Vol. 116, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1983 Pages 244-250

THE INCORPORATION OF IRON INTO APOFERRITIN AS MEDIATED BY CERULOPLASMIN

Rodney F. Boyer* and Barbara E. Schori

Department of Chemistry, Hope College
Holland, Michigan 49423 USA

Received August 26, 1983

SUMMARY: Ceruloplasmin, a copper ferroxidase, promotes the incorporation of Fe(III) into the iron storage protein, apoferritin. The product formed is identical to ferritin as judged by polyacrylamide electrophoresis and iron/protein measurements. Of several proteins examined, only apoferritin accumulates the Fe(III) produced by ceruloplasmin. When ceruloplasmin was replaced by tyrosinase, which we have shown to have ferroxidase activity, no iron incorporation into apoferritin was observed. It is proposed that Fe(III) is transferred directly and specifically to apoferritin. These data support a more specific role for ceruloplasmin in iron metabolism than has previously been proposed.

The biological function of ferritin is to maintain iron in a soluble and metabolically accessible form. Ferritin performs this role by storing hydrated polymeric ferric oxide within a central cavity of the protein (1).

The mechanism of iron incorporation into the ferritin core has been extensively investigated, but, many questions remain. Synthetic ferritins were first prepared by the incubation of apoferritin with Fe(II) in the presence of O_2 (2-4). It was later discovered that apoferritin accelerates the rate of Fe(II) oxidation, in the presence of O_2 or KIO_3/ $Na_2S_2O_3$, with accompanying incorporation of FeOOH into the protein shell (5). Therefore, some investigators consider the role of apoferritin as catalytic and have even labeled it a 'ferroxidase' (6-9). There is no evidence that O_2 plays a direct role as the physiological electron acceptor, in fact, a recent investigation of the fate of

*To whom correspondence should be addressed.

0006-291X/83 $1.50

$^{18}O_2$ during ferritin iron incorporation revealed that only 3-4% of the oxygen atoms in the ferritin core was derived from O_2 (10).

In order to clarify the roles of O_2 and apoferritin in iron incorporation, we have studied the reaction of ceruloplasmin- and tyrosinase-generated Fe(III) with apoferritin. We find that ceruloplasmin, in the presence of Fe(II) and O_2, promotes the incorporation of iron into apoferritin.

MATERIALS AND METHODS

Human ceruloplasmin (EC 1.12.3.1) and mushroom tyrosinase (EC 1.10.3.1) were obtained from Sigma Chemical Co. and treated with Chelex-100 prior to use. Horse spleen apoferritin, obtained from Sigma, contained less than 10 g-atoms of iron per mole of protein. Ascorbate oxidase (curcurbita, EC 1.10.3.3) bovine serum albumin, γ-globulin, apotransferrin and ferrous ammonium sulfate were used as obtained from Sigma. Chelex-100, 200-400 mesh, was purchased from Bio-Rad Laboratories, Inc.

Rates of iron incorporation into apoferritin were estimated according to Collawn, et al. (11). The assays were carried out in 0.1 M HEPES buffer, pH 7.0, at 30°C. Reaction aliquots were removed at one minute intervals, treated with Chelex-100 and the absorbance at 310 nm measured on a Beckman DB spectrophotometer. All data points are the result of at least three measurements. Ferroxidase activities were determined according to Osaki, et al (12), except that the buffer was 0.1 M HEPES, pH 7.0. Ferritin iron was measured as described by Crichton, et al (13). Polyacrylamide gel electrophoresis was completed as previously described (11). The gels were stained for protein with Ponceau S and for iron with potassium ferrocyanide (9).

RESULTS

The rate of iron incorporation into apoferritin in the absence and presence of ceruloplasmin is shown in Figure 1. Clearly, ceruloplasmin enhances the initial rate of iron incorporation into apoferritin. The effect is greater with ceruloplasmin pretreated with Chelex-100 to remove nonenzymic copper and other trace metals.

The product was examined by polyacrylamide gel electrophoresis and shown to be identical to standard ferritin. It was also demonstrated by iron/protein measurements that the ferritin produced in the presence of ceruloplasmin contained more iron as indicated by the increase in A_{310}.

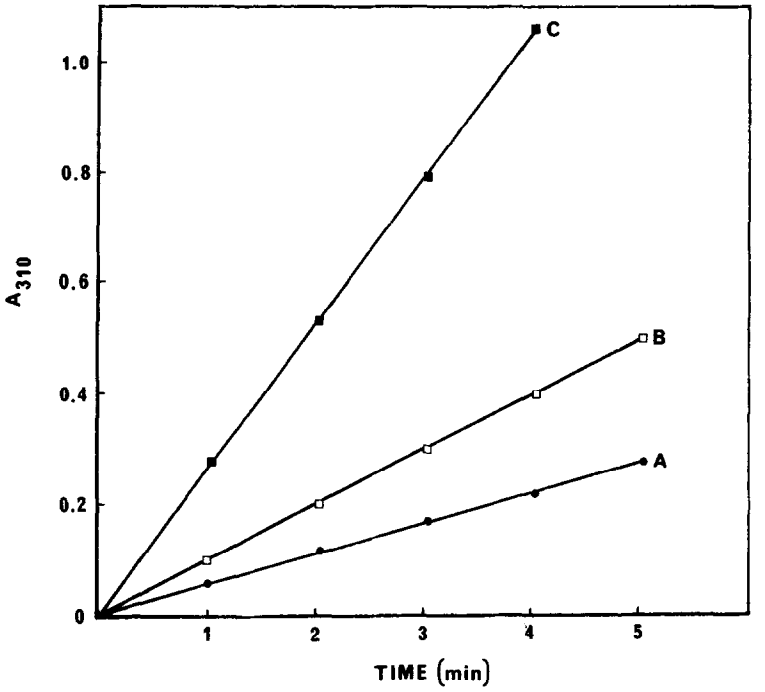
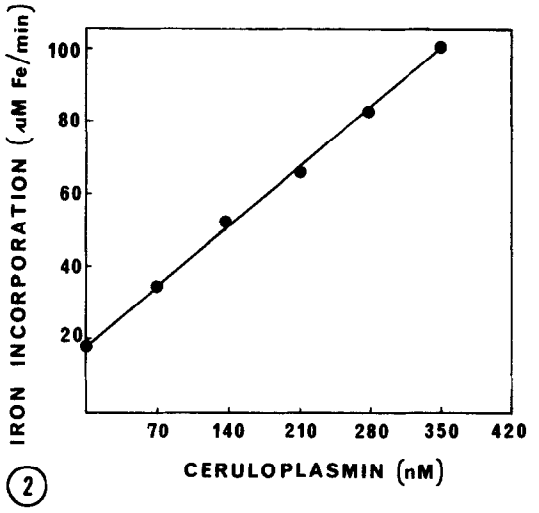


Figure 1. Incorporation of iron into apoferritin. Reaction mixtures contained ferrous ammonium sulfate (0.5 mM) and apoferritin (25 μg/ml) in 0.1 M HEPES, pH 7.0 and were run at 30°C. Curve A, no ceruloplasmin; Curve B, 280 nM ceruloplasmin; Curve C, 280 nM Chelex-treated ceruloplasmin.

The initial rate of iron incorporation into apoferritin is a linear function of the ceruloplasmin concentration (Figure 2). The plot does not go through the origin (0 nM ceruloplasmin) because apoferritin alone enhances the rate of iron accumulation. The initial rate of iron incorporation as a function of apoferritin concentration displays saturation as shown in Figure 3. The K_M and V_{max} are estimated to be 12 nM and 90 μM Fe/min, respectively. The influence of initial Fe(II) concentration on the rate of iron incorporation is shown in Figure 4. Concentrations of Fe(II) greater than 1 mM led to high rates of autooxidation and polymerization of Fe(III) outside the apoferritin. Table I summarizes other characteristics of the reaction system. Proteins other than apoferritin were added to the assay to test for nonspecific incorporation of Fe(III). A slow uptake of iron



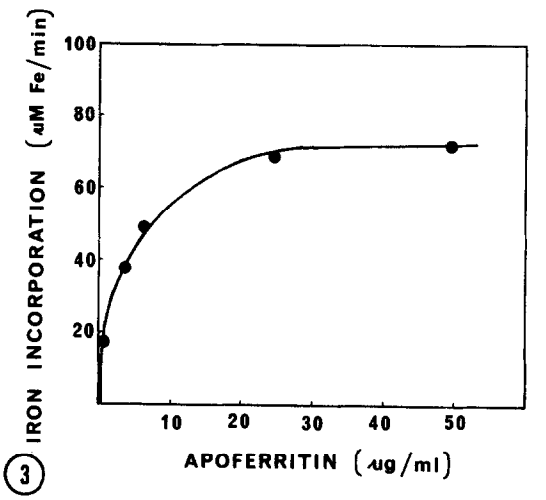


Figure 2. The rate of iron incorporation into apoferritin as a function of ceruloplasmin concentration. Reactions were conducted with apoferritin (25 µg/ml) and ferrous ammonium sulfate (0.5 mM) in HEPES at 30°C.

Figure 3. The rate of iron incorporation into apoferritin as a function of apoferritin concentration at 30°C. Reaction mixtures contained ceruloplasmin (280 nM) and ferrous ammonium sulfate (0.5 mM) in HEPES.

was noted for bovine serum albumin, but, this same rate occurs in the absence of ceruloplasmin. When ceruloplasmin was replaced by two other copper oxidases, mushroom tyrosinase or ascorbate

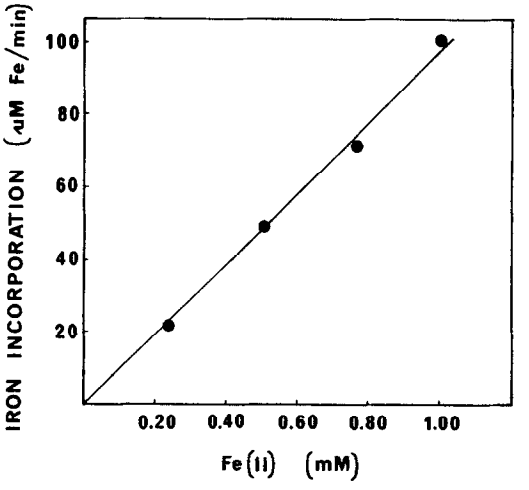


Figure 4. The rate of iron incorporation into apoferritin as a function of ferrous ammonium sulfate concentration at 30°C. Reactions conducted in HEPES contained 140 nM ceruloplasmin and 25 µg/ml apoferritin.

Table 1. Incorporation of iron into apoferritin as mediated by ceruloplasmin. The complete system contained ceruloplasmin (280 nM), apoferritin (25 μg/ml), and ferrous ammonium sulfate (0.5 mM) in HEPES. The observed change in absorbance at 310 nm for the complete system at 30°C was 0.18 per min. All other rates are expressed as percentages of this change.

System	Relative Activity (%)
Complete	100
- ceruloplasmin	39
- apoferritin	22
- Fe(II)	6
+ 25 μg/ml BSA, - apoferritin	28
+ 25 μg/ml γ-globulin, - apoferritin	28
+ 25 μg/ml BSA, - ceruloplasmin	11
- ceruloplasmin, - apoferritin	6
- O_2	0
+ 10 mM azide	67
+ 5 mM Cu(II)	6
+ 4 mM Zn(II)	39

oxidase, no enhancement of iron incorporation was observed (Table 2). Our results show comparable levels of ferroxidase activity for tyrosinase and ceruloplasmin, but, only ceruloplasmin stimulates iron deposition into ferritin. Ascorbate oxidase at several concentration levels was inactive in both assays.

Table 2. A comparison of reaction characteristics of three copper oxidases. Rates of iron incorporation into apoferritin were measured with 50 μg/ml apoferritin and 0.5 mM ferrous ammonium sulfate in HEPES at 30°C as described in Materials and Methods. Ferroxidase assays contained 30 μM ferrous ammonium sulfate and 55 μM apotransferrin in HEPES, pH 7.0.

Enzyme	Iron Incorporation into Apoferritin (μM Fe/min)	Ferroxidase Activity (μM Fe/min)
ceruloplasmin		
140 nM	70	37
280 nM	112	70
tyrosinase		
0 nM	27	0
112 nM	27	9
280 nM	27	23
560 nM	27	50
ascorbate oxidase		
0 nM	27	0
34 nM	27	0
150 nM	27	0
300 nM	27	0

DISCUSSION

The results strongly suggest that iron oxidation and incorporation into apoferritin are mediated by ceruloplasmin. A review of the results provides the following evidence to support a role for ceruloplasmin: (1) linearity of initial rate of iron incorporation vs ceruloplasmin concentration, (2) identification of ferritin product, (3) saturation curve for apoferritin, (4) of several proteins tested, only apoferritin showed iron incorporation, (5) iron incorporation was slowed by N_3^-, a ceruloplasmin inhibitor (12), (6) O_2 was required for iron oxidation and incorporation but is not itself incorporated, and (7) of three copper oxidases studied, only ceruloplasmin promoted iron oxidation and incorporation. The biochemical function of ceruloplasmin, a plasma protein, is still uncertain, but, it is thought to be involved in copper and iron transport (14).

We propose the following scheme for ceruloplasmin-mediated iron incorporation into apoferritin:

$$\text{E-Cu(II)} + \text{Fe(II)} \longrightarrow \text{Fe(II)-E-Cu(II)}$$

$$\text{Fe(II)-E-Cu(II)} \longrightarrow \text{Fe(III)-E-Cu(I)}$$

$$\text{Fe(III)-E-Cu(I)} + \text{apoferritin} \longrightarrow \text{ferritin-Fe(III)} + \text{E-Cu(I)}$$

$$4\text{E-Cu(I)} + O_2 + 4H^+ \longrightarrow 4\text{E-Cu(II)} + 2H_2O$$

Fe(II) oxidation is catalyzed by ceruloplasmin (E-Cu(I)/Cu(II)) which is recycled by O_2 (15). We envision a direct transfer of Fe(III) from ceruloplasmin to apoferritin. This is supported by the observation that, of the copper enzymes tested, only ceruloplasmin stimulates iron incorporation. Even though tyrosinase has measurable ferroxidase activity, it is not able to mediate Fe(III) transfer to apoferritin. Gel filtration and spectrophotometric experiments have not demonstrated complex formation between apoferritin and ceruloplasmin.

Our results indicate a specific interaction between ceruloplasmin and apoferritin, but, we cannot completely rule out the

possibility that Fe(III) is released to the medium by ceruloplasmin, bound to apoferritin, and then incorporated into the protein. Recent studies have demonstrated that Fe(III) binds to and is retained by apoferritin, but the rate of incorporation is slower then for Fe(II) (16,17).

This is, to our knowledge, the first report that mushroom tyrosinase has ferroxidase activity. Tyrosinase also catalyzes the oxidation of monophenols and diphenols (18). Characterization of the interesting ferroxidase activity of tyrosinase is in progress in our laboratory.

ACKNOWLEDGMENT: This research was supported by Grant No. 13781-B3-C, Petroleum Research Fund, administered by the American Chemical Society.

REFERENCES

1. Aisen, P. and Listovosky, I. (1980) Annu. Rev. Biochem. 49, 357-393.
2. Bielig, H.T. and Bayer, E. (1955) Naturwissenschaften 42, 125-126.
3. Loewus, M.W. and Fineberg, R.A. (1957) Biochim. Biophys. Acta 26, 441-443.
4. Pape, L., Multani, J.S., Stitt, C. and Saltman, P. (1968) Biochemistry 7, 606-612.
5. Treffry, A., Sowerby, J.M., and Harrison, P.M. (1979) FEBS Lett. 100, 33-36.
6. Macara, I.G., Hoy, T.G. and Harrison, P.M. (1972) Biochem. J. 126, 151-162.
7. Melino, G., Stefanini, S., Chiancone, E., Antonini, E. and Finazzi, A.A. (1978) FEBS Lett. 86, 136-138.
8. Niederer, W. (1970) Experientia 26, 218-220.
9. Bryce, C.F.A. and Crichton, R.R. (1973) Biochem. J. 133, 301-309.
10. Mayer, D.E., Rohrer, J.S., Schoeller, D.A. and Harris, D.C. (1983) Biochemistry 22, 876-880.
11. Collawn, J.F. Jr., Priest, D.G. and Fish, W.W. (1982) Anal. Biochem. 127, 105-111.
12. Osaki, S., Johnson, D.A. and Frieden, E. (1966) J. Biol. Chem. 241, 2746-2751.
13. Crichton, R.R., Roman, F. and Roland, F. (1980), J. Inorg. Biochem. 13, 305-316.
14. Frieden, E. (1971) in "Bioinorganic Chemistry", (Gould, R.F., ed.) American Chemical Society, 100, 292-321.
15. Osaki, S. and Walaas, O. (1967) J. Biol. Chem. 242, 2653-2657.
16. Treffry, A. and Harrison, P.M. (1979) Biochem. J. 181, 709-716.
17. Chasteen, N.D. and Theil, E.C. (1982) J. Biol. Chem. 257, 7672-7677.
18. Ullrich, V. and Duppel, W. (1975) in "The Enzymes" (Boyer, P.D., ed.) Academic Press, XIIB, 253-297.