STUDIES ON THE MECHANISM OF FERRITIN FORMATION

Superoxide dismutase, rapid kinetics and Cr^{3+} inhibition

Mireille WAUTERS, A. Michael MICHELSON and Robert R. CRICHTON

Unité de Biochimie, Université Catholique de Louvain, Place Louis Pasteur 1, B-1348 Louvain-la-Neuve, Belgium and *Institut de Biologie Physico-Chimique, Fondation Edmond de Rothschild, Rue P. et M. Curie 3, F-75005 Paris, France*

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1. Introduction

The involvement of Fe^{2+} oxidation in the deposition of iron in ferritin, the principal iron storage protein of mammals was shown in pioneering studies [1] and a role for apoferritin (the protein part of the ferritin molecule) in the catalysis of iron oxidation was subsequently established [2–4]. However, the nature of the other products besides Fe^{3+} in the oxidation step remains unclear. Assuming that molecular oxygen is the most likely electron acceptor in a physiological context, the product of one or two electron oxidations would be respectively O_2^- or O_2^{2-}. Further, if the two electron oxidation mechanism is involved, as has been recently suggested [5–7], we would expect that the initial velocity of iron oxidation would be dependent on the second order of the iron concentration. Further we might expect that additional information on the mechanism could be obtained from inhibition studies with other transition metal ions.

We have attempted to detect formation of O_2^- concomitant with ferritin iron oxidation and deposition in ferritin but were unable to establish its presence. The oxidation of Fe^{2+} by apoferritin was studied by rapid kinetic techniques (stopped-flow) and a second order dependence of the initial velocity as a function of iron concentration consistent with a mechanism involving fixation of two iron atoms was found. The inhibition by Cr^{3+} was greater than 80% when 0.5 g atoms Cr^{3+} were bound per polypeptide chain, again indicative of the involvement of two polypeptide chains in the active site of ferritin. The effect of a number of other metal ions was also examined. These results are discussed in terms of our present concepts of iron deposition in ferritin [7,8].

2. Materials and methods

Horse spleen ferritin was prepared as in [10] with a final step of crystallization from 5% $CdSO_4$. Apoferritin was prepared from ferritin by reduction for 16 h in 1% (v/v) thioglycollic acid in 0.25 M sodium acetate buffer, pH 5.4, followed by extensive dialysis at 4°C against 0.5% w/v NH_4HCO_3. The protein was finally dialysed into 0.1 M sodium borate–cacodylate buffer, pH 5.8. Protein concentrations were determined by amino acid analysis after 16 h hydrolysis with 6 N HCl at 110°C using a Locarte amino acid analyser (Locarte Co., London). Ferrous ammonium sulphate was from BDH Chemicals Ltd., Poole, Dorset.

The oxidation of Fe^{2+} catalysed by apoferritin was followed at 310 nm using a Durrum stopped-flow spectrophotometer (Durrum Instruments, Palo Alto, CA). Apoferritin (0.5 mg/ml in 0.1 M sodium borate–cacodylate buffer, pH 5.8) was in one syringe while the other contained ferrous ammonium sulphate in 0.1 M sodium borate–cacodylate buffer, pH 5.8 at 0.63–50.9 mM (corresponding to the addition of 50 atoms Fe^{2+}/apoferritin molecule to 4000 atoms/molecule). Initial velocities were calculated by drawing tangents.

Superoxide radical anion (O_2^-) was detected using reduction of cytochrome *c*, tetranitromethane and nitrobluetetrazolium or oxidation of luminol and luciferin [11,12]. The sensitivity of detection of these

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systems was established using the system xanthine oxidase/hypoxanthine to generate O_2^- [11]. Superoxide dismutase from *Photobacterium leognathi* was isolated as in [12]. Superoxide dismutase from bovine erythrocytes was prepared as in [11].

The effects of Cr^{3+} as $CrCl_3$ were studied by incubation of apoferritin in 0.1 M sodium borate–cacodylate buffer, pH 5.8, for 18 h at 20°C with concentrations of $CrCl_3$ in the range 5×10^{-4} M to 5×10^{-2} M. The samples were subsequently dialysed against several changes of the same buffer to remove the excess of Cr^{3+}. The final concentration of protein for the apoferritin solutions was determined by A_{280} [4] and the Cr^{3+} concentration was determined by atomic absorption. The activity of the samples was controlled at 310 nm using a Beckman DB-GT spectrophotometer (Analis, Namur, Belgium) after addition of ferrous ammonium sulphate. Other transition metals (Cr^{2+}, Ru^{2+} and Co^{2+}) were studied by the same techniques.

3. Results and discussion

A series of studies was effected to detect the possibility of production of O^{2-} during the oxidation of Fe^{2+} by oxygen catalysed by apoferritin. Table 1 shows the results obtained with different methods for O_2^- detection. It can be rigorously confirmed that if free O_2^- is produced during the oxidation of ferrous ions catalysed by apoferritin, such a mechanism involves less than 1 part in 10^4 of the concentrations of Fe^{2+} used, given the sensitivity of the methods of detection used. We also used an indirect method to see if superoxide radical ions were formed during the reaction. Both native and denatured superoxide dis-

Table 1
Detection of superoxide and effect of addition of superoxide dismutase and catalase in apoferritin-catalysed iron oxidation

Addition to the system	Rate of iron deposition	Limit of detection of O_2^-
–	100	–
Luminol	100	10^5 quanta/s/ml
Luciferin	100	10^6 quanta/s/ml
Cytochrome *c*	97	0.001 A_{550}/10 min
Tetranitromethane	100	0.001 A_{350}/10 min
Nitro blue tetrazolium	100	0.001 A_{560}/10 min
Superoxide dismutase (50 μg/ml) (bovine erythrocytes)		
nature	114	–
denatured	189	–
Superoxide dismutase (50 μl) *(Photobacterium leognathi)*	105	–
Catalase (bovine erythrocytes)		
0.6 μg/ml	125	–
6.0 μg/ml	111	–
H_2O_2 (10^{-4} M)	7	–

For the systems luminol and luciferin addition of hypoxanthine and xanthine oxidase at concentrations of 10^{-4} M and 0.03 mg/ml we measured 2×10^7 quanta/s/ml and 7×10^8 quanta/s/ml, respectively. For the same concentrations of hypoxanthine and xanthine oxidase we could detect changes in absorption of 0.025/min at 550 nm, 0.1/min at 350 nm and 0.05/min at 560 nm, respectively. Denaturation of the bovine superoxide dismutase was by heat denaturation at 100°C for 10 min

mutase from both bovine erythrocytes and from the marine bacterium *Photobacterium leognathi* were added to apoferritin in sodium borate–cacodylate buffer 100 mM, pH 5.8, with a concentration of ferrous ammonium sulphate corresponding to 2000 atoms iron added/apoferritin molecule. The results obtained are given in table 1. In order to ensure that the presence of iron did not affect the detection of O_2^-, we carried out control experiments with hypoxanthine/xanthine oxidase as a source of O_2^- in the presence of Fe^{2+} and apoferritin. Superoxide was detected with cytochrome *c* and with tetranitromethane. In both systems of detection the production of O_2^- by xanthine oxidase was unaffected by the presence of Fe^{2+} and apoferritin.

From the results of table 1 we can affirm that if free O_2^- is produced during the oxidation of Fe^{2+} catalysed by apoferritin, such a mechanism involves less than 1 part in 10^4 of the concentration of Fe^{2+} used, given the sensitivity of the system of detection employed. The stimulatory effect observed with the Cu/Zn superoxide dismutase (table 1) was even more marked when denatured enzyme was used. In contrast, no stimulation was observed with the Fe-superoxide dismutase from *Photobacterium leognathi.* We have observed the Cu^{2+} exerts a considerable stimulatory effect on the deposition of iron in apoferritin as measured by stopped-flow techniques [13] and it may be that the effect of bovine superoxide dismutase is due to the presence of Cu. We also verified the effects of catalase on iron deposition (table 2) and, in confirmation of the results in [14], we found very little effect. (Addition of H_2O_2 at a final conc. 10^{-4} M almost completely inhibited iron deposition in ferritin in agreement with the results in [14].) We conclude from these results that if O_2^- or O_2^{2-} is involved in iron oxidation, the intermediate involved is either tightly bound to the protein or else is inaccessible to the reagents used for its detection.

The initial velocity of the oxidation of Fe^{2+} catalysed by apoferritin was determined at 310 nm using a stopped-flow spectrophotometer for a range of iron concentrations from 0.63–50.9 mM. Figure 1 gives the result obtained when v was plotted against $[S]$, the concentration in ferrous iron added. The S-shaped curve can be linearised by plotting $1/v$ against $1/[S]^2$. The theoretical curve generated from the linearisation is also given in fig.1. These results can best be explained by assuming that the concentration of Fe^{2+} enters into the rate equation at least to the second power. This leads us to conclude that at least two iron atoms are bound at each active site for iron oxidation, in accord with the model that has recently been proposed [5–7]. This interpretation receives strong confirmation from the inhibition studies with Cr^{3+} (table 2). In a range of Cr^{3+} concentration from 10^{-4}–5×10^{-2} M

Table 2
Inhibition of ferritin formation by metal ions

Metal ion added	Final conc.	Inhibition ferritin formation	Amount of metal fixed (g atoms/18 500 daltons)
–	–	0	–
Ru^{3+}	10^{-3} M	0	–
Ru^{3+}[a]	10^{-3} M	0	–
Cr^{2+}	5×10^{-3} M	92	–
Co^{2+}	5×10^{-3} M	41	–
Cr^{3+}	10^{-4} M	83	0.54
Cr^{3+}	10^{-3} M	83	0.61
Cr^{3+}	5×10^{-3} M	83	0.49
Cr^{3+}	10^{-2} M	84	0.77
Cr^{3+}	5×10^{-2} M	82	0.62

[a] Ru^{3+} + ascorbic acid

After preincubation of apoferritin with different metal ions the percentage of inhibition of iron deposition from ferrous ammonium sulphate in sodium cacodylate–borate buffer, pH 5.8, was determined. In the case of Cr^{3+} the amount of metal bound to the protein was determined by atomic absorption spectroscopy

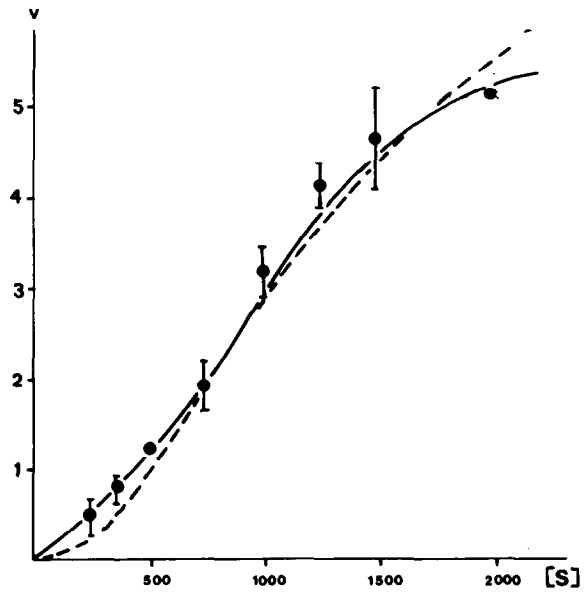

Fig.1. Initial velocity of ferritin formation as a function of iron concentration. The absorbance changes at 310 nm were measured in a Durrum stopped flow spectrophotometer as described in section 2. The initial velocity v is expressed in arbitrary units/s and the iron concentration $[S]$ as the number of iron atoms added/molecule of apoferritin. The experimental values are given by the filled circles, in some cases with error bars. The dashed curve is calculated from the linearisation of the experimental curve (see text).

a relatively constant value of 0.5 g atoms Cr^{3+} are bound/subunit, associated with a considerable loss of activity in Fe^{2+} oxidation. Thus we conclude that the catalytic site of apoferritin consists of two adjacent polypeptide chains which each bind an Fe^{2+} atom prior to the fixation of molecular oxygen and the oxidation of the iron, in accord with the model [5–7]. Of the other metals tested (table 2) Cr^{2+} gave 92% inhibition and Co^{2+} 59% inhibition at the concentrations employed. For these metals the amount of metal bound was not determined. Previous studies have established that apoferritin-catalysed iron oxidation is inhibited by Zn^{2+}, Ni^{2+}, Hg^{2+}, Cd^{2+}, Co^{2+}, Mg^{2+} and Cu^{2+} [2,13,15,16]. Indications that Tb^{3+} binding also results in reduction of availability of Fe^{2+} binding sites have also been reported [17]. In a recent crystallographic study [17] it was inferred that Tb^{3+} binds to Fe^{2+} binding sites thus inhibiting iron uptake. These Tb^{3+} sites are close to an axis of 2-fold symmetry. We have also observed (E. P. Pâques and R. R. C., unpublished observations) that Cr^{3+} binds close to a 2-fold symmetry axis of the apoferritin molecule. These results, together with the results of detection of intermediates, the rapid kinetic studies and the Cr^{3+} binding and inhibition reported above, support the two site model proposed [5–7].

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