In vitro characterization of iron-phytosiderophore interaction with maize root plasma membranes: evidences for slow association kinetics

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Abstract

As an attempt to characterize iron(III)-phytosiderophore transport across plant membranes in vitro, a rapid filtration approach was set up in which plasma membrane vesicles from maize roots were incubated with ^{55}Fe − labelled deoxymugineic acid (DMA). Fe-DMA, and not Fe-EDTA, could associate with plasma membrane vesicles. The rate of Fe-DMA association decreased with a half time of 15 min. The initial Fe-DMA association rate, estimated from the amount of Fe-DMA associated after 10 min incubation, exhibited a saturation curve as a function of Fe-DMA concentration. This curve could be satisfactorily fitted to the Michaelis–Menten model ($K_M = 600$ nM, $V_{max} = 2$ nmol min^{-1} mg^{-1} protein). The association rate of Fe-DMA with control liposomes remained negligible and constant in a pH range from 4 to 8, whereas it strongly increased at acidic pH with plasma membrane vesicles. However, the specific association of Fe-DMA to root plasma membrane could not be explained by a vesicle-filling process because: (i) lowering the vesicle volume by decreasing the osmotic potential of the assay medium with sorbitol did not decrease ^{55}Fe labelling of the vesicles, (ii) creating inside-out vesicles by a Brij-58 treatment had almost no effect on Fe-DMA association to vesicles, (iii) ^{55}Fe labelling is reversible by EDTA and excess free DMA, and (iv) ^{55}Fe labelling was the same using plasmalemma vesicles prepared either from wild type maize or from the *ys1* maize mutant deficient in iron-phytosiderophore transport. A model is proposed to account for the observed Fe-DMA association as the result of very slow binding kinetics onto membrane proteins. This model was validated by its ability to describe quantitatively both Fe-DMA association as a function of time and of substrate concentration. A prediction of the model was that association of Fe-DMA to plasma membranes might overcome a high activation energy barrier. Indeed, the Arrhenius plot for the association rate constant was linear with an activation energy of 64 kJ mol^{-1}. © 1998 Elsevier Science B.V.

Keywords: Root; Iron transport; Deoxymugineic acid; (Maize)

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1. Introduction

To overcome iron insolubility in the presence of oxygen, organisms have evolved two principal mechanisms for iron solubilization and subsequent uptake: reduction or chelation. Yeast and most higher plants, solubilize ferric iron by acidification of their environment due to proton extrusion, and then reduce enzymatically iron(III) by membrane-bound reductases [1], enabling the subsequent uptake of ferrous iron by Fe(II) transporters [2], or by oxidase-permease complexes [3]. In contrast, most bacterial and mammalian cells chelate extracellular ferric iron by siderophores or proteins, and transport Fe(III)-complexes across membranes via Fe(III)-siderophore transporters [4], or receptor-mediated endocytosis [5]. As an exception, graminaceous plant species acquire iron by a strategy sharing similarities with bacterial systems, involving ferric iron chelation by low-molecular weight secondary amino acids called phytosiderophores. As a response to iron-deficiency stress, phytosiderophores are synthesized from methionine as a precursor via nicotianamine to the phytosiderophore mugineic acid family. These compounds function as hexadentate cation chelators [6]. When released from plant roots, phytosiderophores can chelate sparingly soluble iron, as from Fe hydroxides or phosphates, by the formation of an Fe(III)-phytosiderophore complex [7]. Phytosiderophores efficiently chelate iron from the soil, even at high pH and high concentrations of bicarbonate, whereas iron solubilization mediated by proton release and by concomitant increase in root reducing capacity is inhibited by bicarbonate. This explains the ecological advantage of grasses, compared to nongraminaceous plant species, when grown on bicarbonate buffered soil [8].

Uptake of the Fe(III)-phytosiderophore complex across the root plasma membrane has been proposed to occur via specific transport proteins [9], which are well described at the physiological level [10,11]. Although considerable progress has been made towards the molecular and biochemical characterization of the mechanisms contributing to ferric iron solubilization and uptake in microbial, yeast and mammalian systems, the corresponding plant genes and proteins are still poorly described.

As an attempt to characterize iron(III)-phytosiderophore transport, an in vitro assay was developed using isolated maize root plasma membrane vesicles and ^{55}Fe – labelled phytosiderophores, namely deoxymugineic acid (DMA). Following methods that successfully monitored the uptake of radioactively labelled nutrients into plasma membrane vesicles [12–14], a specific interaction between ^{55}Fe – DMA and root plasma membrane vesicles was quantified. This association followed very slow kinetics and could not be explained by a vesicle-filling process. A model has been proposed which fits the experimental data and describes quantitatively both Fe-DMA association as a function of time and of substrate concentration. A prediction of the model is that association of Fe-DMA to plasma membranes must overcome a high activation energy barrier, and this has been experimentally verified.

2. Material and methods

2.1. Plant culture and plasma membrane isolation from maize roots

Seeds of Fe-efficient maize (*Zea mays* L. cv. Alice) and from iron-phytosiderophore uptake deficient mutant (*ys1*) were placed between filter paper moistened with saturated $CaSO_4$ solution and germinated at 25°C in the dark. After 3 days, seedlings were transferred to a continuously aerated nutrient solution [15]. Plants were precultured with 10^{-4} M Fe-EDTA for 6 days and then continued to grow on Fe-deficient nutrient solution to promote Fe-phytosiderophore uptake activity in the roots [11]. The plants were grown for 14 days in a growth chamber at 60% relative humidity, a light intensity of 250 μmol m^{-2} s^{-1} at plant hight and in a 16 h-25°C, and 8 h-22°C, day-night regime.

Plasma membrane vesicles were isolated according to the methods of Faraday and Spanswick [16] and Fischer-Schliebs et al. [17] using aqueous polymer two-phase partitioning [18]. All operations were conducted at 4°C. Maize roots were washed in ice-cold distilled water and homogenized with a Waring blender in a buffer containing 50 mM BTP (adjusted to pH 7.8 by dry MES), 250 mM sucrose, 2 mM EGTA (pH 7.8), 10% (w/v) glycerol, 2 mM DTT, 1 mM PMSF (added after filtration of the homogenate), 2 mM MgSO$_4$, 2 mM ATP, 5 mM mercaptoethanol, 4% (w/v) choline-Cl, 10 mM Na glycerophosphate.

The final ratio of medium to roots was 3 ml g^{-1} fresh weight. The homogenate was filtered through 2 layers of crosswise placed cheesecloth, and the filtrate was centrifuged at $26,000 \times g$ (R_{max}) for 25 min in a Beckman JA-14 rotor. The supernatant was filtered through gauze (63 and 38 μm) and re-centrifuged in a Kontron TFT$_{45.94}$-rotor at $85,000 \times g$ for 35 min. The drained pellet was resuspended in 250 mM sucrose, 5 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 7.8), 3 mM KCl (7 ml mg^{-1} protein) and gently homogenized. The repelleted and resuspended microsomes were loaded onto a two-phase system containing 6.2% (w/w) Dextran T-500, 6.2% (w/w) polyethylene glycol (PEG) 3350, 250 mM sucrose, 5 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 7.8) and 3 mM KCl. After phase separation, the PEG-enriched upper phase was removed and repartitioned two times on a fresh lower phase. The plasma membrane-enriched upper phase was diluted threefold in dilution buffer containing 250 mM sucrose, 5 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (pH 7.8) and 1 mM PMSF and centrifuged at $170,000 \times g$ for 45 min. Membranes were resuspended in conservation buffer (5 mM BTP-MES (pH 7.8), 250 mM sorbitol, 20% (w/v) glycerol) and stored under liquid N$_2$ until required. Protein contents were determined according to Schaffner and Weissmann [19] and a typical yield of 30–40 μg protein g^{-1} fresh weight was obtained.

2.2. Measurement of ATP hydrolysis and H$^+$ pumping

ATPase activity was measured in an assay medium containing 50 mM MES-Tris (pH 6.5), 100 mM KCl, 3 mM MgSO$_4$, and 10 μg membrane protein. To assess vesicle orientation 0.05% (v/v) Brij-58 was added [20]. Further modifications of the reaction mixture included 120 mg l^{-1} lysophosphatidylcholine, 0.25 mM vanadate, 30 mM nitrate, 5 mM azide, 1 mM molybdate or 3 mM IDP or PNPP instead of ATP. The reaction was started by the addition of 3 mM ATP (pH 6.5), conducted at 37°C for 20 min and liberated P_i was measured every 5 min according to Ames [21].

Proton transport into vesicles (100 μg protein ml^{-1}) was measured as the fluorescence quenching of the permeant amine aminochloromethoxy acridine (ACMA) (1 μM) in 5 mM ATP-BTP (pH 6.5), 300 mM sorbitol, 50 mM KCl, 50 mM KNO$_3$, 0.1 mM valinomycin [22] with or without 0.05% (v/v) Brij-58 to invert right side-out plasma membrane vesicle. The reaction was started by the addition of 5 mM MgSO$_4$ and conducted at 30°C under stirring.

2.3. Fe-phytosiderophore binding assay

Phytosiderophores were collected from root exudates of hydroponically cultured wheat plants, partially purified by cation-exchange chromatography and verified for Fe transport activity as described elsewhere [11,23]. As determined by HPLC, phytosiderophores consisted solely of desoxy-mugineic acid (DMA), the same species which is also synthesized by maize. An aliquot of ^{55}FeCl$_3$ (5 TBq mol^{-1} Fe) was mixed with DMA (10% excess ligand) and MES to give a final pH of 4.5. To retain Fe-precipitates, the solution was then filtered through 0.2 μm cellulose nitrate filters. ^{55}Fe − labelled EDTA was prepared by the same procedure.

Liposome vesicles were prepared from l-alpha-phosphatidylcholine (type IVb from soybean, Sigma P 3644), which was vortexed in conservation buffer and in the presence of glass beads for 10 min under argon in order to minimize oxidation processes. Liposomes were then sonicated for 10 min at room temperature in a bath sonicator until the solution became clear.

Vesicles or liposomes (100 μg protein or lipids, respectively) were diluted in 20 volumes of equilibration buffer (5 mM BTP-MES pH 4.5, 100 mM sorbitol, 50 mM KCl), sonicated for 20 s and equilibrated on ice for 10 min. Aliquots of 10 μl vesicle mix were preincubated with 8 volumes of assay buffer at 26°C for 3 min. If not indicated otherwise, the assay buffer consisted of 50 mM BTP-MES (pH 4.5), 300 mM sorbitol, 50 mM KCl, 800 μM ferrozine, 200 mM ^{55}Fe − phytosiderophores and 1 μg 100 μl^{-1} vesicle proteins or liposomes. The reaction was started by adding 10 μl ^{55}Fe − labelled phytosiderophores, flicking the tube and incubating at 26°C. The reaction was stopped by diluting the assay mix in 400 μl ice-cold washing buffer (50 mM BTP-MES (pH 4.5), 250 mM sucrose), and the diluted assay mixture was immediately filtered through prewashed 0.45 μm cellulose nitrate filters (Sartorius, Goettingen, Germany) under a slight vacuum. The

membranes were rinsed with 3 ml washing buffer, mixed with scintillation cocktail and assayed for ^{55}Fe activity by liquid scintillation spectrophotometry. Membrane retained Fe activity was expressed in nmol vesicle-associated Fe mg^{-1} vesicle protein or mg^{-1} liposomes. Treatments were conducted in four replicates and significant differences were verified by Scheffé-test at $P < 0.05$ when ANOVA generated a significant F-value. Presented values are means $\pm$ standard deviation. If no error bars are visible, standard deviations were smaller than the plot symbol.

3. Results and discussion

3.1. Characterisation of the root plasma membrane vesicles

Tight plasma membrane vesicles were obtained after phase-partitioning, as shown by the high H$^+$-pumping activity observed upon activation of the H$^+$-ATPase after reversion of right side-out vesicles using the detergent Brij-58 [20] (Fig. 1). In the
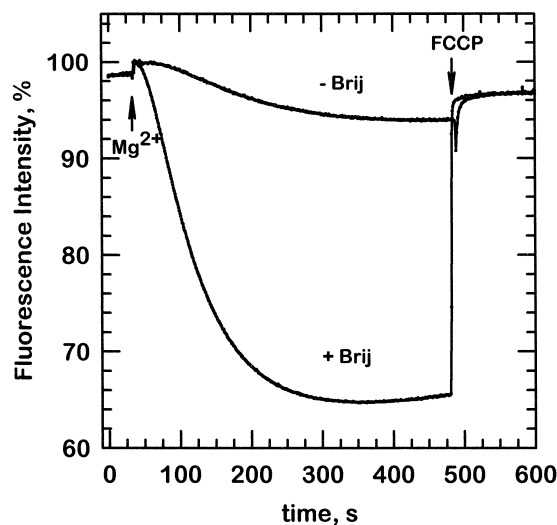
Table 1
Percentage of ATP hydrolytic activity of plasma membrane vesicles from maize roots in presence of different inhibitors or substrates

Treatment	%	SD
Control	26.3	± 1.4
+ Brij 58	100.0	± 13.8
+ Lyso-PC	224.5	± 42.5
+ Vanadate	22.3	± 5.4
- Magnesium	12.2	± 5.6
+ Nitrate	100.8	± 6.1
+ Azide	103.2	± 8.5
+ Molybdenum	98.5	± 1.8
+ IDP	12.2	± 1.6
+ PNPP	6.8	± 2.9

The specific activity in the presence of Brij-58 amounted 1.16 ± 0.16 μmol P_i min^{-1} mg protein^{-1} and was set as 100%.

Fig. 1. H$^+$-ATPase acidification of maize root plasma membrane vesicles. Traces represent the fluorescence of the pH probe ACMA. H$^+$-ATPase was activated by adding a concentrated MgSO$_4$ aliquot (5 mM Mg^{2+} final at the indicated arrow) to an assay medium containing 5 mM ATP. The proton gradient was collapsed by adding the protonophore FCCP (0.5 μM) at the indicated arrow. Trace ' + Brij': 0.05% (v/v) of the detergent Brij 58 was added prior to H$^+$-ATPase activation in order to revert right side-out vesicles [20].

absence of Brij, the sidedness of membrane vesicles was essentially right side-out. This was demonstrated by the stimulation of the H$^+$ pumping rate upon Brij addition. Incubating the vesicles in 0.05% Brij-58, which has been shown to uniformly create inside-out vesicles [20], resulted in a specific ATPase activity of 1.16 μmol P_i mg protein^{-1} min^{-1} (Table 1). Twenty six percent of this activity was still measured in the absence of any detergent, which indicates that 74% of the obtained root membrane vesicles should be right-side out oriented (Table 1). These values are close to those in other reports, irrespectively of whether plasma membranes were purified by two-phase partitioning [16] or by sucrose gradient centrifugation [17].

Lysophosphatidylcholine strongly increased ATPase activity (Table 1), which might be explained by an interaction with the C-terminal autoinhibitory domain of the enzyme [24]. The presence of nitrate, azide and molybdenum did not influence ATP hydrolytic activities indicating that contamination of the plasma-membrane enriched fraction by membranes from intracellular organelles was very low. This was confirmed by the residual P_i formation found when ATP was exchanged by other phosphorylated substrates such as IDP or PNPP (Table 1). Seventy eight percent of the ATP hydrolysis was inhibited by vanadate and 88% was dependent on magnesium, indicating that the majority of the obtained membrane fraction originated from the plasma membrane, and was
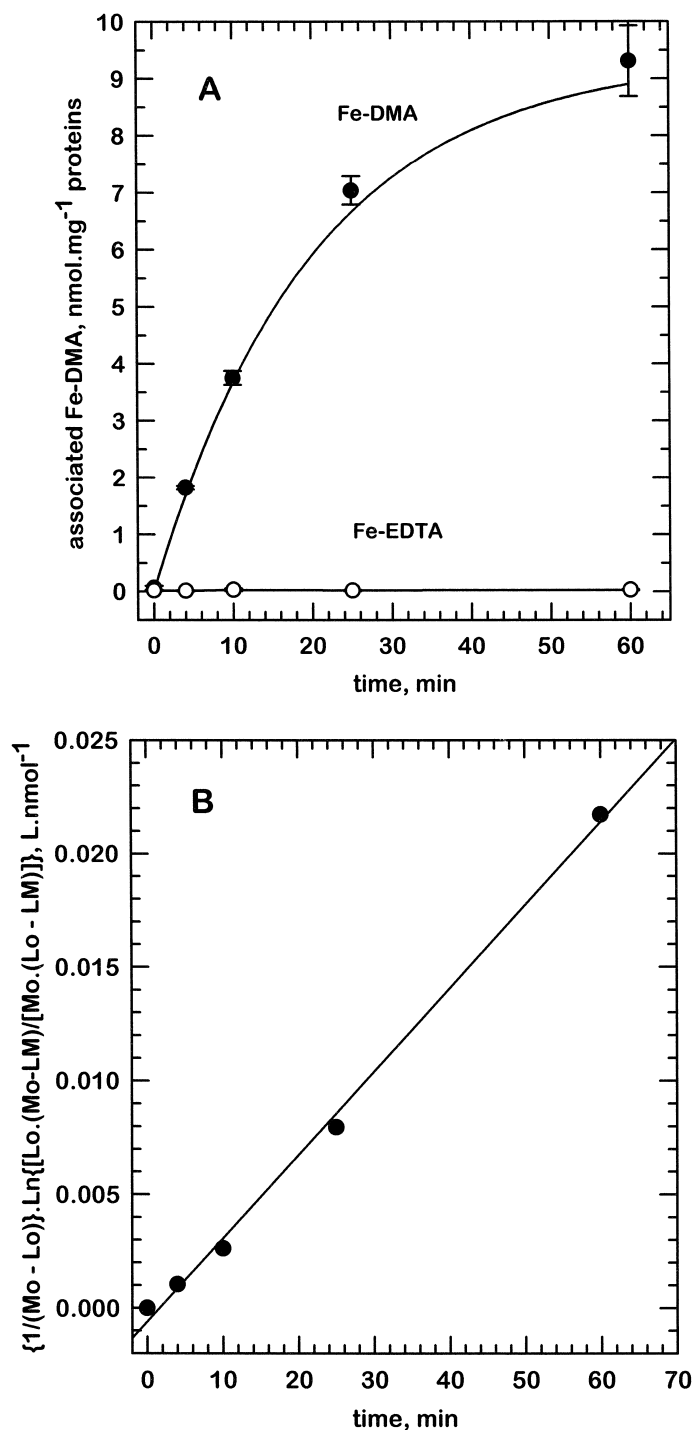
Fig. 2. Kinetics of Fe-DMA and Fe-EDTA association to maize root plasma membrane vesicles. Assay medium contained 200 nM [55]Fe − chelates (pH 4.5, 26°C). The curve in A was obtained from a non-linear regression fit (SigmaPlot, Jandel Scientific) on the basis of a second order association reaction (relation 7 in Section 3, $L_o = 9.8$ nmol mg^{-1} protein, $k = 3 \times 10^{-4}$ L nmol^{-1} min^{-1}). In B: second order reaction kinetics transform (relation 6 in Section 3) applied to Fe-DMA data in A; slope of the linear regression fit gave $k = 3.7 \times 10^{-4}$ L nmol^{-1} min^{-1}.

thus suitable for Fe-phytosiderophore transport assays.

3.2. Kinetics of the interaction between maize root plasma membrane vesicles and iron-phytosiderophores

After rapid filtration of the incubation mix Fe-DMA associated with plasma membrane vesicles, whereas Fe-EDTA did not (Fig. 2A). The rate of Fe-DMA association decreased slowly with a half time of approximately 15 min. In the following, the initial association rate was determined from the amount of Fe-DMA associated after 10 min incubation. Since it was linear with the concentration of plasma membrane protein (Fig. 3A), the initial rate could be expressed in specific units (nmol Fe-DMA min^{-1} mg^{-1} protein). When plotted as a function of Fe-DMA concentration in the medium, the initial rate of Fe-DMA association exhibited saturation kinetics, which could be satisfactorily fitted with the Michaelis–Menten model (Fig. 3B, $K_M = 600$ nM, $V_{max} = 2$ nmol min^{-1} mg^{-1} protein). Fe-DMA association to membrane vesicles was strongly pH dependent, with a large increase at pH lower than 6 (Fig. 4). Noteworthy, Fe-DMA association with control liposomes remained negligible, and was constant with pH (Fig. 4).

Fe-DMA association to plasma membrane vesicles from maize roots seemed specific, therefore, of the iron chelate. The observed influence of time and substrate concentration on ^{55}Fe − labelling of the membrane vesicles might reflect a filling process of the membrane vesicles as mediated by a membrane carrier. In that case, the increase of vesicle-associated

Fe-DMA at low pH (Fig. 4) could indicate the pH optimum of the carrier and/or proton-coupled cotransport. However, Fe-DMA association rates remained constant by increasing the pH of the vesicle interior (data not shown), indicating that only the external pH promoted Fe-DMA association, and not a pH gradient across the vesicle membrane.
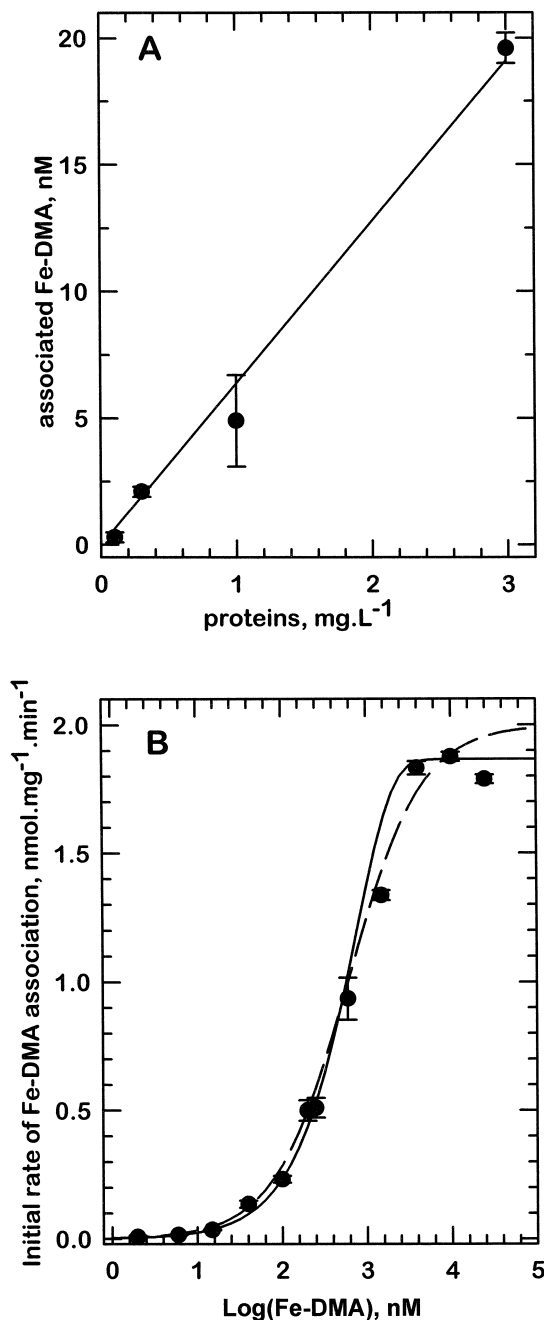

Fig. 3. Fe-DMA association to maize root plasma membrane vesicles as a function of protein (A) or Fe-DMA (B) concentration. (A) Associated Fe-DMA was measured after 10 min incubation (initial association rate) in the presence of 200 nM Fe-DMA and the indicated concentration of protein. (B) Initial rate of Fe-DMA association in the presencce of 1 mg protein ml^{-1} and the indicated Fe-DMA concentration. Curves in B were obtained from a non-linear regression fit (SigmaPlot, Jandel Scientific) of observed initial rates of Fe-DMA association by (dashed-line) the Michaelis–Menten model ($K_M = 600$ nM, $V_{max} = 2$ nmol min^{-1} mg^{-1} protein), or by (continuous line) the second order association model (relations 7 and 8 in Section 3, with Lo = 9.8 nmol mg^{-1} protein, $k = 3 \ 10^{-4}$ L $nmol^{-1}$ min^{-1} and $t_i = 6$ min).
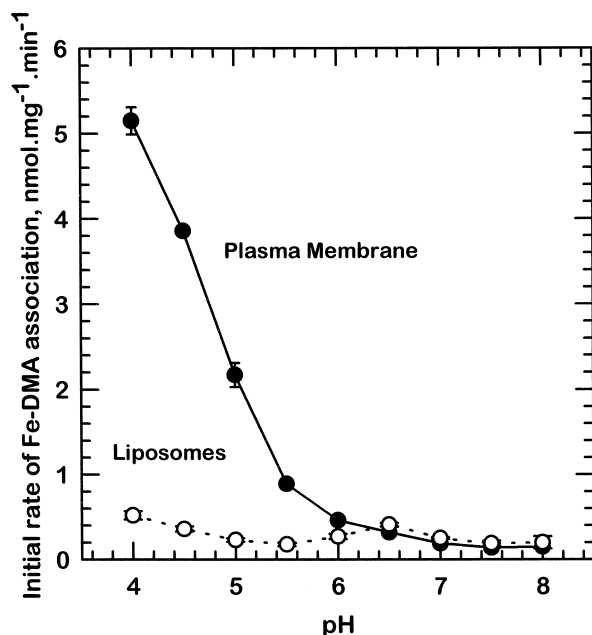
Fig. 4. pH dependence of the initial rate of Fe-DMA association to maize root plasma membrane vesicles (full circles). Except pH, experimental conditions were the same as in Fig. 2 and in Section 2. As a control, the same experiment was performed with liposomes (open circles, dashed line). The experiment was repeated with two different vesicle preparations (four repeats per preparation), and also conducted at 20 μM Fe-chelates and 10 μg vesicle proteins or liposomes yielding similar patterns.

Although adsorption of small molecules is known to be a fast process, usually studied in special devices such as stopped-flow and at low temperatures, it could not be ruled out that $^{55}Fe - DMA$ labelling of the vesicles was due to adsorption at the vesicle surface, without transport inside the vesicle cavity. In this case, pH dependence of Fe-DMA association should be related to electrostatic interactions at the negatively charged surface of biological membranes. Indeed, Fe-phytosiderophore complex behaves as an anion at pH 7 [25] and adsorption of various mineral or organic anions on biological membranes is well known to be strongly hampered by local electrostatic repulsion [26]. Noteworthy, the net charge of the Fe-phytosiderophore complex could decrease upon protonation in this pH range (von Wirén et al., unpublished data), thereby raising the chance for adsorption to the membrane surface. In a pH range from 4 to 8, 75%, 90% or 100% of iron (200 nM) is found chelated with DMA when mixed in a molar ratio, in a

two fold excess of chelate, or in a 50 fold excess of chelate, respectively (speciation plots determination; data not shown). Furthermore the pH dependence of this association, as illustrated in Fig. 4, cannot be attributed to a splitting of the iron-DMA complex between pH 4 and 6 since this complex exhibits the same stability between pH 4 and 8 (data not shown).

3.3. $^{55}Fe - DMA$ labelling of maize root plasma membrane vesicles cannot be explained by a vesicle-filling process

In order to discriminate between the two possibilities mentioned above (i.e., Fe-DMA uptake into the vesicles vs. adsorption to the vesicle surface) the vesicle volume was decreased by increasing the osmotic potential of the assay buffer via addition of sorbitol. Increasing sorbitol concentrations up to 1.2 M did not decrease the initial rate of Fe-DMA association with root plasma membrane vesicles (Fig. 5), which is in contrast to other reports, for example a 6-fold decrease of the initial rate of $Cd-^{35}S-$ phytochelatin uptake by tonoplast vesicles observed in a comparable experimental setup [27]. Indeed, sorbitol slightly stimulated the $^{55}Fe - DMA$ labelling of vesicles (Fig. 5). This could be explained by the
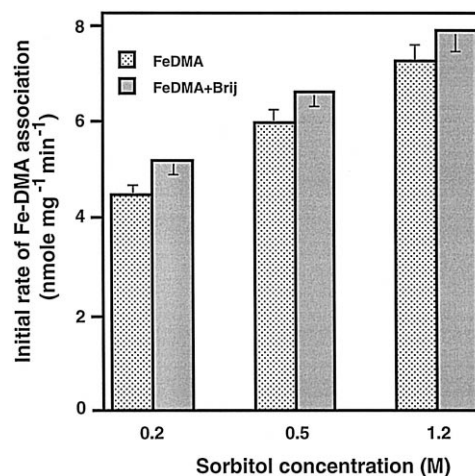

Fig. 5. Effect of increasing sorbitol concentrations on the rate of Fe-DMA association to maize root plasma membrane vesicles. Except the osmoticum concentration, experimental conditions were the same as in Fig. 2 and in Section 2. The experiment was repeated with three different vesicle preparations, and four repeats for each preparation were performed.

water binding activity of sorbitol which might increase the spatial contact between Fe-chelate and vesicle surface. Another experimental evidence was obtained by use of Brij-58, known to revert right side-out vesicles (see Fig. 1), which did not significantly affect the association of Fe-DMA to plasma membrane vesicle (Fig. 5). This result argued against uptake into the vesicles since most membrane transport systems display asymmetric properties. In order to further document Fe-DMA interaction with root vesicles, this association was challenged with either EDTA (Fig. 6) or various free DMA concentrations (Fig. 7). At various time points of the $^{55}Fe - DMA$ interaction with vesicules, aliquots were treated with 800 μM EDTA for 2 min. As a result, $^{55}Fe - DMA$ labelling of the vesicles decreased by 10 to 30% according to the time point considered (Fig. 6). Excess free DMA in the reaction mixture has even a more drastic effect on $^{55}Fe - DMA$ labelling of the vesicles (Fig. 7A). In presence of 100 μM free DMA, $^{55}Fe -$ binding to the vesicles is decreased by 80%. DMA association to the root vesicles, appears, therefore, to be reversible, arguing against a transport process. An additional piece of evidence in favor of an iron association to the vesicule surface rather than

Fig. 7. Kinetics of Fe association from Fe-DMA to root plasma membrane vesicles in the presence of various amount of free DMA. Maize root plasma membrane vesicles were prepared from the wild type genotype Alice (A) or from the *ys1* mutant deficient in iron-phytosiderophore uptake (B) Experimental conditions were: pH 4.5; 200 nM $^{55}Fe - DMA$; 0, 1, 10 or 100 μM free DMA; 1 μg vesicle proteins; $t = 0$, 4, 10, 25 or 60 min, $n = 4$. The experiment was repeated three times with different vesicle and Fe-chelate preparations and similar results were obtained.
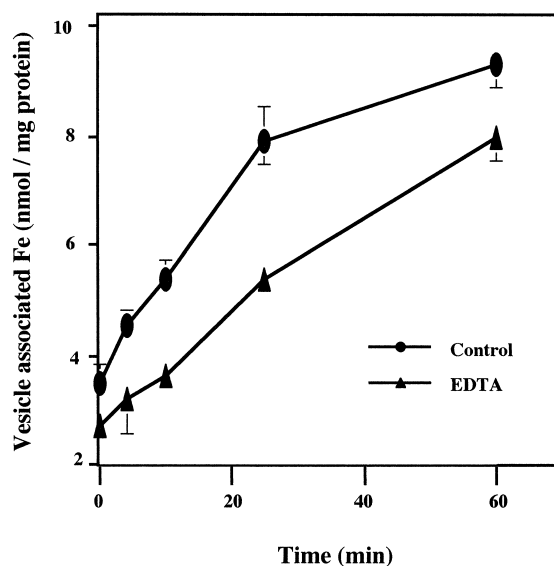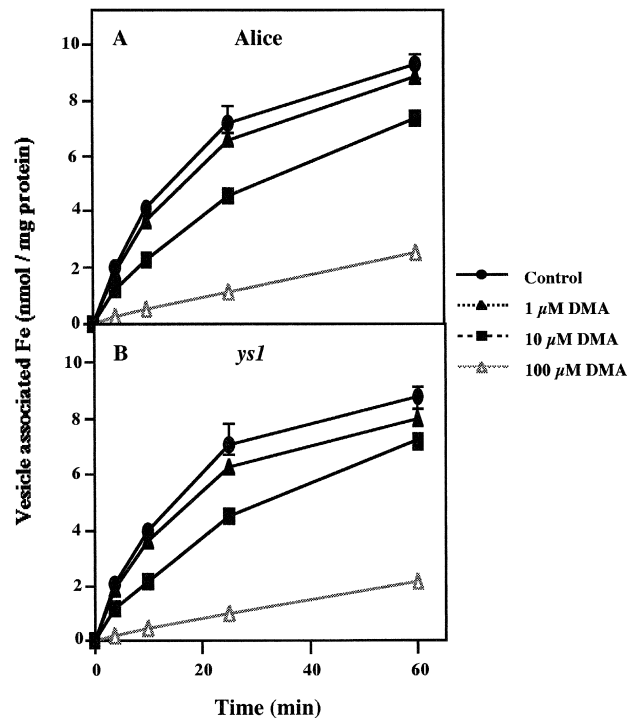

Fig. 6. EDTA effect on Fe-DMA association to maize root plasma membrane vesicles. At various time points of an Fe-DMA binding assay, aliquots of the reaction mixture were incubated for two min with 800 μM EDTA prior to filtration as described in material and methods. $n = 4$.

a transport mechanism came from an experiment using root vesicles prepared from the *ys1* maize mutant, which is deficient in iron-phytosiderophore uptake in vivo [15]. Kinetics and amount of ^{55}Fe vesicle labelling, as well as excess free DMA ability to compete with this interaction, were identical between the *ys1* mutant and the iron efficient Alice genotype (Fig. 7A and B). In vitro $^{55}Fe - DMA$ labelling of the vesicles is, therefore, not altered when using root plasmalemma vesicles from the *ys1* mutant. This observation agrees with an iron association to the vesicle surface, and not with a vesicle filling mechanism.

Estimation of the internal Fe-DMA concentration after 60 min incubation in 200 nM Fe-DMA argues also against a putative transport of Fe-DMA. Assum-

ing an average vesicle volume of 10 μl mg^{-1} protein [28], the internal concentration of Fe-DMA should increase up to 400 μM (Fig. 2). However, a thousand-fold accumulation was very unlikely since neither a pH gradient and a membrane potential, nor an ATP- or reductant-dependent driving force had been built up experimentally. Also Fe-DMA reduction was most likely not involved in ^{55}Fe labelling of vesicles because the large excess of ferrozine in the assay medium would have chelated ferrous iron prior to adsorption, and Fe-DMA association was similar in the presence and absence of 250 mM NADH (data not shown).

In conclusion, although extended time kinetics, as observed here, are usually found for substrate transport into reconstituted and energized vesicles, as reported for drug, sugar, or amino acid transport [29–31], transport of Fe-DMA through the plasma membrane of vesicles was most probably not responsible for the ^{55}Fe labelling of these vesicles. Therefore, the observed extended time kinetics are more likely attributable to Fe-DMA association to the vesicle surface. It has been reported that iron-chelates were able to bind to specific phospholipids, especially phosphatidylserine (PS) [32,33]. Indeed, PS is the only phospholipid for which the reported pK_a of 3.5 [34] would be compatible with the pH dependency of Fe-DMA association to maize root vesicles (Fig. 4). However, PS accounts for about 1.5% in both corn root plasma membrane phospholipids [35] and in soybean phospholipids contained in the lipid mixture used in the present study to form control liposomes (Pr. A.J. Dorne, Grenoble University, France, personal communication). Therefore, the difference in Fe-DMA binding observed between corn root plasma membrane and liposomes appears merely attributable to the proteic moiety of the former. Consistent with this statement, and with the pH dependency of Fe-DMA association to maize root vesicles (Fig. 4), adsorption of various ionic ligands has been shown to strongly depend on pH between 4 to 6, due to protonation of carboxy-groups of proteins [36]. However, we failed to detect plasmalemma membrane ^{55}Fe − labelled proteins by SDS- or native-gel electrophoresis followed by autoradiography. This could be due to a too low ^{55}Fe specific radioactivity.

Fe-DMA adsorption onto membrane vesicles was therefore further analysed in order to propose a model

accounting for the slow association kinetics observed in Fig. 2.

3.4. The association model

A diffusion-controlled adsorption could generate saturable kinetics if binding sites or the substrate become limited. In the present investigation maximum Fe-DMA depletion did not exceed 50% after 60 min (Fig. 2). Substrate depletion is therefore unlikely to account for the observed time-dependent kinetics in Fe-DMA association. The input of liposomes and root vesicles in the assay was adjusted assuming a phospholipid/protein ratio (w/w) of 1 in plasma membranes. As the initial rate of Fe-DMA association was about 10-fold higher with plasma membranes than with liposomes (Fig. 4), the protein fraction accounted for the major part in Fe-DMA association. Assuming 40 kDa as an average molecular weight of plasma membrane proteins, about 25 pmol proteins would face 20 pmol Fe-DMA in 100 μl assay volume. Thus, almost every protein could theoretically interact with one Fe-DMA molecule; such a molar ratio supports the view that only a limited number of protein binding sites could cause the observed saturable kinetics.

To develop an association model which takes into account the slow association kinetics observed in Fig. 2 requires at least two assumptions: (i) initial conditions should be far from equilibrium conditions; practically, only the association reaction of Fe-DMA with membrane vesicles was considered (dissociation reaction being neglected); (ii) a plateau should be reached because reagents should become limiting. As already mentioned above, the association could be limited only by the concentration of free sites. Thus, the plateau should correspond to an equilibrium in which free sites are strongly depleted: practically, we considered that the level of the plateau gave approximately the total concentration of Fe-DMA binding sites.

From the first hypothesis above, only association reaction should be considered:

$$L + M \to LM \tag{1}$$

where *L*, *M* and *LM* are the concentration of free sites, free Fe-DMA and associated Fe-DMA, respec-

tively. The rate of formation of the complex ($\mathrm{d}LM/\mathrm{d}t$) should be given by:

$$\mathrm{d}LM/\mathrm{d}t = kLM \qquad (2)$$

Since $\mathrm{d}LM = -\mathrm{d}L$:

$$\mathrm{d}L/L = -kM\mathrm{d}t \qquad (3)$$

If associated Fe-DMA cannot be neglected compared to total Fe-DMA (i.e., M decreased during the association reaction), a true second order kinetics has to be considered. Noting that $L_0 - L = M_0 - M$, Eq. (3) gives:

$$\mathrm{d}L/\mathrm{d}t = -kL(B_0 - L) \text{ where } B_0 \text{ stands for } M_0 - L_0 \qquad (4)$$

Eq. (4) can be integrated:

$$(1/B_0)\ln\{L/(B_0 + L)\} = -kt + (1/B_0)\ln(L_0/M_0) \qquad (5)$$

Expressing relation (5) as a function of the measured variable LM (from $L_0 = L + LM$) gives:

$$\{1/(M_0 - L_0)\}.Ln = \{[L_0.(M_0 - LM)]$$
$$/[M_0 L_0 - LM)]\} = t \qquad (6)$$

This second order kinetics predicts that a linear plot with time should be obtained from the measured LM values, the slope of which gives the value of the association rate constant. Alternatively, relation (6) permits to express LM as:

$$LM = \{\exp[(M_0 - L_0)kt] -\}/\{\exp[(M_0 - L_0)kt]$$
$$/L_0 - 1/M_0\} \qquad (7)$$

The association rate $\mathrm{d}LM/\mathrm{d}t$, measured in Fig. 2, could be calculated by derivating relation (7). For experimental reasons, initial association rates were estimated from the amount of Fe-DMA associated after incubation for 10 min, assuming that association was linear with time, which was not strictly the case. Therefore, the capacity of the above described associ-

ation model to fit experimental association rates (v_a) vs. Fe-DMA concentration was examined using the following relation, where is t_i the incubation time experimentally used for estimating initial association rate:

$$v_a = LM_{(t_i)}/t_i \qquad (8)$$

where is $LM(t_i)$ the value of LM for $t = t_i$, predicted by Eq. (7).

3.5. Application of the association model

The efficiency of the model described above was evaluated first from its capacity to describe quantitatively Fe-DMA association as a function of time (Fig. 2A) and of substrate concentration (Fig. 3B), using same L_0 and k values (M_0 being fixed in the experiment).

According to the second assumption mentioned above, which is required to develop the association model, the extent of the Fe-DMA association reaction should be actually limited by the concentration of the ligand (L_0). Indeed, the value of the plateau was given by L_0 (protein) (Fig. 3A). Therefore, the value of the plateau reached in time dependent experiments (Fig. 2A) should give directly the L_0 value (9.8 nmol mg^{-1} proteins). This L_0 value allowed to calculate the order 2 transform plot (Eq. (6)) from measured LM values (Fig. 2B). The order 2 transform plot was actually linear with time, and the linear regression slope gave the value of the association rate constant ($k = 3.7 \ 10^{-4}$ L nmol^{-1} min^{-1}). Direct non-linear curve fitting (Sigma Plot software, Jandel Scientific) of data in Fig. 3A, using relation (7), gave $k = 3.0 \ 10^{-4}$ L nmol^{-1} min^{-1}. Finally, the association model with the above given parameters values allowed to describe quantitatively the experimental kinetics (see theoretical line in Fig. 3B).

The parameters used above allowed to describe the association rate as a function of Fe-DMA concentra-

Fig. 8. Temperature dependence of association kinetics of Fe-DMA to maize root plasma membrane vesicles. Except temperature, experimental conditions were the same as in Fig. 2 and in Section 2. Curves in A were calculated according to the second order association model (relation 7) with $L_0 = 9.8$ nmol mg^{-1} protein, and the k values determined from the slope of linear regression fits of second order transforms (relation 6) showed in B ($k = 3.5 \ 10^{-4}$, $1.3 \ 10^{-4}$ or $0.37 \ 10^{-4}$ L nmol^{-1} min^{-1} at 26°, 13° or 2°C, respectively). Linear regression of the Arrhenius plot from these latters (in C) gave an activation energy of 64 kJ mol^{-1}. The experiment was repeated with two different vesicle preparations, and four repeats for each preparation were performed.

tion, using relation (8). As shown by the comparison of the theoretical curves in Fig. 3B, the second order association model provided a better fit to the experi-

mental data than the Michaelis–Menten one (with K_M and V_{max} of 600 nM and 2 nmol mg^{-1} min^{-1}, respectively), at the higher Fe-DMA concentration,
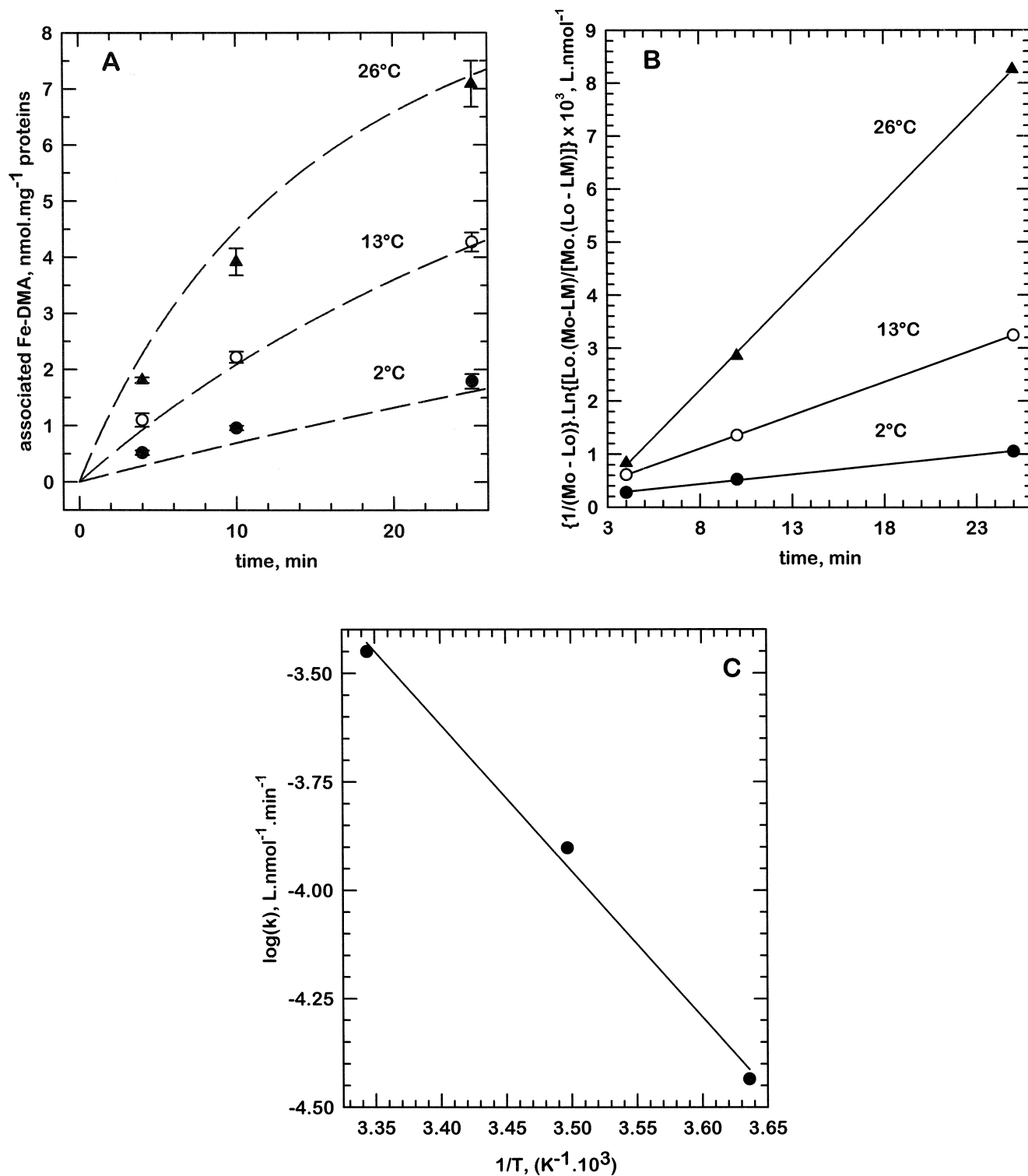
where association can be simplified to a pseudo first order kinetics (since $LM \ll M$).

Very low constant rates of Fe-DMA association to membranes were used to account for observed slow kinetics (Fig. 2), suggesting that Fe-DMA association to vesicles should have to overcome a high activation energy barrier. This point was tested experimentally. Association of Fe-DMA to plasma membrane vesicles was measured at 2°C, 13°C and 26°C (Fig. 8A). The three corresponding order 2 transform plots (relation 6) were linear, and their slopes gave the values of the association rate constant at the different temperatures (Fig. 8B). These latters gave in turn a linear Arrhenius plot (Fig. 8C), corresponding, effectively, to a high activation energy (64 kJ mol^{-1}).

4. Concluding remarks

The physiological characterization of Fe-phytosiderophore uptake in intact plants provided strong evidence that Fe-phytosiderophores cross the root plasma membrane of graminaceous plant species by an active transporter-mediated process [9–11]. With the perspective to characterize in vitro this putative Fe-phytosiderophore transporter, an attempt was made to set up an experimental protocol allowing to measure in vitro a specific ^{55}Fe − phytosiderophore labelling of maize root plasma membrane vesicles. Indeed, using this assay Fe-DMA, but not Fe-EDTA, could associate with plasma membrane vesicles (Fig. 2A), and not with liposomes (Fig. 4). This is in contradiction with a report by Mihashi et al. [37], showing that there is no binding capacity difference between plasmamembrane vesicles and liposomes. The following points could account for this discrepancy. We used a rapid filtration method allowing to determine a blank value (i.e., ^{55}Fe − DMA sticking onto the filter in absence of a lipidic phase). Mihashi et al. [37] used centrifugation through an oil phase, without giving any background value in their paper. Since vesicles passed through a lipid phase in their assay, this might change the amount of adsorbed Fe-DMA. High Fe-DMA association between pH 4 and 6 might be caused by coprecipitation of ^{55}Fe − DMA through the oil, so that an additional loosely bound Fe-DMA fraction would have been determined. In our case, such a loosely associated Fe-DMA

was removed by repeated washings. In addition, Mihashi et al. [37] determined ^{55}Fe − DMA association at different pHs relatively to the binding at pH 0.5. This ratio is questionable because Fe-DMA dissociates below pH 3.5 (von Wirèn, unpublished observation). Therefore, these authors expressed Fe-DMA association at higher pHs relative to free Fe^{3+} association, which has a totally different meaning than what we did.

The equilibrium of Fe-DMA association was reached after 60 min (Fig. 2A). However, the specific interaction between ^{55}Fe − DMA and root plasma membranes was insensitive: (i) to the osmotic potential of the assay medium, (ii) to the Brij-58 treatment of the vesicles (Fig. 5), (iii) to EDTA or excess free DMA challenge (Figs. 6 and 7), and (iv) to a mutation affecting iron-phytosiderophore transport (Fig. 7B). This indicates that this interaction cannot be explained by a vesicle-filling process. Data presented here favour, therefore, the hypothesis that Fe-DMA associates specifically to a protein moiety at the surface of plasma membrane vesicles, and is not transported inside these vesicles. Likewise, adsorption kinetics of iron to reticulocyte endocytic vesicles showed a similar dependence on substrate concentration and on excess of free ligands [38].

The Fe-DMA association to vesicles follows very slow kinetics, and a model fitting these data is proposed. This model was validated by its ability to describe quantitatively both Fe-DMA association as a function of time and of substrate concentration. A prediction of the model is that association of Fe-DMA to plasma membranes must overcome a high activation energy barrier, and this has been verified experimentally since the values of the association rate constant obtained at three different temperatures gave a linear Arrhenius plot corresponding to a high activation energy of 64 kJ mol^{-1} (Fig. 8). The reason for such a high activation energy barrier is unclear. Two possibilities have to be considered. First, if ^{55}Fe labelling measured in vitro is due to non-specific binding, a high energy barrier could prevent this interaction in vivo. As a result, Fe-DMA concentration in the apoplasm would increase, favouring the specific interaction between Fe-DMA and its carrier. Second, and in contrast, if ^{55}Fe labelling measured in vitro corresponds to a specific interaction with one component of the transporter, a strong driving force

would be necessary to overcome the high energy barrier, in order to achieve the transport.

Advances to understand the molecular structure-function relationship of Fe-DMA transport will largely depend on the availability of in vitro functional assays. Future attempts to set up such assays will have to take into account the slow association process of Fe-DMA to root palsmalemma vesicles.

Acknowledgements

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