Cytosolic Aconitase and Ferritin Are Regulated by Iron in Caenorhabditis elegans*

Received for publication, October 9, 2002, and in revised form, November 12, 2002
Published, JBC Papers in Press, November 15, 2002, DOI 10.1074/jbc.M210333200

Brett L. Gourley, Samuel B. Parker, Barbara J. Jones, Kimberly B. Zumbrennen, and Elizabeth A. Leibold†‡

From the Eccles Program in Human Molecular Biology and Genetics and Department of Medicine, Divisions of Hematology and Oncology, University of Utah, Salt Lake City, Utah 84112

Iron regulatory protein-1 (IRP-1) is a cytosolic RNA-binding protein that is a regulator of iron homeostasis in mammalian cells. IRP-1 binds to RNA structures, known as iron-responsive elements, located in the untranslated regions of specific mRNAs, and it regulates the translation or stability of these mRNAs. Iron regulates IRP-1 activity by converting it from an RNA-binding apoprotein into a [4Fe-4S] cluster protein exhibiting aconitase activity. IRP-1 is widely found in prokaryotes and eukaryotes. Here, we report the biochemical characterization and regulation of an IRP-1 homolog in Caenorhabditis elegans (GEI-22/ACO-1). GEI-22/ACO-1 is expressed in the cytosol of cells of the hypodermis and the intestine. Like mammalian IRP-1/aconitases, GEI-22/ACO-1 exhibits aconitase activity and is post-translationally regulated by iron. Although GEI-22/ACO-1 shares striking resemblance to mammalian IRP-1, it fails to bind RNA. This is consistent with the lack of iron-responsive elements in the C. elegans ferritin genes, ftm-1 and ftm-2. While mammalian ferritin H and L mRNAs are translationally regulated by iron, the amounts of C. elegans ftm-1 and ftm-2 mRNAs are increased by iron and decreased by iron chelation. Excess iron did not significantly alter worm development but did shorten their life span. These studies indicated that iron homeostasis in C. elegans shares some similarities with those of vertebrates.

Iron is an essential element required for growth and survival of most organisms. The importance of iron is implicit in the role it plays in oxygen transport and heme synthesis as well as its ability to serve as a cofactor for enzymes involved in a variety of biological processes including DNA synthesis, energy production, and neurotransmitter synthesis. Abnormally high concentration of cellular iron is toxic due to its ability to catalyze the generation of free radicals that damage DNA, lipids, and proteins. In humans, the accumulation of excess cellular iron can result in cirrhosis, arthritis, cardiomyopathy, diabetes mellitus, and increased risk of cancer and heart disease. To provide adequate iron for cellular needs yet prevent the accumulation of excess iron, the concentration of iron within cells is tightly controlled.

In vertebrates, the iron regulatory proteins 1 and 2 (IRP-1 and IRP-2)1 regulate iron homeostasis. IRPs are cytosolic RNA-binding proteins that regulate the translation or the stability of mRNAs encoding proteins involved in iron and energy homeostasis4. IRPs bind to RNA structures, known as iron-responsive elements (IREs), that are located in either the 5′- or 3′-untranslated regions (UTRs) of specific mRNAs. These mRNAs encode proteins involved in iron storage (ferritin), iron utilization (erythroid aminolevulinate synthase and mitochondrial aconitase), and iron transport (transferrin receptor and divalent metal transporter-1). When iron is scarce, IRP binding to the 5′ IRE in ferritin mRNA represses translation, whereas IRP binding to the 3′ IREs in the transferrin receptor mRNA stabilizes this mRNA. When iron is abundant, IRPs lose affinity for the IREs, leading to enhanced ferritin synthesis and to the rapid degradation of transferrin receptor mRNA. By regulating the amount of iron taken up by transferrin receptor and the amount of iron sequestered by ferritin, cellular iron concentration is maintained, and iron toxicity is avoided.

Iron regulates the RNA binding activity of IRP-1 and IRP-2, but the mechanism of regulation differs. In the presence of iron, a [4Fe-4S] cluster assembles in IRP-1, converting it from an RNA-binding protein into a cytosolic aconitase. Aconitases are [4Fe-4S] cluster proteins that are found in the cytosol, mitochondria, and glyoxysomes and catalyze the reversible isomerization of citrate and isocitrate via cis-aconitate (5). Despite information regarding the role of aconitase in mitochondria and in the glyoxylate cycle in microorganisms and plants, the function of cytosolic aconitase in higher eukaryotes is not clear. Unlike IRP-1, IRP-2 lacks a [4Fe-4S] cluster and consequently lacks aconitase activity (6). Rather, iron regulation of IRP-2 involves iron-induced IRP2 degradation by the proteasome (7–9).

IRP-1s have been identified in a wide variety of organisms, including bacteria (10–12), plants (13–15), and animals (16, 17). IRP-1s share a high degree of amino acid identity among different species. For example, mammalian IRP-1s are 90% identical and are highly homologous to IRP-1s from other organisms, including Caenorhabditis elegans (64% identity) (16), Arabidopsis thaliana (59% identity) plants (13), Trypanosoma brucei (64% identity) (17), and Escherichia coli (52% identity) (10). In contrast, IRP-1s share only ~20% amino acid identity to mitochondrial aconitases. Although these IRP-1s show striking similarity to mammalian IRP-1 and in most cases exhibit aconitase activity, only vertebrate and insect IRP-1s (18–21) bind RNA. Exceptions are Bacillus

* This work was supported by National Institutes of Health Grant GM45201 (to E. A. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: University of Utah, HMBG, 15 N. 2030 E. Rm. 3240, Salt Lake City, UT 84112 Tel.: 801-585-5002; Fax: 801-585-5501; E-mail: betty.leibold@hmbg.utah.edu.
‡ The abbreviations used are: IRP, iron regulatory protein; IRE, iron-responsive element; UTR, untranslated region; NGM, nematode growth medium; FAC, ferric ammonium citrate; DF, deferoxamine; GFP, green fluorescent protein.
Expression of GEI-22/ACO-1 in Yeast and Generation of Antibodies—A His, tag was cloned onto the N terminus of pACO-1FLAG, and the insert was subcloned into the KpnI-XbaI sites of pYES2 (Invitrogen), yielding the yeast expression construct pYhis-ACO-1FLAG. pYhis-ACO-1FLAG was transformed into yeast strain JEL1 (25). Yeast were grown overnight in synthetic complete medium minus uracil (SC-Ura) containing 2% dextrose. Yeast were then diluted 1:100 into SC-Ura containing 3% glycerol, 2% lactic acid and grown to an A600 of 0.8. 32P-galactose was added to the culture for 10–18 h to induce GEI-22/ACO-1 expression. Yeasts were harvested, and the pellet was resuspended in 3 ml/g, wet weight, lysis extraction buffer (0.3 M NaCl, 0.05 mM NaH2PO4, pH 8.0, 10 mM β-mercaptoethanol, 0.025% Nonidet P-40, 0.1 mM EDTA) containing a protease inhibitor tablet (Roche Molecular Biochemicals). Cells were disrupted by vortexing with glass beads and centrifuged at 27,000 × g for 30 min. The lysate was mixed with 2 ml of equilibrated Ni2+-nitrilotriacetic acid resin (Qiagen) for 1 h at 4 °C. The slurry was poured into a column and was washed with column buffer (0.3 M NaCl, 0.05 mM NaH2PO4, pH 8.0) containing 0.07 mM imidazole. GEI-22/ACO-1 was eluted with column buffer containing 0.2 mM imidazole, and fractions containing GEI-22/ACO-1 were dialyzed against assay buffer (0.05 mM Tris, pH 7.3, 0.1 mM NaCl, 1 mM diethio-ritol) for antibody preparation. GEI-22/ACO-1 was further purified by preparative 8% SDS-PAGE. The protein was visualized using a cold solution of 2.5 mM KCl, and the band containing GEI-22/ACO-1 was excised and used to inject rabbits. The polyclonal antibodies detected a band with a molecular mass of ~100 kDa, which corresponds to the size of the predicted gene product of gei-22/aco-1 mRNA. Preimmune serum did not detect this band.

Expression of GEI-22/ACO-1 in Human Embryonic Kidney 293 Cells—A 10-cm plate of HEK 293 cells was cotransfected with 8 μg of pACO-1FLAG and 2 μg of pEGFP (Clontech) DNAs. The cells were equally split after 5 h to six 35-mm plates, and duplicate plates received either FAC (50 μg/ml) or DF (50 μg/ml) or no addition. The cells were incubated for 16 h and then harvested in 0.125 ml of lysis buffer (0.02 mM HEPES, pH 7.6, 0.025 mM KCl, 1 mM diethio-ritol, 0.25% Nonidet P-40) containing a protease inhibitor tablet (Roche Molecular Biochemicals). The lysate was centrifuged at 15,000 × g for 20 min at 4 °C, and the supernatants were assayed for protein using the Coomassie Blue Plus Protein Reagent (Pierce).

Isolation of C. elegans Protein Extracts—Worms were harvested from 500-ml cultures containing 0.33 mg/ml FAC or 100 μM DF. The worms were washed free of bacteria by sucrose flotation and suspended in a 2× volume of homogenization buffer (0.05 mM Tris–HCl, pH 7.9, 25% glycerol, 0.1 mM EDTA, 0.32 mM NH4SO4) containing a protease inhibitor tablet (Roche Molecular Biochemicals). The lysates were homogenized on ice using a Brinkmann Instruments Polytron homogenizer at full power for 15 s, repeated seven times. The homogenates were centrifuged at 160,000 × g for 1 h, and the supernatants were assayed for protein using the Coomassie Blue Plus Protein reagent (Pierce).

RNA-binding Gel Shift and Aconitase Assays—RNA-binding assays were performed as described (26), using protein (12 μg) from yeast, HEK 293 cells, or C. elegans lysates and a 32P-labeled rat ferritin L-IRE (R. norvegicus fer-I) (25) or a C. elegans ferritin-2 (ftn-2) RNA. The ftn-2 RNA was synthesized from DNA that corresponded to 530 nt 5′ to the start codon of ftn-2 gene (C. elegans cosmid D1037.3). The forward primer contained a T7 promoter sequence (18 nt) followed by 20 nt of ftn-2 sequence (5′-TAATACGACTCACTATAGGGGTTGATACACTATTAGGCTTAATGAC-3′) and the reverse primer contained the first 20 nt 5′ to the start site in ftn-2 (5′-GGATACTTGGTGGTGCTTGAAGC-3′). After incubation of the RNA-protein complexes for 20 min, RNase T1 (1 unit) was added to the reaction for 5 min followed by the addition of 1.5 μg of heparin. The RNA sample was separated by 3% native polyacrylamide gel was used to resolve the RNA-protein complexes.

Aconitase assays were performed using lysates obtained from worms and from HEK 293 cells transfected with pACO-1FLAG. Aconitase activity was assayed by the addition of cis-aconitate (0.2 mM final concentration) to 50 μg of protein in 0.5 ml of aconitase buffer (0.05 mM HEPES, pH 7.5, 0.1 mM NaCl, 1.5 mM MgCl2) and 0.25% BSA. The reaction rate was measured at 240 nm over time. For aconitase assays in worms, four separate experiments were carried out. The differences between FAC- and DF-treated worms were determined by paired Student’s t test, and p < 0.05 was considered significant.

Immunoblot Analysis—Protein from HEK 293 cells (50 μg), yeast (20 μg), and C. elegans worms (50 μg) were co-injected separated by 8% SDS-PAGE, and the protein was transferred onto nitrocellulose membranes. Membranes were incubated with the following primary antibodies: rabbit anti-Flag (Sigma) (1:5,000), rabbit anti-GEI-22/ACO-1 (1:5,000), mouse anti-GFP (1:2,000), chicken anti-Rattus norvegicus IRP-1 (1:16,000) (6).
and rabbit anti-R. norvegicus IRP-2 (1:8,000) (6). The appropriate goat horseradish peroxidase-conjugated secondary antibodies (Pierce) were used at 1:10,000. Antibodies were visualized using the Renaissance detection system (PerkinElmer Life Sciences).

**Northern Blot Analysis**

Total RNA was isolated from age-synchronized L4 worms grown on FAC (0.003–6.6 mg/ml)-supplemented or DF (100/H9262 M)-supplemented NGM plates for 4 days. Worms (100 mg) were homogenized in TRIzol (1 ml) using a Dounce homogenizer. Total RNA (25/H9262 g) was resolved using a 1.2% formaldehyde agarose gel and transferred to a nylon membrane. The membrane was hybridized with 32P-labeled C. elegans ftan-1, ftan-2, gei-22/aco-1, and act-1 DNA probes prepared by the amplification of worm genomic DNA. DNA templates for amplification were prepared by washing worms in PCR lysis buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.2, 2.5 mM MgCl2, 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% gelatin). The worms were placed in 25/H9262 l of lysis buffer containing proteinase K (1 mg/ml) for 1 h at 65 °C followed by a 15-min incubation at 95 °C. The lysate (1 l) was added to a PCR (50 l) containing 20 M of the appropriate forward and reverse primers. Forward and reverse primers, respectively, for PCR were obtained from sequences of the following C. elegans cosmid clones: ftan-1 (C54F6.14), 5’-ACCTGAGCTCATTCGCCC-3’ and 5’-CTCCGAGCTTGAGCGCC-3’; ftan-2 (D1037.3), 5’-TCCGAGGGTTAAGCTGCC-3’ and 5’-CGGAAAAGGGTCCTGGCC-3’; and act-1 (TO4C12.6), 5’-GACAATCAGTCCGAGGC-3’ and 5’-GCGGAGTGGTGAGGAGG-3’.

**RESULTS**

**Cloning and Expression of a GEI-22/ACO-1 Homolog in C. elegans**—The C. elegans genome possesses two aconitase genes, which are designated gei-22/aco-1 and aco-2. gei-22/aco-1 encodes a protein that shares 63% identity with mammalian IRP-1 (16), D. melanogaster IRP-1A and 1B (16), and A. thaliana (13) and T. brucei (17) IRP-1s while sharing only 24% identity to porcine (28) and C. elegans mitochondrial aconitases. aco-2 encodes a protein that shares 74% identity to human and porcine mitochondrial aconitases.

To study the iron homeostasis in C. elegans, a gei-22/aco-1 cDNA was generated by reverse transcription-PCR using sequences obtained from the C. elegans cosmid clone ZK455.1.

**Iron Regulation in C. elegans**

Iron is essential for numerous processes in C. elegans, including the development of the nervous system, the immune response, and the maintenance of the gut microbiome. Iron is acquired from the diet and transported into cells via iron transporters. Once inside the cell, iron is stored in ferritin or used for oxygen-dependent processes. Iron deficiency can lead to developmental defects, while iron overload can be toxic. The study of iron homeostasis in C. elegans provides insights into the regulation of iron uptake, storage, and utilization, which are important for understanding iron metabolism in other organisms as well.
Iron Regulation in C. elegans

GEI-22/ACO-1 Does Not Bind RNA—Mammalian IRP-1 exhibits the mutually exclusive activities of RNA binding and aconitase. Iron causes IRP-1 to switch from an RNA-binding apoprotein form to a non-RNA binding aconitase form containing a [4Fe-4S] cluster. The switch between these forms occurs without changes in IRP-1 protein levels. Since GEI-22/ACO-1 shows significant amino acid identity with mammalian IRP-1, we questioned whether GEI-22/ACO-1 binds RNA. The 5’ sequences flanking the C. elegans genes encoding ferritin-1 (C. elegans Ftn-1) and ferritin-2 (C. elegans Ftn-2), succinate dehydrogenase, and ACO-2 were inspected for IREs, since 5’ IREs are found in homologous genes in other organisms (19, 34–36). No consensus IREs was identified in these genes. It was possible, however, that nonconsensus IREs might be present in the 5’-UTRs of these genes. To test for this, IRP binding activity was measured in extracts of worms grown in FAC or the iron chelator DF using 32P-labeled RNAs corresponding to sequences located ~500 nt upstream of the start site of the ftn-1, ftn-2, aco-2, and succinate dehydrogenase genes. Since C. elegans exons and introns are generally shorter than in vertebrates (37), we reasoned that if these genes harbor 5’-IREs, then they should be present within these sequences.

Worms were grown in FAC- or DF-supplemental medium, because previous studies showed that FAC and DF can decrease and increase, respectively, IRP-1 RNA binding activity (1, 2, 4). The concentration of FAC and DF was chosen based on testing the growth of worms in different concentrations of FAC and DF (see “Experimental Procedures”). Fig. 3B shows that no specific RNA binding activity was detected in worm extracts by RNA-band shift gels with the 32P-labeled ftn-2 RNA or with other 32P-labeled C. elegans RNAs (data not shown), although GEI-22/ACO-1 was detected in these extracts by immunoblotting using an anti-GEI-22/ACO-1 antibody (Fig. 3C). As controls, HEK 293 cells were treated with FAC or DF, and IRP RNA binding activity was measured using the 32P-labeled R. norvegicus fer-l IRE (Fig. 3A). DF increased IRP RNA binding activity as expected, whereas FAC had little effect on IRP RNA binding activity due to the high iron concentration in these cells. No RNA-protein complexes were formed with a 32P-labeled R. norvegicus fer-l IRE in worm extracts (Fig. 3A).

C. elegans extracts spiked with R. norvegicus IRP-1 showed that 32P-labeled R. norvegicus fer-l IRE bound to R. norvegicus IRP-1, indicating that an inhibitor of RNA binding activity was not present in these extracts (data not shown).

To confirm that GEI-22/ACO-1 lacks RNA binding activity, we expressed His/FLAG-tagged GEI-22/ACO-1 in yeast using a galactose-inducible promoter. The advantage of yeast is that they do not express endogenous IRPs, which might interfere with the detection of small amounts of RNA binding activity. As controls, yeast strains expressing His-tagged R. norvegicus IRP-1 and IRP-2 were also assayed for RNA binding activity. Galactose induced the expression of GEI-22/ACO-1, R. norvegicus IRP-1, and R. norvegicus IRP-2, but only R. norvegicus IRP-1 and R. norvegicus IRP-2 bound to the R. norvegicus fer-l IRE (Fig. 4, A and B). Taken together, these data indicate that GEI-22/ACO-1 does not bind to a mammalian consensus IRE or to C. elegans RNAs that might be expected to harbor functional IREs.

GEI-22/ACO-1 Is an Aconitase—The data indicate that GEI-22/ACO-1 lacks RNA binding activity. The 24 active sites in mitochondrial aconitases (29–31) are present in GEI-22/ACO-1, suggesting that GEI-22/ACO-1 is an aconitase. To determine whether GEI-22/ACO-1 exhibits aconitase activity and whether it is regulated by iron, total aconitase activity was measured in HEK 293 cells transfected with FLAG-tagged GEI-22/ACO-1 or pcDNA3 control. Some cells were treated with either FAC or DF for 16 h before assaying aconitase activity. Cells transfected with FLAG-tagged GEI-22/ACO-1 showed a ~2-fold increase in total aconitase activity compared with pcDNA3-transfected cells (Fig. 5A). When cells were treated with FAC, total aconitase activity increased ~4-fold in GEI-22/ACO-1-transfected cells compared with pcDNA3-transfected cells. Endogenous aconitase activity did not signif-

Fig. 2. Expression patterns of gei-22/aco-1::GFP reporter construct in C. elegans. A, structure of the gei-22/aco-1::gfp gene. White boxes, exons; black box, gfp fused to sequences in exon 8. The gei-22/aco-1 regulatory region is indicated. B, GFP expression in embryos. C, L2 larval stage worms. D, adults. Hypodermis (Hyp), seam cells (Seam), and intestine (Int) are indicated.

Fig. 3. C. elegans extracts lack IRE binding activity. HEK 293 cells were grown in medium containing FAC (100 μg/ml), DF (100 μg/m), or no treatment (NT) for 16 h. L4 larval stage worms were grown in liquid cultures containing FAC (1 mM), DF (100 μg/ml), or no treatment for 4 days. Cytoplasmic extracts were prepared from HEK 293 cells and C. elegans cultures, and protein (12 μg) was incubated with either a 32P-labeled rat ferritin L-IRE (R. norvegicus fer-l; Rnfer-l) (A) or a 32P-labeled C. elegans ftn-2 RNA (Ceftn-2 RNA) (B). After 20 min, RNase T1 (1 unit) was added to the RNA band shift reactions to degrade non-specific RNA (A and B, lanes 2–7). The 32P-labeled RNA-protein complexes were analyzed by 8% native PAGE. IRP-1-IRP-2 RNA complexes comigrate in HEK 293 cells; free RNAs are indicated. C, protein (25 μg) from extracts in A and B were analyzed by 8% SDS-PAGE using anti-rat IRP-1 (R. norvegicus IRP-1 Ab) or anti-GEI-22/ACO-1 (GEI-22/ACO-1 Ab) antibodies.
Iron Regulation in C. elegans

Iron Regulates Aconitase Activity in C. elegans—To determine whether aconitases are regulated by iron in worms, aconitase activity was measured in lysates from mixed stage worms grown for 4 days in liquid cultures supplemented with either 0.33 mg/ml FAC or 100 μM DF. We compared FAC and DF treatments because these conditions represent the extremes of iron concentrations. In these experiments, total aconitase activity (GEI-22/ACO-1 and ACO-2) was measured, because the cytosol was routinely contaminated with mitochondrial proteins. Total aconitase activity from four separate experiments showed base-line variability; however, in all experiments a significant reduction (12–16%) was observed in DF-treated worms compared with FAC-treated worms (p < 0.003). Protein (20 μg) in this experiment and one of the experiments in A were analyzed by 8% SDS-PAGE, and immunoblots were carried out using anti-GEI-22/ACO-1 antibody (Ab).

Iron Regulates C. elegans ftn-1 and ftn-2 mRNAs—The lack of discernible IREs in the C. elegans genome and the absence of GEI-22/ACO-1 RNA binding activity suggested that C. elegans ferritin genes might be iron-regulated by mechanisms other than translation. C. elegans expresses two ferritin-like proteins, which we designated C. elegans FTN-1 and FTN-2. C. elegans FTN-1 and FTN-2 are more homologous to R. norvegicus FER-H (54 and 61%) than to R. norvegicus FER-L (45 and 50%), and both proteins contain ferroxidase active-site residues that are characteristic of ferritins and other proteins. We synthesized 50 μg of each protein (20 μg) in the presence of 0.33 mg/ml FAC or 100 μM DF. We compared FAC and DF treatments because these conditions represent the extremes of iron concentrations. In these experiments, total aconitase activity (GEI-22/ACO-1 and ACO-2) was measured, because the cytosol was routinely contaminated with mitochondrial proteins. Total aconitase activity from four separate experiments showed base-line variability; however, in all experiments a significant reduction (12–16%) was observed in DF-treated worms compared with FAC-treated worms (p < 0.003). Protein (20 μg) in this experiment and one of the experiments in A were analyzed by 8% SDS-PAGE, and immunoblots were carried out using anti-GEI-22/ACO-1 antibody (Ab).

Iron Regulates C. elegans ftn-1 and ftn-2 mRNAs—The lack of discernible IREs in the C. elegans genome and the absence of GEI-22/ACO-1 RNA binding activity suggested that C. elegans ferritin genes might be iron-regulated by mechanisms other than translation. C. elegans expresses two ferritin-like proteins, which we designated C. elegans FTN-1 and FTN-2. C. elegans FTN-1 and FTN-2 are more homologous to R. norvegicus FER-H (54 and 61%) than to R. norvegicus FER-L (45 and 50%), and both proteins contain ferroxidase active-site residues that are characteristic of R. norvegicus FER-H subunits (38).

To determine whether ftn-1 and ftn-2 mRNAs are regulated by iron, worms were grown on agar plates supplemented with FAC (0.003–6.6 mg/ml) or without FAC (100 μM) for 4 days, and ftn-1 and ftn-2 mRNAs were measured by Northern blots. Fig.
Effect of Iron on the Development and Life Span of C. elegans—Since little is known about iron homeostasis in worms, we determined the concentration of iron that causes toxicity in worms. First, we tested whether the development of worms was affected by iron. Embryos were grown on FAC-supplemented (0.033–6.6 mg/ml) NGM plates. All worms grew into adults within 4 days, indicating that iron did not affect worm development (data not shown). We then tested whether the life span of worms was altered when grown on iron plates. L4 larval stage worms were placed on agar plates supplemented with FAC (0.033–6.6 mg/ml), and worms were counted each day until all were dead. Fig. 8 shows that the mean life spans ± S.E. of worms grew on 3.3 and 6.6 mg/ml FAC plates were 12.9 ± 2.0 and 11.1 ± 1.2 days, respectively, compared with 15.8 ± 0.70 days for worms grown on control plates. These data indicated that excess iron does not affect the development of worms but is toxic when worms are exposed to iron during their lifetime.

**DISCUSSION**

Here, we investigate the iron-dependent regulation of GEI-22/ACO-1 and the regulation of iron homeostasis in C. elegans. We show that GEI-22/ACO-1 is similar to other vertebrate IRP-1 proteins in that it exhibits aconitase activity and is post-translationally regulated by iron. Unlike vertebrate IRP-1 proteins, GEI-22/ACO-1 does not bind RNA. Our data show that GEI-22/ACO-1 is expressed in intestine and hypodermis, which are organs that absorb and store nutrients, and is consistent with its role in energy homeostasis. We show that high concentrations of iron reduce the life span of worms. Finally, we demonstrate that iron regulates the amounts of ftu-1 and ftu-2 mRNAs, unlike in vertebrates, where ferritin H and L mRNAs are primarily regulated by translation by the IRP-IRE system.

Aconitases are conserved [4Fe-4S] cluster proteins found in prokaryotes and eukaryotes that catalyze the reversible isomerization of citrate and isocitrate in the Krebs and glyoxylate cycles. Aconitases can be subdivided into several groups (5). One group is represented by IRP-1, which shares >47% identity with mammalian IRP-1, whereas the other group is represented by mitochondrial aconitase, which shares only ~24% identity with IRP-1. Since GEI-22/ACO-1 and other IRP members are so similar to vertebrate IRP-1s, which are RNA-binding proteins, it was expected that GEI-22/ACO-1 also binds RNA. Our data show that GEI-22/ACO-1 lacks RNA binding activity, which is consistent with the lack of discernible IREs in the C. elegans genome. Other IRP-1s, such as T. brucei (13), and Nicotiana tabacum (15), were shown to be aconitases, but RNA binding activity was not reported, and it is probable that these proteins also do not bind RNA. However, some nonvertebrate IRP-1s bind RNA. For example, P. falciparum IRP-1 was reported to bind a mammalian consensus IRE (22), and B. subtilis aconitase binds not only to a mammalian consensus IRE but also to IRE-like stem-loop structures present in bacterial genes (11). Whether these interactions are functional in vivo is not clear. Overall, these studies show that RNA binding is not a requisite feature of the IRP-1 family.

Iron post-translationally regulates GEI-22/ACO-1 activity. In GEI-22/ACO-1-transfected HEK 293 cells, FAC and DF increased and decreased aconitase activity, respectively. In worms, total aconitase activity was 14% lower in worms grown in DF compared with FAC. Because FAC and DF have no effect on the amounts of GEI-22/ACO-1 protein or mRNA, this sug-
gested that iron regulates GEI-22/ACO-1 activity by the assembly and disassembly of the [4Fe-4S] cluster. These studies also show that the [4Fe-4S] cluster machinery in human cells is efficient in assembling a cluster in GEI-22/ACO-1. Although we cannot attribute the changes in aconitase activity solely to GEI-22/ACO-1 because of contamination with mitochondrial ACO-2, the data indicated that altering the iron concentration in worms could regulate aconitase activity. Our data show that in worms grown in the presence of DF, the decrease in aconitase activity was much less than in HEK 293 cells transfected with GEI-22/ACO-1. The reasons for these differences are not clear, but we suspect that DF uptake in worms grown in liquid cultures might not be as efficient as DF uptake into cultured cells.

Despite information regarding the role of aconitase in mitochondria and in the glyoxylate cycle, the function of cytosolic aconitase in higher eukaryotes is not clear. The glyoxylate pathway takes place in plants and microorganisms, allowing these organisms to convert lipids to glucoseogenic precursors for the synthesis of carbohydrates. In plants, IRP-1 participates in the glyoxylate pathway (14, 39), where its expression increases with the growth of seedlings and coincides with the increase in activity of other glyoxylate enzymes (14). A recent study shows that aconitase along with other glyoxylate and Krebs cycle enzymes are increased in *A. thaliana* during infection with *Pseudomonas syringae*, suggesting a role for aconitase in plant defense against pathogens (40). In *T. brucei*, IRP-1 expression is developmentally regulated, increasing in procyclic stage when Krebs cycle enzymes also increase (17). Whether IRP-1 functions in a glyoxylate pathway in *T. brucei* is unclear. In mammals, the role of IRP-1 was hypothesized to be important in maintaining redox control during periods of iron excess (41). Finally, some studies have hypothesized that the glyoxylate pathway may be operative in mammals during periods of stress, such as during starvation (42) and alcohol consumption (43); however, this has yet to be tested.

Because the glyoxylate cycle is operative in *C. elegans* (44, 45), it is plausible that GEI-22/ACO-1 is involved in this pathway. In *C. elegans*, the activities and expression of the glyoxylate cycle protein increase when carbohydrates are low and fatty acids are elevated, which would occur in embryos and in larvae during fasting (46–48). We examined the expression of GEI-22/ACO-1 in L1 to L4 larval stages and in adults and found no differences in amounts of GEI-22/ACO-1.2 It is possible, however, that GEI-22/ACO-1 activity might be altered during these conditions. Other functions for GEI-22/ACO-1 are suggested by our data showing that the expression patterns of GEI-22/ACO-1 are not identical with glyoxylate cycle protein. For example, glyoxylate cycle protein is expressed in body wall muscle and intestine (47, 48), whereas GEI-22/ACO-1 is expressed in hypodermal seam cells and intestine but not in body wall muscle. Furthermore, a recent study showed that GEI-22/ACO-1 interacted with GEX-3 in a yeast two-hybrid screen (49). GEX-3 is thought to be a Rac-interacting protein, which is essential for proper cell migration and cell morpholology, and in its absence, hypodermal cells fail to become organized (50). The localization of GEI-22/ACO-1 to hypodermal cells and its interaction with a GEX-3 suggest that GEI-22/ACO-1 might have a distinct function in development in hypodermis. However, when *gei-22/aco-1* expression was inhibited by RNA interference, mutant worms showed no developmental abnormalities (49). These data suggested that *gei-22/aco-1* may have other roles in hypodermal cells or that *aco-2* can substitute for *gei-22/aco-1* function.

To understand how *C. elegans* controls iron homeostasis, the regulation of *C. elegans* *ftn-1* and *ftn-2* mRNAs was studied. Ferritins are conserved iron storage proteins found in bacteria, plants, and animals that can store up to 4,500 iron atoms and, by so doing, prevent iron toxicity (38, 51). Vertebrate ferritins are generally composed of H and L subunits (FER-H and FER-L), whereas those of plants and bacteria contain mainly H subunits. FER-H contains amino acids that are required for ferroxidase activity, whereas the FER-L functions in enhancing iron nucleation and long term iron storage. In mammalian cells, FER-H and FER-L are regulated at the translational level by the IRP-IRE system. Iron-dependent transcriptional regulation of ferritin has been described in mammalian cells (52), but this plays a minor role in iron regulation.

Since GEI-22/ACO-1 does not bind RNA, and *C. elegans* lacks obvious IREs, we hypothesized that *ftn-1* and *ftn-2* mRNAs might be regulated by iron through processes other than translation. Our data showed that *ftn-1* mRNA increased ~2-fold in worms grown in the presence of iron, whereas *ftn-2* mRNA showed a small but consistent ~0.5-fold increase. In contrast, DF treatment decreased *ftn-1* and *ftn-2* mRNAs ~10-fold and ~2-fold, respectively. One explanation for the ~2-fold increase in *ftn-1* mRNA by iron is that worms are grown in iron-replete medium and fed *E. coli* and are iron-sufficient. The data also show that *ftn-1* mRNA is more responsive to iron concentration than *ftn-2* mRNA. A similar situation occurs in *A. thaliana*, where the fer-1 and fer-3 mRNAs are more responsive to iron than fer-2 or fer-4 (53), and in maize, where fer-1, but not fer-2, is iron-regulated (54–56). Although our data do not conclusively show that *ftn-1* and *ftn-2* genes are transcriptionally regulated by iron in worms, this mechanism would be consistent with the observed transcriptional regulation of ferritin in plants (53, 56, 57) and in insects (58).

The response of *C. elegans* to heavy metals, such as copper, arsenate, and cadmium (59–64), has been investigated, but less is known about iron regulation and toxicity in worms. Our first experiment was to determine the effect of iron on the development of worms. No significant change was observed in the timing of development of worms grown on FAC-supplemented plates. High concentrations of iron, however, did decrease the life span of worms. The mean life span of worms grown on 3.3 and 6.6 mg/ml FAC plates was 12.9 ± 2.04 and 11.1 ± 12 days, respectively, compared with worms grown on control plates (15.8 ± 0.70 days). Although the mechanism responsible for iron-mediated decrease in life span is not known, it is possible that worms maintained on high iron would accumulate iron as they age, leading to increased iron-catalyzed free radical formation. An elevation in the concentrations of free radicals would overwhelm the antioxidant defense machinery, leading to cellular damage and eventual cell death. Studies show that when reactive free radicals are decreased (65, 66) or antioxidant enzymes, such as catalase and superoxide dismutase, are increased (67–69), the life span of the organism can be increased. The toxicity of iron in worms may be akin to iron overload diseases in humans, where the accumulation of iron over a lifetime can result in cirrhosis, cardiomyopathy, diabetes mellitus, neurodegeneration, and increased risk of cancer (70). These studies show that *C. elegans* may serve as a model system for the study of iron homeostasis in mammals.

Acknowledgments—We thank Drs. Susan Mango and Adam Blaszczak and Mike Portereiko for advice on worm culture and for scientific discussion. We thank Drs. Eric Hanson and Bruce Bamber for critical reading of the manuscript and Diana Lim for assistance with figures. We acknowledge the *C. elegans* Genetics Center for cosmids and strains and the University of Utah DNA Core Facility for oligonucleotides. We thank Dr. Chris Rodesch at the University of Utah Cell.

---

2 B. L. Gourley and E. A. Leibold, unpublished observation.
Iron Regulation in *C. elegans*

3234

Iron regulation in *C. elegans* is a topic of interest in understanding the role of iron in cellular processes. Here, we provide a list of references that explore various aspects of iron metabolism and its regulation in this model organism.

**REFERENCES**

1. Hentze, M. W., and Kuhn, L. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 8175–8182.
2. Eisenstein, R. S. (2000) *Annu. Rev. Nutr.* **20**, 627–662.
3. Schneider, B. S., and Leibold, E. A. (2000) *J. Biol. Chem.* **275**, 49065–49066.
4. Peyret, P., Perez, P., and Alric, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 21645–21651.
5. Guo, B., Yu, Y., and Leibold, E. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8175–8179.
6. Alen, C., and Sonenshein, A. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 10412–10417.
7. Kohler, S. A., Henderson, B. R., and Kuhn, L. C. (1995) *J. Biol. Chem.* **270**, 21645–21651.
8. Iwai, K., Klausner, R. D., and Rouault, T. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5350–5357.
9. Prodromou, C., Artym, P. J., and Guest, J. R. (1992) *EMBO J.* **11**, 5822–5831.
10. Iwai, K., Drake, S. K., Wehr, N. B., Weissman, A., LaVaute, T., Minato, N., Klaask, R. D., Levine, R. L., and Rouault, T. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 4924–4928.
11. Alen, C., and Sonenshein, A. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 10412–10417.
12. Mengaud, J. M., and Horwitz, M. A. (1993) *J. Bacteriol.* **175**, 5666–5676.
13. Hayashi, M., DeBelli, L., Alpi, A., and Nishimura, M. (1995) *Plant Cell Physiol.* **36**, 669–680.
14. Navarre, D., Wendeboe, D., Durner, J., Noad, R., and Kessig, D. F. (2000) *Plant Physiol.* **122**, 573–582.
15. Muller, E., and Kuhn, L. C. (1999) *Nucleic Acids Res.* **17**, 1175–1189.
16. Hentze, M. W., and Kuhn, L. C. (1995) *J. Biol. Chem.* **270**, 21645–21651.
17. Saas, J., Ziegelbauer, K., von Haeseler, A., and Fast, B. (2000) *J. Biol. Chem.* **275**, 2745–2755.
18. Rothammer, S., Muller, E., and Kuhn, L. C. (1999) *Nucleic Acids Res.* **17**, 1175–1189.
19. Koler, S. A., Henderson, B. R., and Kuhn, L. C. (1995) *J. Biol. Chem.* **270**, 20781–20786.
20. Gray, N. K., Pantopoulos, K., Dandekar, T., Ackrell, B. A., and Hentze, M. W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 4925–4930.
21. Zhang, D., Albert, D. W., Kohiipp, P., D’Pham, D. Q., and Winnier, J. P. (2001) *Insect Mol. Biol.* **10**, 531–539.
22. Royovsky, M., Lavaste, T., Allanson, C. R., Stearman, R., Kassim, O. O., Cooperman, S., Gordey, V. R., and Rouault, T. A. (2001) *Blood* **15**, 2555–2563.
23. Sulston, J. E., and Brenner, S. (1974) *Genetics* **77**, 95–104.
24. Sulston, J., and Hodgkin, D. J. (1988) *In The Nematode Caenorhabditis elegans* (Wood, W. B., ed) pp. 603–605, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. Lindsley, J. E., and Wang, J. C. (1993) *J. Biol. Chem.* **268**, 8096–8104.
26. Kohler, S. A., Henderson, B. R., and Kuhn, L. C. (1995) *J. Biol. Chem.* **270**, 30781–30786.
27. Kohler, S. A., Henderson, B. R., and Kuhn, L. C. (1995) *J. Biol. Chem.* **270**, 20781–20786.
28. Gray, N. K., Pantopoulos, K., Dandekar, T., Ackrell, B. A., and Hentze, M. W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 4925–4930.
29. Zhang, D., Albert, D. W., Kohiipp, P., D’Pham, D. Q., and Winnier, J. P. (2001) *Insect Mol. Biol.* **10**, 531–539.
30. Royovsky, M., Lavaste, T., Allanson, C. R., Stearman, R., Kassim, O. O., Cooperman, S., Gordey, V. R., and Rouault, T. A. (2001) *Blood* **15**, 2555–2563.
31. Sulston, J. E., and Brenner, S. (1974) *Genetics* **77**, 95–104.
32. Sulston, J., and Hodgkin, D. J. (1988) *In The Nematode Caenorhabditis elegans* (Wood, W. B., ed) pp. 603–605, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
33. Lindsley, J. E., and Wang, J. C. (1993) *J. Biol. Chem.* **268**, 8096–8104.
34. Kohler, S. A., Henderson, B. R., and Kuhn, L. C. (1995) *J. Biol. Chem.* **270**, 30781–30786.
35. Gray, N. K., Pantopoulos, K., Dandekar, T., Ackrell, B. A., and Hentze, M. W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 4925–4930.
36. Zhang, D., Albert, D. W., Kohiipp, P., D’Pham, D. Q., and Winnier, J. P. (2001) *Insect Mol. Biol.* **10**, 531–539.
37. Royovsky, M., Lavaste, T., Allanson, C. R., Stearman, R., Kassim, O. O., Cooperman, S., Gordey, V. R., and Rouault, T. A. (2001) *Blood* **15**, 2555–2563.
38. Sulston, J. E., and Brenner, S. (1974) *Genetics* **77**, 95–104.
39. Sulston, J., and Hodgkin, D. J. (1988) *In The Nematode Caenorhabditis elegans* (Wood, W. B., ed) pp. 603–605, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
40. Lindsley, J. E., and Wang, J. C. (1993) *J. Biol. Chem.* **268**, 8096–8104.
41. Kohler, S. A., Henderson, B. R., and Kuhn, L. C. (1995) *J. Biol. Chem.* **270**, 30781–30786.
42. Gray, N. K., Pantopoulos, K., Dandekar, T., Ackrell, B. A., and Hentze, M. W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 4925–4930.
43. Zhang, D., Albert, D. W., Kohiipp, P., D’Pham, D. Q., and Winnier, J. P. (2001) *Insect Mol. Biol.* **10**, 531–539.
44. Royovsky, M., Lavaste, T., Allanson, C. R., Stearman, R., Kassim, O. O., Cooperman, S., Gordey, V. R., and Rouault, T. A. (2001) *Blood* **15**, 2555–2563.