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Coordinating responses to iron and oxygen stress with DNA and mRNA promoters: The ferritin story

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Abstract Combinations of DNA antioxidant response element and mRNA iron responsive element regulate ferritin expression in animals in response to oxidant and iron stress, or normal developmental signals. Ferritins are protein nanocages, found in animals, plants, bacteria, and archaea, that convert iron and oxygen to ferric oxy biominerals in the protein central cavity; the mineral traps potentially toxic reactants and concentrates iron for the future synthesis of other iron/heme proteins. Regulatory signals and the nanocage gene products are the same throughout biology, but the genetic mechanisms, DNA versus DNA + mRNA, vary. The number of genes, temporal regulation, tissue distribution in multicellular organisms, and gene product size (maxiferritins have 24 subunits and mini-ferritins, or Dps proteins, have 12 subunits and are restricted to bacteria and archaea) suggest an overwhelming diversity and variability. However, common themes of regulation and function are described which indicate not only that the three-dimensional protein structure and the functions of the ferritins are conserved, but also that broad features of genetic regulation are conserved relative to organismal and/or community needs. The analysis illustrates the centrality of the ferritins to life with iron and oxygen and models how Nature harnesses potentially dangerous chemistry for biology.

Keywords Oxygen · Ferritin · Dps protein · Antioxidant response (ARE) genes · mRNA (IRE) regulation

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Introduction

Iron has been central to metabolism since the beginning. In contrast, the harnessing of dioxygen reactions to increase the efficiency of energy metabolism is a more recent event. Side reactions between iron and dioxygen have two products incompatible with normal metabolism: oxy radicals, such as hydroxyl radicals that damage many biomolecules such as nucleic acids and lipids, and iron that precipitates (''rusts'') and/or participates in chain reactions called Fenton chemistry. Other metals important in biology, such as copper, have similar reactions with oxygen, but the copper product is soluble and is also present at much lower concentrations than iron in living cells. Ferritins are central to the effective control of

iron and dioxygen chemistry (Lewin et al. 2005; Liu and Theil 2005). The soluble ferritin protein nanocages form a ferric oxy biomineral in the central cavity, consuming dioxygen (and sometimes peroxide) in the process and reversibly concentrating iron.

The protein nanocages that form the ferritin family are a unique combination of activities and structures found in several protein classes, and appear to be evolutionarily ancient; conserved ferritin structures occur from archaea to humans. Remarkably, the overall amino acid sequences can vary as much as 80% with the folded active structures conserving the positions of key amino acid residues in three-dimensional space (Liu and Theil 2005). Three protein families share structures with the ferritin family: di-iron oxygenases, gated ion channels, and spherical viruses.

Di-iron oxygenases

Di-iron oxygenases use the chemistry of two iron atoms with dioxygen during the catalytic cycle. In the classical reaction, electrons are transferred from an iron cofactor in the diferrous state to dioxygen, which is cleaved with activation of the cofactor. Examples include methane monooxygenase, ribonucleotide reductase, and stearoyl-acyl carrier protein delta 9 desaturase (Liu and Theil 2005). In the case of the ferritins, the two ferrous ions are substrates, rather than part of a cofactor site, and react with either dioxygen or hydrogen peroxide to initiate biomineralization by producing diferric oxy mineral precursors.

Ion channels

Gated ion channels alter concentrations of ions such as Na, Ca, and K or neutral molecules such as glucose on either side of a membrane. Examples include voltage gated and acetylcholine gated channels. In ferritin, two sets of amino acids, conserved in microorganisms, higher plants and animals, control (''gate'') the reversible folding/unfolding of a channel formed at the junction of three subunits to increase or decrease access of reductants to the ferritin mineral, and to change rates of removing iron from ferritin; the biological molecules that control ferritin gates are not fully identified but urea concentrations in the physiological range (1–10 mM) alter ferritin pore folding and iron removal rates (Liu et al. 2003).

Spherical viruses

Spherical protein nanocages occur in a number of small viruses, such as parvovirus and satellite tobacco necrosis virus, and share high thermal and chemical stability with the ferritins. However, ferritin nanocages have the relatively unusual property of containing only α-helices, built from 12 or 24 subunits folded in four helix bundles, and have a very large central cavity that is stable whether filled with biomineral or ''empty,'' that is, filled with buffer. Ferritins with 12 subunits are called mini-ferritins or Dps proteins, reflecting their initial discovery in *Escherichia coli* as DNA protection during starvation proteins (Almiron et al. 1992; Grant et al. 1998), and have outer diameters of ~10 nm with central cavities of ~5 nm in diameter, ~10% of the total volume. Mini-ferritins are found in bacteria and archaea. Ferritins with 24 subunits are called maxi-ferritins, have outer diameters of ~12 nm with central cavities of ~8 nm in diameter, ~30% of the total volume. Maxi-ferritins are found in bacteria, and in many cells of plants and animals where multigene families lead to cell specific ferritin protein sequences (Grotz and Guerinot 2002; Liu and Theil 2005).

Ferritin regulation in animals

Transcription

Genetic regulation of ferritin has been more extensively studied in animals, compared to higher plants and bacteria, because of abnormalities associated with genetic diseases such as hereditary hemochromatosis, certain cataracts, several hemoglobin mutations and adult-onset basal ganglia disease reviewed in (Hentze et al. 2004; Hintze and Theil 2006). Until recently, the emphasis in studies of ferritin regulation has been on ferritin mRNA translation, because of the unusual linkage, through a combinatorial array of

noncoding RNA structures, to a number of other genes of iron metabolism (Theil and Eisenstein 2000). In fact, ferritin gene transcription, the better understood mode of regulation for most genes, has been much less studied in animals, although it is the sole step known for genetic regulation of ferritin in plants (Lescure et al. 1991; Kimata and Theil 1994) and bacteria (Andrews 1998; Cartron et al. 2006). Such studies of ferritin in mRNA translation have emphasized the role of ferritin in responding to the potential damage and stress of excess iron.

Recent studies, by contrast, show that the sensitivity of ferritin gene transcription to inducers of the antioxidant response was large (Torti and Torti 2002), and greater by as much as 80-fold compared to iron (Hintze and Theil 2005), even in animals. Older experiments, where iron effects were shown to induce ferritin gene transcription, required very high and/or chronic exposure to iron (Dickey et al. 1987; Leggett et al. 1993); in retrospect, it appears that the effects of chronic exposure to higher levels of iron reflected oxidative damage initiated by iron, rather than a direct response to iron.

Classical antioxidant response inducers such as t-butylhydroquinone target ferritin gene transcription as effectively as the genes for thioredoxin reductase 1 and quinone reductase. All three genes share a transcription element, the antioxidant response element (ARE) (Torti and Torti 2002; Nguyen et al. 2003; Hintze and Theil 2005). ARE elements are recognized by Bach1 and maf protein transcription regulators (Kitamuro et al. 2003; Nguyen et al. 2003), which when bound to each other decrease transcription. Heme also induces transcription of all three genes (Hintze and Theil 2005). Bach1 binds maf in the absence of heme (Sun et al. 2004). Maf binding is disrupted by heme binding to Bach1; maf is a basic, leucine zipper protein in the AP1 family of transcriptional regulators. Gene specific variations in the ARE sequence influence the Bach1/DNA interaction (K. J. Hintze and E. C. Theil, unpublished observations). Rather than ferritin causing oxidative damage, as is sometimes supposed because of the reaction of iron and dioxygen, ferritin is actually part of the mechanism cells use for self-protection (Balla et al. 1992; Torti and Torti 2002; Hintze and Theil 2006) from oxidants, by sequestering both iron and dioxygen reaction products as a solid mineral.

Translation

The control of ferritin mRNA by cellular iron has been known for decades (Zahringer et al. 1976; Shull and Theil 1982). A specific family of mRNA structures, iron regulatory elements (IREs) (Harrell et al. 1991; Addess et al. 1997; Gdaniec et al. 1998) are required for binding of repressor proteins called IRP1 and IRP2. Each natural IRE member binds IRP proteins with different stabilities in vitro (Ke et al. 1998; Gunshin et al. 2001; Erlitzki et al. 2002), that can be detected in vivo (Ke and Theil 2002). IRE RNA structure has a larger effect on IRP2 binding, compared to IRP1. The IRP/IRE complex blocks ribosome binding in ferritin H, ferritin L, mt-aconitase, ferroportin, and some aminolevulinate synthase mRNAs, or blocks nuclease attack on TfR1 mRNA (Theil and Eisenstein 2000; Hentze et al. 2004).

IRP proteins, much more studied than the IRE RNA, are related to aconitases. An iron signal for IRP1 is direct and is a 4Fe–4S cluster (Rouault and Tong 2005), which changes the classical aconitase structure considerably, and changes apo-IRP1 conformation as well (Dupuy et al. 2006). However, iron signals for IRP2, which are as yet unidentified, cannot be the FeS cluster because of amino acid substitutions at the FeS binding site. Both IRP1 and IRP2 are regulated by iron-induced proteasomal degradation (Pantopoulos 2004; Clarke et al. 2006), which for IRP2 is also oxygen dependent (anoxia) (Hanson et al. 2003). Thus, the mechanism for IRP2, and some IRP1 degradation, in response to iron is likely to be indirect, as is the effect of NO and hydrogen peroxide (Pantopoulos 2004). Heme binds directly to IRP1 (Goessling et al. 1994) and IRP2 (Goessling et al. 1998; Jeong et al. 2004), changing protein degradation rates; whether indirect effects of heme also signal degradation is unknown. IRE/IRP complexes influence other protein/RNA interactions, e.g., mRNA and eIF–4F, suggesting that IRP/IRE complexes (Muckenthaler et al.

1998), IRPs or IREs alone may have other binding partners.

In the IRE/IRP complex, sequences in both the RNA and protein are required for specific fits in the complex as shown by RNA and protein mutagenesis reviewed in (Theil and Eisenstein 2000; Pantopoulos 2004). IRP2 is the more selective in IRE binding of both natural and mutated IRE structures (Hirling et al. 1994; Butt et al. 1996; Ke et al. 1998; Gunshin et al. 2001; Erlitzki et al. 2002). The RNA and protein structure in the IRE/IRP2 complex is hard to predict because the selectivity of IRP2 for different IREs is high, and even the less selective IRP1 complexed to an IRE RNA in the recently completed crystal structure of the ferritin IRE (Selezneva et al. 2006; Walden et al. 2006), showed that both the RNA and protein changed with amazing twists and turns to find a close fit.

The biological significance of the variations among members of the IRE RNA family and IRP proteins is the graded response each member of the IRE family can display in response to the same changes in cellular iron. Thus, mt-aconitase mRNA, which has a relatively weak interaction with IRP2 compared to the ferritin mRNA (Ke et al. 1998) and will be only partly repressed under most conditions, should have a much more constant rate of translation with fluctuating iron concentrations, compared to ferritin. Such predicted differences have been observed in whole animal studies (Chen et al. 1998). The natural combinatorial array of slightly different mRNA ''promoter'' sequences and binding proteins with iso-IRE selectivity and cell-specific concentrations sensitive to iron and oxygen signals, mirrors the combinatorial regulation of DNA promoter families and multiple transcription factors, e.g., glucocorticoid sensitive genes (Darimont et al. 1998). How widespread are combinatorial arrays of mRNA promoters or DNA promoters and proteins is a problem for 21st century exploration.

What happens when a gene encodes a DNA and an mRNA regulatory element with the potential for signal overlap? Heme is a signal for both ARE-DNA (Sun et al. 2004) and IRE-mRNA (Goessling et al. 1998; Jeong et al. 2004), whereas iron salt signals target mainly the IRE in mRNA and t-butylhydroquinone signals target the ARE in DNA. When active ARE-DNA and IRE-mRNA elements are combined, the activation of the ferritin gene is much higher than with either the ARE-DNA or IRE-RNA alone (Hintze and Theil 2005), likely because both protein repressors, Bach1 and IRP1 or 2 bind heme. Since theTRR-ARE is also regulated by heme (Hintze and Theil 2005) and, as a selenocysteine protein is encoded in an mRNA with a translation regulatory structure, the selenocysteine insertion sequence (SECIS), it shares with ferritin the possibility of synergy by effectors that target both DNA and mRNA regulation pathways. Figure 1 is a cartoon of a model that shows the power of combining DNA and RNA structures that each bind protein repressors with sites for the same signal.

Ferritin regulation in plants and bacteria

DNA is the only genetic target of ferritin regulation in higher plants and bacteria. As in animals, the signals are iron or oxidants (e.g., hydrogen peroxide). In both plants and bacteria, there are multiple ferritin genes, which in general, respond to different environmental and/or developmental signals. In animals, both ferritin H and L genes respond to the same environmental signals. However, the apparent contrast between ferritin regulation in animals, compared to plants and bacteria, may be deceiving since the ferritin gene products, H and L ferritin subunits, are regulated by development and cell differentiation, and coassemble in vivo and in vitro, reviewed in (Liu and Theil 2005), to create cell specific ferritins with varied responses to environmental change. The final ferritin gene products in animals can mirror the multi-gene ferritin responses in plants and bacteria.

Plant ferritins

Ferritin transcription in plants is induced by iron or NO during antioxidant responses, as well as by changes in iron concentrations associated with symbiosis during nitrogen fixation (Ragland and

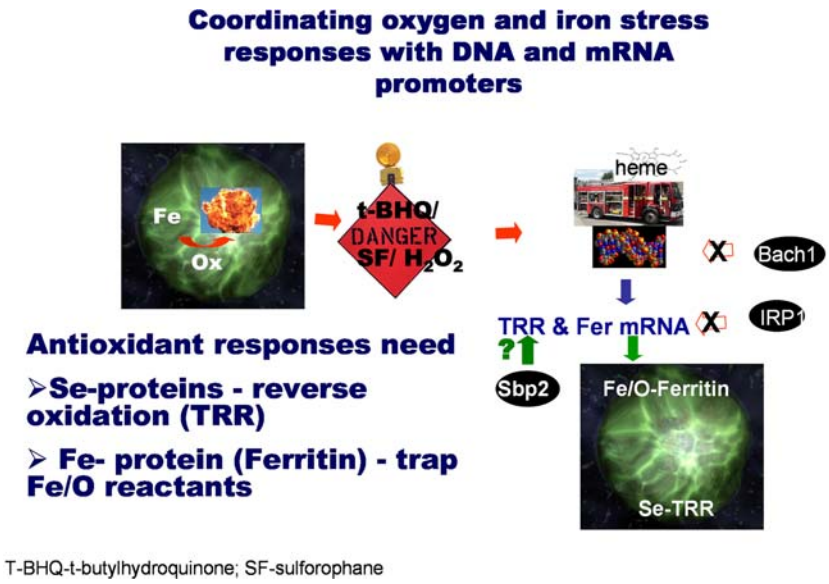

Fig. 1 The ferritin model for the impact of combining DNA ARE antioxidant response element and mRNA IRE iron responsive element structures, recognized by protein repressors, that bind the same signal molecule (heme). Thioredoxin reductase1 is an ARE gene regulated by heme (Hintze and Theil 2005) which also has an mRNA regulatory structure recognized by a regulatory protein SBP2; ''?'' indicates that the identities of small molecules which target the SBP2/TRR/mRNA interactions have not been identified. TRR thioredoxin reductase1, Bach1 heme binding repressor of ARE gene transcription; IRP1 (or IRP2) heme binding repressors of IRE mRNA translation or mRNA degradation

Theil 1993; Wei and Theil 2000; Fourcroy et al. 2004; Dellagi et al. 2005). The metal specificity is high: 300–500 μM (NaCl, $CuCl_2$, $ZnCl_2$, $MnCl_2$) had no effect on ferritin expression, whereas 50 μM ferric hexacitrate increased ferritin 50–60-fold (Wei and Theil 2000). The iron responsive promoter sequences in plant ferritin genes lack similarity to other promoters and vary among plants [*Arabidopsis thaliana* and *Zea mays*–IDRS (Arnaud et al. 2006); *G. max*-FRE (Wei and Theil 2000)].

Siderophores, microbial-specific iron chelate complexes released during iron deficiency, also induce plant ferritin gene transcription during infection even without iron, apparently as a defense mechanism that targets a different regulatory sequence than the IDRS (Dellagi et al. 2005). During bacterial infection/inflammation in animals, siderophores are also released by pathogenic bacteria, and endogenous ferritin also accumulates in cells of the animal immune system as if in parallel to plants, but the effect is most often attributed to changes in hepcidin secretion and changes in cell concentrations of iron, reviewed, e.g., in (Nemeth et al. 2003). How close is the molecular parallel between pathogen-siderophore release and host ferritin accumulations in plants and animals, remains to be investigated.

Bacterial ferritins

Maxi-ferritins and mini-ferritins (Dps proteins) in bacteria share with plants and animals genetic regulation by iron or the general stress associated with stationery phase, or with metals and oxidants during active growth. For bacterial maxi-ferritins, such as *E. coli* maxi-ferritin FTNa used to store iron, and heme containing BFr, a scavenger of iron and oxidant protector during stationary phase, the Fur–Fe^{2+} complex appears to activate transcription of both maxi-ferritin genes (Andrews 1998; Cartron et al. 2006). In addition, the regulatory RNA RyhB has effects on induction of Bfr maxi-ferritin RNA, but the effects are smaller than on some other genes of iron metabolism and may be indirect (Masse et al. 2005).

Mini-ferritins/Dps proteins in bacteria or archaea are particularly important during oxidant stress or, when protein turnover releases iron from degraded proteins as environmental nutrients are depleted (Altuvia et al. 1994; Chen et al. 1995; Antelmann et al. 1997; Moore et al. 2005; Reindel et al. 2006). Regulation of mini-ferritins is exemplified by *E. coli* and *Bacillus subtilis*.

The Dps protein regulatory pattern that emerges in *E. coli*, temporally couples Dps mini-ferritin induction by peroxide via OxyR with the iron storing maxi-ferritin FTNa during log phase (Altuvia et al. 1994). Dps mini-ferritin induction via both Sigma S- and IHF, a histone-like protein, is coupled to iron scavenging maxi-ferritin Bfr, during stationary phase (Altuvia et al. 1994).

Bacillus subtilis, and many other *Bacillus* spp., have two mini-ferritin/Dps genes but no recognizable maxi-ferritin gene. Both Dps mini-ferritin genes are expressed during exponential growth and during the transition from exponential growth. Dps2/MrgA mini-ferritin is induced by peroxide via modification of the PerR transcription factor by an Fe^{2+}/H_2O_2 catalyzed reaction (Lee and Helmann 2006), and Dps1/DpsA mini-ferritin is induced by nonspecific oxidative stress (heat, salt, ethanol stress, and glucose starvation), that is Sigma B-dependent during exponential growth and Sigma B-independent during transitional growth (Antelmann et al. 1997). Different, complementary regulation of the two Dps mini-ferritin genes in *B. subtilis* during both the exponential and transition culture phases of *B. subtilis* growth suggest that the two proteins complement each other and fulfill the roles of both maxi-ferritin (forming iron oxy minerals) and mini-ferritin (DNA protection). Such a conjecture is supported by the differential reactivity of oxidants (O_2 and H_2O_2), with the two *Bacillus* spp. mini-ferritins (Liu et al. 2006). The regulation of a Dps mini-ferritin protein from the archaea, *Halobacterium salinarum*, diverges from that of bacterial ferritins and Dps mini-ferritin genes since the single gene is regulated both by excess iron and by hydrogen peroxide (Reindel et al. 2006). Whether or not the regulation of Dps mini-ferritins in *H. salinarum* or in other archaea reflects the absence of other Dps mini-ferritin genes or maxi-ferritin genes, is unknown.

From the organismal point of view, the developmental regulation of ferritin expression has common features in animals, plants, and bacteria: accumulation of iron in ferritin for the next generation of organisms or for other cells in the community. Examples include the recycling of iron from embryonic erythrocytes to heme in the first generation of adult red cells (Theil 1976; Brown and Theil 1978; Theil and Brenner 1981), and from legume nodules to seeds (Burton et al. 1998). The accumulation of ferritin in proerythroblasts for use in red cell heme synthesis (Peto et al. 1983) and accumulation of ferritin in the senescent, yellowing sections of maize leaves, which will fertilize the sites of seed germination (Theil and Hase 1993), appear to be additional examples. Finally, the accumulation of maxi- and mini-ferritins during stationary and/or transitional phases of bacterial cultures appears to be a similar phenomenon. The regulatory signals for such developmental changes in ferritin expression may extend beyond iron and oxidants, as suggested by hormone and growth factor and myc regulation of ferritin in animals (Theil 1987; Wu et al. 1999).

Perspective

The regulation of ferritin expression by iron and/or oxidants is universal in animals, plants, bacteria, and archaea; since ferritin mineralization consumes both iron and dioxygen, such regulation is a type of metabolic feedback. However, both developmental and environmental signals provide ferritin iron for other cells in the community (stationary culture phase in bacteria, redistribution of nodule iron to seeds, release of iron from heptalytes to erythroid cells). The multiplicity of ferritin genes and biomineralizing nanocage gene products (isoforms of maxi-ferritins in animals, plants and bacteria, and mini-ferritins in bacteria), the differential use of dioxygen and hydrogen peroxide substrates in mineralization (mini-ferritins and maxi-ferritins), and additional protein nanocage functions such as gated pores and DNA protection, are sufficiently complex that the

similarities of structure/function and regulation are sometimes obscured. Adding the multiplicity of genetic targets and signals that integrate ferritin DNA with RNA regulation with related genes such as antioxidant response genes (DNA) and iron transport genes (RNA) may, on the one hand, seem overwhelming. On the other hand, the centrality to biology of the family of iron and oxygen mineralizing protein nanocages, the ferritins, becomes transparent. And the challenge of understanding the structure/function and gene regulation of the ferritin family is both exciting and promising as a model for taming dangerous chemistries for the benefit of biology.

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