Inhibition of Heme Biosynthesis Prevents Transcription of Iron Uptake Genes in Yeast*

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Yeast are capable of modifying their metabolism in response to environmental changes. We investigated the activity of the oxygen-dependent high-affinity iron uptake system of Saccharomyces cerevisiae under conditions of heme depletion. We found that the absence of heme, due to a deletion in the gene that encodes δ-aminolevulinic acid synthase (HEM1), resulted in decreased transcription of genes belonging to both the iron and copper regulons, but not the zinc regulon. Decreased transcription of the iron regulon was not due to decreased expression of the iron sensitive transcriptional activator Aft1p. Expression of the constitutively active allele AFT1-1m was unable to induce transcription of the high affinity iron uptake system in heme-depleted cells. We demonstrated that under heme-depleted conditions, Aft1p-GFP was able to cycle normally between the nucleus and cytosol in response to cytosolic iron. Despite the inability to induce transcription under low iron conditions, chromatin immunoprecipitation demonstrated that Aft1p binds to the FET3 promoter in the absence of heme. Finally, we provide evidence that under heme-depleted conditions, yeast are able to regulate mitochondrial iron uptake and do not accumulate pathologic iron concentrations, as is seen when iron-sulfur cluster synthesis is disrupted.

The insolubility of Fe3+ in aqueous solution and the toxicity of iron-generated oxygen radicals has led to the assumption that the use of iron in biochemical reactions initially occurred in an anaerobic environment. While many iron-requiring biochemical reactions in bacteria occur anaerobically, most eukaryotic iron-requiring biochemical reactions involve electron-mediated oxygen binding or transfer. Examples of these reactions include nucleic acid reduction, lipid desaturation, and sterol synthesis (1). Such reactions do not occur anaerobically, and yeast have developed non iron-dependent alternatives (e.g. ribonucleic acid reduction). Other reactions, (ergosterol synthesis) do not occur in the absence of oxygen and yeast become auxotrophic for the products of those reactions (1). Yeast also show a reduced ability to accumulate iron anaerobically, as the activity of the high-affinity iron transport system is oxygen-dependent (2). Many of the genes that encode iron-dependent enzymes are regulated by heme-sensing transcriptional activators (Hap1p, Hap2,3,4,5 complex) or repressors (Rox1p, Mot3p) (3). In this communication we demonstrate that in the absence of heme, transcription of the iron and copper regulons is inhibited, while the zinc regulon is unaffected.

Mitochondria are the site of two critical points in iron metabolism: the iron chelation step of heme biosynthesis, and Fe-S cluster synthesis. Inhibition of Fe-S cluster synthesis or export in either yeast or mammals leads to excessive mitochondrial iron accumulation (for review, see Ref. 4). In humans with sideroblastic anemia, inhibition of heme biosynthesis has been shown to result in pathological iron accumulation in the mitochondria of erythroid precursor cells (for review, see Ref. 5). We demonstrate that inhibition of heme biosynthesis in yeast prevents transcription of the high-affinity iron transport system, precluding mitochondrial iron accumulation.

MATERIALS AND METHODS

Strains and Growth Media—DY150 [MATα, uro3-52, leu2-3,112, trp1-1, his3-11, ade2-1, can1-100(oc)] and DY1457 [MATα uro3-52, leu2-3,112, trp1-1, his3-11, ade6, can1-100(oc)] were derived from the W303 strain of S. cerevisiae. The Δhem1 strain was constructed by insertion of the LEU2 gene into the HEM1 locus of the DY1457 strain [MATα uro3-52, trp1-1, his3-11, ade6, can1-100(oc), hem1::LEU2]. The FET3-lacZ strain was constructed by insertion of FET3-lacZ reporter construct into the HO locus of the DY150 strain [MATα, uro3-52, leu2-3,112, trp1-1, his3-11, ade2-1, can1-100(oc)], HO::FET3-lacZ. The Δhem1 strain and the FET3-lacZ strain were crossed, tetrads were dissected, and spores from this cross were used for the work in this study. All strains used in this study are listed in Table 1. Strains were generated that contained an integrated plasmid that expressed FET3 and FTP1 under dual constitutively active PGK promoters (21), a gift from Dr. Caroline Philpott (National Institutes of Health, Bethesda, MD).

The Δhem1 strain was maintained in either YPD or DM media supplemented with 50 μg/ml 5-aminolevulinic acid (ALA) (Frontier Scientific, Logan, UT). To deplete heme, cells were washed three times in an equal volume of H2O, and re-inoculated into media containing a source of unsaturated fatty acids, sterols, and methionine (TEM media). 100× Tween/ergosterol supplement was made by mixing 50:50 ethanol/ Tween 80; followed by addition of ergosterol to final concentration of 1.2 mg/ml. 500× methionine stock was made at 27.8 mg/ml. Cells were grown for 10 h in TEM to deplete heme before being used in experiments. An alternative approach to deplete heme was to incubate Δhem1 cells in low (0.5 μg/ml) concentrations of ALA.

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### TABLE I

| Strains used in this study | Source |
|---------------------------|--------|
| DY150 MATa, ura3–52, leu2–3, 112, trpl–1, his3–11, ade2–1, can1–100(oc) | W303 WT strain |
| DY1457 MATa, ura3–52, leu2–3, 112, trpl–1, his3–11, ade6, can1–100(oc) | W303 WT strain |
| Δhem1 FY1547, hem1::LEU2 | This study |
| ΔFET3-lacZ FY1547, H0::FET3-lacZ | This study |
| Δhem1 (6D) hem1::LEU2, trpl–1, his3–11, ura3–52, can1–100(oc) | This study |
| Δhem1 (1A) hem1::LEU2, H0::FET3-lacZ, trpl–1, his3–11, ura3–52, can1–100(oc) | This study |
| Δhem1 (3B) Δhem1 (3B), ade2–1::3PDC/FET3/FT1 | This study |
| Δyfh1 FY1540, yfh1::HIS3, pMETYFH1 | This study |
| Δhem1/Δyfh1 Δhem1 (3B), yfh1::HIS3, pMETYFH1 | This study |
| Δhem1/Δyfh1/FET3/FT1 Δhem1/Δyfh1, ade2–1::3PDC/FET3/FT1 | This study |

### β-Galactosidase Reporter Assay

A β-galactosidase reporter construct containing the promoter sequence of FET3 has been described previously (6). A zinc-responsive β-galactosidase reporter construct containing the promoter sequence of ZRT1 was a gift from Dr. Dennis Winge (University of Utah, Salt Lake City, UT). A heme-sensitive β-galactosidase reporter construct containing the promoter sequence of HMG2 was a gift from Dr. Jasper Rine (UC, Berkeley). β-Galactosidase activity was assayed as described previously (6).

### S1 Nuclease Protection Assay

S1 nuclease protection assay protocol was performed as described previously (7). The oligonucleotides used for this assay were as follows: FET3 oligonucleotide (78 bp); 5′-GGC GCA ATT GGA CAT TGC GTC GTC AAG AAG GCC ACA CGG TCC ATA GAG GGC GTT CCG TTT TGG AAG AAG CGG TGG GGC CAT-3′, FIT1 oligonucleotide (61 bp); 5′-GAT GGT GGT GGT GAT ACT CTC AGC, CMD1 oligonucleotide (46 bp); 5′-GGG CAA AGG CTG TGA ATT CAG CAA TTT GGT CGG AGC CAC-3′.

### Iron Uptake and Subcellular Fractionation

Cellular iron accumulation was assayed in the presence of ascorbate as described previously (8). Accumulation of 59Fe in mitochondria was assayed by S1 nuclease protection assay protocol (6). Accumulation of 59Fe in mitochondria was assayed as described (7).

### Subcellular Localization of Aft1p

An amino terminus GFP epitope-tagged Aft1p was constructed in the high copy pRS426 under control of the AFT1 promoter (1000 bp). This plasmid was sequence-verified and complemented a sft1 cell strain (data not shown). Cells were grown in CM media in the presence of either ALA or TEM. After 10 h of growth, each 50 μg FeSO4 or 80 μg BPS was added to the cell cultures. 10 μl samples of live cells in mid-log phase were mounted onto slides for microscopy. Fluorescence microscopy was performed using an Olympus microscope with the 100× objective lens and a GFP filter. Data was collected using MagnaFire software package.

### Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed using an established protocol (10). Chromatin was immunoprecipitated using an antibody against an HA epitope-tagged Aft1p (PAFT1-HA1x2) generated as described previously (11). Rabbit polyclonal anti-HA antibody was a gift from Dr. Tom Stevens (University of Oregon, Eugene, OR). The primers used for the Aft1p binding region of the FET3 promoter produced a 287-bp fragment: FET3-F 5′-GGG CCA ATA AAT GGT CTT CTC CGA-3′, FET3-R 5′-CAT ACT CCC TCG AAG GAT CGA CTA CT-3′. The primers used for CMD1 promoter produced a 252-bp fragment: CMD1-F 5′-GCC TTC TCT CTA ATT CCC AAA GT-3′, CMD1-R 5′-GTC ATG TAG GAC ACT CTC CAA GG-5′. Immunoprecipitated DNA was PCR-amplified, run on a 3% agarose gel, and imaged using a BioRad phosphorimager.

### RESULTS

**The High-affinity Iron Transport System Is Inactive in the Absence of Heme**—We examined the effect of mutations in the heme biosynthetic pathway on cellular iron uptake to define the relationship between heme synthesis and cellular iron metabolism. Yeast with a mutation in HEM1, which encodes aminolevulinic acid synthase, can be maintained through the addition of high concentrations of aminolevulinic acid (ALA). Removal of ALA results in the loss of heme, and the cells become auxotrophic for unsaturated fatty acids, ergosterol, and methionine (12). In the absence of high concentrations of ALA (50 μg/ml), HEM1 cells can be depleted of heme by incubation in media containing low ALA (0.5 μg/ml); a concentration of ALA that preserves cell viability (14). If the products of the heme-dependent reactions are supplied, (Twen 80, a source of unsaturated fatty acids; ergosterol; and methionine, TEM), HEM1 cells can grow fairly normally in liquid media for extended periods of time. Growth of heme-depleted cells in TEM media, however, is highly strain-dependent (13). The strains employed in this study are from a W303 background and are capable of growing in the absence of heme in TEM media.

Loss of heme can be demonstrated through use of a reporter construct containing the promoter region of the HMG2 gene. HMG2 encodes a hydroxymethylglutaryl-CoA reductase that is induced under anaerobic conditions. Expression of this gene is inhibited by the presence of heme. The data in Fig. 1A confirms previous studies (14), which demonstrate that when Δhem1 cells grown in the absence of ALA (TEM media) cellular heme concentration decreases and HMG2 is induced. Depletion of at least a regulatory pool of heme is rapid, as there is a constant rate of β-galactosidase accumulation after only 2 h of incubation in ALA-free media. Even though there is a dramatic change in HMG2 transcription under heme-depleted conditions, there is little change in cell growth. As shown in Fig. 1B, there is no difference in growth rate of Δhem1 cells grown in either ALA or TEM. When Δhem1 cells were not supplemented with either ALA or TEM, growth was significantly impaired.

Wild-type and Δhem1 cells grown in high ALA showed similar induction in iron transport when media is made iron-limited (data not shown). However, when Δhem1 cells were grown in TEM media, there was minimal induction of the high-affinity iron transport system in response to iron deprivation (Fig. 1C). Similar results were obtained when cells were incubated in low ALA to deplete heme (data not shown). The high-affinity iron transport system is comprised of a multicyper oxidase (Fet3p), and a transmembrane permease (Ftr1p) (15, 16). Expression of these proteins is regulated by the iron-sensing transcription factor Aft1p (17). Analysis of FET3 mRNA by S1 ribonuclease protection assay showed that Δhem1 cells placed in low ALA and low iron demonstrate a reduced level of transcripts (Fig. 1D). In addition to a lack of FET3 mRNA, there was decreased expression of mRNA for FIT1, which encodes a GPI-linked cell wall protein that is another member of the iron regulon (18).

A similar inhibition of iron transport activity and FET3 transcription was seen using a Δhem15 strain. HEM15 encodes ferrochelatase, which catalyzes the final step in the heme biosynthetic process: the insertion of Fe2+ into the porphyrin macrocycle. Δhem15 cells can be grown in the presence of heme. Upon removal of heme, there was no induction of the high-
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The decrease in \textit{FET3} and \textit{FIT1} transcript levels in the absence of heme can be explained by either reduced transcription or decreased mRNA stability. We utilized a \textit{FET3-LacZ} reporter construct to determine whether the absence of heme affects \textit{FET3} transcription. \textit{Δhem1} cells grown in high ALA-containing media, showed a robust expression of \(\beta\)-galactosidase when incubated in low iron media (Fig. 2A). In the absence of ALA, however, there was a significant reduction in \(\beta\)-galactosidase activity in iron-limited \textit{Δhem1} cells. To determine if the absence of heme affects the expression of genes involved in the metabolism of other metals, we utilized metal-sensitive reporter constructs. The absence of heme-reduced induction of genes regulated by the copper regulon, as shown by decreased \(\beta\)-galactosidase induction in cells containing a copper-sensitive \textit{CTR1-LacZ} reporter construct (Fig. 2B). Conversely, there was little inhibition of zinc-regulated genes in heme-depleted cells, as shown through use of a zinc-sensitive \textit{ZRT1-LacZ} reporter construct (Fig. 2C).

Inducibly coupled plasma atomic absorption spectrometry was performed to determine metal concentrations of \textit{Δhem1} cells under different growth conditions. Consistent with the lack of induction the iron and copper regulons, there was a decrease in the metal concentration of iron and copper in heme-depleted cells when compared with either wild-type cells or \textit{Δhem1} cells grown in high ALA (Fig. 3). There was no significant difference in zinc concentration between the wild-type cells and the \textit{Δhem1} cells.

\textit{Synthesis and Translocation of Aft1p Is Not Affected by Heme Depletion}—We considered the possibility that heme-depleted cells are functionally \textit{AFT1} null due to lack of synthesis of Aft1p. Analysis of \textit{AFT1} mRNA levels by S1 ribonuclease protection assays demonstrated that \textit{AFT1} transcripts were unaffected by heme depletion (Fig. 4A). Further, Western analysis of cells transformed with a plasmid containing an epitope-tagged Aft1p showed that in the heme-depleted cells there was no significant difference in concentration of Aft1p when compared with wild-type cells (Fig. 4B). We note that there was increased expression of Aft1p in low iron cells, which was not affected by the absence of heme.

It is possible that in the absence of heme, iron can accumulate in cells and inhibit transcription of \textit{AFT1}-regulated genes. This was suggested by Hassett et al. (19) to explain the lack of \textit{FET3} transcription during anaerobiosis. To test this possibility, we took advantage of the dominant allele \textit{AFT1}–\textit{1up}, which is iron insensitive and constitutively activates transcription of the iron regulon (17). In \textit{AFT1}–\textit{1up} cells, there was minimal expression of the \textit{FET3-LacZ} reporter construct in the absence of heme (Fig. 4C). This result suggests that inhibition of tran-
scription in heme-depleted cells cannot be ascribed to accumulation of cytosolic iron.

The iron-sensing transcription factor Aft1p is cytosolic in the presence of iron (11). In the absence of iron, Aft1p is translocated into the nucleus where it activates transcription of the iron regulon. It may be possible that Aft1p is synthesized but cannot translocate into the nucleus in the absence of heme. To test that possibility, we employed an \( \text{AFT1-GFP} \) fusion construct carried on a high copy plasmid. Genetic experiments confirmed that this construct could complement \( \Delta \text{hem}1 \) cells, permitting their growth on low iron media (data not shown).

When \( \Delta \text{hem}1 \) cells carrying the \( \text{AFT1-GFP} \) construct were incubated in high ALA in the presence of iron, fluorescence was cytosolic (Fig. 5A), whereas when cells were incubated in iron-depleted media, fluorescence was localized to the nucleus (Fig. 5B). This result confirms previous studies that utilized an HA-tagged Aft1p (17). When \( \Delta \text{hem}1 \) cells were depleted of heme by growth in TEM media and grown in either iron-replete (Fig. 5C) or iron-limiting conditions (Fig. 5D), the cellular distribution of Aft1p-GFP was similar to that seen in either wild-type cells or in \( \Delta \text{hem}1 \) cells grown in high ALA. Taken together, the data in Figs. 4 and 5 suggest that the lack of transcription of the iron regulon is not the result of either the absence of Aft1p or its inability to localize to the nucleus.

Translocation of Aft1p from cytosol to nucleus may be independent of DNA binding and transactivation, i.e. nuclear localized Aft1p may not necessarily bind to DNA and induce transcription. To determine whether the Aft1p binding region of the \( \text{FET3} \) promoter is occupied by Aft1p in the absence of heme and iron, we performed a chromatin immunoprecipitation assay. There was minimal \( \text{FET3} \) promoter occupancy when \( \Delta \text{hem}1 \) cells were grown in high ALA in the presence of high iron. Upon iron depletion, however, Aft1p occupies the \( \text{FET3} \) promoter (Fig. 6), resulting in transcriptional activation (Fig. 2A). When \( \Delta \text{hem}1 \) cells were depleted of heme by incubation in TEM...
media, the FET3 promoter occupancy of Aft1p was identical to that seen in hem1 cells grown in high ALA (Fig. 6). The ability of Aft1p to occupy the FET3 promoter in the absence of iron and heme implies that either a co-activator is absent or that a specific repressor is present. To ensure that the PCR analysis was performed within the quantitative range, several input chromatin dilutions were made and used for amplification reactions (10). Results showed that the amount of PCR product was linear with respect to the input chromatin dilution, and that the amount of immunoprecipitated chromatin PCR product was within this linear range (data not shown).

Iron Does Not Accumulate in the Mitochondria of hem1 Cells—The low rate of iron accumulation in hem1 cells grown under heme-depleted conditions (Fig. 1C) can be explained by the inhibition of FET3/FTR1 transcription. We and others (4) reported that mutations in genes that affect Fe-S cluster formation result in excessive mitochondrial iron accumulation. Reduction of cytosolic iron prevents excessive mitochondrial iron accumulation (8, 20). A conclusion from those studies is that iron cannot accumulate in mitochondria when cytosolic iron is low. We devised a test to determine if iron could accumulate in mitochondria when cytosolic iron was high. A hem1 mutant was constructed that constitutively expresses the constitutively active FET3 promoter (negative control). The mock immunoprecipitation (IP) was performed in the absence of antibody.

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Fig. 4. Heme depletion does not affect AFT1 expression but prevents transcriptional activation. A, wild-type or hem1 cells, grown in either high or low ALA, were incubated for 6 h in either CM or CM made iron limited by the addition of 80 μM BPS. Cells were harvested, mRNA extracted, and the mRNA probed for FET3, AFT1, and CMD1. Lane 1, wild type, high ALA; lane 2, wild type, low ALA; lane 3, hem1, high ALA; lane 4, hem1, low ALA. B, hem1 cells, transformed with a plasmid containing an HA epitope-tagged Aft1p, were grown in either high ALA or TEM, with either 50 μM FeSO₄ or 80 μM BPS. Aft1p was visualized in cell extracts by Western analysis: lane 1, hem1 cells grown in high ALA, 50 μM FeSO₄; lane 2, hem1 cells grown in high ALA, 80 μM BPS; lane 3, hem1 cells grown in TEM, 50 μM FeSO₄; lane 4, hem1 cells grown in TEM, 80 μM BPS. C, hem1 cells were transformed with either an empty plasmid (black bar), a plasmid expressing wild-type AFT1 (light gray bar), or a gain of function allele of AFT1 (AFT1-GFP construct, white bar). Cells were incubated in iron-deprived CM for 6 h, and fluorescence was examined. Nomarski differential interference contrast (DIC) image is shown for each panel.

Fig. 5. Heme does not affect the subcellular distribution of Aft1p-GFP. hem1 cells, transformed with a plasmid containing a AFT1-GFP construct, were grown either in high ALA media (A and B), or in TEM media (C and D). The cells were either supplemented with 50 μM FeSO₄ (A and C) or with 80 μM BPS (B and D). The cells were grown for 6 h, and fluorescence was examined. Nomarski differential interference contrast (DIC) image is shown for each panel.
both wild-type (high ALA media) and heme-depleted conditions (TEM media), uptake of $^{59}$Fe was high. Ascorbate was added as a reducing agent to bypass the requirement for the heme-dependent ferrireductase. Compared with $\Delta hem1$ cells grown in ALA and 80 $\mu$M BPS, iron uptake in the $\Delta hem1$ strain grown in TEM and 80 $\mu$M BPS was increased 2.5-fold (Fig. 7A). This result indicates that heme depletion does not affect the activity of Fet3p/Ftr1p when the genes are transcribed from iron-independent promoters. When the $\Delta hem1$-FET3/FTR1 strain was grown in TEM media in the presence of 50 $\mu$M FeSO$_4$, the $^{59}$Fe uptake was approximately equal to $\Delta hem1$ cells grown in ALA and 80 $\mu$M BPS.

We note in Fig. 7A that there was a difference in iron uptake when the $\Delta hem1$-FET3/FTR1 strain was cultured in iron-replete or iron-limiting conditions. Otherwise, we demonstrate that this difference is due to iron-induced post-translational degradation of cell surface Fet3p/Ftr1p. The difference in iron uptake of the $\Delta hem1$-FET3/FTR1 strain, when grown in ALA compared with TEM is likely a consequence of the anaerobic response, although the exact mechanism is unknown.

To investigate the subcellular distribution of $^{59}$Fe in $\Delta hem1$-FET3/FTR1 cells, cultures were incubated with $^{59}$Fe for 10 min followed by 2 h of incubation in $^{59}$Fe-free media. Cells were disrupted, and the homogenate was separated into a cytosol and membrane fraction. In $\Delta hem1$-FET3/FTR1 cells grown in TEM, most $^{59}$Fe was present in the cytosol, with only a small fraction associated with membranes (Fig. 7B). The membrane fraction was resuspended and applied to an Iodixanol gradient to separate organelles based upon density. The membrane-associated $^{59}$Fe in the $\Delta hem1$-FET3/FTR1 strain was not concentrated in any particular fraction (Fig. 7C). This result indicates that even when cytosolic iron level is high heme-depleted mitochondria do not accumulate excessive amounts of iron.

As shown previously, in the absence of Yfh1p, most accumulated $^{59}$Fe was membrane-associated, and when analyzed by Iodixanol gradients, was found to be associated with mitochondria (7, 20). To determine if the absence of heme prevents mitochondrial iron accumulation, cells were constructed that were $\Delta hem1$-FET3/FTR1 and which contained a regulated YFH1 ($\Delta yfh1$, pMET3/YFH1). The total amount of cellular iron present in these cells was similar in both the presence and absence of Yfh1p (data not shown). However, in the absence of Yfh1p there was a greater amount of iron present in the membrane-associated fraction (Fig. 7B). When analyzed by Iodixanol gradient, much of the increased iron was found associated with mitochondria (Fig. 7C) (7, 20). The reason the percentage of radioactivity in membranes appears less in the $\Delta hem1/yfh1$ cells than in $\Delta yfh1$ cells is due to the significantly increased total cellular iron accumulation resulting from the expression of the constitutive FET3/FTR1. When the data is normalized to membrane-associated radioactivity, the percentage in mitochondria is similar. These results indicate that in the absence of heme, yeast still have a functional mitochondrial iron import mechanism.

**DISCUSSION**

Yeast are facultative anaerobes, capable of modifying their metabolism as a function of oxygen tension. Yeast utilize both respiratory and fermentative carbon catabolism when oxygen is replete. Yeast satisfy their energy requirements entirely by glycolysis under anaerobic conditions. Heme, which is used in a variety of oxygen-dependent reactions, is dispensable under anaerobiosis. Under anaerobic conditions, heme synthesis is decreased and cells remodel their metabolism to dispense with the need for heme-based enzymes. A major difference between prokaryotes and eukaryotes is that much of eukaryotic iron-based metabolism, like heme-based metabolism, is oxygen-dependent. Many of the same oxygen-based metabolic pathways that require heme also require inorganic iron. Examples of such pathways include respiration, sterol synthesis, and lipid metabolism. In response to the lowered respiratory activity, the synthesis of many iron-containing enzymes is dramatically decreased. Similar arguments pertain to enzymes that utilize copper, another redox active transition metal. Copper enzymes

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$^a$ J. Kaplan, unpublished data.
that either utilize oxygen (cytochrome c, Fet3p/Fet5p) or metabolize oxygen radicals are either ineffective or unnecessary under anaerobic conditions. Decreased expression of copper and iron containing enzymes results in a decreased concentration of these redox active metals. It is not surprising that in the absence of heme there is reduced expression of the transport systems for redox metal acquisition resulting from decreased transcription of both the iron and copper regulons.

Decreased expression of FET3 due to anaerobiosis has been seen previously (19). That study suggested that decreased expression resulted from increased cytosolic iron, particularly Fe2+, which would repress FET3 transcription by deactivating the iron-dependent transcription factor Aft1p. Support for this hypothesis was based on reversal of the anaerobic repression of FET3 transcription by a permeable Fe2+ chelator, BIP, and by expression of the iron-independent AFT1-1 allele. However, the anaerobic induction of FET3/FTR1 by Aft1-19 only leads to a fraction of the level of FET3 mRNA seen aerobically. In the absence of heme, as shown in this study, there is a dramatic reduction in both iron transport activity and FET3 mRNA; only 10–15% of message activity is recovered by the AFT1-19 allele. It is important to note that there is a methodological difference between Hasset et al. (19) and the study presented here. The former study incubated cells under anaerobic conditions using an argon/nitrogen blanket, while we examined the effects of heme depletion using a hem1 strain grown in ambient oxygen.

The absence of heme is commonly considered to be an indicator of anaerobiosis. In addition to its role as an oxygen carrier in metabolic reactions, heme also plays a role in signaling. Many of the transcriptional changes required for metabolic remodeling are based on heme-dependent transcription factors. Most notable are Hap1p and Hap2/3/4/5p, well-described transcription factors that either activate or repress genes required for aerobic or anaerobic growth. In the absence of heme, many of the physiological changes seen are similar to those seen in anaerobiosis although the absence of heme may not be identical to anaerobiosis.

We have explored the mechanism by which the absence of heme prevents transcription of the iron regulon. The decrease in transcriptional activity does not result from decreased expression of AFT1. In the absence of heme, Aft1p acts in an iron-responsive manner, not only in its ability to translocate from the cytosol to the nucleus in response to iron, but also to occupy the FET3 promoter site as defined by chromatin immunoprecipitation analysis. We conclude that in the absence of heme the behavior of Aft1p is unaffected. Our studies also determined that Hap1p and Hap2/3/4/5p do not affect heme-based transcriptional inhibition of the iron regulon. Deletion of HAP1 or HAP2 did not prevent induction of the iron regulon, nor did deletion of these genes reverse the inhibition of transcription resulting from the absence of heme (data not shown). We also found that deletion of transcriptional repressors Rox1 or MOT3 did not alter the heme-dependent repression of the iron regulon (data not shown). These latter results are supported by published microarray studies that demonstrate that while anaerobiosis had large effects on FET3 and other members of the iron regulon (22), deletion of HAPI, ROX1, or overexpression of HAP4, had little effect on the iron regulon (22–24). Deletion of ROX1 increases expression of the low affinity iron transporter FET4 (25, 26). In Δrox1 cells, there is a reduction in the ability to induce FET3 transcripts (26). We ascribe this reduction to increased cytosolic iron brought in by the increased expression of Fet4p. Beyond a 2-fold change in the ability to induce FET3 reporter constructs when exposed to low iron media, heme depletion still prevented transcription of FET3 in Δrox1 cells. We conclude that there are undescribed genes that affect the transcription of the iron regulon in the absence of iron. Recent studies of oxygen-based transcription and oxygen response elements have suggested that the effects of anaerobiosis cannot be accounted for by the known transcriptional activators or repressors (27, 28).

In yeast, defects in mitochondrial Fe-S cluster synthesis result in increased mitochondrial iron accumulation due to an inhibition of mitochondrial iron export. Studies using mammalian cells also suggest that defects in Fe-S cluster synthesis leads to mitochondrial iron accumulation. This conclusion is based on the observation of increased mitochondrial iron in humans and mice with defects in either frataxin (29, 30) or ABC7 (31, 32), the mammalian homologues of YFH1 and ATM1, respectively. Both Atm1p (4) and Yih1p (7, 33, 34) have been shown to play a role in Fe-S cluster synthesis and export in yeast. Heme deficiency in mammals leads to excessive mitochondrial iron accumulation in erythroid precursors but not in other tissues (35). The absence of heme does not, however, lead to mitochondrial iron accumulation in yeast. We initially explained this result as a consequence of the decreased transcription of the iron regulon. Deficient Fe-S cluster synthesis only results in mitochondrial iron accumulation when cytosolic iron levels are high (20, 36). Decreased cytosolic iron, resulting from either media iron deprivation, deletion of iron transporters or increased vacuolar iron storage, prevents excessive mitochondrial iron accumulation. Since decreased heme synthesis prevents transcription of the high-affinity iron transport system, cellular iron levels are low and may preclude mitochondrial iron accumulation. To test this possibility we expressed FET3/FTR1 independent of the iron regulon using a constitutive promoter. In the absence of heme, cells expressing Fet3p/Ftr1p accumulate high levels of iron when given reduced iron. The fact that these proteins are expressed and functional indicates that the effect of heme is directed at transcription of the transport system. Even when heme-deficient cells are iron-replete there is no excessive iron accumulation in mitochondria. Heme deficiency, however, does not prevent iron-replete Δyfh1 cells from accumulating iron within mitochondria.

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