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Fate of Oxygen during Ferritin Iron Incorporation†

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ABSTRACT: The deposition of Fe(II) into ferritin using $^{18}O_2$ as the oxidant was investigated. Only 3-4% of the oxygen atoms in the FeOOH core of ferritin were derived from the oxidant. This was true whether large (1200-1900 Fe/molecule) or small (220-240 Fe/molecule) amounts of iron were added to apoferritin or when iron was added to ferritin already containing 1000 Fe/molecule. Experiments using $H_2{}^{18}O$ in the solvent showed that nearly all of the oxygen atoms in the core were derived from solvent. The stoichiometry of the reaction was close to 2 Fe(II)/O_2, instead of the expected value of 4 Fe(II)/O_2. Reactions of $^{18}O_2$ with Fe(II) in the absence of apoferritin had a similar ^{18}O distribution and stoichiometry. Our results are most consistent with the crystal growth model of ferritin iron deposition.

The iron-storage protein, ferritin, is found all through the animal and plant kingdoms (Crichton, 1973; Aisen & Litowsky, 1980; Harrison, 1977). It has a hollow spherical protein shell with a molecular weight near 450 000. Inside is a core containing from 0 to about 4300 iron atoms in the form FeOOH, with some associated phosphate. The protein shell has an inner diameter of 7-8 nm and an outer diameter of 12-13 nm. It is comprised of 24 subunits arranged with 432 symmetry so that there are six channels piercing the shell along Cartesian axes (Banyard et al., 1978). Each channel has a diameter of about 1 nm, allowing small molecules to pass through to the core.

Although the core stores Fe(III), it is generally believed that mobilization and deposition involve Fe(II). While thioglycolic acid is most commonly used in the laboratory to reduce ferritin to apoferritin, the most rapidly acting reductants are flavins (Sirivech et al., 1974; Jones et al., 1978). In the presence of oxygen and Cu^{2+}, ascorbic acid may be an important physiologic reductant (Bienfait & Van Den Briel, 1980). In contrast, Fe(III) mobilization by chelating agents is not as fast as reductive removal (Crichton et al., 1980a; Pape et al., 1968; Tufano et al., 1981). Although apoferritin rapidly accumulates thousands of iron atoms per molecule in a solution containing Fe(II) and O_2, Fe(III) is taken up slowly and to a much smaller extent (Treffry & Harrison 1979).

When a solution of apoferritin and Fe(II) is exposed to oxygen, iron accumulates inside the protein shell in the form FeOOH (Macara et al., 1972). The simplest equation that can be written for this process leads us to expect that 4 mol of Fe(II) will react with 1 mol of O_2:

$$4Fe^{2+} + O_2 + 6H_2O \rightarrow 4FeOOH + 8H^+$$

The present study was undertaken to learn the fate of the molecular oxygen involved in Fe(II) uptake by ferritin.

Experimental Procedures

Ferritin. Horse spleen ferritin was purchases from Miles Laboratories (6 times crystallized, Cd removed) or from Sigma Chemical Co. (Type I). It was either used as received or purified by passage through an ascending column (2.6 × 80 cm) of Sepharose 6B eluted with 0.1 M Tris,[1] pH 7.0 (Niitsu & Listowsky, 1973). Apoprotein was prepared by dialysis against three portions of 0.1 M thioglycolic acid–0.1 M sodium acetate at its own pH (4.5) or against the same reagent at pH 5.0 if the protein precipitated. (At pH 5.0 the ferritin redissolves.) The reduction was followed by extensive dialysis

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; STP, standard temperature (0 °C) and pressure (1 atm).

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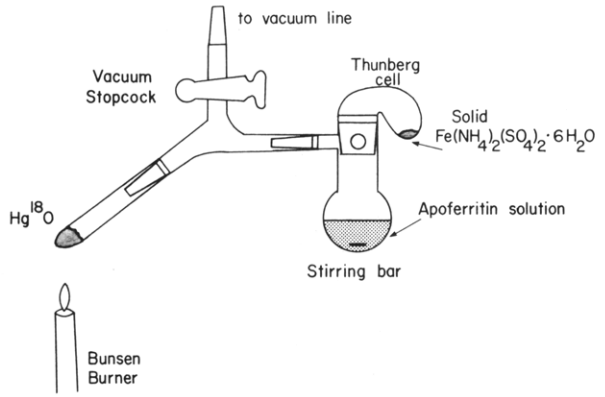

FIGURE 1: Apparatus used for oxidation studies with $^{18}O_2$.

against 0.02 M KCl. The absorbance of 10.0 mg of apoferritin/mL in a 1-cm cuvette at 280 nm was assumed to be 9.0 (Macara et al., 1972). The molecular weight of apoferritin was taken to be 450 000 for all calculations. Ferritin iron analyses were conducted as described previously (Harris, 1978).

$Hg^{18}O$. So that small quantities of $^{18}O_2$ could be produced in a closed system, $Hg^{18}O$ was prepared (Dostrovsky & Samuel, 1962). A stock $H_2{}^{18}O$ solution containing ~40% ^{18}O was prepared from $H_2{}^{18}O$ purchases from Yeda Research and Development Co. (Rehovot, Israel). To 0.10 g (4.3 mmol) of sodium metal in a N_2-filled tube capped with a serum stopper was added 0.8 mL of $H_2{}^{18}O$. This solution was slowly added to 0.40 g (1.5 mmol) of $HgCl_2$ in 0.8 mL of $H_2{}^{18}O$ in a N_2-filled centrifuge tube maintained at 80 °C in a water bath. The precipitated yellow HgO was left in contact with the mother liquor at 70 °C overnight, centrifuged, washed 3 times with 0.2-mL portions of $H_2{}^{18}O$, and dried in vacuo (3 mtorr) at ambient temperature. The ^{18}O content of two separate samples dissolved in 3 M HCl was 38.1 and 38.3%.

Oxidative Deposition of Fe into Ferritin with $^{18}O_2$. Approximately 0.4 mL of apoferritin (~18 mg) and 0.9 mL of buffer (1.0 M Hepes–1.0 M Mes–0.10 M imidazole, pH 7.0) were placed in the round-bottom flask of the apparatus in Figure 1. Appropriate quantities of solid ferrous ammonium sulfate hexahydrate and mercuric oxide were placed in the cap and side arm, respectively. The apparatus was lubricated with Apiezon H stopcock grease and oxygen was removed by repeated exposure to vacuum and Ar. Traces of oxygen were removed from the Ar by passage through a manganese oxide column (Brown et al., 1962). After the mixture was degassed, the apparatus was filled with ~0.7 atm of Ar and the stopcock closed. The HgO was decomposed to O_2 and Hg with a small Bunsen burner, giving an oxygen pressure of ~0.3 atm in the apparatus. The glassware was then tipped and tapped gently to add the ferrous ammonium sulfate to the liquid, one crystal at a time, over a 4-h period. This method of addition resulted in little or no precipitation of hydrous ferric oxide. After the mixture was allowed to stand for 16 h at 20 °C an aliquot was removed under a blanket of Ar and analyzed for unreacted Fe(II) as described below. The reaction liquid was then withdrawn and centrifuged to remove any precipitate. (There was usually none.) The solution was lyophilized, and the solid and liquid portions were separately analyzed for ^{18}O content.

A control experiment showed no significant leakage of air into the apparatus. Apoferritin and ferrous ammonium sulfate were mixed under 0.7 atm of Ar in the apparatus. After 16 h an aliquot was withdrawn and added to a solution of ferrozine [50 mg of ferrozine [disodium 4,4′-[3-(2-pyridinyl)-1,2,4-triazine-5,6-diyl]bis(benzenesulfonic acid)] plus 3.0 g of acetic acid in 50 mL of water, adjusted to pH 5.5 with NaOH]. The absorbance at 562 nm indicated that 98.6% of the Fe(II) remained unoxidized.

^{18}O Analysis. The ^{18}O content was determined by isotope ratio mass spectrometric analysis after equilibration of a solution with CO_2 (Epstein & Mayeda, 1953). A 0.75-mL solution was equilibrated with 1 mL (STP) of CO_2 at 25 °C for at least 48 h. The CO_2 was removed under vacuum and purified cryogenically by using cold traps of −100 °C to remove water and −196 °C to isolate CO_2. The isotope ratios were measured on a dual-inlet Nuclide 3-60 ratio mass spectrometer (State College, PA). The results were expressed as atom percent excess relative to the solvent blank (Campbell, 1974). A typical solvent blank contained 0.1967 atom % ^{18}O [$\equiv(100 \times {}^{18}O)/({}^{18}O + {}^{16}O)$]. Final sample enrichment was generally 0.005–0.010 atom % excess above that of the blank. The standard deviation of the analysis was 0.0001 atom % excess.

In a typical experiment a whole ferritin reaction was lyophilized to separate the liquid and solid. The volume of liquid was measured and a sample was used for ^{18}O analysis. The entire solid residue was redissolved in 1 reaction volume of $H_2{}^{16}O$ to wash away loosely bound $H_2{}^{18}O$. The solution was lyophilized again and the dry residue treated with 0.75 mL of 3 M HCl for 1 h at 100 °C in a sealed tube. An aliquot was analyzed for Fe and the remainder was used for ^{18}O analysis. A great deal of solid remained undissolved by the HCl, but the amount of Fe found in the liquid was near the value expected from the amount of iron in the experiment.

For Fe analysis 25.0 μL of the solution of Fe in HCl was diluted to 25.0 mL. Then 1.00 mL was withdrawn and treated with 0.50 mL of reducing agent (0.25 g of ascorbic acid plus 0.50 g of 12 M HCl in 25 mL of water, freshly prepared) for 10 min. To the colorless solution was added 1.00 mL of colorimetric reagent (50 mg of ferrozine plus 3.0 g of acetic acid in 50 mL of water, adjusted to pH 5.5 with NaOH), and the absorbance was measured at 562 nm after 20 min. Standards were prepared from Fe wire dissolved in HCl.

Calculations. A typical sample for ^{18}O analysis contained a few milligrams of ferritin dissolved in 0.75 mL of 3.09 M HCl, with a great deal of suspended organic matter (buffer and some protein). The incorporation of ^{18}O in ferritin was calculated from the ^{18}O enrichment of the water in the 3.09 M HCl–ferritin solution and the number of exchangeable oxygen atoms in the solution. The ^{18}O enrichment was the difference between the atom percent of ^{18}O in the HCl–ferritin solution and an HCl blank. The number of exchangeable oxygen atoms was the sum of the oxygen in the water, which was calculated from the molarity and density of the HCl solution, and the oxygen in the ferritin core, which was taken as twice the number of iron atoms found in the solution. The oxygen contributed by the ferritin core was about 0.2% of the total. Finally, the incorporation of the starting material into the core was calculated from the total ^{18}O divided by the enrichment of starting material. For example, if the HgO used to generate O_2 was enriched to 38.2 atom % excess and the solution contained 0.030 atom of ^{18}O/atom of iron, the incorporation was 0.030/0.382 = 0.079 atom/atom of iron.

To find the total ^{18}O content of a given reaction, we also analyzed the lyophilized liquid (pure water). The blank for this determination was a mixture of the same buffer and ferritin solutions used in the reaction. Reaction blanks and HCl blanks were usually very close to each other (within 0.0003 atom % excess).

Problems with Sample Preparation for ^{18}O Analysis. The procedure for measuring ^{18}O content required the sample to

Table I: Oxidative Deposition of Fe^{2+} into Ferritin with $^{18}O_2$ in $H_2{}^{16}O$

ferritin concn (μM)	Fe/molecule	buffer concn[a] (M)	% Fe^{2+} unreacted	mol of Fe^{2+} in reaction/mol of $^{18}O_2$ available	mol of Fe^{2+} oxidized/mol of $^{18}O_2$ reacted	mol of ^{18}O in ferritin/mol of Fe in ferritin
			Heavy Loading of Apoferritin			
23	1900	0.77	0.8	0.34	1.7	0.084
34	1900	0.12	6.6	0.37	2.2	0.057
31	1200	0.77	0.4	0.38	2.1	0.081
			Reaction of ~200 Fe^{2+} with Apoferritin			
21	220	0.20	1.4	0.31	2.1	0.071
50	240	0.50	0.3	0.35	2.2	0.051
			Reaction of 1000 Fe^{2+} with Iron Ferritin			
4.4[b]	1000	0.13	0.2	0.24	1.5	0.074

[a] Buffer contained Hepes, Mes, and imidazole in a 10:10:1 mole ratio, at pH 7.0. Buffer concentration refers to the Hepes concentration. [b] Reconstituted iron ferritin containing about 1000 Fe/molecule was isolated from a CsCl density gradient centrifugation (Harris, 1978).

be dissolved in an aqueous solution. We expected that ferric ion in strong acid would exchange its aquo ligands with solvent because the first-order rate constant for $Fe(H_2O)_6{}^{3+}$ ligand exchange with water is 2.4×10^4 s^{-1} (Connick & Stover, 1961). It was hoped that ferritin samples could be ignited in the air to convert them to Fe_2O_3, which would be dissolved in HCl solution.

To test this procedure, we prepared and analyzed an authentic sample of $Fe_2{}^{18}O_3$. $Na^{18}OH$ solution was prepared by dissolving 25 mg (1.1 mmol) of Na in 1.5 mL of water enriched ~8% with ^{18}O. This was added slowly to a solution containing 60 mg (0.22 mmol) of $FeCl_3 \cdot 6H_2O$ in 1.5 mL of the same solvent to precipitate hydrous ferric oxide. All vessels were capped with serum stoppers. The precipitate was centrifuged and washed twice with the same solvent. Samples were then heated in the air or under vacuum to convert them to Fe_2O_3. The final product was weighed and dissolved in 0.75 mL of 3 M HCl in a sealed tube at 100 °C for 1 h. A portion of the liquid was removed and subjected to Fe analysis, and the remainder was equilibrated with CO_2 for ^{18}O analysis.

Several samples of ferric oxide heated in the air at 250–360 °C for 2–24 h were found to contain 16–72% of the expected amount of ^{18}O, based on their mass and the formula Fe_2O_3. Their iron content was 96–100% of the expected value. Two samples heated under vacuum for 2 h at 350 °C contained 105 and 98% of the expected quantity of ^{18}O. We believe that heating $Fe_2{}^{18}O_3$ in the air leads to ^{18}O exchange with atmospheric oxygen.

Since it was not feasible to ignite ferritin samples in the air, we tried heating under vacuum. This led to the formation of intractable tars which retained much of their iron when heated with HCl. We therefore resorted to lyophilizing and using whole reaction mixtures for ^{18}O analysis.

Results

Origin of Oxygen in the Ferritin Core. Table I shows the outcome of experiments in which apoferritin was treated with Fe(II) in the presence of $^{18}O_2$. Under all conditions investigated, the ratio of ^{18}O/Fe in the core was 0.05–0.08. This was true whether large (1200–1900 Fe/molecule) or small (220–240 Fe/molecule) amounts of iron were added to apoferritin or when iron was added to ferritin already containing 1000 Fe/molecule. Assuming that the core composition is FeOOH (Michaelis et al., 1943; Granick & Hahn, 1944), only about 3–4% of the oxygen in the core was derived from the oxidant. To confirm that the remainder came from solvent, and to check our expeimental method, we also deposited iron in ferritin in the presence of $^{16}O_2$ and $H_2{}^{18}O$. Table II shows that nearly 2 mol of ^{18}O/mol of Fe was found in ferritin under these circumstances. The entries in Table II and the fourth and fifth entries in Table III show no clear trend in the ^{18}O/Fe ratio after repeated 5-min washes or after a 24-h wash. We believe that washing does not lead to a significant decrease in the ^{18}O/Fe ratio, within our experimental variations.

Table II: Oxidative Deposition of Fe^{2+} into Ferritin with $^{16}O_2$ in $H_2{}^{18}O$

ferritin concn (μM)	Fe/molecule	buffer concn (M)	% Fe^{2+} unreacted	mol of ^{18}O in ferritin/mol of Fe in ferritin	no. of washes[a]
36	2200	0.54	0.5	1.78	1
				1.64	2
				1.80	3
				1.58	4
23	2200	0.50	0.2	2.00	1
				1.75	1[b]

[a] Washes were for 5 min with 1 reaction volume of $H_2{}^{16}O$. [b] This sample was washed for 24 h.

Table III: Oxidative Precipitation of Ferrous Ammonium Sulfate

Fe^{2+} concn (μM)	buffer concn (M)	workup conditions	mol of Fe^{2+} in reaction/mol of $^{18}O_2$ reacted	mol of ^{18}O in precipitate/mol of Fe in precipitate
		Reaction with $^{18}O_2$ in $H_2{}^{16}O$		
45	0.69	lyophilized, washed 5 min, lyophilized	1.5	0.01
38	0.66	heated to 350 °C for 2 h under vacuum	3.1	0.05
		Reaction with $^{16}O_2$ in $H_2{}^{18}O$		
36	0.50	lyophilized, washed 5 min, lyophilized		1.69
53	0.50	lyophilized, washed 5 min, lyophilized		2.14
		lyophilized, washed 24 h, lyophilized		2.26
		heated to 350 °C for 24 h in air		0.30

Oxidation in the Absence of Ferritin. Table III displays results of experiments analogous to those in Tables I and II, but with no protein present. The results were similar to those of ferritin iron oxidation. Nearly all of the oxygen is derived

from the solvent and very little came from the oxidant.

Stoichiometry of Oxidation. We can learn how much $^{18}O_2$ has been consumed by measuring the total ^{18}O content of the ferritin and the solvent. Tables I and III show that approximately 2 mol of Fe was oxidized for each mol of O_2 consumed in the presence or absence of ferritin. As a test of our analytical method, we compared the ^{18}O content of the whole reaction mixture to the ^{18}O contents of the lyophilized liquid and residual solid. In three experiments the sum of the ^{18}O contents of the liquid and solid agreed to within 1, 3, and 16% with the ^{18}O content of the whole reaction mixture.

Discussion

We begin this discussion by reviewing the two mechanisms that have been proposed for ferritin iron oxidation.

Crystal Growth Model. In the crystal growth model of ferritin iron deposition, the bulk of Fe(II) is oxidized to FeOOH on the surface of the growing FeOOH crystallite (Macara et al., 1972, 1973a; Harrison et al., 1974; Treffry et al., 1978, 1979). Before a substantial crystallite has formed, oxidation is presumed to occur at catalytic sites on the interior surface of the protein shell. This model was first proposed (Macara et al., 1972) when sigmoidal progress curves for iron uptake were observed. These were interpreted in terms of a rate that increased as the surface area of the growing crystallite increased and then decreased after the exposed surface passed through a maximum. Perhaps the strongest support for the crystal growth model is that the rate of uptake and release reaches a maximum when the surface area is expected to be maximal (~1000–1500 Fe/molecule) (Harrison et al., 1974; Harris, 1978; Jones et al., 1978).

Treffry et al. (1979) have presented very strong evidence that the mechanism of iron uptake by ferritin changes after the initial stage of uptake by apoferritin. They found that when apoferritin was presented with Fe(II) in the presence of O_2, the rate of iron uptake was much faster than that obtained with KIO_3 as the sole oxidant. In a second experiment they added Fe(II) and oxidant to ferritin already containing 515 Fe/molecule. In this case KIO_3 and O_2 each produced equally rapid rates of uptake. This experiment suggests that in the early stages of iron uptake KIO_3 is relatively ineffective but in the later stages it is a fully competent oxidant. Therefore, the initial mechanism of iron uptake is different from that of the later stages. We repeated this experiment and observed similar results.

Protein Catalysis Model. The key feature of this model is that the protein is involved in iron oxidation at all stages of iron uptake (Crichton & Roman, 1977, 1978; Crichton, 1979; Crichton et al., 1980b). It has been proposed that two ferrous ions bound at each catalytic site react with a molecule of oxygen to form a μ-peroxo (Fe–O–O–Fe) intermediate, which somehow goes on to produce FeOOH. Tb^{3+} binds to apoferritin and inhibits iron uptake. X-ray diffraction shows that each Tb^{3+} is bound to the inner surface of the protein shell (not in the six channels) close to a 2-fold symmetry axis and just 0.43 nm from its symmetry-related partner (Banyard et al., 1978). These 12 pairs of metal-binding sites are obvious candidates for catalytic oxidation sites. Tb^{3+}, Zn^{2+}, Ni^{2+}, and Co^{2+} all competitively inhibit iron deposition in ferritin (Macara et al., 1973b; Vandamme & Crichton, 1980). Close to 24 ions/ferritin molecule are bound in the cases of Zn^{2+}, Ni^{2+}, and Co^{2+}. Apoferritin is reported to bind about 12 Cr^{3+}/molecule, leading to an 83% decrease in the rate of iron uptake (Wauters et al., 1978). Kinetic data have been interpreted to indicate that the rate of iron uptake by apoferritin is proportional to $[Fe^{2+}]^2$ (Wauters et al., 1978), although the experimental error was large enough for other interpretations to be possible.

In support of the Fe–O–O–Fe intermediate, it was found that incubation of triphenylphosphine with ferritin produced 0.97 mol of triphenylphosphine oxide/subunit and released 15–30 Fe(II)/molecule (Crichton et al., 1980b). Bipyridyl removed iron from ferritin in a biphasic reaction with an initial burst of activity leading to the release of about 22 Fe(II)/molecule (Crichton & Roman, 1978). It has been suggested that the μ-peroxo intermediate is a logical choice for production of triphenylphosphine oxide or bipyridine *N*-oxide. An argument against involvement of one-electron reduction of O_2 is that no O_2^- could be detected outside the ferritin molecule during iron uptake (Wauters et al., 1978).

The fact that KIO_3 is as effective an oxidant as O_2 (after some iron core has formed) implies that the formation of an Fe–O–O–Fe bridge cannot be the exclusive oxidative pathway. It has been found that apoferritin binds 12 VO^{2+}/molecule and that these ions compete with Fe(II) for binding sites (Chasteen & Theil, 1982). This observation contradicts the expectation of 24 Fe(II) binding sites in the Fe–O–O–Fe hypothesis.

Implications of the Present Results. Our results do not rule out either model of ferritin iron oxidation, but they lend support to the crystal growth model. We find similarities between ferritin iron oxidation and inorganic iron oxidation in both the fate of the $^{18}O_2$ and the stoichiometry. Very little $^{18}O_2$ is incorporated into the oxidized iron product in either case, and neither reaction had the expected 4 Fe/O_2 stoichiometry.

The μ-peroxo species proposed in the protein catalysis model might be expected to lead to the incorporation of ^{18}O into the ferritin core. We did not observe such incorporation, and we showed that this was not due to exchange of core oxygen atoms with solvent. It cannot be ruled out that ^{18}O is initially attached to Fe at a catalytic site and then dissociates or exchanges with solvent prior to reaching the FeOOH crystallite.

Stoichiometry of Oxidation. We observed a stoichiometry near 2 Fe/O_2 (Table I) instead of the expected value of 4 Fe/O_2. This was true whether or not protein was present. Using one-tenth of our ferritin concentration and a pH of 6.4, Melino et al. (1978) found a stoichiometry near 4 Fe/O_2. Treffry et al. (1978) reported a variable stoichiometry, ranging from about 1.5 to 3.5 Fe/O_2, under a variety of conditions.

It is difficult to rationalize a stoichiometry of 2 Fe/O_2 because we do not believe that H_2O_2 is a product of the reaction. Our ^{18}O analyses involved exchange of oxygen atoms with CO_2, and presumably $H_2{}^{18}O_2$ could not do this. Also, H_2O_2 reacts so quickly with Fe(II) (Melino et al., 1978) that we expect it to be consumed as rapidly as it could be formed. We used the $KMnO_4$ assay for H_2O_2 (Chance & Maehly, 1955) to show that authentic H_2O_2 does not react with our buffer in 10 min. Therefore, buffer cannot be a sink for H_2O_2 in our reactions. One might suppose that H_2O_2 is formed and catalytically disproportionates to $^1/_2O_2 + H_2O$. This, however, would put half of the ^{18}O back in the form $^{18}O_2$ and could not give the observed results.

If ferritin iron oxidation does proceed through H_2O_2 or O_2^-, it might be expected that the protein has catalase or superoxide dismutase activity to protect itself. We could find no catalase activity in iron ferritin using the $KMnO_4$ method (Chance & Maehly, 1955), and it has been reported that ferritin has little superoxide dismutase activity (Marklund, 1980).

At this time we cannot account for the observed stoichiometry of the reaction. There must either be another ox-

idation product present or the reduction of $^{18}O_2$ to the 2- oxidation level is accompanied by oxidation of an ^{16}O species to release $^{16}O_2$.

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Registry No. Iron, 7439-89-6; oxygen, 7782-44-7.

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