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The Incorporation of Iron into Chicken Apoferritin in the Presence of Ceruloplasmin

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After assaying the appropriate conditions for the experiments, the oxidation of iron with incorporation into chicken apoferritin was studied in the presence of ceruloplasmin, analysing the roles of iron, apoferritin and ceruloplasmin. The results show that the process is hastened by both apoferritin and ceruloplasmin. The dependence of the rate with respect to iron, apoferritin and ceruloplasmin concentrations was in general linear in the studied range. However, for low concentrations of iron or apoferritin the behaviour deviated from the linearity, suggesting that significant changes can happen in the mechanism of iron incorporation into apoferritin when the ratio of iron to apoferritin varies, which is in accordance with previous works. Finally, some differences found in the influence of the species on the process, with respect to an earlier report, open the possibility of differences in the affinity for iron between avian and mammalian apoferritins.

Key words: Iron, Ferritin, Ceruloplasmin, Chicken.

Ceruloplasmin seems to perform an essential role in the organism while promoting, through its activity as a serum ferroxidase, the mobilization of iron, allowing its binding and transport all over the body, as transferrin (4, 11). On the other hand, recent works (BOYER, R. F. and SCHORI, B. E., personal communication, 1984) have dealt with this ferroxidase ability coupled with iron incorporation into apoferritin, the protein responsible for storing this metal as a stock for the organism. Those studies have shown that ceruloplasmin catalyzes the process of iron (II) oxidation and incorporation into horse spleen apoferritin.

In order to investigate this process in birds, and thus to know more about the fixation of iron by ferritin, we have studied iron incorporation into chicken apoferritin in the presence of ceruloplasmin, by analysing the influence exerted by presence, in different concentration, of ceruloplasmin, apoferritin and iron (II).

Materials and Methods

Obtaining of apoferritin. — Ferritin was isolated from chicken liver as described by LINDER and MUNRO (6), with slight modifications. Apoferritin was ob-

tained from it by reducing iron with thioglycolic acid and removing it by dialysis; briefly, this was done in the following way: dialysis, during 24 h, against 3 or 4 successive portions of a solution containing 0.1 M sodium acetate, 0.1 M thioglycolic acid, pH 4.4; dialysis, in a similar way, against a 0.1 M KCl solution; and extensive dialysis against distilled water.

The purity of the apoferritin preparation thus obtained was checked by polyacrylamide gel electrophoresis. The gels were stained for protein with Coomassie Blue and for iron with potasium ferrocyanide. Iron content was evaluated through the bathophenanthroline method (5), and showed to be less than 13 iron atoms per apoferritin molecule. If this content was in some case greater than this value, the dialysis procedure was repeated. Apoferritin concentration was measured according to LOWRY *et al.* (7).

Rate of iron oxidation. — The rate of iron oxidation and incorporation into apoferritin was estimated through the change with time in the absorbance at 310 nm. Samples were prepared by putting the desired amount of solutions of chicken apoferritin and human ceruloplasmin (Sigma) in 0.1 M Hepes-NaOH buffer (Hepes = 4-(2-hydroxy-ethyl)-1-piperazine ethanesulfonic acid). Then, a small amount (40 to 80 μl for a total volume of 1.6 ml) of freshly prepared 0.01 or 0.02 M ferrous ammonium sulfate solution was added, the sample quickly mixed, and the absorbance at 310 nm continuously recorded along the first five minutes.

A Varian Techtron 634 double beam spectrophotometer was used, with a graphic recorder. The buffer solution was employed as the blank. All the assays were carried out at a buffered pH and to constant temperature.

The variation of absorbance throughout the time fitted into a straight line rather well. The slope of that line (dA_{310}/dt) was employed as the measure of initial iron oxidation rate. In order to make more expressive the values of this rate, they were put in terms of the amount of iron oxidized per time and volume units, using an approximate value of 45 ml · cm^{-1} · $(mg\ Fe)^{-1}$ for the extinction coefficient at 310 nm of polynuclear ferric iron inside the ferritin molecule (1).

Results and Discussion

The UV-VIS absorption spectra of samples of chicken liver ferritin and apoferritin were recorded. Their patterns were found to be very similar to those reported for equine ferritin (9), characterized by a very strong peak in the UV region for ferritin, due to its ferric core, which masks the absorption of the protein at 280 nm. The great extension of this peak into the visible region allows to employ 310 nm or even higher wavelengths to monitor the contents of iron (III) of the chicken ferritin.

In fig. 1 the rate of iron oxidation in solution, in the absence of protein, in different conditions of pH, temperature and iron concentration can be seen. The great influence exerced by these three factors, especially pH is clear. This fact agrees with previous reports for mammalian ferritin (1, 8).

According to these results and in order to employ conditions in which the autoxidation and polymerization of iron outside the apoferritin molecule would not interfere with the measuring of iron oxidation with incorporation into the protein, the following conditions were chosen for the subsequent experiences: 30°C, pH = 6.5 and 0.5 mM Fe. The rate of iron autoxidation, observed in the absence of protein, was subtracted from the ones observed in the presence of apoferri-

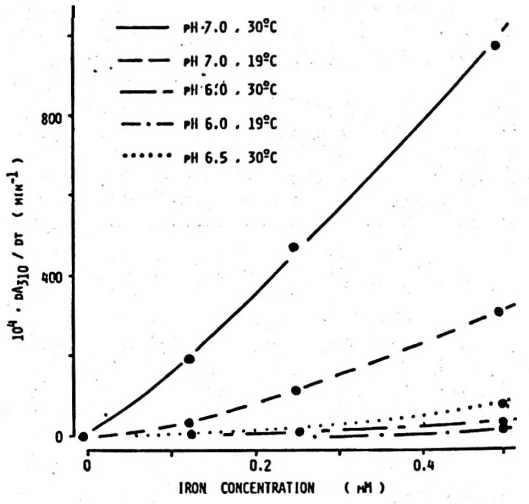

Fig. 1. *Uncatalyzed iron (II) oxidation rate at different conditions of pH, temperature and iron concentration.*

Solution contained ferrous ammonium sulphate in 0.1 M Hepes-NaOH buffer. The oxidation rate (dA_{310}/dt) was evaluated as described in «Materials and Methods», except that neither apoferritin nor ceruloplasmin were put in the sample. The mean of three independent measures is represented.

tin, in order to discount its contribution from the measured value.

The effect of ceruloplasmin on the rate of iron incorporation into apoferritin was investigated doing assays in the absence of ceruloplasmin and in the presence of several concentrations of it. In the conditions employed, ceruloplasmin accelerates iron incorporation into apoferritin, between 14 and 95% with respect to that observed in the absence of the former (fig. 2A). This, together with the linearity obtained when increasing ceruloplasmin concentration, indicates that this latter protein catalyzes the process, either just hastening iron (II) oxidation outside the apoferritin molecule, according to its well-known ferroxidase ability (4), or favouring also in some way the incorporation, as it has already been proposed (BOYER, R. F. and B. E. SCHOR, personal communication, 1984). Experiments were

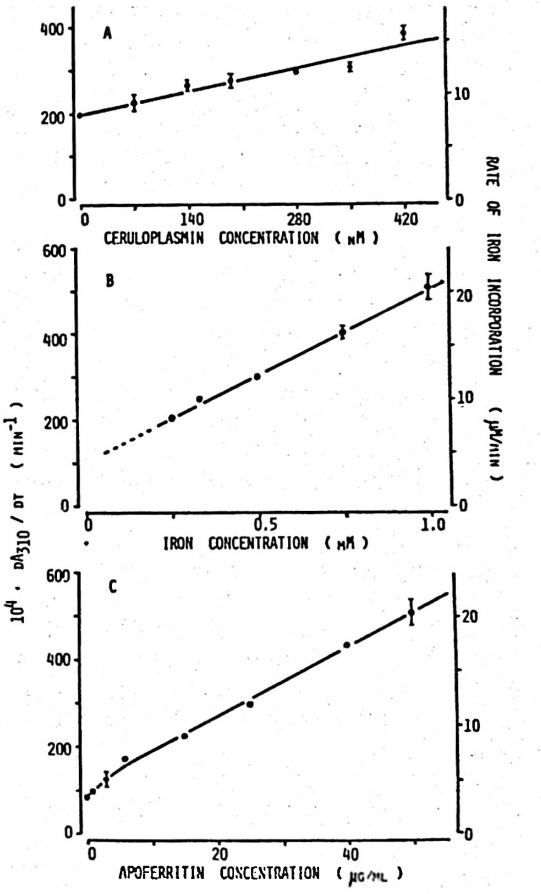

Fig. 2. *The rate of iron incorporation as a function of ceruloplasmin, iron and apoferritin concentrations.*

The assays were made in 0.1 M Hepes-NaOH buffer, pH 6.5, at a constant temperature of 30°C. The mean ± standard deviation (error bars) of at least three measures is represented. (A) The influence of ceruloplasmin concentration; samples contained 25 µg/ml apoferritin, 0.5 mM Fe (II) and variable concentrations of ceruloplasmin. (B) The influence of iron concentration; the reaction mixture contained 25 µg/ml apoferritin, 280 nM ceruloplasmin and the indicated concentrations of iron (II).The dashed line represents the extrapolation of the linear pattern outside the measured range (see the text). (C) The influence of apoferritin concentration: the samples contained 280 nM ceruloplasmin, 0.5 mM Fe (II) and increasing concentrations of apoferritin.

done varying iron concentration, from 0.25 to 1 mM (fig. 2B). The oxidation-incorporation rate thus obtained increases linearly with iron concentration, at least in the studied range. This accounts for the logical fact that the greater the availability of substratum is, the faster the incorporation can take place.

Fig. 2C shows the results obtained for the rate of iron incorporation in the presence of different concentrations of apoferritin. Clearly, apoferritin enhances the rate of the process, as previous studies had already pointed out in the absence of ceruloplasmin. As it can be seen, for apoferritin concentrations between 6 and 50 μg/ml the rate increases linearly. However, when smaller amounts of this protein are used the behaviour changes, deviating from the linearity. This performance suggests that when the concentration of apoferritin is less than 6 μg/ml, the addition of supplementary amounts diminishes its affinity for iron, while once exceeded that value the affinity is not affected by further additions.

Several works carried out before (in the absence of ceruloplasmin) showed that iron concentration, iron to apoferritin ratio, and pH all have a marked effect on both the behaviour of apoferritin for the incorporation and the stoichiometry of iron oxidation (2, 12). This fact points clearly to a change in the mechanism when varying any of these three parameters. It may be expected that in the presence of a fixed concentration of ceruloplasmin those factors will also influence the mechanism. Since when a constant iron concentration is kept and the concentration of apoferritin is changed (fig. 2C), and vice versa (fig. 2B), the iron/apoferritin ratio is changed, it may explain why the affinity of apoferritin for iron changes with apoferritin concentration, giving in fig. 2C linear and non-linear stretches. This idea may also explain the fact that the prolongation of the straight line obtained in fig. 2B does not approach the origin, enabling the mechanism, with iron concentrations below the ones employed to change and the rate of incorporation not to follow that line, but to draw near the origin more quickly. Apart from this, such a distance from the line to the origin points to the high affinity of apoferritin for iron, allowing relatively small amounts of iron to be oxidized and fixed with remarkable speed.

There are some related reports to this one, some employing ceruloplasmin while, most of them working in its absence. Some differences have been found in our results with respect to them, specifically in the pattern of influence of apoferritin and iron concentrations, and smaller rates of incorporation. Such differences can be due to the variety of measuring methods, and specifically to the different conditions used (pH, iron and apoferritin concentrations, iron/apoferritin ratio), which are established to exert a marked influence on the process. A significant divergence in the performance owing to intrinsic differences in the affinity for iron between mammalian ferritins, em-

Table I. *Effect of the absence of each component and the presence of bovine serum albumin (BSA, 25 μg/ml) on the oxidation/incorporation rate of iron.*

The assays were performed at 30°C in 0.1 M Hepes-NaOH buffer pH 6.5. Mean ± SD values of at least three measures are shown. The complete system was formed by 0.5 mM Fe (II), 280 nM ceruloplasmin and 25 μg/ml apoferritin.

	$10^4 \times \frac{dA_{310}}{dt}$ (min^{-1})	Relative activity (%)
Complete system	329 ± 1	100
— Fe (II)	0 ±	0
— ceruloplasmin	229 ± 5	70
— apoferritin	116 ± 6	35
— ceruloplasmin — apoferritin	29 ± 2	9
+ BSA, — ceruloplasmin	217 ± 3	66
+ BSA, —apoferritin	116 ± 4	35
+ BSA, — ceruloplasmin— apoferritin	20 ± 2	6

ployed in the aforementioned works, and the avian ferritin used in this one, is also possible.

In order to analyse the contribution of each species to the process of iron oxidation and incorporation, this process has been studied in the absence of each of them.

As it has been stated (table I and fig. 2A), the lack of ceruloplasmin reduces the rate of incorporation in one third (with respect to a concentration of 280 nM ceruloplasmin), which shows the catalytic effect of this protein on the process.

On the other hand, in the absence of apoferritin a residual activity remains, owing to the formation of iron (III) in solution as a result of the ferroxidase power of ceruloplasmin. This activity can be observed in table I and fig. 2C. The ability of ceruloplasmin to oxidize iron is known since 1960 (3), although it was studied thoroughly later and its biological importance would be established (4, 11).

The fact that iron oxidation in the absence of apoferritin tends to increase in the absorption at 310 nm, as it happens when iron enters into the ferritin molecule, agrees with the similarities found in several studies (10, 12) for the stoichiometry, mechanism and structure of the products formed by oxidation of iron in the absence and presence of apoferritin.

When studying the process without apoferritin or ceruloplasmin (table I), the uncatalyzed oxidation by the oxygen present in solution is observed, the magnitude of which had previously been shown to be adequately low in the work conditions chosen (see above).

Finally, the replacement of apoferritin, ceruloplasmin, or both by albumin shows that this latter protein does not affect the rate of oxidation-incorporation of iron (table I), and therefore the catalytic abilities of both proteins in this process are specific, not due merely to their proteic nature.

Resumen

Se estudia la oxidación del hierro con incorporación a la apoferritina de pollo en presencia de ceruloplasmina, analizando los papeles del hierro, la apoferritina y la ceruloplasmina. Los resultados muestran que el proceso es acelerado tanto por la apoferritina como por la ceruloplasmina. La dependencia de la velocidad con respecto a las concentraciones de hierro, apoferritina y ceruloplasmina fue en general lineal en el rango estudiado. Sin embargo, para bajas concentraciones de hierro o de apoferritina el comportamiento se desvió de la linealidad, lo que sugiere que pueden tener lugar cambios significativos en el mecanismo de incorporación de hierro a la apoferritina cuando varía la relación de hierro a apoferritina, lo cual está de acuerdo con trabajos anteriores. Algunas diferencias encontradas en la influencia de las especies sobre el proceso abren la posibilidad de diferencias en la afinidad por el hierro entre las apoferritinas de ave y de mamífero.

Palabras clave: Hierro, Ferritina, Ceruloplasmina, Pollo.

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