Revisiting the Metal-Binding Chemistry of Nicotianamine and 2′-Deoxymugineic Acid. Implications for Iron Nutrition in Strategy II Plants

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Recently, von Wirén et al. (1999) published a study of the metal-binding properties of two ligands important for Fe physiology in higher plants, nicotianamine (NA) and 2′-deoxymugineic acid (DMA; Fig. 1). However, they failed to address two key aspects of the Fe ligand chemistry that we will address here, and that have important implications for Fe physiology and transport in plants, particularly "Strategy II" species (Ma and Nomoto, 1996).

NA is ubiquitous throughout the plant kingdom (Scholz et al., 1992). It acts as a general internal chelator rather than by stereospecific binding (Ripperger et al., 1982), and has been implicated in cellular Fe homeostasis and regulation (Liu et al., 1998; Pich et al., 2001). Although most evidence argues against a major role for NA in long-distance transport of Fe within plants, NA appears to be important for Cu translocation in Strategy I species (Pich et al., 1994). In Strategy II species, NA has been implicated as a possible precursor in the synthesis of phytosiderophores, but conclusive evidence is lacking (Mori, 1994). Deoxymugineic acid is representative of the family of phytosiderophores that are excreted by Strategy II species into the rhizosphere in response to Fe deficiency (Ma and Nomoto, 1996). Phytosiderophores increase the solubility of Fe^{III} by chelation, and are absorbed as the intact Fe^{III} ligand by Strategy II plants (Romheld and Marschner, 1986; Grusak et al., 1999).

von Wirén et al. (1999) measured and/or reaffirmed some metal ligand stability constants, and then performed chemical speciation modeling and empirical testing that suggested two important results: First, NA is able to out-compete DMA for Fe^{III} at cytoplasmic pH; and second, although NA has a greater thermodynamic affinity for Fe^{III} than for Fe^{II}, complexes of the latter possess some unusual kinetic stability that, as an example, protects chelated Fe^{II} from rapid oxidation by molecular O_2. However, a closer inspection of the pertinent equilibrium chemistry shows these two conclusions to be incorrect.

CASE 1. COMPETITION BETWEEN NA AND DMA FOR FE^{III}

von Wirén et al. (1999) concluded that NA would out-compete DMA for Fe^{III} at physiological pH values, and we have reproduced their calculated speciation (Fig. 2a) using GEOCHEM-PC (Parker et al., 1995) and the constants provided in Tables I and II. However, von Wirén et al.'s computations only considered the formation of the neutral, 1:1 complexes. With DMA, Murakami et al. (1989) clearly demonstrated the presence of a very stable Fe^{III} complex of the type $FeL(H_{-1})^{-}$ (stoichiometrically indistinguishable from $FeLOH^{-}$, the representation used in most chemical equilibrium models), and attributed the loss of the extra proton upon binding with Fe^{III} to deprotonation of the hydroxyl group on the 3″ carbon. Significantly, the negatively charged $FeL(H_{-1})^{-}$ complex was found to dominate at pH values greater than about 3.0 (see Fig. 2 in Murakami et al., 1989). Using high-voltage paper electrophoresis, von Wirén et al. (1999) detected only a complex with a single negative charge at pH 7.0, confirming the importance of this $FeL(H_{-1})^{-}$ complex. Inexplicably, however, they failed to detect or consider this complex when conducting competitive spectrophotometric titrations that purported to yield almost the same formation constant for the neutral FeL complex as that obtained by Murakami et al. (1989; Table I). As a consequence, von Wirén et al.'s (1999) subsequent calculations of Fe^{III} speciation in the presence of DMA failed to consider the formation of this dominant $FeL(H_{-1})^{-}$ complex.

We have recalculated von Wirén et al.'s (1999) speciation of Fe^{III} in presence of both NA and DMA, but incorporating the formation constant for the $FeL(H_{-1})^{-}$ complex of DMA (Table I). As before, the total Fe concentration was 1 μM, and NA and DMA were each at 10 μM, with a constant ionic strength of 0.1 M. When the $FeL(H_{-1})^{-}$ complex is properly considered, it is clear that NA cannot effectively compete with DMA for Fe^{III} at any relevant pH value (Fig. 2b), in marked contrast to the "crossover" that occurs at approximately pH 6 in the original simulations conducted by von Wirén et al. (1999; Fig. 2a).

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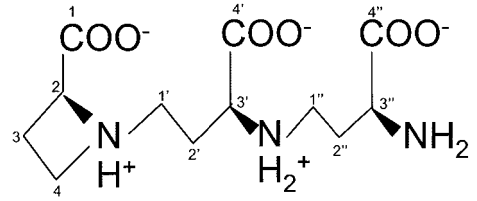
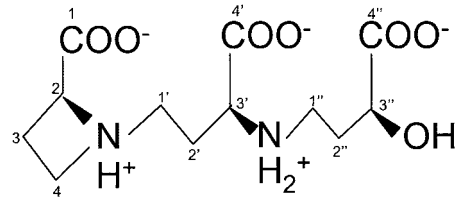


(a) Nicotianamine



(b) 2'-Deoxymugineic acid

Figure 1. The structures of NA (a; pH approximately 8) and DMA (b; pH approximately 7).

CASE 2. AFFINITY OF NA FOR Fe^{II} AND Fe^{III}

von Wirén et al. (1999) noted that, based on their relative stability constants (Table I), NA should form much more stable complexes with Fe^{III} than with Fe^{II}. However, when they then empirically tested the stabilities by mixing equimolar amounts of Fe^{II}, Fe^{III}, and NA (i.e. ratio of total Fe:NA was 2:1) at pH 7.0, and then separated the complexes by capillary electrophoresis, only Fe^{II}NA was detected. This led them to conclude that "NA will preferentially scavenge Fe^{II} even in the presence of Fe^{III}, and that the Fe^{II}NA complex will persist by virtue of its kinetic stability."

When we modeled the same equimolar concentrations of Fe^{II}, Fe^{III}, and NA at pH 7.0 using GEOCHEM-PC (Parker et al., 1995) and the stability constants in Tables I and II, the NA is about equally distributed between Fe^{II} and Fe^{III} if precipitation reactions are not allowed to occur (Table III). This similarity in stability reflects the highly hydrolytic nature of Fe^{III}, which, in effect, creates competition for the limited quantity of NA from a second ligand, the hydroxyl ion. Thus, the conditional stability constants at pH 7.0 that account for the hydrolytic properties of the metal (Stumm and Morgan, 1996) are about the same for Fe^{II} and Fe^{III}, even though the calculated free-ion activity of the latter is some 7 orders of magnitude lower (Table III). von Wirén et al. (1999) attempted to use similar differences in Fe^{2+} and Fe^{3+} activity as an indicator of the comparative strength of metal ligand associations, an ill-advised approach if metal hydrolysis is not properly accounted for.

When the same simulation was run but with the formation of solid-phase $Fe(OH)_3$ (Table II) allowed to occur, the majority of the Fe^{III} is predicted to precipitate (Table III), thus liberating all of the NA to react with Fe^{II}. This scenario most likely explains von Wirén et al.'s experimental result using capillary electrophoresis where, because [Fe^{II} + Fe^{III}] was present in molar excess of NA, there was little to prevent the rapid precipitation of Fe^{III} hydroxides.

von Wirén et al. (1999) also attempted to draw some inferences about the redox activity of Fe-NA complexes. As has been done previously (e.g. Norvell et al., 1993), the redox activity of a given Fe^{III} complex can be computationally assessed by considering the sum of following three reactions:

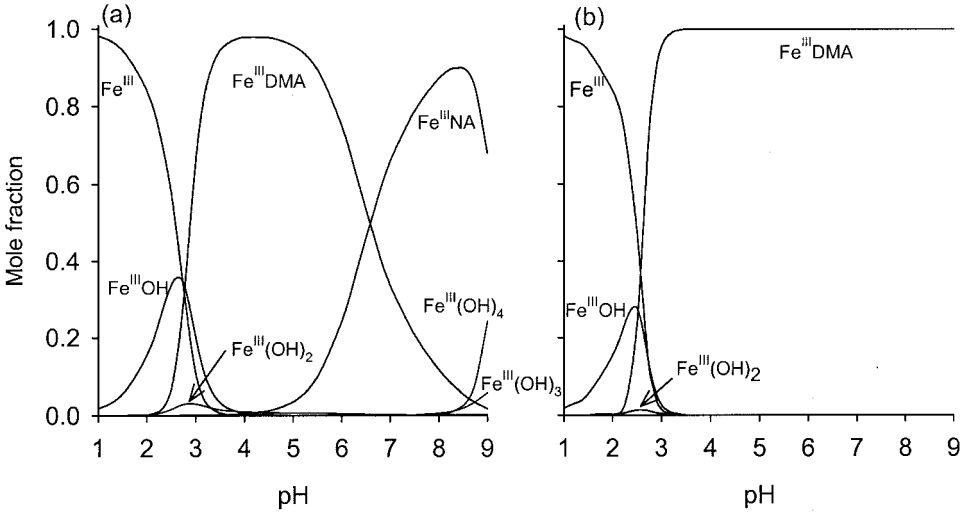


Figure 2. GEOCHEM-PC simulations of the pH-dependent competition between NA and DMA for Fe^{III} binding in a, as per Figure 7C in von Wirén et al. (1999); and b, as per a, plus including Murakami et al.'s (1989) stability constant for the $Fe^{III}DMA(H_{-1})^-$ complex. In both cases, the total Fe^{III} concentration was 1 μM, NA and DMA were present at 10 μM each, the ionic strength was 0.1 M, and precipitation reactions were not allowed.

Table I. *Published formation constants (I = 0.1 M) for the complexation of Fe by NA, DMA, and EDTA*

Reaction	log *K*, NA	log *K*, DMA	Reference
$Fe^{3+} + L^{3-} = Fe(III)L$	20.6	18.1[a]	von Wirén et al. (1999)
$Fe^{3+} + L^{3-} = Fe(III)L(H_{-1})^{-} + H^{+}$	–	16.3	Murakami et al. (1989)
$Fe^{2+} + L^{3-} = Fe(II)L^{-}$	12.8	10.4	von Wirén et al. (1999)
$H^{+} + L^{3-} = HL^{2-}$	10.1	9.6	von Wirén et al. (1999)
$2H^{+} + L^{3-} = H_2L^{-}$	19.2	17.3	von Wirén et al. (1999)
$3H^{+} + L^{3-} = H_3L$	26.2	20.7	von Wirén et al. (1999)
$4H^{+} + L^{3-} = H_4L^{+}$	29.0	23.5	von Wirén et al. (1999)
		log *K*	
$Fe^{3+} + EDTA^{4-} = Fe(III)L^{-}$	–	25.1	National Institute of Standards and Technology (1998)
$Fe^{3+} + EDTA^{4-} = Fe(III)L(OH)^{2-} + H^{+}$	–	17.7	National Institute of Standards and Technology (1998)
$Fe^{2+} + EDTA^{4-} = Fe(II)L^{2-}$	–	14.3	National Institute of Standards and Technology (1998)
$H^{+} + EDTA^{4-} = HL^{3-}$	–	10.1	National Institute of Standards and Technology (1998)
$2H^{+} + EDTA^{4-} = H_2L^{2-}$	–	15.7	National Institute of Standards and Technology (1998)
$3H^{+} + EDTA^{4-} = H_3L^{-}$	–	18.4	National Institute of Standards and Technology (1998)
$4H^{+} + EDTA^{3-} = H_4L$	–	20.4	National Institute of Standards and Technology (1998)

[a] Murakami et al. (1989) reported a similar log *K* of 18.4 for this reaction.

$$\begin{aligned} Fe^{III}L &\rightleftharpoons Fe^{III} + L \\ Fe^{III} + e^{-} &\rightleftharpoons Fe^{II} \\ Fe^{II} + L &\rightleftharpoons Fe^{II}L \\ \hline Fe^{III}L + e^{-} &\rightleftharpoons Fe^{II}L \end{aligned}$$

By using the appropriate values at $I = 0.1$ M from Table I, one can compute effective stability constants (Morel and Hering, 1993) for Fe^{III}-DMA and for Fe^{III}-EDTA that account for the important mixed complexes [$FeL(H_{-1})^{-}$ and $FeL(OH)^{2-}$, respectively]. When combined with the appropriate log *K* for the second reaction (12.5 at $I = 0.1$ M; Morel and Hering, 1993), followed by conversion to E_H notation, the corresponding constants for the net reaction are 0.278, 0.089, and 0.053 V for NA, EDTA, and DMA, respectively. As with any so-ordered ranking of standardized redox potentials, the higher E_H value for NA implies that its Fe^{III} complex is a comparatively good oxidant, and thus more readily reduced by strong reductants such as NAD(P)H (Morel and Hering, 1993). Conversely, its Fe^{II} complex is a relatively poor reductant, and is less susceptible to oxidation by molecular O_2. In contrast, the Fe^{III} chelates of EDTA, and especially DMA, are poorer oxidants, and their high affinity for Fe^{III} leads to rapid, spontaneous oxidation of chelated Fe^{II} in the presence of ambient O_2 (von Wirén et al., 1999). The E_H rankings provided by von Wirén et al. (1999) were based on some earlier, empirical measurements made before all of the needed stability constants were known, and do not conform to the ranking given here (i.e. the E_H for NA is too low). The reasons for this discrepancy are not known, but most likely reflect experimental difficulties with the earlier electrochemical measurements. Thus, von Wirén et al. (1999) correctly concluded that Fe^{II} is relatively stable when complexed by NA, but for the wrong reasons: This comparative stability can be satisfactorily explained based on the pertinent thermodynamics, and kinetic considerations are not needed.

Table II. *Hydrolysis and precipitation constants (I = 0.1 M) for Fe^{III} and Fe^{II} used in the GEOCHEM-PC calculations of Fe speciation*

The Fe^{III} constants are from Lindsay (1979), whereas the Fe^{II} constants are from the National Institute of Standards and Technology (1998).

	log *K*, Fe^{III}	log *K*, Fe^{II}
$Fe^{n+} + water = FeOH^{n-1} + H^{+}$	−2.6	−9.6
$Fe^{n+} + 2H_2O = Fe(OH)_2^{n-2} + 2H^{+}$	−6.4	−20.7
$Fe^{n+} + 3H_2O = Fe(OH)_3^{n-3} + 3H^{+}$	−13.8	−29.0
$Fe^{n+} + 4H_2O = Fe(OH)_4^{n-4} + 4H^{+}$	−22.0	−43.6
$Fe^{3+} + 3H_2O = Fe(OH)_3$ (amorphous) $+ 3H^{+}$	−2.8	na

na, Not applicable.

PHYSIOLOGICAL IMPLICATIONS

Our revisiting of von Wirén et al.'s findings may have important implications for internal transport of Fe and the role of NA in plants. Because the Fe^{III}-DMA complex is seemingly absorbed intact by Strategy II species (Romheld and Marschner, 1986; Grusak et al., 1999), NA and DMA might compete for Fe^{III} in the cytoplasm, and von Wirén et al. (1999) suggested the transfer of Fe^{III} from DMA to NA upon entry into the cytoplasm. However, inclusion of the Fe^{III}-$DMA(H_{-1})^{-}$ complex in the chemical modeling implies that Fe^{III} would remain approximately 100% complexed to DMA in the cytoplasm. Hence, for transfer of Fe to NA to occur, reduction of Fe^{III} in the cytoplasm, by an as yet unknown mechanism, would almost certainly be a requisite step. However, the majority of Fe present within normal plants seems to occur as Fe^{III} (Goodman and DeKock, 1982; Yoshimura et al., 2000), so it seems unlikely that Fe^{III} would be reduced for transport; reduction to Fe^{II} may only need to occur when required for metabo-

Table III. *Simulations with GEOCHEM-PC of the competition between Fe^{II} and Fe^{III} for binding by NA (1 μM for all three components) at pH 7.0 and I = 0.1 M*

Precipitation Allowed?	Fe(II)NA	Fe(III)NA	$Fe(III)(OH)_{3(s)}$	log (Fe^{2+})	log (Fe^{3+})
	μM	μM	μM		
No	0.40	0.55	–	−6.2	−13.9
Yes	0.73	0.0057	0.99	−6.6	−16.5

lism. In Strategy II species, substantial amounts of phytosiderophores have been found in both the xylem and phloem (Mori et al., 1991; Kawai et al., 2001). This, combined with the proper inclusion of the Fe^{III}-$DMA(H_{-1})^{-}$ complex into speciation considerations, supports a role for phytosiderophores such as DMA, but not NA, in long-distance transport of Fe in Strategy II plants.

The significance of the comparative stability of the Fe^{II}-NA complex remains unknown. von Wirén et al. (1999) argued that it could protect Fe^{II} from the rapid spontaneous oxidation by O_2 that occurs in vitro with strong Fe^{III} chelators such as EDTA, but living cells also contain abundant strong reductants (e.g. NADH) that could favor Fe^{II}. von Wirén et al. (1999) did show that Fe complexed by NA exhibited lower activity as a Fenton reagent than EDTA complexes or phosphate salts. However, no direct comparisons were made with Fe-binding polypeptides that are found in plants (e.g. ferritin) and are known to limit the Fe-catalyzed production of the hydroxy radical ($OH^{\cdot}$) from hydrogen peroxide (Becana et al., 1998), so the comparative activity of Fe-NA has not been quantified. In theory, the strong affinity of NA for Fe^{II} (relative to Fe^{III}) should inhibit initiation of the Fenton reaction. However, the multiple reactions affecting Fe^{II} levels make a priori prediction of Fenton activity based on Fe^{II}-/Fe^{III}-binding strength risky. Thus, whether NA's affinity for Fe^{II} plays a major role in protecting plant cells from peroxidative damage (von Wirén et al., 1999) remains a matter of speculation and a topic for future research.

In summary, the singular feature of NA seems to be that, in contrast with ligands such as the phytosiderophores and microbial siderophores that are highly selective for Fe^{III}, it has more comparable affinities for Fe^{II} and Fe^{III} at physiological pH. This is in agreement with evidence suggesting that NA is principally a cytoplasmic Fe regulator. The specificity of DMA for Fe^{III} is consistent with its role as a scavenger of Fe in the rhizosphere of alkaline, oxic soils, and possibly as an internal Fe transporter in Strategy II plants.

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