The Binding of Ferric Iron by Ferritin

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Equilibrium-dialysis experiments with ^{59}Fe -labelled Fe(III) chelate solutions show that ferritin is capable of binding a limited number of Fe(III) atoms. Some of this Fe(III) is readily removed, but up to about 200 Fe(III) atoms/molecule remain bound after extensive washing. Some exchange of labelled Fe(III) with endogenous unlabelled ferritin Fe occurs during prolonged dialysis against ^{59}Fe (III)-citrate, but there is a net binding of Fe(III). Bound Fe(III) resembles endogenous Fe(III) in several respects. It appears to be attached to the micelle and not to the protein component of ferritin. Although the physiological mechanism of Fe incorporation into ferritin is unknown, our experiments suggest the possibility that some iron finds its way into ferritin as Fe(III) chelate.

Iron has a marked tendency to form complexes with a variety of ligands, and this has been exploited in biology to give compounds with different functions. The affinity for ligands depends on the oxidation state of the iron, ferric iron having a particularly high affinity for oxy-ligands, including water. As a result, at physiological pH ferric iron tends to be precipitated as polynuclear 'ferric hydroxide'. The iron-storage compound ferritin is such a polymeric hydrolysate of ferric iron, but the polymer is prevented from precipitation by the presence of a soluble protein coat. The solution chemistry of ferric iron presents both the cell and the experimenter with a problem: how to keep ferric iron soluble and monomeric, so that it can get inside the protein shell of ferritin and bind to it. Fe(III) can be kept in solution by chelation, but the chelator must not have too high an affinity for the cation or it will not release it to the protein. Very little is known about ferritin biosynthesis, but the evidence suggests that it is a cumulative process starting from empty apoferritin shells (Drysedale & Munro, 1966; Hoy & Harrison, 1976; Lee & Richter, 1977). One way of getting round the problem of the insolubility of Fe(III) that has been found successful *in vitro* is to start with ferrous iron (Bielig & Bayer, 1955). In the presence of apoferritin and O_2 or other oxidants ferrous iron is bound by the protein and converted to the ferric hydrolysis polymer, indeed apoferritin accelerates this process (Niederer, 1970; Macara *et al.*, 1972, 1973; Bryce & Crichton, 1973). We have made a detailed study of the oxidative formation of ferritin *in vitro* (Macara *et al.*, 1972, 1973; Harrison *et al.*, 1974), but there is no clear evidence for an oxidative pathway *in vivo*. If ferritin is formed by this pathway, the question arises as to how Fe(II) is protected from oxidation in the cell before incorporation into ferritin. Several workers

(e.g. Drysdale & Munro, 1966; Hoy & Harrison, 1976) have shown that iron injected as Fe(III) is incorporated into ferritin, but it is uncertain whether or not the iron must undergo reduction and reoxidation during ferritin formation. Hoy & Harrison (1976) also showed that if rat liver ferritin is incubated *in vitro* with ferric ammonium citrate carrying a ^{59}Fe label then some counts are retained by ferritin. In the present paper we attempt to characterize the binding of Fe(III) by ferritin and apoferritin more fully, since this could provide an alternative or additional means of iron accumulation.

Materials and Methods

Horse spleen ferritin (twice-crystallized, Cd-free) was purchased from Miles Laboratories (Stoke Poges, Slough, Bucks., U.K.). Ferritin fractions of different iron contents were obtained by centrifugation through sucrose gradients as described by Hoy & Harrison (1975). Apoferritin was prepared from ferritin by dialysis against 0.1 M-thioglycollic acid/0.1 M-sodium acetate mixture, pH 4.25, followed by exhaustive dialysis against glass-distilled water and precipitation with 35% (w/v) $(\text{NH}_4)_2\text{SO}_4$. All chemicals were AnalaR grade (BDH Chemicals, Atherstone, Warwickshire, U.K.). Ferric iron in ferritin was measured as its absorbance at 420 nm ($A_{420}^{1\% \text{Fe}} = 100$; Treffry & Harrison, 1978). Ferrous iron was measured as the absorbance of its bipyridine complex at 520 nm, its concentration being read from a standard curve. $^{59}\text{FeCl}_3$ and $[1,5\text{-}^{14}\text{C}]\text{citric acid}$ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The required label was added either directly to ferric citrate or to stock $\text{Fe}(\text{NO}_3)_3$ solutions to the desired radioactivity

(0.2–0.5 $\mu\text{Ci/ml}$). Solutions were usually equilibrated overnight before use.

Radioactivity was measured in a Triton/toluene scintillation fluid (Turner, 1968) with a Packard model 3385 liquid-scintillation spectrometer. Where applicable, samples were first bleached with thio-glycollate to prevent colour quenching. A chemical quench curve was used for the correction of c.p.m. to d.p.m.

Protein concentrations were measured by the method of Lowry *et al.* (1951), with apoferritin as standard, and apoferritin was measured by its absorbance at 280nm ($A_{1\text{cm}}^{1\%} = 9.0$). Spectrophotometric measurements were made in either a Zeiss M4QIII spectrophotometer or a Unicam SP.1800 double-beam spectrophotometer.

Fe(III)-chelator solutions

Ferric citrate was prepared by dissolving $\text{Fe}(\text{NO}_3)_3$ in a trisodium citrate solution so as to give Fe/citrate in a molar ratio of 1:20. Spiro *et al.* (1967) has previously shown that this molar excess of citrate is sufficient to prevent the polymerization of 1mM-Fe(III). The concentration given for these solutions refers to the concentration of ferric ions. Other Fe(III)-chelator solutions were prepared by adding the required amount of 10mM- $^{59}\text{Fe}(\text{NO}_3)_3$ to the chelator solution.

Fe(II) uptake

With iron added as Fe(II), iron uptake was measured at 310 or 420nm as described by Macara *et al.* (1972, 1973). To provide Fe(II), solutions of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ were prepared in distilled water through which N_2 had been bubbled for 1 h.

Iron release

Rates of iron release were measured in 20mM-sodium acetate/20mM-thioglycollic acid solution adjusted to pH 5.0. The reaction mixture contained 6.4mM- α -bipyridine and the iron released was measured as the absorbance at 520nm of its bipyridine complex.

Patterns of release of ^{59}Fe and total iron were studied with the aid of an ultrafiltration cell as described by Treffry & Harrison (1978). Total iron was assayed by the bipyridine method, and ^{59}Fe by radioactivity counting.

Equilibrium dialysis

Experiments were carried out at 25°C in an MSE Dianorm equilibrium-dialysis apparatus with a cell capacity of 2×1 ml. The buffer solution used in each half-cell contained 0.2% (w/v) NaN_3 and 0.1M- NaNO_3 . The protein solution was placed in one-half of the cell and the Fe(III) chelate solution in the other half. The two cells were separated by a spectrapore 2 membrane (MSE, Manor Royal, Crawley, Sussex, U.K.). Although, in the absence of protein,

equilibrium between the two halves of the cell was established within 24h, in the presence of protein, ^{59}Fe counts in the protein compartment increased continuously, but slightly, over 6 days. Unless otherwise stated cells were left for 7 days before each half was emptied and a proportion was taken for radioactivity counting. The amount of bound ^{59}Fe was obtained from the difference in counts in the two half-cells. In various experiments in which pH and other conditions were altered, the fraction of ^{59}Fe bound ranged from 0.02 to 0.96. To compare amounts of iron and citrate binding parallel experiments were set up. In one set of cells the Fe(III)-citrate was labelled with ^{59}Fe and in the other with $[1,5\text{-}^{14}\text{C}]\text{-citrate}$. When the effect of Tb on the Fe binding was studied, 0.2mM- TbCl_3 was added to both the protein and the buffer compartment.

Fe(III) binding in large volumes

When relatively large amounts of ferritin containing bound $^{59}\text{Fe(III)}$ were required for further analysis, ferritin solutions were incubated in bulk with labelled Fe(III)-citrate under conditions that had been shown by equilibrium dialysis to result in high binding. Usually 150–250 μg of ferritin/ml was incubated with 0.25–0.5mM-Fe(III)-citrate in 0.02M-glycine/NaOH buffer, pH 9.4, containing 0.02% (w/v) NaN_3 . After incubation for 3–6 days the sample was concentrated in an Amicon ultrafiltration cell (50ml capacity). Free Fe(III) was removed by passing through at least 5 vol. of water. Samples to be fractionated by sucrose-density-gradient centrifugation were further concentrated in an Amicon 8MC ultrafiltration cell and washed with 5 vol. of density gradient buffer.

Results

Fe(III)-binding studies by equilibrium dialysis

Effect of pH and chelator. Among various Fe(III) chelates tested, binding of $^{59}\text{Fe(III)}$ to ferritin was observed with oxalate, citrate or nitrilotriacetate as chelator. Table 1 shows the extent of Fe(III) binding to unfractionated native ferritin after 24h of dialysis. The concentration of Fe(III)-oxalate (at molar ratios of 1:10, 1:20 or 1:50, Fe/oxalate) could not be raised above 0.1 mM because of precipitation at the higher concentrations. Binding from Fe(III)-nitrilotriacetate was relatively insensitive to pH in the range 7.4–9.4. With Fe(III)-citrate, binding was markedly higher at pH 9.4 than at pH 7.4 (Table 1), and the extent of binding was also dependent on the buffer used (Table 2). Relatively high binding was obtained in glycine/NaOH or NaHCO_3 buffers at pH 9.4, compared with sodium borate at the same pH. The same concentration of glycine or bicarbonate, buffered at pH 7.4 with Tris/HCl, gave much lower binding.

Table 1. *Effects of chelators on Fe(III) binding to ferritin*

The Table shows the extent of binding of $^{59}\text{Fe(III)}$ to unfractionated ferritin after 24h in an equilibrium-dialysis cell. Protein and iron were placed in separate compartments at the start of the experiments. The buffer concentration was 20mM. Abbreviation: NTA, nitrilotriacetate.

Fe(III)-chelator (concs., mM)	Buffer	Protein concn. (mg/ml)	Fraction of Fe(III) bound	Fe(III) atoms bound/molecule
Fe-citrate (0.5 : 10.0)	Glycine/NaOH, pH9.4	1.27	0.72	126
Fe-citrate (0.5 : 10.0)	Tris/HCl, pH 7.4	1.27	0.05	9
Fe-NTA (0.5 : 0.5)	Glycine/NaOH, pH9.4	1.27	0.39	68
Fe-NTA (0.5 : 0.5)	Tris/HCl, pH 7.4	1.27	0.31	54
Fe-NTA (0.1 : 0.5)	Tris/HCl, pH 7.4	0.13	0.13	8
Fe-oxalate (0.1 : 5.0)	Tris/HCl, pH 7.4	0.50	0.62	41

Table 2. *Effects of buffers on Fe(III) binding to ferritin*

The Table shows the extent of binding of $^{59}\text{Fe(III)}$ to unfractionated ferritin after 24h in an equilibrium-dialysis cell. Protein and iron were placed in separate compartments at the start of the experiments. The buffer concentration was 20mM.

Fe(III)-citrate concs. (mM)	Buffer	Fraction of Fe(III) bound	Fe(III) atoms bound/molecule
0.25 : 5.0	Glycine/NaOH, pH9.4	0.91	100
0.25 : 5.0	Sodium borate, pH9.4	0.13	14
0.50 : 10.0	Glycine/NaOH, pH9.4	0.63	140
0.50 : 10.0	Tris/glycine, pH 7.4	0.05	11

Fig. 1. *Effect of Fe(III)-citrate concentration on Fe(III) binding to native ferritin*

Dialysis was carried out for 24h in 20mM-glycine/NaOH buffer, pH9.4, in the presence of 0.1M- NaNO_3 . Unfractionated ferritin (1mg/ml) was placed in one-half of a 2×1 ml equilibrium-dialysis cell and ^{59}Fe -labelled Fe(III)-citrate in the other half, together with a 20-fold excess of citrate over the concentration of Fe(III)-citrate used.

Dependence of binding on Fe(III)-citrate concentration

In an experiment in which the protein concentration was kept constant at 1mg/ml the binding of labelled Fe(III) to ferritin showed an unusual dependence on Fe(III)-citrate concentration as shown in Fig. 1. At higher concentrations binding was diminished. This may be due to increasing removal of Fe(III) from ferritin by citrate, since the excess of free citrate is increased from 1.8 to 36.0mM as iron concentration is raised from 0.1 to 2.0mM on the assumption that the Fe(III) forms a $[\text{Fe}(\text{Cit})_2]^{5-}$ complex (Spiro *et al.*, 1967).

In a parallel experiment the binding of labelled citrate followed a pattern similar to that of Fe(III).

Dependence of binding of Fe(III)-citrate on protein concentration

Fig. 2 shows the dependence on protein concentration of Fe(III) binding at two separate Fe(III)-citrate concentrations. In each case more Fe(III) atoms are bound/protein molecule at the lower protein concentrations (higher Fe(III)-citrate/protein). At the higher protein concentrations ($>200 \mu\text{g/ml}$) more Fe(III) is bound/molecule at the higher Fe(III)-citrate concentrations, whereas at protein concentrations below $200 \mu\text{g/ml}$ the situation is reversed and more Fe(III) is bound from 0.25mM than from 0.5mM-Fe(III)-citrate. This again suggests competition of citrate with protein for Fe(III) at the higher citrate/protein ratios.

Since highest levels of binding were observed with Fe(III)-citrate, subsequent experiments were carried out with this chelate in glycine/NaOH or NaHCO_3 buffer at pH9.4.

Net Fe(III) binding or exchange? Effects of added Tb(III)

The results described above do not indicate whether the binding of labelled Fe(III) represents a net binding or an exchange with Fe(III) already present within the ferritin molecule. This was checked by analysing both the binding of $^{59}\text{Fe(III)}$ in the ferritin

Fig. 2. Effect of protein concentration on Fe(III) binding to native ferritin

Dialysis was carried out for 24h in 20mM-glycine/NaOH buffer, pH9.4, in the presence of 0.1 M-NaNO₃. ●, 0.5 mM-Fe(III)-citrate; ▲, 0.25 mM-Fe(III)-citrate; other conditions were as in Fig. 1.

Table 3. Effect of Fe(III) exchange on net Fe(III) binding to ferritin

Dialysis was carried out in 2×1 ml of equilibrium-dialysis cells for 5 days in buffer solution containing 20mM-glycine/NaOH, pH9.4, 0.1M-NaNO₃ and 0.02% (w/v) NaN₃. At the start of the dialysis compartment 1 contained one of the following: (a) 100 µg of ferritin in buffer solution, (b) 100 µg of ferritin plus 0.2mM-TbCl₃ in buffer, (c) buffer solution alone. Compartment 2 contained 0.25mM- ^{59}Fe -labelled Fe(III)-citrate plus 5mM-sodium citrate in buffer solution at the start and was analysed for ^{59}Fe and total Fe at the end of the experiment. The $^{59}\text{Fe(III)}$ or total Fe(III) remaining in compartment 2, after dialysis against ferritin solution (a) or (b), expressed as a fraction of that remaining after dialysis against buffer alone (c), is listed in columns 2 and 3.

Fe(III) in compartment 2 measured as ^{59}Fe	Relative amount of Fe in compartment 2 after dialysis against (a), (b) or (c)	
	(a)/(c)	(b)/(c)
$^{59}\text{Fe(III)}$	0.59	0.90
Total Fe(III)	0.80	1.10

compartment and the amount of Fe(III) in the buffer compartment at the end of an equilibrium-dialysis experiment. The results were also compared with those of a control experiment in which Fe(III)-citrate was equilibrated in the absence of protein and with a third experiment in which the effect of adding Tb(III) on Fe(III) binding by the protein was observed. Table 3 shows a net loss of 40% of the $^{59}\text{Fe(III)}$ to the protein compartment as compared with 20% of the Fe(III), or a net addition of Fe(III) to ferritin corresponding to half the number of $^{59}\text{Fe(III)}$ atoms bound. In the presence of Tb(III) there appears to be a net loss of Fe(III) from ferritin, although 10% of the $^{59}\text{Fe(III)}$ is bound. Since the net loss is apparently the same with or without Tb(III), it seems that Tb(III) inhibits the uptake of iron into, but not its removal from, the protein.

Fe(III)-binding patterns as a function of molecular iron content

Fig. 3 shows the binding of Fe(III) by fractions of different molecular iron content plotted either as Fe(III) atoms bound/molecule (Fig. 3a) or Fe(III) atoms bound/Fe atoms present (Fig. 3b). The upper curves are the results obtained in equilibrium-dialysis experiments in which solutions of fractions (150 µg of protein/ml) were dialysed against 0.5mM-Fe(III)-citrate in 0.02M-glycine/NaOH buffer, pH9.4, containing 0.1 M-NaNO₃. The results represent total $^{59}\text{Fe(III)}$ bound rather than net Fe(III). The lower curves were obtained as follows. Native unfractionated ferritin was incubated with $^{59}\text{Fe(III)}$ -citrate solutions under the same conditions as in the equilibrium-dialysis experiments. The unbound $^{59}\text{Fe(III)}$ was removed by ultrafiltration and the sample was then fractionated by sucrose-density centrifugation. Comparison of the two binding patterns suggests that a considerable portion of the Fe(III) is bound only loosely and is removed on washing.

In a parallel equilibrium-dialysis experiment a ferritin fraction containing 3500 Fe atoms/molecule (protein concentration 50 µg/ml) dialysed against 0.25mM-Fe(III)-citrate appeared to bind 1750 $^{59}\text{Fe(III)}$ atoms. After washing with 7 vol. of water in an ultrafiltration cell, only 600 $^{59}\text{Fe(III)}$ atoms/ferritin molecule remained bound and these could not be removed by further washing. In another experiment both $^{59}\text{Fe(III)}$ and [1,5- ^{14}C]citrate binding were measured before and after washing. Parallel cells containing the same concentrations of protein (unfractionated ferritin, 0.25mg/ml) and Fe(III)-citrate (0.02mM), carrying either a ^{59}Fe or a ^{14}C label, were equilibrated for 5 days. Approximately equimolar quantities of Fe(III) or citrate were bound per ferritin molecule before washing (200 atoms or molecules respectively), but after exhaustive washing, whereas 160 $^{59}\text{Fe(III)}$ ions remained bound, only 34

Fig. 3. *Binding of Fe(III) to ferritin fractions*

Net binding is expressed in (a) as Fe(III) atoms bound/ferritin molecule or in (b) as Fe(III) atoms bound/ferritin iron atoms. Data points (▲) were obtained from equilibrium-dialysis experiments in which ferritin fractions of different iron contents (concn. $150\ \mu\text{g}$ of protein/ml) were dialysed for 7 days at 25°C against $0.5\ \text{mM}$ -Fe(III)-citrate labelled with ^{59}Fe in $20\ \text{mM}$ -glycine/NaOH buffer, pH 9.4, containing $10\ \text{mM}$ -sodium citrate, $100\ \text{mM}$ -NaNO₃ and 0.02% (w/v) NaN₃. Data points (●) were obtained in a separate experiment in which unfractionated ferritin (concn. $150\ \mu\text{g}$ of protein/ml) was incubated for 7 days at 25°C with $0.5\ \text{mM}$ -Fe(III)-citrate labelled with ^{59}Fe in buffer solution as used for equilibrium dialysis. After incubation, unbound Fe(III)-citrate was washed off by ultrafiltration, and the ferritin was then fractionated by density-gradient centrifugation. Samples were analysed for ^{59}Fe and total Fe.

Fig. 4. *Release patterns of ^{59}Fe added to ferritin either as Fe(III) or as Fe(II)*

The ferritin fraction used in these experiments contained $1560\ \text{Fe}$ atoms/molecule and, in addition, either $168\ ^{59}\text{Fe(III)}$ atoms bound by incubation with Fe(III)-citrate, or $500\ ^{59}\text{Fe(III)}$ atoms bound by addition of Fe(II)(NH₄)₂(SO₄)₂ in the presence of an oxidant, as described in the Materials and Methods section. Fe release was carried out in an ultrafiltration

cell with $20\ \text{mM}$ -thioglycolic acid/ $20\ \text{mM}$ -sodium acetate, pH 4.3. The volume of the ferritin solutions was kept constant at $5\ \text{ml}$, and the eluate was collected in $1\ \text{ml}$ fractions, and each fraction was assayed for ^{59}Fe by radioactivity counting and for total Fe by the bipyridine method. Iron added as $^{59}\text{Fe(II)}$ and released $4\ \text{h}$ (▲), $72\ \text{h}$ (○) or $30\ \text{days}$ (□) after addition to fraction. Iron added as $^{59}\text{Fe(III)}$ and released $72\ \text{h}$ (●) or $30\ \text{days}$ (■) after addition. Line B represents the theoretical pattern expected if all the added ^{59}Fe is released before any of the ^{56}Fe originally present in the ferritin fraction. Line A + B represents the expected pattern if ^{59}Fe and ^{56}Fe were randomly mixed.

The binding patterns in Fig. 3 show that apoferritin does not bind $^{59}\text{Fe(III)}$ from ferric citrate under the

cell with $20\ \text{mM}$ -thioglycolic acid/ $20\ \text{mM}$ -sodium acetate, pH 4.3. The volume of the ferritin solutions was kept constant at $5\ \text{ml}$, and the eluate was collected in $1\ \text{ml}$ fractions, and each fraction was assayed for ^{59}Fe by radioactivity counting and for total Fe by the bipyridine method. Iron added as $^{59}\text{Fe(II)}$ and released $4\ \text{h}$ (▲), $72\ \text{h}$ (○) or $30\ \text{days}$ (□) after addition to fraction. Iron added as $^{59}\text{Fe(III)}$ and released $72\ \text{h}$ (●) or $30\ \text{days}$ (■) after addition. Line B represents the theoretical pattern expected if all the added ^{59}Fe is released before any of the ^{56}Fe originally present in the ferritin fraction. Line A + B represents the expected pattern if ^{59}Fe and ^{56}Fe were randomly mixed.

conditions used. This was confirmed in separate experiments with apoferritin, in none of which was any binding of Fe(III) observed. The finding that iron must already be present inside the molecule for binding lends further support to the conclusion that Fe(III) is attached to the iron core. This is also suggested by the results discussed below.

Comparison of Fe-release patterns from ferritin with Fe added as Fe(II) or Fe(III)

In parallel experiments ^{59}Fe was added to a ferritin fraction (containing 1560 Fe atoms/molecule) either as Fe(III)-citrate or as $\text{Fe(II)(NH}_4)_2(\text{SO}_4)_2$ in the presence of an oxidant. The amount of iron added in each case represented approx. 500 Fe atoms/molecule. After removal of free iron the ferritin fraction retained 168 ^{59}Fe atoms (10% of total ferritin Fe) from the Fe(III)-citrate and 500 ^{59}Fe atoms (25% of total ferritin Fe) from iron added as Fe(II). Iron was released with thioglycollate in an ultrafiltration cell as described in the Materials and Methods section, and patterns of ^{59}Fe release as a function of total Fe release obtained at various times after ^{59}Fe addition are shown in Fig. 4. As discussed previously (Hoy *et al.*, 1974b), if all the ^{59}Fe was released first, it should follow line B in Fig. 4, and the remaining ^{56}Fe release should follow the horizontal line A. Such a theoretical pattern would only be expected if either the added iron was very much more easily removed than that present in the native molecule, or the added iron completely covered the native iron micelle, so that it had to be removed first. The latter condition is more likely to

apply to the case when iron was added as Fe(II), since a much larger number of Fe atoms was taken up, and indeed Fig. 4 shows that it more closely approximates to the theoretical model in the initial stages of release. Release patterns would not be expected to follow the simple model after the initial stages, since, once a covering layer of ^{59}Fe has been moved from one part of the micelle, the reagent can release the pre-existing micellar ^{56}Fe . Hence the pattern would be expected to approach the A+B line (the theoretical line for complete isotope mixing) as more Fe was released. The release patterns of Fe added by the two methods are broadly similar, and both suggest the 'last-in-first-out' principle expected for iron added to a micelle, i.e. the added Fe is placed on the micelle surface and does not mix rapidly with Fe already present in the molecule (Hoy *et al.*, 1974b).

Fig. 4 shows that if the preparations are left at 4°C for several days before release is started, the release pattern moves slightly towards the A+B line, suggesting a small extent of exchange of ^{59}Fe and ^{56}Fe atoms. However, complete equilibration did not occur even after 30 days. This result differs from that of Hoy *et al.* (1974b), who reported complete mixing after 3 days at 37°C. The difference in results may in part be due to differences in the experimental procedures. Harris (1978) has also found no equilibration of ^{59}Fe taken up into ferritin micelles after 14 days at 4°C when transferrin was the source of Fe. In another experiment release patterns of two reconstituted ferritins were compared (Fig. 5). In Fig. 5(a) iron was added in the order $^{56}\text{Fe(II)}$ followed by $^{59}\text{Fe(III)}$. In Fig. 5(b) a third addition of $^{56}\text{Fe(II)}$ was made. When

Fig. 5. Release patterns from reconstituted ferritins containing ^{59}Fe

Reconstituted ferritins used in these experiments were made by (a) reconstitution to 1400 Fe atoms/molecule with $^{56}\text{Fe(II)}$ followed by addition of 350 Fe atoms/molecule from $^{59}\text{Fe(III)}$ citrate; (b) as (a) but with the further addition, as $^{56}\text{Fe(II)}$, of 1500 Fe atoms/molecule. Reconstitution was as described in the Materials and Methods section and release as described in Fig. 4. The theoretical release pattern if the iron was released in order of addition would be CBA. Line A+B+C represents the expected pattern if isotopes were completely mixed.

release was followed, the label was released first in Fig. 5(a) but not in Fig. 5(b), confirming that in Fig. 5(b) the radioactive isotope was present in the micelle between two ^{56}Fe layers.

Effect of bound Fe(III) on initial rates of release or uptake of further iron

Native ferritin was incubated with Fe(III)-citrate under conditions that gave, after washing, an average of 10% of its iron bound from this source. In a control experiment native ferritin was treated with citrate alone. After washing, the samples were fractionated by sucrose-density centrifugation. Initial rates of release by thioglycollate were estimated spectrophotometrically and the results for the two samples are shown in Fig. 6. Although there is a considerable amount of scatter in the data, the release rates appear to be the same, i.e. the added iron is not obviously released faster than the native micellar iron. The release patterns in Fig. 6 show a maximum initial rate for fractions containing about 2000 Fe atoms/molecule, or molecules that are approx. one-half full. This result is similar to that previously reported when 1,10-phenanthroline was used for Fe release (Hoy *et al.*, 1974a).

A similar pattern was obtained when initial rates of further Fe addition were measured with ferritin

fractions that had been incubated with Fe(III), the further Fe being added as Fe(II)(NH₄)₂(SO₄)₂. Prior incubation with Fe(III)-citrate had little or no effect on rates of addition of Fe(II) to fractions of similar Fe content that had not been so treated. These results indicate that iron incorporated into ferritin as either Fe(II) or Fe(III) gives products that are similar to each other and to native ferritin. They lend further circumstantial evidence to the conclusion that the firmly bound Fe(III) lies inside the molecule attached to its iron core.

Discussion

Studies by Miller & Perkins (1969) and Harris (1978) have shown that iron can be transferred from transferrin to ferritin in the presence of mediators. Miller & Perkins (1969) found that reducing agents such as ascorbic acid were essential for removal of iron from transferrin and incorporation into ferritin, but that chelators enhanced transfer. Harris (1978) on the other hand has found that transfer can be effected in the absence of reductants, provided that a suitable chelator (citrate or ATP) was present. The net flow, however, was always from ferritin to transferrin in his experiments. Our present experiments show that Fe(III) can be bound by ferritin [from $^{59}\text{Fe(III)-citrate}$] and, although some Fe(III) was also lost from ferritin under the conditions used, a small net gain of ferric iron occurred. The product closely resembles native ferritin in several respects. We also find that there are some differences in iron incorporation when iron is added as Fe(III), or as Fe(II) in the presence of an oxidant. In the formation of ferritin from apoferritin and Fe(II) the first step is almost certainly the binding of Fe(II) by the protein (Macara *et al.*, 1972, 1973). In the present experiments we found no evidence of Fe(III) binding by apoferritin. This could have been due to the chelate molecules used, which may have been incapable of forming suitable ternary complexes with apoferritin. We also found that only a limited amount of Fe(III) was bound by ferritin from the citrate complex, whereas much more iron can be taken in as Fe(II) giving molecules that are full, or nearly full, of iron. The binding of Fe(III) was also relatively slow compared with uptake as Fe(II). The slow and relatively small net binding of Fe(III) may be partly a consequence of the simultaneous binding of small amounts of citrate, which may interfere with further iron uptake. The uptake of Fe(III) by ferritin fractions (Fig. 3) also differs, in its dependence on iron content, from that observed with Fe(II) uptake (Harrison *et al.*, 1974). Fe(III) uptake increased continuously with iron content, whereas Fe(II) uptake was maximal for molecules approximately one-third to one-half full. This may partly be explained if the slow binding of Fe(III) occurs not only on readily

Fig. 6. Rates of Fe release from ferritin fractions, with and without additional bound Fe(III)

Ferritin fractions of different iron contents were prepared by sucrose-density-gradient centrifugation, after unfractionated ferritin (250 μg of protein/ml) had been incubated for 7 days with either Fe(III)-citrate [0.25 mM-Fe(NO₃)₃ plus 5 mM-sodium citrate] in 20 mM-glycine/NaOH buffer, pH 9.4, containing 0.02% (w/v) NaN₃ ($\blacktriangle$) or in the same solution as above but without Fe(NO₃)₃ ($\bullet$).

available micelle surface sites but if there is time for some Fe(III) to find its way also into some interstitial irregularities or discontinuities between neighbouring crystallites. These factors, in addition to the relatively low number of $^{59}\text{Fe(III)}$ atoms bound compared with $^{59}\text{Fe(II)}$ may contribute to the differences in release patterns shown in Fig. 4 for iron supplied in the two valence states. Iron supplied as Fe(II) most closely follows the 'last-in-first-out' principle (Hoy *et al.*, 1974b). Williams *et al.* (1978) have examined by Mössbauer spectroscopy a single native ferritin fraction containing approx. 2000 Fe atoms (av.) to which a further 200 $^{57}\text{Fe(III)}$ atoms were bound from Fe(III)-citrate as described here. According to their results the additional 10% Fe slightly altered the micelle-size distribution compared with the native fraction principally by increasing the size of the smaller particles present.

Our experiments *in vitro* unfortunately tell us little about how iron is incorporated into ferritin *in vivo*. They do indicate that, although Fe(II) may be required for the initial nucleation stage of ferritin formation (Macara *et al.*, 1972), some binding of Fe(III) to ferritin is possible. Our experiments also confirm that citrate mediates the slow release of iron from ferritin. Whether there is a net gain or a net loss depends on the interaction of several factors in this complex system. Evidence has been provided that some cellular iron is present as a labile pool (Jacobs, 1977) which may be available for synthesis of haem, iron-containing enzymes or ferritin. It is not known whether this pool is in the form of Fe(II) or Fe(III), although at physiological pH, Fe(III) is the more stable state (Aisen, 1977). It is possible that ferritin acquires iron from different sources in different forms. Conceivably ferritin could acquire iron alternatively as Fe(II) or Fe(III); for example, it might obtain iron from transferrin after reduction at membrane receptor sites, and Fe(III) could also be added through the chelatable pool. However, much more work needs to be done to establish intracellular iron pathways with confidence.

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