Iron release from ferritin by flavin nucleotides

Galina Melman a, Fadi Bou-Abdallah b,⁎, Eleanor Vane a, Poli Maura c, Paolo Arosio c, Artem Melman a,⁎⁎

Abstract

Background: Extensive in-vitro studies have focused on elucidating the mechanism of iron uptake and mineral core formation in ferritin. However, despite a plethora of studies attempting to characterize iron release under different experimental conditions, the in-vivo mobilization of iron from ferritin remains poorly understood. Several iron-reductive mobilization pathways have been proposed including, among others, flavin mononucleotides, ascorbate, glutathione, dithionite, and polyphenols. Here, we investigate the kinetics of iron release from ferritin by reduced flavin nucleotide, FMNH2, and discuss the physiological significance of this process in-vivo.

Methods: Iron release from horse spleen ferritin and recombinant human heteropolymer ferritin was followed by the change in optical density of the Fe(II)–bipyridine complex using a Cary 50 Bio UV–Vis spectrophotometer. Oxygen consumption curves were followed on a MI 730 Clark oxygen microelectrode. Results: The reductive mobilization of iron from ferritin by the nonenzymatic FMN/NAD(P)H system is extremely slow in the presence of oxygen and might involve superoxide radicals, but not FMNH2. Under anaerobic conditions, a very rapid phase of iron mobilization by FMNH2 was observed.

Conclusions: Under normoxic conditions, FMNH2 alone might not be a physiologically significant contributor to iron release from ferritin.

General significance: There is no consensus on which iron release pathway is predominantly responsible for iron mobilization from ferritin under cellular conditions. While reduced flavin mononucleotide (FMNH2) is one likely candidate for in-vivo ferritin iron removal, its significance is confounded by the rapid oxidation of the latter by molecular oxygen.

Keywords: Reductive mobilization Iron core Ferritin FMN/NAD(P)H Oxygen consumption Iron release

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1. Introduction

Iron is an essential metal for virtually all living organisms and the easy inter-conversion between its oxidation states is crucial for a plethora of biochemical redox processes. However, under aerobic conditions, free iron cations catalyze the formation of reactive oxygen species (ROS) which are capable of inducing oxidative stress [1–3]. The toxicity of iron cations is minimized by their intracellular storage as insoluble iron(III) hydroxide inside ferritin, the major iron storage protein [4]. Mammalian ferritins consist of 24 similar subunits of two types, H and L. These units co-assemble in various ratios to form a shell-like structure surrounding a cavity of ~8 nm in diameter that is capable of accommodating up to 4500 iron atoms per ferritin molecule [5]. Iron(II) cations can enter and exit the cavity of animal ferritins via the eight hydrophilic three-fold channels [6,7] (~4 Å wide) and are rapidly oxidized at conserved di-iron centers on the H-subunits to form the mineral iron(III) hydroxide core [8].

Whereas considerable progress has been achieved towards understanding the process of iron deposition into ferritin [5,7] the mechanism of iron mobilization from ferritin, as well as many other surface-limited phenomena of mineral redox and dissolution, remains poorly understood. The ferritin iron core is a relatively stable entity with a very low iron dissociation rate in the absence of reducing agents. Several pathways of iron release from ferritin have been proposed. For instance, mobilization of iron(II) cations from ferritin can occur through reduction by flavin mononucleotide [9,10] ascorbate [11,12] glutathione [10] sodium dithionite [13] polyphenols [14,15] superoxide [16,17] and other [18,19] agents. Alternatively, iron(III) cations can be released from ferritin by hydroxamates [20] catechols [15] and other synthetic chelators [6] with or without intermediate reduction to iron(II) cations [21]. Finally, iron(III) cations can be released through proteolytic degradation of the ferritin shell by proteasome or in lysosomes [22,23]. These findings do not explain which of these iron release pathways is predominantly responsible for the release of iron cations from ferritin in cells under physiological conditions. Because FMNH2 can be produced from...
FMN and NAD(P)H by cellular flavin reductases [10] one of the most likely candidates of iron release from ferritin in-vivo is the reductive mobilization by reduced flavin mononucleotide (FMNH$_2$). Treatment of ferritin with FMNH$_2$ under oxygen-limited conditions results in a rapid iron release either via direct diffusion of FMNH$_2$ to the ferritin iron core through the four-fold channels [7,24] or via a long range electron transfer across the protein shell [25]. However, free FMNH$_2$ is rapidly oxidized by molecular oxygen resulting in the formation of FMN and H$_2$O$_2$ [26–30]. Consequently, the relevance of the aforementioned process to living cells under normoxic environments has not been proven [10]. Here, we report on the reaction rates and competition between iron release from ferritin by FMNH$_2$ and its rapid oxidation by molecular oxygen and discuss the role of FMNH$_2$ in these processes.

2. Reagents and methods

Flavin mononucleotide, NADH and horse spleen ferritin (HoSF) were obtained from Fisher Scientific and used without further purification. Human recombinant heteropolymer apoferritin (p21H- and 3L-subunits) was prepared as described elsewhere and was manually loaded with 500 iron atoms in the presence of oxygen [31]. Unless otherwise stated, all experiments were conducted at 22 °C, in 100 mM MOPS buffer and 50 mM NaCl, pH 7.0. UV–vis spectra were measured either on Cary 100 (Fig. 2), Varian Cary 50 Bio (Fig. 5), or Lambda 35 spectrophotometers [all other measurements]. The concentration of the reductive mobilization of iron cations from ferritin was measured by following the absorption of the Fe(II)–bipyridine complex at 530 nm (ε = 8650 M$^{-1}$ cm$^{-1}$). Concentration of oxygen was measured by a MI 730 Clark oxygen microelectrode. Before each measurement, the electrode was calibrated at 21% oxygen in distilled water and 0% oxygen in a 5 mM dithionite solution. The iron content of horse spleen ferritin was determined experimentally and found to be ~2250 iron(III)/ferritin.

2.1. FMN reduction by NADH and iron release kinetics

The reaction between NADH (0.5–2 mM) and FMN (50–500 μM) was followed spectrophotometrically at pH 7.0 between 300 and 500 nm. The iron release kinetics by NADH alone were performed in a 4 mm-path quartz cuvette open to ambient air by mixing horse spleen ferritin samples (0.2–0.4 μM) with solutions of NADH (2 mM) and 2,2′-bipyridine (2 mM) in pH 7.0 buffer. The iron release kinetics by the non-enzymatic FMN/FMN system was followed by mixing solutions of NADH (2 mM), 2,2′-bipyridine (2 mM), FMN (200 μM) with either horse spleen or human recombinant heteropolymer ferritin (0.2–1.0 μM) in buffer at pH 7.0. The experiments were performed in 1 mm path quartz cuvette fitted with a septum to avoid oxygen diffusion into the cell. The change in absorbance was monitored at 530 nm every 25 s for 80 min. Experiments with horse spleen ferritin were conducted at 22 °C and with those with recombinant human H/L ferritin at 28 °C.

2.2. Combined UV–vis and oximetry kinetics of iron release from horse spleen ferritin

Solutions of NADH (2 mM), 2,2′-bipyridine (2 mM), FMN (2 mM), catalase (2650 U/ml) and horse spleen ferritin (0.6 μM) were mixed and then rapidly transferred to a 4 mm path quartz cuvette fitted with a septum and the oxygen electrode. The oxygen concentration curve and the change in absorbance at 530 nm were monitored simultaneously with an integration time of 0.2 s.

3. Results

The data in Fig. 1 show that immediately after the reagents are mixed, the concentration of dissolved oxygen starts to decrease at a constant rate due to the high concentrations of FMN (2 mM) and NADH (2 mM). During the initial lag phase, no appreciable release of iron is detected (Fig. 1). Following the complete disappearance of oxygen from the solution, a short intermediate phase of less than 1 min is observed followed by a constant rate of slow iron release from ferritin at an oxygen concentration close to 5 μM. This intermediate period is of particular interest for determining the rate of iron release since the rates of iron release by FMNH$_2$ and oxidation of the latter by molecular oxygen are comparable; the shorter the period, the higher the ratio between the rate of FMNH$_2$ oxidation by O$_2$ and the rate of iron mobilization by FMNH$_2$.

3.1. Iron mobilization from horse spleen ferritin by NADH

Despite strongly negative reduction potential, the NAD$^+$/NADH system alone is not an efficient reducing agent for iron(III) cations. However, because our experiments were conducted in the presence of relatively high concentrations of NADH, we felt it was necessary to determine whether a reductive mobilization of iron from ferritin can be solely induced by NADH. Incubation of HoSF in a buffered solution at pH 7.0 containing 2 mM NADH and 5 mM 2,2′-bipyridine resulted in a very slow and gradual release of iron from ferritin. The observed iron release kinetics were extremely slow ($5.9 \times 10^{-8}$ M/min for 0.2 μM ferritin and $1.1 \times 10^{-8}$ M/min for 0.4 μM ferritin) and constituted approximately 0.5% of total ferritin iron after 800 min (Fig. 2).

3.2. FMN reduction by NADH

The non-enzymatic reaction of FMN with NADH was performed aerobically in an open cell and followed spectrophotometrically between 300 and 500 nm. Fig. 3 shows that at lower concentrations of NADH (400 μM) and FMN (50 μM), the change of the concentration of NADH at 340 nm over the course of the reaction is $1.56 \times 10^{-6}$ M/min without a simultaneous decrease of the FMN peak at 440 nm indicating that the concentration of FMN did not change despite the consumption of NADH. At higher NADH and FMN concentrations (2 mM and 200 μM, respectively) the rate of NADH disappearance was about 3 times faster ($4.34 \times 10^{-6}$ M/min) while the concentration of FMN remained constant for the first 8 min of the reaction and then dropped about 80% of the initial absorbance value followed by an 8% increase. These results clearly indicate a non-enzymatic reaction between NADH and FMN with rapid re-oxidation of produced FMNH$_2$ by molecular oxygen. For
the lower NADH and FMN concentrations, the re-oxidation of FMNH₂ did not significantly alter the initial concentration of FMN in solution. At higher concentrations of NADH (2 mM) and FMN (200 μM), the depletion of dissolved oxygen resulted in the accumulation of FMNH₂ and a decrease in the concentration of FMN until the rate of FMNH₂ production became equal to the rate of oxygen diffusion to form FMN (Fig. 3B and C). This oxidation–reduction process was corroborated by repeating the experiment in an air-tight septum to prevent diffusion of oxygen into the solution. The kinetics of iron release were monitored by the absorption of the Fe(II)–bipyridine complex at 530 nm.

3.3. Reductive mobilization of iron from ferritin by NADH/FMN

The reductive release of iron from ferritin was studied in buffered solutions containing 0.2 to 1.0 μM holo-HosF (2250 Fe/shell), 2 mM NADH, 200 μM FMN, and 5 mM of 2,2′-bipyridine in 100 mM MOPS, 50 mM NaCl, pH 7.0 and 22 °C. All solutions initially contained dissolved oxygen at a concentration of 220–250 μM. To avoid side reactions from hydrogen peroxide produced from the reaction of FMNH₂ with oxygen, all experiments were performed in the presence of 2650 U/ml catalase. Additionally, the reaction cell was fitted with an air-tight septum to prevent diffusion of oxygen into the solution. The kinetics of iron release were monitored by the absorption of the Fe(II)–bipyridine complex at 530 nm.

Fig. 4 (left) shows the kinetic profile of iron release as a function of ferritin concentrations. In all cases, the iron release kinetics showed biphasic behavior featuring an initial lag phase lasting 30–40 min followed by a second phase of rapid iron release. During the lag period, the small increase in absorption at 530 nm indicates a slow iron release process. The amount of iron released during the lag phase increased with increasing ferritin concentration from 10 μM for 0.2 μM ferritin to 50 μM for 1.0 μM ferritin corresponding to approximately 2% release of total ferritin iron.

Following the lag phase, a brief transition period was observed for which the rates of iron release dramatically increased as shown in the right panel of Fig. 4. At all ferritin concentrations employed in this experiment, the iron release kinetics achieved a maximal rate in less than 3 min after the end of the lag phase and exhibited a similar increase in absorbance change (i.e. 0.008–0.009 per min) at 530 nm corresponding to the release of 9.2–10.3 μM of iron(II) cations per minute. At 0.2 μM ferritin concentration, iron release was essentially
complete in about 1 hour but was considerably slower at the higher concentration of 1.0 μM (Fig. 4).

The kinetics of iron release were further investigated using 0.2–0.4 μM human recombinant heteropolymer ferritin manually loaded with 500 Fe(III)/shell. Similar biphasic iron release profiles to those with HosF were observed (Fig. 5, left). The somewhat higher rates of iron release (i.e. 0.010–0.011 absorption units per minute corresponding to 12–13 μM of iron(II) cations), and the shorter duration of the lag period can be attributed to the 6 °C temperature difference between the two experiments. Furthermore, the kinetic data with the heteropolymer human ferritin sample showed a faster drop in the rate of iron release due to the lower iron content of the sample.

To examine the role of dissolved oxygen, the reductive iron release kinetics from HosF was repeated with the simultaneous monitoring of dissolved oxygen concentration and Fe(II)–bipyridine complex formation at 530 nm. This setup involved the use of a 2.5 ml quartz air-tight reaction cell containing 0.6 μM HosF, 2 mM NADH, 2 mM FMN, and 2 mM 2,2′-bipyridine at pH 7.0. Because the iron release kinetics involve a relatively slow lag phase, all reagents were rapidly mixed in a polypropylene laboratory transport tube from Perfector Scientific and then transferred as a homogeneous mixture to the reaction cell fitted with a septum and the oxygen electrode. The use of a higher concentration of FMN was necessary to accelerate the reaction and to avoid potential errors due to limited time stability of the oxygen electrode.

4. Discussion

Reduction of flavin mononucleotide by NAD(P)H is catalyzed by flavin reductases [32,33] which are expressed in bacterial and mammal cells. However, the enzyme is not commercially available, and the non-enzymatic reaction between NADH and FMN can be conveniently used for modeling the process of iron release in cells [34]. To achieve sufficient rates of iron reduction, relatively high concentrations of NADH and FMN are required. Under these conditions (Figs. 1, 3–5) iron release proceeds rapidly and exhibits a biphasic characteristic with an extremely slow initial iron release step followed by a transition to a much faster step. The transition between these two phases is somewhat rapid and occurs within less than three minutes. As evidenced in Fig. 1, significant iron release starts soon after much of the dissolved oxygen has essentially been depleted. The duration of the lag phase depends on the concentration of dissolved oxygen as well as the concentrations of NADH and FMN used. These results can be easily rationalized considering that essentially all FMNH₂ generated in the reaction between FMN and NADH is re-oxidized back to FMN by dissolved molecular oxygen without an appreciable reductive mobilization of iron from ferritin. This re-oxidation reaction is very fast even at a low concentration of dissolved oxygen suggesting that the rate of FMNH₂ oxidation by oxygen is much higher than that of iron reduction (Scheme I).

![Fig. 4. Left: Reductive mobilization of iron from HosF containing 2250 Fe/shell. Conditions: 0.2–1 μM ferritin, 2 mM NADH, 200 μM FMN, 2 mM 2,2′-bipyridine at pH 7.0 and 22 °C. Right: Change of the iron(II)–bipyridine release rate versus time for different concentrations of HosF.](image)

![Fig. 5. Left: Reductive mobilization of iron from human recombinant heteropolymer ferritin (0.2 and 0.4 μM) loaded with 500 Fe/protein in the presence of 2 mM NADH, 200 μM FMN, 2 mM 2,2′-bipyridine, 2650 U/ml catalase at pH 7.0 and 28 °C. Right: Change of the iron(II)–bipyridine release rate versus time for different concentrations heteropolymer ferritin.](image)
Nevertheless, during the lag phase where dissolved oxygen is still present in solution, iron release from ferritin proceeds at a slow rate and is proportional to the concentrations of ferritin, FMN, and NADH. This rate is approximately ten times higher than that observed in the absence of FMN (Fig. 2). Because the rate of iron release remained constant during oxygen depletion (from ~250 μM to almost zero), it can be assumed that the mechanism of iron release does not involve a direct competition between FMNH₂ oxidation by O₂ and Fe(III) reduction by FMNH₂ and therefore does not involve the accumulation of free FMNH₂ in solution. We anticipate that the initial reduction reaction during the lag phase might involve superoxide radicals [16,17,35] as a result of the reaction of flavin semiquinone (formed via a rapid reaction between FMNH₂ and FMN [27]) with molecular oxygen [36–38] (Scheme I). Alternatively, iron reduction in ferritin might involve a reaction between the FMN-ferritin complex and NADH as previously suggested [25], although other mechanisms are possible. Nonetheless, this process is unlikely to be physiologically relevant because the NADH and FMN concentrations employed in this study (and elsewhere) are almost one order of magnitude higher than cellular concentrations of NAD(P)H (200 μM) and FMN (25 μM) [39]. Extrapolating the observed iron release rates to physiological concentrations of FMN and NADH would yield much lower rates of iron release. However, the possibility that other cellular components and the in-vivo milieu of the cell favor iron release from ferritin even at the low NAD(P)H and FMN cellular concentrations, or that reduced flavins might be delivered to ferritin by chaperones and/or transporters is not ruled out by the present study.

The data in Fig. 1 demonstrate that iron mobilization occurs soon after dissolved oxygen is almost completely depleted from the solution, suggesting that the apparent second order rate constant k₂ (reaction 3) is much higher than that of the iron reduction by FMNH₂ (reaction 2) and that under normoxic conditions iron release from ferritin by FMNH₂ is negligible. However, it is possible that some Fe²⁺ might get re-oxidized by molecular oxygen thus delaying the appearance of the Fe²⁺-bipyridine complex. This result is in agreement with literature values of iron mobilization from HosF (with an iron core of 2300 Fe/shell) by FMNH₂ [10] (10.8 M⁻¹ s⁻¹) and of FMNH₂ oxidation by molecular oxygen [27] (8 × 10⁻⁷ M⁻¹ s⁻¹) whose apparent second order constants differ by about 4 orders of magnitude. When extrapolating to physiological conditions (i.e. up to 500 μM total ferritin iron and 4 μM oxygen [40,41]), the rate of iron release from ferritin would amount to less than 2% of its corresponding rate under oxygen-limited conditions, according to reactions 2 and 3. However, it is conceivable that in-vivo iron reduction by FMNH₂ can still occur to a significant extent at even lower oxygen pressure. Indeed, under conditions of severe hypoxia, considerable increases in the concentration of labile iron has been reported [42], presumably because of an increase in reductive mobilization of iron from reduced flavins and superoxide [43,44] (reactions 2 and 7). A potential site for the rapid reductive mobilization of iron by reduced flavins is the mitochondria, which can operate at an oxygen concentration of less than 1 μM [40]. Interestingly, the rate of iron release from ferritin by glutathione is about 60 times slower than that by FMNH₂ [10]. However, glutathione concentration in cells is about 2 orders of magnitude higher than FMNH₂, which would result in a 3-fold higher rate of iron mobilization overall.

5. Conclusions

Our results demonstrate that the reductive mobilization of iron from ferritin by the non-enzymatic FMN/NAD[P]H system proceeds via two distinct phases. In the presence of oxygen, a very weak reductive mobilization process that may involve superoxide radicals, but not FMNH₂, is observed initially at high oxygen concentrations. When dissolved oxygen concentration is below 5 μM, a rapid reductive mobilization of iron from ferritin mediated by FMNH₂ is observed. Under oxygen-limited conditions, a very rapid phase of iron mobilization by FMNH₂ is observed. Nonetheless, these estimates do not take into account the effects of chaotropes [34] or short peptides [45] on the iron release rates. Further studies of iron mobilization from ferritin by reduced flavins in the presence of these (and other) agents are needed for a better understanding and new perspectives on cellular iron homeostasis.

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References


