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# Ferritin-dependent radical generation in rat liver homogenates

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ABSTRACT

The hypothesis of this study was that mammalian ferritin (FER) has the ability of releasing Fe in the tissue to catalyze the generation of free radicals, such as ascorbyl ($A^{\bullet}$) and hydroxyl radical ($^{\bullet}OH$), that might lead to the damage of FER itself. The rat liver homogenates exhibited an electron paramagnetic resonance (EPR) signal with the spectral features ($a_H$ = 1.88 G, $g$ = 2.0054) of $A^{\bullet}$. The addition to the reaction medium of isolated rat liver FER increased by 3-fold the EPR signal, as compared to the recorded value in its absence. Isolated microsomes from rat liver incubated during 10 min showed a signal with the spectral features ($a_H$ = 15 G, $g$ = 2.0062) of $^{\bullet}OH$. The addition of FER in the presence of either ethylenediaminetetraacetic acid (EDTA) or adenosine-5′-triphosphate (ATP) significantly increased the recorded spectra. The labile Fe pool (LIP) in the homogenate was assessed by EPR. The rat liver homogenates exhibited an EPR signal with the spectral features ($g$ = 4.3) of the $Fe^{2+}$ and was significantly increased by the addition of FER (3-fold). The oxidation profile of the isolated FER from rat liver was analyzed after incubation with 10 mM ascorbate ($AH^-$). A drastic increase in the width of the band suggested alterations to the protein structure. The FER content of tryptophan (Trp) and thiols was significantly lower when the incubation was performed in the presence of $AH^-$ as compared to the recorded effect in its absence. The data in tissue homogenates presented here showed that radical generation is associated to FER Fe release, and moreover that the FER protein itself was affected during this process.



## 1. Introduction

The amount of Fe within the cell is carefully regulated in order to provide an adequate level of micronutrient while preventing its accumulation and toxicity. Fe is transported and stored in specific proteins (transferrin, lactoferrin, and ferritin (FER)) (Galatro et al., 2007). Fe is sequestered in FER, the main intracellular Fe-storage protein. However, there are indications that FERs may have other functions in addition to the well assessed role in storing intracellular Fe. FER is a nanobox protein designed to contain and maintain in solution up to four thousands Fe atoms, which otherwise would aggregate in toxic precipitates (Arosio and Levi, 2002). FER is mostly cytosolic but it is also found in mammalian mitochondria and nuclei (Arosio et al., 2008). Higher eukaryotes often have two major FER genes that encode subunits with different properties, named H (heavy) and L (light) and co-assemble to form heteropolymers (Arosio et al., 2008). FER has evolved a molecular design that limits the Fe chemistry within its interior, avoiding nonspecific Fe oxidation and hydrolysis reactions from occurring within the cytosol of the cell. In this way, other proteins and nucleic acids are protected from the toxic effects of labile Fe. However, previous data from *in vitro* chemical studies indicate that fully Fe-loaded proteins can release some Fe (Arouma and Halliwell, 1987), and O'Connell and Peters (1987) reported that Fe-storage proteins release Fe to a range of chelators and reducing agents and that the released Fe promoted both hydroxyl radical ($^{\bullet}OH$) formation in the presence of hydrogen peroxide, and lipid peroxidation in liposomes. Moreover, since FER Fe is reduced effectively by reductants with redox potentials more negative than about −200 mV, superoxide anion ($O_2^-$) (redox potential −300 mV) has the potential to reduce Fe from FER in a hydrofobic environment. Data from Puntarulo and Cederbaum (1993) suggested a role for microsomal cytochrome $P_{450}$ on the release of catalytic active Fe from FER, and the induction of cytochrome $P_{450}$ isozymes increased FER-dependent dye oxidation and chemiluminescence (Puntarulo and Cederbaum, 1996). On the other hand, FER can also stimulate the inactivation of enzymes, such as microsomal glucose-6-phosphatase, by a reaction which appeared to reflect FER stimulated lipid peroxidation resulting in cellular damage (Puntarulo and Cederbaum, 1994).

The cytosolic Fe pool (LIP) is a transitory, catalytically active compartment that has been implicated in cell Fe homeostasis and in metal-induced cytotoxicity. It has been postulated that whereas FER levels provide an index of long-term or cumulative Fe loading, LIP measurements provide an "instantaneous" parameter of Fe availability within hepatocytes (Zanninelli et al., 2002). Moreover, this intracellular pool of low molecular weight Fe compounds

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acts as an intermediate between extracellular Fe and a wide variety of intracellular processes, and it is in equilibrium with Fe in storage and Fe in enzymes (Jacobs, 1977). Transition metals catalyze the formation of oxygen radicals by the Fe-catalyzed Haber–Weiss reactions (Haber and Weiss, 1934). Grady et al. (1989) and Van Eden and Aust (2001) showed the production of •OH during the oxidative deposition of Fe into horse spleen FER. Hydroxyl radical ($E_0$ = 2.31 V) is a potent oxidant that reacts at diffusion limited rates ($10^8$–$10^9$ $M^{-1}$ $s^{-1}$) with almost every type of molecule found in living cells including sugars, amino acids, nucleic acids, lipids and organic acids (Van Eden and Aust, 2001). No reports of direct experimental evidence concerning FER-dependent •OH generation by Fe released from FER in tissues are available.

Non-enzymatic antioxidants are the primary protectants against oxidative damage (Hubel et al., 1997). Ascorbate ($AH^-$) is found in rat liver tissue at a concentration of 0.6 mM (Skrzydlewska and Farbiszewski, 1997), and during its antioxidant action undergoes two consecutive one electron oxidations to dehydroascorbic acid (DHA) with intermediate formation of the ascorbyl radical (A•) (Hubel et al., 1997). A• has a relatively long lifetime (approximately 50 s) compared with other free radicals (Buettner and Jurkiewicz, 1993), and it is easily detectable by electronic paramagnetic resonance (EPR) even at room temperature in aqueous solution. It is well known that transition metals, such as Fe, catalyze the oxidation of $AH^-$ *in vitro* (Buettner and Chamulitrat, 1990), leading to the generation of A•. Moreover, *in vivo* studies showed that the A• radical content increased significantly in plasma of Fe-overloaded rats after Fe-dextran administration (Galleano et al., 2002).

The hypothesis of this study was that mammalian FER has the ability of releasing Fe in the tissue to catalyze the generation of free radicals, such as A• and •OH, that might be a clue factor leading to the damage of FER itself that could have metabolic relevance. The content of radicals and the LIP was assessed by EPR in liver homogenates added with rat liver FER. The damage to FER was analyzed as the oxidation of the protein, the decrease in the content of tryptophan (Trp), the formation of bi-tyrosines and the decrease in the content of thiols in the isolated FER during the release of Fe.

## 2. Materials and methods

### 2.1. Experimental preparations

Male Wistar rats, weighing 170–200 g, were starved overnight. Rat livers were excised and homogenized for 30 s in a blender with 40 mM potassium phosphate buffer (pH 7.4). Liver microsomes were isolated as previously described (Klein et al., 1983) by differential centrifugations, utilizing a buffer containing 0.25 M sucrose–0.01 M Tris, pH 7.4. The microsomes were washed with 125 mM KCl, suspended in 125 mM KCl, and stored at −70 °C.

All reagents were of the highest grade available. Chemicals: ammonium sulfate and sodium chloride were purchased from Mallinckrodt (Paris, KY, USA). β-Mercaptoethanol, potassium phosphate buffer (PBS) and potassium chloride were purchased from Merck (Darmstad, Germany). Western blotts reagents were obtained from Invitrogen, CA, USA. All other reagents were obtained from Sigma–Aldrich Chemical Co. The buffers and the water used to prepare all solutions were passed through columns containing Chelex-100 resin to remove metal contaminants.

### 2.2. Purification of rat liver FER

FER from rat liver was isolated according to Thomas et al. (1985) with modifications. The excised livers were homogenized in a blender in 2 volumes of extraction buffer (25 mM sodium acetate, 20 mM ethylenediamine-tetraacetic acid (EDTA), pH 4.8) at 4 °C. The homogenate was centrifuged for 20 min at 1500 × *g* at 4 °C. The supernatant was filtered through 53 μm nylon filter and added to an equal volume of 50% (w/v) saturated ammonium sulfate with continuous stirring for 15 min, and allowed to settle overnight. The pellet was dissolved in 25 mM sodium acetate buffer with 20 mM EDTA, pH 4.8, and centrifuged at 100,000 × *g* for 1 h 30 min. The pellet was suspended in PBS (0.02 M phosphate buffer, pH 7.4, containing 0.1 M sodium chloride), and the non-solubilized material was removed by centrifugation at 10,000 × *g* for 1 h. The supernatant was centrifuged again at 100,000 × *g* for 90 min. The pellet containing the FER was resuspended in 20 mM PBS buffer (pH 7.4) with 0.1 M NaCl, and the supernatant was then loaded on a Sephacryl S-300 column (1.6 cm × 35 cm), and equilibrated in the same buffer. Fractions were collected and proteins and Fe concentrations were assessed. The Fe-rich fractions were pooled and then concentrated through filters with 30,000 nominal molecular weight limit (Centricon YM30). The FER pellets were dissolved in 40 mM potassium phosphate buffer, 0.15 M NaCl, pH 7.4 and stored a 4 °C until use. Protein content in the sample was measured according to Bradford (1976) using bovine albumin (Sigma) as standard. Total Fe content was determined spectrophotometrically after reduction with thioglycolic acid measuring the absorbance at 535 nm in the presence of bathophenanthroline (Brumby and Massey, 1967). FER purity was verified by an SDS-page (14% (w/v) total acrylamide gel) and ran at room temperature under conditions of constant electrophoretic voltage (150 V) for 60 min. Prior to use, isolated FER samples were incubated on ice in the presence of 10 mM EDTA for 60 min and passed through a Sephadex G-25 column equilibrated with 0.3 M NaCl (pH 7.0) to remove loosely associated Fe (Saito et al., 1985). The Fe content of FER was usually about 2300 atoms per FER shell equivalent to 5 μmol non-heme Fe per mg protein.

### 2.3. Rate of release of Fe from FER

Fe release from purified FER was measured spectrophotometrically using the ferrous chelator ferrozine as a chromophore by measuring the absorbance at 562 nm ($\varepsilon$ = 27.9 $mM^{-1}$ $cm^{-1}$) (Hynes and Coinceanainn, 2002). The reaction mixture (1 ml final volume) contained 100 μg FER, 60 mM Hepes buffer (pH 7.0), 500 μM ferrozine, and reactions were initiated by the addition of $AH^-$ at the indicated concentration. The increase in absorbance at 562 nm was continuously monitored using a Beckman DU Series 7000 diode array spectrophotometer during 20 min at 37 °C.

### 2.4. Determination of A• content in rat liver homogenates by EPR

Rat liver homogenates were incubated for 1 min under the conditions indicated in each assay, then an equal volume of dimethyl sulfoxide (DMSO) was added, and the sample was immediately transferred to a Pasteur pipette for EPR detection. A• spectra were measured at room temperature on a Brucker ECS 106 EPR, equipped with a ER 4102ST cavity, operating at the following conditions: microwave power, 20 mW; microwave frequency, 9.75 GHz; modulation amplitude, 1 G; centerfield at 3487 G; time constant, 163.84 ms; sweep width, 15 G; and modulation frequency, 50 kHz. The quantification was performed using an aqueous solution of 2,2,6,6-tetramethyl piperidine-N-oxyl (TEMPO) introduce into the same sample cell. EPR spectra for both, sample and TEMPO solutions, were recorded at exactly the same spectrometer settings and the first derivative of the EPR spectra were double integrated to obtain the area intensity, calculating the concentration of A• according to Kotake et al. (1996).

### 2.5. Determination of •OH generation by EPR

Isolated microsomes (0.5 mg protein/ml) were incubated in the presence of 100 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 0.5 mM azide, and 40 mM potassium phosphate (pH 7.4). Reactions were started by the addition of 0.5 mM NADPH and the samples were immediately transferred to a Pasteur pipette for direct observation of the reaction in a Brucker ECS 106 EPR spectrometer operating at 9.75 GHz. The EPR spectra were recorded at room temperature as a function of time employing the instrumental settings as follows: microwave power, 20 mW; modulation amplitude, 0.475 G; time constant, 327.68 ms; scan time, 2684 s; and modulation frequency, 50 kHz. The cursor was fixed at 3478 G. The increasing height of the peak was recorded during 8 at 16 min to assess the rate of generation of •OH.

### 2.6. Determination of LIP in rat liver homogenates

The LIP was determined by EPR at 77 K, according to Woodmansee and Imlay (2002) with modifications. Rat liver samples were homogenized in 10 mM Tris–HCl buffer, 120 mM KCl (pH 7.4), and 1 mM deferoxamine (DF). Samples were incubated at room temperature for 10 min, and then frozen with liquid nitrogen in a syringe. EPR spectra were recorded under the following experimental conditions: 9.75 GHz, microwave frequency; 20 mW, microwave power; 50 kHz, modulation frequency; 4.759 G, modulation amplitude, 1600 G, centered field; 81.92 ms, time constant, and 800 G, sweep width.

### 2.7. Determination of the content of carbonyl groups in proteins

Carbonyl groups in proteins were derivatized as described by Levine et al. (1994). Samples were mixed with an equal volume of SDS (12%, w/v) and then with 2 volumes of 20 mM dinitrophenylhydrazine dissolved in 10% (v/v) trifluoracetic acid. This mixture was incubated for 25 min at room temperature, and the reaction was stopped by adding 1.5 sample volumes of 2 M Tris–HCl, 30% (v/v) glycerol. Proteins (0.3 μg per well) were loaded in 12% (w/v) acrylamide concentration mini-gels and ran at room temperature under conditions of constant electrophoretic voltage (150 V) for 1 h. For western blotting, proteins were electro-transferred to nitrocellulose membranes at 120 V for 1 h. Blots were blocked with 5% (w/v) non-fat dry milk dissolved in PBS-T (10 mM potassium phosphate buffer pH 7.4, 150 mM NaCl, 0.1%