We have investigated the mechanism of frataxin, a conserved mitochondrial protein involved in iron metabolism and neurodegenerative disease. Previous studies revealed that the yeast frataxin homologue (mYfh1p) is activated by Fe(II) in the presence of O2 and assembles stepwise into a 48-subunit multimer (α48) that sequesters >2000 atoms of iron in 2–4-nm cores structurally similar to ferritin iron cores. Here we show that mYfh1p assembly is driven by two sequential iron oxidation reactions: A ferroxidase reaction catalyzed by mYfh1p induces the first assembly step (α → α6), followed by a slower autoxidation reaction that promotes the assembly of higher order oligomers yielding α48. Depending on the ionic environment, stepwise assembly is associated with accumulation of 50–75 Fe(II)/subunit. Initially, this Fe(II) is loosely bound to mYfh1p and can be readily mobilized by chelators or made available to the mitochondrial enzyme ferrochelatase to synthesize heme. Transfer of mYfh1p-bound Fe(II) to ferrochelatase occurs in the presence of citrate, a physiologic ferrous iron chelator, suggesting that the transfer involves an intermolecular interaction. If mYfh1p-bound Fe(II) is not transferred to a ligand, iron oxidation, and mineralization proceed to completion. Fe(II) becomes progressively less accessible, and a stable iron-protein complex is formed. Iron oxidation-driven stepwise assembly is a novel mechanism by which yeast frataxin can function as an iron chaperone or an iron store.

Mitochondria require micromolar concentrations of iron to support the heme and the iron-sulfur cluster biosynthetic pathways (1, 2). Making this iron bioavailable while limiting its participation in free radical reactions is an essential function accomplished by mechanisms that remain largely uncharacterized (2–4). The importance of these mechanisms is exemplified by Friedreich ataxia (FRDA), a severe neuro- and cardio-degenerative disease (5) in which mitochondria lack the ability to handle iron properly (reviewed in Ref. 6). FRDA is caused by mutations in FRDA and results in the loss of frataxin, a conserved nucleus-encoded mitochondrial protein of as yet unknown function (6, 7). Previous studies in Saccharomyces cerevisiae have shown that the loss of frataxin results in accumulation of iron in mitochondria, widespread oxidative damage to mitochondrial and nuclear DNA via Fenton chemistry, and impaired respiratory function (8–11). This phenotype can be explained by new findings that yeast frataxin is required for the biosyntheses of iron-sulfur clusters (12–16) and heme (17), two processes critical for maintenance of mitochondrial iron homeostasis (18, 19).

An open question is how frataxin influences two different iron-dependent pathways and also provides protection from iron toxicity. We have proposed that such diverse roles could be reconciled if the basic function of frataxin were to bind and store iron in a bioavailable and nontoxic form (20). Our studies with recombinant yeast frataxin have shown that the protein is activated by Fe(II) in the presence of O2 and forms an oligomeric species (α6) that catalyzes Fe(II) oxidation (21). When the Fe(II) concentration exceeds the iron-loading capacity of α6, stepwise assembly of α6 oligomers yields a 48-subunit multimer (α48) that sequesters ~2,400 atoms of ferric iron. The multimer is a regular spherical particle with a hydrodynamic radius of ~11 nm and contains small iron cores of 2–4 nm (22) with Fe-O and Fe-Fe interactions similar to those found in ferritin iron cores (23). Similarly, recombinant human frataxin assemblies during expression in Escherichia coli yielding regular spherical particles of ~1 MDa and ordered polymers of these particles that sequester up to 10 atoms of iron per subunit in small cores structurally identical to the yeast frataxin iron cores (23). High molecular weight forms of frataxin can be detected by gel filtration and Western blotting in yeast cells or mouse cardiac tissue, and the native protein binds stoichiometric amounts of 55Fe in metabolically labeled yeast cells (24, 25). These previous findings support the idea that frataxin, like ferritin, has an iron storage role. Here, we have tested if frataxin might also serve as a reservoir of bioavailable iron. We describe the coupled stepwise-assembly/iron-oxidation reaction of yeast frataxin and show that this mechanism is compatible with both iron chaperone and storage functions.

EXPERIMENTAL PROCEDURES
Reagents, Solutions, and Purified Proteins—HEPES, ferrous ammonium sulfate, potassium chloride, α,α′-bipyridine (BIPY), EDTA, dithionite, deuteroporphyrin IX, pyridine, sodium citrate, and bovine

Yeast Frataxin Sequentially Chaperones and Stores Iron by Coupling Protein Assembly with Iron Oxidation*

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Mitochondria require micromolar concentrations of iron to support the heme and the iron-sulfur cluster biosynthetic pathways (1, 2). Making this iron bioavailable while limiting its participation in free radical reactions is an essential function accomplished by mechanisms that remain largely uncharacterized (2–4). The importance of these mechanisms is exemplified by Friedreich ataxia (FRDA), a severe neuro- and cardio-degenerative disease (5) in which mitochondria lack the ability to handle iron properly (reviewed in Ref. 6). FRDA is caused by defects in frataxin, a conserved nucleus-encoded mitochondrial protein of as yet unknown function (6, 7). Studies in Saccharomyces cerevisiae have shown that the loss of frataxin results in accumulation of iron in mitochondria, widespread oxidative damage to mitochondrial and nuclear DNA via Fenton chemistry, and impaired respiratory function (8–11). This phenotype can be explained by new findings that yeast frataxin is required for the biosyntheses of iron-sulfur clusters (12–16) and heme (17), two processes critical for maintenance of mitochondrial iron homeostasis (18, 19).

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EXPERIMENTAL PROCEDURES
Reagents, Solutions, and Purified Proteins—HEPES, ferrous ammonium sulfate, potassium chloride, α,α′-bipyridine (BIPY), EDTA, dithionite, deuteroporphyrin IX, pyridine, sodium citrate, and bovine

The abbreviation used is: BIPY, α,α′-bipyridine.
Iron Chaperone and Storage Properties of Yeast Frataxin

serum albumin were from Sigma, and bovine brain calmodulin from Calbiochem. All buffers and solutions were made with milli-Q-deionized water (18 MΩ). Stock solutions of ferrous ammonium sulfate (2–10 mM) were freshly prepared in water previously deaerated with purging with argon (>0.2 ppm O₂). Calmodulin and albumin were desalted into the appropriate buffer using NAP-25 columns (Amersham Biosciences).

The mature forms of yeast frataxin (mYfh1p and mYfh1p[C98A]) and yeast ferrochelatase were expressed in E. coli (24, 26) and purified as previously described (22, 27). The construct for expression of mYfh1p[C98A] was created via PCR-mediated site-directed mutagenesis as previously described (24). Human H- and L-chain apoferritin homopolymers (28) (designated H- and L-apoferritin) were a generous gift of P. Arosio (Brescia University, Brescia, Italy) and S. Levi (Ospedale San Giovanni, Milan, Italy). Protein concentration was determined from the absorbance and extinction coefficient (ε₂₈₀ nm = 20.000/4.200, 27,900/34,000 m⁻¹ cm⁻¹ for mYfh1p, bovine serum albumin, H- and L-chain apoferritin, respectively, and ε₂₈₀ nm = 3.000 m⁻¹ cm⁻¹ for calmodulin). Iron concentration was either directly measured by inductively coupled plasma mass spectrometry (ICP-MS) at the Mayo Metals Laboratory or deduced from the concentration of Fe(BIPY)₃⁺ (ε₃₅₀ nm = 9.000 m⁻¹ cm⁻¹) (21).

Electrode Oximetry, Ultrafiltration, Gel Filtration, and Fluorescence Measurements—Measurements of dissolved O₂ concentration were performed with a MI-730 micro-O₂ electrode (Microelectrodes, Inc.) (21). The drift of the electrode was <2 μM/min at 30 or 40 °C. Iron binding by apo-proteins and other proteins were measured by ultrafiltration with a molecular mass cutoff of 5 kDa (21). To analyze stepwise assembly of mYfh1p during the iron oxidation reaction described (21, 40). We used citrate/total iron ratios ranging from 0.06:1 to 166:1, and citrate/ferrochelatase ratios ranging from 1:1 to 2500:1, which encompass and exceed the ratios used in anaerobic ferrochelatase assays (citrate/Fe = 1:1; citrate/ferrochelatase = 28:1) (35). EDTA/total iron ratios ranged from 0.33:1 to 7:1 and EDTA/ferrochelatase ratios from 5:1 to 100:1.

RESULTS

Stepwise Assembly of mYfh1p Is Coupled with Two Sequential Iron Oxidation Reactions—At Fe(II)/mYfh1p ratios <0.5, mYfh1p catalyzes Fe(II) oxidation with a stoichiometry of ~2 Fe(II)/O₂ and production of H₂O₂ (ferroxidase reaction) (21). A ~50-kDa oligomer (α₂₈) is responsible for this activity suggesting that three mYfh1p subunits form one binuclear ferroxidation site (21). Here, we have analyzed the iron oxidation reaction of mYfh1p at concentrations of iron (40–75 Fe(II)/mYfh1p) that encompass the iron loading capacity of mYfh1p (50–75 Fe(III)/mYfh1p depending on the ionic environment) and result in stepwise assembly of α₈ to yield iron-loaded α₄₈ (22, 24). Fig. 1A shows representative O₂ consumption curves recorded when 100 μM Fe(II) was incubated in 10 mM HEPES-KOH, pH 7.3, in the absence or presence of 2 μM mYfh1p (Fe(II)/mYfh1p = 50/1). In buffer without protein there was an initial lag phase due to the time required to generate sufficient hydroxylated Fe(III) to initiate autoxidation of Fe(II) (39) (Fig. 1A, black plot). The final Fe(II)/O₂ stoichiometry was 3.7 ± 0.3 (n = 3) as expected for autoxidation (39). In the presence of mYfh1p, the initial rate of O₂ consumption was faster compared with buffer, consistent with ferroxidase activity, but became slower after the first 4 min (Fig. 1A, red plot). The final Fe(II)/O₂ stoichiometry was 3.6 ± 0.3 (n = 3), indicating that ferroxidation was rapidly overcome by autoxidation. We were unable to detect any H₂O₂ released into the solution, which should be expected given the high Fe(II)/mYfh1p ratio used in these experiments (21, 40).

In Fig. 1B, gel filtration was used to determine the speciation of mYfh1p during the iron oxidation reaction described above. Experimental conditions were similar to those employed for electrode oximetry in Fig. 1A except that both the protein and the iron concentrations were increased 5-fold to enable detection of mYfh1p by absorbance measurements. Such an increase is not expected to change the rate of α₈₄ assembly to a significant degree (22, 24), and therefore the gel filtration data (Fig. 1B) can be correlated with the two phases in the O₂ consumption curve of mYfh1p (Fig. 1A). The initial faster phase (Fig. 1A, 0–4 min, red plot) was associated with the assembly of an oligomer of ~50 kDa (Fig. 1B, 3 min), while the subsequent slower phase (Fig. 1A, 4–50 min, red plot) was associated with stepwise assembly of higher order oligomers (Fig. 1B, 6, 10, and 30 min), in agreement with the previously described progression, α → α₃ → α₉ → α₁₂ → α₁₆ → α₂₄ → α₄₈ (22, 24). The A₂₅₀ of assembled mYfh1p species increased in a time-dependent manner (Fig. 1B).

We showed previously that the mature form of Yfh1p is generated by cleavage of an N-terminal mitochondrial targeting signal between residues 51–52 (24, 41). This cleavage eliminates one cysteine residue at position 32. The mature form of the protein (amino acids 52–174), which is the form used in our experiments, contains one cysteine residue at position 98. Thus, an alternative explanation for the results in Fig. 1B could be that chelation of iron by cysteine residues from different mYfh1p subunits leads to formation of metal-thiolate ag-
ggregates (42). However, others have reported that when yeast frataxin is treated with iodoacetamide to block any exposed cysteine residues and subsequently incubated with 20 equivalents of Fe(II), iron-dependent oligomerization is not affected (43). We obtained similar results using a mYfh1p variant in which cysteine 98 was replaced by an alanine residue (data not shown). We therefore conclude that mYfh1p assembly is driven by iron oxidation: A ferroxidase reaction catalyzed by mYfh1p is associated with the first assembly step ($a \rightarrow a_2$), followed by a slower autoxidation reaction associated with assembly of higher order oligomers to ultimately yield $a_{48}$.

The Ferrous Iron Sequestered by mYfh1p Is Bioavailable—The time required to complete the iron oxidation reaction of mYfh1p is in the order of hundreds of seconds (Fig. 1A), much longer than the iron oxidation reaction of ferritin, which is in the order of tens of seconds (Ref. 40 and data not shown). At the beginning of its reaction, however, mYfh1p rapidly sequesters iron. The time required to complete the iron oxidation reaction of ferritin has 24 dinuclear ferroxidation sites (28, 47). As a negative control we used calmodulin, a calcium-binding protein with a molecular mass and an isolectric point similar to those of mYfh1p (17 versus 14 kDa, and 4.09 versus 4.34). Reactions were started by addition of a fixed concentration of Fe(II) (30 $\mu$M) to buffer in the absence or presence of protein (0.4 $\mu$M; Fe(II)/subunit $= 75:1$ for all proteins tested). At successive time points, an aliquot was withdrawn and divided in two parts that were immediately incubated with either BIPY (2 mM) or ferrochelatase (2 $\mu$M) and deuteroporphyrin IX (118 $\mu$M). The half-life of BIPY-accessible iron estimated from a single exponential fitting was 21.5 min in the presence of mYfh1p compared with 1.0, 4.0, 6.7, and 8.8 min in the presence of H-apoferritin, L-apoferritin, buffer only, and calmodulin, respectively (Fig. 2A). Similarly, ferrous iron was more accessible to ferrochelatase in the presence of mYfh1p relative to buffer or calmodulin (Fig. 2B). BIPY can bind Fe(III) and/or reduce Fe(III) to Fe(II) although with lower affinity compared with Fe(II) (48–50), suggesting that the BIPY accessible iron mobilized from mYfh1p could represent a mixture of both ferrous and ferric iron. However, the concentrations of BIPY-accessible iron at successive time points in the presence of mYfh1p (Fig. 2A) were in the same order as the concentrations of ferrochelatase-accessible iron measured under similar conditions (Fig. 2B). We therefore conclude that the iron mobilized by direct chelation (i.e. BIPY accessible iron) during mYfh1p assembly is largely in ferrous form, becoming progressively less accessible as it is oxidized to Fe(III). The Fe(II) that can be mobilized by direct chelation can also be donated to ferrochelatase. This should involve a direct mYfh1p-ferrochelatase interaction given that there was no deuteroheme synthesis in samples containing Fe(II), mYfh1p, and deuteroporphyrin IX but not ferrochelatase (data not shown).

Transfer of Fe(II) from mYfh1p to Ferrochelatase Occurs in the Presence of Citrate—Copper transfer from a metallochaperone to its target protein is not affected by the presence of the physiologically relevant Cu(I) binding agent, glutathione, indicating that the transfer occurs via an intermolecular interaction (31). We investigated if transfer of Fe(II) from mYfh1p to ferrochelatase can occur in the presence of a physiologically relevant Fe(II) binding agent, citrate (Refs. 19 and 34 and Refs. therein). In Fig. 2C, reactions were started by addition of a fixed concentration of Fe(II) (30 $\mu$M) to buffer in the presence of 0.4 $\mu$M mYfh1p, under the conditions used in the time courses of Fe(II) availability in Fig. 2, A and B. Three different sets of additions followed. In set 1, 2 $\mu$M ferrochelatase was added to mYfh1p-Fe(II) after 15 min of incubation, and the incubation continued for another 5 min to allow putative protein-protein interactions to take place. Then, citrate and deuteroporphyrin IX (120 $\mu$M) were added in rapid sequence and the incubation continued for an additional 20 min, after which deuteroheme levels were measured. In set 2, Fe(II) was incubated with mYfh1p for 15 min as above, after which citrate was added and...
Yeast frataxin promotes Fe(II) availability. Time courses of A, Fe[BIPY]$_3^{2+}$ and B, deuteroheme synthesis were started by adding 30 μM Fe(II) to samples containing 0.4 μM mYfh1p, calmodulin, L-apoferritin, or H-apoferritin, or buffer without added protein. Conditions were 10 mM HEPES-KOH, pH 7.3, at 30 °C. BIPY was added at a final concentration of 2 mM. Yeast ferrochelatase and deuteroporphyrin IX were added at final concentrations of 2 μM and 118 μM, respectively. The levels of Fe[BIPY]$_3^{2+}$ and deuteroheme were determined as described under “Experimental Procedures.” The bars represent the mean ± S.D. of 3 (each protein) or 5 (buffer) independent measurements. The traces show single exponential fittings to the data. C, heme synthesis assays were performed under the conditions used in B. Three sets of assays were analyzed: mYfh1p-Fe(II) + FC + [Cit + PPIX], mYfh1p, and Fe(II) were incubated for 15 min, ferrochelatase (FC) was added for 5 min, followed by citrate (Cit) and protoporphyrin IX (PPIX), and the incubation continued for 20 min (n = 1); mYfh1p-Fe(II) + Cit + FC + PPIX, same as above except that citrate was added to mYfh1p-Fe(II) for 5 min, followed by ferrochelatase and protoporphyrin IX (n = 1); mYfh1p-Fe(II) + Cit + FC + PPIX, mYfh1p and Fe(II) were incubated for 20 min, then citrate, ferrochelatase, and protoporphyrin IX were added in this order (n = 1). Heme levels measured at the end of each assay are plotted versus the citrate concentration.

The incubation continued for 5 min to allow the chelator to access mYfh1p-Fe(II) prior to the putative docking of ferrochelatase onto mYfh1p. Then, ferrochelatase and deuteroporphyrin IX were added in rapid sequence. In set 3, Fe(II) was incubated with mYfh1p for 20 min after which citrate, ferrochelatase, and deuteroporphyrin IX were added in rapid sequence. The time courses in Fig. 2, A and B indicate that 20 min after addition of 30 μM Fe(II) to 10 mM HEPES-KOH, pH 7.3, at 30 °C, little residual Fe(II) is present in buffer without mYfh1p (<3 μM; Fig. 2A, black plot) while ~16 μM Fe(II) is still available in the presence of mYfh1p (Fig. 2A, red plot). Therefore, in all the three sets described above, heme synthesis will largely depend on ~16 μM mYfh1p-Fe(II). In all cases, the small size of citrate (<9 Å) may allow this compound to penetrate the protein and directly access mYfh1p-Fe(II), similar to mobilization of ferritin-iron by direct-chelation (44). However, at neutral pH and in the presence of atmospheric O$_2$, which are the conditions used in these assays, citrate will promote rapid autoxidation of Fe(II) (37). Therefore, any mYfh1p-Fe(II) mobilized by citrate will be rapidly oxidized and excluded from the reaction. If ferrochelatase has a high affinity for binding to mYfh1p, as would be expected for a specific intermolecular interaction, the yield of the transfer reaction should be the same between set 1 and set 3. If docking of ferrochelatase onto mYfh1p hampers the ability of citrate to penetrate mYfh1p and directly chelate Fe(II), the yield of the transfer reactions in set 2 should be lower compared with set 1 and set 3. In set 1, the addition of 2–500 μM citrate (Fe(II)-binding constant = 10$^4$ M$^{-1}$) resulted in a ~6–25% drop in heme levels; however, increasing the citrate concentration to 2 and 5 mM (corresponding to a 66–166-fold molar excess over the total iron concentration and a 1000–2500-fold molar excess over the ferrochelatase concentration) did not cause any significant additional decrease (Fig. 2C, gray plot). Compared with set 1, heme synthesis was further decreased in set 2 (Fig. 2C, pink plot) but remained unchanged in set 3 (Fig. 2C, blue plot). These results can be explained by two parallel reactions: (i) transfer of Fe(II) from mYfh1p to ferrochelatase yielding heme; (ii) direct chelation of mYfh1p-Fe(II) by citrate. It appears that mobilization of mYfh1p-Fe(II) by citrate increased with increasing chelator concentrations up to a maximum limited value that was augmented if the chelator was added to mYfh1p-Fe(II) 5 min before the addition of ferrochelatase (Fig. 2C, pink plot). We will show below that a fraction of mYfh1p-Fe(II) can be released into the solution during ultrafiltration. The fraction of mYfh1p-Fe(II) accessible to citrate most likely corresponds to this labile
mYfh1p-Fe(II) pool (see Table II, 10 min). Thus, in the three sets of assays shown in Fig. 2, the Fe(II) consumed by citrate affected the yield of the transfer reaction. However, once chelation of Fe(II) by citrate reached its maximum, heme levels did not change significantly even in the presence of a large excess of citrate. This indicates that the transfer reaction is mostly independent of the presence of a physiologic ferrous iron chelator, suggesting that the transfer occurs via a specific intermolecular interaction. Two alternative ways by which Fe(II) transfer might occur include (i) release of Fe(II) from mYfh1p of citrate. This indicates that the transfer reaction is mostly independent of the presence of a physiologic ferrous iron chelator, suggesting that the transfer occurs via a specific intermolecular interaction. Two alternative ways by which Fe(II) transfer might occur include (i) release of Fe(II) from mYfh1p.
into the solution, and (ii) release of Fe(II) from mYfh1p via general pores on the protein surface. In these scenarios, ferrochelatase and citrate would directly compete for the same Fe(II) pool or the same docking sites. Under either condition, heme synthesis would be expected to decrease proportionally to an increase in the citrate concentration which is not observed in Fig. 2C. Therefore, the results in Fig. 2C better fit a model where ferrochelatase and citrate access mYfh1p-Fe(II) via different paths. Furthermore, the higher levels of heme detected in set 1 and set 3 compared with set 2 suggest that docking of ferrochelatase unto mYfh1p may hamper the ability of citrate to penetrate mYfh1p. Addition of 10–200 μM EDTA (Fe(II)-binding constant = 10^{14} M^{-1}) (38) resulted in a marked inhibition of heme synthesis (−30% heme synthesized relative to assays without EDTA). However, this strong chelator is expected to mobilize most mYfh1p-bound Fe(II) as is the case for BIPY (Fig. 2A; see also Fig. 3). EDTA could also chelate Fe(II) from ferrochelatase after the transfer and/or destabilize heme, as has been reported for thiol reagents (36).

The Ferrous Iron Sequestered by mYfh1p Is Loosely Associated with the Protein—To analyze the interaction between mYfh1p and iron during stepwise assembly, Fe(II) (30 μM) was incubated in the absence or presence of mYfh1p or L-apoferritin (Fe(II)/subunit = 75:1) under conditions similar to those used in the BIPY and ferrochelatase assays described above. After 10 or 60 min of incubation, each sample was subjected to ultrafiltration with a molecular mass cutoff of 5 kDa (21). In the absence of protein, extensive precipitation of insoluble ferri oxyhydroxides was observed at both time points as expected (Table I). In the presence of L-apoferritin, most iron was recovered in a protein-bound form after either 10 or 60 min of incubation and only very little free iron was observed (Table I), reflecting rapid oxidation of Fe(II) within the protein shell as also seen in Fig. 2A. After 10 min of incubation in the presence of mYfh1p, similar levels of iron (−12 μM) were detected in protein-bound and free form (Table I). After 60 min of incubation, the mYfh1p-bound iron increased to 19 μM, corresponding to a Fe/mYfh1p stoichiometry of −50/1 (22, 24), while free iron decreased to −3 μM (Table I). Under the conditions used in this experiment, the iron sequestered by mYfh1p consists of mostly Fe(II) after 10 min of incubation and Fe(III) after 60 min (see Fig. 2, A and B). Therefore, a significant proportion of the Fe(II) sequestered by mYfh1p was released into the solution during ultrafiltration (Table I, 10 min), indicating that this Fe(II) is loosely bound to the protein. However, if Fe(II) was allowed to oxidize inside mYfh1p prior to ultrafiltration, iron release was greatly reduced (Table I, 60 min). We conclude that Fe(II) is loosely bound to mYfh1p but it is not released into the solution unless the binding equilibrium is perturbed as it occurs during

### Table I

| Analysis of iron binding by assembled mYfh1p | 10 min | 60 min |
|--------------------------------------------|--------|--------|
| Buffer                                    |        |        |
| Concentrate                               | 0.9 ± 0.3 | 1.7 ± 1.1 |
| Flow-through (free iron)                  | 9.0 ± 1.1 | 0.7 ± 0.1 |
| Insoluble iron                            | 18.0 ± 1.0 | 24.5 ± 0.9 |
| L-apoferritin                             | 21.0 ± 1.7 | 29.8 ± 5.6 |
| Concentrate (protein-bound iron)          | 20.1 ± 2.0 | 29.8 ± 5.6 |
| Flow-through (free iron)                  | 1.5 ± 0.2 | 0.8 ± 0.1 |
| Insoluble iron                            | 5.9 ± 2.3 | 7.0 ± 5.4 |
| mYfh1p                                    | 12.5 ± 1.0 | 19.2 ± 2.6 |
| Concentrate (protein-bound iron)          | 12.2 ± 0.4 | 3.0 ± 0.7 |
| Flow-through (free iron)                  | 2.4 ± 0.4 | 3.5 ± 2.3 |

* Purified mYfh1p monomer and L-apoferritin (0.4 μM subunit concentration) were incubated with 30 μM Fe(II) for 10 or 60 min, at the Fe(II)/mYfh1p ratios of 75:1, in HEPES-KOH, pH 7.3, at 30°C.

Fig. 4. mYfh1p catalyzes oxidation of Fe(II) at physiologic ionic strength. Oxygen consumption curves were recorded in 10 mM HEPES-KOH, pH 7.0, 150 mM KCl, at 20 °C for 96 μM mYfh1p or buffer (red and black plot, respectively), or 96 μM albumin (inset) upon addition of 48 μM Fe(II).
ultrafiltration. Once Fe(II) is oxidized to Fe(III), the iron is more tightly bound to the protein.

Iron Oxidation Stabilizes mYfh1p Assemblies—To analyze iron mobilization from mYfh1p, samples containing 40 μM protein and 1.6 mM Fe(II) were incubated at 30 °C. These concentrations changed the reaction kinetics as compared with Fig. 1B, such that mYfh1p was formed within 2 min of incubation. At different time points, one sample was rapidly cooled down to 4 °C to stop assembly (22) and immediately analyzed by gel filtration. A duplicate sample was first treated with EDTA (20 mM; EDTA/Fe(II) = 12.5), a chelator that binds both Fe(II) and Fe(III) with high affinities (10^{14} and 10^{25} M^{-1}, respectively) (38), incubated for an additional 60 min at 30 °C, and finally analyzed by gel filtration. In samples that had not been treated with EDTA, mYfh1p monomer (α) assembled into mYfh1p 48 and there was a progressive increase in the A280 of this species at successive time points (Fig. 3, black plots). Addition of EDTA after 2 min of incubation resulted in disassembly of mYfh1p 48 back to smaller assembly intermediates, with a concomitant decrease in the A280 due to mobilization of mYfh1p-bound iron by direct chelation (Fig. 3A, red plot). A similar result was obtained upon addition of EDTA after 10 min of incubation, although the shift from mYfh1p 48 to smaller assembly intermediates was less pronounced and the levels of EDTA-accessible iron were significantly decreased compared with the 2-min sample (Fig. 3B, red plot).

Upon addition of EDTA after 1 or 16 h of incubation, protein disassembly was no longer observed and there was a time-dependent decrease in the levels of EDTA-accessible iron (Fig. 3, C–D, red plots). These results are consistent with a model in which iron oxidation and mineralization are an integral part of mYfh1p assembly (23). The Fe(II) sequestered by mYfh1p 3 is progressively oxidized and incorporated into a ferrihydrite crystallite. As the crystallite increases in size, stepwise assembly of trimers is promoted by the alignment and binding of one crystallite to another. As mineralization proceeds, the proportion of iron that can be mobilized by direct chelation decreases, while the stability of mYfh1p assemblies increases. In agreement with this model, EDTA caused time-dependent disassembly of mYfh1p 48 into smaller oligomers, whereas the reducing agent, dithionite, caused quantitative disassembly of mYfh1p 48 back to monomer in a time-independent manner (Fig. 3, E and F, orange plots). Others and we have found that the cysteine residue present in the mYfh1p sequence is not required for stepwise assembly (Ref. 43 and data not shown). These observations exclude the possibility that the disassembly induced by treatment with dithionite was due to reduction of metal-thiolate aggregates.

mYfh1p Catalyzes Oxidation of Fe(II) in Different Ionic Environments—To investigate a recent report that iron binding by frataxin takes place only at very low ionic strength (43), we analyzed whether mYfh1p exhibits ferroxidase activity in the
presence of 150 mM KCl, close to the concentration believed to exist in mitochondria (43, 51). Fig. 4 shows representative 02 consumption curves recorded when 48 μM Fe(II) was incubated in 10 mM HEPES-KOH, pH 7.0, 150 mM KCl, in the absence or presence of 96 μM mYfh1p (Fe(II)/mYfh1p = 0.5). Except for the presence of 150 mM KCl, these are standard conditions to detect the ferroxidase activity of mYfh1p (21). O 2 consumption was significantly slowed down in the presence of 150 mM KCl compared with buffer without added protein (Fig. 4). This was the case for samples containing 96 μM albumin instead of mYfh1p (Fig. 4, inset). A stoichiometric Fe(II)/O2 ratio of 2.1 ± 0.3 (n = 3) was determined for the completed reaction of mYfh1p (Fig. 4 and data not shown), consistent with the presence of ferroxidase activity (47, 52). Stoichiometric Fe(II)/O2 ratios of 3.8 ± 0.3 (n = 2) and 4.6 ± 0.2 (n = 2), consistent with autoxidation, were otherwise measured for the completed reactions of albumin and buffer without added protein, respectively (Fig. 4 and data not shown). These results demonstrate that mYfh1p binds and catalyzes oxidation of Fe(II) at physiologic concentrations of salt.

In aqueous solution, the rate of spontaneous Fe(II) oxidation is influenced by the ionic strength as well as the interaction of Fe(II) with different anions (53–55). Upon addition of 100 μM Fe(II) to 10 mM HEPES-KOH, pH 7.3, O2 consumption was significantly slowed down in the presence of 150 mM KCl compared with buffer without added salt (compare black plots in Figs. 1A and 5A) as expected (55). Fig. 5A shows a representative O2 consumption curve for 2 μM mYfh1p upon addition of 100 μM Fe(II) (Fe(II)/mYfh1p = 50) in 10 mM HEPES-KOH, pH 7.3, 150 mM KCl. There is an initial phase (0–2 min) during which Fe(II) oxidation is much faster compared with buffer without protein, consistent with ferroxidase activity, followed by a prolonged phase during which Fe(II) oxidation proceeds at a similar slow rate as in buffer without protein (Fig. 5A). The Fe(II)/O2 stoichiometry for the completed reactions was 3.7 ± 0.3 (n = 4) for mYfh1p and 4.4 ± 0.8 (n = 4) for buffer. A two-phase O2 consumption curve was similarly recorded for 2 μM mYfh1p upon addition of 100 μM Fe(II) in 10 mM HEPES-KOH, pH 8.0, 150 mM KCl (Fig. 5B) or 10 mM HEPES-KOH, pH 7.3, 10 mM MgCl2 (Fig. 5C), conditions that were previously reported to prevent iron binding by frataxin (43). These results confirm that two sequential iron oxidation reactions take place at high Fe(II)/mYfh1p ratios in different ionic environments: A faster reaction catalyzed by mYfh1p is followed by a slower autoxidation reaction. In addition, a comparison of the O2 consumption curves for mYfh1p in Fig. 1A and 5, A–C indicates that both reactions are influenced by the ionic environment, which may depend on salt effects on the protein fold (see below) and the reactivity of Fe(II) toward O2 (53–55).

Salt Affects the Rate of Stepwise Assembly but Not the Iron Binding Capacity of mYfh1p—Evidence reported above (Figs. 1, A and B and 3) and elsewhere (23) is consistent with a model in which iron oxidation and biominalization are integral parts of mYfh1p assembly. Thus, the report that iron-dependent self-assembly of frataxin is inhibited at physiologic concentrations of salt (43) might be explained by salt effects on the kinetics of iron oxidation, and thus on the kinetics of mYfh1p assembly. We analyzed a time course of mYfh1p assembly under conditions similar to those used in Fig. 1B except for the presence of 150 mM KCl during assembly. At the end of a 10-min incubation we could only detect low levels of α2 and larger oligomers, with α48 becoming detectable at 20 min (Fig. 5D). The rate of ferrihydrite mineral accumulation (estimated from the increase in the A260 of α48 at successive time points) was slower in the presence of 150 mM KCl compared with buffer without added salt (compare Figs. 1B and 5D), consistent with the respective O2 consumption curves (Figs. 1A and 5A, red plots). In addition, the levels of residual monomer (peak α) decreased only minimally at successive time points in the presence of 150 mM KCl (Fig. 5D). One possible interpretation of these results is that the presence of salt inhibits iron binding by mYfh1p, hence iron-dependent protein aggregation is also inhibited (43). On the other hand, iron binding by mYfh1p is expected to occur efficiently in the assembly reactions analyzed in Fig. 5D because the protein exhibits ferroxidase activity under similar experimental conditions (Figs. 4 and 5A). Thus, an alternative explanation is that the salt slows down stepwise assembly of α2 to α48 due to the inhibitory effect of KCl on the rate of Fe(II) autoxidation as discussed above. This results in the release of Fe(II) during gel filtration leading to protein disassembly, similar to what we observed in Table I and Fig. 3. If this explanation is valid, the iron loading capacity of α48 assembled in the presence of 150 mM KCl should not be impaired. We therefore analyzed assembly of α48 under conditions similar to those employed in Fig. 5D except that the protein and the iron concentration were increased 10- and 8-fold, respectively, to enable determination of the Fe/mYfh1p stoichiometry. Upon gel filtration, fractions corresponding to α48 were analyzed for protein concentration by SDS/PAGE and for iron concentration by ICP-MS, and a stoichiometric ratio of 70–75 Fe/mYfh1p was determined (n = 2), which is higher than the 100/1 ratio determined for α48 samples assembled in the absence of added salts (Refs. 22 and 24 and Table I, 60 min).

![Fig. 6. Salt effects on the availability of mYfh1p-Fe(II). A, time courses of Fe(BPP)2+ synthesis were started by adding 100 μM Fe(II) to samples containing 2 μM mYfh1p or buffer without added protein. Conditions were 10 mM HEPES-KOH, pH 7.3, at 30 °C in the absence or presence of increasing concentrations of KCl. The bars represent the mean ± S.D. of at least three independent measurements. The traces show single exponential fittings to the data. B, tryptophan fluorescence intensity was measured at 25 °C for 2 μM mYfh1p in 10 mM HEPES-KOH, pH 7.3, in the absence (F0) or presence (F) of increasing concentrations of KCl.](http://www.jbc.org/Downloadedfrom)
The need to maintain a supply of bioavailable iron while avoiding iron toxicity is a central problem in biology (57). In aqueous solutions under aerobic conditions, iron toxicity depends on its tendency to catalyze production of free radicals as illustrated by the iron-catalyzed Haber-Weiss reaction (57) shown in Equations 1 to 3.

\[
\text{Fe(II)} + \text{O}_2 \rightarrow \text{Fe(III)} + \text{O}_2^2^- \quad (\text{Eq. 1})
\]

\[
2\text{O}_2^2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (\text{Eq. 2})
\]

\[
\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{OH}^- + \text{OH}^- \quad (\text{Eq. 3})
\]

Our work indicates that the mature form of the yeast frataxin homologue (mYfh1p) has a mechanism to control iron oxidation and availability in vitro. Initial studies showed that in the presence of Fe(II) and O$_2$, mYfh1p monomer assembles stepwise yielding $\alpha_3 \rightarrow \alpha_6 \rightarrow \alpha_{12} \rightarrow \alpha_{24} \rightarrow \alpha_{48}$. The assembly intermediates in this progression were found to accumulate increasing Fe(III) levels, reaching an apparent maximum loading capacity of 50 Fe(III) per subunit in $\alpha_{48}$ (22, 24). We have recently shown that two different iron oxidation reactions take place during the initial assembly step ($\alpha \rightarrow \alpha_6$). A ferroxidase reaction with a stoichiometry of $-2$ Fe(II)/O$_2$ is evident at Fe(II)/mYfh1p ratios $\leq 0.5$, while an autoxidation reaction with a stoichiometry of $-4$ Fe(II)/O$_2$ becomes predominant at ratios between 0.5 and 1.5 (21). At Fe(II)/mYfh1p ratios $\leq 0.5$, only a small fraction of the H$_2$O$_2$ expected from the ferroxidase reaction (47, 52) is detected in solution, and concomitant oxidative degradation of mYfh1p suggests that most H$_2$O$_2$ reacts with the protein itself (21). Here, we have analyzed the reaction of mYfh1p at saturating concentrations of iron (40–75 Fe(II)/mYfh1p). These ratios were chosen because they encompass the iron loading capacity of mYfh1p and result in stepwise assembly of $\alpha_6$ to yield iron-loaded $\alpha_{48}$ (22, 24). Under these conditions, a faster initial phase is rapidly overcome by a slower phase, with a final stoichiometric Fe(II)/O$_2$ ratio of $-4$ and no detectable H$_2$O$_2$ released in the solution, consistent with ferroxidase activity being rapidly overcome by autoxida-
The initial phase correlates with assembly of \( \alpha_3 \), while autoxidation is associated with assembly of higher order oligomers yielding \( \alpha_{48} \) (Figs. 1B and 5D). Oligomerization enables mYfh1p to rapidly sequester up to 50–75 Fe(II)/subunit depending on the ionic environment. Initially, Fe(II) is loosely bound to the protein and can be readily mobilized, whereas ferric iron is more tightly bound (Tables I and II; Figs. 2, 3, and 6A). We therefore postulate that the mYfh1p reaction is as follows (at Fe(II)/mYfh1p ratios \( \geq 0.5 \)) in Equation 4,

\[
(mYfh1p)_n + 4\text{Fe(II)} + \text{O}_2 + 4\text{H}^+ \rightarrow (mYfh1p)_n - 4\text{Fe(III)} + 2\text{H}_2\text{O} \quad \text{(Eq. 4)}
\]
Iron Chaperone and Storage Properties of Yeast Frataxin

where \((\text{mYfh1p})_n\) represents any of the species formed during stepwise assembly.

This reaction would appear to provide two main advantages compared with spontaneous Fe(II) oxidation in solution (see Equation 1): (i) \(\text{H}_2\text{O}\) is expected to represent the predominant product of \(\text{O}_2\) reduction; (ii) the iron bound to \(\text{mYfh1p}\) is in a readily accessible form until it is converted to a water-soluble ferrihydrite mineral (23), which is stored within the assembled protein.

One limitation is that our analyses were carried out under controlled conditions of pH (7, 7.3, or 8), temperature (20 or 30 °C), and ionic strength (0.01–0.15 M), which are different from the much more complex mitochondrial matrix environment in living cells. The mitochondrial matrix has an alkaline pH of ~8 (58) and contains significant concentrations of certain salts (2 mM \(\text{CaCl}_2\), 0.8 mM \(\text{MgCl}_2\), and 100 mM \(\text{KCl}\)) and iron-chelating molecules (51, 59). These factors are known to influence the rate of Fe(II) oxidation (33, 60) and could interfere with the iron oxidation reaction of \(\text{mYfh1p}\). However, the ferroxidase activity of \(\text{mYfh1p}\) (Figs. 4 and 5, A–C), as well as its iron loading capacity and ability to enhance iron availability (Fig. 6A) were conserved at salt concentrations and pH values close to those believed to exist in the mitochondrial matrix. This suggests that what the protein can do in vitro most likely reflects its function in vivo.

Current reports strongly suggest that yeast frataxin controls the iron required for the in vivo biosyntheses of iron-sulfur clusters (13–16) and heme (17). A recent study further shows that human frataxin functions as an iron donor for assembly of \([2\text{Fe}-2\text{S}]\) clusters in ISU-type proteins in vitro (61). Here, we performed deuteroheme synthesis assays to assess the availability of the Fe(II) bound to \(\text{mYfh1p}\) using a physiologic Fe(II) chelator, i.e. the mitochondrial enzyme ferrochelatase. Given that there was no deuteroheme synthesis in samples lacking ferrochelatase (data not shown), we postulate that Fe(II) is near the outer surface of \(\text{mYfh1p}\) whereby it is transferred to ferrochelatase. We have shown that this transfer can occur in the presence of an excess of a physiologic ferrous iron chelator (Fig. 2C) and at physiologic ionic strength (Table II), and becomes more efficient as \(\text{mYfh1p}\) assembles into progressively larger oligomers (Table II). These data strongly suggest that transfer of Fe(II) from \(\text{mYfh1p}\) to ferrochelatase involves an intermolecular interaction. This conclusion is in accord with a recent report showing that: (i) zinc-protoporphyrin, not heme, is synthesized in yeast cells lacking \(\text{Yfh1p}\), consistent with a specific role of frataxin in making iron available to ferrochelatase, and (ii) \(\text{Yfh1p}\) and ferrochelatase physically interact with each other in Bicore experiments (17).

The ability of yeast frataxin to provide iron to such diverse proteins as ISU-type proteins and ferrochelatase indicates that frataxin is different from copper chaperones, which deliver copper ions to specific partners (62). Copper chaperones contain the motif M(T/H)CXXC, also present in their target proteins, that bind metal ions via the two cysteine residues (62). Metal transfer requires the docking of the chaperone and target protein with their metal binding domains close to each other, followed by metal exchange via formation of intermediates that involve the cysteine residues from both proteins (62). It would appear that yeast frataxin is a different type of metallochaperone, acting as a general reservoir of Fe(II) atoms and making them available to different users perhaps via hydrophobic interactions mediated by the conserved neutral surface found on the protein (43). Importantly, if the Fe(II) is not transferred to iron users, it is oxidized and stored in a water-soluble mineral within the assembled protein (23).

Our previous findings (21–24) together with the results reported here support the following mechanism for yeast frataxin (Fig. 7). Monomer is activated by Fe(II) in the presence of \(\alpha_5\) and forms an oligomer, \(\alpha_n\), with a negatively charged inner surface (63, 64) that sequesters Fe(II) from the solution (Fig. 7A). The oligomer catalyzes oxidation of Fe(II) to Fe(III) and further promotes nucleation of a ferrihydrite crystallite at the negatively charged surface. If the Fe(II) concentration exceeds that of the ferroxidase sites on the protein (>0.5 FeII/subunit), ferroxidation is rapidly overcome by a slower autoxidation reaction at the surface of the growing crystallite. At high Fe(II)/mYfh1p ratios, the initial ferrihydrite crystallite grows into a larger particle (Fig. 7B). Nichol et al. (23) have proposed that the iron core of frataxin forms via a process similar to bacterial biomineralization (65). According to this model, alignment and binding of one ferrihydrite particle to another leads to the interaction of trimeric clusters, which further facilitates biomineralization (Fig. 7B). During this process, frataxin acts as a chaperone, donating residual Fe(II) to ferrochelatase or ISU-type proteins to support heme and iron-sulfur cluster biosynthesis, respectively (Fig. 7B). Interestingly, the Fe(II) chaperone function appears to have been separated from the Fe(III) storage function in human frataxin. Ongoing studies in our laboratory show that the human frataxin monomer acts as a Fe(II) donor to ferrochelatase, consistent with a recent report that human frataxin monomer acts as an iron donor to ISU-type proteins in vitro (61). On the other hand, the assembled form of human frataxin (25) has ferroxidase activity^2 and is able to store Fe(III) in iron cores structurally identical to the yeast frataxin iron cores (Ref. 23).

Observations we have made previously in vivo support this model. When native frataxin is analyzed by gel filtration, the protein is detected as a distribution of species over a broad molecular mass range (from ~13 to >600 kDa) corresponding to monomer and progressively larger molecules (24, 25). This suggests that stepwise assembly occurs in mitochondria and that monomer may be in equilibrium with higher order oligomers. Further support comes from structural studies. Scanning transmission electron microscopy data (22) and extended x-ray absorption fine structure analysis (23) indicate that yeast and human frataxin iron cores are composed of small ferrihydrite crystallites. The three-dimensional structures of human and bacterial frataxin show a highly conserved negatively charged surface similar to the anionic surface involved in the iron storage mechanism of ferritin (63, 64, 66). This surface could be involved in iron oxidation and nucleation, and facilitate biomineralization by keeping the growing ferric iron crystallites in a soluble form. Point mutations of carboxylate residues in this surface compromise stepwise assembly of bacterial frataxin (43) as well as the ferroxidase activity and stepwise assembly of yeast frataxin.2 A second highly conserved uncharged surface predicted to be involved in protein-protein interactions (63, 64) could mediate interactions between frataxin and iron users. Indeed, yeast frataxin and ferrochelatase were found to interact with each other by Bicore studies (17). This evidence and our work support the hypothesis that frataxin could work both as a chaperone for Fe(II) when mitochondrial iron is limiting, and as a storage compartment for Fe(III) when iron is in excess.

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Yeast Frataxin Sequentially Chaperones and Stores Iron by Coupling Protein Assembly with Iron Oxidation
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