OXIDANT SPECIFICITY IN FERRITIN FORMATION

Amyra TREFFRY, Janet M. SOWERBY and Pauline M. HARRISON
Department of Biochemistry, The University, Sheffield, S10 2TN, England

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1. Introduction

The iron-storage protein, ferritin, is capable of accumulating up to 4500 Fe atoms. Iron enters the large cavity inside the protein through inter-subunit channels [1] and is deposited as microcrystalline hydrous ferric oxide [2]. Reconstitution can be achieved by adding Fe(II) to apoferritin in the presence of an oxidant. The protein acts as a catalyst and successfully competes with Fe(II)-oxidation outside the protein shell [3–6].

Alternative mechanisms of iron accumulations have been discussed [4–7]. The main point of difference between them is whether the catalytic action of the protein is confined to the initial stages of oxidation and the provision of nucleation centres, on which further iron can be deposited directly, or whether there are active sites on the protein at which all Fe(II) entering the molecule is oxidised. Such catalytic centres have been proposed [8] to contain a pair of Fe(II) atoms, which co-operatively reduce O_2 to peroxide. This seems to suggest a requirement for O_2 as oxidant. The 'nucleation' model was preferred [4]. In their kinetic studies an excess of the oxidising system $KIO_3/Na_2S_2O_3$ was used rather than O_2 alone, so that rate-limiting effects of O_2 depletion could be avoided. O_2 was not excluded from the solutions used, although the amount present was usually well under that required to account for the Fe(II)-oxidized. We now compare the effectiveness of $KIO_3/Na_2S_2O_3$ in the absence of O_2 with that of O_2 alone. We find that oxidation is more rapid with O_2 at the initial stages of Fe(II)-oxidation, but, when some iron is already present in the molecule, the two oxidants are equally effective.

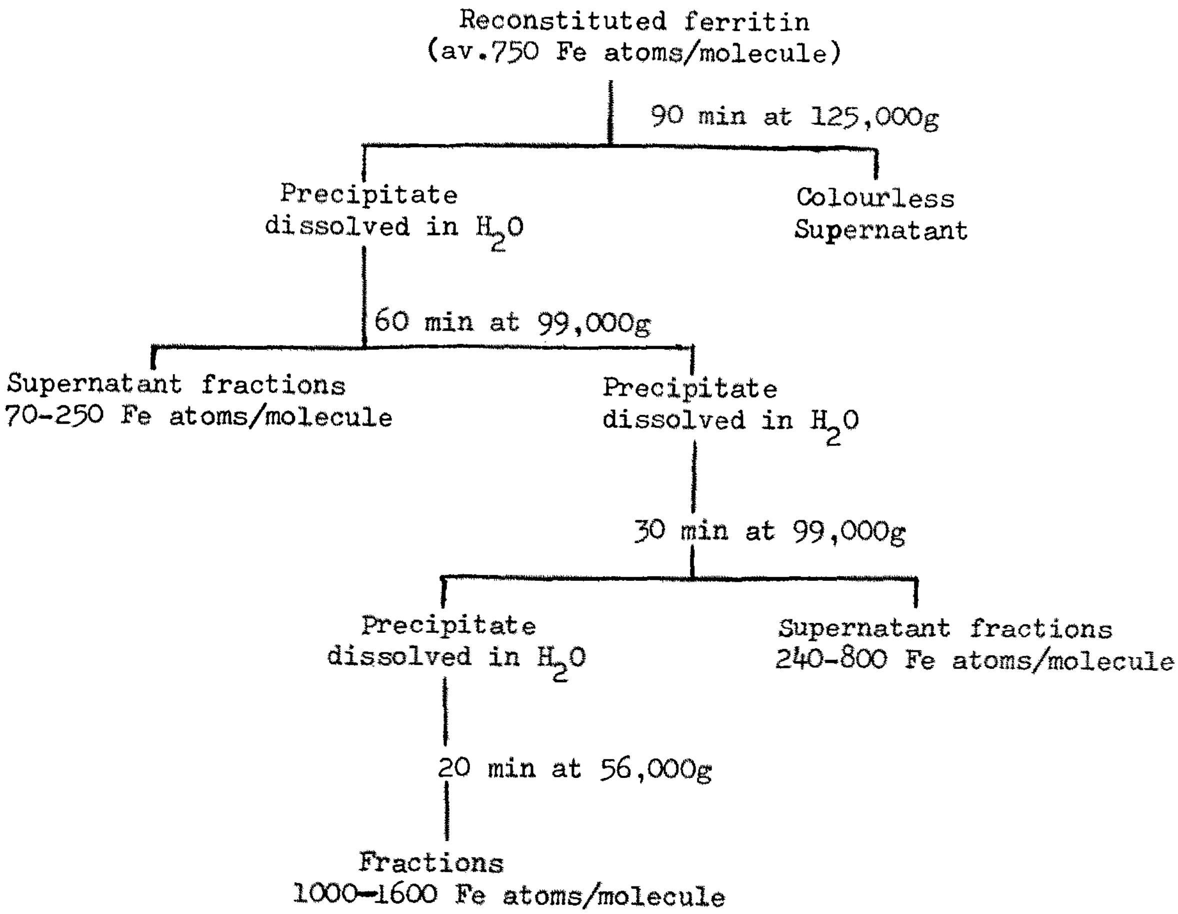
2. Materials and methods

Horse spleen ferritin was isolated from tissue homogenates by heat treatment followed by high speed centrifugation [9] and further purified by gel filtration on Sepharose 6B. Apoferritin was prepared from ferritin by reduction with thioglycollic acid [10]. Chemicals used were AnalaR grade.

Ferritin fractions containing a small amount of iron were obtained from ferritin reconstituted to an average Fe content of 750 Fe atoms/molecule. The reconstitution procedure used was that in [10]. After concentration, fractionation of the reconstituted ferritin solution was carried out in an MSE Superspeed 50 centrifuge (10 × 10 ml rotor) according to scheme 1. After each centrifugation step the supernatant was divided into fractions by careful withdrawal of solution from the top of the centrifuge tube with a syringe.

Fe(II) solutions were prepared by adding solid $Fe(II)(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$ to distilled H_2O through which N_2 had been bubbled for at least 1 h. Solutions were kept stoppered at 4°C. Fe-uptake into apoferritin or ferritin solutions in 20 mM Hepes–NaOH (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, was monitored in a recording spectrophotometer by use of the increasing A_{420} due to the production of red-brown ferric micelles. The procedure for Fe-uptake in the absence of O_2 was as follows: Deaerated buffer, with or without 2 mM KIO_3/8 mM $Na_2S_2O_3$ was placed in a cuvette and Silicone DG antifoam MS A compound (Hopkin and Williams, Chadwell Heath, Essex) was layered on top. Air was excluded with a suba seal and O_2-free N_2 [11] was bubbled through the buffer for 30 min.

Scheme 1



Procedure used for fractionation of reconstituted ferritin containing an average of 750 Fe atoms/molecule

Protein was then added with a syringe to give a concentration of 1 mg/ml (2.2 μM) and bubbling of N_2 continued for a further 15 min. The cuvette was placed in the spectrophotometer (Unicam SP1800) and the Fe(II) solution added with a microsyringe. The control sample containing no oxidant was also covered with a layer of silicone antifoam, but N_2 bubbling was omitted.

3. Results

Figure 1 shows the time course of the oxidation by apoferritin of 1.5 mM $Fe(II)(NH_4)_2(SO_4)_2$ in the presence of atmospheric O_2, with and without the addition of $KIO_3/Na_2S_2O_3$ (4 mM/16 mM).

It can be seen that the initial rate of oxidation is faster in the absence of added $KIO_3/Na_2S_2O_3$ but that when the O_2 in solution (about 0.25 μmol [13]) has all been used up the reaction stops. At this stage about 1 μmol out of the 1.5 μmol Fe(II) present has been oxidized, or about 4 Fe(II)/O_2 molecule. Oxidation continues when further small amounts of O_2 are admitted (arrows) and again proceeds until O_2 is depleted. Although the initial rate is slower in the presence of the oxidizing system, it continues until all the Fe(II) has been oxidized and does not stop when O_2 has been depleted. The reactions

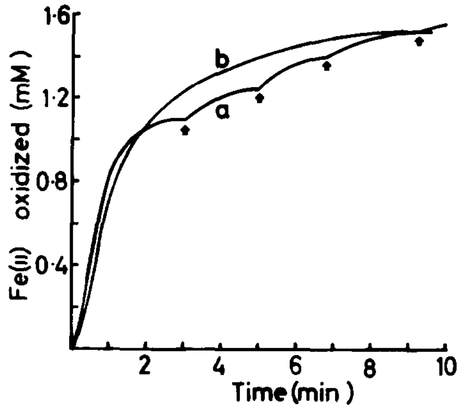


Fig.1. Progress curves of Fe(II)-uptake by apoferritin. Reactions were started by addition of Fe(II)$(NH_4)_2(SO_4)_2$ (to 1.5 mM) to apoferritin (0.5 mg/ml) in 0.02 M Hepes, NaOH buffer (pH 7.4) and followed by recording the increase in A_{420}. Curve (a) atmospheric O_2 the only oxidant, arrows indicate remixing; curve (b) oxidation in the presence of 3 mM KIO_3/12 mM $Na_2S_2O_3$.

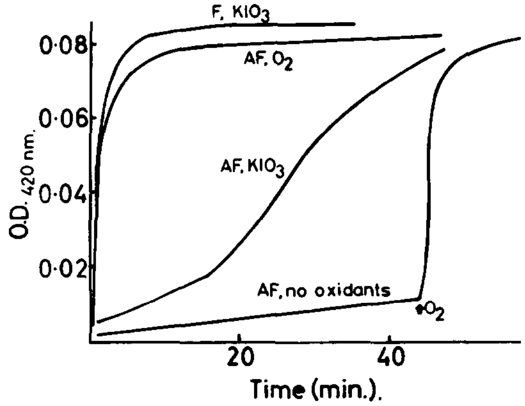


Fig.2. Progress curves of Fe(II)-uptake by ferritin and apoferritin. Reactions were started by addition of Fe(II)$(NH_4)_2$ $(SO_4)_2$ (to 0.2 mM) to ferritin or apoferritin (1 mg/ml protein), giving 90 Fe atoms/molecule added, and followed by recording the increase in A_{420}. AF, no oxidant: apoferritin without O_2 or KIO_3/$Na_2S_2O_3$, the arrow indicates opening of the cuvette to air. AF, KIO_3: apoferritin plus 2 mM KIO_3/8 mM $Na_2S_2O_3$, oxygen excluded. AF, O_2: apoferritin control open to air. F, KIO_3: ferritin fractions containing 515 Fe atoms/molecule plus 2 mM KIO_3/8 mM $Na_2S_2O_3$, oxygen excluded.

shown in fig.1 were carried out at pH 7.4, the pH used in [4], although the buffer used in the present study was different (Hepes rather than imidazole). At lower pH values (6.2–7.2) the difference in the initial rates with and without the oxidizing system is even more pronounced and again the curves cross.

These results show that even when dissolved O_2 has been used up, oxidation proceeds, the oxidizing equivalents being provided by the KIO_3/$Na_2S_2O_3$ system.

The relative effectiveness of the oxidizing system in the initial and secondary stages of Fe(II)-oxidation (by apoferritin and a ferritin fraction containing 500 Fe atoms/molecule) are shown in fig.2, which also demonstrates the relative effectiveness of KIO_3 and O_2 as oxidants in the initial stages. The control shows little Fe(II)-oxidation in the presence of apoferritin under anaerobic conditions in the absence of KIO_3. However, when KIO_3/$Na_2S_2O_3$ is added anaerobically oxidation does proceed, although slowly. When air is admitted to the control, oxidation occurs much more rapidly than with KIO_3/$Na_2S_2O_3$ alone. However, when the apoferritin is replaced by a ferritin fraction containing a small Fe(III)-hydrous oxide nucleus (about 500 Fe atoms/molecule), oxidation is just as fast with the oxidizing system and no O_2, as with apoferritin and oxygen alone (fig.2).

4. Discussion

The results described above indicate that O_2 is not a specific substrate for the oxidation of Fe(II) by apoferritin or ferritin. The mechanism proposed [8] involving specific binding sites for O_2 between 2 bound Fe(II) atoms is therefore not only unnecessary, but can certainly be excluded, where oxidation occurs in the absence of O_2.

These lend further support to the two-stage oxidation mechanism [4]. Thus O_2 appears to be a more effective oxidant at the initial stage (nucleation) and, indeed, this stage, believed to represent oxidation at specific binding sites on the apoferritin shell, is actually inhibited by KIO_3/$Na_2S_2O_3$, whereas at the second, 'crystal growth' stage, KIO_3 is just as efficient as O_2 in promoting the crystal build-up. This strongly suggests that oxidation no longer takes place on protein sites, but directly on the crystallite surface itself, as proposed [4]. The crystal growth model was originally derived from kinetic studies in which the oxidizing system was used, and was based on the observation that sigmoid progress curves were obtained, when relatively large amounts of Fe(II) were added (~ 500 atoms/molecule). These could be broken down into hyperbolic curves of increasing initial rates, if the iron was added in successive small increments [4].

It has been suggested that the sigmoid progress curves result from different steps in the same Fe(II)-oxidation mechanism becoming rate limiting [12]. However, the results here taken together with those in [4,5,13] suggest that oxidation occurs at different sites in the two stages of ferritin formation, whether O_2 or $KIO_3/Na_2S_2O_3$ is used as the oxidant. We have shown [13] that the ratio of Fe(II) oxidized/O_2 molecule varies from 1.5, when a very low Fe(II) concentration, or a very small number of Fe(II) atoms/apoferritin molecule, have been added, to 4, at a higher Fe(II) concentration and a larger number of Fe(II) atoms/added protein molecule. The greater effectiveness of $KIO_3/Na_2S_2O_3$ in the presence of a ferritin fraction, as compared with apoferritin, may also result from the greater availability of electrons at the many Fe(II)-binding sites on the micelle surface. The superiority of O_2 over the $KIO_3/Na_2S_2O_3$ system at low Fe/apoferritin ratios, may be due to its ability to act as a 1 or 2 electron acceptor in the absence of sufficient Fe(II) ions to reduce it to water.

Acknowledgement

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