BioMetals 1995, **9**, 303–309

Dynamic equilibria in iron uptake and release by ferritin

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Received 10 June 1995; accepted for publication 17 October 1995

The function of ferritins is to store and release ferrous iron. During oxidative iron uptake, ferritin tends to lower Fe^{2+} concentration, thus competing with Fenton reactions and limiting hydroxy radical generation. When ferritin functions as a releasing iron agent, the oxidative damage is stimulated. The antioxidant versus pro-oxidant functions of ferritin are studied here in the presence of Fe^{2+}, oxygen and reducing agents. The Fe^{2+}-dependent radical damage is measured using supercoiled DNA as a target molecule. The relaxation of supercoiled DNA is quantitatively correlated to the concentration of exogenous Fe^{2+}, providing an indirect assay for free Fe^{2+}. After addition of ferrous iron to ferritin, Fe^{2+} is actively taken up and asymptotically reaches a stable concentration of 1–5 μM. Comparable equilibrium concentrations are found with plant or horse spleen ferritins, or their apoferritins. After addition of ascorbate, iron release is observed using ferrozine as an iron scavenger. Rates of iron release are dependent on ascorbate concentration. They are about 10 times larger with pea ferritin than with horse ferritin. In the absence of ferrozine, the reaction of ascorbate with ferritins produces a wave of radical damage; its amplitude increases with increased ascorbate concentrations with plant ferritin; the damage is weaker with horse ferritin and less dependent on ascorbate concentrations.

Keywords: ferritin, iron, Fenton reaction, radical damage

Introduction

Ferritin, an ubiquitous iron storage protein, is able to accomodate up to 4000 ferric iron atoms in its mineral core (Harrison & Lilley 1989). Ferrous iron is the precursor form of ferritin iron and its storage is the result of its oxidation by molecular oxygen. In addition, iron from ferritin is released in the Fe^{2+} form after reduction of the ferric ions within the protein (Harrison & Lilley 1989). Ferritins are found in the plastids of plants, in the cytoplasm of animal cells and in blood. Reducing agents are able to promote both the uptake and the release of ferritin iron *in vitro*. Using plant ferritin and ascorbate, the exchange has been shown to occur simultaneously in both directions (Laulhère & Briat 1993).

Too high levels of free ferrous iron are responsible for the generation of oxygen radicals from O_2 reduction. The hydroxyl radicals, for example, are highly harmful for almost any biological molecule (Crichton 1991). As a consequence, under conditions where ferritin functions in uptaking iron, radical damage is expected to be limited (Grady *et al.* 1989, Sun & Chasteen 1992). Ferritin thus behaves as an antioxidant. On the other hand, under conditions where ferritin functions in releasing iron, radical damage is stimulated. Ferritin behaves as a pro-oxidant. *In vivo*, both aspects have been observed: in vascular endothelium, 16 h after oxidative stress induced by haem and H_2O_2, a 10-fold increase in ferritin content has been observed which correlates with a cytoprotective effect (Balla *et al.* 1992). Nevertheless, a recent work has shown that when pro-oxidant conditions are induced by injections of phorone to rats, liver ferritin represents either a pro- or an anti-oxidant in a time-dependent manner (Cairo *et al.* 1995). It thus seems important to understand how one of these functions can predominate in reaction mixtures containing ferritin, iron, a reducing agent, an oxidant and a target for radical damage.

The delicate dynamic balance between the anti-oxidant and pro-oxidant properties of ferritins has not been investigated specifically *in vitro*. Here we study the effect of ferritin on H_2O_2-dependent damage to DNA under aerobic conditions in the presence of various concentrations of exogenous iron and of ascorbate (as a reducing agent).

Ascorbate was chosen since its role in iron metabolism

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has been emphasized: *in vitro*, in the presence of Fe^{2+} scavengers, which inhibit iron uptake, ascorbate is known to promote iron release from ferritins (Bienfait & Van Den Breil 1980, Boyer *et al.* 1988). In the absence of Fe^{2+} ligands, ascorbate allows a iron uptake from transferrin, which has been observed *in vitro* (Miller & Perkins 1969) as well as *in vivo* (Bridges & Hoffman 1986).

Damage to DNA can be quantitated from the relaxation of supercoiled DNA (Floyd 1981, Toyokuni & Sagripanti 1992). DNA is not expected to enter ferritin molecules and its relaxation is a proper probe for free ferrous iron. Moreover, the DNA relaxation assay may serve to study kinetically the balance between ferritin iron uptake and release, and how it is modified by changes in the medium (e.g. addition of Fe^{2+} or addition of ascorbate). Here we show that pea ferritin and horse spleen ferritin control the radical damage done to their environment by buffering external Fe^{2+} concentrations in the micromolar range.

It is remarkable that the concentration of free iron at equilibrium neither depends on the starting concentration of Fe^{2+} nor on the presence of ascorbate.

Materials and methods

All chemicals were analytical reagent grade. Ferrous sulphate heptahydrate was obtained from Aldrich (Saint Quentin, France). MES (2-(*N*-morpholino)ethanesulphonic acid) was purchased from Sigma (Saint Quentin, France).

Pea seed ferritin was purified as previously described (Laulhère *et al.* 1988). The isolated protein population contained 2000 Fe atoms per ferritin molecule. Horse spleen ferritin was purchased from Boehringer Mannheim (Meylans), it had 1800 Fe atoms per ferritin molecule. Apoprotein from pea and horse was obtained as previously described (Laulhère & Briat 1993) with thioglycolate salt, followed by ultrafiltration and dialysis against 0.1 M MES buffer, pH 6.5, during 24 h. Protein concentrations were determined by the Bradford method. Before all experiments, ferritins were treated with Chelex-100 (Sigma) under agitation during 5 min, centrifuged and used immediately. Iron was determined at 560 nm as the Fe^{2+}–ferrozine complex ($E = 28\,000\ M^{-1}\ cm^{-1}$), after reduction of ferritin iron. PUC 8 plasmic DNA was purified by equilibrium centrifugation on CsCl/ethidium bromide gradients.

Evaluation of Fe^{2+}-dependent radical damage to DNA

Radical damage done to supercoiled DNA, as a target molecule for hydroxy radicals, was produced by Fenton-type reactions. The quantification of the damage was done by measuring the transition from supercoiled to relaxed DNA. This change occurs with the first nick of one of the two strands of the plasmid (Toyokuni & Sagripanti 1992).

The reaction mixture (20 μl total volume) contained: (i) the reaction buffer (0.1 M MES, pH 6.5), (ii) 94 ng supercoiled PUC 8 plasmid, (iii) 1 mM H_2O_2 and (iv) iron as 1 μM ferritin or ferrous sulphate standard solutions. After incubation for various time periods (as stated in the corresponding figure legends) at room temperature and in the dark, the reaction was stopped with 4 mM ferrozine in 50% glycerol and 0.025% bromophenol blue (volume added: 5 μl).

Aliquots of 14 μl of the reaction mixture were then analysed by electrophoresis on 1% agarose gel in 90 mM Tris–borate/EDTA buffer, pH 8.0 for 45 min ($12\ V\ cm^{-1}$). After electrophoresis, the gel was stained with ethidium bromide ($0.5\ \mu g\ ml^{-1}$) for 12 h and the fluorescence of DNA bands was recorded using a CCD camera (Apligene 'Imager') on a UV transilluminator. The recorded image was analysed using the NIH computer program 'Image 1.44b11'. The area of each band in the fluoregram was measured and the percentage of supercoiled DNA relative to the total DNA in each lane was quantified. Blanks without iron and with ferrozine as a Fe^{2+} scavenger were used in controls.

Quantitative relationship between Fe^{2+} oxidation and radical damage

Fresh stock solutions of 10 mM ferrous sulphate heptahydrate were prepared in MilliQ Millipore water, previously saturated with argon for 24 h. Oxygen-free diluted solutions of Fe(II) (1 and 0.1 mM) were prepared by pipetting the ferrous stock solution through a layer of paraffin oil which covers the surface and by transferring the aqueous solutions with oxygen-free, gas-tight syringes. Pure Chelex-100 treated water, little volumes, argon-saturated solutions, extemporaneous preparation and quick gas-free transfers are required to obtain reproducible data; no quantitative control of residual oxygen in standard solutions is sensitive enough to be used.

To obtain the calibration plots, Fe(II) solutions in the range 0–40 μM were incubated with supercoiled DNA (94 ng) for 20 min in the dark.

Radical damage after Fe^{2+} or ascorbate addition to ferritins

For Fe(II) uptake experiments, ferritin was incubated with ferrous solutions in the dark, at several Fe^{2+}/protein ratios. To determine kinetically the remaining non-ferritin Fe(II), supercoiled DNA and H_2O_2 were added to aliquots of the reaction at various times. After 20 min, the mixture was used for DNA relaxation measurements as described above. Similarly, after reductive mobilization of iron from ferritin by ascorbate, exogenous Fe^{2+} was quantified by measuring the damage done to the supercoiled DNA during a 5 min incubation time.

Iron release measurements

Independent of radical damage measurements, iron release was also quantified after ascorbate treatment by a spectrophotometric method using ferrozine as an Fe^{2+} chelator. The absorbance of the Fe^{2+}–ferrozine complex in 0.1 M MES buffer, pH 6.5, 1 μM ferritin, 4 mM ferrozine was determined at 560 nm using an Uniscan (II) spectrophotometer (Labsystems, Paris, France).

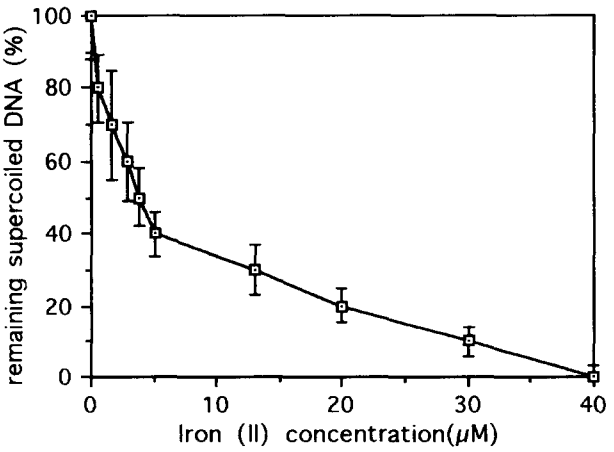

Figure 1. Standard curve of supercoiled DNA relaxation as a function of Fe(II) concentration. The points on the curve are mean values of remaining percentages of supercoiled DNA from five independent experiments. Conditions: 94 ng supercoiled DNA; 0–40 μM ferrous sulphate; 1 mM H_2O_2; 0.1 M buffer, pH 6.5; 20 min incubation time in the dark.

Results

Quantitative correlation between Fe^{2+} concentration and relaxation of supercoiled DNA

A sensitive assay for ferrous iron in neutral aqueous solutions was set up. This was based on the ability of Fe^{2+} to rapidly generate, in the presence of H_2O_2, oxygen radicals that transform the supercoiled forms of a plasmid DNA into a relaxed form. The reaction yield was determined after separation of both forms by gel electrophoresis. As shown in Figure 1, in the 10–40 μM iron concentration range, and with 1 mM H_2O_2, a sensitive and reproducible correlation between Fe^{2+} concentration and radical damage to DNA was obtained, with the damage to DNA being iron concentration dependent. Only in the 1–5 μM range was the uncertainty of the assay significant (± 1 μM). Such a standard curve is much more sensitive than colorimetric measurements. It will serve for assaying extraferritin Fe^{2+}, specifically, in ferritin-containing aqueous solutions. Fe^{3+} was unable to transform supercoiled DNA to relaxed DNA over the same concentration range.

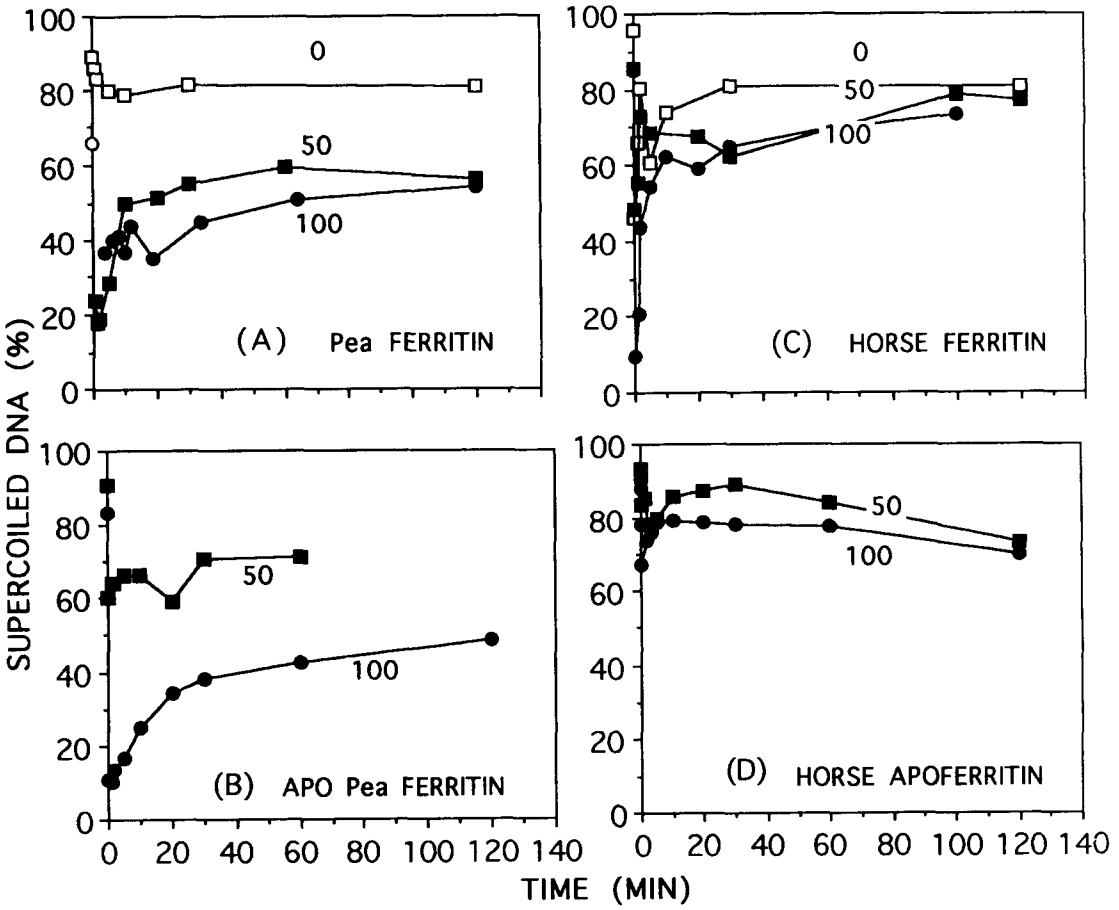

Figure 2. Radical damage done by external Fe^{2+} to supercoiled DNA in ferritin solutions. Ferritins are first treated with Chelex-100 resin for 5 min; then 1 μM ferritin solutions receive 0 (□), 50 (■) or 100 μM (●) Fe^{2+}, in 0.1 M MES buffer, pH 6.5. After a time period of iron exchange (indicated in abcissa), 94 ng supercoiled DNA and 1 mM H_2O_2 are added to the reaction mixture. Radical damage is allowed for 20 min, then stopped with 4 mM ferrozine and the remaining percentage of supercoiled DNA is quantified as in Materials and methods. The white circle (**A**) corresponds to native ferritin, before Chelex treatment. Dark squares and circles (**B**) correspond to pea apoferritin before iron addition. This type of experiment has been repeated three times.

Changes in external Fe^{2+} concentration during the addition of iron to ferritin

When pure pea seed ferritin 1 μM ferritin, 2 mM iron was incubated with supercoiled DNA, 25% of the DNA was relaxed after 20 min reaction, indicating that free Fe^{2+} was spontaneously released from that ferritin preparation, at a total concentration of 3 μM, which is about 10^{-3} times the ferritin iron concentrations. Part of that iron can be eliminated by Chelex treatment. Actually, when pea ferritin, treated with Chelex-100 in the dark, was incubated with DNA, only 10% of the DNA was relaxed after 20 min reaction.

The same Chelexed ferritin was allowed to stand in buffer for a period of time in the absence of DNA. At various time intervals, DNA and H_2O_2 were added to assay for the presence of free Fe^{2+}. As shown in Figure 2(A), relaxation of DNA increased with time and reached a limit value at about 20%. Such a limit was obtained after 10 min and was unchanged after 2 h reaction.

When the Chelexed protein (1 μM) was supplemented with 50 or 100 μM Fe(II), the time-dependent variation of the Fe^{2+} concentration could be determined by assaying for DNA relaxation by the reaction mixture at various time intervals. As shown in Figure 2(A), the damage to DNA decreased with incubation time, reflecting the time-dependent uptake of active Fe^{2+} and its conversion to inactive Fe^{3+} by ferritin.

The oxidation was fast compared with the ferritin-free control. As determined from the standard curve (Figure 1), only about 5 μM Fe^{2+} was still present after 10 min incubation. However, the Fe^{2+} concentration did not reach 0 μM but stabilized to about 3 μM, whatever the initial Fe^{2+}/protein ratio (50 or 100) was.

Figure 2(B) shows the results of a parallel experiment with pea apoferritin. Comparison with Figure 2(A) shows that the final stable concentration of external ferrous iron, during incubation of ferritin with Fe^{2+}, is not dependent on the presence of an iron core in the protein. Moreover, the kinetics of Fe^{2+} oxidation with the apoferritin (Figure 2B) are comparable with those obtained with ferritin (Figure 2A)—most of the added Fe^{2+} being oxidized after the first 10 min.

When the same experiment was carried out with horse ferritin and horse apoferritin (Figure 2C and D), basically the same results were obtained. It may be seen, however, that the initial kinetics of iron uptake is significantly faster for the animal ferritin than for the plant ferritin.

Changes in external free Fe^{2+} concentration during the addition of ascorbate

Ascorbic acid, a natural reducing agent, is known to promote ferritin iron release *in vitro*. The kinetics of iron release in the presence of ascorbate (5–500 μM) was measured spectrophotometrically. Released Fe^{2+} is trapped by ferrozine, a very efficient Fe^{2+} chelator and inhibitor of iron uptake reactions, and the concentration of the resulting stable ferrous complex is quantified from its absorbance at 560 nm

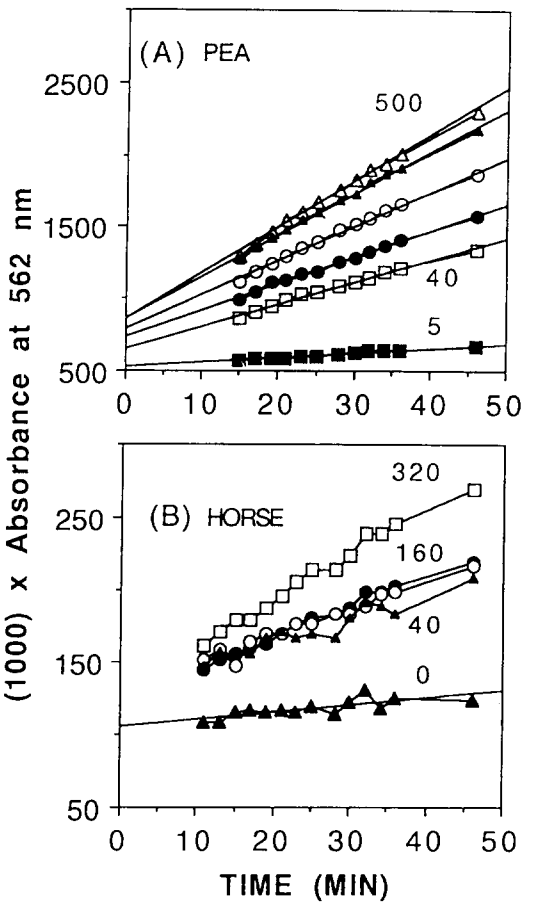

Figure 3. Reductive release of Fe(II) by ascorbate treatment in the presence of ferrozine. (**A**) Pea seed ferritin; (**B**) Horse spleen ferritin. Native 1 μM ferritin solutions in 0.1 M MES buffer, pH 6.5, and 4 mM ferrozine received 0, 5, 40, 80, 160, 320 or 500 μM sodium ascorbate. The Fe^{2+}–ferrozine complex was quantified by its absorbance at 562 nm. In (**A**) the slopes are significantly different, except 320 from 500 μM. In (**B**) the slopes are not significantly different, except 0 μM.

(Figure 3). On the other hand, the free Fe^{2+} concentration resulting from simultaneous iron uptake–release in the presence of ascorbate and in the absence of ferrozine is determined from the DNA relaxation assay (Figure 4).

The rates of iron release from ferritins were stable for more than 2 h (only the first hour is shown in Figure 3) and were a function of ascorbate concentration only in the case of plant ferritin. Rates of iron release from horse spleen ferritin were about 10 times lower than those from pea ferritin and very little affected by variations of ascorbate concentrations in the physiological concentration range (40–160 μM) (*cf.* Figure 3A and B).

The time dependence of iron-dependent radical damage was studied for different concentrations of ascorbate using plant ferritin (Figure 4a and b). The damage increased during the first few minutes and was maximal after about 20 min of reaction, as shown by the DNA relaxation test, applied to the reaction mixture at various time intervals. The maximal damage reflecting the maximal free Fe^{2+}

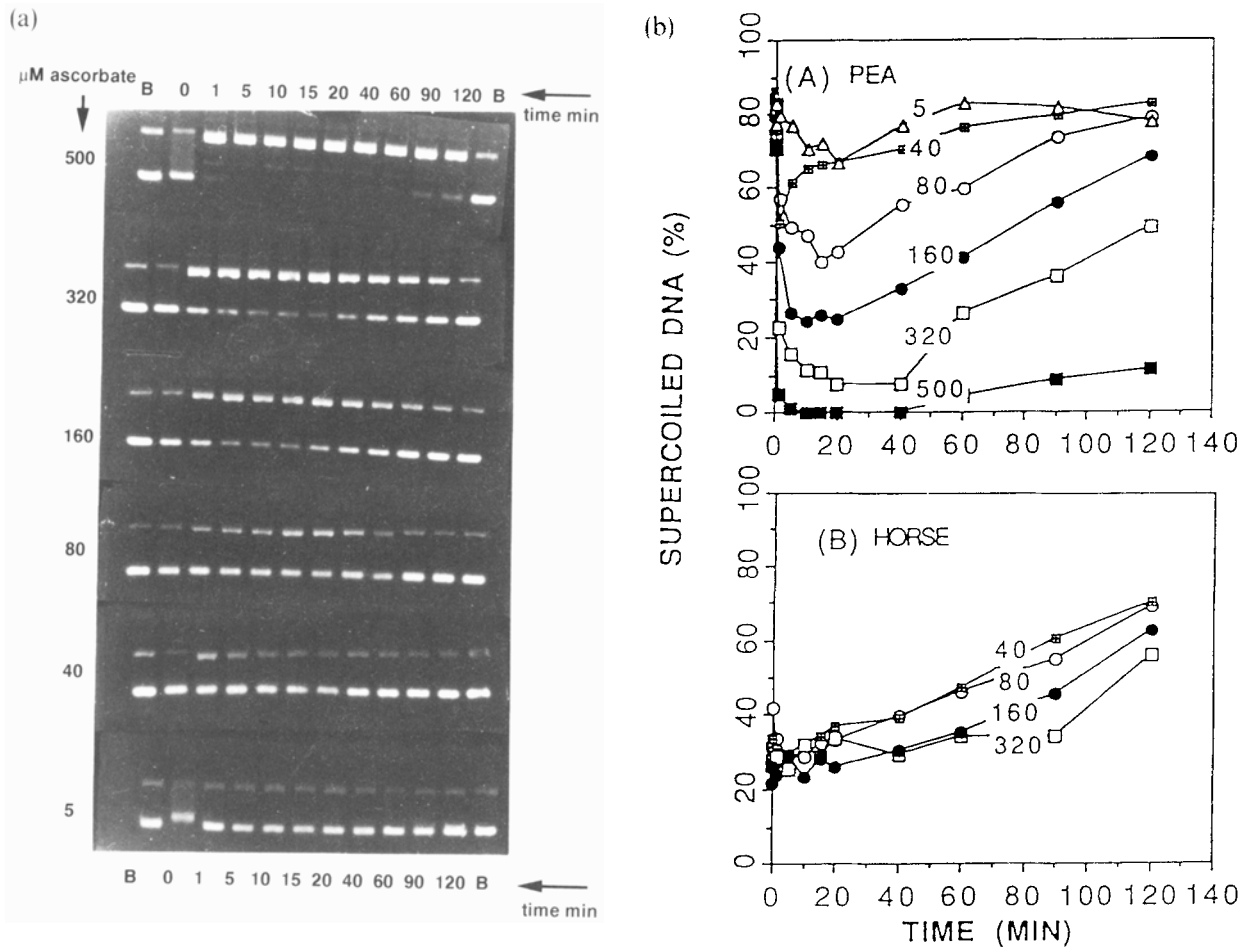

Figure 4. (**a**) Relaxation of supercoiled DNA after addition of ascorbic acid to phytoferritin solutions. Agarose gel electrophoresis of DNA after DNA relaxation tests: lanes 1 and 12 (labelled B), no ascorbate; lane 2 (labelled 0), simultaneous ascorbate and ferrozine addition; lanes 3–11, addition of supercoiled DNA and H_2O_2 after 1, 5, 10, 15, 20, 40, 60 and 120 min of ascorbate treatment. Duration of the relaxation test: 5 min. (**b**) Quantification of the radical damage done by ascorbic acid treatment of ferritins. Native 1 μM protein from pea (**A**) and horse spleen (**B**) was incubated in 0.1 M MES buffer, pH 6.5, and in the presence of 0, 5, 40, 80, 160, 320 or 500 μM ascorbic acid. The proportion of remaining supercoiled DNA was quantified from the fluorometry of DNA bands after electrophoresis. This experiment is the most complete among three comparable repeats.

concentration was a function of ascorbate concentration. Then DNA damage diminished, reflecting a continuous decrease in aqueous free iron due to an iron uptake process faster than the iron release. The free iron concentration lowers to a limit of about 2–5 μM, at which iron uptake and release tends to equilibrate, after more than 2 h of reaction. The time required to reach this equilibrium increases with increased ascorbate concentration.

When horse ferritin was used instead in similar experiments, the external iron concentration was maximal (11 μM) after 2 min and then decreased continuously to the equilibrium state. Varying the ascorbate concentration had no effect.

Figure 5 shows the dependence of both the rate of iron(II) release and the maximal free ferrous iron concentration on ascorbate concentration for plant (Figure 5A) and horse (Figure 5B) ferritin to emphasize the differences between the two ferritins. Only in the case of plant ferritin was the free Fe^{2+} concentration dependent on ascorbate concentration. Moreover, the rate of iron release is much faster with the plant ferritin.

Discussion

Ferritins have an essential iron storage function: they act as a dam across the metabolic flow of Fe^{2+} and compensate for the lack of an iron excretion mechanism. Thus they behave as anti-oxidants (Balla *et al.* 1992). In response to excess iron loading or to oxidative stress, their *in vivo* synthesis is stimulated (Cairo *et al.* 1995, Briat *et al.* 1995).

On the other hand, ferritins have the ability to generate Fe^{2+} and here we show that they are Fenton reagents, i.e. they promote radical damage to DNA, which can be

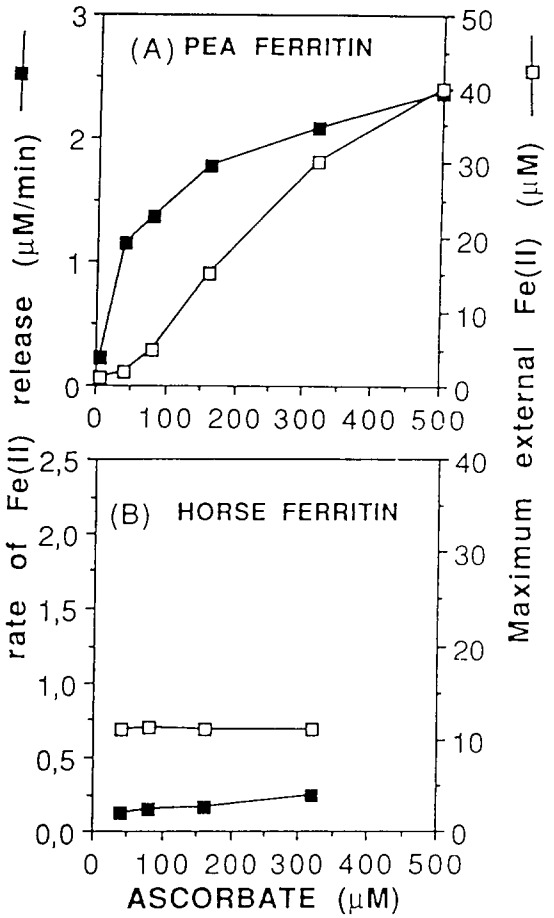

Figure 5. Effect of ascorbic acid concentrations on the rate of iron release (ordinates on the left) and the maximum external Fe^{2+} concentration released by ferritins (ordinates on the right). Rates of iron release are determined spectrophotometrically in the presence of 4 mM of ferrozine at 562 nm. Maximal external Fe^{2+} concentrations are calculated from data in Figure 4, using the calibration curve in Figure 1.

inhibited by strong Fe^{2+} scavengers (Aruoma & Halliwell 1987). In the oxidative incorporation of iron into ferritin, the stoichiometry (Fe^{2+}/O_2) of the reaction varies from 2 during the formation of the core to 4 (Treffry *et al.* 1979). The first reaction involves H_2O_2 production (Xu & Chasteen 1991) and then potentially generates hydroxyl radicals. Nevertheless, since greater than 99% of the H_2O_2 disproportionates to O_2 and H_2O (Sun & Chasteen 1992), little radical damage is produced inside the protein during iron uptake. Most of the damage occurs during iron release, at the external surface, on exposed peptides (Laulhère *et al.* 1990). One difficulty of this work was the selective measure of external damage. Since DNA, the target molecule, is too large to penetrate into the interior of the protein shell and to interact with the iron core, the damage is due to the reductive or spontaneous release of Fe^{2+} in solution. It is conceivable that this free Fe^{2+} originates from protein coats which have been shown to retain stable ferrous iron (Rohrer *et al.* 1987, 1989).

We found a good correlation between the extent of relaxation of supercoiled DNA and the concentration of free Fe^{2+}. Relaxation of DNA thus served as a sensitive and specific assay for free Fe^{2+}. It allowed us to quantitate the variation of free Fe^{2+} concentrations during addition of Fe^{2+} or ascorbate to ferritin solutions.

Spontaneously released Fe^{2+}, in equilibrium with ferritin, accounts only for a few micromolars, to be compared to the millimolar concentrations of ferritin iron. Most of the iron is bound to the protein and made inactive for Fenton reactions. After addition of exogenous iron, the initial equilibrium was restored within a few minutes. The uptake kinetics for pea and horse ferritin are comparable. With either 50 or 100 μM starting external Fe^{2+} concentration, 1–5 μM final free Fe^{2+} concentration was obtained at the end of the reaction and remained stable. Similar equilibrium values were found with ferritin or apoferritin, from pea or from horse spleen. These values are small but large enough to generate oxidizing species in the presence of H_2O_2.

In the presence of reducing agents like ascorbate, the release of Fe^{2+} from ferritin is accelerated. However, as shown from the DNA relaxation assay, free Fe^{2+} does not accumulate continuously. During the first few minutes the Fe^{2+} concentration increases, but decreases later, demonstrating again that under those conditions, free iron results from the balance between simultaneous uptake and release (Laulhère & Briat 1993). Horse and pea ferritins differ significantly in terms of their reactivity towards ascorbate: (i) iron release from horse ferritin is much slower than that from pea ferritin and (ii) ascorbate-dependent DNA damage by horse ferritin is much less sensitive than that by pea ferritin to variations in ascorbate concentrations.

These differences may reflect structural differences between pea and horse ferritin (Andrews *et al.* 1992). In pea ferritin, the mineral core is rich in phosphorous, not crystalline (Wade *et al.* 1993), and could be more accessible to reduction. A network of stacked aromatic residues is expected to allow long distance electron transfer through the protein shell (Lobréaux *et al.* 1992). On the contrary, in horse ferritin, ascorbate could reduce only a limited amount of iron (Figure 3).

These results suggest that the role of ascorbate in iron mobilization from ferritins is more important in plants than in animals. In plants, ferritins are abundant in proplastids. During conversion of proplastics to chloroplasts and greening of thylakoids, ferritin iron is liberated (Lobréaux & Briat 1991). In mature chloroplasts, no ferritin iron can be found (van der Mark *et al.* 1981) and ascorbate reaches concentrations which would promote iron release *in vitro* (10 mM; Law *et al.* 1983). The actual fate of ferritin iron in greening chloroplasts is consistent with expectations from its *in vitro* behaviour with the same ascorbate concentrations. Nevertheless, ascorbate is not proved to be the reducing agent of iron *in vivo*, but it is shown able to release it in similar conditions *in vitro*. In plants, this pro-oxidant aspect of ascorbate is balanced by its function in ascorbate peroxidase, which is known to destroy hydrogen peroxide (Halliwell 1987) and therefore participates in the prevention of Fenton reactions. In contrast, ascorbates is present in

animals in low amounts (40–160 μM in blood; Ames *et al.* 1981). In this range of concentration, both iron release and hydroxyl radical generation are relatively limited.

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