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Uptake and Release of Ferritin Iron

SURFACE EFFECTS AND EXCHANGE WITHIN THE CRYSTALLINE CORE

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The uptake and subsequent release of iron by apoferritin and ferritin was studied by using labelled iron (^{59}Fe). The experimental results are consistent with predictions arising from a model system developed in the interpretation of previous experiments. In this model, uptake and release of ferritin iron is controlled by the available surface area of the small crystalline particles of hydrous ferric oxide found within the ferritin molecule. Evidence is also presented for the exchange of Fe^{3+} ions among the various cation sites within these crystallites.

Ferritin serves as an iron store by sequestering up to 4500 Fe(III) atoms/molecule as ferric oxyhydroxide-phosphate (Granick, 1946; Fischbach & Anderegg, 1965; Harrison & Hoy, 1973). This material occurs as small crystalline particles, or micelles, surrounded by a multi-subunit shell of protein (Farrant, 1954; Harrison, 1959; Haggis, 1965; Harrison *et al.*, 1967). The mechanism of iron uptake into ferritin *in vitro*, and probably also *in vivo*, involves the oxidation of Fe(II) (Beilig & Bayer, 1955; Loewus & Fineberg, 1957) and the incorporation of Fe(III) into a ferric oxyhydroxide lattice. The kinetics of ferritin formation has been studied by a number of research workers and evidence has been provided that apoferritin catalyses the iron oxidation–hydrolysis process (Neiderer, 1970; Macara *et al.*, 1972, 1973*a*,*b*; Bryce & Crichton, 1973).

A model for ferritin formation has been proposed by Macara *et al.* (1972, 1973*a*). The present experiments were designed to provide further tests for this model described below. Ferrous iron enters the apoferritin molecule through inter-subunit channels. It is bound at sites inside the protein, where it is oxidized to Fe(III). Nuclei of hydrous ferric oxide start to grow either at these sites or at some other 'nucleation' sites on the protein. Ferrous iron can be deposited and oxidized directly on the surface of these nuclei and, once stable nuclei are formed and growing, it is the crystallite surface area that controls the rate at which iron is accumulated by ferritin. Molecules may contain more than one nucleus, but once one of these has grown above a certain size it will compete successfully for further iron at the expense of other nuclei by virtue of its larger and increasing surface area.

The model of iron uptake implies that Fe^{3+} ions, together with oxide and hydroxide anions, are laid down layer by layer, as indeed would be expected in any crystallization process. A natural corollary of this hypothesis is that iron release occurs by a reverse process involving the stripping of the crystallite layer by layer from its surface. It can immediately be seen that the principle 'last-in-first out' (or 'first-in-last-out') should apply to the iron-uptake and iron-release process, and it is this principle that the present experiments have been designed to test. This was done by reconstituting ferritin molecules sequentially with labelled and unlabelled iron and then measuring the rates at which the two species are released on reduction with thioglycollate. Evidence that crystallite surface area is an important factor in the release process has been provided in a previous set of experiments in which the rate of iron release to 1,10-phenanthroline was studied as a function of the iron content of ferritin molecules (Hoy *et al.*, 1974). Ferritin fractions containing less than their full complement of iron are again used as the starting point in some of the present experiments. These can be separated from 'full ferritin' on a density gradient, by virtue of their differences in density (Fischbach & Anderegg, 1965).

Materials and Methods

Horse spleen ferritin (twice crystallized, cadmium-free) was purchased from Pentex Inc., Kankakee, Ill., U.S.A., and fractionated into samples, which were relatively homogeneous in iron content, by the following procedure. After dialysis against 0.02M-sodium phosphate buffer, pH7.0, a preliminary centrifugation for 2h at 39000rev./min in a Beckman Spinco SW39 rotor was used to sediment ferritin molecules of greater than 65S (full ferritin approx. 75S, apoferritin, 17.6S). The supernatant was made up to a density of 1.52g/ml with CsCl in 0.02M-sodium phosphate buffer, pH7.0, and centrifuged for 64h at

35000rev./min in a SW41 rotor at 20°C. This produced a linear gradient with densities from 1.30 to 1.75g/ml in all tubes. Samples, from the density-gradient centrifugation, with densities between 1.48 and 1.68g/ml were combined to give a fraction whose iron content/protein molecule was 700–1900 iron atoms. The mean of this range corresponds to ferritin molecules containing roughly one-third of the maximum iron content (4500 iron atoms). The CsCl and all other chemicals were of AnalaR grade (BDH Chemicals Ltd., Poole, Dorset, U.K.). Apoferritin was prepared from ferritin by dialysis against either 3% (w/v) sodium dithionite in 1M-sodium acetate buffer, pH4.8, or 0.1M-sodium acetate in 0.1M-thioglycollic acid, pH4.25. Ferric iron in the micelle was measured by its absorbance at 420nm ($E_{1cm}^{1\%Fe} = 100$) and ferrous iron was measured by the absorbance of its complex with 1,10-phenanthroline at 510nm ($\varepsilon = 11500$). ^{59}Fe was obtained as $FeCl_3$ from The Radiochemical Centre, Amersham, Bucks., U.K., equilibrated with a 5000-fold excess of $^{56}Fe^{2+}$ and counted for radioactivity in a Triton–toluene scintillation fluid (Turner, 1968) with a Packard model 3385 liquid-scintillation spectrometer.

Iron uptake

Incorporation of ferric iron into ferritin was achieved by adding ferrous ammonium sulphate and $Na_2S_2O_3$ to a solution of ferritin or apoferritin followed by a concentrated solution of KIO_3. The amount of $(NH_4)_2Fe(SO_4)_2$ required was calculated and $Na_2S_2O_3$ and KIO_3 were added in the proportions KIO_3:$(NH_4)_2Fe(SO_4)_2$:$Na_2S_2O_3$ (1:2:4, by weight). This provides a twofold excess of oxidant. The conditions used in the experiments were chosen so that all the added iron was incorporated into ferritin molecules as shown previously (Macara *et al.*, 1972). The reaction was virtually complete within 5min, but the mixture was allowed to stand for a further 20min before dialysis against 1% (w/v) $(NH_4)_2SO_4$ and then several changes of water. Approx. 1.6×10^4d.p.s. of ^{59}Fe was used per reconstitution.

Iron release

Iron was released from ferritin by adding sodium acetate and thioglycollic acid in equimolar proportions and leaving the solutions for 6h. The amounts of reducing agent were adjusted so as to give different percentage reduction in different samples at the end of this period. The amount of iron remaining was measured by the change in absorbance at 420nm. Separation of the Fe^{2+} ions from the protein solution was done by dialysing 2ml of this solution against 2ml of water overnight in a dialysis cell. Samples (0.5ml) of the diffusate were then removed for counting ^{59}Fe radioactivity, and 0.05ml samples for measurements of total iron released, measured as a complex with 1,10-phenanthroline. Pilot experiments indicated that 97% of the equilibrium value was obtained in the dialysis cells overnight. Recorded radioactivity (c.p.s.) was corrected for quenching by using a standard curve, and for natural decay. Corrected values (d.p.s.) were in the range 15–1500±5–1.5, background values being typically 0.5d.p.s. These errors were well within the reproducibility of the overall results (±5%).

Results

Release of iron from a fraction of native apoferritin into which further labelled iron had been incorporated

To the selected ferritin fraction (containing on average about 1300 iron atoms/molecule) sufficient labelled iron was added so that the iron content of the average molecule was approximately doubled. Since no molecules were more than half full of iron in the starting fraction, it was expected that very few molecules would be completely filled in the product. This was confirmed by analytical ultracentrifugation in parallel iron-incorporation studies with unlabelled iron. Subsequent reductions of the fractions enriched with labelled iron were carried out by starting (*a*) 4h after incorporation of labelled iron and (*b*) 72h after incorporation, the sample having stood at room temperature. The results are shown in Fig. 1, in which the percentage of ^{59}Fe released is plotted against the percentage of total iron released. Experiment (*a*) showed that, as predicted, radioactively labelled iron released initially accounts for virtually all of the iron released, but the proportion of ^{59}Fe of the total released subsequently declined. In experiment (*b*), however, labelled and unlabelled iron were released at approximately the same rate.

Release of iron from doubly reconstituted ferritin

Starting from apoferritin two doubly reconstituted ferritins were made in which the iron was added in two separate amounts. In one case the first iron uptake was made with labelled iron (L) and the second with unlabelled iron (U). In the other case the order of addition of labelled and unlabelled iron was reversed. These will be referred to as LU and UL respectively. Reductions were carried out after standing at 0°C for (*a*) 4h and (*b*) 72h periods after the second iron incorporation. The results are shown in Fig. 2. Once again in (*a*) ^{59}Fe is released first from the UL system, whereas from the LU system it is released after ^{56}Fe. When there has been a period of 72h before reduction as in (*b*), ^{59}Fe is mobilized at nearly the same rate as ^{56}Fe.

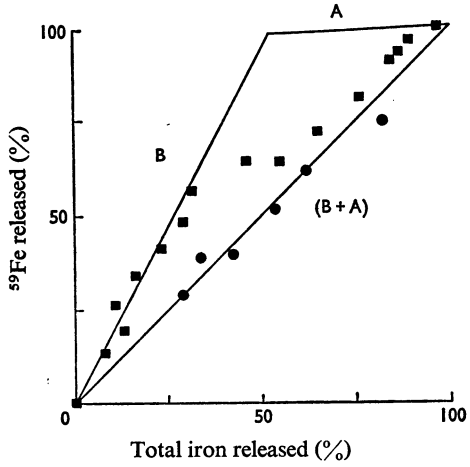

Fig. 1. *Release of labelled iron as a function of total iron from a native ferritin fraction to which an equal amount of labelled iron has been added*

In Fig. 3, A would represent the native micelle and B the added labelled iron. ■, Release initiated 4h after incorporation of labelled iron; ●, release initiated 72h after incorporation of labelled iron. Lines B and A represent predicted release of labelled iron from compartments B and A of the segment shown in Fig. 3(*a*), assuming that release is always from the plane surface of the segment and there is no mixing of iron between compartment A and B. Line (B+A) represents release of labelled iron from the segment assuming that the label has become uniformly distributed between the two compartments before release.

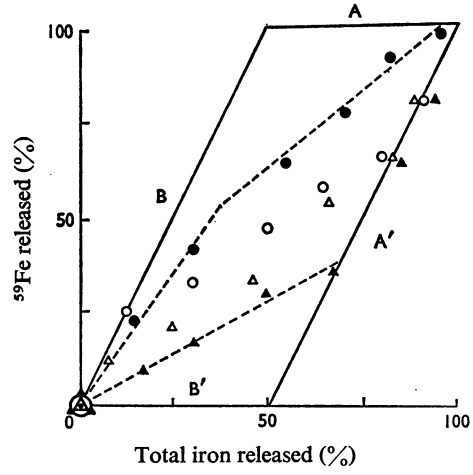

Fig. 2. *Release of labelled iron as a function of total iron from doubly reconstituted ferritins containing equal amounts of labelled and unlabelled iron, added consecutively*

Circles represent molecules with unlabelled iron in compartment A (added first) and label in compartment B (added second) (see Fig. 3). Triangles represent molecules with label in compartment A and unlabelled iron in compartment B (see Fig. 3). ●, ▲, Release initiated 4h after second iron incorporation; ○, △, release initiated 72h after second iron incorporation. Lines B and A represent release of labelled iron from compartment B and A, where B contains label and A unlabelled iron. Lines B′ and A′ represent release of labelled iron from compartments B and A, where B contains unlabelled and A labelled iron. Line (B+A) is omitted for clarity.

Interpretation of results

Previous experiments on the kinetics of iron uptake by ferritin (Macara *et al.*, 1972, 1973*a*) and the release of iron from ferritin (Hoy *et al.*, 1974) have been interpreted in terms of the crystal growth model described in the introduction. A computer simulation based on an idealized picture of crystal growth inside a spherical molecule was found to account reasonably well for the types of progress curves obtained under a variety of conditions. Such a model also provides an explanation for the results of the experiments reported here. It was assumed in the simple model that the hydrous ferric oxide micelle in ferritin can be represented as the segment of a sphere and that growth of this micelle occurs by addition to the plane surface of this segment (see Fig. 3*a*).

In the experiments described above the molecules contain an approximately equal amount of labelled and unlabelled iron at the end of the reconstitution. This can be represented as present in two compartments, A and B, with labelled and unlabelled iron as open and full circles. If iron is removed only from the plane surface of the segment in an exact reversal of reconstitution, then removal of iron from compartment B will precede removal of iron from compartment A, so that if

$$K=\frac{\%\text{ of Fe released from B}}{\%\text{ of Fe released from A+B}}$$

and, if the amounts of iron in compartments A and B are equal, then K will have a value of 2 until all the iron in B is removed and then a value of 0 until all that in A is removed. Similarly, if

$$K'=\frac{\%\text{ of Fe released from A}}{\%\text{ of Fe released from A+B}}$$

then $K'=0$ until all in B is removed, when it becomes equal to 2 until all in A is removed. In the experiments described above percentage release from A+B equals release of total iron where compartments A and B may contain unlabelled (U) and labelled iron (L) or vice versa as appropriate. Examination of Fig. 1 (the experiment in which compartment A represents the micelle of a native fraction and labelled iron was added into compartment B) shows that reductions starting 4h after incorporation into

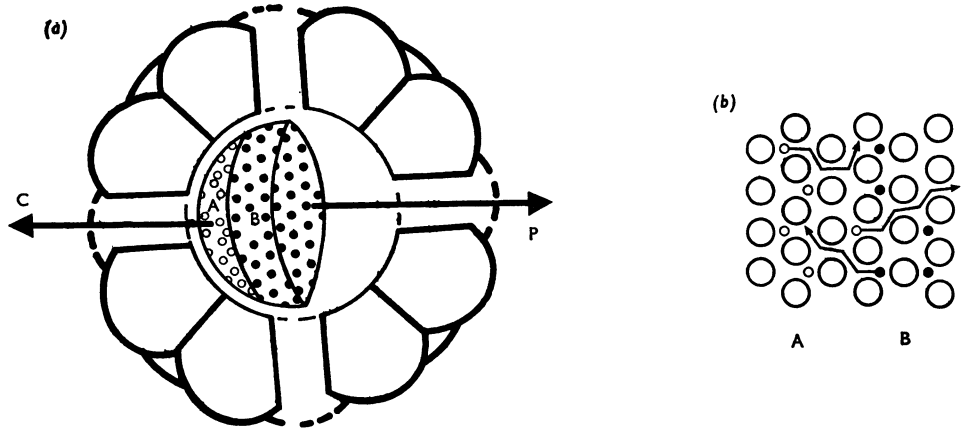

Fig. 3. *Diagrammatic representation of a ferritin molecule seen in cross-section*

(*a*) The molecule consists of a multi-subunit shell with inter-subunit channels, through which iron can enter or leave (e.g. by routes P or C), surrounding an inner core of hydrous ferric oxide. The core is shown here as a hemisphere representing a molecule containing half of its full complement of iron. The hemisphere is divided into two compartments, A and B, which, in the experiments described in the text, may contain either labelled or unlabelled Fe^{3+}. In the simple model iron is added to or released from the molecule only at the plane surface of the segment. Deviations from the simple model found experimentally may be due to some loss of iron from the curved surface (route C). (*b*) Schematic, enlarged drawing of a portion of the core, showing the boundary between compartments A and B. The core structure consists of layers of close-packed oxygen atoms (large open circles) with interstitial Fe^{3+} ions (small circles). Not all the interstitial sites are occupied by Fe^{3+}. Compartments A and B can contain either unlabelled or labelled iron, represented as open and full circles. A may contain labelled iron and B unlabelled iron (or vice versa in different experiments). Diffusion of Fe^{3+} occurs across the boundary between A and B (arrows) so that eventually the labelled iron is mixed throughout the entire segment.

compartment B give releases approximating to the theoretical model in the initial stages, although departure from the model occurs as more iron is removed. A possible explanation for this effect is that iron does not leave the micelle exclusively from the plane surface (route P, Fig. 3*a*). If some iron also leaves by route C (as suggested by Hoy *et al.*, 1974), another surface is formed on the crystallite and an increasing amount of the iron in compartment A will be removed before all from compartment B is released. This would result in K decreasing from the value of 2 as observed. In the double reconstitution experiments in which U was followed by L or L by U, release does not strictly follow the simple model even in the initial stages. A 'last-on-first-off' tendency is, nevertheless, observed. Departure from the idealized model may be the result of heterogeneity of iron contents in the population of molecules produced after the first reconstitution.

In the experiments in which release of iron was delayed until 72h after incorporation, the theoretical two-compartment model is no longer even approximately obeyed. A probable explanation for this is that ^{59}Fe and ^{56}Fe gradually mix by a process of self-diffusion across the boundary between compartments A and B as shown in Fig. 3(*b*). Such a process is known to occur in other crystal lattices (Zhdanov, 1965). The atomic structure of the ferritin micelle consists of layers of hexagonally packed oxygen atoms with iron atoms in interstitial positions (Towe & Bradley, 1967; Harrison *et al.*, 1967). Such an arrangement contains both octahedral and tetrahedral sites, only one-sixth of which are occupied by Fe^{3+} ions. Although it is not certain that both types of site are equally available to Fe^{3+} in the ferritin micelle, the structure is a relatively open one and diffusion can easily take place as is indicated diagrammatically in Fig. 3(*b*). The linear release plot shown in Fig. 1 indicates that, after 72h at 20°C, the Fe^{3+} ions originally in compartment B and A have completely mixed. Since diffusion in crystals is an exponential function of temperature it is easy to see why the samples kept at 20°C reach equilibrium faster than those kept at 0°C (shown in Fig. 2).

Discussion

Ferritin iron atoms are both structurally and functionally heterogeneous in the sense that they occupy either surface or interior sites in the micelle structure,

and only those atoms that happen to be at the surface at any given time are immediately available for release (Hoy *et al.*, 1974). Exchange between the sites appears to occur fairly freely over a period of time. The time taken for added ^{59}Fe to mix completely with ^{56}Fe already present in the molecule must presumably also depend on the size of the pre-existing micelle. This was kept constant (on average) in the experiments described above and the same amount of labelled iron was added in each case. Ferritin is more heterogeneous *in vivo*. In the present experiments the rate of diffusion was slow relative to the rate of release. Diffusion is dependent on temperature and equilibrium would be expected to be reached more rapidly at 37°C than at 20°C or at 0°C.

In a study on storage iron in the pregnant rat, Wyllie & Kaufman (1971) found that on day 20 of gestation the amount of ^{59}Fe in liver ferritin was lower 24h after injection than at 2h. They concluded that 'iron newly-incorporated into ferritin in late pregnancy, is withdrawn for utilization shortly after'. This might be a consequence of the 'last-in-first-out' principle described here, but in view of the exchange observed *in vitro*, the explanation may be that newly added ^{59}Fe is taken up by molecules of relatively low iron content and it is these molecules that also give up their iron most rapidly (Hoy *et al.*, 1974).

Although the model used in the interpretation of the above experiments is oversimplified [for example single micelles have been assumed, whereas it is likely that some molecules contain more than one crystallite (Haggis, 1965; Crewe & Wall, 1970)] the reasonably good agreement between the experimental results and the predictions of the model provides further verification of a stepwise growth mechanism. The exchange process, which appears to have occurred, itself implies that new iron is added to existing crystallites rather than going to form independent crystallites elsewhere inside the molecule. It also provides additional evidence that the added iron is incorporated inside ferritin, since exchange is unlikely to have occurred if the micelles had formed outside the ferritin molecules.

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