

Oxidative Damage of DNA Induced by Ferritin and Hydrogen Peroxide

Jung Hoon Kang

Department of Genetic Engineering, Cheongju University, Cheongju 360-764, Korea. E-mail: jhkang@cju.ac.kr
Received July 7, 2010, Accepted August 23, 2010

Excess free iron generates oxidative stress that may contribute to the pathogenesis of various causes of neurodegenerative diseases. Previous studies have shown that one of the primary causes of increased brain iron may be the release of excess iron from intracellular iron storage molecules. In this study, we attempted to characterize the oxidative damage of DNA induced by the reaction of ferritin with H₂O₂. When DNA was incubated with ferritin and H₂O₂, DNA strand breakage increased in a time-dependent manner. Hydroxyl radical scavengers strongly inhibited the ferritin/H₂O₂ system-induced DNA cleavage. We investigated the generation of hydroxyl radical in the reaction of ferritin with H₂O₂ using a chromogen, 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS), which reacted with ·OH to form ABTS^{•+}. The initial rate of ABTS^{•+} formation increased as a function of incubation time. These results suggest that DNA strand breakage is mediated in the reaction of ferritin with H₂O₂ via the generation of hydroxyl radicals. The iron-specific chelator, deferoxamine, also inhibited DNA cleavage. Spectrophotometric study using a color reagent showed that the release of iron from H₂O₂-treated ferritin increased in a time-dependent manner. Ferritin enhanced mutation of the *lacZ* gene in the presence of H₂O₂ when measured as a loss of α -complementation. These results indicate that ferritin/H₂O₂ system-mediated DNA cleavage and mutation may be attributable to hydroxyl radical generation *via* a Fenton-like reaction of free iron ions released from oxidatively damaged ferritin.

Key Words: Ferritin, DNA, Hydroxyl radical, Mutation

Introduction

Iron is an element essential to cellular metabolism.¹ As long as iron is bound to ferritin, cytotoxic reactions are not expected. However, when iron is improperly regulated or released from ferritin, it is potentially toxic and can lead to cell death. Iron is one of the most transition metals involved in hydroxyl radical formation, owing to its interaction with hydrogen peroxide through Fenton-type reactions.² Mammalian ferritin is a large, iron-storage heteropolymer composed of two equivalent subunit types, light (FTL) and heavy (FTH) polypeptides, which are expressed in most kinds of cells.³⁻⁶ Although both types of polypeptide subunit share a high degree of conformational similarity, they play diverse functional roles. The FTH subunit has a potent ferroxidase activity that catalyzes the oxidation of ferrous iron, whereas the FTL subunit plays important roles in iron nucleation and protein stability, giving ferritin the dual functions of iron detoxification and iron storage.³

It has been reported that iron can be released from ferritin by various exogenous,⁷⁻¹⁰ and endogenous substances *via* reductive mechanisms.^{11,12} If iron is released from ferritin, low molecular iron complexes may undergo redox reactions resulting in cytotoxic damage of macromolecules.^{13,14} Oxidants, including H₂O₂, have been considered mostly as damaging entities that mediate pathogenic processes. H₂O₂ has been implicated in ischemia and reperfusion within the brain,¹⁵ cancer¹⁶ and neurodegenerative disease.¹⁷

This study found that the ferritin/H₂O₂ system resulted in DNA strand breakage. The results indicated that ferritin, as a source of iron, might facilitate oxidative DNA damage.

Experimental Section

Materials. pUC19 plasmid DNA was prepared and purified

from *E. coli* cultures by using QIAGEN plasmid kit (Santa Clara, USA). Ampicillin, bathophenanthroline sulfonate, deferoxamine and ethidium bromide were purchased from Sigma Chemical Co. (St. Louis, MO). Ferritin was purchased from Calbiochem (Darmstadt, German). The commercial equine spleen ferritin was purified by gel filtration chromatography by using Superose 6 FPLC column (Pharmacia, Sweden) for a further purification. Chelex 100 resin (sodium form) was obtained from Bio-Rad. All solutions were treated with Chelex 100 resin to remove traces of transition metal ions.

Analysis of DNA cleavage. DNA single strand breakages were assayed by measuring the conversion of supercoiled plasmid DNA (form I) to nicked circular DNA (form II) and linear DNA (form III). pUC19 DNA (0.5 - 1.0 μ g) in 10 mM potassium phosphate buffer (pH 7.4) was incubated for 3 h at 37 °C with different concentrations of H₂O₂ and ferritin in a total volume of 20 μ L. The reaction was stopped at -80 °C. The loading buffer (0.25% bromophenolblue, 40% sucrose) was added and samples analyzed by electrophoresis in 0.8% agarose in TBE buffer (2 mM EDTA, 89 mM boric acid and 89 mM Tris at pH 8.3). The gel was stained with ethidium bromide. Bands of DNA were detected and photographed under UV light in a dark room.

Measurement of hydroxyl radical. ·OH was measured with a chromogen, 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS).¹⁸ ABTS is water-soluble and has a strong absorption at 340 nm with a molar extinction coefficient ϵ_{340} of $3.66 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. On oxidation, ABTS forms a stable blue-green product presumed to be the cation radical, ABTS^{•+} is conveniently followed at λ_{max} at 415 nm ($\epsilon_{415} = 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).¹⁹ The assay mixture contained 10 mM potassium phosphate buffer (pH 7.4) and 50 μ M ABTS and 0.1 mM H₂O₂ and 1.0 μ M protein in a total volume of 1 mL. The reaction was initiated by addition of H₂O₂ and the increase in absorbance at 415 nm was measured with a UV-vis spectrophotometer (Shimadzu 1601).

Determination of free iron concentration. The concentration of iron ions released from oxidatively damaged ferritin was determined by using a bathophenanthroline sulfonate in the method described previously.²⁰ The reaction mixture contained 1 μ M ferritin and 1 mM H_2O_2 and a 10 mM potassium phosphate buffer (pH 7.4) in a total volume of 0.5 mL. The reaction was incubated for various periods at 37 °C. After the incubation, the mixture was then placed into an Ultrafree-MC filter and centrifuged at 13,000 rpm for 1 h. The colorimetric reagent was added into the filtrate and analyzed by a UV-vis spectrophotometer (Shimadzu, UV-1601) at 535 nm. The final concentrations of the color reagent were 1% ascorbate, 0.02% bathophenanthroline sulfonate and 1% acetic acid-acetate buffer (pH 4.5).

Mutagenicity assay. Plasmid DNA was transformed in *Escherichia coli* DH5 α competent cells. The treated plasmid pUC19 carrying the *lacZ* gene with or without 1 μ M ferritin and 0.1 mM H_2O_2 was incubated with 50 μ L of competent cells at 0 °C for 10 min. SOC medium was added, and the cells were incubated at 37 °C for 1 h. Transformed cells were spread on LB agar plates containing 0.8 mg of X-gal, 2 mg of IPTG and 0.01% ampicillin. The plates were inverted and incubated for 24 h at 37 °C before counting colonies.

Statistical analysis. Values are expressed as the means \pm S.D of three to five separate experiments. The statistical differences between the means were determined by the Student *t*-test.

Results and Discussions

When pUC19 DNA was incubated with 0.1 mM H_2O_2 or 1 μ M ferritin alone, it did not cause significant strand breakages (Fig. 1 lanes 1-10). However, a substantial increase in the proportion of nicked circular form (form II) and linear form (form III) occurred with concomitant loss of the supercoiled one (form I) in a time-dependent manner when DNA was incubated in a mixture of H_2O_2 and ferritin (Fig. 1, lanes 11-15). This indicates that both ferritin and H_2O_2 were required to produce strand breaks in DNA. Deoxyribose and ribose are fragmented by $\cdot OH$, yielding dozens of products. All positions are susceptible to hydrogen abstraction by $\cdot OH$, forming carbon radicals. In the presence of O_2 these concert rapidly to sugar peroxy radicals, which undergo a series of reactions including disproportionate,

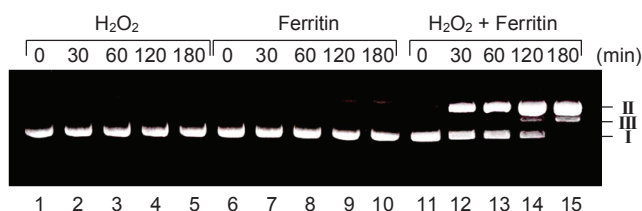


Figure 1. DNA cleavage after incubation with H_2O_2 and ferritin. pUC 19 DNA (1 μ g) was incubated with H_2O_2 and ferritin in a 10 mM potassium phosphate buffer (pH 7.4) at 37 °C during various incubation periods. The reaction mixture contained 1 mM H_2O_2 alone (lane 1-5), 10 μ M ferritin alone (lane 6-10), 1 mM H_2O_2 and 10 μ M ferritin (lane 11-15). Reaction was stopped by -80 °C deep freezing. Loading buffer was added to the samples and analyzed by electrophoresis in 0.8% agarose. I, II and III indicate the position of the supercoiled, circular and linear DNA plasmid forms, respectively.

rearrangement, elimination of water and C-C bond fragmentation, to yield a variety of carbonyl products. Some sugar products remain within DNA or constitute end groups of broken DNA strands, whereas others are released.²¹ It has been reported that H_2O_2 generates ROS through redox cycling that can be facilitated by transition metal ions, such as copper and iron.² The present result suggested that ferritin might facilitate H_2O_2 -induced DNA damage.

The role of free radicals in DNA damage caused by the ferritin/ H_2O_2 system was studied by examining the protective effect of free radical scavengers. When plasmid DNA was incubated with H_2O_2 and ferritin in the presence of azide, formate, mannitol, ethanol, and thiourea at 37 °C for 3 h, all scavengers significantly prevented DNA cleavage (Fig. 2). The sugar alcohol mannitol, azide and ethanol often used hydroxyl radical scavengers in laboratory experiments, but these scavengers can react with other species of oxygen radicals.² In this study, $\cdot OH$ generation occurring during the reaction of ferritin with H_2O_2 was assessed by using a chromogen, 2,2'-azino-bis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS), which reacts with $\cdot OH$ to form $ABTS^{+ \cdot}$.^{22,23} When ABTS was incubated with ferritin and H_2O_2 , $ABTS^{+ \cdot}$ formation monitored for 30 min increased in a time-dependent manner (Fig. 3). However $ABTS^{+ \cdot}$ formation did not occur when ABTS was incubated with 1 μ M ferritin or 0.1 mM H_2O_2 alone. The result suggests that $\cdot OH$ may participate in the ferritin/ H_2O_2 -mediated DNA cleavage.

The H_2O_2 *in vivo* is probably a direct product of O_2^- dismutation and various oxidase reactions. The rates of H_2O_2 and O_2^- formation under physiological conditions have been measured. Considering the volume actually used and the amount of products formed, one obtains a rate 90 μ M H_2O_2 /min in liver²⁴ and 340 μ M O_2^- /min in glucose fed *Escherichia coli* cells.²⁵ The local concentration of H_2O_2 in the immediate vicinity of SOD must be much larger than these values. The production of O_2^- will be much larger as the concentration of oxygen increases during exposure to hyperoxia or when the respiratory chain becomes inhibited leading to an increased concentration of reducing equivalents.²⁶ Recently it has been reported that hyperoxia induced H_2O_2 production increased in human U87 glioblastoma

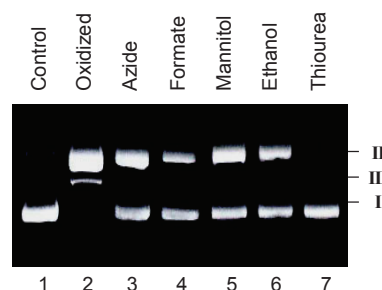


Figure 2. The effect of free radical scavengers on the DNA cleavage by the reaction of ferritin with H_2O_2 . pUC 19 DNA was incubated with 1 mM H_2O_2 and 10 μ M ferritin in a 10 mM potassium phosphate buffer (pH 7.4) at 37 °C for 3 h, in the absence and the presence of 20 mM scavengers at 37 °C. Lane 1, plasmid DNA alone; lane 2, no addition of scavenger; lane 3, azide; lane 4, formate; lane 5, mannitol; lane 6, ethanol; lane 7, thiourea. Agarose gel electrophoresis was performed in 0.8% agarose. I, II and III indicate the position of the supercoiled, circular and linear DNA plasmid forms, respectively.

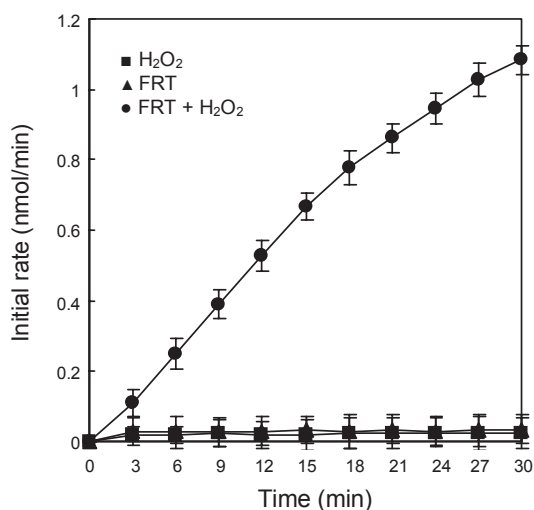


Figure 3. The rates of hydroxyl radical formation determined by using a chromogen, ABTS. 50 μ M ABTS was incubated with 0.1 mM H₂O₂ and 1 μ M ferritin in a 10 mM potassium phosphate buffer (pH 7.4) at 37 °C during various incubation periods. The reaction mixture contained 0.1 mM H₂O₂ alone (■), 1 μ M ferritin alone (▲), 0.1 mM H₂O₂ and 1 μ M ferritin (●).

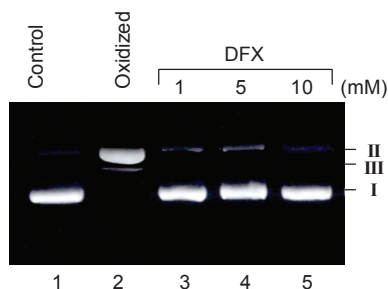


Figure 4. The effect of an iron chelator on the DNA cleavage by the reaction of ferritin with H₂O₂. pUC 19 DNA was incubated with 10 μ M ferritin and 1 mM H₂O₂ in potassium phosphate buffer (pH 7.4) at 37 °C for 3 h in various concentrations of deferoxamine (DFX). Lane 1, plasmid DNA alone; lane 2, no addition of DFX; lane 3, 1 mM DFX; lane 4, 5 mM DFX; lane 5, 10 mM DFX. Agarose gel electrophoresis was performed in 0.8% agarose. I, II and III indicate the position of the supercoiled, circular and linear DNA plasmid forms, respectively.

cells.²⁷ Thus, at least 0.1 mM/min H₂O₂ will be produced continuously under physiological conditions and at a much higher rate under adverse conditions such as hyperoxia or ischemia and reperfusion.

Trace metals such as copper and iron, which are present in biological systems, may interact with hydrogen peroxide, and which causes damage biological molecules including DNA.²⁸⁻³³ In cultured cells, raising the level of iron in the culture medium leads to increases in steady-state levels of oxidative DNA damage.³⁴ Previous studies have suggested that iron ions could stimulate a Fenton-like reaction to produce hydroxyl radicals, which mediates DNA strand breakage.^{35,36} We investigated the participation of iron ions in the H₂O₂/ferritin-mediated DNA strand breakages. When DNA reacted with H₂O₂ and ferritin in the presence of the iron-specific chelator, deferoxamine, DNA strand breakage was significantly inhibited (Fig. 4). The results indicated that iron ions were involved in the DNA cleavage by

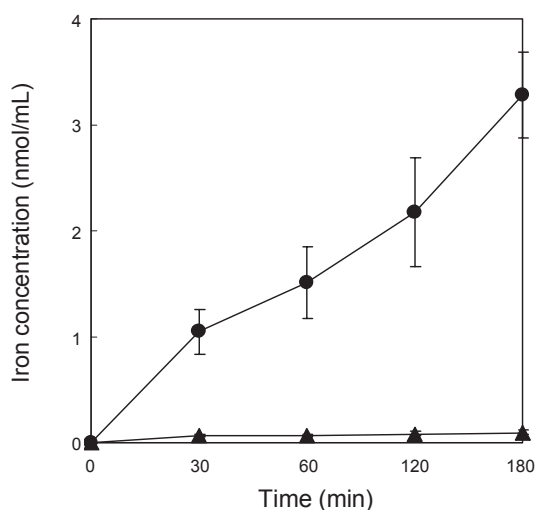


Figure 5. The iron release in the ferritin and H₂O₂ system. 1 μ M Ferritin was incubated with (●) or without (▲) 1 mM H₂O₂ in a 10 mM phosphate buffer at pH 7.4. After incubation, the mixture was then placed into Ultrafree-MC filter and centrifuged at 13,000 rpm for 1 h. The colorimetric reagent was added into the filtrate for analysis by UV-vis spectrophotometry (Shimadzu, UV-1601) at 535nm. The final concentrations of the color reagent were 1% ascorbate, 0.02% bathophenanthroline sulfonate and 1% acetic acid-acetate buffer (pH 4.5).

the ferritin/H₂O₂ system.

H-ferritin subunits have an active ferroxidase site and occur in multiple forms, in humans, animals, plants and bacteria. L-ferritin subunits have a degenerate ferroxidase site and the gene duplication to encode L-ferritin subunits is found only in vertebrate animals. It was shown that the co-presence of both subunits in hybrids make the molecule more efficient for iron incorporation, because of the combined action of the ferroxidase centers on H-chains and of the effective nucleation centers on L-chains.³⁷ X-ray crystallographic data of the mouse L-chain showed that a metal-binding site on the cavity surface, involving residues Glu57, Glu60, Glu61, and Glu64, and in the hydrophilic 3-fold channel involving His118, Glu134, Asp131 and His136³⁸ In the present study, the incubation of H₂O₂ and ferritin led to the release of iron from the protein (Fig. 5). Thus, it is suggested that iron binding sites were modified during the reaction of ferritin with H₂O₂. Consequently, iron became almost free from the ligand and was released from the oxidatively-damaged protein.

It has been previously reported that the mutagenic spectrum of oxygen free radicals is produced by the aerobic incubation of single-strand M13mp2 DNA with iron.³⁹ The biological consequences of DNA damage induced by ferritin/H₂O₂ were investigated. pUC19 plasmid DNA was treated with or without ferritin and H₂O₂. Damaged DNA was transfected into *E. coli* (DH5 α) competent cells, and mutant cells within the nonessential *lacZ* α -gene for β -galactosidase were identified by decreased α -complementation. *E. coli* harboring active β -galactosidase produces dense blue colonies, whereas mutation within the *lacZ* α segment of pUC19 plasmid DNA induced the expression of substantially less active β -galactosidase and yielded light blue or white colonies. The frequency of mutants obtained with ferritin/H₂O₂ was approximately six-fold greater than that ob-

Table 1. Mutagenicity of ferritin/H₂O₂ treated pUC19 plasmid in *E. coli*

Treatment	Relative mutation frequency (fold) ^a
pUC19	1.0
pUC19 + H ₂ O ₂	1.5 ± 0.2
pUC19 + ferritin	1.9 ± 0.4
pUC19 + ferritin/H ₂ O ₂	5.9 ± 0.6

^aMutation frequency was calculated as the ratio between the mutant colonies and total colonies. The results are expressed as a relative mutation frequency and represent the mean ± S.D. from triplicate measurements.

tained with untreated DNA (Table 1). Our results indicated that a multiplicity of DNA lesions produced by the ferritin/H₂O₂ system *in vitro* is promutagenic and could be a source of spontaneous mutations.

In conclusion, the present results indicated that DNA cleavage and mutation were induced by the reaction of ferritin with H₂O₂ involving hydroxyl radicals that were generated by the Fenton-like reaction. Our study suggested that DNA damage by the ferritin/H₂O₂ system could be relatively favored in diseases where ferritin concentration is elevated.

References

- Beard, J. L.; Connor, J. R. *Annu. Rev. Nutr.* **2003**, *23*, 41.
- Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, 4th ed.; Oxford University Press: UK, 2007.
- Harrison, P. M.; Arosio, P. *Biochim. Biophys. Acta.* **1996**, *1275*, 161.
- Theil, E. C.; Matzapetakis, M.; Liu, X. *J. Biol. Inorg. Chem.* **2006**, *11*, 803.
- Theil, E. C. *J. Nutr.* **2003**, *133*, S1549.
- Liu, X.; Theil, E. C. *Acc. Chem. Res.* **2005**, *38*, 167.
- Monterio, H.; Ville, G.; Winterbourn, C. *Free Radic. Biol. Med.* **1989**, *6*, 587.
- Lapenna, D.; de Gioia, S.; Mezzetti, A.; Ciofani, G.; Consoli, A.; Marzio, L.; Cuccurullo, F. *Am. J. Respir. Crit. Care Med.* **1995**, *151*, 431.
- Linert, W.; Herlinger, E.; Jameson, R. F.; Kienzl, E.; Jellinger, K.; Youdim, M. B. *Biochim. Biophys. Acta* **1996**, *1316*, 160.
- Double, K. L.; Maywald, M.; Schmittle, M.; Riederer, P.; Gerlach, M. *J. Neurochem.* **1998**, *70*, 2492.
- Monterio, H.; Winterbourn, C. *Biochem. J.* **1988**, *256*, 923.
- Boyer, R.; Grabill, T.; Petrovich, R. *Anal. Biochem.* **1988**, *174*, 17.
- Halliwell, B.; Gutteridge, J. M. *Mol. Aspects Med.* **1985**, *8*, 89.
- Gotz, M. E.; Kunig, G.; Riederer, P.; Youdim, M. B. *Pharmacol. Ther.* **1994**, *63*, 37.
- Hyslop, P. A.; Zhang, Z.; Pearson, D. V.; Phebus, L. A. *Brain Res.* **1995**, *671*, 181.
- Gius, D.; Spitt, D. R. *Antioxid. Redox. Signal.* **2006**, *8*, 1249.
- Behl, C.; Davis, J. B.; Lesley, R.; Schubert, D. *Cell.* **1994**, *77*, 817.
- Radi, R.; Thomson, L.; Rubbo, H.; Prodanov, E. *Arch. Biochem. Biophys.* **1991**, *288*, 112.
- Childs, R. E.; Bardsley, W. G. *Biochem. J.* **1975**, *145*, 93.
- Pieroni, L.; Khalil, L.; Charlotte, F.; Poynard, T.; Piton, A.; Hainque, B.; Imbert-Bismut, F. *Clin. Chem.* **2001**, *47*, 2059.
- von Sonntag, C. *Free-Radical-Induced DNA Damage and Its Repair*; Springer: Basle, 2006.
- Wolfenden, B. S.; Wilson, R. L. *J. Chem. Soc. Perkin Trans. II* **1982**, 805.
- D'Aquino, M.; Dunster, C.; Wilson, R. L. *Biochem. Biophys. Res. Commun.* **1989**, *161*, 1199.
- Boveries, A.; Oshino, N. *Biochem. J.* **1972**, *128*, 617.
- Imlay, J. A.; Fridovich, I. *J. Biol. Chem.* **1991**, *266*, 6957.
- Turrens, J. F.; Beoni, M.; Brilla, J.; Chavez, U. B.; McCord, J. M. *Free. Radic. Res. Comms.* **1991**, *12*, 681.
- D'Agostino, D. P.; Olson, J. E.; Dean, J. B. *Neuroscience* **2009**, *159*, 1011.
- Goldstein, S.; Czapski, G. *Free Radic. Res. Commun.* **1987**, *51*, 693.
- Gutteridge, J. M.; Halliwell, B. *Biochem. Pharmacol.* **1982**, *31*, 2801.
- Imlay, J. A.; Chin, S. M.; Linn, S. *Science* **1988**, *240*, 640.
- Prutz, W. A. *Radiat. Environ. Biophys.* **1984**, *23*, 7.
- Kang, J. H. *Bull. Korean Chem. Soc.* **2006**, *27*, 1891.
- Kang, J. H. *Bull. Korean Chem. Soc.* **2007**, *28*, 2329.
- Zastawny, T. H. *Free Radic. Biol. Med.* **1995**, *18*, 1013.
- Tachon, P. *Free Radic. Res. Commun.* **1989**, *7*, 1.
- Tachon, P. *Free Radic. Res. Commun.* **1990**, *9*, 39.
- Santambrogio, P.; Levi, S.; Cozzi, A.; Rovida, E.; Albertini, A.; Arosio, P. *J. Biol. Chem.* **1993**, *268*, 12744.
- Granier, T.; Langlois d'Estainot, B.; Gallois, B.; Chevalier, J. M.; Precigoux, G.; Santambrogio, P.; Arosio, P. *J. Biol. Inorg. Chem.* **2003**, *8*, 105.
- McBride, T. J.; Preston, B. D.; Loeb, L. A. *Biochemistry* **1991**, *30*, 207.