The Formation of Ferritin from Apoferritin

KINETICS AND MECHANISM OF IRON UPTAKE

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(Received 13 August 1971)

Ferritin has a high capacity as an iron store, incorporating some 4500 iron atoms as a microcrystalline ferric oxide hydrate. Starting from apoferritin, or ferritin of low iron content, Fe^{2+} and an oxidizing agent, the uptake of iron can be recorded spectrophotometrically. Progress curves were obtained and the reconstituted ferritin was shown by several physical methods to be similar to natural ferritin. The progress curves of iron uptake by apoferritin are sigmoidal; those for ferritins of low iron content are hyperbolic. The rate of iron uptake is dependent on the amount of iron already present in the molecule. The distribution of iron contents among reconstituted ferritin molecules is inhomogeneous. These findings are interpreted in terms of a crystal growth model. The surface area of the crystallites forming inside the protein increases until the molecule is half full, and then declines. This surface controls the rate at which new material is deposited. The experimental results can best be accounted for by a two-stage mechanism, an initial slow 'nucleation' stage, which is apparently zero order with respect to $[\text{Fe}^{2+}]$, followed by a more rapid 'growth' stage. The rate of Fe^{2+} oxidation is increased in the presence of apoferritin as compared with controls. Ferritin can therefore be regarded as an enzyme to which the product remains firmly attached. The protein appears to increase the rate of 'nucleation'. The apparent zero order of this stage suggests the presence of binding sites on the protein, which are saturated with respect to Fe^{2+} . These sites are presumed also to be oxidation sites. The oxidation and subsequent formation of the ferric oxide hydrate may proceed according to one of three alternative models.

Ferritin is well suited to its intracellular role as an iron-storage compound. The protein component, apoferritin, is composed of 20–24 subunits, which form a hollow sphere of internal diameter about 70 Å and total molecular weight approx. 450000 (Harrison, 1964). Within this shell a variable number of iron atoms up to about 4500 may be accommodated as a microcrystalline core, or micelle, of ferric oxide hydrate of approximate composition $(\text{FeOOH})_8$ ($\text{FeO}:\text{OPO}_3\text{H}_2$) (Granick, 1946; Haggis, 1965). The number, size and shape of the hydrous ferric oxide crystallites are variable within the limits imposed by the cavity in the protein molecule. Most molecules contain a single crystallite or a small number of crystallites, and these can be viewed directly in the electron microscope without staining or shadowing (Farrant, 1954; Haggis, 1965; Harrison & Hoy, 1970). The presence of the ferric oxide particles gives ferritin its characteristic red–brown colour (Granick, 1946). Owing to its location inside the protein molecule, the electrophoretic mobility of ferritin is unaffected by its iron content (Mazur & Shorr, 1950). The multi-subunit structure of the protein is an important feature of the molecule, since it allows access to the core by way of inter-subunit channels.

Ferritin iron can be removed by the action of

reducing agents, such as sodium dithionite, leaving apoferritin (Granick & Michaelis, 1943). Ferritin can be reconstituted by the addition to apoferritin of ferrous iron plus an oxidizing agent (Bielig & Bayer, 1955; Loewus & Fineberg, 1957; Harrison *et al.*, 1967), or of ferric iron plus a reducing agent in the presence of molecular oxygen (Loewus & Fineberg, 1957). A preliminary study has shown that provided iron is not added in excess of the apoferritin protein's capacity and the solubility product (10^{-14}) of $\text{Fe}(\text{OH})_2$ is not exceeded, all the added iron can be taken up by the protein. The protein influences both the rate of oxidation of the ferrous iron and the atomic structure of the mineral produced in its interior (Harrison *et al.*, 1967). Both natural and reconstituted ferritin cores resemble the hydrolysis products formed by neutralizing or heating a solution of a ferric salt (Towe & Bradley, 1967), whereas oxidation of ferrous iron at neutral or slightly alkaline pH in the absence of apoferritin gives γ - or α - FeOOH . The atomic structure of the reconstituted ferritin iron core is unaffected by altering the oxidizing agent used (Harrison *et al.*, 1967).

Niederer (1970) has studied the uptake of iron by apoferritin by measuring the rate of loss of ferrous iron and has demonstrated a marked catalysis of the

oxidation rate by apoferritin. He has postulated an active site on the protein which may act as a primary electron acceptor in the oxidation of the ferrous iron. Because of its relevance to the role of ferritin in iron storage the present study was initiated to explore further the kinetics of ferritin reconstitution. The absorption of the micelle at 420nm was used as a direct, sensitive and continuous means of following the reaction in preference to a chemical assay of loss of substrate. Although apoferritin has some enzyme-like properties, the reaction it catalyses is unusual in that the product remains firmly bound to the protein (giving ferritin). Our results show that the rate of reaction depends in a complex way not only on the protein but also on the amount of iron already taken up by the molecule.

Materials and Methods

Horse spleen ferritin (twice crystallized, cadmium-free) was purchased from Pentex Co., Kankakee, Ill., U.S.A., and was reduced to apoferritin by dialysis against 3% (w/v) sodium dithionite, in 1M-sodium acetate buffer, pH4.8, and stored as a precipitate under 30% (w/v) $(\text{NH}_4)_2\text{SO}_4$. Native apoferritin was isolated from ferritin by differential centrifugation (2h at 260000g).

Crystallized bovine plasma albumin was purchased from Armour Pharmaceutical Co. (Eastbourne, Sussex, U.K.) and all other reagents were of AnalaR grade (BDH Chemicals Ltd., Poole, Dorset, U.K.).

All glassware was steeped for several hours in 5M-HCl to remove metal contaminants and rinsed thoroughly in glass-distilled water.

Ferrous iron was determined as its complex with 2,2'-bipyridyl at 520nm.

Protein was determined either by its absorption at 280nm ($E_{1\text{cm}}^{1\%} = 9.0$), or by the method of Lowry *et al.* (1951).

Iron uptake

In the present experiments oxidation was carried out with a solution containing potassium iodate (0.05M) and sodium thiosulphate (0.2M) adjusted to buffer pH. Oxidation with this system was chosen in preference to molecular oxygen, because a large excess of oxidizer could readily be used. The rate of uptake of iron by apoferritin was followed either by measuring the absorption of the ferric oxide hydrate micelle at 420nm ($E_{1\text{cm}}^{1\%} = 100$) in quartz 1cm path-length cells, by using a Cary 14 or a Beckman DB recording spectrophotometer, or, in some experiments, by measuring the rate of loss of ferrous iron (Niederer, 1970).

Kinetic parameters were varied singly. The reaction was started by the addition of ferrous ammonium sulphate to a mixture of apoferritin (0.25–3.0mg/ml) and a large excess of oxidizer,

usually in imidazole buffer (8–130mM), made up to a final volume of 2.65ml. The amounts of iron added gave initial concentrations of ferrous iron in the range 0.1–5.0mM. The temperature was kept constant at 25°C. A few experiments were carried out in other buffer systems, as reported in the Results section, and some with bovine plasma albumin instead of apoferritin. Peak velocities (see below) were estimated by drawing tangents.

Analytical ultracentrifugation

The reconstituted ferritin samples, which varied with respect to both iron content and protein concentration, were dialysed exhaustively against glass-distilled water and concentrated by ultrafiltration to a final volume of about 1ml. They were then dialysed against 50mM-tris-HCl buffer, pH7.4, and adjusted by dilution to identical protein concentrations. The samples were analysed in a Beckman model E analytical ultracentrifuge at 34000rev./min in a cell with a double-sector centre-piece and schlieren optics.

Starch-gel electrophoresis

Electrophoresis was carried out in starch gels in the Shandon apparatus at a constant current of 20mA by using the discontinuous buffer system of Poulik (1957). The sliced gels were stained for iron with 0.3% potassium ferrocyanide in 3% (w/v) trichloroacetic acid, or, for protein, with Amido Black.

X-ray diffraction

Reconstituted ferritin samples made with iron added to give an iron/protein ratio approaching that found in natural ferritin were dialysed against water and crystallized by slow evaporation from 1% CdSO_4 . Single crystals were mounted wet in thin-walled quartz capillary tubes and sealed. X-ray photographs were taken on a precession camera by using either $\text{Cu } K_\alpha$ or $\text{Mo } K_\alpha$ radiation obtained from a Hilger microfocus generator (35kV, 2.8mA).

Electron microscopy

Reconstituted ferritin samples were kindly examined by Dr. G. H. Haggis in an EMI 6B electron microscope without staining or shadowing.

Results

Comparison of reconstituted with natural ferritin

That iron is taken up by apoferritin to give a ferritin-like product was shown in several ways. Since all detectable iron in reconstituted samples migrated with the protein on gel electrophoresis, it could be concluded that the iron was situated inside the protein, as in native ferritin, and did not affect

EXPLANATION OF PLATE I

Ultracentrifuge schlieren patterns of (a) native horse spleen ferritin and (b) and (c) reconstituted ferritins. Pictures were taken at 16 min after reaching a speed of (a) 33450 rev./min, (b) and (c) 34000 rev./min. The slow sedimentation from right to left peak is iron-free apoferritin and the broad, faster peak or shoulder corresponds to ferritin molecules with a range of iron contents. The average number of iron atoms per molecule in each sample is approx. (a) 3000 (b) 2000 (c) 600.

EXPLANATION OF PLATE 2

X-ray-diffraction patterns of native and reconstituted ferritins. (a) and (b), low-angle diffuse patterns obtained from stationary single crystals with X-ray beam parallel to [100] axis of octahedral crystal: (a) native and (b) reconstituted ferritin. (c) High-angle powder patterns given by stationary single crystals showing powder rings due to ferric oxide hydrate. Left: native, right: reconstituted ferritin. Spacing of outermost ring is 0.147 nm (1.47 Å).

its surface charge. Electron microscopy showed the presence of electron-dense micelles, similar in size and appearance to those of native ferritin. When relatively large amounts of iron were added, two major components were detected in the analytical ultracentrifuge, a colourless 18S peak identifiable as apoferritin and a coloured faster-moving peak (about 60S). When small amounts of iron were added the iron-containing component appeared simply as a shoulder on the apoferritin peak. Successive additions of iron produced a progressive increase in the average sedimentation coefficient of the iron-containing components and a decrease in the relative amount of apoferritin. Schlieren patterns of reconstituted and natural ferritin samples are shown in Plate 1.

X-ray-diffraction patterns of reconstituted ferritin single crystals showed on the one hand that the protein configuration and its ability to crystallize remained unchanged as a result of the reconstitution procedure, and on the other hand that the newly formed iron component was comparable in several respects to native ferritin. Samples that gave ultracentrifuge schlieren patterns similar to that in Plate 1(b), gave low-angle diffuse patterns typical of relatively full ferritin molecules (see Plates 2a and 2b) and unlike those of ferritin fractions of low iron content (Fischbach *et al.*, 1969). These patterns suggest that the average shape of the ferritin iron cores in the crystals is determined by the protein. High-angle diffraction with Mo K_α radiation of a large stationary single crystal gave, like natural ferritin, both sharp, discrete reflexions, due to the protein crystal lattice, and broad weak powder lines, due to the atomic structure of the iron cores (Plate 2c). These patterns not only confirm the conclusion, previously based on electron diffraction patterns, that the atomic arrangement within the iron cores of ferritin and its reconstituted counterpart are the same, but also show that, as in native ferritin, this arrangement is not specifically related to the structure of the protein (Fischbach *et al.*, 1969).

Kinetics of iron uptake by apoferritin

Progress curve. The progress curve of iron uptake by apoferritin obtained by following the absorption of the hydrated ferric oxide at 420nm in a recording spectrophotometer was found to be sigmoidal, as shown in Fig. 1. Comparable results were obtained with apoferritin whether isolated from native ferritin by differential centrifugation or prepared by reduction with sodium dithionite. Similar curves were also obtained (Fig. 1) by measurement of the loss of ferrous iron, but in this case a higher buffer concentration was found to be desirable since this decreased the reaction rate (see below), so that the curve could be sampled at closer intervals. This allowed a sigmoidal shape to be detected (a result not obtained by

Fig. 1. Uptake of ferrous iron by apoferritin as a function of time

KIO_3 (0.05M) + $Na_2S_2O_3$ (0.2M) was used as oxidant in imidazole buffer, pH 7.45 at 25°C. The protein concentration was 1.15 mg/ml. The numbers indicated on the curves are the total number of ferrous ions added, either per apoferritin molecule present, or for an equivalent weight of bovine plasma albumin. — Apoferritin iron uptake followed by measuring the E_{420} of the hydrated ferric oxide micelle as it formed; the E_{420} values were converted into ferric iron concentrations. The buffer concn. used was 20mM. — · — · —, Oxidation of ferrous iron in the absence of apoferritin with bovine plasma albumin as a control protein; ferric iron was measured as described above. The buffer concn. used was 20mM. — ● —, Apoferritin iron uptake estimated by differences. The E_{520} of the remaining ferrous iron as a complex with 2,2'-bipyridyl was measured in 50mM-imidazole buffer and corrected by using the results shown in Fig. 4(b) to 20mM-imidazole.

Niederer, 1970). Measurements of the rate of disappearance of ferrous iron showed a marked acceleration in the presence of apoferritin compared with the protein-free control, as found by Niederer (1970). The rate of formation of ferric iron could not be measured in the control by the direct spectrophotometric method, since the product rapidly formed a precipitate. In the presence of bovine plasma albumin,

however, the ferrous iron was oxidized to a red-brown product which remained soluble. The progress curve for this reaction was also sigmoidal, with the initial slow reaction extending over a longer period of time than in the presence of apoferritin (see Fig. 1). These two reactions, without protein or with bovine plasma albumin, are not strict controls for ferritin formation, however, since the atomic structure of the ferric product formed was found to be different from that in ferritin.

Effect of varying iron concentration. The variation with iron concentration of the maximum slope of the progress curve, V_p , measured graphically, is shown in Fig. 2. This curve is also probably S-shaped, although its extension beyond the range of concentrations shown is difficult to estimate because of the high absorption, and because, at high iron concentrations, formation of FeOOH outside the protein

Fig. 2. Effect of varying the initial ferrous iron concentration on the 'peak velocity', V_p , of ferrous iron uptake, i.e. the maximum gradient of curves such as those shown in Fig. 1

The reaction was carried out at a constant protein concn. of 1.15 mg/ml in 20 mM-imidazole buffer, pH 7.45, 25°C.

may lead to precipitation. The rate constant estimated for the linear region is $k = 0.71 \text{ min}^{-1}$ (in 20 mM-imidazole buffer, pH 7.45, 25°C, apoferritin concentration 1.15 mg/ml).

Effect of varying protein concentration. Contrary to the first-order dependence of reaction rate on enzyme concentration found in normal enzyme-catalysed reactions, a much more complex and, as far as is known, unique relationship was discovered (Fig. 3). At low protein concentrations (below 0.25 mg/ml) the reaction is complicated by competitive formation of $\gamma\text{-FeOOH}$ outside the protein (identified by X-ray diffraction). At apoferritin concentrations greater than 1 mg/ml, no iron could be detected outside the protein on completion of the reaction, and the fall in V_p indicates that something other than apoferritin concentration is rate-determining.

Effect of pH and buffer concentrations. The rate of reaction is sensitive to pH changes, as might be predicted, since H^+ ions are released in the formation of the hydrous ferric oxide. The pH dependence is shown in Fig. 4. An order of reaction of about 1.5 can be derived from the log-log plot, which is linear over the pH range shown.

Varying the imidazole concentration (in the presence of 20 mM-barbital, pH 7.4, to maintain buffering) produced the curves shown in Fig. 4(b). At high imidazole concentrations an almost linear plot, with slope 1.5, is obtained.

Incremental addition of substrate. If, instead of a single addition of ferrous iron being made to the apoferritin-oxidizer medium the Fe^{2+} is added stepwise, the results shown in Fig. 5 are obtained. Although the initial uptake of iron follows a sigmoidal curve, subsequent additions do not. Successive amounts of added iron are incorporated at increased rates.

Fig. 3. Effect of apoferritin concentration on the peak velocity, V_p , of iron uptake

Conditions were as described in the Fig. 1. The concentration of ferrous iron was 1.51 mM.

Fig. 4. Effect of pH and imidazole in the peak velocity, V_p , of iron uptake

(a) The buffer was 20mM-imidazole, the ferrous iron concn. 1.45mM, and the apoferritin concn. 1.15mg/ml. (b) The imidazole concn. was between 9 and 132mM, and 20mM-barbital, pH7.4, was added to maintain the pH. Other conditions were as stated in Fig. 1. Ferrous iron concn.: ●, 1.31mM; ■, 0.88mM; ▲, 0.44mM.

Fig. 5. Effect of stepwise addition of Fe^{2+} on iron uptake by apoferritin

Conditions were as stated in Fig. 1. Ferrous iron (50mM-ferrous ammonium sulphate stock solution) was added in successive increments to give initial concentrations of (a) 0.44mM or (b) 0.15mM.

Interpretation of Results

The sigmoidal progress curve shown in Fig. 1 is reminiscent of those obtained for reactions catalysed by allosteric enzymes. This might suggest that initial addition of iron activates the protein in some way that allows further uptake of iron to proceed more rapidly. However, another model was suggested by the observation that the rate of uptake is dependent on the amount of iron already present in the molecule

and by the crystalline nature of the iron core. Crystal growth is known in general to produce sigmoidal rate curves (Zhdanov, 1965; Nielsen, 1964). The rate of growth depends initially on the formation of stable nuclei and, once these nuclei have formed, on the fact that the surface area on which new material is deposited enlarges as the crystal grows.

Although the synthesis of ferritin from apoferritin involves both oxidation of ferrous iron and formation of the micro-crystalline ferric oxide hydrate, it was decided to see first how closely the rate curves could be stimulated by a simple crystal-growth model, omitting oxidation and nucleation as separate stages; that is, by assuming that a major factor determining the rate of uptake of iron is the surface area of the growing crystallites. The rate of loss of substrate can then be expressed by the equation

$$-\frac{d[S]}{dt} = k_G A[S] \quad (1)$$

where k_G = rate constant for crystallite growth, A = surface area of the crystallite at time t min and $[S]$ = ferrous iron concentration at time t min (in mM). In the special case of ferritin synthesis crystal growth is proceeding within the confined space at the centre of the protein molecule. To express A in measurable terms, some assumption must be made about the way in which the micelle grows within the apoferritin shell. For simplicity, growth was represented as the movement of a boundary across a sphere (Fig. 6) the molecule being gradually filled by deposition of successive layers on the available plane

Fig. 6. Schematic model of ferritin reconstitution (not to scale)

The growth of the hydrated ferric oxide microcrystallite within the apoferritin shell (internal radius r) is represented by the growth of a spherical segment, of volume V and plane surface area A . The distance from this surface to the centre of the sphere is ' a '.

surface of the spherical segment. Although this model is doubtless over-simplified, circumstantial evidence for layer growth is provided by low (Fischbach & Anderegg, 1965) and high angle (Fischbach *et al.*, 1969) X-ray diffraction of ferritin fractions of different iron contents. The rate equation can now be expressed as:

$$-\frac{d[S]}{dt} = k_G \cdot A([S_0] - cV) \quad (2)$$

where V = volume of crystallite at time t min, $[S_0]$ = initial substrate (Fe^{2+}) concentration, and c is a scale factor.

Assuming a unit-radius, V can be expressed in terms of ' a ', the distance from the centre of the sphere to the boundary, giving:

$$[S] = [S_0] - c \cdot \pi \left(\frac{2}{3} - a + a^3/3 \right) \quad (3)$$

(a taken as negative when the centre of the shell is contained within the segment).

Differentiating:

$$\begin{aligned} \frac{d[S]}{da} &= c \cdot \pi (1 - a^2) \\ &= c \cdot A \end{aligned} \quad (4)$$

Now:

$$\begin{aligned} \frac{dt}{da} &= \frac{d[S]}{da} \cdot \frac{dt}{d[S]} \\ \therefore dt &= \frac{c \cdot A \cdot da}{k_G \cdot A \cdot ([S_0] - cV)} \end{aligned}$$

or

$$dt = \frac{c}{k_G \{ [S_0] - c \cdot \pi \left(\frac{2}{3} - a + a^3/3 \right) \}} da \quad (5)$$

Assuming values for the constants: $k_G = 0.7 \text{ min}^{-1}$ (approximating to the experimentally determined value; see Fig. 2), $c = 950$ (assuming the maximum value for $V = 4000$ iron atoms and that $V = \frac{4}{3}\pi = 4000/c$) this eqn. (5) can now be integrated

numerically (by using Simpson's rule) for a variety of substrate concentrations, expressing the rate, at t min, in terms of the fraction of the protein molecule which has been filled.

The experimental results were also converted into these terms, and a comparison with the calculated values is shown in Fig. 7. The calculated curve is sigmoidal and the fit between the two sets of curves is fairly good, except for a discrepancy between the maximum gradients. A perfect fit is not to be expected, however, considering the simplifications involved: the micelle is unlikely to grow exactly in the form of a segment of a sphere, and two or more crystallites may be forming simultaneously within the same molecule. A more serious fault with the simple treatment outlined above, however, is that individual molecules only are considered and a naive extension to the system as a whole would give a homogeneous product. That this is not the case is shown in Plate 1. The 'all-or-none' character of the product shown in Plate 1(c) suggests that, for a population of molecules, iron uptake is a competitive phenomenon, there being a higher probability of adding iron to those molecules already containing some iron than to those which contain little or none. To account for this effect, an additional step in the mechanism of ferritin formation is proposed.

This step may be termed 'nucleation', or initiation of crystal growth, and may be thought of as the formation of a stable species above a certain critical size below which re-solution may occur. The physical processes that may be involved in this first step, including the possible roles of the protein, are discussed in a later section. A new expression for the rate of loss of substrate through 'nucleation' may be written

$$-\frac{d[S]}{dt} = k_N [\text{Apo}][S]^n \quad (6)$$

where k_N is the rate constant for nucleation, $[\text{Apo}]$ = apoferritin concentration, $[S]$ = ferrous iron

Fig. 7. Comparison of calculated with experimental progress curves for the uptake of iron by apoferritin

The calculations were based on the model shown in Fig. 6, the uptake being expressed in terms of $[\text{Fe}^{3+}]$, which for this model is a function of 'a'. ----, Calculated progress curve; —, experimental progress curve.

concentration, and n is the order of reaction with respect to $[\text{S}]$ (n need not be an integral number and may be larger than 3, unlike the situation in normal chemical reactions).

Then the time between 'nucleations' can be written:

$$t_N = \frac{1}{k_N [\text{Apo}][\text{S}]^n} \quad (7)$$

Now eqn. (1) can be extended to cover a population of h apoferritin molecules, giving

$$-\frac{d[\text{S}]}{dt} = k_G [\text{S}]^m \sum_{i=1}^{i=h} A_i$$

where A_i is the available surface area of the i th molecule, and m is the order of reaction with respect to substrate, S .

However, it is no longer possible to express $d[\text{S}]/dt$ as a continuous function and to analyse the model further, it was necessary to develop a computer program, considering a set of h apoferritin molecules, plus a variable number of substrate molecules, and to stimulate the reconstitution as follows. (a) At the start of a calculation each molecule in the set can be allocated an initial amount of iron to simulate any distribution required. (b) t_N is then calculated from eqn. (7). (c) The growth of molecules already containing iron is calculated for this period,

$[\text{S}]$ being taken as constant if the total growth is less than 0.1 radius units, or updated each 0.1 of growth until t_N is reached. (d) At this point, if empty molecules exist, a further nucleation is created. (e) If $[\text{S}] > 0$ the program returns to point (b), otherwise the process is terminated with output consisting of: (1) the cumulative time, t , of each nucleation; (2) $[\text{S}]$ at time t , automatically plotted as a rate curve, on a scale allowing direct comparison with the experimental results; (3) a distribution analysis of the iron contents of the set of ferritin molecules on an arbitrary scale from 'empty' to 'full'.

Since the critical size of the nucleus was unknown, the number of ferric ions per nucleus was varied between 1 and 10. No significant difference in the results was apparent, indicating that this parameter is not of primary importance, and in all further calculations for simplicity it was assumed to be unity.

Adjustment of the rate constants k_N and k_G allows simulation of a wide range of experimental conditions, including variation of pH, buffer and oxidizer types and concentrations. Preliminary calculations showed that computed distribution of iron contents in the product was sensitive to the ratio k_N/k_G , whereas the progress curve was sensitive to the magnitudes of k_N and k_G and the orders n and m of the steps of the reaction.

The calculated iron distributions can be converted into theoretical schlieren patterns. Fig. 8 shows the effect of varying k_N/k_G and the amount of iron added on these patterns and compares them with those observed experimentally. The sharp boundaries of the theoretical diagrams are a consequence of the fact that diffusion processes were ignored in the calculations. The forms of the patterns are, however, quite clear. The introduction of a 'nucleation' step into the growth mechanism gives iron-content distributions closely resembling those obtained from reconstituted ferritins. Best agreement is obtained with k_N/k_G about 1. A comparison of the observed and calculated progress curves is shown in Fig. 9, adjusted to give the best fit. This was obtained with orders $n=0$ (for 'nucleation') and $m=1.5$ (for crystal growth). The curves fit fairly well, although deviations increase as the substrate concentration approaches zero. One possible cause of this discrepancy may be inherent in the method by which the problem was tackled. The substrate concentration must be taken as constant during the interval t_N (or during the interval required for each 0.1 r of growth), but since t_N approaches ∞ as $[\text{S}] \rightarrow 0$, the error in assuming constant substrate increases as $[\text{S}]$ decreases. Although improved fit might have been obtained by a further sophistication of the model, this was not considered to be a profitable exercise, since it would have required the introduction of more assumptions into the calculations. Reasonable agreement between theory and experiment was also

Fig. 8. Observed schlieren patterns (top row) showing the distribution of iron in reconstituted ferritins compared with those computed for the final model with different ratios for the kinetic constants k_N and k_G

Sedimentation was from right to left. Experimental conditions were as for Plates 1(b), 1(c) and Fig. 1. The numbers of iron atoms added per molecule of apoferritin are indicated.

obtained when computer-simulated incremental substrate additions (Fig. 10) were compared with the observed curves shown in Fig. 5. Both the loss of sigmoidal character and the dependence of rate of uptake on initial iron content are predicted by the model and suggest that the proposed mechanism is fundamentally correct.

Discussion

The experimental results presented above show that a ferritin-like product is formed from apoferritin and Fe^{2+} in the presence of an oxidizing

agent. Ferritin formation seems to resemble crystallization: it can be accounted for by two stages analogous to nucleation and crystal growth. These processes occur, however, within the limited space available in the interior of the apoferritin shell, which appears to provide a favourable environment. The net result is that the rate of oxidation of ferrous iron, whether measured as the disappearance of Fe^{2+} , or as the formation of the red-brown hydrous ferric oxide, is increased by the presence of apoferritin.

Some idea of the magnitude of the apoferritin 'catalytic' effect can be obtained from the fact that,

Fig. 9. Comparison of the observed progress curves for iron uptake by apoferritin with those computed for the final model for various numbers of iron atoms added per apoferritin molecule (as indicated on curves)

Values of the parameters used in the computed curves were $k_N/k_G = 1$, $n = 0$, $m = 1.5$, where k_N and k_G are the rate constants and n and m are the orders of reaction for the two steps of the proposed mechanism.

under the conditions used in the experiments described above, in which the total internal volume of the apoferritin molecules present in solution is less than one-thousandth of the solvent volume, all the added iron enters and is retained by the protein, and none of it forms $\gamma\text{-FeOOH}$ in the solvent. This suggests that the rate of 'nucleation' inside apoferritin is greater than 10^3 times that in the solvent and lends credence to the view that apoferritin can be regarded as an enzyme.

That the major effect of apoferritin appears to be at this initial slow or 'nucleation' stage can be seen by comparing the progress curves obtained in the presence of apoferritin and of bovine plasma albumin (Fig. 1). A secondary consequence of the formation of large numbers of small but stable nuclei within the apoferritin molecules may be an increase in the surface area available for the deposition of more ferric oxide hydrate as compared with that formed in the

Fig. 10. Computed iron-uptake progress curves for simulated stepwise additions of ferrous iron

Increments equivalent to 0.7 mM- and 0.2 mM-ferrous iron were used for the two curves. Computations were based on the final model, with parameters as for Fig. 9. Compare Fig. 10 with Fig. 5.

absence of protein. Thus, although part of the crystallite surface is protected by protein shell, the latter also prevents the particles from growing or coalescing. Under the conditions used above about half of the 10^{14} molecules present have nucleated by the end of the experiment. When these molecules are half full (hemispheres) they would provide roughly 1000 times as much surface as 1000 equal cubes of total equivalent volume. It could be argued that this very large surface area would increase the tendency for re-solution to take place; however, the insolubility of ferric ions at neutral pH is such that the equilibrium is far over on the side of the aggregated species. Re-solution may be decreased both by the binding of the crystal to the protein and by the relative inaccessibility of the inside of the molecule to the solvent, so that the net effect is to decrease the critical nucleus size.

It is a well-known phenomenon of crystal growth that the energy required to form a nucleus of a new phase is significantly lower if the nucleus is formed on the walls of a vessel or on the surface of an impurity (heterogeneous nucleation) than for spontaneous or homogeneous nucleation (Zhdanov, 1965; Nielsen, 1964). Heteronucleation may then take place at a lower concentration than that required for homonucleation. One of the roles of apoferritin molecules in ferritin formation might therefore be to provide surfaces on which the free energy change of nucleation is lowered, i.e. to act as a site for heteronucleation.

Since the process of nucleation resembles transition through an activated state in ordinary chemical reactions, the apoferritin could be thought of as lowering the 'activation energy' for the formation of the hydrous ferric oxide, giving it another enzyme-like property.

In this discussion so far only nucleation and crystal growth and the possible influences of the protein on these processes have been considered. Apoferritin might also be expected to be involved in the oxidation of the ferrous iron, possibly by providing 'active sites' at which oxidation is catalysed, as suggested by Niederer (1970) and by Harrison & Hoy (1972*a,b*). The protein might also provide a local environment with a relatively high pH, which would effectively lower the redox potential of the iron.

Evidence that iron binding by apoferritin may be the initial step in ferritin synthesis is provided by two types of observation. The first of these is that zinc inhibits iron uptake, which implies that zinc may be competing with iron for binding sites on the protein. Zinc was first noted *in vivo* to give rise to ferritin with a low iron/protein ratio (Coleman & Matrone, 1969), and the inhibition was subsequently confirmed *in vitro* by Niederer (1970) and by the present authors (I. G. Macara, T. G. Hoy & P. M. Harrison, un-

published work). The second is the apparent zero order of the 'nucleation' step with respect to ferrous iron concentration noted above. This could result from saturation by Fe^{2+} of binding sites before the first slow step of ferritin formation.

Three models depicting possible mechanisms of ferritin synthesis are shown in Fig. 11. A section through the protein showing subunits and intersubunit channels is illustrated schematically in this diagram. The channels are considered to be large enough for both iron atoms and oxidizing agent to enter them. The first event in each of the models is the binding of Fe^{2+} to sites either in the interior of the molecule or in the channels. These sites are also oxidation sites. In model (a) the oxidation site is also the site at which the hydrated ferric oxide starts to grow. Oxidation and 'nucleation' can thus be regarded as a single step, the crystallite growing round an iron atom already bound to the protein. In this model the protein can only be involved directly in the original oxidation step, since its 'active site' would soon be covered. Its function would be primarily to initiate growth or 'nucleation', by catalysing the slow first step of ferritin formation. Subsequent oxidation must take place at sites other than the initiation site, and, since no independent oxidation step is suggested by the kinetic results, crystal growth and oxidation must

Fig. 11. Schematic models indicating possible mechanisms for the formation of ferritin

The drawings represent sections through the protein molecule showing subunits and intersubunit channels. In model (a) an Fe^{2+} atom is bound at a site inside the molecule (perhaps to carboxyl group or tyrosine hydroxyl group) where it is oxidized to Fe^{3+} . This then acts as a nucleus around which the micro-crystal of ferric oxide hydrate grows by addition and oxidation of further iron atoms on its surface. In model (b) all Fe^{2+} atoms entering the molecule are bound and oxidized on sites (perhaps containing histidine or amino groups) located in the inter-subunit channels. Fe^{3+} is less firmly bound to these sites than Fe^{2+} . It forms ferric oxide hydrate crystals inside the protein. No specific sites are required for the latter, but the internal environment favours crystal growth. Model (c) is similar to model (b), except that the protein provides specific sites for both nucleation and oxidation.

again be synchronous, i.e. Fe^{2+} is rapidly deposited and oxidized on the growing crystal surface. Model (b) is similar to that proposed by Niederer (1970) except that the oxidation sites are depicted in the channels rather than on the inside surface of the molecule, so that they remain accessible as the molecule is filled. All iron atoms entering the molecule are rapidly bound and oxidized at these sites. Crystal nucleation does not take place at the oxidation sites, which must be presumed to bind Fe^{3+} less firmly than Fe^{2+} . For this model to be consistent with the proposed crystal growth mechanism, the ferric oxide hydrate growing inside the protein must bind ferric ions more strongly than the oxidation sites, thus pulling the ferric ions off these sites and driving the oxidation forward. Models (c) and (b) are essentially the same except that in model (c) specific Fe^{3+} binding or heteronucleation sites are depicted inside the molecule in addition to the oxidation sites, whereas in model (b) the protein could simply provide an 'environment' favourable to crystal formation.

The three models cannot readily be distinguished in the present experiments. Model (a) might be favoured on grounds of simplicity, but one possible difficulty with both models (a) and (c), in which the protein is implicated in the nucleation process, is the apparent lack of structural relationship between the protein and its inorganic core deduced from X-ray-diffraction photographs like that in Plate 2(c). Foreign particles are usually especially efficient at inducing nucleation if their structures are similar to or compatible with that of the crystallizing substance (Zhdanov, 1965; Nielsen, 1964). Either apoferritin does not have a strongly orientating effect on the direction of crystal growth, or conceivably the crystallite, once growing, may shift or even become detached from its original site [it could not escape through the channels once it had grown above about 1.5 nm (15 Å) in size]. The structural resemblance of ferritin mineral to the hydrolysate of ferric salts, rather than to α - or γ - FeOOH obtained by oxidation of Fe^{2+} , may be cited in support of models (b) or (c), although not conclusively. Whichever model is correct it seems unlikely that the protein is the primary electron acceptor in the oxidation or that the oxidizing agent is directly bound to the protein, since oxidants of different structure can be used. Further work is clearly required to elucidate the mechanisms of oxidation in detail.

As a model system for ferritin formation *in vivo*, the conditions used in the present experiments could be criticized on the grounds that they are highly artificial. However, similar products have been found under different conditions, including the use of a different oxidant (molecular oxygen) (Harrison *et al.*, 1967). Change of pH or buffer affects only the magnitude of the rate constants k_G and k_N . The type

of oxidizing agent used is similarly of little importance with respect to the concepts inherent in the proposed mechanism. Change of oxidant and other conditions, which affect the ratio k_G/k_N , may alter the distribution of iron per molecule in the product. Such differences have been noted in other experiments. In general support of the mechanism, experiments by other workers may be cited. Thus in studying the transfer of iron from transferrin to ferritin *in vitro*, in the presence of a reducing agent plus atmospheric oxygen, Miller & Perkins (1969) found that more iron was taken up by ferritin, which was roughly half saturated with respect to iron, than by ferritin of low iron content or by apoferritin. Studies of the uptake of ^{59}Fe by ferritin in HeLa cells showed not only that the molecules were filled progressively by iron, but also that those molecules which were partially filled incorporated iron more rapidly than those which were nearly saturated (Miller, 1968). Progressive uptake of iron by ferritin molecules has also been demonstrated in rat liver after the initial stimulus given to the synthesis of ferritin protein by iron injection (Drysdale & Munro, 1966).

Much more work is required on ferritin formation *in vivo*. Local cellular conditions, such as pH and oxygen tension, together with the relative amounts of iron and ferritin protein present, may be of importance in the control of ferritin iron uptake, and may also affect the distribution of iron between ferritin and haemosiderin. It is possible that the iron *in vivo* is carried to and may even enter the molecule not as free Fe^{2+} but as a chelate complex (Mazur *et al.*, 1960; Miller & Perkins, 1969). The expected effect of this would again be to alter the rate constants rather than to invalidate the basic concepts of the model.

We thank the Medical Research Council for financial support.

References

- Bielig, H.-J. & Bayer, E. (1955) *Naturwissenschaften* **42**, 125
- Coleman, C. B. & Matrone, G. (1969) *Biochim. Biophys. Acta* **177**, 106
- Drysdale, J. W. & Munro, H. N. (1966) *J. Biol. Chem.* **241**, 3630
- Farrant, J. L. (1954) *Biochim. Biophys. Acta* **13**, 569
- Fischbach, F. A. & Anderegg, J. W. (1965) *J. Mol. Biol.* **14**, 458
- Fischbach, F. A., Harrison, P. M. & Hoy, T. G. (1969) *J. Mol. Biol.* **39**, 235
- Granick, S. (1946) *Chem. Rev.* **38**, 379
- Granick, S. & Michaelis, L. (1943) *J. Biol. Chem.* **147**, 91
- Haggis, G. H. (1965) *J. Mol. Biol.* **14**, 598
- Harrison, P. M. (1964) in *Iron Metabolism* (Gross, F., ed.), p. 40, Springer-Verlag, Berlin
- Harrison, P. M. & Hoy, T. G. (1970) *J. Microsc. (Paris)* **91**, 61

- Harrison, P. M. & Hoy, T. G. (1972a) in *Inorganic Biochemistry* (Eichhorn, G. L., ed.), chapter 8, Elsevier, New York and London, in the press
- Harrison, P. M. & Hoy, T. G. (1972b) in *Cellular and Subcellular Aspects of Iron Metabolism in Health and Disease* (Kinney, T. D. & Trump, D. F., eds.), chapter 2, Springer-Verlag, Berlin, in the press
- Harrison, P. M., Fischbach, F. A., Hoy, T. G. & Haggis, G. H. (1967) *Nature (London)* **216**, 1188
- Loewus, M. W. & Fineberg, R. A. (1957) *Biochim. Biophys. Acta* **26**, 441
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265
- Mazur, A. & Shorr, E. (1950) *J. Biol. Chem.* **182**, 607
- Mazur, A., Green, S. & Carleton, A. (1960) *J. Biol. Chem.* **235**, 595
- Miller, A. O. A. (1968) *Biochim. Biophys. Acta* **155**, 262
- Miller, J. P. G. & Perkins, D. J. (1969) *Eur. J. Biochem.* **10**, 146
- Niederer, W. (1970) *Experientia* **26**, 218
- Nielsen, A. E. (1964) *Kinetics of Precipitation*, pp. 1-151, Pergamon, Oxford
- Poulik, M. D. (1957) *Nature (London)* **180**, 1477
- Towe, K. M. & Bradley, W. F. (1967) *J. Colloid Interface Sci.* **24**, 384
- Zhdanov, G. S. (1965) in *Crystal Physics* (Brown, A. F., ed.), chapter 13, Oliver and Boyd, Edinburgh and London