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Iron detoxifying activity of ferritin

Effects of H and L human apoferritins on lipid peroxidation in vitro

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Three recombinant human apoferritin variants were added to ferrous iron and the amount of lipid peroxidation produced by hydrogen peroxide was studied. The H-apoferritin had the strongest inhibitory effect on lipid peroxidation, probably due to its ferroxidase activity. The L-apoferritin inhibited lipid peroxidation slowly and only at neutral pH. The H-mutant 91, deleted of the last 22 C-terminal amino acids, and which is not able to form an iron core, had minimal effects on iron lipid peroxidation. It was concluded that both ferro-oxidase and iron mineralization activities are necessary for ferritin iron detoxifying action.

Recombinant ferritin; Mutant protein; Lipid peroxidation; Iron toxicity

1. INTRODUCTION

Ferritin is composed of 24 subunits of two types (named H and L) which co-assemble in a protein shell enveloping a polynuclear ferric hydroxide similar to the mineral ferrihydrite [1]. The structure suggests that ferritin protein keeps iron in a bioavailable and non-toxic form which does not produce free radicals. However, experimental evidence that ferritin acts as an iron detoxifying agent is elusive: it was shown that ferritin oxidizes and incorporates iron in a reaction that produces few, and probably non-toxic, free radicals [2], while various studies showed that ferritin-iron is available to the catalysis of free radical formation when in the presence of reducing agents [3-7]. The studies of in vivo iron-detoxifying activity are complex as they involve the understanding of the iron forms that generate free radicals. In vitro iron toxicity may be studied by the Haber and Weiss reaction, and by monitoring the peroxidation of lipids in the presence of iron and hydrogen peroxide [5,7]. In vitro and aerobically apoferritin takes up iron(II) by inducing its oxidation and mineralization inside the cavity [1,8-10]. This reaction has not been fully elucidated, and its effects on iron induced lipid peroxidation have not been studied.

We analyzed three variants of human ferritins with different characteristics: the recombinant H-chain, which has a ferroxidase activity [9,11,12], the recombinant L-chain which lacks such activity [10], and an H-chain mutant deleted of the last 22 C-terminal anino acids, named 91, which differs from the two in not being able to form a stable iron core [9]. These apoferritins were studied for their capacity to affect iron-mediated lipid peroxidation. It was found that H-wild-type reduced lipid peroxidation at neutral and acidic pH, the L-wild-type reduced it slowly and only at neutral pH, while the H-mutant 91 was essentially non-effective. The results indicate that ferritin shell has an inhibitory activity on iron induced lipid peroxidation, which is linked to its capacity to oxidize iron and to form a stable iron core.

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Abbreviations: MDA, malondialdehyde; TBA, thiobarbituric acid; TBAR, TBA reactive; NTA, nitrilotriacetate

2. MATERIALS AND METHODS

2.1. *Ferritins and mutants*

Recombinant human L-ferritin with the first two N-terminal amino acids modified from Ser-Ser with Asp-Pro, and previously named rLFo in [10], recombinant human H-ferritin and the H-mutant 91 were overexpressed in *E. coli* and purified as previously described [9,10]. Iron was removed from the purified ferritins by dithionite reduction and chelation as described in [10]. The apoferritins had absorbance ratios at 280:260 nm of more than 1.3.

2.2. *Lipid peroxidation*

The liposomes were prepared according to the procedure described in [13]: egg phosphatidyl choline (Sigma) dissolved in chloroform was dried, and resuspended in water at a concentration of 20 mg/ml. After 60 min resting at 4°C, it was sonicated with 10 pulses of 30 s. The liposomes were stored up to 4 weeks at 4°C. The lipid peroxidation reactions were performed as in [14], in 50 mM Hepes at the stated pH values, and the stock ferrous ammonium sulfate solution in water was prepared immediately before use. The reaction mixtures (0.15 ml) contained 1 mg/ml phospholipid liposomes, 0.1 mM H_2O_2, 0.2 mM Fe(II) and sample. After 10 min incubation at 37°C, to induce liposome peroxidation and formation of malondialdehyde (MDA),

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the reaction was blocked by addition of 0.4 M HCl, 6.25% trichloroacetic acid final concentrations. Then 0.02 mM desferrioxamine, 0.05% butylated hydroxytoluene, 0.33% thiobarbituric acid (final concentrations) were quickly added, the samples incubated for 20 min in boiling water to allow the development of the reaction between malondialdehyde adducts and thiobarbituric acid, chilled in an ice bath for 10 min and centrifuged for 5 min at 10 000 × g. The amount of thiobarbituric acid-reactive oxidation products (TBAR) was detected by optical reading at 532 nm. As reference solutions we used the reaction mixtures without iron.

2.3. *Ferritin iron uptake*

It was performed as described in [10]. The apoferritins (50 μg/ml, 0.1 μM) in 0.05 M Hepes buffer were incubated at 37°C with 0.2 mM freshly made ferrous ammonium sulfate. At intervals, samples were taken and the absorbance was read at 310 nm, or after the addition of 2,2′-bipyridine (Baker), 0.5 mM final concentration, and the absorbance read at 520 nm, or analyzed for MDA formation as described above.

3. RESULTS AND DISCUSSION

3.1. *Effects of apoferritins addition on iron-mediated lipid peroxidation*

As an index of iron toxicity, we analyzed the amount of phospholipid MDA adducts (TBAR) produced by mixtures of iron and hydrogen peroxide [5,7]. The proteins were added to the Fe(II)/H_2O_2 mixture: the H-apoferritin reduced MDA formation by about 30% while L-apoferritin had no detectable effect (Table I). Desferrioxamine reduced by 35% the MDA formation. Desferrioxamine and H-apoferritin had an additive action and together reduced the reaction by about 70%. Bovine serum albumin, used as a protein control, had no effect. Further studies (not shown) indicated that the H-apoferritin inhibitory effect on MDA formation ranged between 12 and 36% in the concentration range 0.02–0.2 mg/ml, the desferrioxamine between 35 and 43% in the range 0.4–4.0 mM, while L-ferritin and albumin showed no significant effects up to a concentration of 0.2 mg/ml. A complete inhibition of MDA formation was obtained by previous incubation of apo H- and L-ferritins with Fe(II) for 18 h at pH 7.0, i.e. by reconstitution of the iron cores, and in absence of Fe(II) (rHF-Fe and rLFo-Fe in Table I). These findings indicate that ferritin-iron does not induce lipid peroxidation, and thus is not toxic in aerobic conditions, and that the H-ferritin-linked ferroxidase activity has an inhibitory effect on MDA formtion, in keeping with the lower reactivity of Fe(III) than Fe(II) in the Heber and Weiss reactions.

Table I

Effects of ferritins on iron-mediated lipid peroxidation

Samples	A_{532nm}	Lipid perox. (%)
rHF-Fe	0.006 (±0.003)	3
rLFo-Fe	0.003 (±0.002)	1
Fe^{2+}	0.204 (±0.023)	100
Fe^{2+} + Apo-rHF	0.142 (±0.005)	71
Fe^{2+} + Apo-rLFo	0.208 (±0.014)	>100
Fe^{2+} + BSA	0.230 (±0.011)	>100
Fe^{2+} + DFO	0.132 (±0.014)	65
Fe^{2+} + DFO + Apo-rHF	0.061 (±0.002)	30
Fe^{2+} + DFO + Apo-rLFo	0.153 (±0.002)	75
Fe^{2+} + DFO + BSA	0.184 (±0.004)	90

Reaction mixtures in 50 mM Hepes, pH 7.0, containing 0.2 mM ferrous ammonium sulfate, 0.1 mM H_2O_2, 1 mg/ml phospholipids, were added to 50 μg/ml of recombinant L (rLFo), recombinant H (rHF) apoferritins, bovine serum albumin (BSA) or to 0.4 mM desferrioxamine (DFO), incubated for 10 min at 37°C and then treated for TBA reactivity as indicated in the text. The samples rHF-Fe and rLFo-Fe consisted of H and L ferritins previously loaded with 2000 iron atoms. Means ± SE of 4 experiments.

3.2. *Kinetics effects of apoferritins on iron-mediated lipid peroxidation*

Further experiments were performed at pH 6.5 and 6.0, conditions in which iron autoxidation is minimized and the ferritin ferroxidase activity is more evident [9]. We studied the kinetics of iron uptake following the formation of ferric iron core at 310 nm reading, the disappearance of Fe(II) at 520 nm reading after addition of 2,2′-bipyridine and, in addition, we monitored the capacity of the mixture to induce MDA formation by incubating aliquots with H_2O_2 and phospholipids, and then detecting TBAR at 532 nm. In the absence of apoferritin during 30 min incubation, iron was not oxidized and the MDA formation capacity did not change appreciably either at pH 6.5 (not shown) or at pH 6.0 (Fig. 1A). The addition of L-apoferritin did not inhibit the formation of MDA (Fig. 1B). In contrast H-apoferritin readily induced both the decrease of MDA formation and iron oxidation, which reached plateau 30 min at pH 6.0 (Fig. 1C). Thus it appeared that MDA formation decreased with decreasing concentration of Fe(II), suggesting that Fe(II) is the active form.

However, the Haber and Weiss reactions indicate that also Fe(III) is able to react H_2O_2, but with a reactivity dependent upon the type of complex it forms, e.g. Fe(III)-NTA or Fe(III)-EDTA are highly reactive [15,16], while ferrioxamine [17,18] , Fe(III)-transferrin [19,20] or Fe-ferritin (see above) are not reactive. Previous studies identified transient mono(oligo)nuclear Fe(III) forms during the ferritin iron uptake [21,22] of unknown chemical reactivity with H_2O_2. We studied a H-ferritin mutant which lacks the capacity to form a stable iron core. This mutant, named 91, is deleted of the last 22 C-terminal amino acids. We previously showed that it assembles in the 24-mer protein shell, has ferroxidase activity, but is unable to incorporate iron and to keep it in solution [9]. It was expected that by reacting with Fe(II) this mutant induces the accumulation of unstable oligonuclear Fe(III), as suggested by previous finding that upon prolonged incubation with iron the protein precipitates [9].

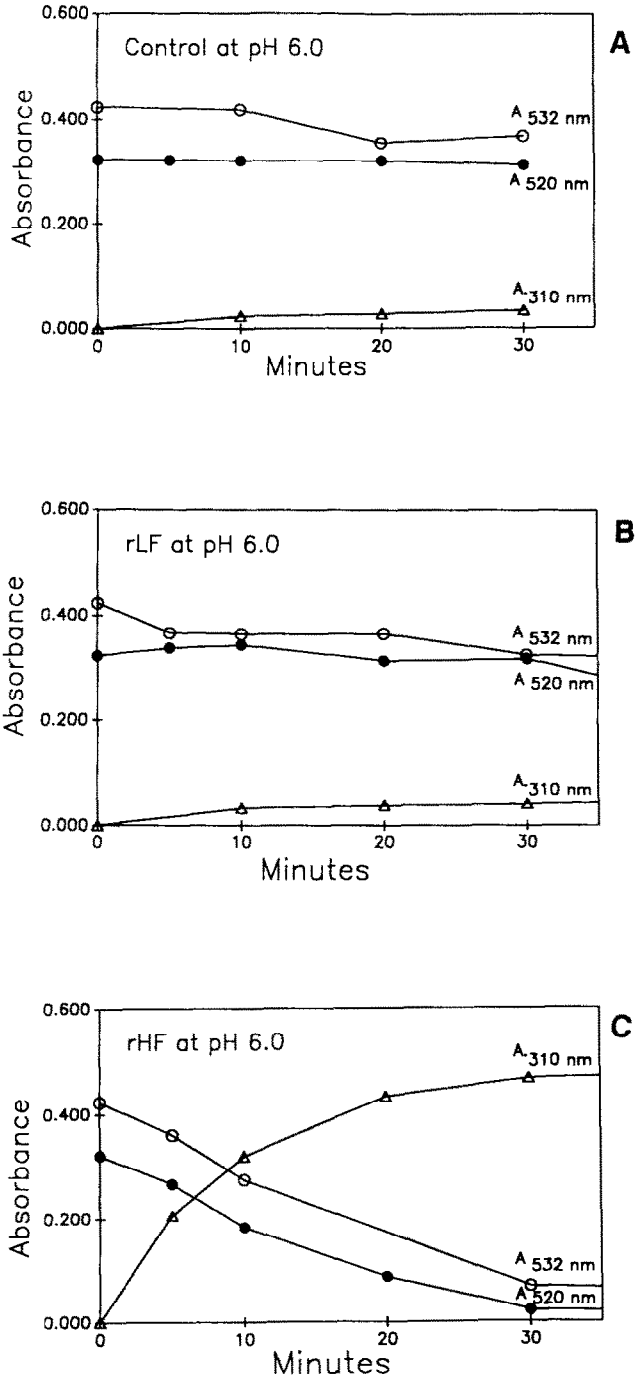

Fig. 1. Kinetics of ferritin iron uptake in 50 mM Hepes, pH 6.0. (A) Control in absence of ferritins. (B) In the presence of L-apoferritin. (C) In the presence of H-apoferritin. The reactions were monitored for iron oxidation by reading absorbance at 310 nm; for the presence of ferrous iron, by chelation with 2,2′-bipyridine and then reading at 520 nm; and for MDA formation after incubation of the aliquots with H_2O_2, phospholipids and TBA, as described in the text, and then reading at 532 nm.

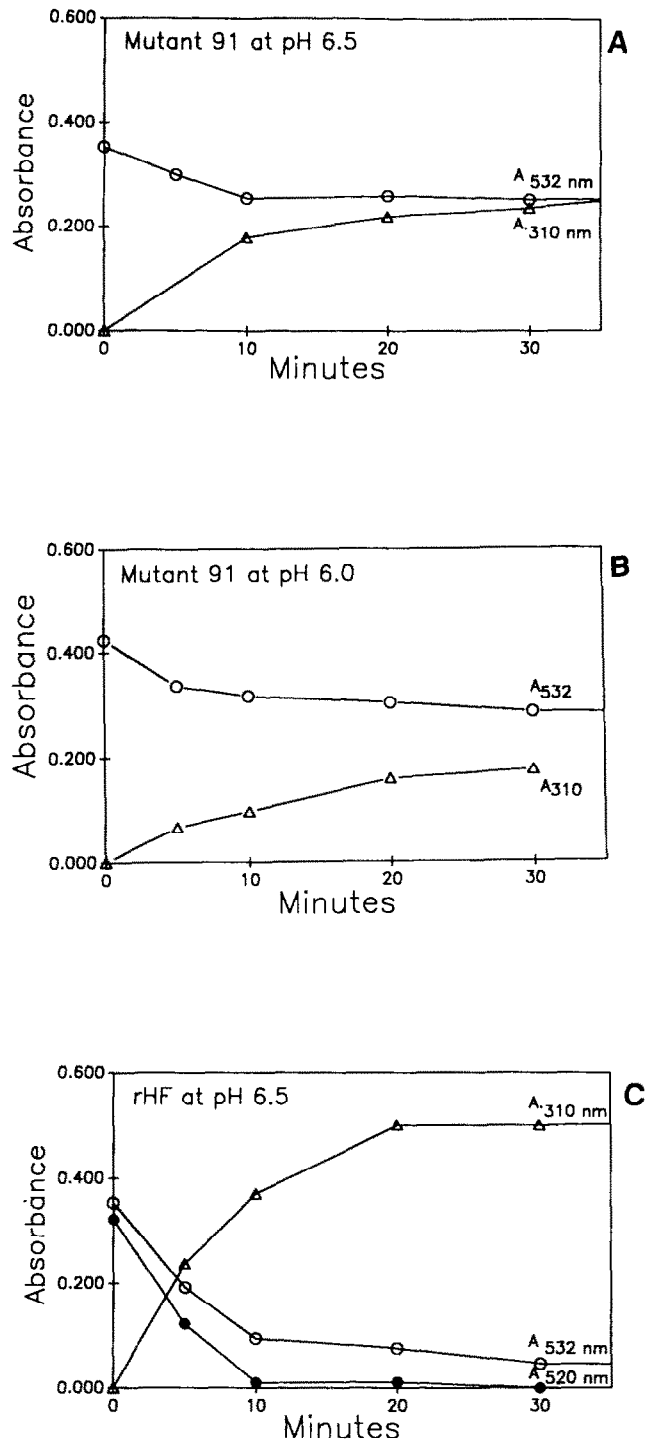

Fig. 2. Experiments as in Fig. 1. (A) H-mutant 91 at pH 6.5. (B) Mutant 91 at pH 6.0. (C) H-Apoferritin at pH 6.5.

Reactions at pH 6.0 and 6.5 showed that mutant 91 induced iron oxidation, even though less effectively than H-wild-type, while it decreased only marginally the iron lipid-peroxidating activity (Fig. 2A and B). Moreover we found transient conditions during the H-ferritin iron uptake (e.g. at 30 min at pH 6.5) in which Fe(II) was undetectable while MDA formation capacity was still present (Fig. 2C). In fact, the addition of 0.1 mM hydrogen peroxide to this sample induced a release of Fe(II) accounting for about 30% of the total iron added, as measured by the increase of 0.090 absorbance

units at 520 nm after 2,2'-bipyridine addition. After a further 18 h of incubation, the addition of hydrogen peroxide had no effect either on MDA formation or the release of Fe(II) (not shown). Thus, the study of mutant 91 of H-wild-type indicated that iron oxidation is not sufficient to abolish its toxicity, since the Fe(III) which is not in the mature iron core can react with hydrogen peroxide and produce lipid peroxidation.

In conclusion, the present study shows that ferritin, while taking up iron, reduces its toxicity, and this reduction is due to both its ferro-oxidase activity and to its biomineralization capacity.

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