The Release of Iron from Horse Spleen Ferritin to 1,10-Phenanthroline

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The rate of release of iron to 1,10-phenanthroline from ferritin fractions of different iron contents has been studied. The experimental results could be interpreted by a simple hypothetical model of the shape of the hydrous ferric oxide micelle at different iron contents, and reasonable correlation obtained between the rate of release and the calculated micelle surface areas. Initial rates of release did not correlate significantly with protein concentration.

The iron-storage protein, ferritin, isolated from liver, spleen and other organs is a heterogeneous material consisting of apoferritin, a multisubunit protein in the form of a roughly spherical hollow shell, and molecules which contain a variable amount of hydrated ferric oxide of approximate composition $(\text{FeOOH})_8$ ($\text{FeO} : \text{OPO}_3\text{H}_2$) stored as a crystalline micelle within the protein shell (Granick, 1946; Farrant, 1954; Harrison, 1964). The iron-containing core, or micelle, measuring 6–7 nm (60–70 Å) diameter, can be seen in the electron microscope without staining or shadowing and consists of either a single crystallite or a small number of crystallites (Farrant, 1954; Haggis, 1965; Harrison & Hoy, 1970). Structures composed of close-packed layers of O atoms with interstitial Fe atoms have been proposed for these crystallites (Towe & Bradley, 1967; Harrison *et al.*, 1967). Layer structures have also been demonstrated by dark-field electron microscopy (Masover & Cowley, 1973).

The release of iron from native unfractionated ferritin to 1,10-phenanthroline has been studied *in vitro* by Jones & Johnston (1967), who found a first-order reaction with respect to ferritin iron concentration under conditions of excess of 1,10-phenanthroline. It has since been shown that the rate of iron uptake by ferritin *in vitro* depends on the amount of iron already present in the molecule and appears to be a function of the surface area of the crystalline micelle (Macara *et al.*, 1972).

An investigation of the hypothesis that the rate of release of iron from ferritin is also a function of iron content is now described. For these experiments horse spleen ferritin was fractionated by equilibrium density-gradient centrifugation in CsCl (Fischbach & Anderegg, 1965) and the rate of iron release studied for sets of fractions adjusted either to equal total iron concentration or to equal protein concentration. The results of these experiments show that the rate of iron mobilization can be correlated with the surface area of the crystalline micelles.

Materials and Methods

Horse spleen ferritin (twice crystallized, cadmium-free) was purchased from Pentex Co. Ltd., Kankakee, Ill., U.S.A. and fractionated by the following procedure into samples homogeneous in iron content. A preliminary centrifugation for 2 h at 39000 rev./min in a Beckman SW39 rotor was used to sediment material greater than 65S. (Full ferritin, 75S; apo-ferritin, 17.5S.) The supernatant was made up to a density of 1.52 g/cm³ with CsCl in 0.02 M-sodium phosphate buffer, pH 7.0, and centrifuged for 64 h at 35000 rev./min in a SW41 rotor at 20°C. This produced an almost linear gradient with densities from 1.30–1.75 g/cm³ in all tubes. The pellets from the preliminary centrifugation were further fractionated from a solution made up to a density of 1.70 g/cm³ in the same manner, which resulted in a gradient ranging from 1.63–1.75 g/cm³ after centrifuging for 96 h at 20000 rev./min in a SW25.1 rotor. CsCl and all other chemicals were of AnalaR grade (BDH Chemicals Ltd., Poole, Dorset, U.K.). All glassware was steeped in 5 M-HCl for several hours to remove metal contaminants and then rinsed in glass-distilled water. Ferric iron in the micelle was estimated by its absorption at 420 nm ($E_{1\%}^{1\text{cm}} = 100$). Protein was estimated by the method of Lowry *et al.* (1951) or from the iron concentration and a knowledge of the protein/iron ratio for fractions isolated at a particular buoyant density (Fischbach & Anderegg, 1965).

The ferrous iron complex with 1,10-phenanthroline was estimated by its absorbance at 510 nm ($\epsilon = 11\,500$).

Iron release

In the present experiments iron release was carried out in the presence of 0.05 M-sodium acetate buffer, pH 5.0, containing 0.05% (w/v) chlorbutol as a bacteriostatic agent and 63 mM-1,10-phenanthroline.

The temperature was maintained at 30°C and the samples were kept under constant illumination since photoreduction of Fe^{3+} to Fe^{2+} occurs in the complex of iron with 1,10-phenanthroline (Jones & Johnston, 1967). Six fractions were selected from the density gradients with the following protein/iron ratios: 45; 13.8; 10.4; 5.6; 3.5; 2.0; corresponding approximately to 170, 530, 720, 1330, 2160 and 3750 Fe atoms/protein molecule [4500 being the maximum number that one molecule can accommodate (Fischbach & Anderegg, 1965)]. As far as was possible with the amount of material available from the gradient each of the six fractions was made up in 0.05M-acetate buffer at pH 5.0 to the following iron concentrations: 0.11, 0.36, 0.48, 0.90, 1.43 and 2.50 mM. This series ensured that one set of fractions was available at constant protein concentration (0.28 mg/ml). The mobilization was initiated by adding 1,10-phenanthroline made up in the same buffer; the samples were then kept in sealed bottles except when being assayed at appropriate intervals.

Results

In each sample an initial amount of iron was complexed to the 1,10-phenanthroline within a few min. This phenomenon was also reported by Jones & Johnston (1967) and was attributed to the presence of iron loosely bound to the protein. The reaction was then followed for up to 1000h by which time most of the samples had released more than 90% of their iron.

None of the progress curves could be interpreted as simple zero, first- or second-order reactions with respect to iron concentration when displayed as the appropriate logarithmic plot. At each constant iron concentration the rate of release was always greatest for the molecules of lower iron content and decreased as the iron content increased. This is shown for one iron concentration (0.48 mM) in Fig. 1. As the protein concentration is an inverse function of iron content for any fixed iron concentration this might be an effect of the protein. However, the set of samples at constant protein concentration did not form a family of curves throughout the reaction (i.e. they crossed). The progress curves for the first 10h of these are shown in Fig. 2. In terms of ascending iron content the initial rates of release from fractions at constant protein are $170 < 530 < 720 < 1330 < 2160 < 3750$ which shows a maximum around a fraction which contains 1330 Fe atoms/apoferritin (i.e. approx. 1/3 full). Hence the reaction is not simply related to protein concentration.

Interpretation of results

It is clear that the observed results could not be correlated independently either with ferritin iron concentration or with protein concentration. The

Fig. 1. Release of iron from ferritin fractions of different iron content at constant initial iron concentration

The reaction was carried out with 63 mM-1,10-phenanthroline in 0.05M-sodium acetate buffer, pH 5.0. The initial total iron concentration for all fractions was 0.48 mM. The values on the curves represent the numbers of iron atoms/molecule in the various fractions.

Fig. 2. Release of iron from ferritin fractions of different iron content at constant protein concentration

Conditions were as described in Fig. 1 except that the fractions were at constant protein concentration (0.28 mg/ml of apoferritin). The values on the curves represent the numbers of iron atoms/molecule in the various fractions.

rate of iron release might, however, be a function of micelle surface area as appears to be the case with iron deposition (Macara *et al.*, 1972). In a computer model of iron uptake by ferritin, the complete iron-containing micelle was taken to be spherical and iron accumulation was treated as successive additions to the plane surface (PA) of a segment of a sphere (see Fig. 3). The spherical surface (SA) was assumed to be unavailable for growth since it was in contact with the protein shell. In the interpretation of the iron-mobilization experiments it was considered that iron might be released both from the plane surface (PA) and from a fraction of the curved surface (SA) via the channels through the protein by routes A or B respectively (Fig. 3). The available surface area could then be represented by $PA + \phi SA$, where ϕ could be varied between 0 and 1, since this fraction was unknown. Since the shape of the micelle after a certain amount of iron had been released was unpredictable, only the initial rates of release were calculated.

In summary, if the initial rate is I and K is a constant then $I = K \times \text{total available surface}$

$$\begin{aligned} &= K[\text{Protein}] \times (PA + \phi SA) \\ &= K[\text{Fe}] \frac{[\text{Protein}]}{[\text{Fe}]} \times (PA + \phi SA) \\ &= K[\text{Fe}] \times \frac{(PA + \phi SA)}{[\text{Fe}]/[\text{Protein}]} \end{aligned}$$

or $I = K^1 [\text{Fe}] \times \frac{\text{surface area of micelle}}{\text{volume of micelle}}$

For any set of fractions at constant iron concentration, I is directly proportional to the micelle surface

Fig. 3. Diagrammatic representation of a ferritin molecule containing half its full complement of iron

The protein forms a roughly spherical multi-subunit shell. The iron-containing micelle is represented as a segment of a sphere (in this case a hemisphere). Iron can be released from its plane surface (PA) by way of inter-subunit channels, such as A, or from the spherical surface (SA) by way of channel B.

area/volume ratio. In Fig. 4 this ratio is plotted against iron content as a band representing the limits in surface area available for release (the top line corresponds to $\phi = 1$ and the bottom line to $\phi = 0$). The calculated initial-velocity band is compared in Fig. 4 with the experimental values. These were derived from the progress curves for the first 10h of the reaction. The experimental initial rates shown in Fig. 4 are the average values for each fraction scaled together by a least-squares procedure. All fractions were normalized to a constant iron concentration. It can be seen that the experimental points fall within the limits set by the simple surface area model used. When all the initial rates were scaled to constant protein, however, a constant value was not obtained. The pooled results confirmed the findings for a single protein concentration shown in Fig. 2.

Discussion

It has been demonstrated that the rate of release of iron from ferritin to 1,10-phenanthroline is a more complicated process than that suggested by Jones & Johnston (1967). A reasonable explanation for the dependence on iron content of the rate of release would appear to be that iron is removed from the surface of the micelle just as iron seems to be

Fig. 4. Initial rate of iron release from ferritin fractions of different iron contents plotted as a function of iron content

Experimental values $\bar{x}$, where bars denote ± 1 s.d. The upper and lower curves represent the limits of the expected initial rates assuming iron release from a spherical micelle as shown in Fig. 3. Lower curve: release from plane surface (PA) only; upper curve: release from plane surface (PA) and spherical surface (SA). The difference between the two curves denotes the range of surface/volume ratios for the simple model.

added to this surface in the reverse process (Macara *et al.*, 1972, 1973).

The heterogeneity in iron content found in native ferritin and the micellar nature of its iron complex thus provides molecules of different affinities for iron. In comparison with a single homogeneous molecular species, this gives ferritin an extra versatility as an iron store.

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