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Releasing iron from ferritin protein nanocage by reductive method: The role of electron transfer mediator

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ABSTRACT

Background: Ferritin detoxifies excess of free Fe(II) and concentrates it in the form of ferrihydrite ($Fe_2O_3{\cdot}xH_2O$) mineral. When in need, ferritin iron is released for cellular metabolic activities. However, the low solubility of Fe (III) at neutral pH, its encapsulation by stable protein nanocage and presence of dissolved O_2 limits *in vitro* ferritin iron release.

Methods: Physiological reducing agent, NADH ($E_{1/2} = -330$ mV) was inefficient in releasing the ferritin iron ($E_{1/2} = +183$ mV), when used alone. Thus, current work investigates the role of low concentration (5–50 μM) of phenazine based electron transfer (ET) mediators such as FMN, PYO - a redox active virulence factor secreted by *Pseudomonas aeruginosa* and PMS towards iron mobilization from recombinant frog M ferritin.

Results: The presence of dissolved O_2, resulting in initial lag phase and low iron release in FMN, had little impact in case of PMS and PYO, reflecting their better ET relay ability that facilitates iron mobilization. The molecular modeling as well as fluorescence studies provided further structural insight towards interaction of redox mediators on ferritin surface for electron relay.

Conclusions: Reductive mobilization of iron from ferritin is dependent on the relative rate of NADH oxidation, dissolved O_2 consumption and mineral core reduction, which in turn depends on $E_{1/2}$ of these mediators and their interaction with ferritin.

General significance: The current mechanism of *in vitro* iron mobilization from ferritin by using redox mediators involves different ET steps, which may help to understand the iron release pathway *in vivo* and to check microbial growth.

1. Introduction

Iron is vital to all organisms owing to its participation in various physiological functions such as electron transfer (ATP synthesis, photosynthesis and N_2 fixation), DNA synthesis and oxygen transport [1–5]. About 10^{-6} M to 10^{-3} M iron is required for all these cellular functions but Fe(III) solubility, under physiological conditions, is only ~10^{-18} M [1]. Ferritins decreases Fe(III) solubility gap inside living cells with protein-coated hydrated ferric oxide minerals ($Fe_2O_3{\cdot}H_2O$) [6]. Ferritins encoded by almost all organisms, are hollow spherical proteins, self-assembled from 24 folded polypeptide subunits [7]. Highly toxic, free Fe(II) are sequestered rapidly (in few msec to sec) through eight hydrophilic 3-fold pores [8–11] and are ferried to the ferroxidase sites *via* conserved transit residues [12], where oxidoreduction reaction takes place [10–16]. Finally these ferric mineral precursors migrate to ferritin central nanocavity for ferrihydrite ($Fe_2O_3{\cdot}xH_2O$) mineral formation [17]. About 2 nm thick, ferritin protein cage keeps the iron mineral safe and in non-toxic form by encapsulating it and prevents unwanted leaking of Fe(II) by cytoplasmic reductants. When required, iron is released in a controlled way for various cellular and metabolic activities [18].

Unlike iron uptake and oxidation/mineralization process that leads

Abbreviations: NADH, β-Nicotinamide adenine dinucleotide; FMN, flavin mononucleotide; PMS, phenazine methosulfate; PYO, pyocyanin; Fz, ferrozine; ROS, reactive oxygen species; ET, electron transfer; $E_{1/2}$, mid-point potential; MOPS, 3-(N-morpholino) propane sulfonic acid; M_{Ox}, oxidized form of mediators; M_{Red}, reduced form of mediators

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to ferritin core formation, mechanism of mineral core reduction/dissolution resulting in iron mobilization is poorly understood [3,19,20]. *In vivo*, what kind of cytoplasmic signals and regulation exists for iron mobilizations from ferritin iron mineral core is not well known. Ferritin protein cages which encapsulate iron mineral are very stable but *in vitro*, selective opening and closing of ferritin pore gates ("gating") are known to accelerate the kinetics of Fe(II) release [1,3,21]. In all these experiments, iron mobilization from ferritin nanocage was performed by reductive approach with physiological electron donor- NADH, 2, 2′-bipyridine (the Fe(II) chelator) and high (0.5–2.5 mM) concentration of ET mediator, FMN. Moreover, the *in vivo* mechanism of iron release by dissolution of mineral core is likely to be carried out by NADH/FMN or GSH [22].

Iron release from ferritin, *in vivo*, *via* NCOA4-mediated ferritinophagy or by proteasomic degradation (ferroprotein mediated) mechanism have provided a new direction [23]. However, these mechanisms are associated with degradation of ferritin protein cage, which may lead to uncontrolled iron release [20,24,25]. The direct removal of Fe(III) from ferritin iron core have also been tried by the non-reductive pathways using chelating ligands (deferiprone and desferrioxamine (DFO)) [26,27]. But the presence of stable ferritin protein nanocage, surrounding the iron mineral not only delayed iron extraction but also required high concentration of the iron (III) chelators.

Similar to ferritin iron core formation (by oxidation of Fe(II)), the core disintegration (by the reduction of Fe(III) mineral) involves electron transfer (ET) steps [28,29]. However, the mechanism of ET and its pathway are yet to be clearly understood [19,22,30]. One of the primary factors on which ET kinetics depends is the mid-point potential difference ($\Delta E_{1/2}$) between donor and acceptor [31]. Presence of suitable mediators can further facilitate the ET process *i.e.* electron relay, by passing electron from electron donors to the acceptors over a long range [3,32,33]. As, mid-point potential of the electron donor (NAD^+/NADH, $E_{1/2} = -330$ mV at pH 7.0) is known, determining the mid-point potential of mineralized ferritin (acceptor) is essential in selecting suitable mediators to facilitate iron release from ferritin core.

Free FMN has been used in the recent past for ferritin iron release studies but at concentrations, invariably higher than normal physiological concentrations [11,21,27,32]. In the current work, we have used low (5–50 μM) concentration of selected phenazine derivatives as ET mediators (Fig. 1): FMN (physiological concentration), PYO (pyocyanin, concentration range during pathogenic condition in sputum) [34,35], and synthetic mediator PMS (phenazine methosulfate). The ET efficacy of these phenazine derivatives were investigated by monitoring the iron release kinetics from mineralized frog M ferritin, initiated by reducing agent, NADH [32,35]. PYO, one of the virulence factor secreted by *Pseudomonas aeruginosa*, the causative agent of lung infection during cystic fibrosis, may act as a suitable ET mediator in the ferritin iron release owing to its excellent redox active properties [34]. An earlier report suggests the ability of PYO as redox active mediator in acquiring iron from transferrin *via* Fe(III) reduction [36]. Moreover, it has been reported that *P. aeruginosa* can acquire iron from mineralized bullfrog M ferritin [34]. Though the mechanism of iron acquisition by *P. aeruginosa* was not well established, but PYO was proposed to be one of the potential factors to be considered. PMS having similar structure (phenazine derivative) but different $E_{1/2}$ value (compared to PYO and FMN) is used to understand the comparative electron relay efficiency and iron release ability of these ET mediators. Herein, we investigate the efficiency of these ET mediators by reporting their relative rate of *in vitro* iron release in the presence and absence of ROS (reactive oxygen species) scavengers, kinetics of NADH oxidation, dissolved O_2 consumption and their interaction on external surface of frog M ferritin. Moreover, efforts have been made to establish the relationship between $E_{1/2}$ of these mediators and their iron release ability from ferritin iron core.

2. Materials and methods

2.1. Materials

Recombinant frog M ferritin protein was expressed in *Escherichia coli* BL21 (DE3) pLysS (Stratagene) and purified as reported [12,37]. NADH [$\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$ at 340 nm], FMN [$\varepsilon = 12.20\ mM^{-1}\ cm^{-1}$ at 450 nm], PMS [$\varepsilon = 26.30\ mM^{-1}\ cm^{-1}$ at 386 nm], $FeSO_4{\cdot}7H_2O$, ferrozine, PYO, SOD, catalase and mannitol were obtained from Sigma Aldrich (Fig. S1).

2.2. Iron mineralization

Fresh $FeSO_4$ solution was prepared in 1 mM HCl, which was then mixed with solution of purified WT frog M ferritin in 100 mM MOPS (pH 7.0) containing 100 mM NaCl, to make mineralized ferritin. After mixing, this solution was incubated for 2 h at room temperature, followed by overnight incubation at 4 °C to complete the mineralization process. Cage concentration was maintained at 2.08 μM containing 1.0 mM of ferritin-caged iron (~480 Fe/cage).

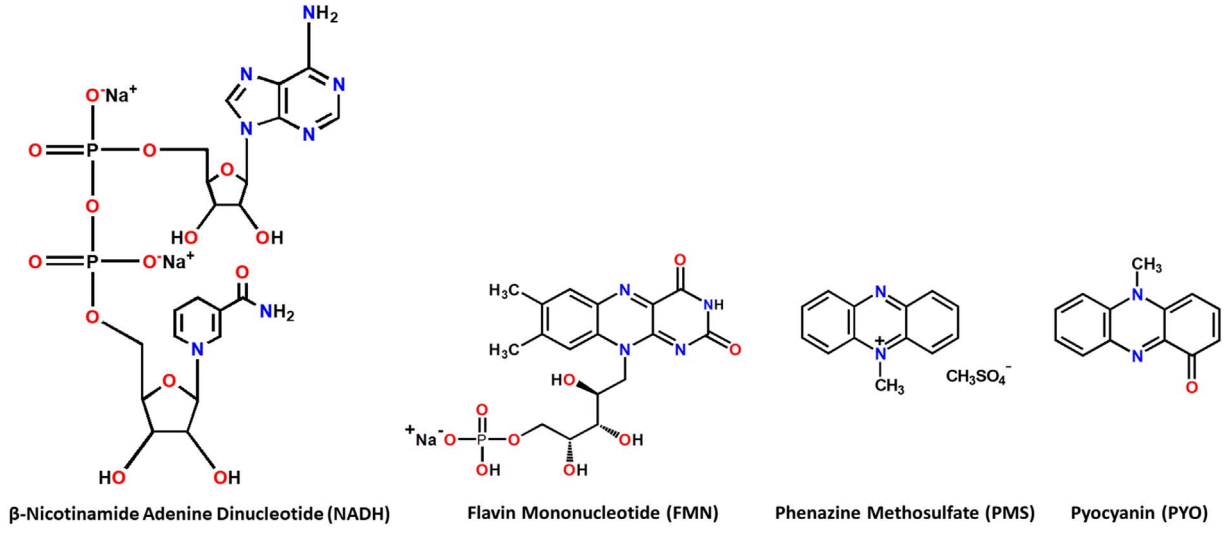

Fig. 1. Structure of electron source (NADH) and electron transfer (ET) mediators (FMN/PMS/PYO) used in the current work.