Photoreduction and incorporation of iron into ferritins

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Pea seed ferritin is able to incorporate ferrous iron *in vitro* into the mineral core. Fe^{2+} may be formed by reduction of exogenous Fe^{3+} with ascorbate or by photoreduction by ferritin and by ferric citrate. In our experimental conditions the bulk of the photoreduction is carried out by ferritin, which is able to photoreduce its endogenous iron. Citrate does not enhance the photoreduction capacity of ferritin, and exogenous ferric citrate improves the yield of the reaction by about 30 %. The mineral core of the ferritin is shown to photoreduce actively, and the protein shell does not participate directly in the photoreduction. Low light intensities and low concentration of reducing agents do not allow a release of iron from ferritins, but induce a 'redox mill' of photoreduction and simultaneous ferroxidase-mediated incorporation. High ascorbate concentrations induce the release of ferritin iron. These reactions are accompanied by the correlated occurrence of damage caused by radicals arising from Fenton reactions, leading to specific cleavages in the 28 kDa phytoferritin subunit. This damage caused by radicals occurs during the oxidative incorporation into the mineral core and is prevented by *o*-phenanthroline or by keeping the samples in the dark.

INTRODUCTION

It is now well established that the occurrence of iron in plants is not sufficient to prevent the syndrome of iron deficiency (Laulhère & Alquier-Bouffard, 1969; DeKock, 1986). Mossbauer spectroscopy on intact plants has shown that green leaves contain both ferrous and ferric iron, whereas chlorotic leaves contain ferric iron only (Machold & Schnorr, 1968). In some cases the addition of reducing agents has been sufficient to induce recovery from chlorosis (Bienfait & van der Mark, 1983). The 'efficiency' of iron is dependent on reductive biochemical steps that control its biochemical availability: for example, the reducing power of the roots (Brown *et al.*, 1961) controls iron nutrition. Iron nutrition, iron biochemical availability and reducing power are linked in a complex interdependent relationship. The major source of plant reducing power is photosynthesis, which is dependent on the availability of iron, at least during chloroplast biogenesis. Both chloroplast function and biogenesis are light-dependent. Hence light, reducing power and iron availability are interdependent through their involvement into photosynthesis.

A very direct relationship of light with iron chlorosis has been pointed out by Brown *et al.* (1979), who showed that in similar conditions of iron nutrition chlorosis may be avoided by a sufficient irradiation with blue and green light, and that this same light was able to photoreduce iron from ferric citrate. Ferric citrate has been identified as the main iron-transporter in stems (Tiffin, 1966).

Recovery from iron chlorosis may not result directly from the photoreduction of ferric citrate, but this photoreduction could participate in the recycling of trapped iron. The iron-transporter ferric citrate is a stable complex and cannot deliver much ionic iron or exchange iron with a weaker ligand (Laulhère *et al.*, 1972; Marx, 1989). Its reduction or photoreduction could liberate the metal for participation in further biochemical functions.

In the present paper we investigate *in vitro* the relationship between light, reducing power and iron exchange by the plant iron-storage protein ferritin.

Ferritins are the main form of iron storage in eukaryotes and some prokaryotes (Theil, 1987). They are hollow spheres built up from 24 protein subunits. They can accommodate several

thousand atoms of iron in the central mineral core of each molecule.

In plants phytoferritins have been characterized from a few plant species (Bienfait & van der Mark, 1983). All the information on iron exchange with plant ferritin results either from electron-microscopic studies or from studies on the iron load of ferritin in various physiological conditions *in vivo* (Seckbach, 1982; van der Mark *et al.*, 1982). *In vitro*, one study on plant ferritin (Szczekani & Joshi, 1987) has shown that a ferroxidase activity allows the uptake of ferrous iron. Unfortunately, this uptake is accompanied by an extensive degradation, and non-physiological iron solutions were used (millimolar concentrations of ferrous iron).

The incorporation of iron into pea seed ferritins *in vitro* is studied in the present work in conditions that are intentionally limited to those that are more likely to occur *in vivo* (i.e. pH and iron concentration ranges, nature and amount of iron transported, presence of O_2 and ascorbate, light intensities etc.).

MATERIAL AND METHODS

Purification of ferritins

Pea seed ferritins and apoferritins were extracted and purified as described previously (Laulhère *et al.*, 1989).

Analysis of ferritins

Protein was determined by the method of Bradford (1976), with BSA as standard. Iron concentration measurements and SDS/PAGE (15% acrylamide) of the subunits have been previously described (Laulhère *et al.*, 1989). Mineral core is prepared as follows. A 1 ml sample of ferritin solution in 10 mM-Tris/HCl buffer, pH 7.5, is incubated at 37 °C for 3 h with 0.25% (w/v) SDS and 0.25 mg of proteinase K/ml. The sample is deposited on a 50% (w/v) glycerol layer and centrifuged at 260 000 *g* for 30 min in a TLA 100.2 rotor (Beckman). The pellet is resuspended in 1 ml of water, centrifuged again and resuspended in the same volume of water.

Incorporation of ^{59}Fe into ferritins

The reagents are added in the following order: trisodium citrate, 50 nmol; $^{59}\text{FeCl}_3$ (1–10 μCi ; Amersham IFS-1),

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13.6 nmol, plus HCl, 1.3 μ mol; Tris base, 1.3 μ mol; Pipes buffer, pH 7.0, 300 nmol, plus phytoferritin, 230 pmol (1850 iron atoms per molecule), or horse spleen ferritin, 110 pmol. When *o*-phenanthroline (25 μ g) or ascorbate (0–1500 nmol) is added, distilled water is added to give a final volume of 50 μ l. After incubation (for various times in these conditions), ferritins are purified by agarose-gel electrophoresis in TBE buffer (89 mM-Tris base/89 mM-boric acid/2 mM-EDTA, pH 8), then, either (i) the ferritin bands are cut out and incubated in 3 ml of 50 mM-sodium acetate buffer, pH 6.0, containing 1% (v/v) thioglycolic acid and 0.02% *o*-phenanthroline until there is a stable absorbance of the ferrous *o*-phenanthroline complex, and absorbance at 510 nm and the radioactivity are quantified, or alternatively (ii) the gel is dried *in vacuo* and autoradiographed, the ferritin band is cut out and its radioactivity is measured in scintillation vials.

In some experiments, after the exchange ferritins are purified by chromatography on Sephadex G-50 mini-columns (0.5 cm $\times$ 9 cm). The ferritin sample (50 μ l) is eluted by SSC buffer (15 mM-NaCl/150 mM-sodium citrate buffer, pH 7.0); fractions containing ferritins are pooled, and iron and radioactivity are quantified to determine the specific radioactivity of the iron.

Quantum-yield measurements of photoreactions

Actinometric studies were done by an adaptation to low volumes of the method of Parker (1968). The actinometric solution is potassium ferrioxalate [$K_3Fe(C_2O_4)_3$]. It has kindly been prepared in the dark and recrystallized three times by Pr. Jardon (Department of Chemistry, Université Joseph Fourier). The concentration of potassium ferrioxalate was 12 mM in 50 mM- H_2SO_4 . The light source was two fluorescent tubes (FL 15S E-30 Toshiba Erythemal) with aluminium reflectors. The light was

filtered through a 7 cm-thick Pyrex cuvette containing a $CuSO_4$ solution, which absorbs heat and high-wavelength radiations. At 20 cm under the tubes a dark-box with six windows contained two 96-well microfiltration plates (Costar no. 3590). Each window was stoppered by a monochromatic light filter (for transmission spectra see Fig. 4). Six compartments under each filter were separated by wells containing black ink. Light leaks were eliminated. Each compartment contains 12 wells of capacity 300 μ l. The volumes and array of potassium ferrioxalate solution and samples were identical (50 μ l). The photon flux was measured by the rate of photoreduction of the actinometric compound corrected for its quantum yield. When photoincorporation was measured, the ferritins were purified by chromatography on Sephadex G-50 minicolumns (see above). Photoreduction was monitored by the absorbance of the ferrous *o*-phenanthroline complex at 510 nm (0.02% added before exposure to light). The absorbance values of dark controls were subtracted to eliminate putative *o*-phenanthroline-driven reductions.

RESULTS

Uptake of exogenous iron in the presence or the absence of ascorbate

The choice of the iron source for iron uptake was important. A previous attempt to load phytoferritin with exogenous ferrous iron has shown that the addition of Fe^{2+} at pH 7 leads to a loss of 35–65% of the protein (Sczekan & Joshi, 1987). This and the instability of Fe^{2+} solutions lead us to a different choice in the chemical conditions for uptake studies *in vitro*. In previous work (Laulhère *et al.*, 1989) we established that damage to ferritin caused by radicals occurs when Fe^{2+} and Fe^{3+} are present

Fig. 1. Influence of ascorbate on the uptake of ^{59}Fe by plant ferritin

(a) Ferric citrate was incubated with various concentrations of ascorbate and phytoferritin at 25 °C for 17 h in natural light. Phytoferritin (PF) samples were then purified by agarose-gel electrophoresis, and the gel was dried and autoradiographed. Lane 1, $^{59}Fe^{3+}$ without citrate or ferritin; lanes 2–8, [^{59}Fe]ferric citrate with phytoferritin and the indicated concentrations of ascorbate. Lanes 9 and 10, without ascorbate. (b) Quantification of a similar experiment in the dark. Total iron was extracted from agarose-gel bands containing phytoferritins by reduction and *o*-phenanthroline chelation. Total iron content (●) was calculated from the absorbance at 510 nm, and ^{59}Fe incorporated (▲) was determined by counting the radioactivity of the same samples. Only high-specific-radioactivity ^{59}Fe was added to samples containing 1850 iron atoms/ferritin molecule.

together with dissolved O_2 . Experimental conditions were therefore chosen to minimize the Fenton reactions that cause these losses of protein. The conditions were chosen to be consistent with those expected to occur *in vivo*, and therefore chelated ferric iron was used. In plants Fe^{3+} ions are in equilibrium with the natural chelate ferric citrate. Conditions that buffer Fe^{3+} ions in similar ranges were used *in vitro* (Laulhère *et al.*, 1972). The incorporation of ferrous iron is studied by using a naturally occurring chloroplast reducing agent, namely ascorbate (Rabinowitch & Fridovich, 1983). All iron solutions were stable, at neutral pH, in the presence of O_2 .

Fig. 1(a) shows the incorporation of ^{59}Fe by ferritin in the presence of increasing amounts of ascorbate. ^{59}Fe is incorporated at ascorbate concentrations lower than 2.5 mM. However, at higher ascorbate concentrations no incorporation occurs, and Fig. 1(b) shows that the uptake of ^{59}Fe is not accompanied by a release of total iron. This uptake corresponds to ascorbate concentrations that allow the reduction of exogenous iron but do not allow release of the iron of the mineral core. After the uptake, treatment with *o*-phenanthroline, EDTA or desferrioxamine B before and during electrophoresis does not allow a significant release of ^{59}Fe , which suggests that it is incorporated into the mineral core after reoxidation by the ferroxidase activity of phytoferritin (Szczekan & Joshi, 1987). The constancy of iron contents in Fig. 1(b) can be explained by the high specific radioactivity of ^{59}Fe that has been added to samples containing 1850 atoms of iron per ferritin molecule. This means that the incorporated iron does not significantly change the overall total iron content.

Photoincorporation of iron into phytoferritins

The clear uptake in the control without ascorbate (Fig. 1a, lanes 9 and 10) was either a direct uptake of Fe^{3+} by ferritins, as observed at a low rate in animal ferritins (Treffry & Harrison, 1979), or an Fe^{2+} uptake after a putative endogenous reduction. Such a reduction was suspected to be due to a photoreduction of ferric citrate (Brown *et al.*, 1979). Experiments were done to investigate a light-dependent reduction of exogenous iron. Fig. 2 shows the incorporation of ^{59}Fe into ferritin and its isolated components, i.e. apoferritin and the deproteinized mineral core. This experiment was done in the light, in the dark and in the presence of inhibitors of Fenton reactions.

In the dark no significant incorporation of exogenous iron

Fig. 2. Photoincorporation of iron from $[^{59}Fe]$ ferric citrate into phytoferritin and the influence of various inhibitors

Isolated mineral core (M), apoferritin (A) and ferritin (F) from pea seeds were illuminated for 3 h at 25 °C with $[^{59}Fe]$ ferric citrate at pH 7, 15 cm under diffused light of a daylight 16 W Sylvania fluorescent tube or placed in the dark. Certain samples contained 20 mM-*o*-phenanthroline (*o*-Ph), 4 mM-desferrioxamine (Dfoa) or 20 mM-phytate (Phy). Samples were then purified by agarose-gel electrophoresis, and the gel was dried and autoradiographed.

Fig. 3. Kinetic investigation of the photoincorporation of iron into phytoferritin

Experimental conditions are as described for Fig. 1(a). Abbreviation: *o*-Ph, *o*-phenanthroline. Quantification in (b) was performed by direct counting of the radioactivity of the ferritin bands in the dried agarose gel. A count of 10^6 c.p.m. corresponds to 35 ng of iron.

occurs into ferritin or into apoferritin or the isolated mineral core. Chelated ferric iron is also not significantly incorporated in the dark. However, in the light Fe^{3+} is incorporated into ferritin, except when inhibitors are present. ^{59}Fe is incorporated to a much smaller extent into the isolated mineral core or apoferritin.

When EDTA, desferrioxamine B or *o*-phenanthroline is added after exposure to light or during the electrophoresis, these chelating agents cannot remove the label from ferritin over a period of 17 h (result not shown). The inhibitors of the photoincorporation are able to chelate ferrous iron (*o*-phenanthroline) or ferric iron (desferrioxamine; Cohen, 1987) or to use up O_2 (phytate; Graf & Empson, 1987). They are also inhibitors of Fenton reactions by preventing further redox reactions (Winterbourn, 1987). The lack of incorporation into apoferritin (Fig. 2, second lane) suggests that a previously nucleated mineral core is necessary for the incorporation. The relatively slow incorporation into the isolated mineral core (Fig. 2, first lane) shows that the protein may accelerate the uptake either in the reducing step or by its ferroxidase activity. Another effect of the protein shell may be to make cores more accessible by preventing their aggregation.

Fig. 3 shows the kinetics of the photoincorporation. It also shows that *o*-phenanthroline inhibits both ascorbate-dependent uptake and photoincorporation.

Studies of the photoreduction

The ability of *o*-phenanthroline to withdraw ferrous iron from the reagents can be used to isolate the first step of the photoincorporation, namely the photoreduction. In the presence of *o*-phenanthroline the incorporation of Fe^{2+} into ferritin or autoxidation by dissolved O_2 (which is spontaneous at neutral pH) are inhibited (see Fig. 1). *o*-Phenanthroline also allows the reduction of Fe^{3+} to be monitored by its absorbance at 510 nm, provided that dark controls are used to subtract light-independent reductions.

A quantitative analysis of the photoreduction of iron requires a precise control of light intensities along the spectrum of the active radiation. This quantification was done by a classical actinometric method of photochemistry, with the potassium ferrioxalate system developed by Parker (1968). This photoreaction allows a precise quantification of the photons received by the reagents under monochromatic filters in the

Fig. 4. Photochemical characteristics of the quantum-yield measurements

(a) Absorption spectra of phytoferritin, ferric citrate and ferrioxalate used as actinometric compound. (b) Transmittance spectra of monochromatic filters used in quantum-yield measurements.

reaction glassware. The actinometric reaction is also a photoreduction of Fe^{3+} . Its quantum yield (i.e. Fe^{2+} ions produced/photon) is very high and precisely known for each wavelength. The quantum yield of the unknown reaction can be determined precisely by reference to the amount of photons per Fe^{2+} ion produced in the actinometric reaction.

Fig. 4(a) shows the absorption spectra of the potassium ferrioxalate, the ferritin and $[\text{Fe}^{59}]\text{ferric citrate}$ that are used in the photoincorporation and the photoreduction. Fig. 4(b) shows the transmission spectra of the monochromatic filters used in this study.

The first question addressed was the definition of the photoreceptors of the photoreduction.

The ferritin solution alone, the ferritin+citrate and the ferritin+citrate+iron were tested for photoreduction (in the presence of *o*-phenanthroline) in the conditions of concentration that were used for photoincorporation (see the experiment illustrated in Fig. 2). The actinometric measurements were made using u.v.-rich fluorescent tubes under six monochromatic filters. Dark controls showed no reduction. Table 1 shows the yield of reduction and the quantum yield of the photoreduction for each filter and for each reagent mixture. It appears that: (i) ferritin alone is able to photoreduce its endogenous iron; (ii) citrate does

not enhance the photoreduction capacity of ferritin; (iii) exogenous ferric citrate improves the yield of the reaction by about 30%.

We conclude that the main photoreceptor of the photoreduction is ferritin. Exogenous ferric citrate is not the main source of Fe^{2+} in the photo-reduction of iron by ferritin. The contribution of ferric citrate in the photoreduction is higher in the wavelength range of the absorbance of ferric citrate (340 nm) than in the absorbance of ferritin (300–500 nm). In order to verify this point, we have measured the quantum yield of ferric citrate under four monochromatic lights. The results (Table 1) confirm that the photoreducing activity of ferric citrate is not restricted to the region of the major absorbance (300–400 nm), but is also active in higher wavelengths.

The quantum yield of ferric citrate reduction alone shows that exogenous ferric citrate is a possible source of Fe^{2+} , which can be further incorporated by oxidation into the mineral core of the ferritin. Nevertheless a comparison of the absorbance of ferritin and ferric citrate (Fig. 4) shows that, in the conditions of our experiment, in a mixture of both components the bulk of the light is absorbed by ferritin, leaving a very limited energy for the exogenous production of Fe^{2+} by ferric citrate.

As ferritin is the molecule responsible for the major photoreduction, the question of the site of the photoreduction is raised: is the mineral core alone capable of photoreduction and does the apoferritin participate by improving the yield of the photoreduction? A quantitative experiment on photoreduction was performed to answer this question. Table 1 shows that the mineral core alone photoreduces more actively than does ferritin having the same iron content. In the case of the mineral core, the dark controls were not negligible. The apoferritin does not seem to participate directly in the photoreduction.

Photoincorporation and damage caused by radicals and reduced forms of oxygen

The inhibitors of the photoincorporation used in Fig. 1 are also known as inhibitors of the Fenton cycle. The Fenton cycle is an oxidative process involving Fe^{2+} , Fe^{3+} , O_2 and the reduced forms of oxygen $\text{OH}^\cdot$, H_2O_2 and $\text{O}_2^{\cdot-}$. We have shown previously (Laulhère *et al.*, 1989) that, when reducing agents are added to ferritin in conditions that allow the release of iron, extensive and specific damage by radicals occurs to the protein shell. Radicals are responsible of specific cleavages around residue 21 at the N-terminal end of the 28 kDa subunit. In order to determine whether damage caused by radicals accompanies the photoincorporation, we have exposed ferritin to high and to low light intensities. In the presence of unfiltered u.v. light from two

Table 1. Quantum yields of the photoreactions

Reaction mixtures were buffered with 10 mM-Pipes buffer, pH 7.0. Wavelengths correspond to the maximum transmittance of the filters used. The quantum yield of photoincorporation is defined as number of Fe^{3+} ions incorporated/photon absorbed. The quantum yield of photoreduction is defined as number of Fe^{3+} ions reduced/photon absorbed. Abbreviation: N.D., not determined.

Wavelength (nm)	Photons absorbed (photons/min)	Quantum yield of photoincorporation by phytoferritin	Quantum yield of photoreduction by:				
			Phytoferritin + ferric citrate	Phytoferritin + citrate	Phytoferritin	Mineral core	Ferric citrate*
339	1.3×10^{14}	0.0091	0.0280	0.0172	0.0200	0.0230	0.0160
369	3.3×10^{14}	0.0057	0.0150	0.0108	0.0084	0.0174	0.0103
387	3.0×10^{14}	0.0047	0.0160	0.0098	0.0093	0.0194	N.D.
417	2.6×10^{14}	0.0030	0.0131	0.0098	0.0094	0.0129	0.0071
460	1.4×10^{14}	0.0031	0.0199	0.0134	0.0132	0.0104	0.0070

* Quantum yield measured in the absence of phytoferritin. The light received in its presence is much lower (see Fig. 4).

Fig. 5. Induction of damage by free radicals in the 28 kDa subunit of pea seed ferritin by photoreduction of endogenous iron

Phytoferritin was exposed to ambient light (lanes 5–7) or kept in the dark (lanes 2–4) at room temperature for 15 days in the presence of *o*-phenanthroline (lanes 3 and 6) or of phytate (lanes 4 and 7). The samples were then denatured and subjected to SDS/PAGE (15% polyacrylamide) before Coomassie Blue staining. Lane 1, molecular-mass markers; lane 8, pea seed ferritin stored at -20°C in the dark.

15 W u.v. fluorescent tubes (Toshiba FL15S-E-30) at 17 cm at room temperature the protein is destroyed in 17 h when incubated with exogenous ferric citrate (result not shown). On the other hand, if ferritin is left in low light (room light of the laboratory) for 15 days at room temperature without exogenous iron, the protein remains functional, but the subunits of the samples exposed to light are nicked (Fig. 5). No loss of protein is detectable. Darkness and *o*-phenanthroline inhibit this degradation completely, but phytate does not. This shows that Fe^{2+} is involved in the process, not during the photoreduction, which is not inhibited by *o*-phenanthroline, but later, i.e. during the oxidative incorporation into the mineral core. *o*-Phenanthroline inhibits this step and also the damage caused by radicals. It is surprising to observe that phytate does not prevent degradation, since it blocks iron incorporation (Fig. 2). A possible explanation could be that phytate acts only at the periphery of ferritin molecules. In Fig. 2, in the presence of ferric citrate, the target of phytate would be accessible, whereas in Fig. 5, without ferric citrate added, oxidoreduction of the mineral core would not be accessible to phytate.

DISCUSSION

Because of biochemical and immunological differences between plant and animal ferritins (Laulhère *et al.*, 1988), we should not accept without evidence that the properties of animal ferritin are valuable for plants. However, knowledge of the properties of other ferritins is a useful guide in this study. In a first approach to iron exchange *in vitro*, we avoid experimental conditions that cannot be found *in vivo* (such as high concentrations of ferrous iron, unchelated Fe^{3+} ions, anoxia or non-physiological pH values). On the contrary, the nature of the iron supply and the concentrations used in our experiments are likely to occur *in vivo*. These experimental conditions are also suitable to minimize external Fenton reactions. The loss of protein is generally negligible.

A direct Fe^{3+} uptake?

An incorporation of Fe^{3+} in the dark always takes place, but its rate is low, much lower than after reduction. The incorporation of Fe^{3+} in the presence of *o*-phenanthroline could correspond to a direct Fe^{3+} uptake, as described for horse spleen ferritin (Treffry & Harrison, 1979), but its rate is relatively low.

Reduction of exogenous and endogenous iron

The reduction of iron from exogenous sources and its subsequent uptake by ferritin have been described for animal ferritins *in vivo* (Bridges & Hoffman, 1986) and *in vitro* (Miller & Perkins, 1969) after treatment with ascorbate. As suggested for animals, reduction could be the necessary step for iron exchange between the transportation and the storage forms of iron in plants. It seems that the oxidative incorporation does not immediately follow the uptake of Fe^{2+} , since a transient pool of Fe^{2+} has been detected within animal ferritins (Rohrer *et al.*, 1987). Independently, the existence of a pool of about 200 molecules of ferric citrate in the periphery of the horse spleen molecule has been observed (Treffry & Harrison, 1979), among which 34 remain bound after extensive washings. Such a pool might be located in the periphery of the plant ferritin molecule, like the Fe^{2+} pool, which remains accessible to *o*-phenanthroline. If such pools exist in phytoferritins, they could represent a potential site of ascorbate or light-dependent reduction, different from the site of the oxidative uptake, which should be close to the mineral core.

As shown in Fig. 1, depending on the amounts of reducing agents an overall uptake or release of iron may occur. Ascorbate should first reduce the external or peripheral Fe^{3+} , then eventually the mineral core, for the higher doses only. At low concentrations, as in lanes 4–10 in Fig. 1(a) we might suspect that the reduction of exogenous iron is followed by a ferroxidase-mediated incorporation into the mineral core. For ascorbate concentrations higher than 2.5 mM, the ferroxidase activity could become of less importance and the reductive release could dominate (Fig. 1a, lanes 2 and 3). The reductive and oxidative steps, although simultaneous, seem to occur in separate sites, since the site of the oxidative incorporation is the mineral core, deeply hidden inside the protein.

The reductive step is not inhibited by *o*-phenanthroline. Ascorbate or light induces the accumulation of Fe^{2+} -*o*-phenanthroline complex, which prevents further oxidative incorporation and the correlated damage by radicals.

If not exported or chelated, the released Fe^{2+} induces a rapid breakdown of the ferritin (Laulhère *et al.*, 1989). *o*-Phenanthroline inhibits this breakdown (Fig. 5), withdrawing the ferrous iron that is necessary for the completion of the Fenton reactions. *o*-Phenanthroline also inhibits the ferroxidase activity and prevents the iron uptake (Fig. 3a). It has been suggested that *o*-phenanthroline not only monitors but also promotes iron reduction, and therefore is not a suitable agent for measuring iron reduction. This objection does not apply in our experiments, since it was used in comparison with dark or ascorbate-free controls.

Photoreduction

When ferritins are exposed to light, even in the absence of exogenous iron, photoreduction occurs. The resulting ferrous iron may feed the transient internal pool of Fe^{2+} (Rohrer *et al.*, 1987) and return to the mineral core by oxidative incorporation. This could be the mechanism of the photoincorporation when exogenous iron is available. This internal 'redox mill' occurs even without overall iron release. The photoreduction by the isolated mineral core shows that it is the site of the photoreduction and that Fe^{3+} is the target for the light. Evidence of photoreduction by synthetic ferrihydrite has been given by Finden *et al.* (1984). The marked differences in photoreduction rates by fresh ferrihydrite as compared with aged samples show that the most active species in photoreduction are transition compounds between Fe^{3+} ions and formed crystals. If a similar situation were to be found in the fine structure of phytoferritins, one could expect that the interface of the protein with the

mineral core carries the photoreduction site, the ferroxidative function, and eventually some electron-transport system, which could couple both functions.

The turnover of the protein could be faster in the light than in the dark (Fig. 5, lanes 2 and 6). The relationship between photoreduction and damage caused by radicals is indirect, since the photoreduction can occur in the presence of *o*-phenanthroline and without damage caused by radicals (Fig. 5, lane 6). We may conclude that damage caused by radicals is a secondary consequence of Fe^{2+} production by light: the $\text{Fe}^{2+}/\text{Fe}^{3+}$ mixture is sufficient to induce Fenton reactions and damage by radicals (Braugher *et al.*, 1986, 1987).

Phytoferritins and storage iron in the plant

The physiological significance of iron photoreduction in ferritin as well as in citrate might be important, particularly in the recycling ionic iron from stable ferric complexes. Ferric iron is strongly bound by stable complexes such as ferritins, ferric citrate etc. One can guess that the biochemical availability of iron is conditioned by its recycling to the benefit of less-stable forms such as ferrous complexes or weaker ferric ligands. Unstable ligands deliver more free ionic iron to their biochemical environment than to stable complexes. In the absence of light or reducing power, strong ferric ligands can be considered as sites for unavailable iron, which remains trapped. Light and reducing agents allow the production of Fe^{2+} , which is not bound by ferric ligands, and allow its transitory release into the free ionic iron pools.

Ferritins are not encountered in adult leaves (Briat *et al.*, 1989), except after an iron overload (van der Mark *et al.*, 1982; Seckbach, 1968). The lack of ferritin in green tissues is unexplained. Proplastids contain ferritins (Bienfait & van der Mark, 1982; Seckbach, 1982), whereas chloroplasts, which are the site of production of reducing power (including ascorbate) do not. During greening, light and ascorbate produced by photosynthesis should induce a release of iron from phytoferritins. This could explain why greening tissues contain degraded forms of ferritin (result not shown) and lose their ferritin iron.

We have obtained preliminary results showing that photoincorporation of iron is not restricted to phytoferritins. Horse spleen ferritin photoincorporates iron from exogenous ferric citrate with the same qualitative features, including inhibition by the same reagents. Horse spleen ferritin is also able to photoreduce iron of its mineral core. However, the conditions of photoreduction and of photoincorporation by horse spleen ferritin have not been optimized. Therefore it is difficult to make a comparison between animal and plant ferritins at this point.

In conclusion, we have shown that the mineral core of pea seed ferritin and ferric citrate can be photoreduced. In a second step, Fe^{2+} produced in this reaction is incorporated into the mineral

core by an oxidation step, which is accompanied by damage caused by radicals arising from Fenton reactions.

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