Iron release and uptake by plant ferritin: effects of pH, reduction and chelation

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Ferritins are iron-storage proteins that accumulate in plastids during seed formation, and also in leaves during senescence or iron overload. Iron release from ferritins occurs during growth of seedlings and greening of plastids. Depending on the concentration of the reducing agent ascorbate, either an overall iron release or uptake by ferritins from iron(III) citrate may occur. We have designed methods to measure these simultaneous and independent uptake and release fluxes. Each individual step of the exchange was studied using different iron chelates and an excess of ligand. It is shown that: (i) the chelated form of iron,

and not ionic Fe^{3+} , is the substrate for iron reduction, which controls the subsequent uptake by ferritin; (ii) iron uptake by ferritins is faster at pH 8.4 than at pH 7 or 6 and is inhibited by an excess of strongly binding free ligands; and (iii) strongly binding free ligands are inhibitory during iron release by ascorbate. When reactions are allowed to proceed simultaneously, the iron chelating power is shown to be a key factor in the overall exchange. The interactions of iron chelating power, reducing capacity and pH are discussed with regard to their influence on the biochemical mobilization of iron.

INTRODUCTION

Iron is an essential element for living organisms, because of its importance in major biological mechanisms such as electron transport, nitrogen fixation and DNA synthesis. In plants, iron deficiency causes a series of biochemical lesions which mainly affect the photosynthetic apparatus, leading to the syndrome of chlorosis. During the growth of seedlings, as well as during the greening of leaves, the mobilization of iron from its storage forms is decisive. Chlorosis may occur in plants which contain large amounts of iron, indicating that iron mobilization from storage forms could be a rate-limiting factor to its biochemical use (De Kock, 1986). The reducing power of its environment may be involved in the mobilization of stored iron.

Plant ferritins are iron-storage proteins that are located in plastids and are especially abundant in seeds. They are used as an iron source during germination (Lobreaux and Briat, 1991). Their function is to buffer the ferrous ion concentration in their environment. Ferritin prevents direct deposition of ferric hydroxide colloids (especially at pH > 6) by oxidizing Fe^{2+} and storing iron within the mineral core. The oxidation is performed by the ferroxidase function of the protein moiety, but is also affected by the catalytic influence of the mineral core; therefore both components (protein and mineral core) influence iron uptake. A specific site for ferroxidase activity has been proposed within the H subunit of animal ferritin (Andrews et al., 1992). Iron may be held in the reaction centre by three amino acids, namely His-65, Glu-62 and Glu-27. L subunits do not carry this site. Amino acids involved in the formation of the ferroxidase centre of plant ferritins are conserved, except in pea where His-62 is found instead of Glu-62 (Andrews et al., 1992). Fe^{2+} is oxidized to Fe^{3+} by oxygen, which is especially abundant in chloroplasts, and both forms of iron may react with reduced forms of O_2 to produce deleterious hydroxyl radicals (Halliwell, 1987). It seems that ferritin, via its capacity to withdraw hundreds

of iron atoms within plastids, participates in the detoxification of iron more efficiently than it contributes to iron toxicity as a direct Fenton reagent (O'Connell et al., 1985; Thomas et al., 1985; Biemond et al., 1986; Grady et al., 1989). Moreover, when free oxygen radicals are generated by iron exchange, plant ferritin behaves as a radical scavenger, leading to cleavage in the N-terminal part of its subunit (Laulhere et al., 1989). Therefore plant ferritins probably participate in protection against oxidative stress, which could be highly deleterious in the photosynthetic cellular compartment.

Very few investigation have been carried out on iron exchange in plant ferritin. From analogies with animal ferritins and from a small amount of experimental data obtained with plant ferritins, we expect that the biochemical pathways shown in Figure 1 occur in plants. Iron uptake (step 2) has been observed qualitatively (Sczekan and Joshi, 1987). We have shown that reducing agents such as ascorbate or light allow the incorporation of iron into plant ferritins in the presence of oxygen (steps 1 and 2). Reducing agents are also known to induce the release of iron from plant ferritin (step 3). The overall direction of the iron exchange was shown to depend on the amount of reducing agent: a concentration of up to 2.5 mM ascorbate resulted in uptake, while release occurred at higher concentrations (Laulhere et al., 1990). Our working hypothesis is that both uptake and release occur simultaneously, with the direction of the net exchange being determined by the relative kinetics of steps 1+2 versus those of step 3. Iron uptake results from the ferroxidase activity of the protein moiety and the crystal growth of the mineral core, and release results from reduction of the mineral core. If both fluxes are simultaneous, the apparent loading of the ferritin will correspond to the difference between the real exchanges.

A major difficulty in the quantitative study of iron exchange is the instability of ionic iron at neutral pH values. When not chelated by a high-affinity organic compound, Fe^{3+} undergoes precipitation by reaction with OH^- or with mineral ions. The

Abbreviation used: NTA, nitrilotriacetate.

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Figure 1 Schematic representation of the hypothetical reactions involved in iron exchange between plant ferritin and ferric chelates

Step 1 is iron reduction from ferric chelates. This step could be either direct (1c) or indirect, involving, firstly, an equilibrium dissociation of iron complexes (1a) followed by reduction of free ferric iron (1b). Step 2 indicates the uptake activity of ferritin, which catalyses oxidation of Fe^{2+} by oxygen and incorporation of iron into the mineral core. Step 3 corresponds to the reduction of Fe^{3+} in the mineral core by ascorbate and the release of Fe^{2+} . Step 4 illustrates chelation of Fe^{2+} which, when it occurs, inhibits the uptake activity; notice that this step was studied *in vitro* by using Fe^{2+} ligands which do not exist naturally in plants (ferrozine and o-phenantroline). DHA, dehydroascorbate; sal., salicylate.

main natural transport form of iron in xylem sap is ferric citrate (Cataldo et al., 1988; Maas et al., 1988). However, it is not known whether citrate is a direct partner for iron exchange with ferritin in the plastids. As an unsaturated ferric chelate, citrate buffers free ionic iron in solution. The concentration of free Fe^{3+} in equilibrium with complexed forms changes according to the ratio of free ligand to chelated Fe^{3+} . If the ionic form Fe^{3+} is a transitory intermediate in an exchange, the amount of substrate involved in this exchange will be dependent on the amount of free ligand. In most biochemical exchanges of iron between molecules, it is not known whether exchange occurs directly between ligands or whether a dissociation is necessary for the exchange. The use of citrate to give various levels of chelation could provide information on the influence of chelation in iron exchanges with ferritins. In this work, the range of chelating conditions will be extended by the use of weaker and stronger ligands than citrate.

Another important condition that affects iron availability is pH, which also controls pFe^{3+} through the OH^- concentration. An increase of 1 pH unit results in a decrease in the equilibrium concentration of free Fe^{3+} by a factor of 10^{-3} (Chaberek and Martell, 1959). The pH is known to vary at least between pH 5 and pH 8 in plastids (Alberts et al., 1983), the subcellular compartment in which ferritin is located in plants.

The aim of this work was to study iron fluxes between citrate and storage molecules, under conditions which are likely to occur *in vivo* in the plastids. Therefore we used physiological iron transporters and a natural reducing agent, ascorbate, within concentration ranges expected to occur *in vivo* (Law et al., 1983). We chose to work with native iron-loaded ferritin in the presence of oxygen, which is naturally present within chloroplasts. pH values and ligand/Fe ratios were selected to fit known physiological values.

MATERIALS AND METHODS

Iron exchange experiments

The absorbance of the mineral core per iron atom at 430 nm is 4.36 times lower for pea ferritin than for horse spleen ferritin, probably because of the high phosphorus content of the former (Wade et al., 1992). Therefore the molar absorption coefficient of

plant ferritin mineral core is not established, and we had to develop non-spectrophotometric methods for the quantification of iron loading.

A typical iron exchange experiment contained: (i) 15 μl of a ligand [citrate, nitrilotriacetate (NTA), EDTA or salicylic acid at a concentration varying from 1.2 to 100 mM according to the desired pFe], (ii) 15 μl of exogenous iron (0.1 mM $\text{Fe}_2(\text{SO}_4)_3$ in 10 mM HCl, made to 5 μCi using $^{59}\text{FeCl}_3$; Amersham, (iii) 50 μl of a H^+ buffer adjusted to the desired pH by mixing different volumes of 200 mM Tris and 200 mM maleic acid with 200 mM NaOH (final concentration 66 mM), (iv) 15 μl of pure pea seed ferritin (0.42 mg/ml) purified as described previously (Laulhere et al., 1990), containing 1800 Fe atoms per molecule [in the experiment of Table 1, pea and horse spleen ferritin concentrations (Boehringer) (1480 and 1460 Fe atoms per molecule respectively) were 15 $\mu\text{g}/\text{ml}$], (v) 15 μl of fresh ascorbate solution to obtain a final concentration of 1 or 10 mM, and (vi) water, to give a final volume of 150 μl .

Exchange occurs at 25 $^{\circ}\text{C}$, and was stopped by electrophoresis as described elsewhere (Laulhere et al., 1990), or by addition of 15 μl of 0.5% (w/v) ferrozine.

Iron loading measurements using $^{59}\text{Fe}^{2+}$

When Fe^{2+} was used directly as the substrate in uptake experiments, strict anaerobic conditions were maintained for the preparation of the substrate, by bubbling vessels, syringes and substrate solutions with argon for at least 1 h. Then $^{59}\text{Fe}^{3+}$ (IFS-1, Amersham) was mixed with 9 times its quantity of FeSO_4 , resulting in a mixture ($\text{Fe}^{2+}/\text{Fe}^{3+} = 9$). Since there is a rapid exchange of electrons between iron isotopes of both valencies, the specific radioactivity of total iron is reduced in the same ratio. The reaction was started by mixing 70 μl of anoxic substrate solution with 80 μl of air-saturated reagents (final volume 150 μl ; 205 mm² interface with air) containing plant ferritin (0.42 mg/ml), H^+ buffers and ligands, as described above, but without exogenous Fe^{3+} . The reaction was stopped by the addition of excess ferrozine. The ferritin $^{59}\text{Fe}^{2+}$ content was determined after electrophoretic purification as described elsewhere (Laulhere et al., 1990).

Iron release measurements

After ascorbate treatment, exogenous Fe^{2+} was measured by the absorbance of Fe^{2+} /ferrozine. Samples of 15 μl of the exchange mixture were pipetted into 100 μl of 0.1% ferrozine solution, in a 96-well EIA plate (Costar, Cambridge, MA, U.S.A.). For short kinetic experiments, samples were mixed and photographed within 5 s. Each plate contained several densitometric calibration standards in 12 wells. With lighting provided by an even transilluminator, the photograph was taken through a monochromatic filter (λ_{min} , 560 nm). The films (Polaroid 665) were scanned using a Hoefer densitometric recorder. Only density values which fell within the linear part of the calibration curve were retained. Alternatively, when only one reading of absorbance at 562 nm $\cdot$ s⁻¹ was required, a Uniscan II spectrophotometer (Labsystems, Paris, France) was used.

Specific radioactivity of ferritin iron

A 15 μl sample of exchange mixture was made to 5% glycerol and loaded on 2% agarose/TBE gels (Laulhere et al., 1990) in plastic tubes (vertical racks of 24 tubes; 12 mm² section $\times$ 80 mm long). After electrophoresis at 100 V, gels were removed from the tubes and the region between 30 and 70% of the migration

distance of Bromophenol Blue was cut out and immersed in 1 ml of solution containing 50 μ l of thioglycolic acid, 100 μ l of 3 M sodium acetate, pH 6, and 30 μ l of 1% ferrozine. After up to 3 days of agitation in the cold, the A_{562} and radioactivity were measured; when stable, these values were recorded.

Simultaneous measurement of kinetics of iron uptake and iron release

The simultaneous and independent measurement of the rates of iron uptake and release was based on differential labelling of the initially internal and external iron. The exogenous iron citrate was labelled. By choosing a high specific radioactivity for exogenous ^{59}Fe -labelled iron citrate, then uptake would not lead to spectrophotometrically detectable increases in ferritin iron content. Consequently, under these experimental conditions, the spectrophotometric estimations of ferritin iron cannot reflect an uptake but rather a release. The increase in radioactivity of ferritin iron reflects uptake, ferritin iron estimations indicate release, and the specific radioactivity reflects the result of both processes.

RESULTS

Reduction of iron from iron chelates

The experiment shown in Figure 2 was designed to discover the actual substrate involved in the reduction by ascorbate (step 1 in Figure 1). To eliminate step 3, ferritin was omitted. Removal and spectrophotometric quantification of Fe^{2+} was made possible by the addition of ferrozine. Different iron(III) sources were used, differing by the stability constant of the ligand and by the ligand/iron concentration ratio. Ligands and ligand/iron ratios were chosen to produce Fe^{3+} buffers which liberate Fe^{3+} over a wide range of concentrations, while the soluble iron concentration is kept constant. The reduction of Fe^{3+} to Fe^{2+} appears to be faster at low pH, and is highly dependent on the nature of the ligand, in the following order of efficiency for reduction by ascorbate: $\text{NTA} \gg \text{citrate} \gg \text{EDTA}$. The results with salicylate are not easily interpreted: at high pH its colour changes, and we cannot claim that iron remains in the same chelated form. The reduction of NTA-Fe and salicylate-Fe is strongly limited at high pH, but that of citrate-Fe is less sensitive to OH^- concentration. Taken together, these results indicate that iron chelates (step 1c in Figure 1), and not ionic ferric iron (steps 1a + 1b in Figure 1), are the substrates for iron reduction, as the reaction kinetics: (i) depended on the nature of the ligand, (ii) were not inhibited by an excess of free ligand, and (iii) did not follow the expected kinetics of dissociation from the iron complexes. The use of ferrozine, which is able to shift the equilibria, could have accelerated the reduction of iron as presented in Figure 2. Nevertheless, the rates would still be influenced by various ligands in the presence and absence of ferrozine. Therefore our conclusion that iron chelates are the substrates for reduction is still valid.

Iron uptake and release from pea seed ferritin

Iron uptake was measured separately from simultaneous iron release (steps 1 and 3 in Figure 1) by adding $^{59}\text{Fe}^{2+}$, as a direct substrate, in the absence of a reducing agent. Autoradiograms of the protein gels have shown that all of the ^{59}Fe migrated with the ferritin protein, and that no label was observed at the level of the wells, indicating that no precipitated iron hydroxide was formed

Figure 2 Kinetics of iron reduction from ferric chelates in the presence of ferrozine

Iron (Fe) concentration was constant at 10^{-4} , and ligand (L) concentrations were variable in order to obtain the following L/Fe ratios: salicylate, 1.2 and 10; citrate, 1.2, 2, 10 and 100; NTA, 2, 10 and 20; EDTA, 1.2, 2 and 10. The reaction was allowed to proceed in the presence of ferrozine for 2, 5, 11 and 20 min, at three pH values (6, 7 and 8.4), using 1 mM ascorbate as reductant (a similar experiment using 10 mM ascorbate was also performed). Instant densitometry of samples was recorded by photography of 562 nm, and quantified by scanning the negatives as described in the Materials and methods section.

Figure 3 Influence of free ligands and of pH on iron uptake by pea seed ferritin

The exchange reaction was carried out for 5 min at 25 $^{\circ}\text{C}$ at three different pH values (6, 7 and 8.4). The ^{59}Fe concentration was 0.42 mM $^{59}\text{Fe}^{2+}$ and pea seed ferritin was present at 0.43 mg/ml. Ligand (L) concentrations as indicated in Figure 2. The incorporated radioactivity was measured after electrophoretic purification of ferritin using one-tenth of the sample.

Figure 4 Influence of pH and of free ligands on the kinetics of iron release from pea ferritin in the presence of 1 mM ascorbate and ferrozine

The absorbance at 562 nm of ferrozine-iron was used to monitor the reduction of iron from the mineral core. Measurements were done by direct spectrophotometry using a Uniskan II (Labsystem, Les Ulis, France). The ferritin concentration was 0.43 mg/ml, containing 1800 iron atoms/molecule. Two ascorbate concentrations were used (shown are results at 1 mM) at pH 6, 7 and 8.4.

in any sample (results not shown). As shown in Figure 3, the rates of iron uptake are much higher at pH 8.4 than at pH 7 or 6. The uptake activity is partially inhibited by unsaturated ligands (citrate, NTA, EDTA).

Iron release (step 3 in Figure 1) was measured independently of its uptake (step 2 in Figure 1) by the use of ferrozine during the reaction, allowing us to quantify the kinetics of the reduction. In absence of reducing agent, iron release was not measurable using ferrozine during the time scale of this experiment (Laulhere et al., 1990). The ligands used previously were added without exogenous iron to study their influence on the release of ferritin iron. As shown in Figure 4, external ligands can influence the reductive mobilization of iron from ferritins. EDTA is strongly inhibitory, particularly at high pH. At pH 8.4, the highest excess of citrate and EDTA (ligand/Fe ratios of 100 and 10) slow down the release of iron from ferritin. Using 10 mM ascorbate, the release is twice as slow at pH 8.4 than at pH 6 (results not shown).

Kinetics of iron uptake and release

In the absence of ferrozine the uptake reactions are not inhibited. When ascorbate is added to ferric citrate and ferritin, ferrous iron is produced from both molecules and can ultimately be

Figure 5 Kinetics of net uptake of iron from iron chelates by pea seed ferritin in the presence of (a) 1 mM ascorbate and (b) 10 mM ascorbate

The specific radioactivities of ferritin iron were measured after electrophoretic purification during the same experiment as in Figure 3. Ferritin iron was extracted from the gel by reduction in the presence of ferrozine. When stabilized, the absorbance of ferrous ferrozine (A_{562}) was measured and the radioactivity in ^{59}Fe was counted.

reincorporated into the ferritin core. Depending on the relative rates of these reactions, an apparent overall exchange results. The release and the uptake of iron, which contribute to the overall exchange, have been discriminated by using a different labelling for iron citrate and ferritin as described in the Materials and methods section.

Using 1 mM ascorbate, the final iron content of ferritins was not significantly changed at any pH. No iron release was observed. With 10 mM ascorbate, however, the amount of iron found in ferritin decreased with time at pH 6 and 7, but not at pH 8.4 (10% and 15% of total iron is released/hour at pH 7 and pH 6 respectively). At pH 6 and 7, the overall flux is, therefore, a release; the rate of release of iron is pH-dependent. Despite of net release, uptake of ^{59}Fe occurred simultaneously (Figure 5). The greatest iron uptake was found at pH 8.4 and using 10 mM ascorbate. The measurements were done on the same samples as those used for the release study.

In the presence of 1 mM ascorbate, the specific radioactivity of ferritin iron increased (Figure 5a). The pH did not greatly influence the specific radioactivity resulting from the iron exchange. Plateau values were comparable: specific radioactivities were stabilized in 1 h. In the presence of 10 mM ascorbate (Figure 5b), the initial rate of ^{59}Fe incorporation during the first 5 min was higher than with 1 mM ascorbate. After 1 h the specific radioactivity of ferritin iron reached a stable level and seemed to be pH-dependent (Figure 5b; see also the Discussion). Iron uptake and release occur simultaneously. With 1 mM

Figure 6 Kinetics of iron release by pea ferritin in the presence of 10 mM ascorbate, oxygen and free ligands

At the end of the incubation, ferrozine was added and the A_{562} was recorded immediately using a Uniscan II spectrophotometer. The ferritin concentration was 0.43 mg/ml, and three pH values (6, 7 and 8.4) were used. Various concentrations of ligands (L) were used in order to vary the L/Fe ratio as indicated.

ascorbate at any pH, and with 10 mM ascorbate at pH 8.4, no release occurred. In contrast, with 10 mM ascorbate at pH 6 or 7, iron release occurred leading to a decrease of the specific radioactivity of exogenous iron. This could explain the lower final specific radioactivity of ^{59}Fe in the presence of 10 mM ascorbate compared with 1 mM ascorbate.

Ascorbate-driven exchanges of ferritin iron between ferritins and various external ligands

The exchanges reflecting steps 1–3 in Figure 1 were measured in the same experiments after 3 h of incubation with 1 or 10 mM ascorbate in the presence of exogenous $^{59}\text{Fe}^{3+}$, without ferrozine. Uptake was studied using the same set of ligands and ligand/iron ratios as in Figures 2, 3 and 4. Addition of ferrozine after different periods of exchange allowed the release of ferrous iron produced during exchange periods to be monitored spectrophotometrically. The results are shown in Figure 6 for release in the presence of 10 mM ascorbate. No release was observed in the presence of 1 mM ascorbate (results not shown). In Figure 7, the specific radioactivity of ferritin iron, resulting from both uptake and release, was measured after treatment with 1 or 10 mM ascorbate under the same conditions as in Figure 6.

Using 10 mM ascorbate, the net iron flux was released at pH 6 or 7. This means that step 3 is faster than steps 2 + 1 (Figure 1). NTA favours iron release, irrespective of the pH. Uptake, indicated by the labelling of ferritin iron in Figure 7, occurred

Figure 7 Specific radioactivity of pea seed ferritin iron after exchange with ^{59}Fe -citrate

(a) In the presence of 1 mM ascorbate; (b) in the presence of 10 mM ascorbate. The reaction proceeded for 3 h at three different pH values (6, 7 and 8.4) and at 25 °C in presence of oxygen and of various free ligands at various concentrations (in mM). Measurements were made after electrophoretic purification of ferritin and reduction of ferritin iron from the gel as described in the text. Abbreviation: Sal., salicylate.

Table 1 Influence of pH and ascorbate concentration on the release and uptake from horse spleen and pea seed ferritins

Each molecule of ferritin contained the same number of iron atoms per molecule (1450). Release is expressed as the percentage of iron remaining in the mineral core of ferritins after 60 min of ascorbate treatment at 25 °C; 100% corresponds to the iron content of the ferritin before treatment with the reducing agent (i.e. 1450 iron atoms per ferritin molecule = 100%). Iron uptake is expressed as the specific radioactivity (c.p.m./60 nM Fe) of mineral core iron after ascorbate treatment for 7 min at 25 °C in the presence of exogenous ^{59}Fe citrate (see the Materials and methods section). Measurements were performed as described in the Materials and methods section. After exchange, ferritins were electrophoretically purified, and the iron remaining in their mineral cores was released by reduction and quantified by spectrophotometry at 562 nm in the presence of ferrozine and by counting the ^{59}Fe incorporated.

	[Ascorbate] (mM)	Pea seed ferritin		Horse spleen ferritin	
		pH 6	pH 8.4	pH 6	pH 8.4
Release	1	88	91	102	101
(%)	10	34	69	97	99
Uptake	1	111 090	112 230	33 290	70 110
(c.p.m./60 nm Fe)	10	45 570	78 050	17 370	29 150

under all tested conditions. After an exchange of 3 h, uptake seemed independent of ascorbate concentration. It was not inhibited by high pH, but was inhibited by strong ligands at high ligand/iron ratios. In the case of net uptake (10 mM ascorbate at pH 8.4 and 1 mM ascorbate at all pH values), the highest specific

radioactivities occurred for lower ligand/Fe ratios and for the lowest stability constants of the ligand towards iron(III). When release prevailed over uptake (10 mM ascorbate at pH 6 or 7), the specific radioactivities tended to equilibrate by a rapid exchange between external and internal iron.

As a first attempt to compare the iron-exchange properties of plant and animal ferritins, we have compared the simultaneous uptake and release capacities of horse spleen ferritin and pea seed ferritin with two ascorbate concentrations and at two pH values. Both ferritins had the same iron content (1500 Fe atoms per molecule). Although less active, horse spleen ferritin is able to take up iron from iron citrate in the presence of ascorbate, but under the same conditions (60 min incubation in the presence of oxygen and the absence of ferrozine), no net release of iron was observed, even at pH 6 using 10 mM ascorbate (Table 1).

DISCUSSION

In this paper it is shown that iron citrate and pea seed ferritin, both carrying ferric iron, are able to exchange iron *in vitro* in the presence of ascorbate. Iron exchanges had not been previously studied *in vitro* using methods allowing the independent measurement of uptake and release. Kinetic studies (Figure 5) show that the uptake of iron into pea seed ferritin and its release are simultaneous, the overall direction of the exchange being determined by the amount of ascorbate and the pH (Figure 6). Iron uptake is rapid over a short period of time (< 1 h), while iron release continues for several hours.

A low pH facilitates Fe^{3+} reduction from iron chelates as well as from ferritins (Figures 2 and 4). In contrast, the uptake of Fe^{2+} is less efficient at low pH (Figure 3). When the substrate is iron(III) citrate, the specific radioactivity of ferritin iron presented in Figure 5(b) suggests that the loading is pH-dependent, i.e. slower at low pH. If the specific radioactivity of external iron was kept constant, these different levels would reflect different loadings, but at pH 6 or 7 simultaneous iron release is observed. The specific radioactivity of the exogenous iron is lowered by the release of unlabelled endogenous iron. Therefore the low specific radioactivity of ferritin iron at pH 6 or 7 does not mean that iron loading is less efficient, but that the sensitivity of its detection is progressively hampered by the lowered specific radioactivity of the external iron. pH-dependence of reduction during iron release has been shown with horse spleen ferritin (Funk et al., 1985).

Ligand/iron ratios have almost no influence on iron reduction, suggesting that an excess of free ligand does not compete for the substrate and that dissociation of iron complexes is not obligatory before reduction. Each chelate has its own kinetics of reduction by ascorbate. The order of efficiency in the isolated step 1 (Figure 1) is: $\text{NTA-Fe}^{3+} \gg \text{salicylate-Fe}^{3+} > \text{citrate-Fe}^{3+} \gg \text{EDTA-Fe}^{3+}$. This is not the order of stability nor of the effective stability constants for Fe^{3+} with these chelates (Chaberek and Martell, 1959). We conclude that iron complexes are directly reduced by ascorbate (step 1c in Figure 1) and are the substrates which release Fe^{2+} , allowing further uptake of iron by ferritins.

Iron uptake and release from ferritin are inhibited by an excess of free ligand (Figures 4 and 7). A similar inhibition of reductive iron release by EDTA from horse spleen ferritin has already been reported (Boyer et al., 1988). We do not know if this inhibition is due to an affinity for ferric or ferrous iron. The expected chelating power for iron(III) is roughly correlated with an inhibition of iron exchange (Figure 7). The stability constants for iron have been considered here to reflect affinities, but they are thermodynamic equilibrium constants and provide no information about the rate at which equilibria are established (see

Pitt and Martell, 1980). Nevertheless, we can generally conclude that strongly binding ligands decrease iron exchange with ferritins. On using citrate, the inhibitory effect is very dependent on the amount of free ligand.

The citrate and iron contents of xylem exudates have been extensively studied in relation to the iron status of the plant (Tiffin, 1966; Brown and Chaney, 1971; Clark et al., 1973; Tiffin and Chaney, 1973; Cataldo et al., 1988). It appears that low citrate/iron ratios are observed under conditions of abundant iron. Our results show that, *in vitro*, this ratio influences the filling of the storage compartment. However, it is not known whether citrate acts as the ultimate carrier bringing ferric iron to ferritins in plastids *in vivo*. Our experiments suggest that if another ferric ligand were involved, it would need to be more stable towards iron than is citrate, and therefore it would slow down further exchanges.

It is interesting to note that the critical concentration of ascorbate which alters the direction of the net exchange is within the concentration range found in chloroplasts (3.5–16 mM; Nakano and Asada, 1981; Law et al., 1983). The increase in reducing power during greening could explain iron release from ferritins for the construction of thylakoids. This would explain the absence of ferritins from mature leaves (Lobreaux and Briat, 1991). In the absence of data on plant ferritin turnover, it cannot be ruled out that ferritin degradation participates in the release of iron in addition to its reduction. Iron deficiency inhibits chloroplast differentiation, which in turn results in a lack of reducing power which is necessary to recycle iron from its ferric forms within plastids. A level of 2 or 3 mM ascorbate seems necessary to release iron from its storage forms. Thus photosynthesis and iron availability seem to undergo a reciprocal interdependence.

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