Research over the past 6 years has identified the primary components of the high affinity iron uptake system in the yeast *Saccharomyces cerevisiae* (1–8). One of the striking features of this pathway is the involvement of a protein, encoded by *FET3*, that is homologous to the multicopper oxidases, ceruloplasmin, and ascorbate oxidase (5, 9). The Fet3 protein is localized to the yeast plasma membrane (7, 8) in association with the *FTR1* gene product, the putative iron permease (8). Ftr1 contains a REGLE motif that is homologous to a functionally essential feature of mammalian ferritin thought to be involved in the trafficking of Fe(III) from the ferroxidase center to the site of iron core formation in that protein (10, 11). The apparently obligate role of Fet3 in high affinity iron accumulation in yeast directly links the iron status of the cell to its copper status: copper-deficient yeast are also iron-deficient because the apo-Fet3 found in copper-deficient cells is inactive with respect to its essential role in iron uptake (4, 5, 8). This insight has provided significant clues to the molecular interactions between iron and copper in mammals (12, 13). That is, the identification of the role that Fet3 appears to play in iron accumulation in yeast and its homology to ceruloplasmin has provided support for the hypothesis that the ferroxidase activity exhibited by ceruloplasmin is integral to this protein’s role in mammalian iron metabolism (8, 9, 12, 13).

The initial step in iron uptake in yeast is the reduction of medium Fe(III) to Fe(II) by plasma membrane metal reductases (1–3). The major reductase is encoded by *FRE1*. This Fe(II) is suggested to be the substrate for Fet3, which reoxidizes it to Fe(III) coupled to a four-electron reduction of O$_2$ (7, 9, 13). In this model, the Fe(III) is taken into the cell via Ftr1 (8, 13). Evidence for this model is that yeast plasma membranes containing active Fet3 are reported to consume O$_2$ in an Fe(II)-dependent manner with an Fe(II)/O$_2$ stoichiometry of 4:1, consistent with Equation 1 (7). This equation describes the ferroxidase reaction previously demonstrated to be catalyzed by ceruloplasmin (14). Fet3 also possesses a ferroxidase activity (9).

$$4\text{Fe(II)} + O_2 + 4H^+ \rightarrow 4\text{Fe(III)} + 2H_2O \quad (\text{Eq. 1})$$

This model postulates dioxygen as an obligate co-substrate in iron accumulation in yeast. In this report we test the hypothesis that the state of cell aerobiosis also regulates the expression of iron uptake activity in yeast, for example, it regulates the expression of respiratory function in this organism (15). Indeed, we show that anaerobically grown yeast do not express genes that encode proteins associated with high affinity iron uptake in yeast. The data indicate that this regulation by culture aerobiosis involves Aft1, a putative trans-activator known to regulate the expression of genes associated with high affinity uptake in an iron-dependent manner (16, 17). These genes are proposed to constitute the iron “regulon” in yeast (17). Although the mechanism by which Aft1 modulates expres-
expression of these genes is not precisely known, in vivo DNA footprinting does indicate Aft1 binds to a consensus ciselement in the 5'-untranslated region of these several loci in iron-depleted cells and not in iron-replete ones. This suggests, although does not prove, the simple model that iron binding to Aft1 in iron-replete cells blocks Aft1-DNA binding and resulting trans-activation (17). We show also that the mRNA species corresponding to the proteins of the high affinity iron uptake pathway accumulate within 5 min following reoxygenation of an anaerobic culture, or addition of a membrane-permeant Fe(II) chelator to anaerobic cells. This result, together with the fact that this expression pattern is modulated in part by Aft1 activity, suggests that a small, labile Fe(II) pool in the cell mediates this regulation also. These observations indicate that high affinity iron uptake by yeast is regulated in two ways by dioxygen. Not only is O2 a substrate essential for this uptake, but its absence or presence in the growth medium modulates the expression of this uptake activity.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions—Wild type strain DEY1457 (MATa ade6 can1 his3 leu2 trpl ura3), DEY1442 (DEY1457 fet4::LEU2), and DEY142772 (DEY1457 fet4::LEU2 GAL4-PET74) were obtained from D. Edel (18). The latter strain expresses Fet4 protein from the GAL promoter when grown in galactose but is Fet4+ when grown in glucose (18). ARY1458 (frt1Δ) was constructed in DEY1457 using a fragment of FTR1 disrupted by TRP1 (8) while ARY1459 (aff1Δ) was constructed in DEY1457 using a fragment of AFT1 disrupted by TRP1 (18). Cultures of these strains were grown in YPD (2% yeast extract, 1% peptone, and 2% glucose) to early log phase, prepared by transformation of the two parental strains with plasmid pT14, fAAS, flameless atomic absorption spectrophotometry; BIP, 2,2'-bipyridyl; BPS, bathophenanthroline disulfonate.

Analytical Measurements and Northern Analysis—59Fe uptake and Fe(III) reductase measurements were made at 30 °C as described previously (19) in 0.1 M MES at pH 6.6 which contained 20 mM citrate and 2% glucose. When present, ascorbate was added to 1 mM. This buffer contained <15 nM contaminating iron as determined by flameless atomic absorption spectrophotometry (fAAS). All uptake velocities were measured in synthetic selective medium (minus uracil) containing 10 μM each copper and iron (19). Cultures grown anaerobically under argon or nitrogen were supplemented with 2 μg/mL ergosterol and 0.2% Tween 80 (20). Anaerobic cultures for experiments were prepared using stocks that were themselves grown anaerobically.

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Analysis of Northern Blots—Total RNA isolation and Northern analysis were by standard techniques (21). All samples were taken from early log phase cultures. fAAS analyses were performed using a Perkin-Elmer model 110B with a model 700 graphite furnace on extracts prepared from whole cells digested at 75 °C in 6 M perchloric acid. Accumulation of the complex between 2,2'-bipyridyl (BIP) and Fe(III) in yeast cells was estimated by extracting cells pretreated with BIP (100 μM for 1 h) with benzyl alcohol (22). The rose-colored BIP-Fe(III) complex was quantitated in the benzyl alcohol phase by its absorbance at 520 nm (ε = 11,000 M⁻¹ cm⁻¹ as determined from a standard curve). Statistical analyses were carried out using INSTAT (GraphPad, San Diego, CA), while 59Fe uptake versus time data were graphed and fit by linear least squares using Cricket Graph (Cricket Software, Malvern, PA).

RESULTS

High Affinity Iron Uptake Activity Is Not Expressed in Anaerobically Adapted Cells—The proposed role of dioxygen as a substrate for high affinity iron uptake in yeast suggested that expression of iron uptake activities might be regulated by the state of aerobiosis of the culture. Indeed, the transcripts encoding all three members of the high affinity iron accumulation pathway, Fre1 (metal reductase), Ftr1, and Fet3 were undetectable in anaerobically grown cells (Fig. 1, panels A and B, t = 0). Consequently, these cells exhibited essentially no Fe(III) reductase activity (Fig. 2, panel A, t = 0) nor 59Fe uptake (Fig. 2, panel B, t = 0). On the other hand, these activities quickly recovered when anaerobically grown cultures were resuspended and then grown in air-saturated medium (Fig. 2). This recovery was preceded by the accumulation of the iron-associated mRNA species absent in the initial anaerobic culture (Fig. 1). This recovery of the steady-state transcript level was rapid, occurring within 15 min, with some recovery ≤5 min (Fig. 1, panel B). This rapid increase indicates that the state of aerobiosis as well as the medium iron concentration (2, 5, 8) modulates the expression of these genes, although it does not suggest the mechanism of this apparent transcriptional regulation.

We next assessed the sensitivity of this transcriptional control to [O2]. To do so, cultures were grown anaerobically and at t = 0, air-saturated medium was added to achieve a final concentration of 0.24, 2.4, or 12.2 μM dissolved O2. Cell samples were taken at intervals for isolation of total RNA. This RNA was then analyzed by Northern analysis as above. The results of this analysis are shown in Fig. 3. Addition of O2 to 0.24 μM caused little increase in the FET3 and FTR1 transcript abundance. In contrast, 2.4 and 12.2 μM O2 caused an increase in these species within 5 min with a maximum increase at 15 min. At this time, both transcripts began to decline in the cells exposed to 2.4 μM O2, a decline that was apparent in the cells exposed to 12.2 μM O2 only after 30 min. Our interpretation of this pattern is that the limiting dioxygen added to these latter cultures was consumed by the cells reducing the dissolved [O2].

![Fig. 1. Anaerobic and aerobic growth and expression of genes of the iron regulon.](http://www.jbc.org/)
Regulation of Iron Uptake Activities by Aerobiosis Is Linked to the Aft1 Protein—We next addressed the mechanism of the down-regulation of FTR1 and FET3 expression (the high affinity iron permease complex) in anaerobic cells. We considered two explanations for this expression pattern which are not, however, mutually exclusive. The first was that O₂ was a direct derepressing; the state of oxygenation of the cells modulated the sensitivity of the Aft1-dependent iron-sensing path-

way to medium iron concentration. The latter explanation was reasonable and had precedent in the case of the transcriptional regulators in *Escherichia coli*, Fur, and Fnr (23). Thus, this possibility was tested first by making the Aft1 trans-activation of iron uptake activities relatively iron-independent and determining whether this negated the anaerobic down-regulation. This was accomplished by using transformed strains DEY1457(pT14) and ARY1458(pT14). The plasmid pT14 carries an Aft1 gain-of-function allele, AFT1-1<sup>up</sup> (16), while, as a control, ARY1458 carries a deletion in FTR1, encoding the high affinity iron permease. Because of a Cys to Phe mutation, the DNA binding activity of Aft1<sup>up</sup> protein at otherwise iron-repressible genes, e.g. FTR1 and FET3 is less strongly suppressed by medium iron (16, 17). That is, in an Aft1<sup>up</sup>-carrying strain, the expression of high affinity iron uptake activity is only weakly repressed by 40 μM ferrous sulfate in comparison to the 50% repression observed in wild type (16).

Wild type transformant DEY1457(pT14) was grown both aerobically and anaerobically in synthetic (SC) medium, which is normally repressing for expression of high affinity iron uptake (17, 18). The two cultures were washed, maintaining their respective states of aerobiosis, and were then resuspended in air-saturated buffer to initiate aerobic uptake. Samples were taken also for preparation of total RNA, which was subjected to Northern analysis. The data showed (Fig. 4, Northern analysis) that in air-grown wild type strain DEY1457, as expected, the Aft1<sup>up</sup> protein supported a 4–5-fold higher level of expression of the “iron regulon” (17), which resulted in a comparable fold increase in iron uptake (Fig. 4, uptake values). Consistent with the model being tested, the Aft1<sup>up</sup> protein supported significant expression of this activity in anaerobically grown cells, as well, although the suppression of the anaerobic phenotype was not complete. That is, these cells recovered ~20% of the transcript abundance and uptake activity seen in the air-grown culture of the pT14 transformant, while, as shown above, the untransformed cells exhibited no detectable uptake activity (Fig. 4). In the same experiment, the *ftr1*Δ strain expressing Aft1<sup>1</sup>, ARY1458(pT14), exhibited essentially no high affinity 59Fe uptake, demonstrating that the uptake seen in the wild type transformant grown under nitrogen or air was Ftr1-dependent (data not shown). These results are consistent with the suggestion that the lack of expression of high affinity iron uptake in anaerobic cultures was due at least in part to the putative iron-dependent suppression of Aft1 protein DNA binding (and subsequent trans-activation) (16, 17), a degree of suppression that would not occur in an aerobic culture at a comparable medium iron concentration (e.g. in YPD which is partially derepressing; *cf.* Fig. 7). The conclusion that Aft1 was involved in this expression pattern was also supported by the fact that

![Graph showing aerobic and anaerobic growth and Fe(III) reductase and 59Fe-uptake activities](http://www.jbc.org/)

**FIG. 2.** Anaerobic and aerobic growth and Fe(III) reductase and 59Fe-uptake activities. Early log phase cell samples from anaerobically grown cultures of DEY1457 (wild type) were used for measurement of Fe(III) reduction (panel A, t = 0) and 59Fe uptake (panel B, t = 0). The remainder of the culture was resuspended in air-saturated medium and allowed to double twice while maintaining the culture density by dilution with fresh medium. Samples were taken for reductase and uptake measurements at the times indicated. 59Fe accumulation was measured under air in the absence (open symbols) and presence (filled symbols) of ascorbate. Ascorbate renders iron uptake reductase-independent. The t = 0–4 h values are from two experiments with samples in triplicate; the error bars represent 1 S.E.

| [O₂], μM | 0.24 | 2.4 | 12.2 |
|----------|------|-----|------|
| *FET3*   |   0  | 50  |  100 |
| *FTR1*   |   0  | 50  |  100 |
| *ACT1*   |   0  | 50  |  100 |

**FIG. 3.** Dioxegen dependence of expression of *FET3* and *FTR1* mRNA. Wild type strain DEY1457 was grown anaerobically in YPD to early log phase. Separate cultures were exposed to the concentrations of dissolved O₂ as indicated by addition of air-saturated medium. Cell samples were removed at the times indicated, and total RNA was prepared, size-fractionated, transferred to nitrocellulose, and probed with 32P-labeled DNA fragments from *FET3* and *FTR1*. The autoradiograph of the resulting blot is shown. Even sample loading was confirmed by analysis of mRNA due to *ACT1*.
the up-regulation of the iron-associated genes upon oxygenation of an anaerobic culture was absent in strain ARY1459, a construct carrying a deletion of AFT1 (data not shown). This observation was consistent with the fact that these genes are not expressed in aerobically grown cultures of an afts1Δ-containing strain (16, 17).

Regulation of Iron Uptake Activities by Aerobiosis Is Linked to Cellular Fe(II)—If this anaerobic repression was due to an iron-independent modulation of Aft1 activity, then manipulation of iron availability by chelation would also be expected to alter this anaerobic expression pattern. One iron chelator, bathophenanthrolines disulfonate (BPS) is well documented to induce expression of aerobic iron uptake in yeast by activating the Aft1 protein-dependent expression of FTR1 and FET3, as well as the other genes in the iron “regulon” (16, 17). This activation is linked to the depletion of cellular iron caused by an inhibition of iron uptake. That is, addition of BPS to an Fe(II)-uptake mixture caused an immediate inhibition of uptake, which was relieved if the BPS was washed out (Fig. 5, filled circles). Similarly, addition of BIP to yeast caused a rapid and strong inhibition of Fe(II) uptake, also (Fig. 5, filled triangles). However, BPS, which is anionic, is likely to be impermeant to yeast cells as it is to mammalian cells (22). BIP, in contrast, is membrane-permeant (22) and, unlike BPS, does enter yeast cells as it is to mammalian cells (22). BIP, in contrast, is membrane-permeant (22) and, unlike BPS, does enter yeast cells as indicated by a distinct rose coloration of the BIP-treated cells due to the (BIP)₃Fe(II) complex (λₘₐₓ 520 nm, ε = 11,000 M⁻¹ cm⁻¹). This complex, which was retained by the cells for at least 2 h following the washout of the extracellular BIP (filled triangles), was extracted from the cells by benzyl alcohol treatment (22) and quantitated spectrophotometrically (Fig. 5, inset). BPS-treated yeast cells did not accumulate (BPS)₃Fe(II) as indicated by the lack of any cell-associated absorbance due to this Fe(II) complex (λₘₐₓ 533 nm, ε = 25,000 M⁻¹ cm⁻¹, data not shown). Note that the presence of the (BIP)₃Fe(II) complex in the cells had little effect on resumption of Fe(II) uptake after removal of the BIP remaining in the uptake buffer (filled triangles).

This difference in permeance of BPS and BIP allowed for a test of the hypothesis that a pool of intracellular Fe(II) provided the signal for repression of Aft1-dependent trans-activation of Aft1-target genes, particularly in anaerobic cells. We reasoned that BIP, being membrane-permeant, had the potential to pass into the cells and alter the intracellular iron distribution, perhaps causing a partitioning of iron away from Aft1 protein or from the iron pool sensed by Aft1, leading to trans-activation of

![Fig. 4. Anaerobic repression of high affinity uptake is mediated through the Aft1 protein.](http://www.jbc.org/)

![Fig. 5. Inhibition of high affinity aerobic Fe(II)-uptake by iron chelators and recovery.](http://www.jbc.org/)

Aft1 protein target genes. BPS, on the other hand, could not affect this pool in this manner, since this chelator was membrane-impermeant. The results were consistent with this reasoning in that while addition of BPS to anaerobic cultures caused only a limited induction of the various iron-regulated genes over 30 min (Fig. 6, panel A, lanes 1, 3, 5, and 7), upon BIP addition the quantity of these transcripts showed a significant increase within 5 min and a strong increase by 15 min (Fig. 6, panel A, lanes 2, 4, 6, and 8). This result is consistent with the hypothesis that the regulation of expression of the iron-dependent target genes in yeast is due at least in part to an intracellular pool of iron that is accessible to BIP and which is possibly redox active, as suggested by the effects of anaerobiosis. The similar effect of aerobiosis on the one hand and presumed Fe(II) chelation by BIP on the other suggests it is the Fe(II) in this pool that is directly or indirectly repressing with respect to Aft1 trans-activation.

For this pool to be sensitive to relatively small changes in available iron concentration and for this sensitivity to be translated into tight control of the expression of high affinity iron uptake activities, the pool itself would have to be small, and the level of these activities would have to be controlled by their rate of expression. This latter condition requires that the transcripts for FTR1 and FET3, for example, be short lived, as suggested above (Fig. 3), and that the permease complex be turned over rapidly or its activity be tightly regulated. The first of these requirements was tested by adding iron back to cells induced to express high affinity iron uptake by pretreatment with either BPS or BIP. As shown in Fig. 6 (panel B) FTR1 and FET3 transcripts were >90% degraded within 10 min of exposure of the induced cells to 10 μM iron. This result shows that these mRNAs do, in fact, have relatively short half-lives; for
A potential role of the low affinity Fe(II) transporter, Fet4, in anaerobic iron uptake—Thus, despite the lack of high affinity iron uptake, anaerobically adapted cells accumulated equivalent iron stores in comparison to air grown ones. One possible explanation of this apparent paradox was that this anaerobic iron accumulation was due to the low affinity iron transporter, encoded by the FET4 gene (18). FET4 encodes a protein required for a low affinity iron uptake in S. cerevisiae that is independent of Fet3 (and Ftr1) and which is specific for Fe(II). The $K_m$ value for iron in Fet4-mediated iron uptake is $\sim 30 \mu M$. An additional distinction between the low and high affinity iron uptake systems is that $FET4$ expression is Aft1-independent. The following results supported this possible explanation.

First, in contrast to the transcripts encoding the components of the high affinity iron uptake pathway, the level of $FET4$ mRNA was equivalent in aerobic and anaerobic cells as indicated by Northern analysis of the same RNA preparations used above (data not shown). Thus, anaerobically grown cells did have the capacity to accumulate iron via the Fet4 protein in that these cells did express this activity.

Second, if this uptake were in fact due to Fet4, it should be inhibitable by iron chelation, i.e. by BPS (18). In the experiments above, the effects of BPS and BIP were followed for short times after chelator addition. If accumulated iron stores were involved in the anaerobic repression, growing the cells in BPS should result eventually in iron depletion and expression of high affinity iron uptake even in anaerobic cultures. This proved to be the case. Growth of cells in the presence of BPS did lead to expression of this activity as demonstrated by Northern blot analysis and iron uptake measurement (Fig. 7). As the results show, there was an $\sim 90\%$ recovery of both $FTR1$ mRNA and high affinity iron uptake in the BPS-treated anaerobic culture in comparison to the air-grown one. That BPS treatment did lead to cell depletion of iron was confirmed by fAAS analysis of these cells; growth in BPS reduced the iron content in the anaerobic culture by $70\%$ (from 2.9 to 0.9 pmol$/10^6$ cells). The percentage inhibition by BPS of iron accumulation in an aerobic culture was the same; cell iron was reduced from 3.5 to 1.1 pmol$/10^6$ cells.

Third, previous data had indicated that Fet4 protein levels did modulate the activity of the high affinity uptake system (18). We wished to build upon these observations with the express purpose of demonstrating a role for Fet4-mediated iron uptake more directly in the regulation of high affinity uptake. Thus, DEY1457 and mutant strains DEY1422 (fet4A) and DEY1422T2 were evaluated for this latter activity. DEY1422T2 is fet4A but in addition carries an integrated copy of a GALA promoter:FET4 fusion, putting $FET4$ expression under control of medium galactose (18). The hypothesis that Fet4-dependent iron uptake modulated the
Aft1-dependent expression of the high affinity iron regulon. The iron concentration, is linked to a cellular pool of iron that modulates the cell via Fet4, an uptake activity that is apparently observed was consistent with the proposal that iron enters DEY1422 persisted under anaerobiosis, the significant recovery of 40% of the high affinity iron uptake by DEY1422T would be galactose-dependent, since dioxygen was an obligate substrate for this uptake via the ferroxidase reaction catalyzed by Fet3 (6, 7, 9, 13), its level might also regulate the level of this activity in the cell. The data clearly show that the expression of FTR1 and FET3 (and FRE1) was down-regulated in anaerobically grown cultures. The expression of these genes was known to be regulated by iron, a regulation that was modulated by the putative trans-activator, Aft1; neither of these loci is expressed in an aft1Δ-containing strain (16, 17). The feedback inhibition due to iron appears to arise from the fact that cellular iron inhibits the binding of Aft1 to a specific target sequence in the 5′-noncoding region of the several genes that are iron-regulated through Aft1 (17). The simplest model, therefore, is that iron binding to Aft1 inhibits or alters its binding to these sequences. In this simplest model, Aft1 is itself also a trans-activator when bound to these cis elements whose consensus sequence is PyPuAC-CCPu (17). Only part of this model has been experimentally confirmed; however, while Aft1-DNA binding in an iron-dependent manner has been demonstrated (17), neither the trans-activation by Aft1 nor the mechanism by which the DNA binding is modulated by iron has been characterized.

One model that links the iron-dependent expression of the genes encoding the high affinity uptake activity via Aft1 to the effect of anaerobiosis is that the state of aerobiosis alters the sensing of the cellular iron level or distribution, or redox state as has been suggested in the case of Fur (23, 26), an iron-regulated repressor protein in E. coli (23, 26, 27). Fnr, a positive regulator of many of the same bacterial genes, is postulated also to be an iron-binding protein whose activity is redox sensitive (23, 28). However, the mechanism of action of Aft1 is likely to be the reverse of the postulated mechanism for Fur, for example. Fur is thought to bind Fe(II) and in this metal-bound form binds to a “Fur” box in target genes as a repressor. Raising the redox potential of the cell (making it more oxidizing, presumably displacing the Fe(II)/Fe(III) redox equilibrium toward Fe(III)) or chelating the iron leads to iron dissociation from Fur, relieving the DNA binding and the repression of transcription (23, 26).

Nonetheless, this model of Fur action appears highly relevant to our finding that anaerobically grown yeast did not express the components of the high affinity iron uptake pathway, the expression of which is known to be iron- and Aft1-dependent. Our data showed that the regulation of expression of FTR1 and FET3 by the state of aerobiosis was similarly Aft1-dependent and iron-mediated. This fact was shown most dramatically by two results. First, expression of the gain-of-function AFT1-1α allele effectively although not completely
reversed the down-regulation seen in an anaerobic culture of the AFT1 wild type strain. The AFT1-1up allele is known to largely (although not completely) uncouple expression of these genes from the cellular iron level (16, 17). Although the mechanism of this uncoupling is not known, a reasonable hypothesis is that the mutation in Aft1 associated with the AFT1-1up allele, a Cys to Phe substitution, inhibits or reduces the binding of iron to Aft1 protein. Consequently, the protein’s activity as a trans factor is rendered relatively insensitive to cellular iron levels, since the Aft1up protein binds constitutively to the DNA at medium iron concentration ≤100 μM (16, 17). This is similar to a Fur protein that is mutant at the potential Fe(II) ligands Cys92 and Cys95 and is inactive with respect to DNA binding to a Fur protein that is mutant at the potential Fe(II) ligands Cys92 and Cys95 and is inactive with respect to DNA binding and transcriptional repression, presumably because it exists only in an apo-form (27). On the other hand, evidence of Aft1 protein phosphorylation has been shown, although not with regard to iron-dependent gene regulation (29). That is, the link between cellular iron levels and Aft1 trans-activity might well be more complex than a simple metal-protein binding equilibrium, for example, and remains to be experimentally delineated.

Second, expression of FTR1 and FET3 increased rapidly in anaerobic cells following chelation of intracellular iron specifically. That is, our data showed that in anaerobic cells this rapid chelator effect was seen only with a chelator that was membrane-permeant. Thus, BIP treatment caused a strong induction of expression of FTR1 and FET3 in anaerobically grown cells, while treatment with BPS, which can only work extracellularly, did not. As shown here, chelation of extracellular iron in aerobic cultures by either reagent blocked high affinity uptake, which effect is known to up-regulate these loci (16). However, chelation of extracellular iron would have a limited impact on iron accumulation within the 5–30-min time frame that with BIP treatment led to induction of expression of the iron-dependent genes (Fig. 6, panel A); over short time periods, intracellular iron levels in such cultures would be relatively insensitive to addition of BPS, for example. At long treatment times, however, BPS did cause a level of cellular iron limitation that led to expression of the high affinity iron uptake, even in anaerobic cultures (Fig. 7). This result is consistent with the demonstrated inhibition of iron uptake due to BPS. Bipyridyl is a chelating agent that can diffuse into the cell and thereby have the potential to chelate intracellular “labile” iron. What is significant about BIP, too, is that it is an Fe(II) chelator (22). Therefore, strong and rapid up-regulation of the iron- and Aft1-dependent loci in anaerobic cells treated with BIP (in contrast to those treated with BPS) suggests that these cells contain a chelatable pool of Fe(II) that is directly or indirectly involved in the equilibria that regulate Aft1 protein-DNA binding or trans-activation (17). This pool would be similar to the chelatable or labile iron pool postulated in mammalian cells that plays a significant role in the regulation of expression of ferritin and transferrin receptor biosynthesis (22, 25) or the iron pool in E. coli that regulates Fur activity (26).

Our results do not directly show how large this pool in yeast is; however, based on the 520-nm absorbance of the benzyl alcohol cell extract and assuming a cell volume of 60 μm³ (30), cellular [BIP₂Fe(II)] = 10 μM can be calculated, or 0.5 pmol of chelatable Fe(II)/10⁶ cells. This value compares to the steady-state level of iron in yeast determined in this work, 3 pmol/10⁶ cells, suggesting that ~15% of the total cell iron is chelatable by BIP. This result also can be compared with the chelatable iron pool measured in rat hepatocytes of 18% of the total transferrin iron accumulated by the cells in 30 min (22). On the other hand, these values for “labile” Fe(II) are to be taken with caution, since they are determined by use of a chelator that very likely displaces some equilibrium distribution of iron in the cell. This could involve both the physical redistribution of Fe(II) as well as the distribution between Fe(II) and Fe(III).

Although the data consistently support a model of regulation of FTR1 and FET3 in anaerobiosis via the iron- and Aft1-dependent signal transduction pathway, this support is, in general, qualitative not quantitative. For example, the Aft1up protein supported only 20% recovery of expression of these genes in anaerobic cells (Fig. 4). Similarly, in the test of the hypothesis that Fet4 contributed to the iron pool sensed by Aft1 in anaerobic cells, the data showed that deletion of FET4 resulted in the recovery of only 40% of the expression of high affinity uptake seen in an aerobic culture (Fig. 8, panel B). While it is perhaps not surprising that the repression of expression of these genes observed in anaerobic cells is not completely suppressed by any one of these genetic manipulations, the results do leave open the possibility that an additional pathway contributes to the anaerobic repression. Certainly, the expression pattern could implicate a repression mechanism that functions in anaerobic cells and, in one model, is relieved by oxygen either directly or indirectly. However, no such anaerobic repressor has been identified in yeast. In comparison, the Rox1 protein is a well characterized repressor of otherwise anaerobically expressed genes; the expression of Rox1, which is a short-lived protein, is positively regulated by heme and oxygen, so that Rox1 is produced in aerobic cells but absent in anaerobic ones (31). This pattern of Rox1 level (repressor activity) leads to the opposite expression pattern than that seen for the genes encoding high affinity iron uptake with respect to culture aerobiosis. Nonetheless, an anaerobic repressor function that contributes to the modulation of the expression of these loci cannot be ruled out on the basis of the results presented here.

On the other hand, the data do indicate that the level of high affinity iron uptake activity in yeast is controlled by the rate of expression of this activity at least at the level of transcription. This is shown here by the fact that the mRNAs encoding the high affinity iron uptake proteins are relatively short lived, thus their steady-state level will be determined by their synthesis. This transcriptional activation appears very sensitive to the change in intracellular iron concentration (or the iron concentration in a specific pool), since it is strongly inhibited within 5 min following cell exposure to medium iron. This result is consistent with the notion that the cellular iron pool controlling this expression is small and labile, and may contain Fe(II), as indicated by the effects of BIP.

This latter inference raised an apparent dichotomy between the models presented here and elsewhere (8, 13), namely that Fe(III), following its generation by the ferroxidase reaction catalyzed by Fe(II), is taken into the cell by Ftr3, but Fe(II) is proposed as the species that is sensed by Aft1. It is also likely that the major storage form of iron in yeast is Fe(III), probably as a polyphosphate in the yeast vacuole (32). The question was, then, where does the Fe(II) in the putative regulatory pool come from? One possibility is that all of the Fe(III) brought into the cell via Ftr1 is subsequently reduced, with this Fe(II) being sensed by Aft1. In this model, iron targeted to the vacuole is reoxidized, comparable to the Fe(II)/Fe(III) conversion in the formation of the iron core in ferritin (10, 11). None of our data excludes this mechanism as contributing to Aft1-dependent gene regulation. Another possibility was that the Fet4 iron transporter was a source of the regulatory Fe(II); Fet4 is specific for ferrous iron (18). Our data do support a role for Fet4 iron as part of this regulatory mechanism, although they do not prove it.

This proposition is kinetically appealing. The Kₘ for Fe(II) in Fet4-dependent uptake is 30 μM (18), so that in the iron concentration regime that regulates expression of FTR1, FET3,
and FRE1 (the ferrireductase), 1–10 μM (1, 18), the rate of Fe(II) uptake by Fet4 is strictly first-order in iron concentration. This kinetic situation predicts that the iron concentration in the intracellular pool(s) to which this Fe(II) is targeted is directly proportional to the iron concentration in the medium. Furthermore, the expression of Fet4 uptake is independent of medium iron concentration (18), that is, Fet4-dependent uptake is constitutive. This expression pattern is consistent with what one would predict for a component of a signal transduction pathway. Last, Ftr1-dependent, high affinity iron uptake is induced 2–3-fold in a fet4Δ strain in comparison to wild type (18) (Fig. 8). This fact and the new data on anaerobically grown cultures that we show here (Fig. 8) are all consistent with a model in which Fet4 iron is likely to be an important source of the intracellular Fe(II) pool that regulates expression of high affinity uptake activity via Aft1.

This discussion of the iron uptake capacity of Fet4 relates also to an apparently paradoxical result, namely, the equivalent iron accumulation in wild type cells grown aerobically or anaerobically, 3.5 versus 2.9 pmol of iron/10⁶ cells, respectively, despite the lack of high affinity iron uptake in the latter condition. This result indicates that, in a YPD-grown anaerobic culture, Fet4 iron accumulation alone is equivalent to the iron accumulation under air due to Fet3 and Fet4. This result is actually quite informative about the interplay of gene expression regulation and modulation of enzyme activity by substrate concentration. First, Fet4-mediated iron uptake is likely to be 2–3-fold greater from anaerobic in comparison to aerobic medium. Fe(II) will predominate in the former condition while Fe(III) will predominate in the latter one. Fet4 iron uptake is strongly dependent on the redox state of the exogenous metal as is indicated by the nearly 3-fold increase in Fet4-dependent uptake upon prereducing the metal with ascorbate (18). Second, while aerobic cultures do express high affinity iron uptake activity, in YPD this uptake is about one-third of maximum, as, for example, indicated by the data in Fig. 7. These data showed that BPS treatment (inducing conditions) resulted in an increase in the rate of high affinity uptake from 3.9 to 11.4 pmol/10⁶ cells/min. Thus, although when fully expressed, Fet3 and Fet4 exhibit similar Vₘₐₙₐ values (18), in YPD Fet4, which is not regulated by medium iron, is fully expressed, while Fet3 is not. In other words, this difference in expression indicates that the loss of Fet3 uptake in anaerobiosis would not have the dramatic impact on iron accumulation that might be considered likely at first glance. Last is the fractional saturation of the two systems by iron at the iron concentration present in YPD, 0.5 mg/liter or 9 μM, as determined by FAAS (data not shown). Assuming that some fraction of this iron in a complex medium like YPD was not biologically available for uptake, for example, 50% of the total, even the available “free” iron concentration = 4.5 μM would be 30 times the Kₘₐₜ value for Fet3 uptake (0.15 μM) (3) and 0.15 × Kₘₐₜ for Fet4 uptake (Kₘₐₜ = 30 μM) (18). Given the relative level of expression of Fet3 and Fet4 activity in YPD (above), at this iron concentration, aerobic Fet3 uptake (97% saturation of 30% of expressible transport activity) would be ~3 times Fet4 uptake (13% saturation of 100% of expressible transport activity). In going to the anaerobic state, the loss of the uptake contribution due to Fet3 would be compensated for by the anticipated 2–3-fold increase in Fet4 uptake due to the predominance of Fe(II) in the medium under this condition.

This analysis does not suggest the implication that Fet3 is redundant to Fet4. Aerobic cultures growing in iron-limited medium become iron-starved if they lack Fet3-dependent high affinity iron uptake (5). That is, Fet3 plays an essential role under conditions of iron deprivation that cannot be rescued by Fet4 uptake; it is simply too inefficient at medium iron concentration <1 μM, particularly in aerobic cultures in which condition iron is essentially present only as Fe(III). What the results presented do suggest is that, with a two-tiered mechanism of regulation of high affinity iron uptake, yeast can very tightly control the rate of iron uptake, particularly when going from an anaerobic to an aerobic growth condition. That is, in the yeast system dioxygen, in effect, regulates high affinity iron uptake in two ways. First, it appears to modulate the expression of the genes encoding the components of this pathway, perhaps by altering the sensitivity of the cellular iron-sensing pathway that functions in this gene regulation via the transcription factor, Aft1. Second, as substrate for Fet3, it is an obligate co-substrate for high affinity iron uptake. The first of these modulation effects is somewhat longer term, while the latter is immediate to the process of iron accumulation by the cell.

That this two-tiered regulatory mechanism presumably has selective advantage indicates that repressing the expression of high affinity iron uptake in anaerobiosis has selective advantage. What this advantage is to a facultative anaerobe like yeast remains to be elucidated, but that it is linked to environmental dioxygen level suggests that it may well relate to suppressing metal-dependent oxidative stress (33). It may appear paradoxical that high affinity iron uptake by an anaerobic cell has pro-oxidant character; iron accumulation by aerobic cells would appear to have more potential for cytotoxicity. However, a facultative anaerobe like yeast is not unlike an obligately aerobic tissue that has become ischemic. That such tissues are at risk for significant cell damage upon reoxygenation and that iron, as Fe(II), released during the ischemia appears to be a significant contributor to this reperfusion damage are both well documented phenomena. For example, desferrioxamine-chelatable iron is released by isolated, ischemic lungs, which exhibited several indices of oxidative damage following reperfusion (34). Similar damage was shown in brain tissue following hypoxia-ischemia reperfusion; a marked intracellular translocation of iron during the ischemia could be linked to the oxidative damage caused by the subsequent reoxygenation (35). A delocalization of intracellular iron occurs in ischemia in skeletal muscle, also; furthermore, the oxidative reperfusion injury sustained by this tissue is attenuated by the application of iron chelators in the reperfusion (36). As noted above, in yeast the data suggest that, in the transition from anaerobic to aerobic growth, suppression of an influx of iron via Fet3 is important for cell homeostasis. In the yeast system, this type of prediction can be tested readily. Indeed, understanding the corresponding, albeit implied toxic relationship between dioxygen and iron in yeast indicated by the results reported here may well clarify the role of intracellular, labile iron in a variety of human pathologies (37).

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