Iron Oxidation Chemistry in Ferritin

INCREASING Fe/O₂ STOICHIOMETRY DURING CORE FORMATION*

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The origin of previously observed variations in stoichiometry of iron oxidation during the oxidative deposition of iron in ferritin has been poorly understood. Knowledge of the stoichiometry of Fe(II) oxidation by O₂ is essential to establishing the mechanism of iron core formation. In the present work, the amount of Fe(II) oxidized was measured by Mössbauer spectrometry and the O₂ consumed by mass spectrometry. The number of protons produced in the reaction was measured by "pH stat" titration and hydrogen peroxide production by the effect of the enzyme catalase on the measured stoichiometry. For protein samples containing low levels of iron (24 Fe(II)/protein) the stoichiometry was found to be 1.95 ± 0.18 Fe(II)/O₂ with H₂O₂ being a product, viz. Equation 1.

$$2Fe^{2+} + O_2 + 4H_2O \rightarrow 2FeOOH + H_2O_2 + 4H^+$$ (1)

EPR spin trapping experiments showed no evidence of superoxide radical formation. The stoichiometry markedly increased with additional iron (240–960 Fe/protein), to a value of 4 Fe(II)/O₂ as in Equation 2.

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

As the iron core is progressively laid down, the mechanism of iron oxidation changes from a protein dominated process with H₂O₂ being the primary product of O₂ reduction to a mineral surface dominated process where H₂O is the primary product. These results emphasize the importance of the apoferritin shell in facilitating iron oxidation in the early stage of iron deposition prior to significant development of the polynuclear iron core.

Ferritin is the principal reservoir for iron in the cell. Iron is stored in ferritin in the form of a hydrous ferric oxide mineral encased in a clathrate protein shell of molecular weight 450,000 (1–7). The detailed mechanism of oxidative deposition of iron within the protein, and the formation of the mineral core is poorly understood. However, it is generally believed that early in iron core formation Fe(II) can be oxidized by molecular oxygen to Fe(III) at "ferroxidase" sites on the apoprotein (6, 8–13). Growth of the hydrous ferric oxide crystallites then ensues at "nucleation" sites which may be distinct from or the same as the sites of iron oxidation. According to the crystal growth model, once sufficient mineral surface has formed, Fe(II) oxidation and deposition occurs directly on the growing crystallite (4, 14, 15), whereas in the protein catalysis model, the protein plays a role in iron oxidation at all stages of iron uptake (16).

Knowledge of the stoichiometry of Fe(II) oxidation by molecular oxygen during the formation of ferritin from apoferritin, Fe(II), and O₂ is needed to fully understand the mechanism by which iron is accumulated by the protein. Several laboratories have attempted to measure the stoichiometry of iron oxidation but the results have been conflicting (17–19). Reported stoichiometries range from 1.5 Fe(II)/O₂ to 4.0 Fe(II)/O₂, depending on the conditions of the experiment and the methods of measuring the amounts of Fe(II) and O₂ consumed in the reaction. The various stoichiometric reactions between Fe²⁺ and oxygen which could potentially lead to formation of the FeOOH core of ferritin are summarized in Equations 1–5.

$$Fe^{2+} + O_2 + 2H_2O \rightarrow FeOOH + O_2^- + 3H^+$$ (1)

$$Fe^{2+} + O_2^- + 2H_2O \rightarrow FeOOH + H_2O + H^+$$ (2)

$$2Fe^{2+} + O_2 + 4H_2O \rightarrow 2FeOOH + H_2O_2 + 4H^+$$ (3)

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

$$Fe^{2+} + H_2O_2 + H_2O \rightarrow FeOOH + HO^+ + 2H^+$$ (5)

The involvement of ferritin iron in reactions producing oxo-radicals and its potential role in causing oxidative damage to the cell are areas of much current interest (20–27). Hydroxyl radical is produced in Equation 5 by the reaction of ferrous ion with hydrogen peroxide, i.e. the Fenton reaction. Superoxide radical is produced in Equation 1 by the one-electron reduction of dioxygen. The one-electron reduction steps in Equations 1 and 2 sum to give Equation 3 where hydrogen peroxide is the net product of dioxygen reduction. Equation 4 is the overall reaction for complete reduction of dioxygen to water.

Equations 1, 3, and 4 predict a stoichiometry of one, two, and four Fe(II) oxidized per O₂ consumed, respectively. Thus, by measuring the stoichiometry of iron(II) oxidation, the dominant oxidation reaction in core formation can be established. In the present work, the amount of O₂ consumed was determined by ⁴⁰O mass spectrometry and the extent of Fe(II) oxidation measured directly by ⁶⁷Fe Mössbauer spectroscopy. It is now known that under conditions of high iron concentration not all Fe(II) present in ferritin samples forms a ferrous chromophore complex with o-phenanthroline (28, 29).

The results reported here provide new insight into the mechanism of iron deposition in ferritin. At low iron loading of the protein (24 Fe/protein), the stoichiometry was found to be 2 Fe(II) oxidized per O₂ consumed with H₂O₂ being the product of dioxygen reduction (Equation 3). When additional iron was loaded into the protein (up to 960 Fe/protein), the stoichiometry increased markedly, reaching the theoretical

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limit of 4.0 Fe(II)/O2 (Equation 4). The number of protons released during formation of the iron core was found to be 2H+ per Fe(II) oxidized in keeping with Equations 3 and 4. Spin trapping experiments showed that protein radicals are formed which appear to be secondary radicals produced from HO• (Equation 5) as observed previously (21). Superoxide spin-trapped radical was not observed, however. The implication of these findings for the mechanism of iron deposition in ferritin is discussed.

MATERIALS AND METHODS

Cadmium-free horse spleen ferritin, bovine erythrocyte superoxide dismutase (EC 1.15.1.1), and bovine liver catalase (EC 1.11.1.6) were purchased from Boehringer Mannheim. The enzymes were employed as previously described (21, 35). Apoferritin was prepared by anaerobic dialysis of ferritin against a solution of thioglycolic acid (30). The apoprotein concentration was determined from its absorption at 280 nm, ε280 = 1.78 × 10^4 M⁻¹ cm⁻¹. A subunit molecular weight of 19,824 (32). The 57Fe(II) solution for Mössbauer spectroscopy was prepared from 57Fe foil and sulfuric acid by the method of Bauminger et al. (33) and stored at -120 °C until used. The final concentration was 34.0 mM 57Fe in 0.1 M H2SO4. Since Mössbauer spectroscopy indicated that 95.5% of the iron had oxidized to 57Fe(III), the concentration of 57Fe(II) used in all calculations was taken as 32.7 mM. Mössbauer spectra were measured at 77 K as described previously (34).

The oxidation reaction was carried out in a specially designed aluminum cell with an internal cavity volume of 2.44 ml holding 30 µl of protein solution. Aluminum construction ensured good thermal conduction when freezing the sample by placing the cell on dry ice. The addition of 57Fe(II) and the removal or addition of O2 was carried out through a septum at the top of the cell using a gas-tight microliter syringes. The protein solution was stirred with a small magnetic spin bar which was removed from the solution with a magnet prior to freezing the sample.

The experiments were carried out with the aluminum cell placed in a glove bag under a high purity (>99.95%) N2 gas atmosphere. Prior to each experiment the rubber septa and O-rings for assembling the cell were evacuated in a vacuum desiccator overnight to degas adsorbed oxygen. The glove bag was connected to a Hewlett-Packard HP 5898A/5890A gas chromatograph-mass spectrometer system. The gas chromatograph served as a convenient port for introducing samples to the mass spectrometer and did not itself affect separation of N2 from O2. The Mössbauer cup contained 300 µl of 0.042 mM apoferritin (1 mM subunit concentration) in 0.15 M NaCl, 0.15 M MOPS, pHi 7.22. Before adding known amounts of O2 to the cell, 8.8 µl of 57Fe(II) stock solution was injected into the protein solution to give an 57Fe(II) concentration of 1.00 mM. Then a 2-35 µl sample of O2 gas was withdrawn with a gas-tight syringe from a septum-covered test tube containing pure O2 and injected into the aluminum cell. 8 µl 10-min intervals for GC/MS analysis. When no further decrease in O2 level in the cell was observed, the experiment was terminated and the protein sample frozen for later analysis by Mössbauer spectrometry. Because of the low solubility of oxygen in water, the head space above the protein solution accounted for greater than 95% of the oxygen in the cell and therefore was a reliable indicator of the oxygen consumed in the reaction.

Standard curves were prepared by injecting a known amount (2–35 µl) of dry O2 into the cell containing the apoferritin solution but without Fe(II) present. The cell was reassembled for each data point. The points on the working curve were frequently checked during stoichiometry measurements. Most measurements were made in duplicate. The micromoles of O2 added to the cell were calculated from the ideal gas equation using the ambient temperature and the atmospheric pressure.

Proton release measurements were performed on 2 ml of unbuffered protein solution at a concentration of either 1 or 42 µM, adjusted to pH 7.5 using 0.100 M NaOH and 0.100 M HCl. To this solution was added 20 µl of 0.100 M Fe2+, freshly prepared from FeSO4·7H2O dissolved in deoxygenated water, to give a final iron concentration of 1.00 mM and an Fe2+/protein ratio of either 24 or 960. As protons were released, the pH was maintained near 7.5 with a 50-µl syringe containing 0.100 M NaOH.

Spin trapping experiments were performed using PBN and DMPO by procedures similar to those described previously (21) except the two spin traps were incubated with the apoprotein for 24 and 2 h, respectively, before adding the iron(II).

RESULTS

Mass Spectrometry—Good analytical methodology is needed to determine stoichiometries accurately. Accordingly, considerable attention was paid to sources of oxygen contamination and the reproducibility of the 16O mass spectrometric determinations. Fig. 1A shows typical GC/MS chromatographic traces of the mass 28 and 32 peaks of N2 and O2, respectively. The first peak in the N2 trace corresponds to injection of 5 µl of the glove bag atmosphere into the GC/MS. No corresponding O2 peak is observed, indicating an inert atmosphere (<7 ppm O2) had been attained. The three subsequent peaks in both the N2 and O2 traces correspond to triplicate injections of 8 µl of the atmosphere from the aluminum cell. The cell had been injected previously with 14 µl of O2 and contained the Mössbauer cup with the apoferritin solution in buffer but no iron. The reproducibility of the fractional area of the O2 peak defined as A_{O2}/(A_{N2} + A_{O2}), where A_{O2} and A_{N2} are the integrated areas of the O2 and N2 peaks defined as A_{O2}/(A_{N2} + A_{O2}), respectively.

![Fig. 1. A, chromatograms of mass 28 and 32 peaks following injection of gas samples into the GC/MS. The first mass 28 peak is produced upon injection of 5 µl of the glove bag atmosphere. No corresponding mass 32 peak is seen, indicating an oxygen free (<7 ppm O2) atmosphere. The second through fourth mass 28 peaks and the corresponding three mass 32 peaks are from triplicate injection of 8 µl of the aluminum cell to which 14 µl of O2 had been previously injected. B, standard curve for oxygen determination in gas samples. The curve was prepared by injection of 8 µl of gas from the aluminum cell into the GC/MS. Varying amounts of O2 gas (abscissa) had been previously injected into the 2.44-ml nitrogen atmosphere of the glove bag. The y-axis represents the area of the O2 peak (ordinate) as a function of volume of gas injected.](https://example.com/fig1.png)
mass peaks, was typically ±2.5% at the levels of oxygen employed in most of the experiments.

The standard curve for determining the oxygen level in the head space of the cell is shown in Fig. 1B. The abscissa is the fractional area of the oxygen peak for an 8-μl injection of the cell atmosphere into the GC/MS, and the ordinate is the microliters of pure O₂ previously injected into the cell. Excellent linearity (correlation coefficient 0.999) with a slope of (4.03 ± 0.07) × 10⁻⁴ (relative S.D. of ±1.7%) is obtained over the entire range of oxygen added to the cell.

Mössbauer Spectrometry—A typical ⁵⁷Fe Mössbauer spectrum of a partially oxidized ferritin sample is shown in Fig. 2. The spectrum consists of two overlapping quadrupole doublets from Fe²⁺ and Fe³⁺. Mössbauer spectra were curve-fitted to a pair of doublets having Lorentzian line shapes. The percentage of iron oxidized was calculated from the areas of the subspectra, giving a value of 51.9 ± 2.3% for the sample in Fig. 2; the recoilless fractions of the Fe²⁺ and Fe³⁺ were assumed to be the same. In general, the relative standard error in the determination of the percentage of Fe oxidized from Mössbauer curve fitting was 4–7%.

Oxygen Uptake Experiments—Fig. 3 shows a typical oxygen uptake curve during iron incorporation into apoferritin. In this experiment 2 μl of O₂ was injected into the cell containing the Fe(II)-apoferritin solution (24 Fe(II)/protein) to give a mole ratio of Fe(II)/O₂ of 3.59. An 8-μl sample of the cell atmosphere was then removed at various times and injected into the GC/MS. Fig. 3 shows that the 2-μl O₂ was completely consumed in 80 min at which time the sample was frozen for Mössbauer spectroscopy. For samples containing more than 8 μl of O₂, corresponding to Fe(II)/O₂ ratios less than 0.90, the reaction usually reached completion within 20–40 min, the limiting factor appearing to be the rate of dissolution of the O₂ into the protein solution.

Table I summarizes the Fe(II)/O₂ stoichiometries obtained as a function of the O₂/Fe(II) starting ratio. Only 24 Fe(II)/apoferritin were employed in these experiments. It is evident from the data in Table I that all of the iron(II) becomes oxidized only at starting O₂/Fe(II) ratios exceeding approximately 0.5, i.e. an Fe(II)/O₂ ratio less than 2.0. For the completely oxidized samples the average stoichiometry obtained was 1.95 ± 0.18 Fe(II) oxidized per O₂ molecule consumed which is close to the theoretical value of 2.0 Fe(II)/O₂ expected from Equation 3 (see Introduction). For the two incompletely oxidized samples a somewhat higher stoichiometry, average 2.43 ± 0.40 Fe(II)/O₂, was obtained, suggesting slightly more efficient use of O₂ in situations where dioxygen is limiting.

From the stoichiometry of 2 Fe(II)/O₂, we expect that H₂O₂ should be the principal product of iron oxidation (Equation 3). To test this prediction, the reaction was run in the presence of the enzyme catalase (Cat) which catalyzes Reaction 6.

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{Cat}} \text{O}_2 + 2\text{H}_2\text{O}
\]  

(6)

The measured stoichiometry increased from 1.95 ± 0.18 without catalase to 4.08 ± 0.14 Fe(II)/O₂ with catalase present, a
result confirming that \( \text{H}_2\text{O}_2 \) is a product in the reaction.\(^2\)

The effect of the enzyme superoxide dismutase (SOD) on the reaction stoichiometry was also investigated. Superoxide dismutase catalyzes Reaction 7.

\[
\text{O}_2^- + \text{H}^+ \text{apo} \rightarrow \text{H}_2\text{O}_2 + 2\text{H}_2\text{O}
\]

If superoxide were the final product of the oxidation reaction, a stoichiometry of 1 Fe(II)/\( \text{O}_2 \) would be expected (Equation 1), increasing to 2 Fe(II)/\( \text{O}_2 \) in the presence of superoxide dismutase.\(^3\) A stoichiometry of 2.13 \(\pm\) 0.12 was obtained in the presence of superoxide dismutase versus 1.95 \(\pm\) 0.18 in its absence. Thus, under the conditions of our experiments and within the precision of the stoichiometry measurements, no evidence for superoxide being the final product of iron oxidation in ferritin was obtained.

The effect of the Fe(II)/apoprotein ratio on the stoichiometry of oxidation was also investigated. In these experiments, an excess of oxygen was used (\( \text{O}_2/\text{Fe(II)} \) ratio of 2.10). The concentration of Fe(II) was held at 1 mM, whereas the concentration of protein was varied to give Fe(II)/apoferritin ratios ranging from 24 to 960 for the different samples. In all instances, Mössbauer spectroscopy indicated that complete oxidation of the iron(II) had occurred. Fig. 4 shows the observed Fe(II)/\( \text{O}_2 \) stoichiometry as a function of the Fe(II)/apoprotein ratio. As iron is added to the protein, the stoichiometry increased dramatically from 2 Fe(II)/\( \text{O}_2 \) to the theoretical limit of 4 Fe(II)/\( \text{O}_2 \). Thus at low Fe loading of the protein, \( \text{H}_2\text{O}_2 \) is the principal product of iron(II) oxidation (Equation 3) and at high Fe loading, \( \text{H}_2\text{O} \) is the principal product (Equation 4).

Equations 3 and 4 predict that 2H\(^+\) will be produced per each Fe\(^{3+}\) oxidized. This expectation was confirmed by measurements of protons released when either 24 or 960 Fe\(^{3+}\) were added to the apoprotein and allowed to oxidize while the pH was maintained at pH 7.5 with standard base (“Materials and Methods”). A value of 2.01 \(\pm\) 0.02 H\(^+\) per iron(II) oxidized was obtained in both instances.

**Spin Trapping Experiments—**The production of \( \text{H}_2\text{O}_2 \) at low levels of iron loading of ferritin raises the possibility of hydroxyl radical being produced via the Fenton Reaction 5 with the subsequent formation of protein radicals. Accordingly, radical spin trapping experiments were carried out using the reagents PBN and DMPO under conditions similar to those of the stoichiometry measurements (24 Fe/protein). PBN spin-trapped radicals were observed by EPR as reported previously (21). These protein-derived radicals are presumably produced from the reaction of the protein with hydroxyl radical, since their formation was enhanced by the anaerobic addition of \( \text{H}_2\text{O}_2 \) directly to Fe\(^{3+}\)-apoferitin (Equation 5).

The fact that hydrogen peroxide is produced only in the initial stages of iron deposition (Fig. 4) explains the previous observation that radicals are produced primarily in the beginning stages of iron deposition in the apoprotein (21). No spin-trapped superoxide radical was observed using either PBN or DMPO, a result which is also consistent with previous work (21).

**DISCUSSION**

The results of the present study indicate that the stoichiometry of iron oxidation is variable, ranging from 2 Fe(II)/\( \text{O}_2 \) at 24 Fe/protein to 4 Fe(II)/\( \text{O}_2 \) at 960 Fe/protein (Fig. 4). These findings are in keeping with those of Treffry et al. (18) where a general increase in stoichiometry was also observed as the protein was increasingly loaded with iron. In their work a maximum of 3.5 Fe(II)/\( \text{O}_2 \) was reached at 2220 Fe/protein (18), whereas we obtained a value of 3.92 \(\pm\) 0.13 at 960 Fe/protein. Values near 4.0 Fe(II)/\( \text{O}_2 \) have also been observed by Melino et al. (17) at high loadings of the protein, although lower stoichiometries, \( \sim2.5-2.8 \) Fe(II)/\( \text{O}_2 \), were also obtained at similar loadings. The present work and that of others (17, 18) are at variance with the stoichiometry of \( \sim2 \) Fe(II)/\( \text{O}_2 \) reported by Harris and co-workers (19) for ferritins containing 220–1900 Fe/protein. These workers used rather high concentrations of Fe\(^{3+}\) (4.4–65 mM) and perhaps in their experiments the iron(II) was incompletely oxidized, a situation known to occur when high concentrations of Fe(II) are employed (28, 29).

The fact that the stoichiometry changes as iron is added to the protein (Fig. 4) indicates a change in oxidation mechanism. In these experiments, the Fe(II) concentration was held fixed at 1 mM, and the apoprotein concentration was varied so the observed effect on the stoichiometry has to be due to the protein and not to the concentration of Fe(II) used. (The stoichiometry for simple autooxidation of iron(II) in the absence of protein has been shown to increase with increasing Fe(II) concentration in the solution, reaching a value of 4 Fe(II)/\( \text{O}_2 \) at 0.8 mM Fe(II) (18).)

Recent work (6, 8–10) with recombinant H and L human liver ferritins has lead to the conclusion that all ferroxidase activity resides in the H-subunit of the proteins. A site of ferroxidase activity on the H-subunit has been identified and its structure determined (6). The idea that L-subunits completely lack ferroxidase sites seems at variance with the results in Fig. 4 showing that the horse spleen protein, which consists of 96% L-subunits, plays a role in the mechanism of iron(II) oxidation during the beginning stages of core formation.\(^4\)

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\(^2\)The increase in stoichiometry from 2 to 4 Fe(II)/\( \text{O}_2 \) can be understood by adding Equation 6 to twice Equation 3 to give Equation 4 which has a stoichiometry of 4 Fe(II)/\( \text{O}_2 \).

\(^3\)The increase in the stoichiometry from 1 to 2 Fe(II)/\( \text{O}_2 \) can be understood by adding Equation 7 to twice Equation 1 to give Equation 3 which has a stoichiometry of 2 Fe(II)/\( \text{O}_2 \).

\(^4\)An alternate explanation of the data in Fig. 4 is that the increase in stoichiometry with increased Fe content of the protein may simply reflect a change from small core particles with limited surface sites for binding and oxidation of Fe\(^{3+}\) to large surface area particles with an abundance of Fe\(^{3+}\) sites. When many Fe\(^{3+}\) are bound to the mineral surface, partially reduced oxygen species are more likely to encounter additional Fe\(^{3+}\) leading to complete reduction of dioxygen to \( \text{H}_2\text{O} \). In this model the role of the protein would simply be to control the particle size and hence the stoichiometry.
Stoichiometry measurements on a recombinant L-subunit ferritin homopolymer are needed to help clarify the function of the L-subunits in iron oxidation.

That horse spleen apoferritin plays a role in the early stages of iron oxidation is further supported by a number of studies demonstrating that Fe(II) complexes are formed with the apoprotein (33, 36–39, 41, 42, 45). Recent work (45, 46) indicates that a nitrogen donor ligand, most likely histidine, is involved in binding the Fe(II) at a major site on the protein. Iron(II) oxidation may occur at these Fe(II) sites followed by migration to other sites where crystallite nucleation and growth take place. In support of this idea, EPR data indicate that Fe(III) is translocated to other sites following iron(II) oxidation (37, 39). Fe(II)-protein interactions involving carboxylate-like coordination have been shown by extended x-ray absorption fine structure for samples containing 9.6 Fe(II)/protein molecule (42).

The observation of a stoichiometry of 2 Fe(II)/O₂ at low iron loading (24 Fe/protein) with H₂O₂ being a product of the reaction is of special interest (Table I, Equation 3). Although the observed stoichiometry is in accord with an early proposal for the existence of a binuclear Fe(II)/Fe(II) reaction center on the protein where two electron reduction of O₂ occurs to produce H₂O₂ (16), recent spectroscopic (42) and kinetic (34) data argue against such a proposal. Mössbauer spectroscopy has found no evidence for a Fe(II)-Fe(II) dimer (42). Furthermore, for a binuclear center to be involved in a concerted reaction with O₂, a second-order dependence on ferrous ion concentration, [Fe(II)]², is expected. However, the rate of iron oxidation at the low iron/protein ratio of 12 is first-order in [Fe(II)]³ (34). Thus it seems more likely that iron oxidation occurs in two one-electron steps with the oxidation of the first Fe(II) being rate limiting; the O₂ produced probably reacts rapidly with a second Fe(II), which is perhaps bound nearby, to produce the final product H₂O₂. That would account for the first-order dependence of the rate on [Fe(II)]² as well as our inability to spin trap any “free” superoxide radical in solution. The observation of mixed-valence Fe(II)-Fe(III) dimer at relatively low Fe(II) loading of the protein (30, 38) and under oxygen limiting conditions is a further indication that iron oxidation most likely occurs in one-electron steps.

It is evident from our work and that of others (18) that the efficiency of O₂ reduction increases with increasing Fe/protein ratio, with H₂O being the ultimate reduction product at high iron loading of the protein (Fig. 4, Equation 4). The marked increase in stoichiometry with iron loading of the protein is in accord with the crystal growth model for core formation in which iron(II) oxidation occurs directly on the surface of the growing crystallite once it has been initially formed (14, 15). Although intermediate species of oxygen reduction such as O₂⁺ are likely formed during Fe(II) oxidation on the mineral surface, they are probably rapidly reduced by additional adsorbed Fe(II) before they can diffuse away from the surface, ultimately producing H₂O as the end product of oxygen reduction. Reactions at surfaces are known to be more efficient, since collisions between adsorbed reactants is governed by two-dimensional diffusion rather than three dimensional diffusion as in normal solution kinetics (40, 44). H₂O production could also be a consequence of iron catalytic decomposition of H₂O₂ at the mineral surface since iron salts are known to promote reaction 6 (43). Some of the reported dependence of the stoichiometry on the buffer employed (18) can perhaps be explained by competition between Fe(II) and buffer molecules for binding sites on the mineral surface of the core.

In the present work catalase had a pronounced effect on the stoichiometry, changing it from 2 to 4 Fe(II)/O₂ at 24 Fe/ protein, a result which demonstrates that H₂O₂ is a product of the reaction. Previous work (17, 18) failed to see an effect of catalase on the stoichiometry or on the kinetics of iron uptake. The data in Fig. 4 indicate that H₂O₂ production would not be expected at the iron levels of 2300–3450 Fe/protein used in the work of Melino et al. (17). The iron loading employed in the catalase experiments of Treffry et al. (18) was not specified.

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We have also measured stoichiometries by following dissolved oxygen consumption with an oxygen electrode under the same conditions as in Table I of Treffry et al. (18) (540 Fe/protein, 0.18 mM iron, 0.148 mg/ml protein in 0.1 M buffer, pH 7.4) (S. Sun and N. D. Chasteen, unpublished data). A stoichiometry of 4.00 was obtained with MOPS. Values of 3.24 and 3.70 were obtained with HEPES and Tris, respectively, which compare favorably with the reported value of 3.2 for HEPES but not with the value of 2.5 for Tris (18). In the case of HEPES, the oxygen consumed reached a maximum at approximately 3 min following introduction of the Fe(II) to give the stoichiometry of 5.24 followed by a period of slow O₂ production which leveled off after 16 min to give an apparent net stoichiometry of 4.0 for the reaction. We attribute the observed oxygen evolution to the known iron catalysis of the reaction 2H₂O₂ → O₂ + 2H₂O (43). This time dependence in apparent stoichiometry may also account for some of the variability in stoichiometry reported for samples run in HEPES (18, 19).
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