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The Formation of Ferritin from Apoferritin

CATALYTIC ACTION OF APOFERRITIN

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The iron-storage protein ferritin consists of a protein shell and has an iron content of up to 4500 iron atoms as a microcrystalline ferric oxide hydrate. A study was made of the uptake of ferrous iron by apoferritin in the presence of an oxidizing agent at very low iron:protein ratios. At ratios of less than about 150 iron atoms per apoferritin molecule hyperbolic progress curves were obtained, whereas at higher ratios the curves became sigmoidal under the conditions used. A computer model, developed previously (Macara *et al.*, 1972), was shown to account for this result. The experimental evidence indicates that apoferritin binds ferrous iron and catalyses the initial stage in the formation of the ferric oxide hydrate inside the protein shell. This stage involves the oxidation of sufficient iron within the protein molecule to form a stable nucleus on which the growth of the microcrystalline iron-core particles can proceed. A possible schematic mechanism for the action of apoferritin is suggested.

Ferritin molecules are made up of two components: a protein shell, called apoferritin, and a microcrystalline core or micelle, situated within the hollow shell, of approximate composition $(FeOOH)_8(FeO:OPO_3H_2)$ and containing up to about 4500 iron atoms (Granick, 1946; Harrison, 1964; Harrison *et al.*, 1967). The protein coat consists of 20–24 subunits, which are symmetrically arranged and thus allow access to the inner cavity through inter-subunit channels (Harrison, 1959, 1964; Crichton, 1973). The iron can be removed by the action of reducing agents to leave apoferritin (Granick & Michaelis, 1943), and ferritin can be reconstituted by a reverse procedure from apoferritin, Fe^{2+} and an oxidant (Bielig & Bayer, 1955; Harrison *et al.*, 1967).

In a previous study we have shown that reconstituted ferritin resembles native ferritin as judged by several criteria (Harrison *et al.*, 1967; Macara *et al.*, 1972) and have studied the kinetics of reconstitution by using ferrous ammonium sulphate as substrate and an excess of KIO_3–$Na_2S_2O_3$ as oxidant (Macara *et al.*, 1972). Under the conditions used in this study sigmoidal progress curves were obtained if about 200–600 iron atoms were added per apoferritin molecule at the start of the reaction. However, if the iron was added in successive small increments, hyperbolic curves were obtained, the initial gradients of which increased with each addition. Under conditions giving sigmoidal iron uptake, the iron was distributed unequally in the product, a proportion of the molecules containing no iron at the end of the experiment.

The results were interpreted by the following hypothesis: iron uptake by apoferritin is a process involving two stages; the first is the formation of stable nuclei on which further crystallization can take place, the second stage is one of crystal growth on these nuclei. It was shown that apoferritin itself catalyses 'nucleation', but the subsequent rate of growth of the iron cores seems to be determined at any time by the surface area of their crystallites available for the deposition of more iron oxide hydrate.

The purpose of the present study was to obtain further evidence of the catalytic action of the protein; to do this, the kinetics of iron uptake were followed at very low iron concentrations, at which crystal growth is insignificant compared with nucleation.

Materials and Methods

Horse spleen ferritin (twice crystallized, cadmium-free) was purchased from Pentex Inc., Kankakee, Ill., U.S.A. In some cases it was further purified before use by gel filtration on Sephadex G-200. Apoferritin was prepared from ferritin by reduction either with $Na_2S_2O_4$ (Macara *et al.*, 1972) or with 0.12M-thioglycollate, pH4.6. A ferritin fraction containing nearly 2000 iron atoms per molecule (density 1.67) was isolated by equilibrium density-gradient centrifugation in CsCl (Fischbach & Anderegg, 1965). Crystallized bovine serum albumin was purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K., and all other reagents were of AnalaR grade (BDH Chemicals Ltd., Poole, Dorset, U.K.).

Apoferritin concentrations were determined from the absorbance of solutions at 280nm ($E^{1\%}_{1cm} = 9.0$). Experiments on iron uptake by apoferritin were carried out in imidazole buffer, pH7.45, in a similar

manner to that described previously (Macara *et al.*, 1972). As described before, the oxidant was KIO_3 with $Na_2S_2O_3$ at concentrations of 3.7 and 14.8mM respectively in all experiments except those in which the effect of alteration of the oxidant concentration was being studied, when the KIO_3 was varied from 0.9 to 9.6mM ($Na_2S_2O_3$ 3.6–38.4mM, Fe^{2+} 0.115mM). Very low concentrations of ferrous ammonium sulphate (0.03–0.60mM) were used and the formation of the ferric oxide hydrate product was followed at 310nm ($E_{1cm}^{1\%} = 450$) instead of at 420nm ($E_{1cm}^{1\%} = 100$). The imidazole concentration was normally 18.5–19.2mM, but when the effect of pH on the rate of iron oxidation was being followed a series of buffers was prepared in the range pH6.62–7.75 that had a constant uncharged imidazole concentration of 58mM (since it is the uncharged species that can form a ligand to Fe^{2+}) and had total imidazole concentrations from 69 to 230mM. The ionic strength, I, was varied over the range $I = 0.03$ to $I = 0.188$ by addition of NaCl and the apoferritin concentration was varied from 0.41 to 3.0mg/ml. In most cases the measured rates of iron oxidation were corrected to a value corresponding to $I = 0.1$ and [apoferritin] = 1.0mg/ml. In some experiments apoferritin was replaced by bovine serum albumin or by a ferritin fraction. Bovine serum albumin was used as a 'control' protein, since it had been found previously (Macara *et al.*, 1972) that its protective colloid action prevented the precipitation of the γ-FeOOH formed at relatively high iron concentrations. The reaction could therefore be followed in a spectrophotometer by measuring the increase in absorbance at 420nm due to the formation of the ferric oxide hydrate as for ferritin formation. In other experiments the rate of iron oxidation was followed by measuring the remaining ferrous iron as its 2,2′-bipyridyl complex ($\epsilon = 9100$ litre·mol^{-1}·cm^{-1} at 520nm, estimated from a standard curve) at intervals after the start of the reaction. The pH of the solutions was checked in some cases at the end of the uptake experiments and was found to have remained constant.

Initial rates are designated V and peak rates (in sigmoidal progress curves) as V_p.

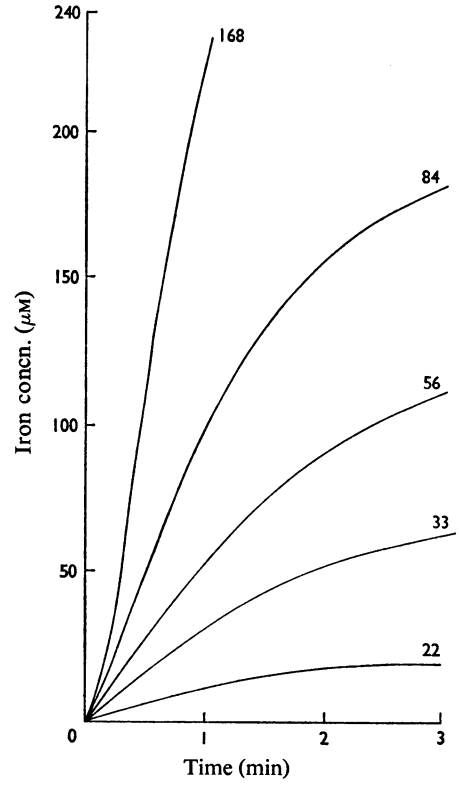


Fig. 1. *Uptake of low concentrations of iron by apoferritin*

Ferrous ammonium sulphate (74–500μM) was added to a solution of 1.5mg of apoferritin/ml and oxidant (3.7mM-KIO_3, 14.8mM-$Na_2S_2O_3$) in 18.5mM-imidazole buffer, pH7.45; the ionic strength I was 0.055. The reaction was followed by measuring the changing absorption of the iron oxide hydrate product at 310nm ($E_{1cm}^{1\%} = 450$). Numbers on the curves give the average number of iron atoms per apoferritin molecule. When this number is greater than about 150 the uptake curves are sigmoidal under the conditions used.

Results

As reported previously (Macara *et al.*, 1972), at high iron or low protein concentration and excess of oxidant, the uptake of iron by apoferritin shows sigmoidal kinetics. When the iron:protein ratio is lower the progress curves become hyperbolic, the transition occurring in the region of 90–150 iron atoms per apoferritin molecule under the conditions used (Fig. 1). Within the limits of error of the experiments the progress curves were found to coincide, whether measured as loss of substrate (remaining Fe^{2+} determined at intervals as the bipyridyl complex) or as formation of product (increase in absorbance at 310nm due to hydrous Fe_2O_3 measured continuously in a recording spectrophotometer). At the low iron concentrations used in the present experiments little or no measurable ferric iron was formed in the absence of apoferritin (with or without bovine serum albumin) during the normal time-course of the experiments (usually about 5min).

Further studies of the ferritin iron-core nucleation process were made by measuring the effects of various conditions on the initial velocity, V, of the hyperbolic progress curves obtained at low

iron:protein ratios. A linear dependence of initial rate on protein concentration was found (Fig. 2). This confirmed the catalytic action of the protein and also enables V in other experiments to be referred to a standard apoferritin concentration (1mg/ml). A double-reciprocal plot of V and iron concentration is also linear (Fig. 3). Extrapolation gives an apparent K_m of about 0.38mM and $k_{cat.}$ of $150min^{-1}$ at pH7.45 and 20°C with the concentrations used of oxidizer and buffer. [The apparent K_m is dependent on the tendency of the buffer to bind Fe^{2+} as a ligand. The relatively large error in measuring V is a consequence of the very small region over which the progress curve is linear (Fig. 1).] A variation in oxidant concentration over a 100-fold range did not significantly alter V after correction to constant ionic strength. Similarly, in experiments in which apoferritin was replaced by a ferritin fraction or by bovine serum albumin, variation in oxidizer concentration was without significant effect on V or V_p respectively.

Changing pH in the range 6.62–7.75 (in imidazole buffer with constant uncharged imidazole concentration) altered V in a non-linear manner (Fig. 4*a*), with a sharp increase occurring at about pH7.2. A similar pH effect was observed with a ferritin fraction, or with bovine serum albumin (Fig. 4*b*), although these experiments were performed with higher ferrous iron concentrations. With bovine serum albumin, higher iron concentrations were necessary for measurable oxidation to occur during the experiments and V_p (the maximum rate of a sigmoidal curve) was measured instead of the initial rate, V, since the latter was close to zero.

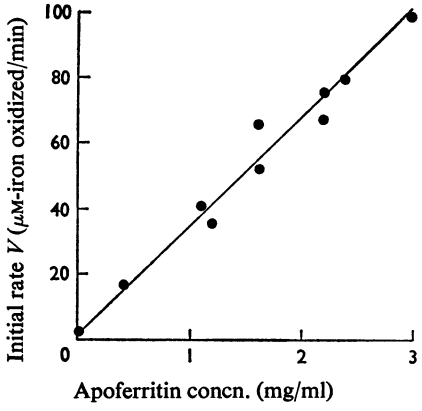


Fig. 2. *Dependence of the initial rate of iron uptake on apoferritin concentration*

The ferrous iron concentration was 74μM, or in some cases 115μM, and the apoferritin concentration was varied over the range 0–3.0mg/ml. All other conditions were as stated in Fig. 1. The measured initial rate, V (μM-iron oxidized/min), was corrected to $I = 0.1$.

Computer simulation

In an earlier paper (Macara *et al.*, 1972) a model was described that simulated all of the phenomena observed during iron uptake by apoferritin at relatively high iron:protein ratios. In this model nucleation begins at a single point within the apoferritin shell and crystal growth proceeds by the addition of successive layers of ferric oxide hydrate to form increasingly large segments of a sphere. The rate of growth is therefore dependent on the available surface area of the crystallites at any given time.

In the computer program used, loss of substrate through nucleation was described by the equation:

$$\frac{-d[S]}{dt} = k_N[Apo][S]^n \quad (1)$$

and the rate of loss through crystal growth in a population of h molecules by:

$$\frac{-d[S]}{dt} = k_G[S]^m \sum_{i=1}^{i=h} A_i \quad (2)$$

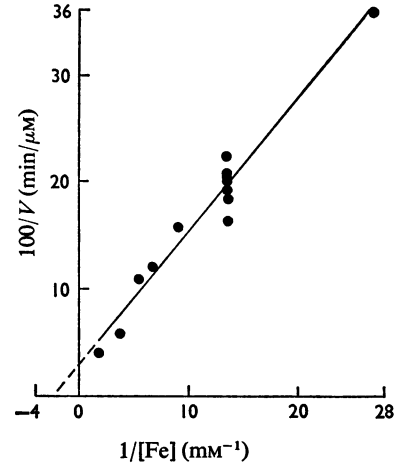


Fig. 3. *Dependence of the initial rate of iron uptake by apoferritin on the substrate concentration*

Ferrous iron was varied within the range 0.037–0.115mM and the protein concentration was in most cases 1.5mg/ml (data from Fig. 2 were also used). Other conditions were as stated in Fig. 1. All rates have been corrected to $I = 0.1$ and protein concentration has been corrected to 1.0mg/ml.

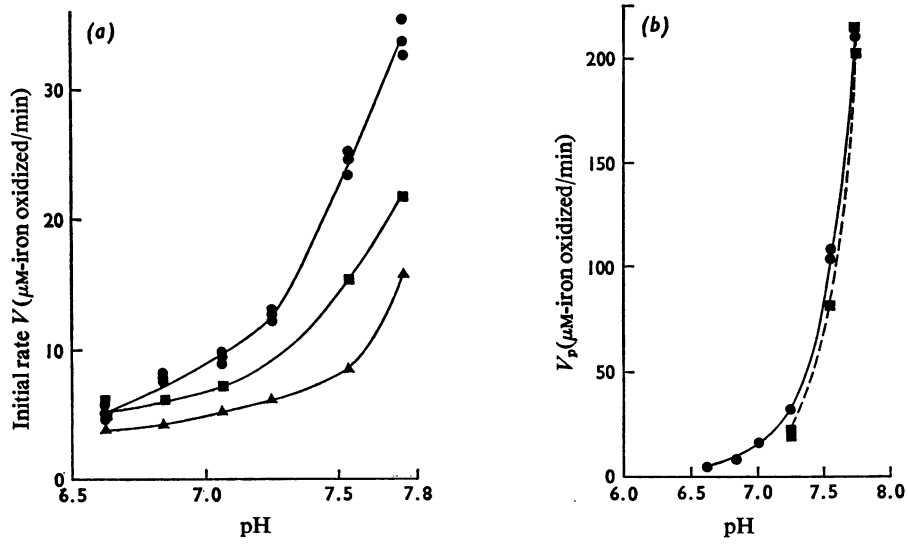


Fig. 4. *Effect of pH on the rate of iron oxidation in the presence of apoferritin, a ferritin fraction, or bovine serum albumin*

Conditions were as stated in Fig. 1, except that the total imidazole concentration was adjusted to each pH to maintain a constant uncharged imidazole species concentration of 58mM. (*a*) Initial rates, V, have been corrected to $I = 0.1$ and apoferritin concentration to 1.0mg/ml. Ferrous iron concentrations were: ●, 194μM; ■, 115μM; ▲, 58μM. (*b*) ●, Ferritin fraction (approx. 2000 iron atoms/protein molecule). Protein concentration 0.31mg/ml, ferrous iron concentration 1.85mM. ■, Bovine serum albumin 1.1mg/ml, ferrous iron concentration 1.85mM. V_p, the peak velocity of the sigmoidal curves, was measured, rather than the initial rate.

where k_N and k_G are nucleation and crystal growth rate constants respectively, [S] and [Apo] are the iron and apoferritin concentrations respectively, n and m are orders of reaction with respect to S, and A_t is the crystallite surface area. Since not all molecules nucleate simultaneously, crystallite size and surface area vary among the different members of the population of h molecules at any time t. The summation in eqn. (2) takes account of this variation.

The model had originally been designed to simulate iron uptake under conditions of relatively high ferrous iron concentrations and gave sigmoidal progress curves under these conditions. The best fit between the calculated and observed sigmoidal progress curves was obtained with $m = 1.5$ and $n = 0$. Without further alteration, the extension of the model to conditions of low iron:protein ratios produced hyperbolic curves similar to those observed.

Eqn. (1) with $n = 0$ can be regarded as the reduction of the more general binding equation

$$\frac{-dS}{dt} = \frac{k_N[\text{Apo}]}{1+(K/[S])} \qquad (3)$$

in the situation in which all binding sites are saturated (K is a binding constant). Use of eqn. (3) instead of eqn. (1) in the computer program made very little difference to the form of the simulated iron-uptake curves even with a 10^6-fold variation in K. The point of transition from sigmoidal to hyperbolic curves depends on the values assumed for the rate constants k_N and k_G, the number of iron atoms added, [S], and the number of iron atoms necessary for formation of a nucleus (see Macara *et al.*, 1972). The results of one set of computer simulations calculated for different values of [S] but with the same values of k_N and k_G are shown in Fig. 5. It should be emphasized that because of the simplification involved in the computer model, comparison of the observed and simulated curves can only be expected to give qualitative agreement.

Discussion

It can now be seen that the hypothesis described previously (Macara *et al.*, 1972) accounts very well for the kinetics of iron uptake by apoferritin under a variety of conditions. The main premise of this hypothesis is that the rate of formation of ferric oxide hydrate within the apoferritin shell (i.e. ferritin for-

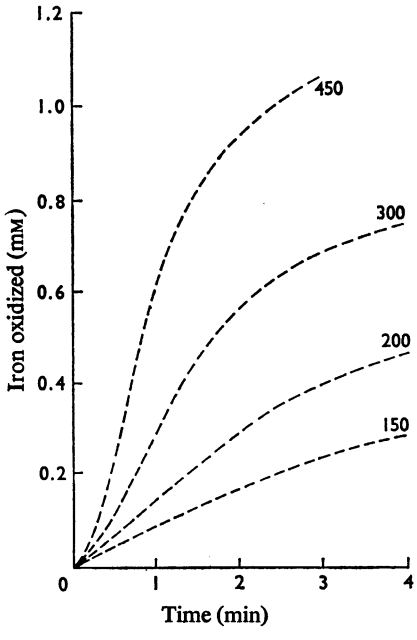


Fig. 5. *Computer simulation of transition from sigmoidal to hyperbolic uptake curves*

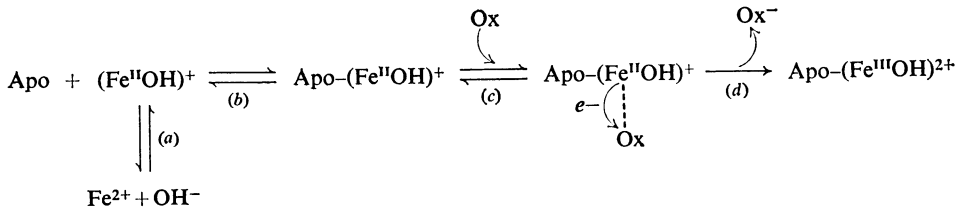
The equations used in the simulation are described in the text. Numbers on the curves give the number of ferrous ions added per apoferritin molecule.

mation) is dependent on the number of sites available either on the protein or on mineral crystallites that have already been formed inside it. When stable crystal nuclei are present the number of sites increases with each addition of 'iron hydroxide', and the process becomes autocatalytic and gives sigmoidal progress curves. In the absence of preformed nuclei, that is under conditions such as those discussed in the present experiments, the process is started by binding ferrous iron to apoferritin. This binding is an essential part of the catalytic activity of the protein, as discussed below.

Although the catalytic action of apoferritin was implied in previous calculations (Macara *et al.*, 1972), since the apoferritin concentration enters directly into the rate equation for nucleation [eqn. (1) above] and although some experimental evidence for this catalysis has already been provided, it has been confirmed more directly by the results reported here. The binding of ferrous iron by apoferritin, as the first step in the reaction, is now indicated both by the finding that the initial rate of iron uptake is proportional to apoferritin concentration (Fig. 2) and by the fact that substrate saturation is observed (see Fig. 3).

The computer model provides a framework in which to explore further the mechanism by which ferrous iron is oxidized and hydrolysed to form Fe_2O_3 within the protein molecule. The model, supported by experimental evidence discussed above and by Macara *et al.* (1972), implies that the rate of growth of the hydrous Fe_2O_3 crystallite can be equated with the rate of loss of ferrous iron, and that free ferric iron is not produced in measurable quantities. The effects of various pH and oxidizer concentrations give some indications of possible reaction mechanisms. The dependence of oxidation rate on pH shown in Fig. 4 suggests that the concentration of OH^- must appear in the overall rate equation, as indeed would be expected, since hydrolysis as well as oxidation is involved in the formation of the product. A similar dependence is found both for the nucleation stage shown in Fig. 4(*a*) and for growth on pre-existing nuclei (the reaction with a ferritin fraction containing nearly half its full complement of iron, shown in Fig. 4*b*). The peak velocity of oxidation of ferrous iron in the presence of bovine serum albumin seems to show a similar pH dependence (Fig. 4*b*). A possible explanation for this effect is that the hydrolysed species $(Fe^{II}OH)^+$ rather than Fe^{2+} is the substrate in the reaction. This species may be bound either by the protein or to the '$Fe^{III}OOH$' crystallite surface forming a ferrous–ferric complex and become oxidized after binding. [$(Fe^{II}OH)^+$ was considered to be the species reacting with oxidant in a study of the mechanism of ferrous iron oxygenation under different conditions by Goto *et al.* (1970).] A 100-fold variation in oxidizer concentration, on the other hand, produced no significant change in the initial rate of iron uptake by apoferritin or by a ferritin fraction, or in the peak rate of oxidation in the presence of bovine serum albumin. This appears to indicate that the oxidation step is not the rate-limiting process in the overall reaction under the conditions used in these experiments [unlike those of Goto *et al.* (1970)]. Another explanation for the apparent zero order of the reaction with respect to oxidizer might be that oxidation is a surface phenomenon and the ferrous iron bound either to the protein or to the crystallite is saturated by the oxidant under the conditions used. It should be recalled that little or no ferrous iron is oxidized in the absence either of apoferritin or of hydrous Fe_2O_3 nuclei.

The catalytic activity of apoferritin on the formation of its iron core may now be explained as follows. Apoferritin binds ferrous iron [or $(Fe^{II}OH)^+$] on its inner surface. Chelation promotes its oxidation, the oxidant reacting directly with the Fe^{II}. [There is no evidence that the oxidizer is bound by the protein or that the latter acts as an intermediate electron acceptor, and this seems unlikely since different oxidants can be used (Harrison *et al.*, 1967; Macara



Scheme 1. *Possible reaction sequence for the initial stages of ferritin formation*

(*a*) substrate formation is dependent on OH^- concentration. (*b*) might involve detachment of a proton from Apo and formation of Apo-(Fe^{2+}-H_2O) [rather than Apo-($Fe^{II}OH)^+$]. (*d*) the Apo-Fe^{III} complex might form a heteronucleation centre for further hydrolysis and polymerization of Fe^{III} ions or it might release the Fe^{III} for nucleation elsewhere in the molecule.

et al., 1972).] One or more 'ferric hydrolysate' nuclei are formed inside each apoferritin molecule from several ferric ions and act as centres for growth of the crystalline mineral particles. The nuclei could grow directly on an Fe^{III} atom retained at its original binding site, so that this site acts as a centre for both oxidation and 'heteronucleation' as suggested in Fig. 11(*a*) of Macara *et al.* (1972). Alternatively, the Fe^{III} might be released from its binding site after oxidation and nucleate elsewhere within the central cavity of the protein, as in Fig. 11(*b*) or 11(*c*) of Macara *et al.* (1972). There seems to be no need, however, to postulate a mechanism involving catalytic oxidation by the protein of all ferritin iron atoms entering the molecule [as in Figs. 11(*b*) and 11(*c*) of Macara *et al.* (1972)], since the whole reaction proceeds rapidly once nuclei have formed (even in the absence of apoferritin). Because apoferritin probably consists of 24 identical polypeptide chains, 24 identical ferrous-iron-binding centres would be expected per molecule (unless the centres occur in symmetry axes and involve more than one subunit, when this number would be decreased). The presence of several iron atoms in the 7 nm cavity in the apoferritin molecule would produce a local high 'concentration' of ferric iron. The hydrolysed ferric ions might be expected to react together rapidly, since the 'mononuclear barrier' (Schubert, 1964) is already exceeded by the presence of a single Fe^{III} atom inside the apoferritin cavity (Harrison & Hoy, 1973). The fact that many ferritin molecules seem to contain only one, or a small number, of iron-core crystallites (Harrison & Hoy, 1970) rather than 24 is a consequence of the probability that once a single stable nucleus has formed its growth will tend to compete successfully with the formation of other nuclei in the same or other molecules (Macara *et al.*, 1972). Nuclei containing more than a few iron atoms will be retained by the protein shell, since they are too large to escape through the inter-subunit channels.

One possible mechanism for the catalytic activity of apoferritin in initiating ferritin formation is summarized in Scheme 1.

The studies described above have been carried out on apoferritin from horse spleen. Linder-Horowitz *et al.* (1970) found that different organs of the same species contain isoferritins that seem to differ both in their amino acid composition and in their iron content. The discovery that apoferritin is actively involved in its iron uptake suggests that the existence of these isoferritins may provide a means of regulating iron storage in different tissues.

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