VARIABLE STOICHIOMETRY OF Fe(II)-OXIDATION IN FERRITIN

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

Received 24 August 1978

1. Introduction

Ferritin is an iron storage protein of wide distribution. The protein, apoferritin, is composed of 24 subunits arranged as a spherical shell with outside diam. 13 nm and inner cavity width ~8 nm [1]. Up to 4500 Fe atoms can be stored in this space in the form of a ferric oxhydroxide microcrystalline core. It has been shown that apoferritin catalyses the oxidation of Fe(II) to Fe(III) [2–5] and the reconstituted ferritin so formed closely resembles native ferritin [3]. The mechanism of Fe(II) oxidation within the ferritin molecules is controversial. It has been proposed [6] that all Fe^{2+} ions entering the molecule are oxidized on specific sites on the protein, and that these sites involve close pairs of Fe(II) atoms, which bind molecular O_2 with the simultaneous transfer of 2 e^- to give a Fe(III)–O_2^{2-}–Fe(III) complex. Such a mechanism was rejected [3,4] in favour of a 'crystal growth' model. In this model Fe(II) is bound by groups on the protein's inner surface, chelation accelerates oxidation, and the Fe(III), which remains bound, forms nucleation centres on which further Fe atoms can be added without the obligatory involvement of oxidation sites on the protein. It is suggested that Fe(II) may be oxidized directly on the surface of the growing crystallites [3,7]. The finding in [1] of close pairs of Tb(III) near to 2-fold axes, might be taken to support the model in [6], since these pairs may also represent Fe sites. However, such sites are not inconsistent with the model in [3], since they occur on the inner surface of the protein shell and could represent nucleation centres. Evidence has been provided [8] that in ferritin formation a single O_2 molecule oxidizes 4 Fe(II) without producing significant amounts of free radical intermediates. The results reported here indicate that, either in the presence or absence of apoferritin, the overall stoichiometry of Fe(II) oxidation by O_2, which probably involves steps of 1 e^- transfer is dependent on the buffer, pH and Fe concentration used and, in the case of ferritin formation, on the relative concentrations of Fe and protein.

2. Materials and methods

Horse spleen ferritin was prepared by heat treatment followed by high speed centrifugation [9] and further purified by gel filtration on Sepharose 6B to remove dimers and trimers. Apoferritin was prepared from ferritin by reduction with thioglycollic acid [10]. Superoxide dismutase was prepared according to [11] and its activity was checked by using the xanthine–xanthine oxidase system [12]. Xanthine, xanthine oxidase (grade I) and catalase (beef liver, crystallized) were obtained from Sigma Chemical Co. (Poole, Dorset). Other chemicals used were Analar grade.

Oxygen consumption during Fe(II) oxidation was measured with an oxygen electrode (Rank Brothers, Cambridge). The oxygen electrode was calibrated by the method in [13] and the oxygen concentration in 0.02 M Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) NaOH buffer (pH 7.0), was found to be 0.254 mM at 25°C. This is within 5% of 0.242 mM given in [13]. Stock $Fe(NH_4)_2(SO_4)_2$ solutions were prepared in distilled water through which N_2 had been bubbled for at least 1 h and the solutions were kept stoppered at 4°C. Fe(II) concentrations were determined as the 2,2'-bipyridine com-

plex. Fe(II) oxidation was measured in 0.02 M Hepes at the pH stated in the text. The effects on the stoichiometry of oxidation of varying Fe atoms/protein molecule at fixed Fe(II) concentration were studied by varying the concentration of apoferritin. The effects of varying the Fe(II) concentration at different fixed Fe atoms/molecule were also found again by varying protein concentrations. In these experiments the protein used was from 0.007–3.2 mg/ml and the Fe from 0.02–0.6 mM. The stoichiometry of Fe(II) oxidation in the absence of protein was also studied. In some experiments oxygen consumption was measured in the presence of excess EDTA.

Rates of Fe uptake into ferritin with oxygen as oxidant were followed in a spectrophotometer by monitoring $A_{420\ nm}$ due to the formation of ferric oxyhydroxide micelles inside the protein [3].

The effects of varying amounts of superoxide dismutase and catalase on ferritin formation were also examined, with respect to both rates of Fe uptake and the stoichiometry of Fe(II) oxidation.

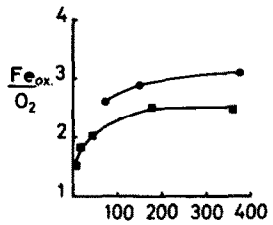

Fig.1.

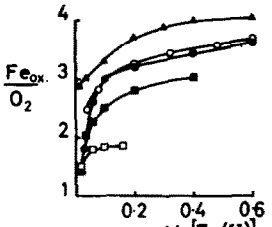

Fig.2.

Fig.1. Dependence of Fe(II) oxidation stoichiometry, during Fe uptake into ferritin, on the numbers of Fe atoms added/protein molecule. The reaction was carried out in 0.02 M Hepes NaOH, buffer (pH 7.0) at either 0.06 mM (■) or 0.5 mM (●) $Fe(NH_4)_2(SO_4)_2$. Apoferritin concentration was varied in the range 0.074–2.96 mg/ml. The ordinate shows the number of Fe atoms oxidized/O_2 molecule consumed.

Fig.2. Dependence of Fe(II) oxidation stoichiometry on Fe(II) concentration during Fe uptake into ferritin or in the absence of protein. The reaction was carried out at the indicated concentrations of $Fe(NH_4)_2(SO_4)_2$, in 0.02 M Hepes, NaOH, buffer at pH 7.0 in the presence of apoferritin or at pH 7.0 (○) or pH 7.8 (▲) in the absence of protein. Apoferritin concentrations were adjusted to give 22 Fe atoms/molecule (□), 222 Fe atoms/molecule (■), or 2220 Fe atoms/molecule (●).

3. Results

In the presence of apoferritin the number of Fe(II) atoms oxidized by one oxygen molecule was found to be variable. When Fe(II) concentration was kept constant, the ratio Fe oxidized/O_2 molecule was dependent on the number of Fe atoms/apoferritin molecule added. Figure 1 shows that at 0.06 mM Fe this ratio varied from 1.5 at 9 Fe atoms/molecule to 2.5 at 200–400 Fe atoms/molecule. The data in fig.2 also shows variable stoichiometry of oxidation at different Fe atoms up to 3.5 Fe/O_2 molecule. Both fig.1 and fig.2 show that the ratio Fe atoms oxidized/O_2 is dependent on the Fe(II) concentration and, as can be seen in fig.2, this is true whether or not apoferritin has been added. With or without protein, the Fe/O_2 ratio was smaller at lower Fe concentrations that at higher Fe concentrations. The Fe/O_2 ratio also showed pH dependence. In the absence of protein, higher ratios were obtained at pH 7.8 than at pH 7.0, see fig.2. Table 1 shows that in the presence of protein, the Fe/O_2 ratio depended both on pH and on the buffer used.

When oxidation was carried out in the presence of excess EDTA and no protein the stoichiometry was found to be independent of Fe at 0.02–0.06 mM, but it was dependent on whether the solution was buffered or unbuffered. In 0.02 M Hepes buffer (pH 7.0) 1.56 Fe atoms were oxidized/O_2 molecule, whereas when buffer was omitted the ratio was increased to 2.03.

No effect was observed on the rate of ferritin formation measured spectrophotometrically when either catalase or superoxide dismutase was added and these enzymes were also without effect on the overall stoichiometry of oxidation in the presence of protein and on the rate of oxygen consumption. In

Table 1
Stoichiometry of Fe(II) oxidation by ferritin in different buffers

Buffer	Fe/O_2
0.1 M Tris, HCl (pH 7.4)	2.5
0.1 M Imidazole, HCl (pH 7.4)	3.7
0.1 M Hepes, NaOH (pH 7.4)	3.2
0.1 M Hepes, NaOH (pH 7.0)	2.9

0.18 mM Fe, 0.148 mg/ml protein (540 Fe atoms/protein molecule)

the absence of apoferritin, catalase affected neither the final ratio of Fe oxidized/O_2 molecule nor the rate of oxygen consumption. Superoxide dismutase had no effect on the Fe/O_2 ratio, although it slightly reduced the rate of oxygen consumption. However the latter was not proportional to the amount of superoxide dismutase added, addition of 5 μg/ml and 100 μg/ml giving 22% and 30% inhibition, respectively.

4. Discussion

The overall similarity in the stoichiometry of oxidation of Fe(II) in Hepes buffer (pH 7.0), at high ratios of Fe atoms/protein molecule and in the absence of protein (fig.2) suggests similarity in oxidation mechanisms. The dependence of the Fe/O_2 ratio on Fe(II) concentration suggests that this ratio depends on the number of Fe(II) atoms an oxygen radical is likely to meet before interacting with an alternative electron donor. In this study, Fe/O_2 ratios $\leqslant$3.5 were observed in the presence of protein. The fact that higher ratios up to 4 Fe/O_2 were found in [8] is probably explained by the higher Fe concentrations, 1.5–6.5 mM Fe, used in that study. In our work, at very low Fe concentrations, the plots such as those shown in fig.1,2 tend to Fe/O_2 ratios of 1. Fe concentrations could not usefully be reduced further than those shown, because at very low values the percentage oxygen consumed was very small (<5% of the total) and variations of as little as 0.5% in oxygen consumption had a relatively large effect on the ratio. Attempts to detect superoxide or peroxide, products of one or two electron transfer to oxygen, respectively, were inconclusive or unsuccessful in our experiments and in those of [8,14]. At low concentrations or low Fe atoms/protein molecule this may be explained by their reaction with species other than Fe(II). The proposal in [6] that ferritin formation involves the concerted oxidation of 2 Fe(II) atoms in the protein by one oxygen molecule does not appear to be substantiated by our results. Our results tend to emphasise the similarity of Fe(II) oxidation with and without protein rather than to require a special mechanism for Fe uptake into ferritin. The picture of ferritin formation that we suggest is as follows. At very low Fe concentrations and Fe atoms/ferritin molecule most of the bound Fe atoms are too far apart to allow oxidation of more than 1 Fe/O_2. As the number of Fe atoms/ferritin molecule is increased concerted oxidation of more than 1 Fe/O_2 becomes possible, perhaps first at the suggested close Fe-binding sites on the inner surface of the protein shell and later on the surfaces of the FeOOH crystallites. The concerted oxidation of more than 2 Fe atoms/O_2 seems to require the presence of $\geqslant$50 Fe atoms/ferritin molecule under the conditions of our study. This suggests that greater efficiency of oxidation is a consequence of the formation of FeOOH nuclei on which further Fe(II) atoms may be bound and oxidized, lending further support to our proposed model [3,4]. Part of the evidence for this model was the observation that at higher additions of Fe per protein, the progress curves of Fe uptake become sigmoidal, whereas at low ratios they are hyperbolic in shape [3,4]. Factors affecting the appearance of sigmoidal behaviour have been studied further [15,16] and it is interesting to note that the conditions favouring increased efficiency of oxidation, or higher Fe/O_2 ratios, are also those which give rise to sigmoidal curves. These conditions include high Fe concentrations, high Fe/protein and high pH. Moreover it has been noted that Fe uptake into ferritin in Tris buffer [15,16] does not give sigmoidal curves and we see in table 1 that Fe/O_2 ratios in Tris buffer are also exceptionally low. Thus it would seem that the results we present here not only support our 'crystal growth' model, but show that increased rates of oxidation depend both on the increasing number of sites available for Fe deposition as the FeOOH crystallities grow and on the fact that this increasing number of sites permits, when Fe is added at sufficiently high concentrations, more efficient Fe(II) oxidation, ultimately reaching Fe/O_2 ratios of 4 with the reduction of oxygen to water.

Acknowledgement

We thank the Medical Research Council for support.

References

[1] Banyard, S. H., Stammers, D. K. and Harrison, P. M. (1978) Nature 271, 282–284.

[2] Niederer, W. (1970) Experientia 26, 218–220.
[3] Macara, I. G., Hoy, T. G. and Harrison, P. M. (1972) Biochem. J. 126, 151–162.
[4] Macara, I. G., Hoy, T. G. and Harrison, P. M. (1973) Biochem. J. 135, 343–348.
[5] Bryce, C. F. A. and Crichton, R. R. (1973) Biochem. J. 133, 301–309.
[6] Crichton, R. R. and Roman, F. (1978) J. Mol. Catal. 4, 75–82.
[7] Harrison, P. M., Hoy, T. G., Macara, I. G. and Hoare, R. J. (1974) Biochem. J. 143, 445–451.
[8] Melino, G., Stefanini, S., Chiancone, E., Antonini, E. and Finazzi-Agro, A. (1978) FEBS Lett. 86, 136–138.
[9] Crichton, R. R. (1973) in: Structure and Bonding, vol. 17, pp. 67–134, Springer-Verlag, Berlin, Heidelberg, New York.
[10] Treffry, A. and Harrison, P. M. (1978) Biochem. J. 171, 313–320.
[11] Weser, U., Bunnenberg, E., Cammack, R., Pjerassi, C., Flohé, G., Thomas, G. and Boelter, W. (1971) Biochim. Biophys. Acta 243, 203–213.
[12] Younes, M. and Weser, U. (1976) FEBS Lett. 61, 209–212.
[13] Robinson, J. and Cooper, J. M. (1970) Anal. Biochem. 33, 390–399.
[14] Crichton, R. R., Wauters, M. and Roman, F. (1975) in: Proteins of Iron Storage and Transport in Biochemistry and Medicine (Crichton, R. R. ed) pp. 287–294, North-Holland, Amsterdam.
[15] Crichton, R. R. and Päques, E. (1977) Biochem. Soc. Trans. 5, 1130–1132.
[16] Treffry, A., Sowerby, J. and Harrison, P. M. (1978) unpublished work.