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Iron release from ferritin by flavin nucleotides

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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.

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(III) 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 proteasoma 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 $FMNH_2$ can be produced from

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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_2O_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 (~21H- 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 spectrophotopmeters (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 ($\varepsilon = 8650\ M^{-1}\ cm^{-1}$). 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 NADH/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 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 consumption 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 (5.9×10^{-9} M/min for 0.2 μM ferritin and 1.1×10^{-8} M/min for 0.4 μM ferritin) and constituted approximately 0.9% 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 1.56×10^{-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 (4.34×10^{-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

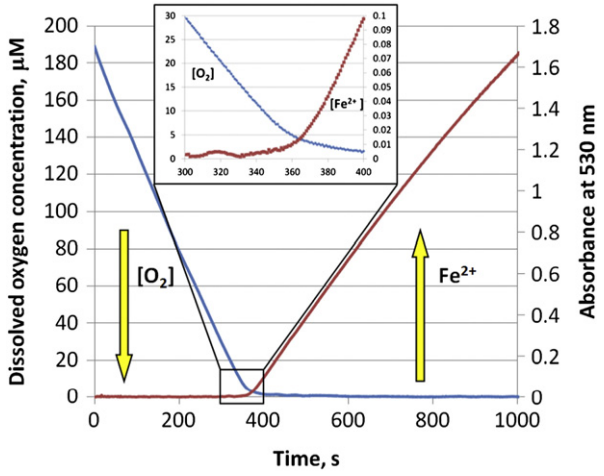

Fig. 1. Simultaneous monitoring of oxygen consumption (blue curve) and Fe(II)–bipyridine complex (red curve) during the reductive release of iron from HosF (0.6 μM) in the presence of 2 mM NADH, 2 mM FMN, and 2 mM 2,2′-bipyridine. During the induction period (lag phase), the reaction between NADH and FMN depletes dissolved oxygen and substantial iron release starts only after complete oxygen removal.