CHEMICALLY AND BIOLOGICALLY HARMLESS VS. HARMFUL FERRITIN/COPPER-METALLOTHIONEIN COUPLES

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Abstract: The simultaneous measurement of the decrease of available Fe^{II} and the increase of available Fe^{III} allowed the analysis of the ferroxidase activity of two distinct apoferritins. Although recombinant human apoferritin (HuFtH) rapidly oxidizes Fe^{II} to Fe^{III}, this is not properly stored in the ferritin cavity, as occurs in horse spleen H/L-apoferritin (HsFt). Fe storage in these apoferritins was also studied in the presence of two Cu-loaded mammalian metallothioneins (MT2 and MT3), a scenario occurring in different brain cell types. For HuFtH, unstored Fe^{III} triggers the oxidation of Cu-MT2 with concomitant Cu^{I} release. In contrast, for HsFt, there is no reaction with Cu-MT2. Similarly, Cu-MT3 does not react during either HuFtH or HsFt Fe reconstitution. Significantly, the combination of ferritin and metallothionein isoforms reported in glia and neuronal cells are precisely those that avoid a harmful release of Fe^{II} and Cu^{I} ions.

Iron is an essential element for organisms but it is highly toxic in excess. It is well known that free Fe^{II} promotes the formation of highly reactive oxygen species capable of causing irreversible cell damage.[1] Organisms have developed chemical machinery based on the ferritin family of proteins to manage the availability of vital, but potentially toxic, free Fe^{II}.[2-4] Thus, every type of tissue must contain an appropriate type of ferritin (Ft) that scavenges for and stores any Fe which is not required for immediate metabolic purposes. Therefore the Fe is rendered non-toxic and yet still available for when it is required by the cell. The structures of many Ft proteins isolated from a wide range of organisms and biological tissues have been determined. The most extensively studied ferritin is that found in horse spleen, traditionally used as a model for mammalian Ft. It consists of a hollow protein shell composed of 24 subunits arranged in cubic symmetry and has a Mr of about 450 kDa. The shell encapsulates an aqueous cavity of 8 nm in diameter, capable of accommodating

thousands of Fe atoms as an Fe^{III} mineral, often described as ferrihydrite, $[Fe^{III}_{10}O_{14}(OH)_2]$. [5]

There are two different types of monomer included in the 24 subunits which compose the Ft shell: the heavy (H) subunits, 178 amino acids and 21 kDa, and the light (L) subunits, 171 aminoacids and 19 kDa, and each have different functionality. Thus, whereas the H monomers play a key role in rapid Fe detoxification, because they contain a catalytic ferroxidase center for Fe^{II} oxidation, the L subunits seem to be associated with Fe nucleation, mineralization and long-term Fe storage in the ferritin cavity.[2] In accordance with this, the H/L ratio in a ferritin shell varies widely in different organisms and different tissues, but it is worth noting that Ft proteins with a combination of H and L subunits are only found in vertebrates. In this subphylum, L-subunit-rich ferritins predominate in Fe storage organs such as the liver and spleen, whereas organs that require Fe detoxification, such as the heart and most brain cells, contain mostly H-rich ferritins. As an example, horse spleen Ft contains about 90% L- and 10% H- subunits. In bacteria, plants and invertebrates, ferritins are exclusively composed of H-like subunits.

To load apoferritin (apoFt), a Ft molecule that does not contain Fe, requires Fe^{II} and not, Fe^{III} as the substrate. In broad terms, it is accepted that Fe^{II} moves through the hydrophilic channels until it finds the ferroxidase center of an H-subunit, here it is catalytically oxidized to Fe^{III} by reaction with cellular O_2. It then moves to the cavity where it is finally stored as a ferrihydrite nanoparticle.[6] The mechanism of the ferroxidase oxidation of Fe^{II} to Fe^{III}, as well as the path of Fe^{III} from the ferroxidase center to the cavity, are currently a point of controversy.[2,6-10] In any case, if any Fe^{III} remains at the ferroxidase center, somehow close to the surface of ferritin, some biomolecules could be able to scavenge it. In fact, Hagen et al. have shown that transferrin, a large protein (80 kDa) that is unable to enter the Ft cavity, scavenges the two Fe^{III} ions of the ferroxidase center.[10] However, in these experiments transferrin just served as an Fe^{III} sensor to confirm the nature of the ferroxidase center and the results have little biological relevance because Ft is basically intracellular, whereas transferrin is the plasma Fe^{III} transport protein.

Considering this background, and with the aims of, (a) gaining further insight

into the biological consequences of ferroxidase center function, and (b) correlating the apoferritin composition resulting Fe^{III} unavailable, we decided to study how Fe^{II} is oxidized by two apoFts with different H/L ratios: recombinant human H-apoFt (HuFtH), a homopolymer of 24 identical H- subunits each with a ferroxidase center, and the commonly studied horse spleen apoFt (HsFt), a heteropolymer formed of approximately 3 H- and 21 L-subunits (L-subunits lack the ferroxidase center). The Fe^{II} to Fe^{III} oxidation activities of HuFtH and HsFt, and their capacities to make the formed Fe^{III} unavailable, were studied by following a very simple protocol. This was based on monitoring the decrease of available Fe^{II}, and the increase of available Fe^{III}, in a batch of 15 fresh samples initially containing Fe^{II} and the respective apoFts in catalytic proportions (approx. 200 Fe^{II}/apoFt). The addition, at different reaction times, of an excess of ferrozine (fz) or apolactoferrin (Lf) serves as an indicator, via measurement of the respective UV-vis absorbances at 562 nm ($[Fe^{II}(fz)_3]^{2+}$) or 464 nm (Fe^{III}-Lf), of the concentration of available Fe^{II} or Fe^{III} at every step of the Ft reconstitution process. The combination of Fe^{II} decrease and Fe^{III} increase availability patterns provides a dynamic picture of the overall Fe uptake process carried out by Ft. Hence, monitoring ($[Fe^{II}(fz)_3]^{2+}$) concentration, i.e., recording the time dependence of A^{562nm} allows evaluation of the capability of each apoFt to oxidize Fe^{II} because the faster is the Fe^{II} oxidation process, the faster is the decrease of the UV-vis absorbance at 562 nm. As shown in Figure 1, the free Fe^{II} concentration decreases in the presence of HsFt (L-rich) to reach a plateau corresponding to a remaining 40% of the initial Fe^{II}, while the decrease of free Fe^{II} in the presence of HuFtH (entirely H-subunits) is significantly higher, so that within a few minutes almost all the initial Fe^{II} is no longer available to react with fz. These results corroborate the idea that the ferroxidase activity of apoFt increases with its H/L ratio, as activity is clearly higher for HuFtH than for HsFt.

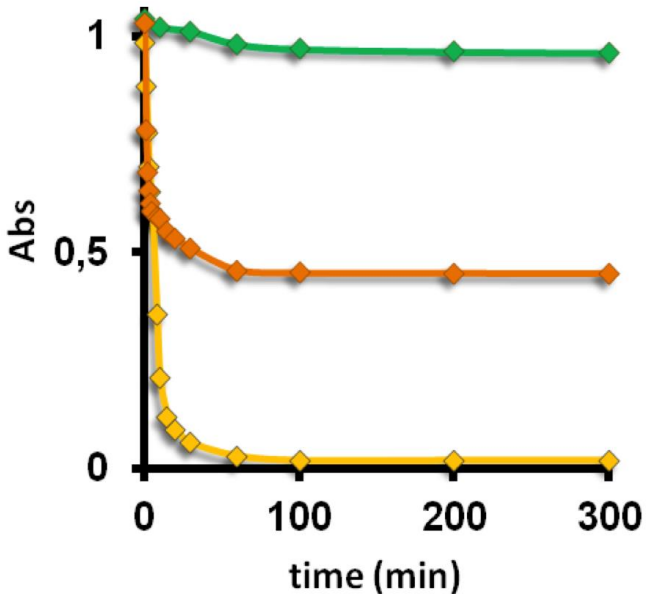

Figure 1. *Fe(II) availability in the presence of two different apoFts: Absorbance at 562 nm, i.e.* $[Fe^{II}(fz)_3]^{2+}$ *concentration, after addition, at different times, of ferrozine to the mixtures HsFt +* Fe^{II} *(orange) and HuFtH +* Fe^{II} *(yellow). Green data correspond to the control (absence of protein). As shown in the control, under our experimental conditions,* Fe^{II} *remains stable in solution at least 300 minutes.*

An analogous batch of experiments was performed but Lf in the presence of bicarbonate was added instead of fz, (see Experimental). Monitoring the UV-vis absorbance of the Fe^{III}-Lf complex at 464 nm provides information about the concentration of available Fe^{III} produced by the Ft reconstitution process. As can be seen in Figure 2, HsFt does not yield any peaks at this wavelength, suggestive of the low availability of Fe^{III} formed by this apoFt, whereas HuFtH gives rise to the development of the UV-vis band at 464 nm typical of Fe^{III}-Lf, and which is independent of the allowed Fe^{II}-HuFtH reaction time. This absorbance correlates with a permanent concentration of 0.16 mM of free Fe^{III}, which corresponds to almost 50% of the Fe^{II} that was allowed to react with HuFtH.

According to previous reports on this subject, we cannot rule out the possibility of a small fraction of the initial Fe^{II} being stabilized in the ferritin cavity and therefore unavailable for fz complexation.[11] However, the rapid increase of

A^{464nm} due to the formation of Fe^{III}-Lf in the presence of HuFtH confirms that for this protein, Fe^{II} is not available for fz complexation because a large proportion of the initial Fe^{II} is actually oxidized to Fe^{III} rather than stabilized within the protein as Fe^{II}. In either case, the presence or absence of a small fraction of stabilized Fe^{II} within the ferritin cavity is neither the focus of this work nor is it crucial for the conclusions which wewill draw, our focus is on the presence/absence of available Fe^{III} during the Ft mineralization process. In this context, our results clearly demonstrate that once Fe^{II} is oxidized by HsFt, the resulting Fe^{III} is in an inaccessible form, probably because these Fe^{III} ions are stored inside the protein cavity and Lf is too large to pass through the Ft channels to bind Fe^{III}. However, although HuFtH oxidizes practically all of the initial Fe^{II}, half of the formed Fe^{III} is available to large biomolecules such as Lf. Furthermore, according to our results and contrasting them with those reported by Hagen et al.,[10] the amount of Fe^{III} complexed by Lf during the Fe reconstitution process of HuFtH is almost double than that corresponding to the number of Fe atoms at the ferroxidase centers (24 x 2 per protein). Here, the protein is present in catalytic quantities, and therefore a large excess of Fe^{II} has been added (a situation that is probably closer to the actual *in vivo* scenario) and so we can conclude that the target of Lf is probably not only the Fe^{III} ions fixed at the ferroxidase centers but also the Fe^{III} cations formed at such centers and which are not really stored by HuFtH within the protein cavity. This protein, therefore, conclusively exhibits high ferroxidase activity but a low Fe storage capacity.

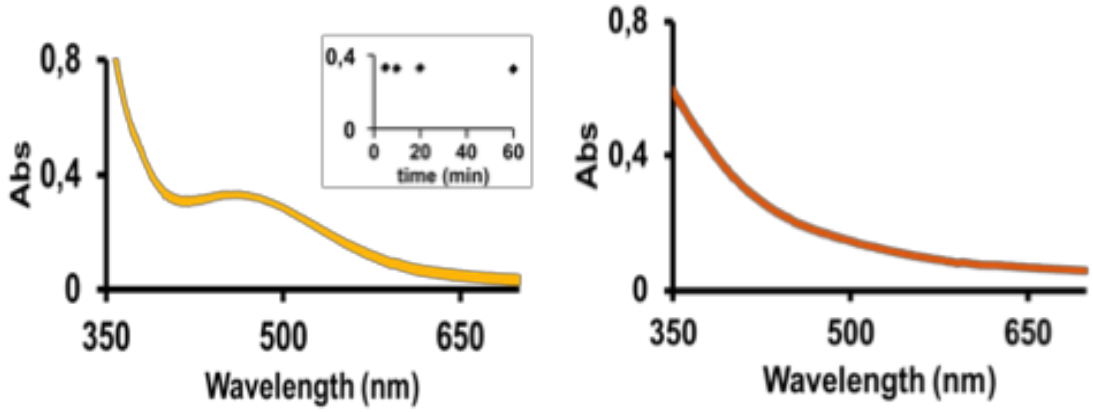

***Figure 2**. Fe^{III} availability after oxidation of Fe^{II} by two different apoFt proteins: UV-vis spectra after addition, at different times, of lactoferrin to the mixtures HuFtH + Fe^{II} (left) and HsFt + Fe^{II} (right). The inset corresponds to the time dependence of the absorbance at 464 nm, i.e. [Fe^{III}-Lf] concentration.*

In contrast, HsFt exhibits low ferroxidase activity but with a high Fe^{III} storage capacity since we did not observe Fe with Lf, thus indicating that although relatively little Fe^{III} is formed, it is genuinely stored within the ferritin cavity (i.e., it is not available for a chelating agent). More interestingly, and in a second stage of this work, we focused on the biological relevance of our results by investigating some of the chemical consequences of the observed Fe^{III} availability during reconstitution of Ft. We tried to go one step further beyond the demonstration that once Fe^{II} has been oxidized at the ferroxidase center of Ft, some Fe^{III} ions can then be scavenged by an Fe^{III} chelator. If, according to the present results and those of Hagen et al.,[10] Fe^{III} produced in the Ft ferroxidase center is available for complexation by a large molecule such as transferrin, it should also be able to act as an oxidant against metallothioneins (MTs).[12] This possibility takes special relevance in view of the Fenton reactivity of $Cu^{I/II}$ ions and the widely assumed presence of Cu-containing MTs in brain cells. Bearing this in mind, we studied the Fe reconstitution of the two different ferritins from our study (HuFtH and HsFt) in the presence of the Cu-loaded forms of two mammalian MT isoforms (MT2 and MT3). MTs are small (6 - 10 kDa) and cysteine rich (33%) metaloproteins. They are naturally found bound to either Zn^{II} and/or Cu^{I} but their biological function is still a matter of debate although they are accepted as

major players in the homeostasis of physiological Zn and Cu.[13,14] We paid particular attention to MT2 and MT3 because the former is predominantly synthesized in astrocytes and microglia, where ferritin is mainly found in the L-rich form, while the latter is a brain-specific isoform constitutively expressed in neurons, where H-rich ferritins are predominant.[15,16] Therefore, the coexistence of certain combinations of Ft and MT (i.e., MT2 with L-rich or MT3 with H-rich ferritins) can be considered to be a direct reflection of the situation encountered in different types of brain cells. In accordance with the Zn-thionein character of MT2[17] and the partial Cu-thionein character of MT3,[18,19] they would constitute mixed Zn, Cu-MT complexes (instead of homonuclear Cu-MT species) when synthesized in the brain, even in the presence of physiologically high Cu concentrations. For this reason we used the metal-MT2 and metal-MT3 preparations that result from the recombinant synthesis of MT2 and MT3 in Cu-supplemented *E. coli* cultures grown under regular aeration,[20] which is the closest approximation to mammalian cell conditions. They yielded a mixture of Zn_1Cu_{10}- and Zn_2Cu_{10}- as major species for MT2[17] and a mixture of Zn_4Cu_6- and Cu_{10}- complexes for MT3[18], which from now on and for the sake of simplicity we shall refer to as the Cu-MT2 and Cu-MT3 complexes.

For these experiments, we prepared a batch of Fe^{II}-apoFt mixtures with HsFt and with HuFtH, and allowed them to react aerobically for 15 minutes. These reaction mixtures were then degassed and saturated with argon prior to the addition of the Cu-loaded forms of MT2 or MT3 with the appropriate chelating agent, either BTA or fz. The Cu^{I} released or the Fe^{II} formed as a result of the oxidation of the Cu-MTs by Fe^{II} could be respectively determined by monitoring the UV-vis absorbance at either 480 nm (Cu^{I}-BTA) or 562 nm ($[Fe^{II}(fz)_3]^{2+}$) with time.

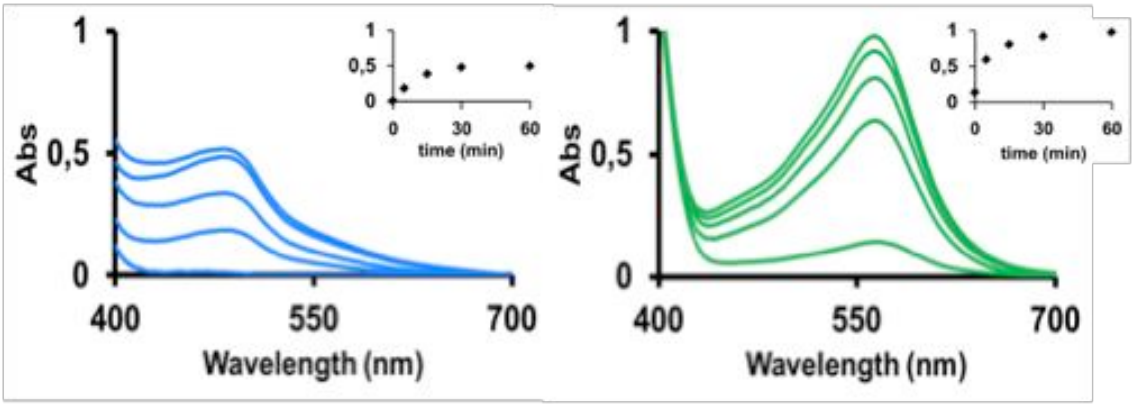

***Figure 3**. UV-vis spectra recorded at different times after the addition of Cu- MT2 + BTA (left) and Cu-MT2 + fz (right) to a mixture of HuFtH + Fe^{II}. Insets correspond to the time dependence of the absorbance at 480 nm (left) and 562 nm (right).*

Based on the initial Cu concentration of the Cu-MT2 sample (41 μM), it can be concluded that almost 90% of Cu was removed from MT2 after 30 min of reaction (35,7 μM final concentration of Cu^{I}-BTA). The same reaction with Cu-MT3 released only 6% of the initially coordinated Cu^{I}. However, the parallel study of HsFt is dramatically different; no absorbance peak developed at 480 nm, highlighting the absence of free Cu^{I} since, in agreement with the high Fe storage capacity of HsFt, there is a very low availability of Fe^{III} in the medium to oxidize either Cu-MT2 or Cu-MT3.

It is therefore reasonable to assume that the Fe^{III} formed after oxidation at the HuFtH ferroxidase center is the agent responsible for oxidizing Cu-MT2 on the basis of three different experiments: (i) The mixtures of HuFtH, Cu-MT2/Cu-MT3 and BTA do not exhibit any significant UV-vis absorption corresponding to Cu^{I}-BTA in the absence of Fe^{II}. This points out that, in the absence of Fe^{II}, neither MT2 nor MT3 are oxidized. (ii) Addition of an Fe^{III} salt, in the same concentration as that generated in the Fe^{II} + HuFtH experiment, to an anaerobic Cu-MT2 solution, but not in one containing Cu-MT3, in the presence of BTA produces Cu^{I}-BTA with a Cu^{I} delivery pattern similar to that of the Fe^{II} + HuFtH + Cu-MT2 experiment (see Experimental). This clearly indicates that Fe^{III} is a powerful oxidant capable of oxidizing the mammalian MT2 isoform in its Cu loaded form, while that of MT3 practically resists this oxidizing environment. (iii) When HuFtH is incubated with Fe^{II} and after 15 min of reaction (once the Fe^{II}

oxidation is almost complete, Figure 1) Cu-MT2 -together with fz- is added to the reaction mixture, the ($[Fe^{II}(fz)_3]^{2+}$) complex forms and its concentration increases with time (Figure 3). This final experiment evidences that after the reduction of the Fe^{II} concentration to almost zero during the Fe reconstitution process of HuFtH, reaction upon addition of Cu-MT2 again raises the Fe^{II} concentration to 59% of the initial value as a consequence of the oxidation of MT2 by Fe^{III}.

In summary, in this paper we demonstrate that part of the Fe^{III} formed at the ferroxidase centers of HuFtH is available, not only for complexation with an Fe^{III} chelator, but also to oxidize Cu-loaded MT2, giving rise to the concomitant release of two toxic metals ions, Cu^{I} and Fe^{II}. The latter reaction does not take place when the apoferritin is a H/L heteropolymer such as HsFt. In this case, all the Fe^{III} formed at the ferroxidase center is unavailable for either Lf complexation or MT oxidation. The chemical scenario we have created is especially significant in the brain, where incorrect Fe uptake by Ft has been suggested to result in the progression of neurological diseases. Hence, Dobson and co-workers proposed that patients with Alzheimer's disease could have a dysfunction in brain Fe storage due to a disruption in the balance of ferritin H- and L- subunit synthesis.[21] In this sense, our results point out that in cells where Cu-MT2 and Ft coexist, there is no danger if the ferritin is L-rich, as it occurs in astrocytes and microglia,[16] and both proteins would preserve their integrity and functionality. However, if this imbalance in L- and H-subunit synthesis leads to the synthesis of pure H apoferritins, and therefore to some events analogous to those we have described here in which the high ferroxidase activity and low Fe storage capacity of a H-rich Ft would provoke Cu^{I} liberation from Cu-MT2, then the presence of two free and toxic metals such as Cu^{I} and Fe^{II}, could produce fatal results for the cell. Interestingly, Cu-MT3 does not react with either HuFtH or HsFt in the presence of Fe^{II} thus making the Cu-MT3 and Ft pair a non-deleterious combination in neurons. This is extremely relevant because in neuron cells Ft is mainly present in H-rich forms,[16] which would lead to a harmful interaction should the predominant MT isoform present be that of MT2. What we wish to underline here is that the process of Fe storage in ferritin affects parallel metal metabolisms depending on the protein shell itself. Following on from that we have shown that an excessive proportion of H-subunits in the ferritin shell

results in a significant decrease in the amount of Fe stored, meaning that there is free Fe^{III} available that is capable of oxidizing Cu-MT2, ultimately leading to the liberation of toxic Fe^{II} and Cu^{I}. As a final conclusion, it is worth highlighting that the current results reconcile the simultaneous presence of ferritins and MT in cells,[12] provided that the correct combination of isoforms is preserved, otherwise metal-related neuronal disorders could be triggered if parting from this ideal combination.

Experimental Section

Materials. Horse spleen apoFt (HsFt) was obtained from Sigma and was exhaustively dialyzed against Milli-Q water using a Spectra/Por Float-A-Lyzer with a molecular weight cut off (MWCO) of 300,000 Da. Recombinant human H-Ft (HuFtH) was prepared as previously described[21,22] and rendered iron free by dialysis against sodium hydrosulfite (dithionite), $Na_2S_2O_4$, and complexation with 2,2'-bipyridyl at pH 6.0[23], to isolate an apoferritin containing 10 Fe/protein. The Cu loaded forms of the mammalian metallothionein isoforms MT2 and MT3 were recombinantly synthesized and characterized (ICP-AES and ESI-MS) as previously reported.[17,18] The final preparations were 0.13 x 10^{-4} M (MT2) and 0.35 x 10^{-4} M (MT3) in 50 mM Tris-HCl, pH 7.5 buffer, and they were deaerated under N_2 flux to avoid the presence of O_2 during the described reactions. Monoferric lactoferrin was purchased from Fonterra. Lf was prepared following the same protocol as reported previously.[24]

Measurement of available Fe^{II}. Batches of 15 fresh solutions for each type of apoferritin, 0.2 μM in 0.15 M HEPES buffer, pH 7.0, and fresh aqueous solutions of $Fe(SO_4)_2(NH_4)\cdot 6H_2O$ (final Fe 0.0353 mM) were mixed. 1 mL of an aqueous ferrozine solution (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p-disulfonic acid monosodium salt hydrate, Aldrich), final concentration 0.935 mM, was added to every sample after 1, 2, 3, 4, 5, 8,10, 15, 20, 30, 60, 100, 200 and 300 min and the UV-visible spectra immediately recorded. Available Fe^{II} concentration was calculated for every sample in the batch from the UV-vis absorbance measured at 562 nm, due to formation of the ($[Fe^{II}(fz)_3]^{2+}$) complex (ε = 27,900 $M^{-1}cm^{-1}$).

Measurement of available Fe^{III}. A batch of four fresh solutions of each apoferritin, 0.2 μM in 0.15 M HEPES buffer containing 10 mM of $NaHCO_3$, pH 7.0, and fresh aqueous solutions of $Fe(SO_4)_2(NH_4)\cdot 6H_2O$ (final Fe 0.353 mM) were mixed. 200 μL of an aqueous solution of Lf (final concentration 200 μM) was added to every sample after 5, 10, 20 and 60 min and the UV-vis spectra recorded. Available Fe^{III} concentration was calculated for every sample of the batch from the UV-vis absorbance measured at 464 nm, due to formation of the $[Fe^{III}(Lf)]$ complex (ε = 2,600 $M^{-1}cm^{-1}$).

Measurement of Cu^{I} during ferritin reconstitution in the presence of Cu-MT. In a parallel experiment, HuFtH and HsFt (0.2 μM) in 0.15 M HEPES buffer, pH 7.0, were incubated with aqueous solutions of $Fe(SO_4)_2(NH_4)\cdot 6H_2O$ (final [Fe] 0.035 mM) at 37 ºC and in aerobic conditions for 15 min. The reaction mixture was then totally deoxygenated under nitrogen flux and kept under an anaerobic atmosphere prior to the addition of Cu-MT2 (100 μL, 5.2 μM, [Cu] 41 μM) or Cu-MT3 (100 μL, 4.7 μM, 40 μM). Then, 10 μL of 0.4 mM BTA (bathocuproinedisulfonic acid disodium salt) was added to the mixture in order to detect Cu^{I} by observation of the UV-vis band at 480 nm due to formation of the $[Cu^{I}(BTA)_2]^{+}$ complex (ε = 14,000 $M^{-1}cm^{-1}$). The same experiments carried out in the absence of Fe^{II} did not produce an absorbance peak at 480 nm for the UV-vis spectra of $[Cu^{I}(BTA)_2]^{+}$.

Measurement of Fe^{III} reduction to Fe^{II} during HuFtH ferritin reconstitution in the presence of Cu-MT2. The previous protocol was followed but using ferrozine instead of BTA as a chelating agent for the purposes of measuring the Fe^{II} concentration. We noted that after 15 min of Fe^{II} reconstitution by HuFtH, the Fe^{II} concentration had dramatically decreased (Figure 1). Therefore the increase of Fe^{II} concentration in this experiment corresponds to the oxidation of Cu-MT2 by Fe^{III} with the subsequent formation of Fe^{II}, which is ultimately detected and quantified by its complexation with fz.

Oxidation of Cu-MT by Fe^{III}. In a parallel experiment, Cu-MT2 (100 μL, 3 μM) and Cu-MT3 (100 μL, 3 μM) were incubated at 37 ºC in a degassed aqueous solution of Fe^{III} acetate (0.02 mM) under Ar. BTA or fz was added to detect and quantify the Cu^{I} or Fe^{II} concentrations.

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Keywords: Ferritin • Metallothionein • Ferroxidase activity • Fe metabolism

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