Oxygen catalyzed mobilization of iron from ferritin by iron(III) chelate ligands†

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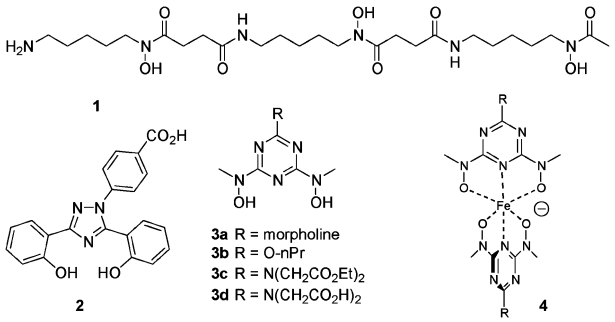
Tridentate chelate ligands of 2,6-bis[hydroxy(methyl)amino]-1,3,5-triazine family rapidly release iron from human recombinant ferritin in the presence of oxygen. The reaction is inhibited by superoxide dismutase, catalase, mannitol and urea. Suggested reaction mechanism involves reduction of the ferritin iron core by superoxide anion, diffusion of iron(II) cations outside the ferritin shell, and regeneration of superoxide anions through oxidation of iron(II) chelate complexes with molecular oxygen.

Iron is an abundant element essential for virtually all forms of life. Easy conversion between the oxidation states of iron is fundamental for catalyzing a plethora of biochemical redox processes. Under aerobic conditions, labile iron cations catalyze the production of reactive oxygen species (ROS) that are toxic for most living organisms.[1–3] The inherent toxicity of iron cations is minimized by their intracellular storage in the form of a macro-inorganic iron hydroxide mineral inside ferritin, the major iron storage protein. Mammalian ferritins consist typically of 24 similar subunits of two types, H and L that co-assemble in various ratios forming a shell-like structure surrounding a cavity of ~8 nm in diameter capable of accommodating up to 4500 iron atoms per ferritin molecule.[4] Iron(II) cations enter and exit the cavity of animal ferritins *via* the eight hydrophilic three-fold channels[5,6] (~4 Å wide) and are oxidized rapidly at conserved di-iron centers to form the mineral core.[4–7]

Whereas considerable progress has been achieved towards understanding the process of iron deposition into ferritin, the mechanism of iron mobilization remains poorly understood. The ferritin iron core is a relatively stable entity with a very slow iron dissociation rate in the absence of reducing agents. However, facile mobilization of iron from the ferritin core has been demonstrated *in vitro* and *in vivo* using a variety of reducing agents such as ascorbate, thioglycolic acid, cysteine, glutathione, and flavins.[8,9] The resultant iron(II) cations were then chelated by selective iron(II) chelate ligands such as 2,2-bipyridine or ferrozine.[10] Physiologically, the more plausible iron release mechanism appears to involve electron shuttling from an electron donor such as NADPH by way of a flavinnucleotide.[11]



Scheme 1 Structures of iron chelators, DFO (**1**), DFX (**2**) and BHT (**3**). BHT–Fe(III) complex (2 : 1 ratio) is also shown (**4**).

Small bidentate iron(III) chelate ligands such as deferiprone,[12] catechols,[13] and hydroxamates[14] are capable of removing iron from the ferritin inorganic core *via* direct extraction followed by diffusion of the Fe(III)-chelate out of the protein shell. However, the relatively low affinity of these ligands to iron requires high ligand concentration (3.5–100 mM) in order to achieve significant iron release. Larger hexadentate iron chelators such as DFO (Scheme 1) can still extract iron from ferritin albeit at a much slower rate,[15] most probably because of the difficulty of DFO to pass through the narrow 3-fold protein channels.

Direct iron release from ferritin is important for the activity of orally bioavailable tridentate selective iron(III) chelator deferasirox (DFX, Scheme 1) that was introduced for treatment of iron overload diseases. Recently reported family of compact pincer tridentate chelators based on bis(hydroxyamino)-1,3,5-triazine motif (BHT chelators) possess similar iron(III) affinity[16–18] to deferasirox making them potential drug candidates for regulation of iron homeostasis.

Herein, we report rapid aerobic mobilization of iron from ferritin by tridentate iron(III) chelators in the absence of reducing agents. Our investigation of iron extraction by BHT ligands focused on a recombinant heteropolymer human ferritin composed of 20.4H and 3.6L subunits, a ratio similar to that found in the ferritin from the human heart. Purified apo-ferritin was loaded aerobically with 1000 Fe atoms per molecule (two 500 Fe(II)/protein injections, 15 minutes apart) and checked spectrophotometrically using a molar absorptivity value of ~3000 $M^{-1}cm^{-1}$. The iron release experiments were conducted in 0.1 M MOPS, 50 mM NaCl, pH 7.0 in the presence of 100 nM ferritin and 400 μM chelators **1–3** (*i.e.* 4 chelator : 1 Fe ratio). The kinetics of iron release from ferritin (Fig. 1) were monitored by the increase in the characteristic MLCT absorption bands of the corresponding iron(III)-chelate complexes (425 nm for DFO, 470 nm for DFX and 535 nm for BHT). The percentage of released iron was calculated using experimentally determined UV-Vis molar absorptivity

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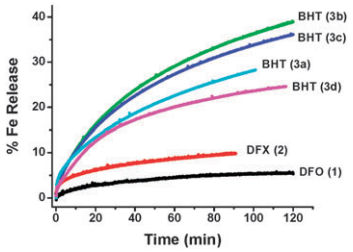

Fig. 1 Kinetic curves of iron mobilization from ferritin by DFO (**1**); DFX (**2**); and BHT (**3a**; **3b**; **3c**; **3d**).

coefficients for each iron(III)-chelate complex at these wavelengths (3500 $M^{-1}cm^{-1}$ for DFO, 3000 $M^{-1}cm^{-1}$ for DFX and 4000 $M^{-1}cm^{-1}$ for BHT).

All chelate ligands **1–3** used in these experiments were capable of extracting iron from ferritin under aerobic conditions. The rate of iron release strongly depended on the type of chelator employed. In line with earlier reports,[12] DFO mobilized less than ~5% of iron from ferritin within 90 min of reaction and ~90% after 48 h. The rate of iron release by the tridentate chelator deferasirox was ~8% of iron mobilization after 90 min and ~66% after 48 h. In contrast, BHT chelators **3a–d** mobilized iron from ferritin much more rapidly releasing ~22–36% of total ferritin iron within 90 min. Notably, the rate of iron release increased with the acidities of hydroxyamino group of free chelate ligands of type **3** in the order of **3d** (22%, pK_a 9.2), **3a** (27%, pK_a 8.8), **3b** (32%, pK_a 8.7), and **3c** (35%, pK_a 8.6).[17]

The iron release kinetics from ferritin by chelate ligand **3a** are shown in Fig. 2 and involve two phases, one fast and the other slow. The first fast phase is ascribed to the presence of a small amount of loosely bound iron cations to ferritin, most probably at sites in or near the 3-fold channels while the second slower phase is attributed to the slow dissociation of iron from the ferritin iron core.[19] Both phases show saturation kinetics consisting of a linear [BHT]-dependent step in the range of 0–200 μM followed by a non-linear step at a BHT concentration > 200 μM.

The observed higher rates of iron release with type **3** ligands are inconsistent with a mechanism involving direct iron

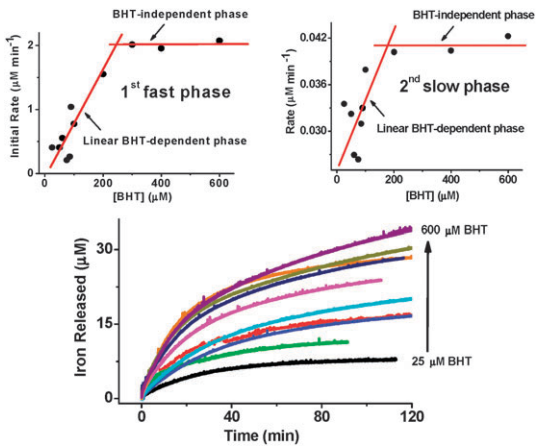

Fig. 2 Kinetics of iron release from ferritin at different concentrations of chelate ligand **3a** (below) and dependence of rates of iron release on the concentration of **3a** for fast and slow processes.

chelation. Although some flexibility and "breathing" of the ferritin channels have been previously reported[20] in the presence of chaotropic agents such as urea, the minimal linear size of chelate ligands type **3** is ~10 Å and is much larger than the reported diameter of the three-fold channels in ferritin (~4 Å) and thus argues against direct iron chelation.

In contrast, with BHT ligands the rate of iron release from ferritin was reduced by ~50% in the presence of 100 mM urea (Fig. 3) suggesting that the iron mobilization mechanism does not involve passage of BHT molecules through the eight hydrophilic three-fold channels. Since urea, a free radical scavenger, has been shown to inhibit iron release from ferritin,[21] other compounds such as mannitol, superoxide dismutase (SOD) and catalase known for their oxygen radical scavenging activities were also tested.

Fig. 3 shows that all of these reagents inhibit to varying degrees the rate of iron release thus indicating the involvement of reactive oxygen species (ROS) in the process of iron removal from ferritin. To corroborate this finding, we conducted iron release experiments under anaerobic conditions (Fig. 3). The data indicate almost complete inhibition of iron release by BHT ligands under these conditions suggesting that molecular oxygen is required to initiate the generation of reactive oxygen species (most likely superoxide anions) which then reduce the mineral core and release Fe(II). Similar inhibition reactions were observed with DFO (*i.e.* 41% inhibition with mannitol, 43% with urea, 33% with catalase and 12% with SOD). In contrast, no appreciable inhibition was observed with DFX suggesting a different reaction mechanism.

Despite the different iron mobilization rates, the fact that similar inhibition mechanisms are observed with BHT and DFO strongly suggest similar iron release pathways. In contrast, iron mobilization by DFX follows a different reaction path since ROS scavengers had no significant effect on the amount or the rate of iron release from ferritin. This difference in reaction mechanisms could be due, at least in part, to the structure of the iron chelates, particularly the iron binding pocket which involves hydroxyl-amino groups in the case of DFO and BHT but not DFX.

Based on these results, we propose a superoxide-mediated iron release mechanism in which superoxide radicals rapidly diffuse through the ferritin shell to reduce the ferrihydrite core and generate soluble iron(II) cations which then diffuse out of the protein shell through the eight hydrophilic three-fold

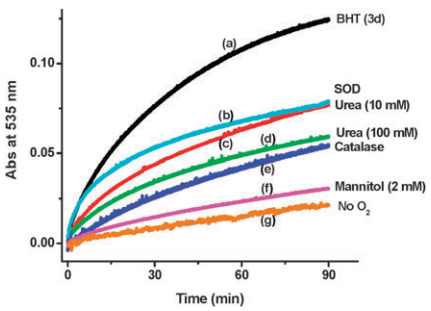

Fig. 3 Kinetic curves of iron mobilization from ferritin by chelate ligand **3d** (a) in the absence of any reagents; (b) in the presence of 3 units mL^{-1} SOD; (c) 10 mM urea; (d) 100 mM urea; (e) 1300 units mL^{-1} catalase; (f) 2 mM mannitol; and (g) under anaerobic conditions.

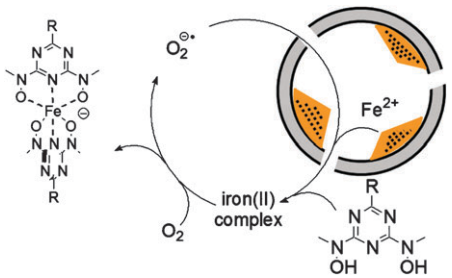

Fig. 4 Proposed mechanism of iron release from ferritin by ligands of type **1** and **3**.

channels where they encounter BHT or DFO ligands to form Fe(II)–BHT or Fe(II)–DFO chelate complexes. These latter are rapidly oxidized by molecular oxygen to produce the more thermodynamically stable Fe(III)–chelates and regenerate superoxide anions thus completing the catalytic cycle (Fig. 4). This process is facilitated by the very low Fe^{2+}/Fe^{3+} reduction potentials of these chelate complexes (−0.80 V)[17] in comparison with the superoxide anion reduction potential (−0.33 V)[22] and has been previously observed with specific iron(II) chelators and sacrificial hydrogen donor such as biphenols.[21]

This catalytic cycle can be initiated by the reaction of BHT or DFO ligands with iron(II) cations that are present in ferritin either as ferrous cations[23a,b] or as magnetite[23c] and is accompanied by other side reactions that replenish superoxide radicals lost due to disproportionation. Inhibition of iron release by catalase, mannitol, and urea that scavenge hydrogen peroxide and hydroxyl radicals indicates that these reactive oxygen species can also participate in catalytic cycle, most probably through a sacrificial hydrogen donation from the anionic form of the BHT ligand.

Under normal conditions, it is unlikely that the superoxide-mediated iron release process from ferritin is physiologically relevant. However, it has been shown that inflammation stimulates polymorphonuclear leukocytes and macrophages to produce large amounts of superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) which then result in the mobilization of iron from human and horse ferritin.[24] While the exact physiological mechanism of this process remains unknown, the continuous production of superoxide anions during inflammation might lead to the release of enough iron which could then catalyze the formation of the more damaging hydroxyl radicals, the underlying cause for a variety of diseases.[25]

In conclusion, chelate ligands of 2,4-bis[hydroxy(methyl)amino]-1,3,5-triazine family are capable of rapidly mobilizing iron from ferritin. Our data suggest that iron release from ferritin is catalyzed by oxygen and involves reduction of the iron core by superoxide anion. The reduced iron diffuses out of the ferritin shell and forms Fe(III)-complexes with BHT with the concomitant production of superoxide anions. A similar iron release process is suggested to occur with the commonly used iron-chelate desferroxamine (DFO) but not with deferasirox (DFX).

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