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# The binding of aluminum to mugineic acid and related compounds as studied by potentiometric titration

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**Abstract** The phytosiderophores, mugineic acid (MA) and epi-hydroxymugineic acid (HMA), together with a related compound, nicotianamine (NA), were investigated for their ability to bind Al(III). Potentiometric titration analysis demonstrated that MA and HMA bind Al(III), in contrast to NA which does not under normal physiological conditions. With MA and HMA, in addition to the Al complex (AlL), the protonated (AlLH) and deprotonated ($AlLH_{-1}$) complexes were identified from an analysis of titration curves, where L denotes the phytosiderophore form in which all the carboxylate functions are ionized. The equilibrium formation constants of the Al(III) phytosiderophore complexes are much smaller than those of the corresponding Fe(III) complexes. The higher selectivity of phytosiderophores for Fe(III) over Al(III) facilitates Fe(III) acquisition in alkaline conditions where free Al(III) levels are higher than free Fe(III) levels.

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## Introduction

Many plants suffer from iron deficiency in alkaline soil conditions, where only a limited level of iron is available due to formation of sparingly soluble iron hydroxides. Graminaceous plants circumvent such conditions through the formation of series of phytosiderophores such as mugineic acid (MA) and epi-hydroxymugineic acid (HMA) (Fig. 1) (Takagi et al. 1984). These compounds are synthesized and secreted in order to chelate and solubilize Fe(III), which in turn is taken up by the plants. The biosynthetic pathway for phytosiderophores has been established (Mori and Nishizawa 1987; Nomoto et al. 1987; Shojima et al. 1990; Ma et al. 1999) and the genes that code the enzymes have been cloned (Higuchi et al. 1999; Takahashi et al. 1999; Kobayashi et al. 2001). In addition to iron uptake, MAs may function to acquire Zn(II), Cu(II), Ni(II) and Co(II), as MA complexes where these metal ions are also transported through a specific transporter (Haydon and Cobbett 2007; Ishimaru et al. 2010).

In contrast to phytosiderophores, nicotianamine (NA), a compound with the structure in which the 3″-hydroxyl group of MA is replaced by an amino group

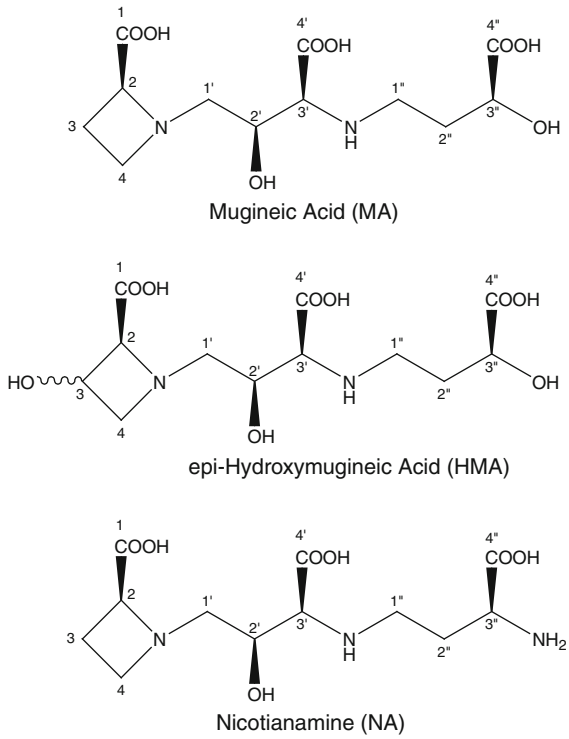


**Fig. 1** Chemical structures of mugineic acid (MA), epi-hydroxymugineic acid (HMA), and nicotianamine (NA)

(Fig. 1), occurs in all plants and is not secreted. NA binds Fe(II), Cu(II) and Zn(II) and plays a role in long-distance transport of these metals in higher plants (Haydon and Cobbett 2007).

Because aluminum is the most abundant metal in the earth's crust and because of the similarity in the chemistry of Al(III) and Fe(III), it is conceivable that Al(III) competes with Fe(III) for the phytosiderophore binding site. Bacterial siderophores are known to possess an appreciable affinity for Al(III) (Evers et al. 1989; Watteau and Berthelin 1994; Rogers et al. 2001); in contrast, it is unclear whether mugineic acids possess the ability to bind Al(III). The equilibrium formation constant for the dehydrated Al(III)–MA complex has been reported to be smaller than that of the corresponding Fe(III) complex by a factor between $10^6$ and $10^8$ (Murakami et al. 1989), although the thermodynamic formation constants have not been reported. Furthermore, no interaction between Al(III) and MA has been reported on the basis of either physiological or NMR investigations (Chang et al. 1988). It is important to clarify whether MAs and NA bind Al(III) under physiological conditions in order to generate a clear understanding of trivalent metal metabolism in higher plants. We have therefore investigated the binding abilities of MA, HMA and NA for Al(III) and present herein the equilibrium constants for formation of the corresponding complexes.

## Materials and methods

MA and HMA were isolated from the root washings of Fe-deficiency-stressed barley (*Horden vulgare* L. cv. Minorimugi) and (*Horden vulgare* L. cv. Ehimehadaka), respectively, according to the method reported by Takagi et al. (1984). NA was kindly supplied by Professor Takayuki Shioiri, Faculty of Pharmaceutical Sciences, Nagoya City University. The chemical structures of the compounds are shown in Fig. 1.

Equilibrium formation constants of NA, MA and HMA with Al(III) were determined using an automated, computerized system. A jacketed titration cell was used to maintain the temperature at 25°C under an argon atmosphere. The compounds (ca. 6.5 μmol) were dissolved in 10.7 ml of 0.1 M KCl, acidified by 3 ml of 0.2 M HCl, and then titrated with 0.2 M KOH. After this initial titration, the solution was acidified with an appropriate volume of 0.2 M HCl (the equivalent amount of KOH used in the first titration), 4 μmol Al(III) was added, and the resulting solution was titrated with 0.2 M KOH. In addition, a solution containing 4 μmol of Al(III) in 10.1 ml of 0.1 M KCl was acidified with 2 ml of 0.2 M HCl and titrated with 0.2 M KOH. The titration curve (pH of solution containing NA, MA, or HMA vs. the volume of 0.2 M KOH added) was analyzed to determine the acid dissociation constants and the second curve (pH of solution containing in addition to Al, NA, MA, or HMA vs. the volume of 0.2 M KOH) to the equilibrium constants for the formation of the Al(III) complexes using the TITREFIT program, a modified version of NONLIN (Taylor et al. 1988).

## Results and discussion

Titration curves of Al(III)–NA system (Fig. 2) were analyzed by monitoring the differential pH values with respect to volume in order to feasibly identify buffer regions, which are reflected by the minimal differential

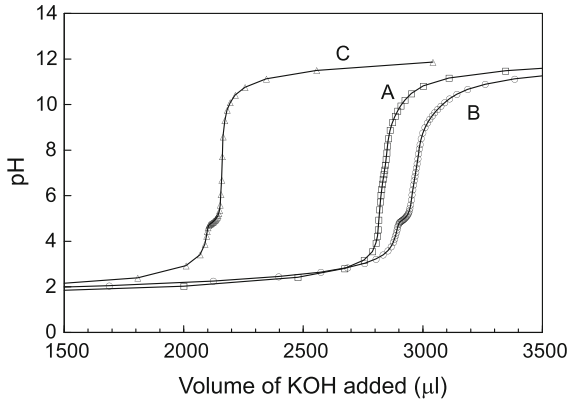


**Fig. 2** Potentiometric titration curve of the solution containing Al(III) and nicotianamine (NA). NA (6.54 μmol) was dissolved in 10.7 ml of 0.1 M KCl, acidified by 3 ml of 0.2 M HCl, and titrated with 0.2 M KOH (**A**). The resulting solution was acidified with 0.2 M HCl (3.855 ml), 4 μmol Al(III) added, and then titrated with 0.2 M KOH (**B**). A solution containing 4 μmol of Al(III) in 0.1 ml of 0.1 M KCl was acidified with 2 ml of 0.2 M HCl and then titrated with 0.2 M KOH (**C**). Only the titration curves with the titrant volumes falling within the range 1,500–3,500 μl are shown

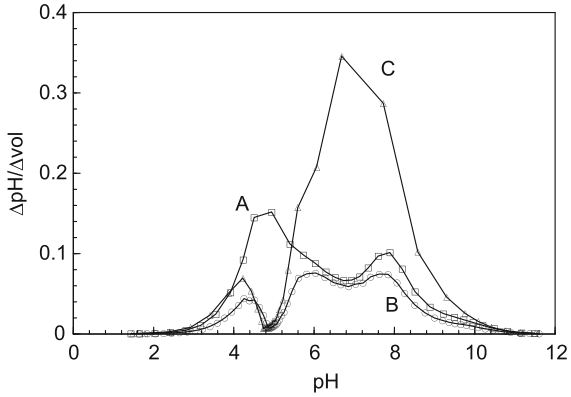


**Fig. 3** Differential pH values for the titration curves of an Al(III)–NA system with respect to the titrant volume as a function of the solution pH. Each of the titration curves presented in Fig. 2 was differentiated. For the experimental details, refer to Fig. 2

values (Fig. 3). A weak buffer region is observed in the pH region close to 7.0 in the titration curve for a solution containing NA alone (6.54 μmol) (Figs. 2A, 3A). After the titrant was acidified with 0.2 M HCl (4,855 μl) and Al(III) (4.0 μmol) added, a second titration was undertaken, in which in addition to the weak buffer region at pH 7.0, which is almost identical to that of the NA solution, a strong buffer region appeared over the pH range from 4.5 to 5.0 (Figs. 2B, 3B). A similar pH region was also identified for the solution containing 4 μmol Al alone (Figs. 2C, 3C). This prominent buffer region is likely to be associated with the hydrolysis of Al(III) aquo ions, as the solubility product of $Al(OH)_3$ (Dean 1999) predicts that an $Al(OH)_3$ precipitate begins to form at pH 4.2 for Al(III) (330 μM). These findings indicate that when simultaneously present, Al(III) and NA were titrated independently, inferring that Al(III) does not bind to NA. This finding is in contrast to Fe(III), where Fe(III) has been demonstrated to bind NA with a binding constant of $10^{20.6}$ (von Wirén et al. 1999).

In contrast, a significant change is induced in the titration curve of MA by the presence of Al(III), where buffer regions emerged at pH 4.0 and at pH 8.0, in contrast to the titration curve for MA alone. These changes are reflected in the induced changes in the buffer regions of the MA titration curve (Fig. 4). A similar behavior was observed with HMA (Fig. 5). These observations indicate that the two phytosiderophores bind Al(III). An analysis of the titration curves yielded the cumulative association constants, $\beta_{11r}$, defined by Eq.(1):

$$\mathrm{Al} + \mathrm{L} + \mathrm{rH} = \mathrm{AlLH_r}, \quad \beta_{11r} = [\mathrm{AlLH_r}]/[\mathrm{Al}][\mathrm{L}][\mathrm{H}]^{\mathrm{r}} \tag{1}$$

where L represents the phytosiderophore with their carboxylate functions being fully deprotonated

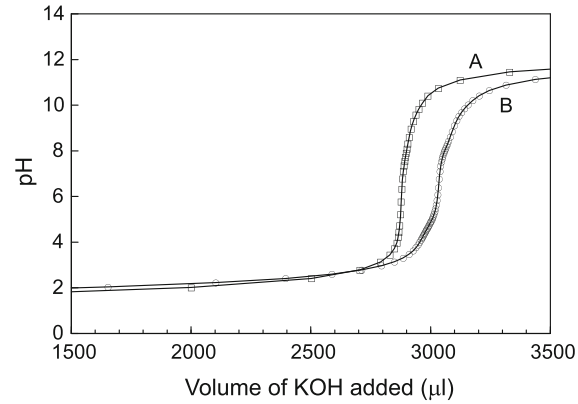


**Fig. 4** Potentiometric titration curve of MA in the presence and absence of Al(III). MA (6.50 μmol) was dissolved in 10.7 ml of 0.1 M KCl, acidified by 3 ml of 0.2 M HCl, and titrated with 0.2 M KOH (**A**). The resulting solution was acidified again with 0.2 M HCl (3.792 ml), added with 4 μmol Al(III), and then titrated with 0.2 M KOH (**B**). Only titration curves with titrant volumes falling between 1,500 and 3,500 μl are presented

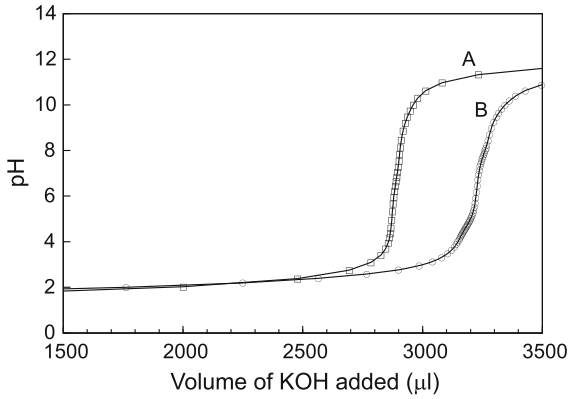


**Fig. 5** Potentiometric titration curve of HMA in the presence and absence of Al(III). HMA (6.19 μmol) was dissolved in 10.7 ml of 0.1 M KCl, acidified by 3 ml of 0.2 M HCl, and titrated with 0.2 M KOH (**A**). The resulting solution was acidified again with 0.2 M HCl (3.572 ml), added with 4 μmol Al(III), and then titrated with 0.2 M KOH (**B**). Only titration curves with titrant volumes falling between 1,500 and 3,500 μl are presented

**Table 1** Cumulative association constants for the Al(III)- and Fe(III)-phytosiderophore complexes

| | $\log \beta_{110}$ | | $\log \beta_{11-1}$ | |
|---|---|---|---|---|
| | Al(III) | Fe(III)[a] | Al(III) | Fe(III)[a] |
| MA | 13.4 | 17.7 | 8.8 | 15.4 |
| HMA | 12.1 | – | 7.3 | 15.6 |

[a] Data for Fe(III) complex adapted from Murakami et al. (1989)

(Table 1). These data demonstrate the presence of two major types of the complex, AlL, and $AlLH_{-1}$. The equilibrium formation constants for the deprotonated Al(III)–MA ($AlLH_{-1}$) and Al(III)–HMA were unequivocally established to be lower than those of the corresponding Fe(III) complexes by factors of $10^{6.6}$ and $10^{8.2}$ respectively. These findings are consistent with the general observation that Al(III) complexes of N- and O-donor ligands possess weaker formation constants than the corresponding complexes with Fe(III). Thus, desferrioxamine B binds Fe(III) $10^6$ times tighter than Al(III) [11], and both EDTA and HEDTA bind Al(III) with a much weaker formation constant than Fe(III) (Martel and Smith 1989).

The X-ray crystallography structure of the MA complex with Co(III) has a nearly-octahedral configuration, with the azetidine nitrogen, secondary amine nitrogen, three carboxylate oxygens and hydroxyl oxygen as donors (Sugiura and Nomoto 1984). Both the Fe(III)–MA and Al(III)–MA complexes are anticipated to have similar configurations (von Wirén et al. 1999). The observation that NA does not form a complex with Al(III) demonstrates that the hydroxyl oxygen of MA (or HMA) is an important factor for the complex formation with Al(III) based on the difference in structure between NA and MA (or HMA). As the affinity of $NH_3$ is much smaller for Al(III) than for Fe(III) (Evers et al. 1989), the replacement of oxygen donor by nitrogen donor in the ligand would be detrimental to Al(III)–NA complex formation. The values for the log $\beta_{11-1}$ constants for the binding of Fe(III) and Al(III) to MA are apparently quite low, namely 15.4 and 8.8, respectively. However, this value does not take account of the alcoholic proton which is fully ionized on coordination to tribasic cations (Murakami et al. 1989; Sugiura and Nomoto 1984). Using the acid dissociation constant of the alcoholic proton, $K_a$, which has been determined, for the related molecule lactic acid, to be 15.1 (Silva et al. 2009), the stability constant ($K_{stab}$) of the deprotonated complex can be expressed by the following equation:

$$K_{stab} = \beta_{11-1}/K_a \quad (2)$$

Thus the overall log stability constants of MA for Fe(III) and Al(III) are 30.5 and 23.9. These values compare well with the corresponding affinity

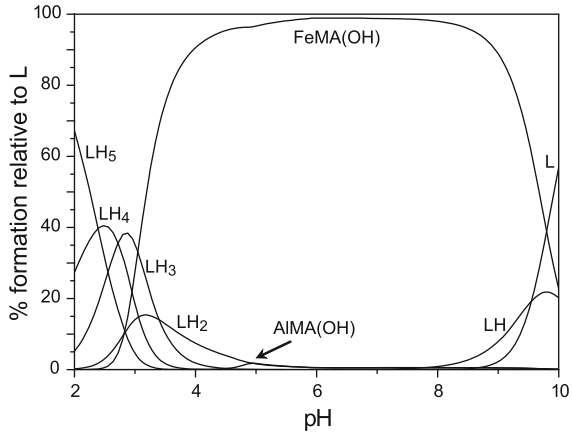


**Fig. 6** Speciation plot of iron(III), Al(III) and MA each at 1 μM. The values of the solubility products of iron(III)$(OH)_3$ and aluminum(III)$(OH)_3$ adopted are $10^{-38.61}$ (Liu and Millero 1999) and $10^{-30.42}$ (Venturini and Berthon 1989) respectively. The plot was constructed by HYSS3 (Alderighi et al. 1999)

constants for the trishydroxamate siderophore desferroximine B, namely 30.6 and 22.0 (Ackrill and Day 1985).

In principle, MA or HMA, when secreted from graminaceous plants, can form complexes with both Fe(III) and Al(III) in the rhizosphere. However, Al(III) is unlikely to compete with Fe(III), since $\beta_{11-1}$ values for Al(III) of MA and HMA are much lower. This difference is highlighted in the speciation plot (Fig. 6). It is clear that when Fe(III) and Al(III) are present together at equal concentrations the Fe(III)–MA interaction dominates over the Al(III)–MA interaction over the entire range.

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