

STOICHIOMETRY OF IRON OXIDATION BY APOFERRITIN

Gennaro MELINO, Simonetta STEFANINI, Emilia CHIANCONE and Eraldo ANTONINI
C.N.R. Center of Molecular Biology, Institute of Chemistry, Faculty of Medicine, University of Rome, 00185 Rome

and

Alessandro FINAZZI AGRÒ
Cattedra di Biochimica Applicata, University of Cagliari, 09100 Cagliari, Italy

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1. Introduction

Ferritin is the iron storage protein of animal organisms; each ferritin molecule can bind as many as 4500 iron atoms/450 000 g protein [1,2]. Iron is present as Fe^{3+} in a dense iron hydroxide core surrounded by a shell of protein chains (apoferritin). It is generally accepted that, while iron is stored in the ferric form, it is exchanged with the surrounding medium as ferrous iron [1,2]. Thus, *in vitro* studies show that, in the presence of air, the protein catalyses the oxidation of Fe^{2+} , which is then incorporated into the shell. In a previous paper [3] we have shown that in anaerobiosis the protein binds some Fe^{2+} and converts it to Fe^{3+} on admission of oxygen. In this respect the protein acts as an oxidoreductase; therefore, it seemed of interest to measure the stoichiometry of the reaction between oxygen and iron as catalysed by apoferritin.

The results indicate that 4 Fe^{2+} are oxidized per oxygen molecule and that the product of oxygen reduction is water. The presence of catalase does not affect the time course nor the stoichiometry of the reaction.

2. Materials and methods

Horse spleen ferritin was obtained from O.T.I. (Parma, Italy) and was purified by filtration on a Sephadex G-200 column. Apoferritin was prepared

by chemical reduction as in [4]. The purity of the preparations was checked in the analytical ultracentrifuge. The micellar iron concentration was calculated from A_{480} using $E_{1\text{cm}}^{1\%} 47$. H_2O_2 solutions were titrated before use with KMnO_4 . Other chemicals were of high purity grade and were used without further purification.

Manometric experiments were performed in a conventional Warburg apparatus using air as the gas phase. The protein samples were diluted with 0.15 M imidazole-HCl buffer, pH 6.4. The remaining Fe^{2+} was determined at the end of the incubation with the α - α' -dipyridil method [5], except that thioglycolic acid was omitted. Spectrophotometric experiments were carried out at 38°C in a Cary 14 apparatus. For the experiments in the absence of air, a Thunberg type cell, sealed with a rubber cap, was employed; reagents were added through the rubber cap with an air-tight syringe. Before and after each addition A_{480} was recorded. The formation of ferritin was checked in the ultracentrifuge or by absorption spectrophotometry after extensive dialysis of the incubation product against citrate containing buffer, in order to remove specifically bound iron. The ultracentrifuge experiments were carried out in a Spinco Model E ultracentrifuge at 36 000 or 40 000 rev/min and 10°C.

3. Results and discussion

The time course of the iron oxidation by oxygen and the stoichiometry of the reaction as determined

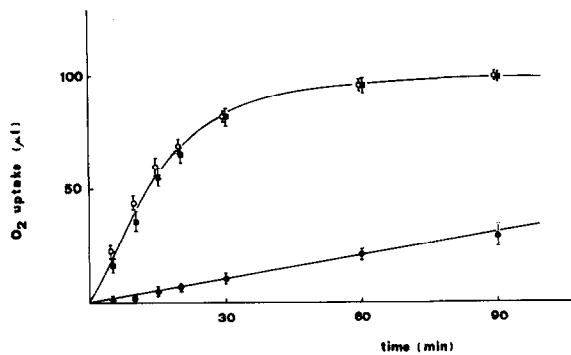


Fig.1. Oxygen uptake during iron incorporation into apoferritin. Apoferritin (2.6 μM) was incubated in 0.15 M imidazole-HCl buffer, pH 6.4, in the presence of 5.95 mM ferrous ammonium sulfate. (○), Apoferritin without catalase; (■), apoferritin with 0.13 μM catalase; (●), blank without apoferritin. The size of the vertical bars indicates the experimental error.

in the Warburg apparatus in the presence and absence of apoferritin are reported in fig.1 and table 1, respectively. Figure 1 clearly shows the well known increase in the rate of iron oxidation brought about

by apoferritin. Parallel spectrophotometric experiments showed that the increase in A_{480} occurred in the same time range of the oxygen uptake measured manometrically. From table 1 it is evident that 4 Fe^{2+} are oxidized/oxygen molecule in the presence of apoferritin, indicating that water is the reduction product. This stoichiometry is the same as that found with free Fe^{2+} and was obtained at Fe^{2+} /protein ratios up to 2500 atoms/apoferritin molecule; under these conditions all the iron is incorporated into apoferritin, as shown by the ultracentrifuge controls. At higher ratios the solutions became turbid during the course of the experiments and the amount of reconstituted ferritin, determined both from the absorption of the iron micelle and by ultracentrifugation, was lower than expected. This resulted in a higher oxygen uptake and a lower $\text{Fe}^{3+}/\text{O}_2$ ratio, probably due to side reactions with the protein of oxygen radicals produced during oxidation of unbound Fe^{2+} . Similar arguments would explain findings of oxygen radicals (O_2^- , H_2O_2) production during iron incorporation into apoferritin [1].

Table 1 and fig.1 also show that addition of catalase does not influence the extent nor the rate of iron

Table 1
Stoichiometry of iron incorporation

Apoferritin (μM)	Fe^{2+} added atoms/450 000 g protein	μmol	Catalase (μM)	O_2 uptake after 1 h (μmol)	Fe^{2+} oxidized (μmol)	Fe^{3+} formed O_2 uptake
0	—	17.9	0	4.2 ^a	17.8	4.2
0	—	17.9	0.13	5.0 ^a	17.8	3.6
1.04	1500	4.8	0	1.1	4.5	4.1
2.60	2300	17.9	0	4.4 ^b	16.2	3.7
2.60	2300	17.9	0.13	4.4 ^b	16.9	3.8
1.04	3060	10.0	0	2.8	10.0	3.5
1.90	3450	20.0 ^c	0	4.3	12.0	2.8
1.90	3450	20.0 ^c	0.13	4.5	11.1	2.5

^a determined at 180 min

^b determined at 90 min

^c a precipitate was present

0.15 M imidazole-HCl buffer, 38°C

incorporation by apoferritin, in agreement with [6,7]. This result seems to exclude the formation of hydrogen peroxide; in contrast, it suggests that apoferritin is a water-producing ferroxidase. This reaction requires a concerted mechanism of oxidation, where 4 Fe^{2+} are cooperatively oxidized without significant production of radical intermediates. An alternative possibility is that intermediates are formed but strongly bound to the protein where they are further reduced to the level of water.

In order to gain information on this point, H_2O_2 was used to oxidize iron in anaerobiosis in the presence or absence of protein; iron oxidation was followed from A_{480} . The measurements showed that in this case the protein does not influence the rate of iron oxidation, which occurs during the dead time of the experiment. Parallel ultracentrifuge runs showed that under these conditions no iron is incorporated into the protein shell and that the increase in absorbance was due to aspecific iron aggregates. On the basis of these results, if free H_2O_2 were formed during iron incorporation in the presence of air, large amounts of unbound Fe^{3+} would also be formed at all Fe/protein ratios and catalase would have affected the stoichiometry of the reaction. Hydrogen peroxide is therefore unable to oxidize the protein- Fe^{2+} complex which is

formed in anaerobiosis [3]. In other words, apoferritin is a good catalyst when oxygen or the thiosulfate-iodate system [4,8] are used as oxidants of Fe^{2+} , while it is unable to incorporate the metal when oxidation is brought about by H_2O_2 .

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