Frataxin is required for maintenance of normal mitochondrial iron levels and respiration. The mature form of yeast frataxin (mYfh1p) assembles stepwise into a multimer of 840 kDa (αm) that accumulates iron in a water-soluble form. Here, two distinct iron oxidation reactions are shown to take place during the initial assembly step (α → α3). A ferroxidase reaction with a stoichiometry of 2 Fe(II)/O2 is detected at Fe(II)/mYfh1p ratios of ≤0.5. Ferroxidation is progressively over- autoxidation at Fe(II)/mYfh1p ratios of >0.5. Gel filtration analysis indicates that an oligomer of mYfh1p, α3, is responsible for both reactions. The observed 2 Fe(II)/O2 stoichiometry implies production of H2O2 during the ferroxidase reaction. However, only a fraction of the expected total H2O2 is detected in solution. Oxidative degradation of mYfh1p during the ferroxidase reaction suggests that most H2O2 reacts with the protein. Accordingly, the addition of mYfh1p to a mixture of Fe(II) and H2O2 results in significant attenuation of Fenton chemistry. Multimer assembly is fully inhibited under anaerobic conditions, indicating that mYfh1p is activated by Fe(II) in the presence of O2. This combination induces oligomerization and mYfh1p-catalyzed Fe(II) oxidation, starting a process that ultimately leads to the sequestration of as many as 50 Fe(II)/subunit inside the multimer.

The two major iron-utilizing processes in the cell, production of heme by ferrochelatase and the iron-sulfur cluster biosynthetic pathway, reside in the mitochondrial matrix. Mitochondria contain micromolar concentrations of chelatable iron (1), i.e. iron that is not yet complexed in heme or iron-sulfur clusters and is bioavailable (2). Keeping this iron pool in soluble and nontoxic form represents a remarkable biological challenge given the alkaline pH (3) and the high production of O2 (4) within mitochondria. Thus, the existence of proteins capable of handling iron safely within mitochondria was first postulated several decades ago (5). Recent studies have identified a handful of inner mitochondrial membrane proteins involved in mitochondrial iron transport (6–8) as well as a mitochondrial matrix ferritin involved in iron storage (9). The mitochondrial matrix protein frataxin has been implicated in mitochondrial iron homeostasis (10, 11), but its precise function is still not known. Frataxin was first identified as the protein deficient in Friedreich ataxia, a neuro- and cardio-degenerative disease (12). Studies in Saccharomyces cerevisiae have shown that defects in yeast or human frataxin are associated with the accumulation of iron in mito- chondria and oxidative damage to both mitochondrial and nuclear DNA as well as to the iron-sulfur centers of mitochondrial aconitase and other respiratory enzymes (10, 14, 15, 34). Similarly, mouse models in which the frataxin gene is selectively inactivated in neuronal or cardiac tissue present multiple respiratory enzyme deficits and accumulate iron in mitochondria in a time-dependent manner (16). In agreement with these observations, evidence of abnormal cellular iron homeostasis, increased oxidative damage, and respiratory enzyme deficits has been reported for the human disease (reviewed in Ref. 17). It has been shown that frataxin defects result in impaired mitochondrial iron efflux (18), defective biosynthesis of iron-sulfur clusters (19, 20), loss of ATP synthesis (21), and/or disabled antioxidant defenses (22), all conditions that could ultimately lead to mitochondrial iron accumulation and increased oxidative damage. We have proposed that the apparent involvement of frataxin in so many diverse processes could be explained if the basic function of this protein were to provide an iron storage mechanism to keep iron in a bioavailable and nontoxic form (17). Indeed, titration of the mature form of yeast frataxin (mYfh1p) with Fe(II) under aerobic conditions results in stepwise assembly of a 48-subunit multimer with a molecular mass of 840 kDa and a hydrodynamic radius of 11 nm that sequesters 50 atoms of iron/subunit and forms iron-rich cores with a diameter of 2–4 nm (23, 24). Similarly, the mature form of human frataxin assembles naturally during expression in Escherichia coli yielding regular multimers of ~1 MDa and ordered polymers of these multimers that sequester ~10 atoms of iron/subunit (25). Frataxin can be detected in a high molecular mass complex under native conditions in yeast or mouse heart, and both the yeast and the human protein bind stoichiometric amounts of 55Fe in metabolically labeled yeast cells (23, 25). In this study, we investigate the iron oxidation reaction of yeast frataxin. Our results support a direct role for frataxin in iron metabolism.

EXPERIMENTAL PROCEDURES

Reagents and Solutions—HEPES, ferrous ammonium sulfate, 2-deoxyribose, thiobisurbitic acid, and α-β-bipyridine were from Sigma, and beef liver catalase was from Roche Molecular Biochemicals. All of the buffers and solutions were made with milli-Q deionized water. Stock solutions of ferrous ammonium sulfate (2–10 mM; pH 3.6) were freshly prepared in water previously deaerated by purging with argon gas (<0.2 ppm O2). Recombinant mYfh1p was expressed in E. coli (23) and purified as previously described (24). The protein concentration was determined from the absorbance and extinction coefficient of mYfh1p monomer (ε270 = 20,000 M−1 cm−1). Iron concentration measurements were carried out by inductively coupled plasma emission spectroscopy at the Metals Laboratory, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN.

Electrode Oxiometry—Measurements of dissolved O2 concentration were performed with a MI-730 micro-O2 electrode connected to an O2/ADPT adapter (Microelectrodes, Inc., NH). A TBX-6ST isothermal terminal block connected to a NI 4350 high precision voltage meter and
a personal computer with the LabVIEW Base Package (National Instruments, Austin, TX) were used for data acquisition. The electrode was calibrated with HEPES-KOH buffer at each experimental pH and temperature, using buffer deaerated by extensive purging with argon gas (<0.2 ppm O2) (0% standard) and air-saturated buffer (21% standard). For anaerobic experiments, the buffer and the protein stock solution were without protein present. The samples (500 μl) containing a few crystals of dithionite and 2 mM KOH, pH 7.0, either before or after the addition of Fe(II), and the O2 electrode was equilibrated in air-saturated water and then quickly plunged into a rapidly stirred 100 mM dithionite solution or a deoxygenated solution, and the electrode output was measured versus time (26). The electrode response followed first-order kinetics with a half-life of 6 s. The drift of the O2 electrode was not significant (~1 μl O2/40 min). O2 consumption was measured in the presence of a few crystals of dithionite that was inserted into the cell through the buffer-filled capillary. HEPES is known to retard Fe(II) oxidation, albeit to a much lower degree compared with other Good's buffers (27). To minimize this effect, a relatively low concentration (10 mM) of HEPES-KOH buffer was used for O2 consumption measurements, which was sufficient to maintain the pH at 7.0–6.97 at all iron concentrations tested.

**Ultronification Assays and Gel Filtration—**An aliquot of Fe(II) stock solution was directly added to 10 mM HEPES-KOH, pH 7.0, with or without protein present. The samples (500 μl) were incubated at 30 °C for different times and immediately transferred to a Ultrafree-0.5 cell (nominal molecular weight limit = 5,000) (Millipore, Bedford, MA) and centrifuged for 5 min at 14,000 × g at 4 °C. The concentrate and the filtrate were transferred to Eppendorf tubes, a few crystals of dithionite and α-α' -bipyridine (final concentration, 2 mM) were added to each sample, and the concentration of Fe[α-α'-bipyridine]2+ (εmax = 9,000 M−1 cm−1) was determined in a DU640B spectrophotometer (Beckman, Fullerton, CA) (28). After removal of the concentrate, insoluble iron was stripped from the Ultrafree membrane by adding 500 μl of buffer containing a few crystals of dithionite and 2 mM α-α'-bipyridine and by mixing vigorously for 1 min, followed by absorbance measurements at 520 nm (as described above). For gel filtration analysis of assembly products, the samples (1 ml) were centrifuged for 5 min at 20,800 × g at 4 °C and loaded onto a Superdex 200 column (23). In anaerobic experiments, the buffer and the protein stock solution were made anaerobic by purging with moistened argon gas (<0.2 ppm O2). Protein and Fe(II) were added to 2-ml vials sealed with a rubber septum (Sherwood Medical, St. Louis, MO) (final volume, 100 μl) by a gas-tight syringe, and the assembly reaction was incubated for 1 h at 30 °C (23). After cooling of the sample at 4 °C to stop any ongoing assembly reactions at 24, 36, or 48 h (Fig. 1A), the stoichiometric Fe(II)/O2 ratios at the end of these two reactions were 2.2 and 2.4 (Fig. 1A). Stoichiometric Fe(II)/O2 ratios of 3.5 and 3.2 were otherwise measured when 24 or 48 μM Fe(II), respectively, was incubated in buffer without mYfh1p (Fig. 1A).

**RESULTS**

**Yeast Frataxin Catalyzes Fe(II) Oxidation at Low Fe(II)/mYfh1p Ratios—**Electrode oximetry was used to study the iron oxidation reaction of mYfh1p at low Fe(II)/mYfh1p ratios. We showed previously that these conditions result in the assembly of an oligomeric species, αρ, predicted to represent the building unit of the mYfh1p multimer (23). Fig. 1A shows the O2 consumption curves recorded when 24, 48, or 144 μM Fe(II) was incubated in 10 mM HEPES-KOH, pH 7.0, at 30 °C, in the absence or presence of 96 μM mYfh1p (final volume, 200 μl) for 30 min at 30 °C. Phosphoric acid (4%) and thiobarbituric acid (1%) were added (200 μl each), and each sample was boiled for 15 min. After cooling of the samples on ice for 3 min, 75 μl of 10% SDS was added, and a malondialdehyde-thiobarbituric acid adduct (ε340 = 1.54 × 105 M−1 cm−1) was measured as described by Halliwell and Gutteridge (31).

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1 The abbreviations used are: HRP, horseradish peroxidase; DNP, 2,4-dinitrophenylhydrazine; mYfh1p, mature form of yeast frataxin.
measurements. The pH remained between 7.00 and 6.97 throughout.

Iron-dependent stepwise assembly of mYfh1p (23, 24). Importantly, Fe(III)/mYfh1p ratios in the concentrate from the three reactions analyzed in Table I were 0.21, 0.43, and 1.2. These ratios are close to the initial Fe(III)/mYfh1p ratio of 0.25, 0.5, and 1.5, respectively, indicating that most added iron was bound to mYfh1p at the end of the oxidation reaction.

To identify the iron-binding species, duplicates of reactions Fe(II)/mYfh1p = 0.5 and Fe(II)/mYfh1p = 1.5 were incubated at 30 °C for 2 or 25 min and immediately analyzed by Superdex 200 gel filtration. In all four samples, most protein was recovered in peak a, corresponding to mYfh1p monomer (Fig. 2). Inductively coupled plasma emission spectroscopy analysis demonstrated that peak a did not contain any significant levels of iron as previously reported (23, 24). An iron-containing peak with an apparent molecular mass of ~50 kDa, corresponding to the α3 oligomer (23), was observed in both reactions at each time point analyzed (Fig. 2). At 25 min, the Fe(III)/mYfh1p ratio in peak a was 0.6 for reaction Fe(II)/mYfh1p = 0.5 and 1.7 for reaction Fe(II)/mYfh1p = 1.5. These ratios are close to those determined by ultrafiltration (see above), suggesting that α3 is the iron-binding species present at the end of the iron oxidation reaction. Consistent with this interpretation, in Fig. 2 there is a small decrease in the A400 of peak a from 2 to 25 min, and a concomitant increase in the A400 of peak a that is not completely accounted for by the decrease in peak a. Nearly identical chromatograms were obtained in two independent gel filtration analyses, one of which is shown in Fig. 2, indicating that these results are reproducible. The time-dependent increase in peak α3 intensity can therefore be explained by progressive conversion of monomer to α3 and, consistent with the time course of O2 consumption in Fig. 1A, by progressive deposition of iron oxides inside α3. These data support the conclusion that α3 forms during, and represents the main iron-binding species at the completion of reactions Fe(II)/mYfh1p = 0.5 and Fe(II)/mYfh1p = 1.5. However, when the completed reactions are analyzed by gel filtration, peak α3 accounts for only a small fraction of the total iron and protein, whereas most protein is eluted from the Superdex 200 column as iron-free monomer (Fig. 2). This suggests that α3 disassembles during gel filtration because it is not stable.

Production of H2O2 during mYfh1p-catalyzed Fe(II) Oxidation—By analogy with H-ferritin (26, 32), the observed stoichiometry of 2Fe(II)/O2 implies that H2O2 is produced during the ferroxidase reaction catalyzed by mYfh1p, according to the following equation (26).

$$2Fe^{2+} + O_2 + 4H^+ \rightarrow 2FeOOH + H_2O_2 + 4H^+ \quad (Eq. 2)$$

This possibility was initially tested by electrode oximetry, measuring O2 evolution in the presence of catalase, as described in Ref. 29. We could not detect any O2 evolution when catalase was added to 96 µM mYfh1p 5 min after the addition of Fe(II) (48 µM) nor any increase in the final Fe(II)/O2 stoichiometry when Fe(II) was added to mYfh1p in the presence of catalase (data not shown). However, we were able to detect

**Fig. 1. Ferroxidase activity of mYfh1p.** A, O2 consumption curves in the presence of 96 µM mYfh1p or buffer without protein added. The conditions were 10 mM HEPES-KOH, pH 7.0, at 30 °C in the presence of 24, 48, or 144 µM Fe(II). B, O2 consumption curves were recorded in the presence of 96 µM mYfh1p and the indicated Fe(II) concentrations. The Fe(II)/O2 stoichiometry determined for each completed reaction is plotted versus the Fe(II) concentration (bottom x axis) and the Fe(II)/mYfh1p subunit ratio (top x axis). The bars represent the means ± S.D. in 2–5 mYfh1p; red plot or 1–11 (buffer only; black plot) independent measurements. The pH remained between 7.00 and 6.97 throughout the reactions both in the absence and the presence of mYfh1p.
Ferroxidase Activity of Yeast Frataxin

TABLE I
Analysis of iron binding by mYfh1p oligomer (α3)

| Fe2+/mYfh1p ratioa | Buffer | Concentrate | Flow-through | Insoluble iron | mYfh1p | Concentrate (protein-bound iron) | Flow-through (free iron) | Insoluble iron |
|---------------------|--------|-------------|--------------|----------------|--------|--------------------------------|-------------------------|--------------|
| 0.25/1 at 24 μM Fe3+ | 0.6 ± 0.1 | 11.2 ± 0.5 | 7.5 ± 0.9 | 20.5 ± 0.3 | 0.5 ± 0.2 | 1.0 ± 0.4 |
| 0.5/1 at 48 μM Fe3+ | 2.7 ± 0.7 | 16.0 ± 1.0 | 23.9 ± 1.0 | 41.6 ± 0.1 | 1.1 ± 0.2 | 2.7 ± 0.3 |
| 1.5/1 at 144 μM Fe3+ | 1.9 ± 1.4 | 5.1 ± 0.7 | 90.5 ± 8.0 | 114.1 ± 2.4 | 1.2 ± 0.1 | 3.9 ± 0.5 |

a A fixed concentration of mYfh1p (96 μM) was incubated with 24, 48, and 144 μM Fe(II) for 25 min under the conditions used for electrode oximetry in Fig. 1. Iron was measured in the three indicated fractions as described under “Experimental Procedures.”
b Mean ± S.D. from three independent experiments (μM iron). The total recovery at 144 μM Fe(II) was only ~63% in the presence of mYfh1p and 67% in buffer without protein due to incomplete solubilization of the precipitated ferric oxides by addition of dithionite.

Some H2O2 production using a more sensitive assay (29) based on the reaction of H2O2 with 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) in the presence of HRP, which yields a fluorescent compound with an emission maximum at 583 nm. Fig. 3A shows the fluorescence intensity spectra obtained from representative reactions in which 24 or 48 μM Fe(II) was incubated for 30 min at 30 °C in the absence or presence of 96 μM mYfh1p, conditions similar to those used for electrode oximetry in Fig. 1. There is a net increase in H2O2 production in the presence of mYfh1p (Fig. 3A). However, the levels of H2O2 detected correspond to only ~0.25 μM/mYfh1p. This is consistent with the stoichiometry of 2 Fe(II)/O2 consumed inside the protein and only low amounts of H2O2 are released into the solution where they can be detected by the Amplex Red/HRP assay. The possibilities that mYfh1p has catalase activity or that mYfh1p uses H2O2 as an oxidant for Fe(II) are inconsistent with the stoichiometry of 2 Fe(II)/O2 observed when 24 or 48 μM Fe(II) was incubated with 96 μM mYfh1p (Fig. 1). On the other hand, the slow rate of the mYfh1p ferroxidase reaction (Fig. 1A) could favor reaction of H2O2 with the protein. Some of the H2O2 produced during the ferroxidase reaction of horse spleen ferritin has been shown to react with the protein (29, 33). This possibility was therefore investigated for mYfh1p by use of a protein oxidation detection assay (13). Reactions containing 96 μM mYfh1p were incubated in the absence or presence of 48 μM Fe(II) as described above, and after derivatization to DNP, protein carbonyls were detected by Western blotting with anti-DNP antibodies (13). The mYfh1p band was detected in all derivatized samples, but its intensity was ~3-fold higher if the protein had been incubated in the presence of Fe(II) prior to derivatization (Fig. 3B, compare lanes 2 and 4). Degradation products of mYfh1p were barely detected by Coomassie Blue staining of purified monomer (Fig. 3C, lane 5), but they were much more intensely decorated than the full-length protein by anti-DNP antibodies (Fig. 3B, lanes 2 and 4). These results indicate that mYfh1p is prone to oxidative degradation, which is enhanced after incubation with Fe(II), possibly because the H2O2 produced during the ferroxidase reaction of mYfh1p reacts with the protein. The protein concentration used in these experiments (96 μM) is in excess of the expected concentration of H2O2, i.e. 24 μM corresponding to only 0.25 μM/mYfh1p. This is consistent with the results shown in Fig. 3D. Here, protein staining of the blot following immunodetection of carbonyls shows that the oxidative damage that occurred to mYfh1p in the presence of 48 μM Fe(II) (Fig. 3B, lane 4) did not result in a measurable decrease in the levels of full-length protein (Fig. 3D, compare lanes 2 and 4). To further investigate the possibility that H2O2 reacts with mYfh1p, 48 μM Fe(II) and 24 μM H2O2 were added to 5 mM
2-deoxyribose in the absence or presence of 96 µM mYfh1p for 30 min at 30°C. We then measured oxidative degradation of 2-deoxyribose to malondialdehyde (31). We detected 2.6 ± 0.3 versus 0.7 ± 0.5 \( (n = 4) \) µM malondialdehyde in the absence versus the presence of mYfh1p (Fig. 3E, bars 3 and 4), indicating that mYfh1p can attenuate Fenton chemistry. This effect could be achieved by the protein via sequestration of Fe(II) and/or reaction with \( \text{H}_2\text{O}_2 \). The relative degree of damage observed in the presence of mYfh1p with addition of Fe(II) only (39%; bar 2/bar 1) does not increase with addition of Fe(II) and \( \text{H}_2\text{O}_2 \) (27%; bar 4/bar 3) (Fig. 3E). This is consistent with the possibility that mYfh1p not only sequesters Fe(II) but also reacts with \( \text{H}_2\text{O}_2 \).

**Fe(II) and \( \text{O}_2 \) Are Both Required for mYfh1p Assembly**—To assess the role of \( \text{O}_2 \) in mYfh1p assembly, monomer was made anaerobic by purging with argon gas. Then monomer (80 µM) was anaerobically incubated with Fe(II) (3.2 mM) at a Fe(II)/mYfh1p ratio of 40/1, expected to result in formation of multimer (23). The assembly reaction was anaerobically analyzed by gel filtration in a TSK-GEL column. This column has a much smaller volume than the Superdex 200 column used above, which facilitated maintenance of nearly anaerobic conditions during sample loading and gel filtration. Most protein was eluted in peak \( \alpha \) corresponding to iron-free monomer (Fig. 4). To exclude the possibility that the argon treatment might have denatured the protein, making it unable to assemble, argon-treated monomer was also aerobically incubated with Fe(II) and analyzed by TSK-GEL filtration. In this case, most protein was eluted as iron-loaded \( \alpha \alpha \) (Fig. 4). This result indicates that both Fe(II) and \( \text{O}_2 \) are required for the activation of mYfh1p.

**DISCUSSION**

Our results support the following mechanism of mYfh1p function (Fig. 5). The protein is activated by Fe(II) in the presence of \( \text{O}_2 \) and forms an oligomeric species that contains a ferroxidation site and catalyzes Fe(II) oxidation. When the Fe(II) concentration exceeds that of the ferroxidase sites on the protein, autoxidation progressively overcomes ferroxidation. Coalescence of \( \alpha \alpha \) to yield higher order intermediates and finally multimer occurs at concentrations of Fe(II) that exceed the iron-loading capacity of \( \alpha \).

Several lines of evidence support this model. A stoichiometry

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**Fig. 3. Production of \( \text{H}_2\text{O}_2 \) during Fe(II) oxidation in mYfh1p.**

A, reactions containing 96 µM mYfh1p and 24 or 48 µM Fe(II) were incubated in 50 mM HEPES-KOH, pH 7.0, for 30 min at 30°C in the presence of Amplex Red/HRP reagent. Immediately afterward, fluorescence intensity was recorded from 570 to 610 nm. The corrected fluorescence intensity curves were recorded, and samples containing buffer plus Amplex Red/HRP were used for background corrections. The corrected fluorescence intensity curves were integrated, and a standard curve was constructed. The correlation coefficient of the fitted line to the data is 0.999. To determine the concentration of \( \text{H}_2\text{O}_2 \) produced in the presence of mYfh1p (see “Results”), samples containing buffer plus 24 or 48 µM Fe(II) and Amplex Red/HRP were used as blanks for background corrections. The corrected fluorescence intensity curves for mYfh1p were integrated, and the \( \text{H}_2\text{O}_2 \) concentration was calculated from the standard curve. A.U., arbitrary units. B, reactions containing 96 µM mYfh1p were incubated for 30 min at 30°C in the absence or presence of 48 µM Fe(II) under the experimental conditions used in the Amplex Red/HRP assays described above. Following incubation with 2,4-dinitrophenylhydrazine (DNPH) to derivatize carbonyl groups to DNP, the samples (7.5 g of total protein) were analyzed by SDS/PAGE and Western blotting using a polyclonal anti-DNP antiserum. C, 2 µg of the purified mYfh1p monomer used in the experiments described in the present study was subjected to SDS/PAGE and Coomassie Blue staining (lane 5). D, following immunodetection, the membrane was subjected to SYPRO Ruby protein blot staining. Arrows d, degradation products of mYfh1p; lane MW, molecular mass standards. E, a mixture of 48 µM Fe(II), 24 µM \( \text{H}_2\text{O}_2 \), and 5 mM 2-deoxyribose (DOR) was incubated in 10 mM HEPES-KOH, pH 7.0, in the presence or absence of 96 µM mYfh1p for 30 min at 30°C, and production of malondialdehyde-thiobarbituric acid (MDA-TBA) \( (\epsilon_{532} = 1.54 \times 10^5 \text{ M}^{-1} \text{cm}^{-1}) \) was measured (31). The indicated controls were analyzed at the same time and treated identically. The bars represent the means ± S.D. of four independent measurements.
Peaks α and α2 represent mYfh1p monomer and multimer, respectively. The A0.5% of peak α2 is much higher than that of peak α because of the absorbance of iron oxides (23). The molecular weight markers (dashed chromatogram) are as in the legend of Fig. 2. The A0.5% of mYfh1p and the molecular weight standards is shown by the left- and right-hand y axes, respectively. V0 denotes void volume as determined by the elution volume of blue dextran (2 MDa).

The observed stoichiometry of 2 Fe(II)/O2 (Fig. 1) implies that H2O2 is produced (26, 32) during the ferroxidase reaction catalyzed by α3. However, given that a change in stoichiometry, from 2 to 4 Fe(II)/O2, occurs at a relatively low Fe(II)/mYfh1p ratio (≤0.5/1) (Fig. 1B), H2O2 is expected to be produced only transiently at the beginning of the assembly reaction. Moreover, three independent assays measuring H2O2 production (Fig. 3A), oxidative degradation of mYfh1p (Fig. 3, B–D), and Fenton chemistry (Fig. 3E) suggest that H2O2 is not readily released into the solution but may instead react with the protein, as has been shown for at least some of the H2O2 produced during the ferroxidase reaction of H-ferritin (29). By use of the OxyBlot assay, we could not precisely determine whether the degree of protein oxidation accounts for all of the missing H2O2.

Two alternative explanations could be that mYfh1p has catalase activity or that mYfh1p uses H2O2 as an oxidant for Fe(II). These possibilities, however, are not consistent with the stoichiometry of 2 Fe(II)/O2 we observed at the Fe(II)/mYfh1p ratio used in Amplex Red/HRP and OxyBlot assays. On the other hand, given that the protein concentration in these experiments was in a 4-fold excess of the expected concentration of H2O2, it could have been possible for most H2O2 to react with the protein. The attenuation of Fenton chemistry observed when both Fe(II) and H2O2 were added to mYfh1p is also consistent with this view. Future studies will be required to determine the mechanism and stoichiometry of the reaction of H2O2 with mYfh1p.

The complete inhibition of multimer assembly that we have observed under anaerobic conditions (Fig. 4) indicates that mYfh1p senses the presence of Fe(II) and O2. This mixture might cause a conformational change that enables mYfh1p to oligomerize, bind Fe(II), and catalyze its oxidation in a protein-protected compartment. In this way, the presence of redox-active iron triggers a safety mechanism mediated by frataxin that can ultimately result in the sequestration of as many as 50 Fe(II) atoms per subunit.
Fe(II)/subunit in the multimer (Fig. 5). In Fig. 1A, the O$_2$ consumption curves at 144 μM Fe(II) (Fe(II)/mYfh1p = 1.5) show that mYfh1p decreases the rate of Fe(II) autoxidation compared with buffer. The data in Table I further suggest that this is due to binding of Fe(II) to mYfh1p, at sites that are presumably different from the ferroxidation sites. The iron concentration used in these experiments was not in excess of the iron-loading capacity of α$_y$, approximately four or five iron atoms/subunit; Ref. 23). Thus, based on the data in Fig. 1 and Table I, it appears that when the Fe(II) concentration exceeds the iron-loading capacity of α$_y$, a slow autoxidation reaction progressively overcomes ferroxidation. We showed previously that when the Fe(II) concentration exceeds the iron-loading capacity of α$_y$, higher order intermediates and multimer are formed (23). Ongoing studies further reveal that Fe(II) is slowly oxidized inside the multimer and can be readily donated to a ferrous iron chelator or the mitochondrial enzyme ferrochelatase to synthesize heme.3 Therefore, by virtue of its ferroxidase activity, frataxin assembles into a macromolecule that is likely to play a direct and central role in mitochondrial iron metabolism.

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