reacting overnight at room temperature *N*,*N*′-bis(*tert*-butyloxycarbonyl) pyrazole-1-carboxamidine (*32*) with 4-chloroaniline in anhydrous THF according to the procedure of Bernatowicz et al. (*32*).

UV−*Visible Studies of the Interaction of MP8 with Various Compounds.* Studies were carried out at room temperature in 1-cm path length cuvettes containing 0.6 μM MP8, and with a UVIKON 942 spectrophotometer (Kontron Biotek). Assayed compounds were first dissolved in DMSO and then in buffer, and equivalent volumes of DMSO and buffer were added to the reference cuvette. Dissociation constants (K_s) of the MP8−ligand complexes were calculated from the plots of $1/\Delta A(\lambda_{max} - \lambda_{min})$ versus 1/[ligand].

Spectrophotometric pK_a Determination. pK_a values of *N*-aryl *N*′-hydroxyguanidines **1**−**8** and **17** were determined on the basis of the absorption spectra (200−400 nm) of 67 μM solutions in 0.1 M PBS buffer between pH 3 and pH 11. The protonated and unprotonated forms have very different absorption maxima, allowing the measurement of the pK_a value as the midpoint pH value for the transition from one form to the other. As the pK_a values for the *N*-(parasubstituted phenyl) *N*′-hydroxyguanidines **1**−**6** were found to follow a classical Hammet relationship ($pK_a = -1.32\sigma + 7.61$, $R^2 = 0.97$), pK_a values for molecules **7** and **8** were extrapolated from the values of the other ones (Hammet's constants taken from ref *33*).

EPR Spectroscopy. EPR spectra were obtained using a Bruker ER 200 spectrometer operating at X-band frequency (9.45 GHz), 100 kHz modulation frequency, microwave power of 10 mW, and equipped with an Oxford Instrument liquid helium probe. Quartz tubes containing MP8 alone (150 μL, 100 μM) or in the presence of the studied compounds were frozen in cold ethanol and then in liquid nitrogen. All spectra were recorded at 7 and 35 K with five to 10 accumulations for each experiment. The method of Taylor (*34*) for crystal field parameters calculation was used with the axis systems described in the text and the following equations giving the rhombic (V) and axial (Δ) ligand field terms in unit of spin−orbit coupling constant ζ.

$$V/\zeta = g_x/(g_z + g_y) + g_y/(g_z - g_x)$$

$$\Delta/\zeta = g_x/(g_z + g_y) + g_z/(g_y - g_x) - V/2\zeta$$

Resonance Raman Spectroscopy. The Raman spectra were recorded at 20 ± 1 °C using a Jobin-Yvon spectrophotometer (HG 2S) with the 413.1 excitation of a Kr^+ laser (Coherent Innova). Using radiant laser powers of 15−40 mW, the RR spectra (2−8 scans) were collected, imported, checked, and analyzed with a Grams/32 software (Galactic industries). Under these conditions, the frequency precision is 0.5−3 cm^{-1}, depending on the intensity of the bands.

RESULTS

Study by UV−*Visible Spectroscopy of the Interaction between MP8-Iron(III) and Various N-Hydroxyguanidines and Related Compounds.* The spectrum of free MP8 in PBS buffer, pH 7.4, containing 20% methanol showed absorption maxima at 397, 530, and 621 nm (Figure 2), as previously reported (*7*). The progressive addition of *N*-(4-methoxyphenyl) *N*′-hydroxyguanidine **1** (see Figure 1 and Table 1 for the numbering of the compounds used in this study) gave rise to a difference spectrum characterized by a minimum at 394 nm and a maximum at 408 nm (Figure 3). This reaction was saturable, and the plot of $1/\Delta A(408-394\text{ nm}) = f(1/[\mathbf{1}])$ led to a dissociation constant (K_s) of the MP8−**1** complex of 35 ± 4 μM (inset of Figure 3). The data were also fitted according to the Hill equation (*35*). The resulting slope (0.98 ± 0.03) was not significantly different from 1, in agreement with the binding of a single molecule of **1** to monomeric MP8, as expected at the low concentration of MP8 used (0.6 μM). When fully saturated with **1**, the solution showed an absolute spectrum with a Soret band at 405 nm and α and β bands at 560 and 535 nm (Figure 2). The spectrum of this *N*-hydroxyguanidine-saturated MP8 solution did not change after 24 h at room temperature.

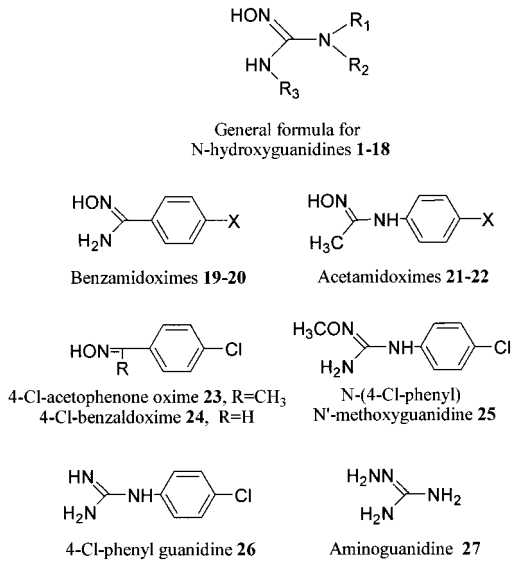

FIGURE 1: Structure of the compounds used in this study. See Table 1 for the numbering of the various compounds.

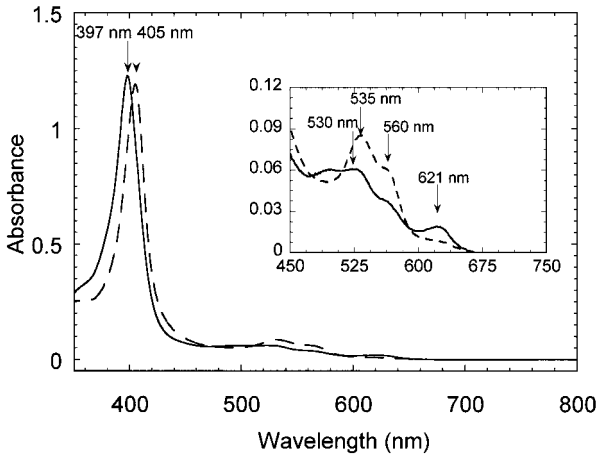

FIGURE 2: UV−visible spectrum of MP8−Fe(III) (8 μM) alone (solid line) and of the complex formed upon addition of 5 mM *N*-(4-methoxyphenyl) *N*′-hydroxyguanidine **1** (dashed line). Spectra were recorded in 0.1 M PBS buffer, pH 7.4, containing 20% methanol and under the conditions described in Materials and Methods.

Table 1: Dissociation Constants Measured for the Complexes of MP8−Fe(III) with *N*-Hydroxyguanidines **1**−**18** and Amidoximes **19**−**22**

N-hydroxyguanidines R_1R_2N-C(=N-OH)-NHR_3

	R_1	R_2	R_3	K_s [a] (μM)	pK_a [b] (±0.1)	$K'_{s7.4}$ [c] (μM)
1	4-CH_3O-C_6H_4-	H-	H-	35±4	7.9	8.4±1.0
2	C_6H_5-	H-	H-	61±5	7.7	22±2
3	4-CH_3-C_6H_4-	H-	H-	48±3	7.8	14±1
4	4-^{t}Bu-C_6H_4-	H-	H-	51±7	7.8	15±2
5	4-Cl-C_6H_4-	H-	H-	95±14	7.3	56±8
6	4-CF_3-C_6H_4-	H-	H-	160±20	6.9	110±14
7	4-NH_2-C_6H_4-	H-	H-	18±4 [d]	8.3 [e]	1.8±0.4
8	4-HO-C_6H_4-	H-	H-	30±6	8.0 [e]	6.0±1.2
9	2-Cl-C_6H_4-	H-	H-	35±2	ND	ND
10	3-Cl-C_6H_4-	H-	H-	70±20	ND	ND
11	n-C_5H_{11}-	H-	H-	106±12	ND [f]	17±2
12	C_6H_5-CH_2-	H-	H-	35±4	ND [f]	5.8±1
13	C_6H_5-$(CH_2)_2$-	H-	H-	48±1	ND [f]	8.0±0.5
14	R1 R2 = 	H-		85±5	ND	ND
15	R1 R2 = 	H-		60±4	ND	ND
16	*iso*-C_3H_7-	H-	*iso*-C_3H_7-	33±2	ND	ND
17	C_6H_5-	H-	C_6H_5-	15±4	6.3	14±4
18	H-	H-	H-	280±40	8.0 [g]	56±8

Amidoximes R_1-C(=N-OH)-NHR_2

	R_1	R_2	K_s [a] (μM)
19	CH_3O-C_6H_4-	H-	570±10
20	Cl-C_6H_4-	H-	2500±400
21	CH_3-	CH_3O-C_6H_4-	180±10
22	CH_3-	Cl-C_6H_4-	470±30

[a] K_s values were calculated from the difference spectra obtained after the addition of increasing amounts of compounds **1**−**22** to 0.6 μM MP8 in PBS buffer, pH 7.4, containing 20% methanol under the conditions described in Materials and Methods. Means ± SD from at least three experiments. [b] pK_a values were obtained by measuring the 200−400 nm UV−visible absorption spectra of compounds **1**−**6** or **17** in 0.1 M PBS buffers of different pH values as indicated in Materials and Methods. [c] $K'_{s7.4}$ values were calculated by multiplying the K_s value by the fraction of deprotonated compound at pH 7.4. [d] The K_s value for **7** was assumed to correspond to the binding of the *N*-hydroxyguanidine group of this molecule to the iron atom, since aniline has a much lower affinity (K_s in the millimolar range (*18*)). [e] pK_a value extrapolated on the basis of Hammet's constants (see Materials and Methods). [f] pK_a of NOHA, another monoalkyl-substituted compound was reported to be 8.1 (*5*). [g] From ref *36*. ND: not determined.

To evaluate the degree of specificity of this new MP8 ligand, we studied the interactions of MP8 with other compounds of related structures. Twenty-five compounds bearing the C═NOH function were prepared for that purpose, according to classical procedures indicated in Materials and Methods.

The progressive addition of any of the *N*-aryl *N*′-hydroxyguanidines **1**−**10** to a solution of MP8 gave rise to similar absolute and difference spectra. The same kind of interaction with MP8 was also observed with *N*-alkyl *N*′-hydroxyguanidines **11**−**13**, and disubstituted hydroxyguanidines **14**−**17**. Dissociation constants for mono- or disubstituted *N*-hydroxyguanidines (Table 1) varied between 15 and 160 μM, with a mean value around 50 μM. The same interaction

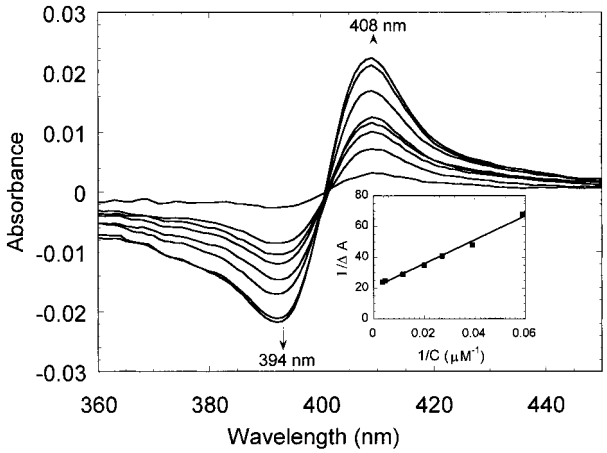

FIGURE 3: Difference spectrum obtained upon addition of increasing concentrations of *N*-(4-methoxyphenyl) *N*′-hydroxyguanidine **1** to free MP8−Fe(III). The two cuvettes contained 0.6 μM MP8 in 0.1 M PBS buffer, pH 7.4, containing 20% methanol. Inset: Plot of $1/\Delta A$(408−394 nm) against 1/[**1**].

Table 2: Effect of pH on the Dissociation Constants (K_s) of Some MP8−*N*-Hydroxyguanidine Complexes[a]

compound	**1**	**5**	**11**
pK_a	7.9	7.3	8.1
K_s at pH 6.0	316 ± 40	265 ± 30	540 ± 30
K_s at pH 7.4	35 ± 4	95 ± 14	106 ± 12
K_s at pH 8.1	13 ± 3	51 ± 1	15 ± 1
ratio $K_{s\,6.0}/K_{s\,8.1}$	24	6	36

[a] K_s values were calculated from the difference spectra observed after the addition of increasing amounts of compounds **1**, **5**, or **11** to 0.6 μM MP8 in PBS buffer, pH 6.0, 7.4, or 8.1 containing 20% methanol, under the conditions described in Materials and Methods. Means ± SD from at least three experiments.

was also observed with *N*-hydroxyguanidine itself (compound **18**), and with the NOS product, NOHA, although with significantly higher K_s values (280 and 220 μM, respectively).

The same kind of spectra were also observed with amidoximes **19**−**22**, but the measured K_s values were much higher (between 180 and 2500 μM, Table 1). *N*-Aryl acetamidoximes **21** and **22**, and benzamidoximes **19** and **20** showed dissociation constants about 5 and 16 times higher, respectively, than those observed for the *N*-hydroxyguanidines bearing the same aryl group. For all the studied *N*-hydroxyguanidines and amidoximes, the spectral characteristics (minima and maxima of the difference spectra, absolute position of the Soret band of the ligand-saturated MP8 solution) were almost identical (data not shown).

On the contrary, no difference spectrum was observed when ketoxime **23**, aldoxime **24**, or *N*-aryl *N*′-methoxyguanidine **25** was added to an MP8 solution, even when using high concentration of these compounds (10 mM). High concentrations (10 mM) of arylguanidine **26** or aminoguanidine **27** also failed to produce any significant difference spectrum with MP8. Finally, simple compounds involving an OH function, such as benzyl alcohol or phenol did not modify the visible spectrum of MP8, even when used at a concentration of 3 mM (data not shown).

Table 2 shows that an increase of the pH of the solution from 6.0 to 8.1 led to a marked decrease of the K_s values measured for the MP8 complexes with *N*-hydroxyguanidines

Table 3: Comparison of the EPR Data of Various Low-Spin MP8−Fe(III) Complexes[a]

ligand	*g* values	Δ/ζ, V/Δ[b,c]	Δ/ζ, V/Δ[b,d]
1	1.86, 2.26, 2.55	5.2, 0.59	4.9, 0.74
5	1.85, 2.26, 2.55	5.1, 0.58	4.8, 0.75
imidazole	1.49, 2.26, 2.98	3.0, 0.79	3.2, 0.55
methionine[e]	1.51, 2.29, 2.92	3.0, 0.71	3.1, 0.62
cyanide[e]	1.16, 1.97, 3.28	2.7, 1.15	3.7, 0.31

[a] EPR spectra of 100 μM MP8−iron(III) in the presence of *N*-hydroxyguanidines **1** or **5** or imidazole were recorded at 35 K under the conditions described in Materials and Methods. [b] Δ/ζ is the tetragonal field expressed in units of spin−orbit coupling constant ζ, and V/Δ is the rhombicity, in an arbitrary scale depending on the convention. [c] Crystal field parameters calculated with the convention originally used by Peisach and Blumberg (*44*), i.e., with $g_y > -g_x > -g_z > 0$. [d] Crystal field parameters calculated with the convention used by Thomson et al. (*47*), i.e., with $g_z > g_y > g_x > 0$. [e] *g* values taken from ref *50*.

1, **5**, and **11**. This pH-dependent variation of K_s appeared to increase with the pK_a of the *N*-hydroxyguanidine. On the contrary, addition of ketoxime **23**, aldoxime **24**, or phenol to MP8 failed to lead to any difference spectrum even at pH 9.8, confirming the very different behavior of these compounds and *N*-hydroxyguanidines toward MP8.

EPR Study of the Interaction of MP8-Iron(III) with N-Hydroxyguanidines and Related Compounds. The EPR spectrum of 100 μM MP8−Fe(III) in PBS buffer pH 7.4 containing 20% methanol displayed three sets of signals at 7 K: a high-spin feature at ca. $g_\perp = 5.7$ ($g_\parallel$ unresolved) and two low-spin features ($g_1 = 3.18$, $g_2 = 2.13$, g_3 unresolved, and $g_1 = 2.9$, $g_2 = 2.3$, g_3 unresolved) (spectra not shown). For higher concentrations of MP8 (500 μM), the relative intensity of the low-spin species increased ($\simeq$ 5 times), as compared to that of the high spin signal. This result strongly supported the interpretation that the low-spin features were related to MP8 aggregates, while the high spin signal at $g_\perp = 5.7$ corresponded to MP8 monomers. Aggregation has already been observed for concentrations of MP8 higher than 10 μM (*8*).

Addition of *N*-hydroxyguanidine **1** (5 mM) to the 100 μM MP8−Fe(III) solution gave rise to a new low-spin species ($g_1 = 2.55$, $g_2 = 2.26$, $g_3 = 1.86$, Table 3) with complete disappearance of *all* the signals of starting MP8 (Figure 4). A similar observation was done when adding *N*-hydroxyguanidine **5** or amidoximes **19** and **21**. In the case of the two latter compounds, disappearance of the signals of free MP8 was not complete, which is in agreement with the lower affinity of these molecules for MP8 previously found by UV−visible spectroscopy (Table 1). In all cases, the *g* values measured for the new MP8−Fe(III) complexes were very similar (data not shown).

On the contrary, ketoxime **23** (5 mM), *N*-methoxyguanidine **25** (5 mM), or *N*-arylguanidine **26** (5 mM) failed to modify the EPR spectrum of a 100 μM solution of free MP8 (data not shown).

These results confirmed the ability of *N*-hydroxyguanidines to act as potent ligands of MP8, and showed that the corresponding complexes are low-spin ferric species.

Resonance Raman (RR) Study of the Complex of MP8−Fe(III) with 1. The high-frequency region (1300−1700 cm^{-1}) of the RR spectrum of free MP8−Fe(III) (100 μM) was very similar to that previously described (*37*) (Figure 5A). The oxidation state marker band, ν_4, was observed at 1376 cm^{-1}, as expected for a ferric complex. The coordination and spin state sensitive bands, ν_2, ν_3, and ν_{10}, were found at 1589, 1508, and 1642 cm^{-1}, respectively. These frequencies were indicative of a hexacoordinated low-spin species (*37*, *38*). However, shoulders were clearly identified at 1574 (ν_2) and 1618 (ν_{10}) cm^{-1}, indicating the presence of a high-spin species.

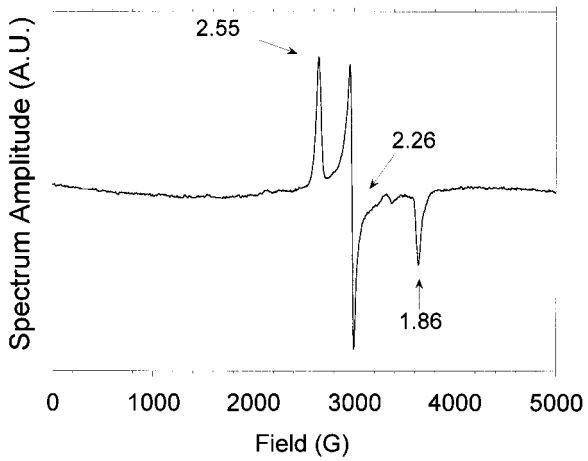

FIGURE 4: X-band EPR spectrum obtained after the addition of 5 mM *N*-(4-methoxyphenyl) *N*′-hydroxyguanidine **1** to a 100 μM solution of MP8 in 0.1 M PBS buffer, pH 7.4, containing 20% methanol. MP8 and ligand **1** were mixed at room temperature, set in an EPR tube, and cooled at 35 K before recording of the spectra under the conditions described in Materials and Methods. a.u.: arbitrary units.

Addition of *N*-hydroxyguanidine **1** (5 mM) to MP8−Fe(III) (100 μM) induced the formation of another Fe(III) hexacoordinated low-spin species with ν_2, ν_3, ν_{10}, and ν_{11} modes at 1590, 1505, 1639, and 1561 cm^{-1}, respectively (Figure 5A and Table 4). The ν_4 mode was observed at 1377 cm^{-1}, indicating that the exogenous ligand had no influence on the redox state of MP8. The high-spin contributions observed in the RR spectrum of free-MP8 were totally absent when *N*-hydroxyguanidine **1** was added, and the ν_{10} band displayed a single component, as observed by Gaussian analysis (data not shown).

The low-frequency region (150−700 cm^{-1}) of RR spectra of MP8 in the absence and in the presence of *N*-hydroxyguanidine **1** exhibited differences for the ν_8 and ν_{50} modes (Figure 5B). After ligand addition, the ν_8 and ν_{50} bands were respectively downshifted from 349 to 347 cm^{-1} and from 362 to 358 cm^{-1}. In the same region, the frequencies of modes involving heme peripheral substituents, i.e., 404 and 420 cm^{-1} (thioether bridges) and 379 cm^{-1} (propionates) (*38*), were not significantly modified by the interaction of MP8 with *N*-hydroxyguanidine **1**.

Finally, under the same conditions, addition of ketoxime **23**, *N*-methoxyguanidine **25**, or *N*-arylguanidine **26** (each 5 mM) to a 100 μM solution of free MP8 did not lead to any significant modification of the RR spectrum of the native hemepeptide (data not shown).

DISCUSSION

The aforementioned results clearly show that *N*-aryl and *N*-alkyl *N*′-hydroxyguanidines constitute a new family of strong ligands for MP8−Fe(III). Their binding to MP8-iron,

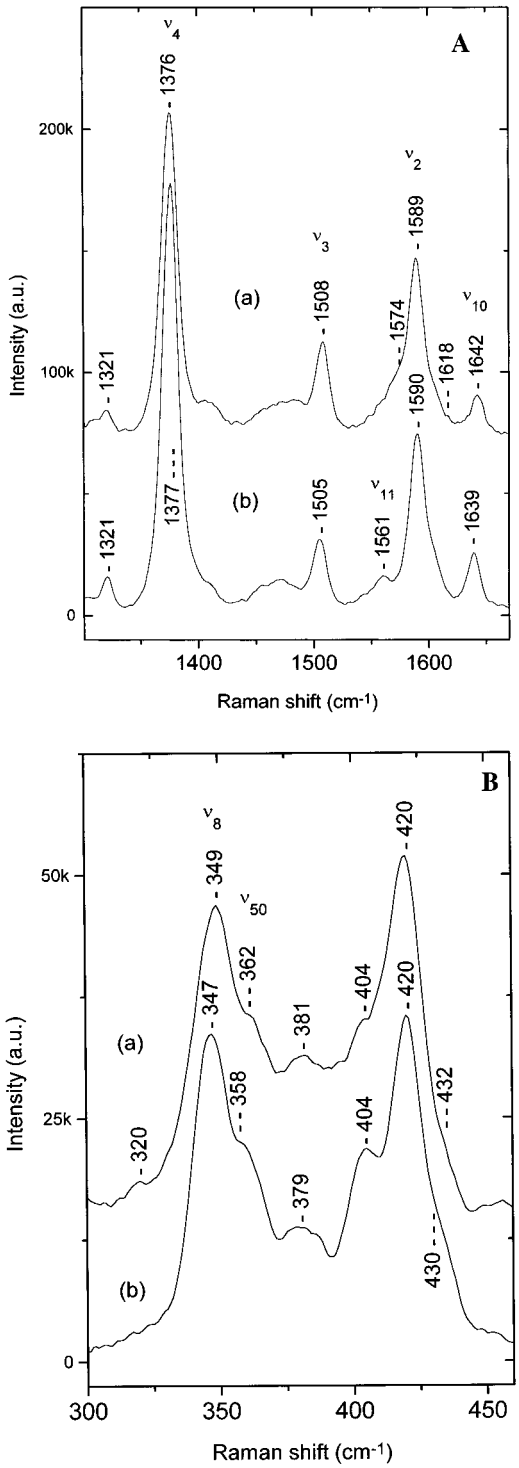

FIGURE 5: High-frequency (A) and low-frequency (B) resonance Raman spectra of 100 μM MP8−Fe(III) in 0.1 M PBS buffer, pH 7.4, containing 20% methanol, alone (a) and in the presence of 5 mM *N*-(4-methoxyphenyl) *N*′-hydroxyguanidine **1** (b). a.u.: arbitrary units.

in trans position to the endogenous imidazole axial ligand, leads to the formation of low-spin Fe(III) complexes, as shown by the EPR spectra (Figure 4) and the high-frequency region of the RR spectra (Figure 5A) obtained after addition of *N*-hydroxyguanidines to MP8−Fe(III). Table 4 compares the positions of the ν_2, ν_3, ν_4, and ν_{10} RR bands of various MP8−Fe(III) and MP8−Fe(II) complexes. It clearly shows that the RR spectrum observed for MP8 after addition of **1** reveals the only presence of a low-spin Fe(III) complex. The

Table 4: Comparison of the High-Frequency RR Modes (in cm^{-1}) of Various MP8 Complexes

exogenous ligand	ν_{10}	ν_2	ν_3	ν_4
1 (this study, Figure 5A)[a]	1639	1590	1505	1377
Low-Spin MP8−Fe(III) Complexes				
imidazole[b]	1637/1641	1587	1504	1377
lysine[b]	1638/1640	1589	1506	1377
N-Ac-methionine[b]	1630/1640	1590	1505	1373
CN^{-}[c]	1639[d]	1591	1506	1375
High-Spin MP8−Fe(III) Complexes				
no added ligand[e,f]	1625/1629	1578	1492	1374
F^{-}[c]	1609[d]	1572	1479	1371
MP8−Fe(II) Complexes				
no added ligand[e,g]	1603	1570	1468	1353
imidazole[f]	1617	1592	1494	1358

[a] RR spectrum of 100 μM MP8−Fe(III) in the presence of 5 mM **1** (see Materials and Methods). [b] Taken from ref *39*. [c] Taken from ref *51*. [d] No precision is given on the broadness of the band. [e] In the presence of the detergent CTABr, MP8 is mainly monomeric. [f] Taken from ref *37*. [g] Taken from ref *52*.

low-spin Fe(III) state of MP8−*N*-hydroxyguanidine complexes is confirmed by the positions of the Soret, α and β bands of their UV−visible spectrum that are very similar to those observed for a typical low-spin ferric complex, MP8−Fe(III)-imidazole (*8*).

In fact, all the mono- and disubstituted *N*-hydroxyguanidines studied in this work, **1**−**17**, are strong MP8 ligands with K_s values between 15 and 160 μM; their affinity for MP8 is similar to that of imidazole (K_s = 26 μM under our conditions) and much higher than that of pyridine (in the mM range (*40*)). Related compounds not containing the N−OH function, such as *N*-aryl *N*′-methoxyguanidine **25**, *N*-arylguanidine **26**, and amino-guanidine **27** fail to give such complexes with MP8. However, amidoximes **19**−**22** also bind to MP8−Fe(III), but with a markedly lower affinity (K_s values between 180 and 2500 μM, Table 1), whereas ketoximes and aldoximes such as **23** and **24** do not interact with MP8 under identical conditions. These results show that formation of MP8 complexes with the aforementioned molecules at least requires the presence of a -C(NHR)═NOH moiety.

Very little data have been reported on the coordination properties of molecules bearing a C═N−OH moiety toward hemes and hemeproteins. Aldoximes, contrary to ketoximes, bind to Fe(II) porphyrins and cytochromes P450−Fe(II) via their nitrogen atom (*41*, *42*). Binding of fluorenone oxime to an iron(III) porphyrin has been recently reported to occur via coordination of its oxygen atom (*43*).

The results reported in this paper, as well as those described in a recent publication about the formation of a complex between *N*-hydroxyguanidine itself and a cytochrome *c* peroxidase mutant (*20*), strongly suggest that *N*-hydroxyguanidines could be a new class of ligands for hemes and hemeproteins. Preliminary results indicate that this is true at least for cytochromes P450 and NOSs (D. Lefèvre-Groboillot, J.-L. Boucher, and D. Mansuy, in preparation).

The nature of the binding mode of *N*-hydroxyguanidines to heme−iron(III) remains to be determined, as the X-ray structure reported for the complex of *N*-hydroxyguanidine with a cytochrome *c* peroxidase mutant did not allow the authors to conclude about the detailed ligand binding mode

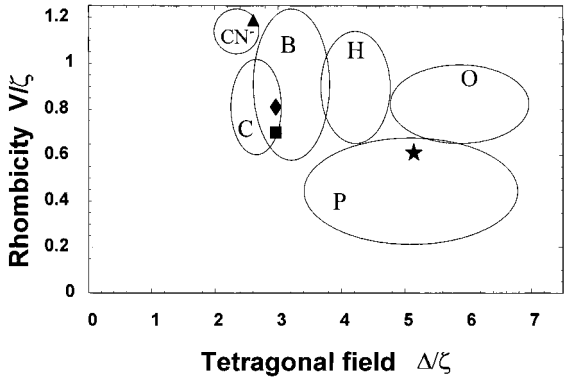

FIGURE 6: Correlation between the tetragonal field and the rhombicity of various low-spin MP8 complexes and positioning of these complexes in a modified Peisach and Blumberg's diagram (*46*). The Peisach and Blumberg's convention for the principal axes (*44*) was used. The B, C, H, O, P, and CN^- zones contain respectively the bis-imidazole, imidazole-methionine, imidazole-imidazolate, imidazole-phenolate, thiolate containing, and cyano-imidazole derivatives according to Tsai et al. (*46*). Positions of the MP8−Fe(III) complexes containing the CN^- (▲), imidazole (◆), methionine (■), or *N*-hydroxyguanidine **1** (★) ligands are indicated.

(*20*). Detailed analysis of the EPR spectra of low-spin ferric complexes of hemeproteins, using Taylor's equations (*34*) and Peisach and Blumberg's diagram (*44*), has been done to get further insights into the coordination mode of the *N*-hydroxyguanidine moiety. Peisach and Blumberg's diagram proved to be useful in assigning ligands of noncrystallized proteins, as in the case of chloroperoxidase for which a thiolate axial ligand was predicted on the basis of EPR data before other structural information became available (*45*).

We have calculated the position of several MP8−Fe(III) low-spin complexes in the Peisach and Blumberg's diagram, constructed from the rhombic (V) and tetragonal (Δ) field strength, and designed with the convention $g_y > -g_x > -g_z > 0$. Values are listed in Table 3 and are located in a Peisach and Blumberg's diagram that has been slightly modified in a recent publication of Tsai et al. (*46*) to include data related to chloroperoxidase and NO synthases (Figure 6). The three previously described MP8−Fe(III) complexes with imidazole, methionine, and CN^- fall in the expected zones, i.e., the B, C, and cyano zones, containing respectively the bis-imidazole derivatives, the methionine-imidazole derivatives, and the cyano-imidazole derivatives. These three complexes display moderate tetragonal field, between 2 and 3. At the opposite, the *N*-hydroxyguanidine-MP8 complexes display a much higher tetragonal field (≃5.2) and fall at the not well-defined border between the O-zone, containing imidazole−hydroxyde and imidazolate−phenolate complexes and the P-zone, containing the P450 or P450-like protein complexes, which have a cysteinate ligand and any other axial ligand.

Another analysis of the EPR spectra of hemeprotein complexes, using the $g_z > g_y > g_x > 0$ convention, and correlating EPR and near-infrared MCD data, has been done by Thomson et al. (*47*). These authors showed that, for hemes having an histidine proximal ligand, the tetragonal field varies with the nature of the sixth ligand in the order: thioether < CN^- ∼ imidazole ∼ RNH_2 < imidazolate < N_3^- < SH^- < phenolate ∼ OH^- ∼ RS^- (*47*). With this axis convention, the MP8−Fe(III) complexes with imidazole, methionine, and CN^- have tetragonal field in the expected range (between 3.0 and 3.7) with the order methionine < imidazole < CN^-, whereas the *N*-hydroxyguanidine-MP8 complexes display a much higher tetragonal field (≃4.9, Table 3), comparable to those observed with hydroxyde, phenolate, or thiolate axial ligands.

Although these plots do not give any precise structural information, the analysis of this new type of MP8−Fe(III) complex with the Taylor's equation is of interest in the understanding of its electronic properties. It indicates that this ligand is able to cause an important axial distortion to the metal center, similar to those observed with very good π donor ligands, such as cysteinate, hydroxyde, or phenolate ligands.

Insights into the environment of the Fe(III) atom are also provided by the ν_8 and ν_{50} modes of the MP8−Fe(III)−**1** complex (Figure 5B). These modes correspond to combination of Fe−N(pyrrole) stretching with Fe−axial ligand stretching (*37*, *48*). ν_8 is sensitive to the heme conformation and ν_{50} to the axial ligation (*37*, *39*, *49*). Their downshifted frequencies compared to the free hemepeptide can be reliably associated with a change in the geometry of the coordination sphere of the iron atom upon binding of **1**. Comparison with previously described low-spin MP8−Fe(III) complexes shows that the ν_8 band of the MP8−Fe(III)−**1** (347 cm^{-1}) is 3 to 4 cm^{-1} higher than those of the MP8−Fe(III)-imidazole, lysine, and methionine complexes (*37*, *39*), whereas the ν_{50} band (358 cm^{-1}) is distinct from the one of the imidazole complex (362 cm^{-1}), and closer to that of the lysine complex (359 cm^{-1}) (*37*, *39*).

As far as the global structure of the heme is concerned, the 2−4 cm^{-1} downshifts displayed by the ν_3 and ν_{10} modes of the MP8−Fe(III)−**1** complex (Figure 5A and Table 4) as compared to free MP8 can be attributed to an increased heme distortion upon binding of **1**. These bands are assigned to skeletal modes [$\nu_s(C_aC_m)$ and $\nu_{as}(C_aC_m)$, respectively] and are sensitive to the porphyrin conformation (*37*−*39*, *49*). The presence of a single ν_{10} band at 1639 cm^{-1} suggests that the interaction between MP8−Fe(III) and **1** produces a rigid complex. This is quite unusual as the RR spectra of all the previously studied iron−(III) and nickel(II)−MP8 complexes exhibited composite ν_{10} modes, which indicate a conformational flexibility of the MP8 *c*-type heme (*37*, *39*, *49*).

The variation of the binding constants of *N*-hydroxyguanidines to MP8 as a function of pH (between 6 and 8) is expected to be informative as *N*-hydroxyguanidines are weak bases with pK_a in the range 7−8 (*36*). The marked decrease of the K_s values of compounds **1**, **5**, and **11** when the pH is increased (Table 2) and the larger variation of these constants with more basic *N*-hydroxyguanidines ($K_{s\ 6.0}/K_{s\ 8.1}$ varies in the order 11 > 1 > 5) suggest that only the basic neutral form of *N*-hydroxyguanidines binds to MP8−Fe(III). The pK_a values of the *N*-aryl *N*′-hydroxyguanidines **1**−**8** and **17** were found (see Materials and Methods) to vary between 6.3 and 8.3 (Table 1). Dissociation constant values corresponding to the binding of the unprotonated forms, RNH−C(NHR′)═NOH, of *N*-hydroxyguanidines to MP8 (at pH = 7.4) were then calculated ($K'_{s7.4}$, Table 1), to take into account the very different percentage of protonation of these *N*-aryl *N*′-hydroxyguanidines at pH = 7.4 (from 7% for **17** to 90% for **7**). The same calculation was done for *N*-hydroxyguanidine itself (pK_a = 8.0 (*36*)) and for the *N*-alkyl

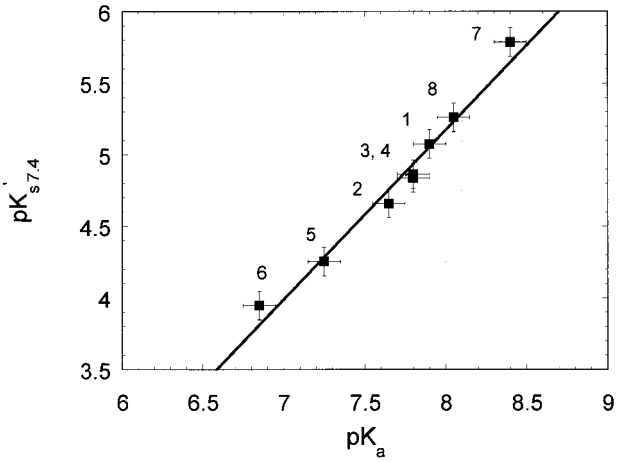

FIGURE 7: Plot of p$K'_{s7.4}$ values against pK_a values for different *N*-aryl *N*′-hydroxyguanidines. The straight line is the best linear fit for *N*-(parasubstituted-phenyl) *N*′-hydroxyguanidines **1**−**8** (p$K'_{s7.4}$ = 1.18 pK_a − 4.29, R^2 = 0.97).

N′-hydroxyguanidines **11**−**13**, assuming that the pK_a values of the *N*-hydroxyguanidine moieties of these three monoalkyl compounds are not significantly different from the pK_a of NOHA (pK_a = 8.1 (*5*)). These $K'_{s7.4}$ values varied from 1.8 μM for compound **7** to 110 μM for compound **6** (Table 1).

Table 1 clearly shows that there is a global relationship between the basicity of the *N*-hydroxyguanidines and their affinity for MP8−Fe(III). For a given class of compounds exhibiting a highly similar structure at the *N*-hydroxyguanidine function, the *N*-aryl *N*′-hydroxyguanidines **1**−**8**, there is a linear relationship between their p$K'_{s7.4}$ and their pK_a, as shown in Figure 7. The unability of the protonated form of *N*-hydroxyguanidines to bind to MP8−Fe(III) is also shown by EPR experiments indicating that addition of 1 mM **1** to 100 μM MP8−Fe(III) at pH 4 does not significantly modify the EPR spectrum of native MP8 (data not shown, compare with the results of Figure 4). Conversely, addition of 1 mM **1** to 100 μM MP8 at pH 9 leads to a complete conversion to the low-spin complex.

Thus, our results show that compounds involving a -C(NHR)═NOH moiety act as good ligands of MP8−Fe(III) with an affinity that depends on the electron richness of this moiety. The EPR spectra of MP8−*N*-hydroxyguanidine complexes, which are similar to those observed for hexacoordinated heme−Fe(III) complexes involving a π-donor anionic axial ligand (HO^-, RO^-, or RS^-), strongly suggest that *N*-hydroxyguanidines and amidoximes bind to MP8-iron via an especially electron-rich oxygen atom. A first possible structure (Figure 8a) would involve a deprotonated oxygen atom. Such a deprotonation should be favored by the binding of the N−OH moiety to the iron in a first step, which makes the hydrogen more acidic, and by the delocalization of the negative charge on the C═N−O^- moiety. Another possible structure (Figure 8b) would involve the weakening of the ligand O−H bond by an intramolecular hydrogen bond with the non-hydroxylated nitrogen. Such intramolecular hydrogen bonds have already been proposed to exist in the free *N*-hydroxyguanidine moiety, on the basis of configuration analysis (*53*). In both cases, the role of the non-hydroxylated nitrogen atom would be to facilitate either the deprotonation of the C═NOH function or the weakening

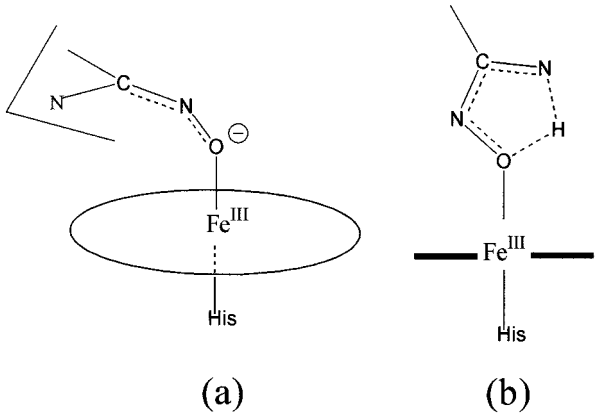

FIGURE 8: Possible binding modes of *N*-hydroxyguanidines or amidoximes to MP8−Fe(III).

of its O−H bond and consequently to strengthen the Fe−O bond. In that regard, it is noteworthy that simple compounds involving an OH function, such as alcohols or phenols, are unable to form such low-spin complexes of MP8.

Further studies are currently being performed on various iron(III) porphyrins and hemeproteins to evaluate the importance of *N*-hydroxyguanidines as iron ligands, to determine the nature of the iron-ligand bond in these new heme−iron(III) complexes, and to understand the molecular origin of the high affinity of *N*-hydroxyguanidines for heme−iron(III).

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