Spin–Lattice Relaxation Time and Temperature Dependence of Fluorine-19 Nuclear Magnetic Resonance Spectra of Cysteine-containing Peptide Iron(II) Complexes†

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The ^{19}F NMR signals of cysteine-containing peptide ligands, such as Z-Cys(1)-Pro-Leu-Cys(2)-Gly-X [Z = $PhCH_2OCO$; X = NH(C_6H_4F-*m*), NH(C_6H_4F-*p*), NH($CH_2C_6H_4$F-*p*) or NH($CH_2CH_2C_6H_4$-F*p*)] were isotropically shifted both down- and up-field by co-ordination to Fe^{II} in $[NEt_4]_2$[Fe(Z-cys-Pro-Leu-cys-Gly-X)$_2$]. The shifted signals show very short spin–lattice relaxation times (T_1) in the range 5–55 ms, while the corresponding free peptide ligands give much longer T_1 values (1500–3500 ms). The temperature dependence of the ^{19}F NMR spectra indicates that the isotropic shifts are caused by the dipolar mechanism when the groups X are NH($CH_2C_6H_4$F-*p*) and NH($CH_2CH_2C_6H_4$F-*p*), while when X = NH(C_6H_4F-*p*) and NH(C_6H_4F-*m*) both contact and dipolar mechanisms are involved. The observed behaviours are explained by the formation of X NH ··· S Cys(2) hydrogen bonds and the presence of π–π interactions between the aromatic group and the sulfur atom of the cysteine residue.

A variety of physical techniques including electronic absorption,[1] NMR,[2] CD,[3] MCD,[4] ESR[5] and Raman[6] spectroscopies have been used to study the properties of native metalloproteins and their models. Especially, NMR spectroscopy has been widely utilized in recent years. For example, the structure around the metal centre of cobalt(II) stellacyanin, the crystal structure of which is still unavailable, was determined on the basis of metal–proton distances obtained by using NMR spectroscopy and the primary structure of the protein.[7] For the high-potential iron–sulfur protein from *Chromatium vinosum*, similar NMR studies[8] were carried out and the structure deduced is consistent with that obtained by X-ray analysis. The unique effect of a paramagnetic metal centre has been widely utilized for structure determination.[9] Detailed mechanisms of the delocalization of unpaired electrons from iron were proposed based on such NMR studies and also on comparison with the available crystal structure.[8b]

The ^{1}H NMR spectra of native *Desulfovibrio gigas* rubredoxin and its models, cysteine (Cys)-containing peptide iron(II) complexes, have been reported recently.[10,11] The Cys $C_\beta H_2$ groups which have the nearest protons to the iron(II) ion were isotropically shifted to much lower field (in the range δ 150–250 referenced to $SiMe_4$) and the signals of such protons were very broad due to the strong paramagnetic effect of iron(II) ($S = 2$).[10,11] Thus, conventional NMR techniques as nuclear Overhauser enhancement (NOE) and two-dimensional measurements are not effective for such iron(II) thiolate systems at present, although these techniques have been applied to the studies of some proteins including iron–sulfur proteins of ferredoxin.[8,12] Furthermore, the spin–lattice relaxation time T_1 of these Cys $C_\beta H_2$ protons was found to be extremely short (less than 1 ms).[11] Similar shortening of T_1 values has been observed for native rubredoxin.[13] In this paper, we present the results of ^{19}F NMR T_1 measurements of cysteine-containing peptide iron(II) complexes with a distal fluoro-substituent.

Fluorine-19 NMR spectroscopy has frequently been used to study the properties and structures of metalloproteins or related metal complexes owing to its high resolution and the high sensitivity of the ^{19}F nucleus to local magnetic environments.[14] For example, fluoro-substituted tyrosine (Tyr) was incorporated into the alkaline phosphatase from *Escherichia coli* and ^{19}F NMR spectroscopy was used to study the protein conformation, local environments of specific residues (*e.g.* Tyr) and the motional properties of proteins.[15] The ^{19}F magnetic relaxation has been employed to examine the binding of fluoride ion to bovine copper–zinc superoxide dismutase.[16] Since no reports have appeared on the ^{19}F NMR study of peptide models of metalloenzymes, the present investigation is expected to give important information on the local peptide structure of the model complexes.

Experimental

Materials.—The cysteine-containing oligopeptide iron(II) complexes used in this study, $[NEt_4]_2$[Fe{Z-cys-Pro-Leu-cys-Gly-NH(C_6H_4F-*m*)}$_2$] **1** (Z = benzyloxycarbonyl, Ph-CH_2OCO), $[NEt_4]_2$[Fe{Z-cys-Pro-Leu-cys-Gly-NH(C_6H_4F-*p*)}$_2$] **2**, $[NEt_4]_2$[Fe{Z-cys-Pro-Leu-cys-Gly-NH($CH_2C_6H_4$-F-*p*)}$_2$] **3** and $[NEt_4]_2$[Fe{Z-cys-Pro-Leu-cys-Gly-NH(CH_2-$CH_2C_6H_4$F-*p*)}$_2$] **4**, were synthesised by reaction of $[NEt_4]_2[Fe(SBu^t)_4]$ with the corresponding deprotected-Cys peptide ligand in tetrahydrofuran (thf) solution as described previously.[17,18] All the operations and measurements were carried out under argon atmosphere. Solvents used for synthesis and spectral measurements such as thf and CD_3CN were distilled and deoxygenated by purging with argon or by repeated evacuation before use.

Physical Measurements.—The 470 MHz ^{19}F NMR spectra of the fluoro-containing cysteine peptide iron(II) complexes were measured on a JEOL JNM-GX 500 Fourier-transform spectrometer. The signal due to $CFCl_3$ was defined as δ 0 and used as external reference. The temperature dependence of the spectra was examined at +30, +10, −10 and −30 °C. Experimental conditions were as follows: about 2560 transients for iron(II) complexes or 256 ones for mixtures of iron(II) and free peptide ligands were collected with a band width of 100 kHz using 16 384 data points. The pulse delay times were 2.0 and 15 s for the cysteine peptide iron(II) complexes and the free peptide ligands, respectively. The inversion recovery method was employed for T_1 measurements of ^{19}F NMR signals using a 180°–τ–90° pulse where τ is the delay time between 180 and

† Throughout this paper, cys indicates cysteine (Cys) deprotonated at the SH group.

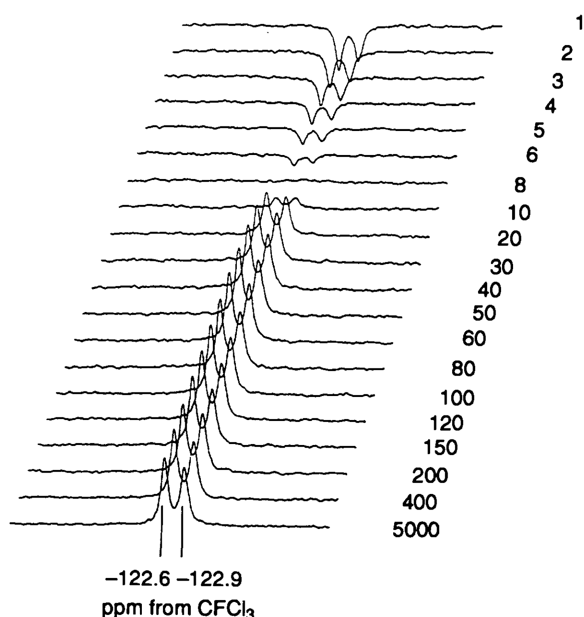

Fig. 1 The ^{19}F NMR spectra from inversion-recovery T_1 measurements of $[Fe\{Z\text{-cys-Pro-Leu-cys-Gly-NH}(C_6H_4F\text{-}p)\}_2]^{2-}$ **2** in CD_3CN at 30 °C. Delay times (ms) between 180 and 90° pulses given at right

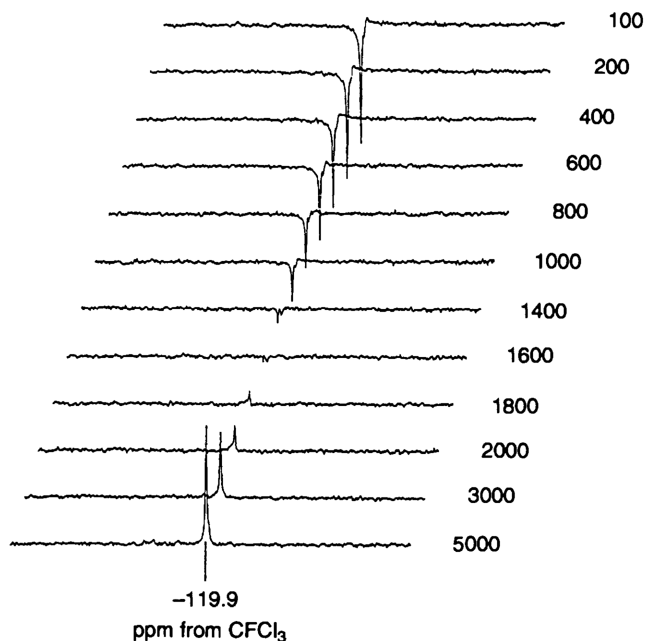

Fig. 2 The ^{19}F NMR spectra from inversion-recovery T_1 measurements of Z-Cys-Pro-Leu-Cys-Gly-NH(C_6H_4F-*p*) in CD_3CN at 30 °C. Delay times (ms) between 180 and 90° pulses given at right

90° pulses.[19] The T_1 values were obtained using a linear least-squares fitting of the signal intensities as a function of τ.

Results

Spin-Lattice Relaxation Time (T_1) *of* ^{19}F *NMR Signals observed for Iron*(II) *Cysteine Peptide Complexes*.—Measurements of T_1 were carried out for complexes **1**–**4** and the corresponding free peptide ligands in CD_3CN at 30 °C. For example, Figs. 1 and 2 show the ^{19}F NMR spectra from inversion-recovery T_1 measurements of complex **2** and the corresponding free peptide ligand Z-Cys-Pro-Leu-Cys-Gly-NH(C_6H_4F-*p*). The T_1 values of the signals at δ −122.6 and −122.9 are 13.3 and 13.2 ms, respectively. As reported previously, these two signals were assigned to the co-ordinated peptide ligands and isotropically shifted to high field due to the influence of the paramagnetic iron(II) ion.[17] The corresponding free peptide ligand gives a signal at δ −119.9 and a T_1 value of 2160 ms. Therefore, a noticeable T_1 shortening occurs for the iron(II) complex through the co-ordination of the sulfurs of the cysteine residues. The result indicates the existence of a strong magnetic interaction between the fluorine and paramagnetic centre due to the formation of a (p-FC_6H_4)NH ··· S Cys(2) hydrogen bond (see below). Similar results were obtained for the other fluoro-containing peptide iron(II) complexes and the T_1 values are listed in Table 1. All of the complexes **1**–**4** exhibit two isotropically shifted ^{19}F NMR signals which have been confirmed to be due to the existence of two isomers (δ and λ) in solution.[20]

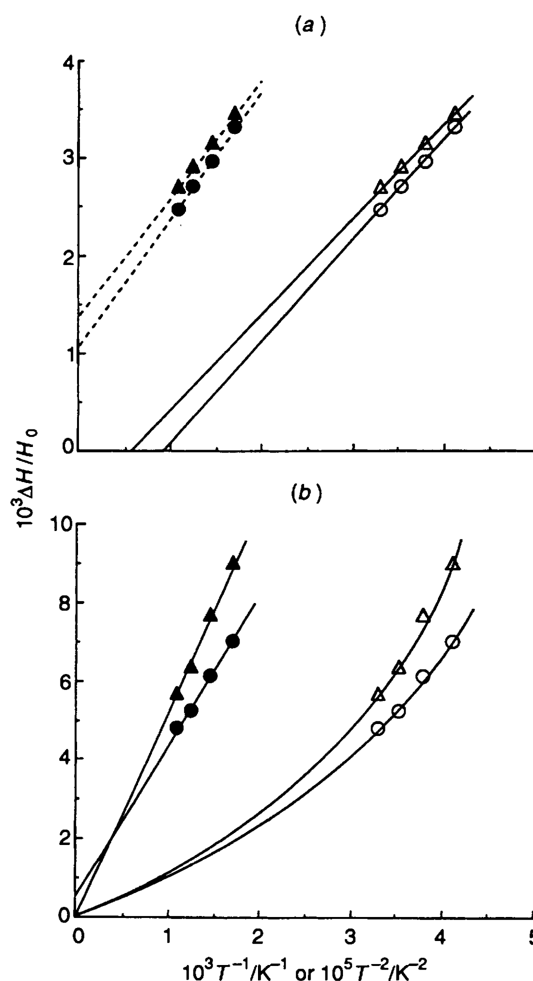

Fig. 3 Plots of $\Delta H/H_0$ against T^{-1} (open symbols) and T^{-2} (filled symbols) for the isotropically shifted ^{19}F NMR signals of (*a*) $[Fe\{Z\text{-cys-Pro-Leu-cys-Gly-NH}(C_6H_4F\text{-}p)\}_2]^{2-}$ **2** (δ −122.9, △, ▲; −122.6, ○, ●) and (*b*) $[Fe\{Z\text{-cys-Pro-Leu-cys-Gly-NH}(CH_2C_6H_4F\text{-}p)\}_2]^{2-}$ **3** (δ −118.7, △, ▲; −118.6, ○, ●)

Temperature Dependence of the Isotropically Shifted ^{19}F *NMR Signals*.—The temperature dependence of isotropically shifted ^1H and ^2H NMR resonances of the iron(II) cysteine peptide complexes was previously examined and plots of $\Delta H/H_0$ *vs.* T^{-1} were found to be linear and to obey the Curie–Weiss law.[11] Now the temperature dependence of the isotropically shifted ^{19}F NMR signals was investigated at 30, 10, −10 and −30 °C. For example, plots of the isotropic shifts ($\Delta H/H_0$) of complexes **2** and **3** against T^{-1} or T^{-2} are shown in Fig. 3.

Table 1 Fluorine-19 spin-lattice relaxation times (T_1) of $[Fe^{II}(Z\text{-cys-Pro-Leu-cys-Gly-X})_2]^{2-}$ and the corresponding free peptide ligands, Z-Cys-Pro-Leu-Cys-Gly-X, in CD_3CN at 30 °C

	T_1/ms (δ)*		
X	Iron(II) complex		Ligand
$NH(C_6H_4F\text{-}m)$	6.8 (−103.5)	8.2 (−108.3)	1670 (−114.0)
$NH(C_6H_4F\text{-}p)$	13.3 (−122.6)	13.2 (−122.9)	2160 (−119.9)
$NH(CH_2C_6H_4F\text{-}p)$	24.1 (−118.6)	33.2 (−118.7)	1810 (−118.1)
$NH(CH_2CH_2C_6H_4F\text{-}p)$	44.1 (−119.8)	52.1 (−120.3)	3200 (−119.3)

* Fluorine-19 NMR spectra of complexes **1**–**4** were reported in refs. 17 and 18.

In the case of complex **2**, linear correlations were obtained for the plots of the isotropic shifts against both T^{-1} and T^{-2} as shown in Fig. 3(*a*) where the T^{-1} dependence is indicated by solid lines and the T^{-2} dependence by broken ones. The intercepts of the T^{-1} and T^{-2} dependences on the isotropic shift axis at infinite temperature are -0.9×10^{-3}, -0.5×10^{-3} and 1.1×10^{-3}, 1.4×10^{-3}, respectively, for the signals at δ −122.9 and −122.6 **2** at 30 °C in CD_3CN. Consequently, the isotropically shifted ^{19}F NMR signals observed for complex **2** are of both contact and dipolar origin. The contact contributions should be predominant since the plots *vs.* T^{-1} give smaller intercepts than those *vs.* T^{-2}.[21] However, the temperature dependence of the iron(II) complex of Z-Cys-Pro-Leu-Cys-Gly-$NH(CH_2C_6H_4F\text{-}p)$ **3**, is quite different from that of complex **2** as shown in Fig. 3(*b*). The isotropically shifted resonances do not conform to a T^{-1} dependence; a linear relationship *vs.* T^{-2} is found which can be extrapolated through the origin within experimental error. The intercepts for the T^{-1} plots are -3.7×10^{-3} and -7.5×10^{-3} when straight lines are drawn instead of the curves in Fig. 3(*b*), while those for the T^{-2} plots are 0.5×10^{-3} and 0 for the signals at δ −118.6 and −118.7. Thus the intercepts for the T^{-2} plots are much smaller than those for the T^{-1} plots. The results indicate that the ^{19}F NMR isotropic shifts of complex **3** are essentially due to dipolar contributions.[21]

Discussion

NH···S Hydrogen Bonding and Interactions of Aromatic Groups with Sulfur in Cysteine Peptide Iron(II) *Complexes.*— Complexes **1**–**4** can be divided into two groups according to the Gly-X fragments. In one the NH of X is conjugated with a benzene ring, *i.e.* when X = $NH(C_6H_4F\text{-}m)$ **1** or $NH(C_6H_4\text{-}F\text{-}p)$ **2**. In the other, namely where X = $NH(CH_2C_6H_4F\text{-}p)$ **3** or $NH(CH_2CH_2C_6H_4F\text{-}p)$ **4**, the NH of X does not conjugate with the benzene ring because of the intervening methylene group(s).

In the conjugated case, complexes **1** and **2**, some electronic charge flows from the sulfur of the co-ordinated cysteine residue, Cys(2), to the benzene ring through the NH···S hydrogen bond as demonstrated by the linear relationship between the redox potentials and the Hammett constant of the *para* substituent Y in the complexes $[Fe^{II}\{Z\text{-cys-Pro-Leu-cys-Gly-}NH(C_6H_4Y\text{-}p)\}_2]^{2-}$ (Y = OMe, H, F or CN).[17] Hence, the isotropically shifted ^{19}F NMR signals observed for **1** and **2** are due to contact contributions mainly through the Fe–S···H–N bonds and the π-conjugating benzene ring as shown in Fig. 4(*a*), *i.e.* the NH···S hydrogen bond would provide a pathway for direct spin delocalization into the aromatic group *via* a contact mechanism. The downfield shift in the *m*-fluoro case and the upfield shift in the *p*-fluoro case were caused by the opposite signs of the spin density at the *meta* and *para* positions.[22]

The distance between the N(H) and sulfur atoms of cysteine residues forming the NH···S hydrogen bonds has been reported to be in the range 3–4 Å for native *Clostridium pasteurianum* rubredoxin from X-ray analysis.[23] A similar

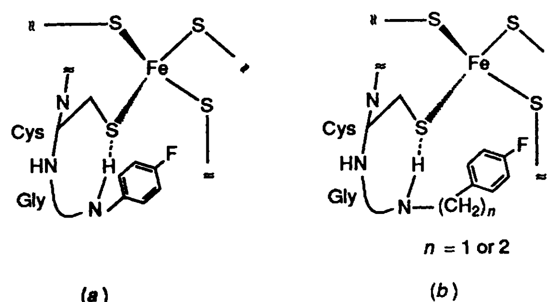

Fig. 4 Schematic structures (Cys-Gly-X part) of (*a*) $[Fe\{Z\text{-cys(1)-Pro-Leu-cys(2)-Gly-}NH(C_6H_4F\text{-}p)\}_2]^{2-}$ **2** and (*b*) $[Fe\{Z\text{-cys(1)-Pro-Leu-cys(2)-Gly-}NH(CH_2)_nC_6H_4F\text{-}p\}_2]^{2-}$ (n = 1 or 2)

distance was also estimated by using BIOGRAF (energy Minimizing program, MSI, USA) energy-minimized calculations for the cysteine peptide iron(II) model complexes.[11] Thus, the benzene ring of the anilide is considered to be quite near to the FeS_4 core. The result is supported by the very short spin–lattice relaxation times of ^{19}F in complexes **1** and **2** as shown in Table 1. This short distance probably results in partial dipolar contributions to the isotropic shifts of ^{19}F NMR signals observed for complexes **1** and **2**. The two kinds of isotropic shifts are explained by both contact and dipolar contributions to the ^{19}F NMR shifts as deduced from the temperature dependence of the ^{19}F NMR spectra of complexes **1** and **2** [Fig. 3(*a*)].

In the case of $[Fe^{II}\{Z\text{-cys(1)-Pro-Leu-cys(2)-Gly-NH-}(CH_2)_nC_6H_4F\text{-}p\}_2]^{2-}$ (n = 1 **3** or 2 **4**), however, the electronic effect does not propagate from the sulfur of Cys(2) to the benzene ring through the NH···S hydrogen bond because of the presence of the intervening methylene group(s) [Fig. 4(*b*)]. This means that the ^{19}F NMR isotropic shifts of complexes **3** and **4** cannot be due to contact interactions through the Fe–S···H–N bonds which occurred in complexes **1** and **2**. The temperature dependence indicates that the isotropic shifts in the spectra of **3** and **4** are essentially of dipolar origin [see Fig. 3(*b*)], *i.e.* through-space interactions directly between the aromatic group and the anisotropic high-spin paramagnet of Fe^{II}.

The observation of upfield shifted ^{19}F NMR signals through a dipolar mechanism in complexes **3** and **4** implies that the ^{19}F nuclei in these complexes are close to the iron(II) ion, as in **1** and **2**. Furthermore, similar ^{19}F T_1 values (see Table 1) were obtained for complexes **2**–**4** which all have a *p*-fluoro-substituent. This also indicates that the distances between the fluorine nuclei and the Fe^{II} are similar in these complexes, and was confirmed to be caused by the presence of π–π interactions between the aromatic group and sulfur atom of the co-ordinated cysteine residue which perhaps co-operated with the formation of the X NH···S Cys(2) hydrogen bond. It is probable that such interactions force the aromatic ring to face the FeS_4 core. The existence of such interactions has already been proposed for native metalloproteins from X-ray analysis, not only for rubredoxin but also for high-potential iron–sulfur and other proteins.[24] For example, the distances between the carbon

atoms of the benzene ring of Phe-49 or Tyr-11 and the sulfur of the cysteine residue (Cys-6, -9 or -39) in native rubredoxin are in a range 3–5 Å.[24]

A large reduction in T_1 is predominantly caused by the proximity of ^{19}F to the paramagnetic iron(II) centre. The complexation of peptide ligands to Fe^{II} also contributes to this since the metal complex is larger than the ligand alone.

In conclusion, the ^{19}F NMR spin–lattice relaxation time (T_1) measurements and the temperature dependence of the ^{19}F NMR spectra of complexes **1**–**4** indicate that the benzene ring of the X fragment in these model complexes is close to the FeS_4 core, and provide further evidence for the existence of interactions between the aromatic group and the sulfur atom of the cysteine residue which we have proposed previously.[18]

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