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High-Resolution Proton Nuclear Magnetic Resonance Analysis of Solution Structures and Conformational Properties of Mugineic Acid and Its Metal Complexes†

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ABSTRACT: Proton nuclear magnetic resonance (^{1}H NMR) spectral studies at 360 MHz have been conducted on mugineic acid and its Zn(II) and Co(III) complexes. Resonance assignments are presented for all the CH protons of mugineic acid on the basis of homonuclear spin decoupling and the *J*-resolved two-dimensional spectroscopic experiments. The conformational analysis using chemical shifts and vicinal coupling constants showed that (1) mugineic acid coordinates to Zn(II) and Co(III) ions in hexadentate fashion by the six functional groups and (2) the C1′–C2′, C1″–C2″, and C2″–C3″ bonds change from mixtures of rotamer populations in free mugineic acid to predominantely gauche–gauche populations in the metal complexes. The structural conformation of the mugineic acid–Co(III) complex in aqueous solution corresponds well to that of its crystal structure determined by X-ray diffraction technique.

Mugineic acid, (2*S*,2′*S*,3′*S*,3″*S*)-*N*-[3-carboxy-3-[(3-carboxy-3-hydroxypropyl)amino]-2-hydroxypropyl]azetidine-2-carboxylic acid, is a unique phytosiderophore that is excreted from the roots of barley (Takagi, 1976; Takemoto et al., 1978). Recent experiments demonstrated that (1) the ^{59}Fe uptake in the rice root is remarkably stimulated by mugineic acid and (2) the addition of mugineic acid to the medium of water-cultured rice at pH 7.0 increases the chlorophyll content (Mino et al., 1983). Other graminaceous plants such as wheat and oats have also produced novel amino acids similar to mugineic acid from their roots in order to effectively absorb iron under the condition of iron deficiency (Nomoto et al., 1979; Fushiya et al., 1980). The coordination chemistry of this novel amino acid is essential to elucidate the mechanism of iron uptake and transport in graminaceous plants and/or the iron chlorosis induced by transition metals such as Cu(II) and Zn(II) ions (Hunter & Vergano, 1953). Although we previously clarified the X-ray crystal structures for the Cu(II) and Co(III) complexes of mugineic acid (Mino et al., 1981, 1983), the solution chemistry of mugineic acid and its metal complexes is uncertain. Proton nuclear magnetic resonance has proven to be of limited value for the study of mugineic acid–Fe(III) and –Cu(II) complexes because of their paramagnetic effects. However, the conformational changes of mugineic acid resulting from metal coordination can be studied by using Zn(II) and Co(III), since mugineic acid forms a tightly bound complex with these diamagnetic ions. Using the chemical shifts and coupling constants highly resolved by 360-MHz proton nuclear magnetic resonance (^{1}H NMR) spectra, in this paper, we have demonstrated the tertiary structure of mugineic acid and the conformational changes associated with metal binding in aqueous solution. The solution structure of the mugineic acid–Co(III) complex has also been compared with the structures determined by X-ray crystallographic analysis. The present information is prerequisite to understanding the conformation of biologically important mugineic acid–Fe(III) complex in aqueous medium and the mechanism of heavy metal induced iron chlorosis.

Experimental Procedures

Materials. Mugineic acid was isolated and purified according to our previously reported procedures (Takemoto et al., 1978) and then was checked by field-desorption (FD) mass and NMR (^{1}H and ^{13}C) spectra. All other reagents were the highest quality available.

NMR Measurements. ^{1}H NMR spectra were obtained at 360 MHz on a Nicolet NT-360 NMR spectrometer equipped with a computer-controlled homonuclear decoupling accessory.

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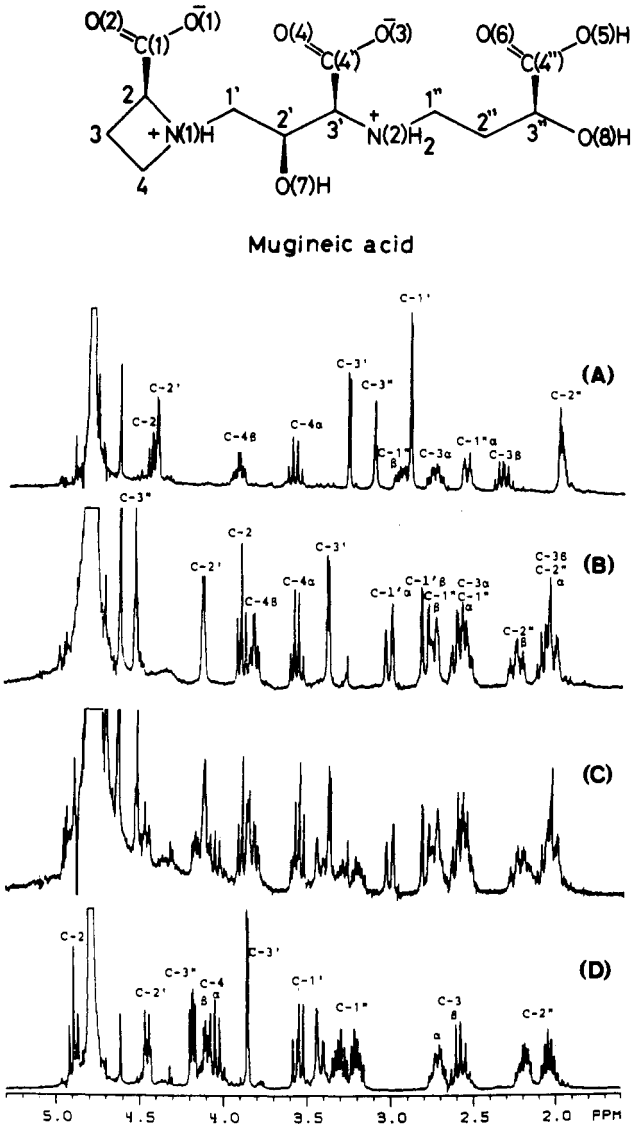

FIGURE 1: 360-MHz FT ^{1}H NMR spectra of 1:1 mugineic acid–Co(III) complex (A), 1:1 mugineic acid–Zn(II) complex (B), 2:1 mugineic acid–Zn(II) complex (C), and ligand only (D) at pD 4.5.

Quadrature detection was employed, and 32 K Fourier transform was obtained with a spectral width of 2074.68 Hz. The 1:1 mugineic acid–Zn(II) complex was prepared by addition of zinc sulfate (50 mM) dissolved in 99.9% D_2O to the solution of mugineic acid (50 mM) in 99.9% D_2O. The 1:1 mugineic acid–Co(III) complex was synthesized by the method previously reported (Mino et al., 1983) and was dissolved in 99.9% D_2O. The pD of the resulting solution was adjusted to the indicated values with either D_2SO_4 or NaOD. Standard electrode correction has been employed: pD = pH meter reading + 0.4. The internal standard sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate (TSP) was used.

Results and Discussion

Assignments. Figure 1 shows the ^{1}H NMR spectra of metal-free mugineic acid and its complexes with zinc(II) and cobalt(III) ions at pD 4.5. The Zn(II) and Co(III) complexes of mugineic acid exhibited sharp and numerous proton signals, indicating the presence of the diamagnetic ion ($S = 0$) in both the metal complexes. The resonance in mugineic acid and its metal complexes were assigned on the basis of the homonuclear spin decoupling and the *J*-resolved two-dimensional spectroscopic experiments. Mugineic acid and these metal complexes have independent three-spin systems in their proton spin networks, namely, a set of four-proton system and two sets of a five-proton system. The five-proton systems are distinguishable from each other because the azetidine ring moiety shows the five-proton spin system, which has a characteristic triplet signal due to the proton at C2 of the rigid ring. The chemical shifts for the resonances in mugineic acid and its metal complexes are summarized in Table I. In the ^{1}H NMR spectrum of mugineic acid, the characteristic triplet signal of the C2 methine proton is observed at 4.88 ppm ($J = 9.7$). This proton signal is coupled with the methylene protons of coupling patterns of dddd at 2.51 and 2.71 ppm, respectively. The methylene protons are further spin coupled to the methylene proton (ddd) at 4.03 and 4.09 ppm. The configurations of each proton on the azetidine ring of mugineic acid are inferred by the comparison of the corresponding coupling patterns in azetidine-2-carboxylic acid benzyl ester, the signal assignments of which have been confirmed by nuclear Overhauser effect (NOE) measurements. The coupling constants of the azetidine ring protons of mugineic acid are very similar to those (C2H–C3Hα, $J = 9.6$ Hz; C2H–C3Hβ, $J = 8.0$ Hz; C3Hα–C4Hα, $J = 9.6$ Hz; C3Hα–C4Hβ, $J = 6.4$ Hz; C3Hβ–C4Hα, $J = 9.6$ Hz; C3Hβ–C4Hβ, $J = 9.9$ Hz) of the corresponding protons of azetidine-2-carboxylic acid benzyl ester (see Table II). Therefore, the signals at 4.88, 2.71, 2.57, 4.03, and 4.09 ppm are assigned to C2H, C3Hα, C3Hβ, C4Hα, and C4Hβ, respectively. Another five-proton system, (3-hydroxy-3-carboxypropyl)amino (C1″–C3″) moiety, is assigned as shown in Table I. The configurations of both methylene protons at C1″ and C2″ are not clear, however, because the carbon skeleton of mugineic acid is not rigid. The remaining four-proton system, 3-carboxy-2-hydroxypropyl (C1′–C4′) moiety, is easily assigned except for the configuration of the methylene protons at C1′.

Table I: Chemical Shifts

		mugineic acid	Zn(II) complex	Co(III) complex
C2H		4.88	3.88	4.40
C3H$_2$	α	2.71	2.55	2.73
	β	2.57	2.07	2.31
C4H$_2$	α	4.03	3.56	3.57
	β	4.09	3.81	3.89
C1′H$_2$	α	3.54[a]	3.00	2.88
	β	3.42	2.80	2.88
C2′H		4.44	4.10	4.37
C3′H		3.84	3.36	3.23
C1″H$_2$	α	3.20[a]	2.59	2.53
	β	3.28	1.71	2.91
C2″H$_2$	α	2.04[a]	2.02	1.97
	β	2.18	2.22	1.97
C3″H		4.18	4.52	3.10

[a] The configuration is uncertain.

The complex molecule of metal–mugineic acid is so rigid that the configuration was determined by using W-type long-range coupling and NOE as shown in Table I. The overlapped signals were also successfully separated by using the two-dimensional *J*-resolved method to make clear the coupling pattern.

pH Studies. Figure 2 shows the pD-dependent shift for all proton resonances of mugineic acid. The protonation at pD 3.5 clearly affects only the chemical shift of the C3″ methine proton, while the protonation of ca. pD 2.5 influences both the chemical shifts of the C2 and C3′ methine protons. The data are consistent with the result of potentiometric pH titration [pK_1(COOH) = 2.39, pK_2(COOH) = 2.76, pK_3(COOH) = 3.40, pK_4(>N$^+$H–) = 7.78, and pK_5(–N$^+$H$_2$–) = 9.55] (Sugiura et al., 1981). Although the proton chemical shifts

Table II: Coupling Constants

		mugineic acid		Zn(II) complex		Co(III) complex	
		Hz	rotamer pop.[a]	Hz	rotamer pop.[b]	Hz	rotamer pop.[b]
C2H-C3H$_2$ α	J_{vic}	9.7		9.7		9.7	
β		9.7		9.7		9.7	
	J_{gem}	11.8		12.7		11.7	
C3H$_2$-C4H$_2$ α-α	J_{vic}	9.7		9.7		9.7	
α-β		4.5		3.8		4.7	
β-α		9.7		9.7		9.7	
β-β		9.7		9.7		9.7	
	J_{gem}	9.7		9.7		9.7	
C1′H$_2$-C2′H α	J_{vic}	9.4[c]	<10% gg	2.6	>90% gg	2.4	>90% gg
β		2.6		2.6		2.4	
	J_{gem}	13.4		14.4		*d*	
C2′H-C3′H	J_{vic}	3.2	<10% trans	5.0	20% trans	4.8	20% trans
C1″H$_2$-C2″H$_2$ α-α	J_{vic}	6.6[c]		3.8			
α-β		7.7	25% trans	4.4	>90% gg	*e*	
β-α		7.8		13.2			
β-β		6.3		3.2			
C1″H$_2$	J_{gem}	12.6		13.2		*e*	
C2″H$_2$	J_{gem}	22.0		17.1		*e*	
C2″H$_2$-C3″H α	J_{vic}	4.6[c]	20% gg	3.8	>90% gg	3.2	>90% gg
β		7.3		3.8		3.2	

[a] Rotamer populations were calculated by using the parameter J_{trans} = 11 Hz and J_{gauche} = 2.5 Hz; gg designates the gauche-gauche rotamer. [b] Rotamer populations were calculated by using the parameter J_{trans} = 11 Hz and J_{gauche} = 3.5 Hz. [c] The configuration is uncertain. [d] Not observed. [e] The protons on C2″ are very close. So, it is very difficult to find out the coupling constants in first order.

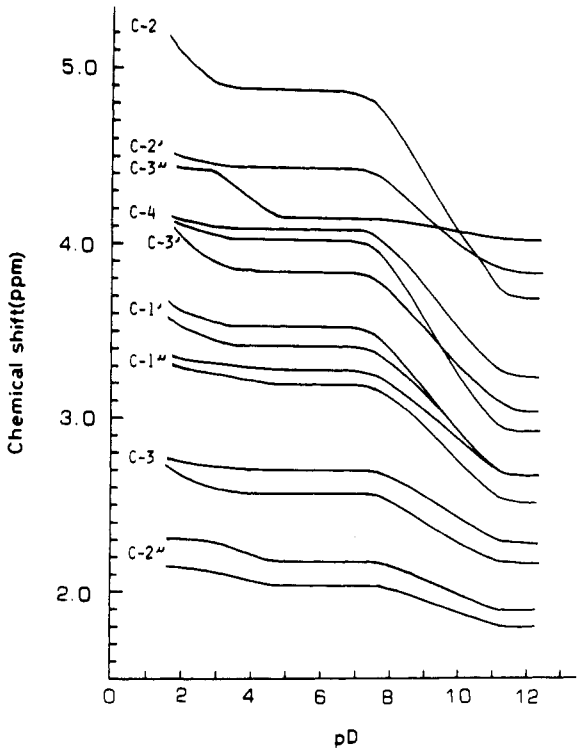

FIGURE 2: pD dependence of proton chemical shifts in mugineic acid.

adjacent to two amino groups were largely changed in the pD range 7–11, the pK_a assignment of the two amino groups was unable to be ascertained by the present ^{1}H NMR study only. On the other hand, the pD changes did not cause any concomitantly large changes in the coupling constants, indicating that the protonations occur without major alterations in the molecular conformation.

Table I compares the proton chemical shifts of mugineic acid, the Zn(II) complex, and the Co(III) complex at pD 4.5. Upon metal complexation, the large changes of the chemical shifts are evidently observed in the proton resonances adjacent to the six functional groups (two amino, three carboxyl, and terminal hydroxyl groups). Indeed, it is known that these groups coordinate octahedrally with the Co(III) ion in the crystal structure (Mino et al., 1983; Sugiura et al., 1981). The direction of these changes was almost upfield, although the binding of Zn(II) to a heterocoordination atom usually induces downfield shift of the adjacent proton resonance. Therefore, this strong shielded resonance indicates that the metal binding causes the juxtaposition of the affected protons with anisotropic environment arising from sources such as carbonyls and heteroatoms. pH-titration studies for both of the metal complexes were also carried out. All of the proton resonances of the complex with Zn(II), as well as Co(III), were not changed in the pD range 4–10, clearly indicating that in this pD range stable complex is formed and the complex molecule undergoes no significant structural alteration. This result corresponds well to the report by Takagi (1976) that mugineic acid–Fe(III) complex is stable in the pH range 4–9. The formation of stable single species is also supported by the fact that the ^{1}H NMR spectrum of the 2:1 mugineic acid–Zn(II) solution is superimposed on the joint spectra of mugineic acid and its 1:1 complex (see Figure 1).

Coupling Constants. Table II summarizes the coupling constants for the resonances in mugineic acid and its Zn(II) and Co(III) complexes. The calculated rotamer populations are also listed in Table II. We used 12 and 3.5 Hz as estimation for the values of the trans and gauche coupling constants in the case of metal complex, respectively (Karplus, 1963; Blackburn, 1970; Lemieux & Lown, 1964).

On the basis of the present conformational analysis, free mugineic acid molecules appear to favor an extended form (N1---C3′, trans; C1′---N2, trans) because other conformations can be ruled out by the steric hindrance between the azetidine ring and intermediate carboxylate. This agrees with the X-ray crystallographic result of mugineic acid by Takemoto et al. (1978). The Zn(II) binding to mugineic acid causes a large conformational change in the ligand molecule except for the azetidine ring, as reflected by alterations in the vicinal coupling constants. The C1′–C2′, C1″–C2″, and C2″–C3″ bonds change from mixtures of rotamer populations in the free mugineic acid to predominantly (>90%) gauche–gauche populations in the metal complex (see Figure 3). On the other hand, the C2′–C3′ bond of the metal complex, as

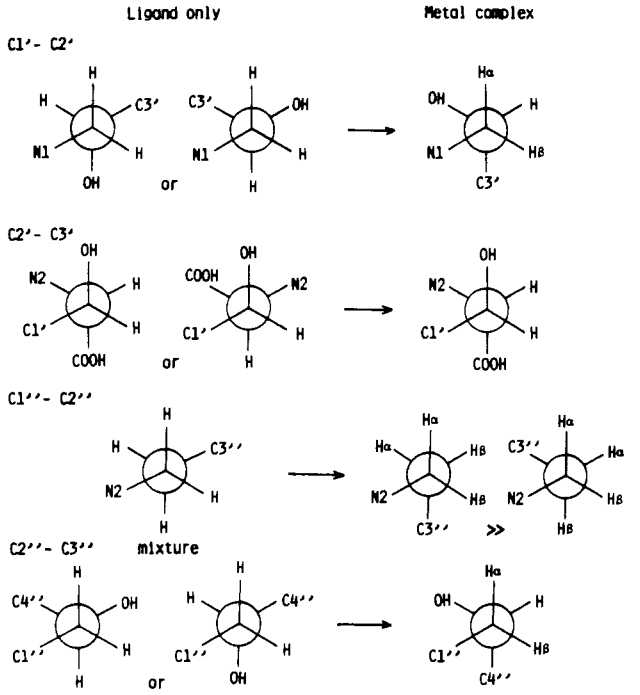

FIGURE 3: Predominant rotamer populations for mugineic acid and its metal complexes.

well as free ligand, seems to adopt a gauche form as shown in Figure 3. Two rotational isomers of the gauche form are not distinguishable from each other on the basis of the conformational analysis, since only a single vicinal coupling constant is available. However, the metal complex undoubtedly adopts one rotational isomer (hydroxylate---carboxylate, trans), while in the free-ligand molecule another isomer (C1'---N2, trans) appears to predominate as mentioned above. This conformation facilitates the simultaneous coordination of two amines and intermediate carboxylate with metal ion, resulting in the formation of two chelate rings, chair-form six-membered ring metal–N1–C1'–C2'–C3'–N2 and nearly planar five-membered ring metal–O3–C4'–C3'–N2. More appropriate proof of this conformation is the observation of a W-type long-range coupling between the α proton of C1' methylene and C3' methine proton. Similarly, the conformation of the C1''–C2''–C3'' chain represents an orientation that is able to form another set of five- and six-membered chelate rings, when the secondary amine and terminal hydroxylate and carboxylate are are involved in the metal coordination.

The present ^{1}H NMR studies confirmed that mugineic acid coordinates with Zn(II) or Co(III) ion as a hexadentate chelator, forming octahedral configuration just as that of the crystal structure of its Co(III) complex (see Figure 4). Both the Zn(II) and Co(III) complexes are structurally analogous to each other as reflected by the similarity of vicinal coupling constants. However, it should be noted that although the C1' methylene protons, as well as the C2'' methylene protons, of the mugineic acid–Co(III) complex give equivalent chemical shifts, those of the Zn(II) complex have individual chemical shifts. In addition, the chemical shifts of the C3'' protons are significantly different between the Co(III) and Zn(II) complexes. These differences in the chemical shift between the metal complexes can be mainly accounted for in the terms of anisotropy on the basis of a temperature-independent paramagnetism of Co(III) ion.

The crystal structure of the biological important mugineic acid–Fe(III) complex has not as yet been established by X-ray

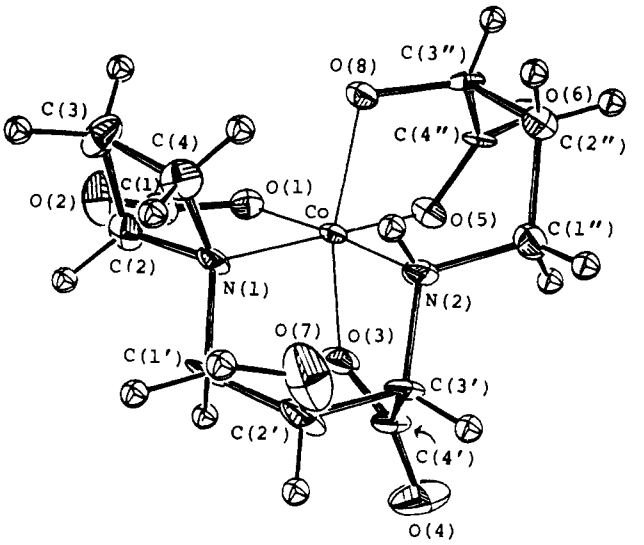

FIGURE 4: A perspective drawing of mugineic acid–Co(III) complex (Mino et al., 1983).

diffraction techniques, and its conformational analysis by ^{1}H NMR spectra has never been accomplished because of paramagnetic effect. The present NMR study of the mugineic acid–Co(III) complex provides valuable information for solution structure and conformational property of biologically important mugineic acid–Fe(III) complex, because of the similarity in the coordination chemistries between Co(III) and Fe(III). It is known that remarkable stimulation of iron uptake is induced by Zn(II) stress in several plants (Ambler & Brown 1969; Warnock, 1970). The present information is also indispensable for elucidating the relationship between iron uptake and heavy metals, especially Zn(II).

Registry No. Mugineic acid, 69199-37-7; mugineic acid–Zn(II), 86727-43-7; mugineic acid–Co(III), 86782-60-7.

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