Ferroxidase activity of ferritin: effects of pH, buffer and Fe(II) and Fe(III) concentrations on Fe(II) autoxidation and ferroxidation

Xiaoke YANG and N. Dennis CHASTEEN[1]

Department of Chemistry, University of New Hampshire, Durham, NH 03824, U.S.A.

It is widely accepted that iron deposition in the iron storage protein ferritin *in vitro* involves Fe(II) oxidation, and that ferritin facilitates this oxidation at a ferroxidase site on the protein. However, these views have recently been questioned, with the protein ferroxidase activity instead being attributed to autoxidation from the buffer alone. Ligand exchange between another protein with ferroxidase activity and ferritin has been proposed as an alternative mechanism for iron incorporation into ferritin. In the present work, a pH stat apparatus is used to eliminate the influence of buffers on iron(II) oxidation. Here we show that the recent experiments questioning the ferroxidase activity of ferritin were flawed by inadequate pH control, that buffers actually retard rather than facilitate iron(II) oxidation, and that horse spleen ferritin has ferroxidase activity when measured under proper experimental conditions. Furthermore, high pH (7.0), a high Fe(II) concentration and the presence of Fe(III) all favour Fe(II) autoxidation in the presence or absence of ferritin.

Key words: ferroxidase site, iron hydrolysis, iron storage, pH stat.

INTRODUCTION

Excess cellular iron is stored reversibly in the protein ferritin as a hydrous ferric oxide mineral core which is physiologically available to the cell as needed. The protein shell of mammalian ferritin is composed of 24 subunits of two types, H and L [1,2], which are assembled to form a cavity capable of storing 4500 iron atoms in the mineral core. *In vitro* studies have shown that the H-subunit facilitates iron oxidation at a dinuclear iron ferroxidase site [3–8], while the L-subunit expedites nucleation and core formation [9–11]. There are two pathways for iron incorporation by the protein, the protein-catalysed (ferroxidation) and the mineral-surface (autoxidation) pathways, with the latter becoming increasingly important as the mineral core develops [12]. Fe(III) is not accumulated directly by the protein to any significant extent *in vitro* [13]; a redox reaction is required.

Aust and co-workers have challenged the widely held view that ferritin has ferroxidase activity, and have presented experiments suggesting that the reported catalytic activity of the protein is actually due to Fe(II) autoxidation facilitated by the buffer. In the absence of a buffer, no ferroxidase activity was observed with horse spleen ferritin [14–16] or with the recombinant H-chain homopolymer of rat liver ferritin [17,18]. In a series of papers, they suggested that ferritin acquires Fe(III) through a ligand-exchange mechanism between ferritin and ceruloplasmin, a protein known to have ferroxidase activity [14–19].

In order to resolve this important issue, we have examined the factors influencing iron autoxidation and incorporation into ferritin under a variety of experimental conditions, using electrode oximetry and a pH stat apparatus for pH control. By controlling the pH with a pH stat, the influence of the buffer itself can be eliminated from the experiment. The data show that ferritin does exhibit ferroxidase activity, as generally believed, but that under conditions resulting in a high Fe(II)/apoprotein ratio ($\sim 500:1$), the activity is masked by the dominant autoxidation reaction occurring at the mineral surface. Moreover, the reported failure of ferritin to display ferroxidase activity in the absence of buffer [14,15] is shown to be due to a drop in pH associated with iron hydrolysis, resulting in a marked decrease in the rate of iron oxidation by the protein. The data further indicate that, in the absence of ferritin, iron(II) autoxidation at pH 7.0 is significantly retarded by Tris and Good's buffers compared with pH control using the pH stat, an effect attributed to Fe(II) complexation by the buffers. Moreover, the autoxidation rate is accelerated by the presence of Fe(III), either as ferritin iron or as hydrolysed inorganic iron, due to surface catalysis on the hydrous ferric oxide. The results of the present study emphasize the importance of using proper experimental conditions when investigating the iron oxidation properties of ferritin.

EXPERIMENTAL

Horse spleen apoferritin (apoHoSF) and Tris buffer were purchased from Sigma (St. Louis, MO, U.S.A.). Ferrous sulphate was purchased from Baker Scientific Inc., and Mes, Hepes and Mops buffers were from Research Organics Inc. (Cleveland, OH, U.S.A.). Protein was dialysed against buffer or saline solution prior to use. Apoprotein concentrations were determined by UV absorbance at 280 nm [20]. All other chemicals used were reagent grade or purer.

Iron deposition and autoxidation were carried out in a specially constructed reaction cell, as described previously [12]. The apparatus can monitor simultaneously the oxygen consumption rate, the proton release rate and the pH of the solution during the reaction. A variety of experimental conditions, such as different buffers, ferrous and ferric ion concentrations, pH and temperatures, were employed. Some of the experimental conditions were identical to or comparable with those used by other investigators [14,15].

Abbreviation used: HoSF, horse spleen ferritin.

[1] To whom correspondence should be addressed (e-mail ndc@christa.unh.edu).

RESULTS

Iron autoxidation at high Fe(II) concentration

The data in Figure 1 (trace A) indicate that, at a high Fe(II)/protein ratio ($\sim 500:1$) in the absence of buffer, iron oxidation is slow, a result confirming the findings of Aust and co-workers [14]. There was an initial fast phase, accounting for only about 1% of O_2 consumption, followed by a slow phase for the remainder of the reaction (Figure 1, trace A). The short-lived fast phase probably arises largely from rapid mineral-surface autoxidation at the initial pH of the solution (pH 7.0); the much slower phase is due to decreasing iron(II) oxidation under the influence of a declining pH (see below). The pH decreased from 7.0 at the beginning of the reaction to 5.2 at the end of the 30 min period of the experiment. Only about 20% of the Fe(II) was oxidized during this time period.

In the presence of 50.0 mM Hepes buffer (pH 7.0), iron(II) oxidation proceeded at a much higher rate both in the absence (Figure 1, trace B) and in the presence (Figure 1, trace C) of apoferritin. In the absence of apoferritin, there was an initial lag phase in the reaction (Figure 1, trace B), which is consistent with previous studies of others showing a similar slow start to the autoxidation reaction [21,22]. The reaction in the presence of apoferritin was faster and lacked the lag phase (Figure 1, trace C) compared with autoxidation in Hepes alone (Figure 1, trace B). In contrast, with the pH stat set at 7.0 and no buffer, Fe(II) oxidation in the presence of apoferritin (Fe/protein = 500:1) was very rapid (Figure 1, trace D), indicating that the Hepes buffer itself inhibits the oxidation reaction (Figure 1, trace C). Under pH stat control, autoxidation largely dominates, since the reaction profiles were not significantly different in the presence or the absence of the apoprotein (cf. Figure 1, trace D, and Figure 4, trace E). The observed stoichiometric ratio of $Fe(II)/O_2 = 4:1$ for the completed reactions (Figure 1, traces B–D) is also consistent with iron autoxidation [23–25], i.e.:

$$4Fe^{2+} + O_2 + 6H_2O = 4FeO(OH)\downarrow + 8H^+ \quad (1)$$

where $\downarrow$ denotes a solid.

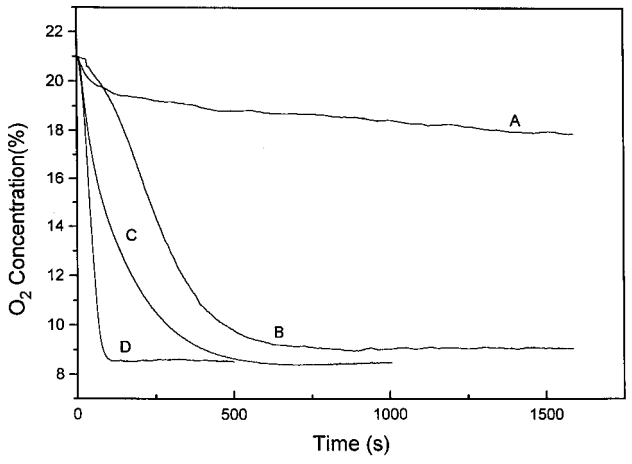

Figure 1 Kinetics of iron incorporation into apoHoSF and iron autoxidation monitored as O_2 uptake

Conditions were 500 μM $FeSO_4$/50.0 mM NaCl, pH 7.0, at 37 °C. Trace A, 1.0 μM HoSF in the absence of buffer, initial pH 7.0; trace B, 50.0 mM Hepes without apoferritin; trace C, 1.0 μM apoHoSF in 50.0 mM Hepes; trace D, 1.0 μM apoHoSF in the absence of buffer with pH stat at 7.0.

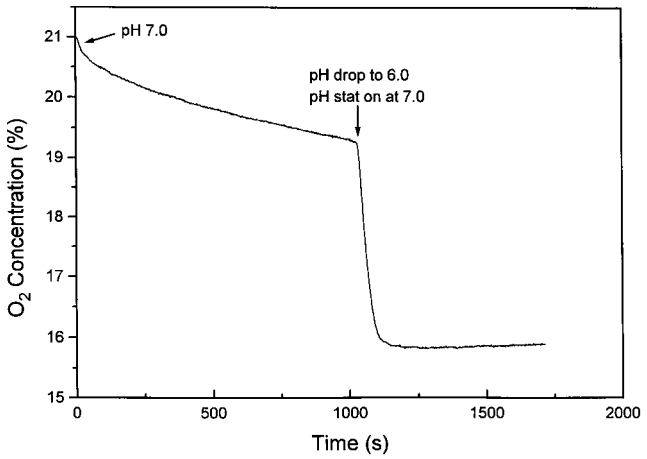

Figure 2 Dependence of Fe(II) autoxidation on pH

Conditions were 300 μM $FeSO_4$ in 50.0 mM NaCl, with an initial of pH 7.0, at 25 °C. The pH stat at 7.0 was turned on when the pH of the solution dropped to 6.0, as indicated.

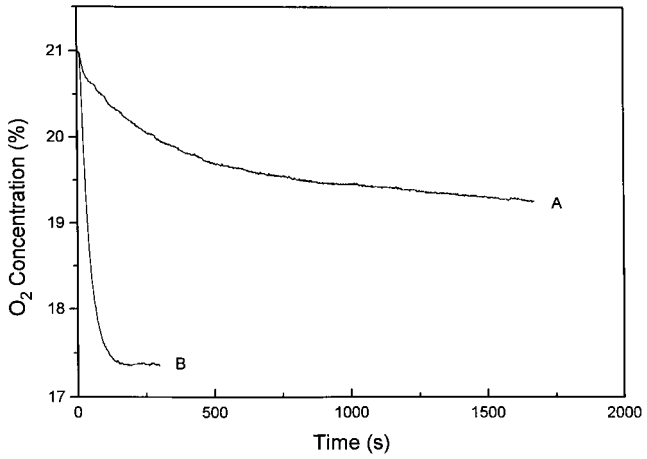

Figure 3 Kinetics of iron incorporation and autoxidation at low Fe(II) concentrations

Conditions were 50.0 mM NaCl, with the pH stat at 7.0, at 25 °C. Trace A, 100 μM Fe(II) only; trace B, 100 μM Fe(II) in the presence of 12 μM apoHoSF.

Effect of pH on iron autoxidation

The effect of declining pH from Fe(III) hydrolysis on the rate of Fe(II) oxidation, as in Figure 1 (trace A), was illustrated further in a pH stat experiment. Figure 2 shows the oxygen consumption rate when 300 μM Fe(II) was added to an unbuffered 50.0 mM NaCl solution in the absence of protein at an initial pH of 7.0. The initial reaction rate was rapid and then quickly slowed as the pH decreased to 6.0. However, upon returning the pH to 7.0 using the pH stat, rapid oxidation was resumed, indicating that autoxidation of Fe(II) is strongly pH dependent, confirming the findings in Figure 1 (trace A). The $Fe(II)/O_2$ stoichiometric ratio observed for the reaction in Figure 2 was again 4:1.

Ferroxidase activity of ferritin

In order to observe the ferroxidase activity of ferritin without the overriding autoxidation reaction, the Fe(II) concentration must

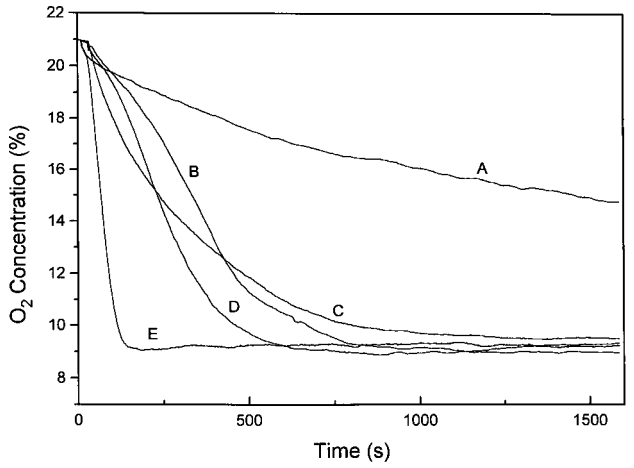

Figure 4 Kinetics of iron autoxidation under the influence of different buffers and pH stat

Conditions were 500 μM $FeSO_4$ in 50.0 mM NaCl, pH 7.0, at 37 °C. Trace A, 50 mM Tris; trace B, 50 mM Mes; trace C, 50 mM Mops; trace D, 50 mM Hepes; trace E, in the absence of buffer with pH stat at 7.0.

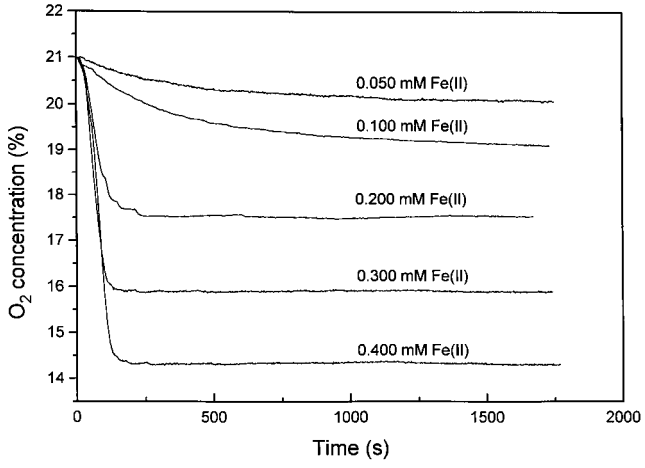

Figure 5 Dependence of kinetics of iron autoxidation on Fe(II) concentration

Conditions were 50.0 mM NaCl, pH stat at 7.0, at 25 °C.

be comparable with that of the protein ferroxidase sites. Figure 3 (trace A) is an oxygen consumption curve for the oxidation of 100 μM Fe(II) at a pH stat of 7.0 in the absence of apoferritin. The reaction was relatively slow. Figure 3 (trace B) is a curve for the same amount of Fe(II) under the same conditions but in the presence of 12 μM apoHoSF (8 Fe/protein). The markedly higher rate of uptake of O_2 demonstrates that the protein itself facilitates iron oxidation when there is no buffer present and when the pH is properly maintained. Furthermore, in this case the observed Fe(II)/O_2 ratio was 2:1 (Figure 3, trace B), a value characteristic of the previously reported protein ferroxidation reaction [24,25]:

$$2Fe^{2+} + O_2 + 4H_2O = 2FeO(OH)\downarrow + H_2O_2 + 4H^+ \quad (2)$$

Effects of buffers on Fe(II) autoxidation

To investigate the influence of buffers on Fe(II) oxidation, reactions in the absence of protein were carried out in the presence of different buffers at pH 7 or with the pH stat set at pH 7.0. Figure 4 (trace A) is the O_2 uptake curve for 500 μM Fe(II) in 50.0 mM Tris and 50.0 mM NaCl. Traces B, C and D are the corresponding curves for reactions carried out in 50.0 mM Mes, Mops and Hepes respectively (all containing 50.0 mM NaCl). The fastest oxidation was observed for the 50.0 mM NaCl solution in the absence of buffer with the pH stat at 7.0 (Figure 4, trace E). Thus all the buffers tested retarded iron(II) autoxidation compared with the pH stat solution, with Tris retarding the oxidation to a much greater degree than the others. All reactions in Good's buffers proceeded with a Fe(II)/O_2 stoichiometric ratio of 4:1, indicative of autoxidation. The value for Tris was not measured, since the reaction was not followed to completion because of its slow rate (Figure 4, trace A).

Effect of Fe(II) concentration on Fe(II) autoxidation

The dependence of Fe(II) autoxidation on Fe(II) concentration is presented in Figure 5. The half-life of the reaction for the lower Fe(II) concentrations of 0.050 and 0.100 mM was about 250 s, and this decreased to about 70 s for the higher concentrations of 0.200–0.400 mM Fe(II). There is clearly a change in the nature of the reaction kinetics with changing Fe(II) concentration; however, no simple rate law is apparent. Again, the Fe(II)/O_2 stoichiometric ratio was 4:1.

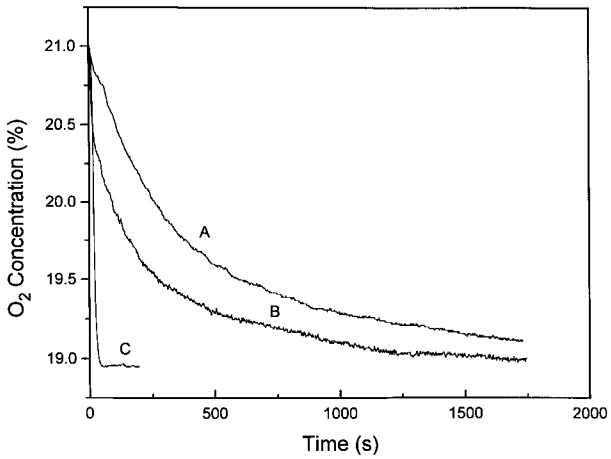

Figure 6 Effect of Fe(III) on the kinetics of Fe(II) autoxidation

Conditions were 100 μM $FeSO_4$ in 50.0 mM NaCl, with pH stat at 7.0, at 25 °C. Trace A, in the absence of Fe(III); trace B, in the presence of 300 μM Fe(III) as holoferritin; trace C, in the presence of 300 μM Fe(III) in the form of $FeCl_3$.

Effect of Fe(III)

To confirm that Fe(III) has a catalytic effect on Fe(II) oxidation, experiments were carried out in the presence of Fe(III) either as holoferritin or as added ferric chloride. Figure 6 (trace A) is the curve for the autoxidation of 100 μM Fe(II) in the absence of Fe(III) with the pH stat set at 7.0. Trace B is the same reaction in the presence of 300 μM Fe(III) as holoHoSF, and trace C is the reaction when an anaerobic mixture of 100 μM Fe(II) and 300 μM Fe(III) (pH 3.5) was injected into the reaction cell (pH stat 7.0). When the Fe(II)/Fe(III) mixture was injected into the reaction cell, immediate Fe(III) hydrolysis occurred (prior to O_2 consumption), as indicated by rapid titration from the pH stat autotitrator (Figure 6, trace C). The data show that Fe(II)

autoxidation is accelerated by the presence of Fe(III), in the form of either holoferritin or hydrolysed Fe(III). In addition, the presence of Fe(III) at the start of the reaction eliminated the lag phase, a further indication of its catalytic effect on the reaction rate.

DISCUSSION

The chemical versatility of iron makes it an important element in biology. However, its properties are also a source of complexity in its chemistry. The results of the present investigation emphasize several aspects of iron oxidation that are relevant to ferritin research, and demonstrate that it is critically important to choose proper experimental conditions for studies of iron incorporation into this protein. The findings are in disagreement with the hypothesis that ferritin does not possess ferroxidase activity and that iron acquisition by ferritin in the absence of ceruloplasmin is due to autoxidation facilitated by buffer [14,15]. At the high Fe(II)/protein ratio employed in previous work [14], the autoxidation rate was high, and this would mask the protein ferroxidation reaction (Figures 1 and 3). High Fe(II) concentrations are known to cause saturation of the ferroxidase sites and to result in a change to the mineral-surface autoxidation reaction [8,24,25]. Furthermore, the ferroxidase reaction primarily initiates the core-formation process. Without proper pH control, the drop in pH due to the hydrolysis of Fe(III) causes oxidation to slow considerably, leading to the mistaken notation that the buffer, and not the protein, is the source of ferroxidation. A decreased pH stabilizes Fe(II) relative to Fe(III) and decreases the rate of Fe(III) hydrolysis; both are driving forces for iron autoxidation.

To observe the ferroxidase activity of ferritin, the Fe(II) concentration must be comparable with that of the ferroxidase sites [$\sim$ 8 Fe(II)/apoHoSF, i.e. 2 Fe(II)/H-subunit), the Fe(II) concentration must be relatively low (100 μM or lower) and the pH should be maintained between 6.5 and 7.0 throughout the reaction. Under these conditions, the autoxidation rate is relatively low (Figure 3, trace A) and the ferroxidase activity of ferritin is readily apparent (Figure 3, trace B).

Buffers slow Fe(II) oxidation kinetics in the presence or absence of ferritin compared with pH stat solutions (cf. Figures 1 and 4). Good-type buffers widely used in biological studies were once considered to have very low affinity for biologically important metal ions [26]. However, later studies have shown this assumption to be invalid [27,28]. Even though there has been no study of the affinity of Good's buffers for ferrous iron, investigations have shown that they do have appreciable affinity for other bivalent metals, such as Zn(II), Co(II) and Cu(II) [27]. Tris has a stronger affinity for bivalent cations [29] and retards iron oxidation the most (Figure 4, trace A), a result consistent with previous work [30].

Previous studies have shown that Fe(II) autoxidation does not follow any specific reaction order, although the kinetics are dependent on the Fe(II) concentration ([22], and references cited therein). At higher concentrations of Fe(II), autocatalysis is enhanced (Figures 5 and 6). With a relatively low Fe(II) concentration (100 μM), the autoxidation rate is low (Figure 6, curve A); however, in the presence of Fe(III) (Figure 6, curve C), the reaction is very rapid, confirming the catalytic effect of hydrolysed Fe(III). The rate is intermediate in the presence of Fe(III) in the form of the mineral core of holoferritin (Figure 6, curve B), suggesting that this form has less catalytic activity than the newly hydrolysed Fe(III). The presence of the protein shell, less mineral surface area or the aged ferrihydrite core may be responsible for the lower activity seen with ferritin.

In conclusion, the data presented here confirm the widely accepted view that ferritin possesses ferroxidase activity. The ferroxidase activity associated with the H-chain is identified not only from its ability to catalyse Fe(II) oxidation, but also from the stoichiometry of the ferroxidation reaction indicated by eqn. (2), which is distinct from that of the autoxidation or mineral-surface reaction of eqn. (1). Once an incipient core is formed, the reaction pathway shifts from the ferroxidase to the mineral-surface mechanism. Nevertheless, the results do not exclude the possibility that iron incorporation *in vivo* proceeds via another mechanism different from the one generally assumed or under conditions different from those commonly used for *in vitro* studies.

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