Supplementary Material (ESI) for Chemical Communications

Oxygen Catalyzed Mobilization of Iron from Ferritin by Iron(III) Chelate Ligands

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Supplementary Materials

1 Synthesis of chelate ligands 3a-d

Chelate ligands **3a**, **3c**, and **3d** were prepared according to J. Gun, I. Ekeltchik, O. Lev, R. Shelkov and A. Melman, *Chem. Commun*., 2005, 5319-5321. Chelate ligand **3b** was prepared according the following procedure:

A solution of 2,4,6-trichlorotriazine (923 mg, 5 mmol) in acetone (10 mL) at -2 °C under vigorous stirring was added dropwise to a solution of isopropanol (285 mg, 5 mmol) and 2,6-lutidine (530 mg, 5 mmol) in acetone (5 mL). The reaction mixture was stirred for 30 min. The solution was allowed to warm to room temperature and was stirred overnight. The reaction mixture was then filtered, the filtrate was poured into crushed ice and filtered again to give 2, 6-dichloro-4-*n*-propyloxy-1,3,5-triazine which was dried and recrystallized from isopropanol (665 mg, 3.2 mmol). To a suspension of 2, 6-dichloro-4-*n*-propyloxy-1,3,5-triazine (624 mg, 3 mmol) in dioxane at 0 ºC (9 ml) was added a solution of MeNHOH hydrochloride (942 mg, 12 mmol) and NaOH (0.54 g, 12 mmol) in water (3 mL). The reaction mixture was stirred for 14 h, evaporated to dryness, and triturated with water to give **3b** (1.5 mmol, 0.35 g).

2 Iron loading into ferritin

Reagent grade ferrous sulfate was obtained from Baker Scientific Inc. (Phillipsburg, NJ) and Mops hemisodium buffer from Research Organics (Cleveland, OH). The heteropolymer H/L ferritin was a gift from Professor Sonia Levi at *Vita-Salute San Raffaele University, Milano, Italy.* The protein was rendered iron free by anaerobic reduction using 55 mM sodium dithionite in 0.1 M Mes (2-(*N*-morpholino) ethanesulfonic acid), pH 6.0, followed by aerobic dialysis against 0.1 M Mops (3-(*N*-morpholino) propanesulfonic acid) at pH 7.0. The apoprotein concentration was determined by its absorbance at 280 nm using a molar absorptivity value of 672000 $M^{-1}cm^{-1}$. Iron was typically loaded into ferritin via two additions of 500 Fe(II) per shell that were followed kinetically at 310 nm where the iron ferritin core absorbs. The Fe(II) solution was prepared in pH 2.0 DI water.

3 Iron mobilization from ferritin in the absence of reducing agents

Purified apo-ferritin was loaded aerobically with 1000 Fe atoms per molecule using two additions of 500 Fe(II)/protein, 15 minutes apart. The iron-loaded protein was checked spectrophotometrically using a molar absorptivity value of ~ 3000 $M^{-1}cm^{-1}$ at 305 nm where the iron-oxo complex mineral core absorbs. The iron release experiments were conducted in 0.1M MOPS, 50 mM NaCl, pH 7.0 in the presence of 100 nM ferritin and 400 uM BHT chelators at a a ratio of 4 BHT per Fe. The kinetics of iron release 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 percent of iron release from ferritin was calculated using experimentally determined UV-Vis molar absorptivity 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.

The anaerobic iron release experiments were performed under the exact conditions but in the absence of oxygen. Oxygen-free protein and BHT solutions were thoroughly deoxygenated overnight and before the experiment using high purity argon gas (99.995%, <5 ppm O_2).

4 Iron mobilization from ferritin in the presence of the physiological reductant NADH/FMN

The reductive release of iron from ferritin consisted of 2 phases: a lag phase followed by a rapid phase. Iron release was triggered in ferritin following the addition of 400 uM BHT ligand, 2 mM FMN, and 2 mM NADH to 0.1 uM holoferritin (1000 Fe(III)/shell) solution in 0.1 M MOPS, 50 mM NaCl pH 7.0. The absorbance of the Fe(III)-BHT complex was followed at 535 nm.

Note that in the absence of NADH/FMN, two kinetic processes are observed following the addition of BHT to holoferritin with the following rate constants: $k_1 \sim 0.06$ min^{-1} and $k_2 \sim 0.008$ min^{-1} (Fig. S1**)**. However, in the presence of NADH/FMN, only one kinetic process is observed following a lag phase over a period of 10-12 min (Fig. S1**)**. The rate constant of this process is similar to that of the rapid phase observed in the absence of NADH/FMN (i.e. $k_1 \sim 0.07$ min^{-1}). After 80 min have elapsed and in the absence of NADH/FMN, about 30 % of the total iron present in ferritin has been released. However, in the presence of NADH/FMN, about 50 % of the iron is released in a matter of 40 min.

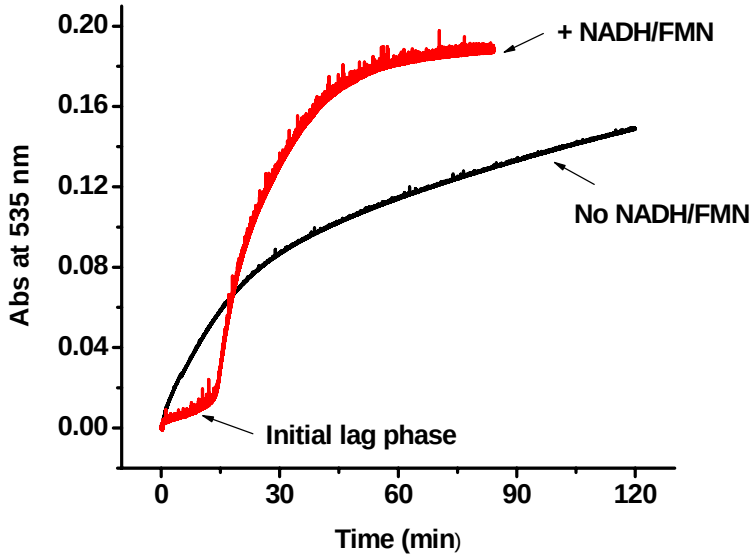

Fig. S1: Kinetic curves of iron release from ferritin in the presence and absence of NADH/FMN.

It is of interest to note that similar iron release kinetics were observed with *Listeria innocua* Dps, a ferritin-like protein, in the presence of NADH/FMN (Bellapadrona, G. et al., *J. Biol. Chem.*, 2009, 284 (28), 19101–19109). When DFO and DFX ligands were used in the presence of the physiological reductant NADH/FMN, similar iron release kinetics to those obtained with BHT were observed and are summarized in Table S1 below:

In the absence of NADH/FMN						
	DFO	% Fe Release	DFX	% Fe Release	BHT	% Fe Release
k1	0.23 min^{-1}		0.20 min^{-1}		0.08 min^{-1}	
k2	0.018 min^{-1}	5 %	0.015 min^{-1}	8 %	0.01 min^{-1}	30 %
In the presence of NADH/FMN						
k	0.15 min^{-1}	20 %	0.15 min^{-1}	50 %	0.08 min^{-1}	50 %

Table S1: Iron release rates from ferritin by BHT, DFO and DFX in the presence and absence of NADH/FMN. Conditions: 0.1 uM holoferritin containing 1000 Fe(III)/shell, 400 uM chelates, 2 mM FMN, 2 mM NADH in 0.1 M MOPS, 50 mM NaCl pH 7.0.

5 Analysis of the iron release kinetics

The initial rates were obtained from the linear A_1 term of a fifth-order polynomial curve fitted to the experimental data, namely $Y= A_0 + A_1t + A_2t^2 + A_3t^3 + A_4t^4 + A_5t^5$ and $dY/dt = A_1 + 2A_2t + 3A_3t^2 + 4A_4t^3 + 5A_5t^4$ (at t = 0, $(dY/dt)_{t=0} = A_1$). We found that a fifth-order polynomial fits better the observed kinetics. Here, t is the time in minutes and Y is the change in absorbance. The rate constants of the second slow phase following the initial rapid phase were obtained by fitting the kinetic curves to a single exponential function of the form:
$y = A_{1*} \exp(x/t_1)$ where A_1 is a pre-exponential factor (amplitude) and t_1 the rate constant.