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Review

The ferritins: molecular properties, iron storage function and cellular regulation [1]

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Contents

1. Summary . . . 162

2. Introduction . . . 162
   2.1. Iron metabolism and the importance of iron storage . . . 162
   2.2. Iron metabolism in humans: an outline . . . 163
   2.3. Scope of the review . . . 164

3. Ferritin molecules: their distribution and structures . . . 164
   3.1. Ferritin protein shells of animals, plants and bacteria . . . 164
   3.2. Ferritin iron-cores . . . 171

4. Iron storage processes . . . 171
   4.1. Iron-loading of apoferritin shells – the catalysis of Fe(II)-oxidation by apoferritin in vitro . . . 171
   4.2. The catalytic sites on H-chains: the ferroxidase centres . . . 175
   4.3. The mechanism of Fe(II) oxidation at ferroxidase centres . . . 176
   4.4. Fe(III)-tyrosinate and tyrosine radicals . . . 177
   4.5. The dinuclear Fe centres of ferritins and other proteins compared . . . 177
   4.6. Role of third iron site? . . . 178
   4.7. Why heteropolymers? – the role of L-chains and the disadvantage of too many H-chains . . . 178
   4.8. The route of Fe(II) entry into ferritin protein shells . . . 179
   4.9. Iron oxidation in the absence of dioxygen . . . 180
   4.10. The effect of phosphate on iron-core formation . . . 180
   4.11. Can ferritin store iron as Fe(II)? . . . 181
   4.12. Can ferritin bind Fe(III)? The transfer of Fe(III) between ferritin molecules . . . 181
   4.13. Model complexes for ferritin iron-cores . . . 182

5. Haemosiderin . . . 182

6. Ferritin as a source of iron . . . 183
   6.1. In vitro studies of iron release from ferritin and bacterioferritin and their biological relevance . . . 183
   6.2. Ferritin as a donor of iron to hepatocytes: its involvement in their cycling of red cell iron . . . 184

7. Regulation of iron storage . . . 185
   7.1. Ferritin biosynthesis . . . 185

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[1] This review is dedicated to the memory of Hamish N. Munro, a pioneer in the study of iron metabolism, especially ferritin biosynthesis and its regulation. A great scientist, who is sadly missed since his death in 1995.

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7.2. Iron induction of ferritin synthesis in animals . . . 186
7.3. Translational regulation of ferritin by iron . . . 187
7.4. Haemin as the ferritin inducer: an alternative model of regulation . . . 189
7.5. The untranslated regions of ferritin on RNA and the repression of translation: the involvement of IRE flanking regions? . . . 189
7.6. Co-ordinated regulation of iron metabolism . . . 191
7.7. Non-iron mediated induction of ferritin synthesis . . . 191

8. The ferritin genes . . . 192

9. Other biological functions associated with ferritins . . . 193
9.1. Ferritin and oxidative damage . . . 193
9.2. Regulators of protein synthesis? . . . 194
9.3. Extracellular ferritins . . . 194
9.4. Ferritin and the regulation of cellular proliferation . . . 194
9.5. Genetic hyperferritinaemia . . . 195

10. Conclusions and perspectives . . . 195

1. Summary

The iron storage protein, ferritin, plays a key role in iron metabolism. Its ability to sequester the element gives ferritin the dual functions of iron detoxification and iron reserve. The importance of these functions is emphasised by ferritin's ubiquitous distribution among living species. Ferritin's three-dimensional structure is highly conserved. All ferritins have 24 protein subunits arranged in 432 symmetry to give a hollow shell with an 80 Å diameter cavity capable of storing up to 4500 Fe(III) atoms as an inorganic complex. Subunits are folded as 4-helix bundles each having a fifth short helix at roughly 60° to the bundle axis. Structural features of ferritins from humans, horse, bullfrog and bacteria are described: all have essentially the same architecture in spite of large variations in primary structure (amino acid sequence identities can be as low as 14%) and the presence in some bacterial ferritins of haem groups. Ferritin molecules isolated from vertebrates are composed of two types of subunit (H and L), whereas those from plants and bacteria contain only H-type chains, where 'H-type' is associated with the presence of centres catalysing the oxidation of two Fe(II) atoms. The similarity between the dinuclear iron centres of ferritin H-chains and those of ribonucleotide reductase and other proteins suggests a possible wider evolutionary linkage. A great deal of research effort is now concentrated on two aspects of ferritin: its functional mechanisms and its regulation. These form the major part of the review. Steps in iron storage within ferritin molecules consist of Fe(II) oxidation, Fe(III) migration and the nucleation and growth of the iron core mineral. H-chains are important for Fe(II) oxidation and L-chains assist in core formation. Iron mobilisation, relevant to ferritin's role as iron reserve, is also discussed. Translational regulation of mammalian ferritin synthesis in response to iron and the apparent links between iron and citrate metabolism through a single molecule with dual function are described. The molecule, when binding a [4Fe-4S] cluster, is a functioning (cytoplasmic) aconitase. When cellular iron is low, loss of the [4Fe-4S] cluster allows the molecule to bind to the 5'-untranslated region (5'-UTR) of the ferritin m-RNA and thus to repress translation. In this form it is known as the iron regulatory protein (IRP) and the stem-loop RNA structure to which it binds is the iron regulatory element (IRE). IREs are found in the 3'-UTR of the transferrin receptor and in the 5'-UTR of erythroid aminolaevulinic acid synthase, enabling tight co-ordination between cellular iron uptake and the synthesis of ferritin and haem. Degradation of ferritin could potentially lead to an increase in toxicity due to uncontrolled release of iron. Degradation within membrane-encapsulated 'secondary lysosomes' may avoid this problem and this seems to be the origin of another form of storage iron known as haemosiderin. However, in certain pathological states, massive deposits of 'haemosiderin' are found which do not arise directly from ferritin breakdown. Understanding the numerous inter-relationships between the various intracellular iron complexes presents a major challenge.

2. Introduction

2.1. Iron metabolism and the importance of iron storage

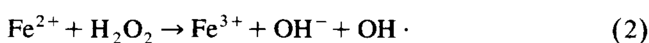
The importance of iron in biology reflects both its chemical versatility and its abundance. It is the second most abundant metal in the earth's crust (after aluminium). One of the properties of iron (not shared by aluminium), which has been widely exploited, is its ability to change valence. This ability can be subtly modified by a variety of organic ligands to provide electron transport proteins with a wide range of redox potentials. Iron is also found at the active centres of many enzymes and of oxygen carrier proteins.

However, there are problems in the physiological management of iron which have arisen from the increase in atmospheric oxygen since early times. One such problem is that despite its overall abundance, usable iron is in short supply. This is because, at physiological pH under oxidising conditions, iron is extremely insoluble. Several strategies must therefore be adopted for its acquisition. Bacteria and fungi mainly acquire iron by production of 'siderophores' – small organic molecules with a very high affinity for Fe(III), which act as scavengers [1–3]. The synthesis of these compounds, the uptake of the Fe(III) complexes, and the release of this iron inside the cell require the action of a complex array of proteins [4–8]. Plants adopt several strategies for iron assimilation from soil (reviewed in Ref. [9]). They may exploit siderophores produced by symbiotic micro-organisms, synthesize and release their own chelating molecules, release protons or organic acids leading to localised lowering of soil pH, or synthesise reductants, which convert Fe(III) to the more soluble Fe(II). Roots also contain specific plasma membrane translocating systems. Higher animals, e.g., man, acquire iron from foodstuffs. Here bioavailability may again be limited. Plants tend to be a poor source of iron, because of the presence of phosphates, phytates and polyphenols, all of which inhibit absorption by formation of insoluble complexes. Meat is a better source of iron, because haem is readily absorbed.

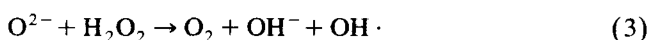
The functional importance of iron, coupled with its generally poor bioavailability, make it essential for living organisms to husband this element. Virtually all life forms require iron except for some strains of lactobacillus, where the role of iron may be assumed by another metal such as Co or Mn. The iron-storage molecule, ferritin, is also ubiquitous. A second form of storage iron, haemosiderin, is found in both plants and animals. It is generally associated with high cellular iron levels.

Most of the iron present in living organisms is tightly complexed in proteins, although it may also be present in a soluble 'pool' of low molecular weight complexes such as ferric citrate and Fe(III) ATP [10]. Free iron concentrations are low: not more than about 10^{-18} M Fe(III) and about 10^{-8} M Fe(II) [11]. Uncomplexed iron together with superoxide (which reduces Fe(III), Eq. (1)) and hydrogen peroxide (which is decomposed by the Fenton reaction, Eq. (2)) provide a lethal mixture containing reactive hydroxyl radicals. The sum of these two reactions is the so-called iron-catalysed Haber-Weiss reaction [12] (Eq. (3)). Fe(III) produced as in Eq. (2) can also be reduced by ascorbate leading to further radical production.

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2 \quad (1)$$



$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot \quad (2)$$



$$O^{2-} + H_2O_2 \rightarrow O_2 + OH^- + OH\cdot \quad (3)$$

The hydroxyl radical is extremely reactive causing lipid peroxidation, DNA strand breaks and degradation of other biomolecules [13]. In vertebrates defence against the toxic effect of Fe and oxygen mixtures is provided by two specialised iron-binding proteins: the extracellular transferrins and the intracellular ferritins. Both retain iron in a safe Fe(III) form, which, unless mobilised, will not catalyse the production of free radicals. Iron is stored mainly intracellularly, where its potentially damaging effects are greatest. Hence the design and regulation of the major iron storage protein are of great importance. Ferritin's protective ability is based on its capacity to transform the highly toxic Fe(II) into the less toxic Fe(III) and to sequester it in its cavity. The major role of ferritin in vertebrates is in iron storage, whereas in prokaryotes it may be more important as an iron detoxifier and in efficient iron management functions associated with its ability to oxidise and incorporate iron.

2.2. *Iron metabolism in humans: an outline*

Human iron metabolism and absorption has been the subject of a recent review [14]. Normal human males contain 3–5 g of iron (often less in females) and, of this, two-thirds is in circulating red cells as haemoglobin and 15–25% in storage as ferritin and haemosiderin [15,16]. The remaining iron is in muscle myoglobin (about 8%) and in cytochromes and iron-containing enzymes. Plasma transferrin accounts for only 3 mg Fe, but the daily exchange of iron through plasma transferrin is ten times this amount. Transferrin therefore plays a central role in iron distribution. It accepts iron from reticuloendothelial cells (22 mg daily released by destruction of effete red cells), from parenchymal tissues (7 mg/day) and from the mucosal cells of the intestine (1 mg/day) and delivers it to the erythroid marrow (22 mg daily) and other tissues for synthesis of 'functional' iron proteins and for storage. Iron delivery by transferrin to erythroid and many non-erythroid cells involves interaction of transferrin with specific receptors followed by endocytosis and recycling of apotransferrin and the receptor [17–19]. These receptors are present in low amounts on phagocytic cells which receive their iron from degraded red cell haemoglobin. In the case of hepatocytes it has been proposed that iron is released by reduction at the plasma membrane [20] rather than by receptor-mediated endocytosis, but there is no evidence for a diferric reductase in rat liver plasma membranes [21]. Under pathological conditions (e.g., iron overload or serum transferrin deficiency) the amount of iron not bound to transferrin may become important. This is distributed to organs in an inappropriate manner leading to iron overload in parenchymal organs such as liver and pancreas [22]. Fe(III) bound to citrate, nitriloacetate or other complexes is efficiently taken up by cultured cells. Ferritin may also carry significant amounts of iron between liver Kupffer cells and hepatocytes [23]. How iron is recycled to the blood stream is obscure. Serum transferrin appears to have an important

role in this recycling, and animals or patients with congenital hypotransferrinaemia develop iron overload. Particularly interesting is the recent observation that subjects with a congenital absence of ceruloplasmin also develop severe iron overload [24], supporting the hypothesis that the copper protein is involved in the transfer of iron to transferrin, possibly because of its ferroxidase activity.

The major sites of iron storage are the liver (about one-third), spleen and bone marrow. Muscle is also quantitatively important because of its large mass, although the actual concentration of storage iron (40 mg/kg) is low [25]. In the gastrointestinal mucosa, ferritin plays a role in the regulation of iron entry into the body by sequestering unneeded iron which is lost when the cells are desquamated [14,26]. However, the level of ferritin in the gut of subjects with chronic iron overload is not exceptionally high [27]. Only about 1 mg Fe/day is absorbed into the body in normal healthy adults, this amount representing only about 10% of food iron. Although not all of the latter is readily absorbable there are mechanisms regulating intake. These are poorly understood, but intake is responsive to total body iron stores such that subjects with low storage iron (e.g., in iron deficiency) absorb more. An increased rate of erythropoiesis also leads to increased absorption. Absorption inappropriate to the amount of storage iron does occur. Dietary iron overload is uncommon, but 'Bantu siderosis', due to repeated consumption of beer brewed in iron containers and manifested by increased storage iron in liver, spleen and bone marrow, has been well characterised [15]. Genetic (idiopathic or hereditary) haemochromatosis is one of the most common genetic disorders in western populations, particularly among Celtic peoples [28–30]. This disease is associated with greatly increased (sometimes 50-fold) deposits of storage iron, predominantly as haemosiderin, in the liver and other tissues due to abnormally high absorption from the gut, although there seems to be little or no ferritin in the duodenal absorption cells [31]. Genetic haemochromatosis has an autosomal recessive mode of inheritance with a gene locus tightly linked to the HLA-A locus on chromosome 6 of the histocompatibility antigen complex [28]. In spite of intense research, the biochemical nature of the primary defect has not been established. Abnormally high iron stores are also found as a secondary effect arising from ineffective erythropoiesis (e.g., the defective globin synthesis of the thalassaemias) [30]. These 'iron-loading anaemias' are associated with increased absorption and are also treated by blood transfusion. The excess iron cannot be eliminated and must be stored. A characteristic feature of humans is that their means of excretion is limited. Only about 1 mg/day is lost, mainly through the gut and to a less extent through the urine (0.1 mg) and skin. A fine internal balance must be maintained between iron released from macrophages and iron incorporated into developing erythroid cells.

Elevated body iron leads to increased iron in storage (and not to increases in haemoglobin, myoblobin, etc., except in the treatment of iron deficiency anaemia). Under normal iron loading, ferritin is the major storage form, but, in diseases of iron overload, the capacity to synthesize ferritin levels off and haemosiderin becomes predominant [32]. Ferritin can be seen by electron microscopy as tiny dense particles dispersed in the cytosol [33]. Haemosiderin may also be visualised by electron microscopy. Together with some ferritin, it is found in membrane-bound bodies or 'siderosomes' derived from secondary lysosomes, although clusters of electron-dense material without membranes, or only partially enclosed by membranes, are also observed [33]. Haemosiderin is also revealed by Perl's staining of liver sections, whereas the dispersed ferritin gives only a faint Prussian blue background. In most forms of iron overload, storage iron is increased relatively in Kupffer and other phagocytic cells, whereas in primary (genetic) haemochromatosis there is a preferential loading of liver hepatocytes. Both forms of storage iron are mobilisable. Treatment of genetic overload is usually by phlebotomy and of iron-loading anaemias by treatment with iron chelating agents. In iron deficiency, storage iron is decreased and may be totally absent.

2.3. Scope of the review

This review concentrates on the following aspects of iron storage:
1. how does the molecular architecture of ferritin enable it to store iron? To what extent is this architecture conserved?
2. what are the iron storage mechanisms?
3. what are the roles of the different subunits in vertebrate ferritins?
4. how is ferritin iron made available?
5. how is ferritin biosynthesis regulated?
6. what is the relationship between ferritin and haemosiderin in iron overload?
7. does the iron-storage molecule, ferritin have other biological functions?

3. Ferritin molecules: their distribution and structures

3.1. Ferritin protein shells of animals, plants and bacteria

3.1.1. Definition, distribution, primary structures, isoferritins

The ferritin molecule is a hollow protein shell (outside diameter 12–13 nm, inside 7–8 nm, M_r about 500000), composed of 24 polypeptide chains and capable of storing up to 4500 Fe(III) atoms as an inorganic complex [34,35]. It has been isolated from a wide range of sources.

Among vertebrate ferritins those from man, horse, sheep, pig, rabbit, rat, mouse, chicken and tadpole have been extensively characterised with complete or partial amino

acid sequences (see Refs. [36,37] for review). Two ferritins from the snail *Lymnaea stagnalis* (located in either soma or yolk) have been isolated and their cDNA and amino acid sequences determined [38]. Two ferritins of different primary structures have been found in the parasite *Schistosoma mansoni*, one more prevalent in females and the

Table 1
Amino acid sequences of some ferritins

	A … B
HumH	------------□□□□□□□□□□□□□□□□□□□□□□□□□□□-------□□□□□□□□□□□□□□□□□□□□□□□□□□□□□------------....
	1 . . . . * . . 77
EcBFR	MKGDTKVINYLNKLLGNELVAINQYFLHARMFK..NWGLKRLNDVEYHESIDEMKHADRYIERILFLEGLPNLQDLGKL....
AvBFR	MKGDKIVIQHLNKILGNELIAINQYFLHARMYE..DWGLEKLGKHEYHESIDEMKHADKLIKRILFLEGLPNLQELGKL....
MlBFR	MQGDPDVLRLLNEQLTSELTAINQYFLHSKMQE..NWGFTELAERTRVESFDEMRHAEAITDRILLLDGLPNYQRIGS.....
MaBFR	MQGDPEVLRLLNEQLTTQLTAINQYFLHSKMQD..NWGFTELAEHTRAESFDEMRHAEAITDRILLLDGLPNYQRLFSL....
BmBFR	MKGEPKVIERLNDALFLELGAVNQYWLHYRLLN..DWGYTRLAKKEREESIEEMHHADKLINRIIFFEGFPNLQTVSPL....
EcFTN	MLKPEMIEKLNEQMNLELYSSLLYQQMSAWCSYH..TFEGAAAFLRRHAQEEMTHMQRLFDYLTDTGNLPRINTVESP....
HpFTN	MLSKDIIKLLNEQVNKEMNSSNLYMSMSSWCYTH..SLDGSGLFLFDHAAEEYEHAKKLIVFLNENNVPVQLTSISAP....
Soy	TAPQVSLARQNYADECESAINEQINVEYNASYVYHSLFAYFDRDNVALKGFAKFFKESSEEEREHAEKLMKYQNTRGGRVVLHPIKNVPSEF
Mail	QSPDKSLARHKFVDDCEAALNEQINVEYNASYAYHSLFAYFDRDNVALKGFAKFFKESSDEEREHAEKLMEYQNKRGGRVRLQSIVTPLTEF
Sch1	SLCRQNYHEECEAGVNKQINMELYASYVYMTMAFHFNRDDVALNGFYKFFLNESEEERQHAIKLMTYQNMRGGRIVLQDISAP....
Sch2	KTMSSSRARQSFATECENAINKQINVELQAAYDYMAFFTYFDRDDVSFPKAAEFFRKASHEEREHAEKLAKYQNKRVGRVQYSDIKCP....
Lym1	MSVSQARQNYHAESEAGINRQINMELYASYSYQSMAYYFDRDDVALPGFHKFFKHQSEEEREHAEKLMKYQNKRGGRIVLQDIKKP....
Lym2	KEFVATVRQNYKENINQLLEQQIQKELAASYIYQAYASYFQRADVSLPGIKKFFSDASSEERDDAQSLIDYINQRGGHVQYDKIDLK....
Sal1	MTSQVRQNFHQDCEAAINRQINLELYASYVYLSMAYYFDRDDQALHNFAKFFKNQSHEEREHAEKLMKVQNQRGGRIFLQDVKKP....
Sal2	MESQIRQNYHHDCERAINRMINMEMFASYTYTSMAFYFSRDDVALPGFAHFFKENSEEEREHADKLLSFQNKRGGRILLQDIKKP....
BfMF	MVSQVRQNYHSDCEAAVNRMLNLELYASYTYSSMYAFFDRDDVALHNVAEFFKEHSHEEREHAEKFMKYQNKRGGRVVLQDIKKP....
BfHF	MDSQVRQNFHRDCEAAINRMVNMELYASYTYLSMAFYFDRDDIALHNVAKFFKEQSHEEREHAEKLMKDQNKRGGRIVLQDVKKP....
ChHF	MATPPSQVRQNYHQDCEAAINRQINLELYASYVYLSMSYYFDRDDVALKNFAKYFLHQSHEEREHAEKLMKLQNQRGGRIFLQDIKKP....
MoHF	TTASPSQVRQNYHQDAEAAINRQINLELYASYVYLSMSCYFDRDDVALKNFAKYFLHQSHEEREHAEKLMKLQNQRGGRIFLQDIKKP....
HuHF	TTASTSQVRQNYHQDSEAAINRQINLELYASYVYLSMSYYFDRDDVALKNFAKYFLHQSHEEREHAEKLMKLQNQRGGRIFLQDIKKP....
BfLF	MESQVRQNFHQDCEAGLNRTVNLKFHSSYVYLSMASYFNRDDVALSNFAKFFRERSEEEKEHAEKLIEYQNQRGGRVFLQSVEKP....
MoLF	TSQIRQNYSTEVEAAVNRLVNLHLRASYTYLSLGFFFDRDDVALEGVGHFFRELAEEKREGAERLLEFQNDRGGRALFQDVQKP....
HoLF	SSQIRQNYSTEVEAAVNRLVNLYLRASYTYLSLGFYFDRDDVALEGVCHFFRELAEEKREGAERLLKMQNQRGGRALFQDLQKP....
HuLF	SSQIRQNYSTDVEAAVNSLVNLYLQASYTYLSLGFYFDRDDVALEGVSHFFRELAEEKREGYERLLKMQNQRGGRALFQDIKKP....
	1 88

	C … D … E
HumH	·----- □□□□□□□□□□□□□□□□□□□□□□□□□□□□□--□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□----□□□□□.□□□□□□---------
	78 158
EcBFR	..NIGEDVEEMLRSDLALELDGAKNLREAIGYADSVHDYVSRDMMIE.ILRDEEGHIDWLETELDLIQKMG.....LQN.YLQAQIREEG
AvBFR	..LIGEHTKEMLECDLKLEQAGLPDLKAAIAYCESVGDYASRELLED.ILESEEDHIDWLETQLDLIDKIG.....LEN.YLQSQMDE
MlBFR	..RVGQTLREQFEADLAIEYEVMSRLKPGIIMCREKQDSTSAVLLEK.IVADEEEHIDYLETQLALMGQLG.....EEL.YSAQCVSRPPS
MaBFR	..RIGQTLREQFEADLAIEYEVMDRLKPAIILCREKQDSTTATLFEQ.IVADEEKHIDYLETQLELMDKLG.... VEL.YSAQCVSRPPS
BmBFR	..RIGQNVKEVLEADLKGEYDARASYKESREICDKLGDYVSKQLFDE.LLADEEGHIDFLETQLDLLAKIG.....EER.YGQLNAAPADEAE
EcFTN	.FAEYSSLDELFQETYKHEQLITQKINELAHAAMTNQDYPTFNFLQW.YVSEQHEEEKLFKSIIDKLSLAG..KSGEGL.YFIDKELSTLDTQN
HpFTN	.EHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKGKDHATFNFLQW.YVSEQHEEEVLFKDILDKIELIG..NENHGL.YLADQYVKGIAKSRKS
Soy	EHVEKGDALYAMELALSLEKLVNEKLLNVHSVADRNNDPQMADFIESEFLSEQVESIKKISEYVAQLRRVG...KGHGV.WHFDQRLLD
Mail	DHFEKGDALYAMELALALEKLVNEKLHNLHGVATRCNDPQLTDFIESEFLEEQGEAINKISKYVAQLRRVG KGHGV WHFDQMLLEEEA
Sch1	PQLSWNSGLHAMQDALDLEKKVNQSLMELVAVGERHRDTHFCDFINNEYLEIQVQSMKKLSDYITNLIRVG...NGLGE.YTFDKETLHGESQ
Sch2	TKTEFSSLEDAMNTALGMEKAVSKSLLELHEVASKNNDPALADFIESEFLHEQEDAIKQFADYLTETQRVG...KGLGE.YLFDKLTLNE
Lym1	DRDEWGTGLEAMQVALQLEKSVNQSLLDLHKLCTSHDDAQMADFLESEFLEEQVKSIKELSDYITNLKRVG...PGLGE.YIFDKETLSSSS
Lym2	ERSDWKEGLIAFEDTLAIERYVNAQLLDIHKKADDEKDAHLTHILEHEFLEEQVSSINKIAHAITRLRSFE...QGSGNNYKLGRVYLRPTPQISH
Sal1	EKDEWGSGVEALESSLQLEKSVNQSLLDLHKVCSEHNDPHMCDFIETHYLDEQVKSIKELGDTPDNLRRMGAPQNAMAE.YLFDKHTLGKEST
Sal2	ERDEWGNGLEAMQCALQLEKNVNQALLDLHKIASDKVDPHLCDFLETHYLNEQVEAIKKLGDHITNLTKMDAVKNKMAE.YLFDKHTLG-GQS
BfMF	ERDEWGNTLEAMQAALQLEKTVNQALLDLHKLATDKVDPHLCDFLESEYLEEQVKDIKRIGDFITNLKRLGLPENGMGE.YLFDKHSVKESS
BfHF	ERDEWGNTLEAMQAALQLEKTVNQALLDLHKVGSDKVDPHLCDFLETEYLEEQVKSIKQLGDYITNLKRLGLPQNGMGE.YLFDKHTMGESS
ChHF	DRDDWENGLTAMECALHLEKNVNQSLLELHKLATEKNDPHLCDFIETHYLDEQVKAIKQLGDHVTNLRKMGAPKYGMAE.YLFDKHTLGESDS
MoHF	DRDDWESGLNAMECALHLEKSVNQSLLELHKLATDKNDPHLCDFIETYYLSEQVKSIKELGDHVTNLRKMGAPEAGMAE.YLFDKHTLGHGDES
HuHF	DCDDWESGLNAMECALHLEKNVNQSLLELHKLATDKNDPHLCDFIETHYLNEQVKAIKELGDHVTNLRKMGAPESGLAE.YLFDKHTLGDSDNES
BfLF	ERDDWANGLEALQTALKLQKSVNQALLDLHAVAADKSDPHMTDFLESPYLSESVETIKKLGDHITSLKKLWSSHPGMAE.YLFNKHTLG
MoLF	SQDEWGKTQEAMEAALAMEKNLNQALLDLHALGSARTDPHLCDFLESHYLDKEVKLIKKMGNHLTNLRRVAGPQGSLGE.YLFERLTLKHD
HoLF	SQDEWGTTLDAMKAAIVLEKSLNQALLDLHALGSAQADPHLCDFLESHFLDEEVKLIKKMGDHLTNIQRLVGSQAGLGE.YLFERLTLKHD
HuLF	AEDEWGKTPDAMKAAMALEKKLNQALLDLHALGSARTDPHLCDFLETHFLDEEVKLIKKMGDHLTNLHRLGGPEAGLGE.YLFERLTLKHD
	89 182

The table shows primary structures of five bacterial haemoferritins (BFRs), two bacterial non-haem ferritins (FTNs), and examples of ferritins from plants, invertebrates and vertebrates.

Key: BFRs from *E. coli* (Ec [62]), *A. vinelandii* (Av [64]), *M. leprae* (Ml [66]), *M. avium* (Ma [68]), *B. melitensis* (Bm [67]); FTNs from *E. coli* (Ec [63]), *H. pylori* (Hp [66]) and other H-type ferritins from soybean (Soy [54]); maize (Mail [56]), *Schistosoma mansoni* (Sch1 and Sch2 [39]), *Lymnaea stagnalis* (soma, Lym1 and yolk, Lym2 [38]); salmon (Sal1, Sal 2, [474]), bullfrog (BfMF and BfHF [36]), chicken (ChHF [307]), mouse (MoHF [396]), horse (HoLF [475]) and human (HuLF [74]).

For further sequences consult Ref. [37]. The numbers above the sequences correspond to those of the BFRs and those below to HuHF.

Residues in bold indicate those which have been identified as metal ligands (at the ferroxidase centres) in EcBFR [97,175], EcFTN [86] or HuHF [82]. The starred methionine is the haem iron axial ligand of BFRs [85,98].

Residues appearing in helices in HuHF are shown as □.

Note that certain sequences contain N-terminal extensions or insertions not shown in the table: N-terminal 'germination' extension, Soy = STVPLTGVIFEPFEEVKKSELAVP and Mail = AAGKGKEVLSGVVFQPFEEIKGELALVP; Lym2 has an N-terminal signal sequence MNSVLFLTLAVCSSLAYG and an insertion DACETVMKFVTSDTSGLEEFRDRRMCICGFVATKTINDNCG between residues 88 and 89; MoLF has an 8-residue insertion QPAQTGAP between 161 and 162 (HuHF numbering).

second in males [39]. Ferritins from chitons and limpets [40,41] and from the earthworm *Octolasium complanatum* [42] have been described in some detail. Examples of ferritins from fish and insects that have recently been characterised are from the rainbow trout [43], from the lamprey [44], from larvae of the insect *Calpodes ethluis* [45] and from midgut-epithelial cells by *Philaenus spumarius* [46]. Although in vertebrates, ferritins are mainly located in the cytoplasm, low concentrations (123 ng/ml in human males, 56 ng/ml in females) are also found in blood plasma [47]. Insect ferritins are present within the vacuolar system, lumen of the endoplasmic reticulum or secreted in the haemolymyph [45,46,48]. Chitons and limpets have high concentrations of ferritin in the haemolymph (0.4 mg/ml in chitons), where it seems to play an iron transport role [40]. Plants store iron as ferritin within the plastids, chiefly in tissues which show low photosynthetic activity [49,50]. It is absent from roots and leaves but accumulates in seeds [51]. Its synthesis and degradation are developmentally regulated [52].

The amino acid (cDNA) sequences of ferritins from pea seeds [53], soya bean [54], French bean [55] and maize (2 sequences with 96% identity) [56] have been determined. Ferritins are also found in fungi [57], yeast [58] and in bacteria [59–63]. Seven complete amino acid sequences of bacterial ferritins are known: *two* of these are from *Escherichia coli* [62,63] one each from *Azotobacter vinelandii* [64], *Mycobacterium leprae* [65], *Helicobacter pylori* [66] *Brucella melitensis* [67] and *Mycobacterium avium* [68]. Partial amino acid sequences have been determined for ferritins from *Nitrobacter winogradskyi* [69], *Mycobacterium paratuberculosis* [70], the cyanobacterium *Synechocystis* [71] and from the obligate anaerobe *Bacterioides fragilis* [72]. Except for the latter, for one of the ferritins from *E. coli* designated FTN [63], and for that reported for *H. pylori* (designated PFR (prokaryotic ferritin) [66] but here referred to as FTN because of its similarity to the FTN of *E. coli*), the ferritins so far characterized from microbial sources are haemoferritins. They are generally known as bacterioferritins or BFRs. Haem contents, as isolated, are usually between about 0.25 and 0.5 haem per subunit.

Selected examples of ferritin amino acid sequences are given in Table 1, including *A. vinelandii* (AvBFR) [64], *H. pylori* FTN [66], BFRs of *M. leprae* [65], *M. avium* [68], *B. melitensis* BFR [67] and maize ferritin [56]. For a more complete list (but not including the most recent sequences), see Andrews et al. [37]. Note that, in animals, more than one sequence may be found (e.g., two in humans, three in tadpoles). In mammalian ferritins (Fig. 1) the two sequences known as H and L show about 54% identity, whereas about 90% of H-chain residues and about 85% of L-chains are identical [37,73,74]. The three tadpole and four invertebrate sequences are closer to H-chains of mammals (60–70% and 37–69% identities respectively) than to their L-chains (50–65% and 31–54%). Plant ferritin sequences, aligned with those from animal ferritins, are all more similar to mammalian H-chains than to their L-chains. Pea seed ferritin, for example, gives 49% identity with human H-chain and 40% with human L-chain. Bacterial ferritins diverge further from animal ferritins being only 22–24% identical to H-chains and 18–21% identical to L-chains of mammals, and the two *E. coli* ferritins, BFR (haemoferritin) and FTN show only 14% identity to each other [37,75]. However, it is clear that the ferritin family of iron storage proteins includes members of bacterial origin [37,64,75].

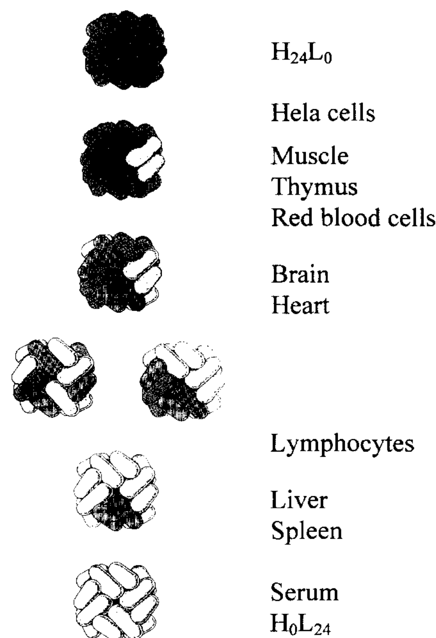


Fig. 1. Schematic diagram of human 'isoferritins' of different subunit compositions. Each ferritin subunit is depicted as a 'sausage' and subunits are packed in a symmetrical shell. Twelve of the 24 subunits are visible. Mammalian ferritins are composed of two subunits of different primary structures, known as H and L. In the diagram stippled subunits represent human H-chains and plain subunits, L-chains. H-chain and L-chain homopolymers are shown respectively at the top and bottom of the figure, and heteropolymers of descending H content are placed between the homopolymers. Coassembly of the two subunit types is possible because many of the inter-subunit contact residues are conserved. The sources of various ferritins are listed in the right-hand column, such that their average subunit compositions are indicated (e.g., muscle ferritin about 20 H: 4 L, liver ferritin about 2–3 H: 22–21 L). Most of the data were obtained by immunoassay using H- or L-chain-specific antisera [476]. The composition of human brain ferritin was measured after subunit separation by gel electrophoresis [477]. The pair of molecules in the fourth position indicates that subunits may be clustered differently in molecules of the same composition. In vitro assembly experiments indicate that dimers are a first assembly intermediate and it is likely that these dimers are antiparallel pairs formed by association of subunits along their along axes as shown here. It may be supposed that H-chains (or L-chains) forming on their polysomes would associate into homodimers before co-assembly into heteropolymers, although there is no evidence for this.

Ferritins isolated from mammalian tissues consist of a mixture of isoferritins with a range of subunit composi-

tions and iron contents (Fig. 1) [73]. Twenty-five isoferritins of defined composition are possible, with subunit ratios: $H_{24}L_0$, $H_{23}L_1$ $H_{22}L_2$-H_0L_{24}, but isoferritins $H_{22}L_2$ to H_2L_{22} are heterogeneous given that different distributions of subunits may be possible within the shell. In general L-rich ferritins are characteristic of organs storing iron (liver and spleen) and these ferritins usually have a relatively high average iron content (1500 Fe atoms/molecule or more). H-rich ferritins which are characteristic of heart and brain have relatively low average iron contents (less than 1000 Fe atoms/molecule). No H-chain homopolymers have been isolated but human serum ferritin is devoid of H-chains [76] and an L-chain homopolymer fraction has been extracted from horse spleen ferritin [77].

Some ferritin subunits have amino acid insertions or N-terminal extensions relative to mammalian chains. Plant ferritins are synthesized as precursors with an N-terminal extension of over 70 residues that is absent from the mature ferritin [52–54,78]. The first part of this extension is a plastid targetting (PT) sequence (about 40 residues) which is lost on entry into the organelle. The second part (extension peptide, EP) is lost after molecular assembly either in vivo on germination or in vitro during iron exchange [78]. Pea seed ferritins, with and without PT and EP, have been cloned and expressed in *E. coli* [79]. Assembly to 24-mer was obtained only after PT deletion.

3.1.2. Three-dimensional structures

The known three-dimensional structures of ferritins are more highly conserved than their primary structures. Each of the 24 subunits consists of a bundle of 4 long helices, a fifth short helix and a long extended loop (overall dimensions about $25 \times 25 \times 50$ Å^3, Fig. 2). The subunits are arranged to give a hollow, symmetrical shell with outside and inside diameters 125 and 80 Å respectively (Figs. 1–3). X-ray structures have been derived for the following ferritins: native horse spleen (HoSF-85% L-chain) [34,80], native rat liver (67% L-chain) [81,82], recombinant rat L-chain (RaLF) [81,82], recombinant human H-chain (HuHF) [82], recombinant bullfrog L-chain (BfLF) [83,84], recombinant *S. mansoni* (type 1, Yewdall, Hirzmann, Artymiuk and Harrison, unpublished work), *E. coli* BFR (EcBFR) [85] and *E. coli* FTN (EcFTN, now in course of refinement, Ref. [86] and unpublished work of Hempstead, Yewdall, Hudson, Andrews, Artymiuk, Guest and Harrison). The structures are closely similar, e.g., most main chain atoms of human H, rat L, horse L, *S. mansoni* 1 and EcFTN (H) apoferritins superpose within about ± 1.0 Å. The root mean square difference in backbone atomic positions of HuHF and BfLF is 0.97 Å [83] and that for 156 equivalenced alpha carbons of EcBFR and horse spleen L ferritin is 2 Å (Dautant, Meyer, Yariv, Kalb (Gilboa), Frolow and Precigoux, personal communication). There is no X-ray structure as yet of a plant ferritin, but computer modelling suggests that these ferritins can adopt conformations resembling those of mammalian ferritins [53].

Each apoferritin (iron-free) molecule is assembled from 24 structurally equivalent subunits. In mixed subunit 24-mers (heteropolymers) H- and L-subunits have the same conformations and the many identical or similar residues in H-H, H-L and L-L inter-subunit contact regions enable the formation of heteropolymers with the complete range of subunit compositions. It has been proposed, however, that like chains cluster preferentially within the polymer [87] and this may apply particularly to dimer pairs. The apoferritin shell is notable for its stability to heat (treatment for 5–10 min at 70°C is routinely used as a preparative step) and to urea or guanidinium chloride. The large numbers of intra- and inter-subunit salt bridges and hydrogen bonds contribute to this stability. Features of the

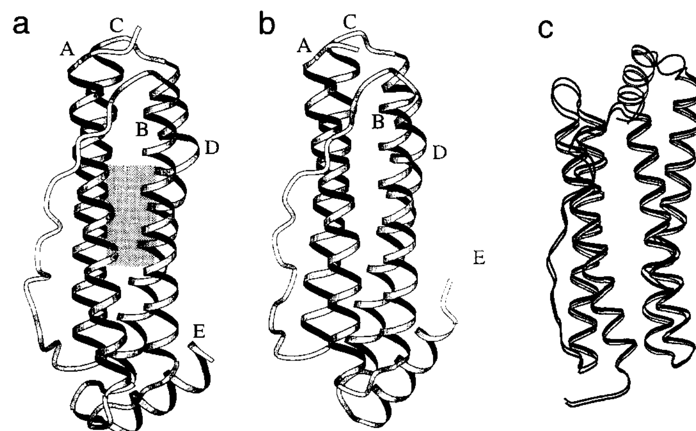


Fig. 2. Ribbon diagrams of ferritin subunits showing conservation of subunit fold. (a) HuHF; (b) EcFTN; (c) superposition of HuHF (black) and BfLF (grey). Subunits are folded as 4-helix bundles with a fifth short helix lying at an angle of about 60° to the bundle axis. Identities and similarities in amino acid sequences are respectively: HuHF and EcFTN, 24% and 51%; HuHF and BfLF 61% and 86%; EcFTN and BfLF, 25% and 54%. HuHF structure as in [82], EcFTN as in [86] and BfLF as in [84]. The stippled area in (a) indicates a hydrophilic region which is detailed in Fig. 6. We thank P.D. Hempstead for (a) and (b) and N.M. Allewell for (c). Helices in (a) and (b) are labelled A, B, C, D, E. Accession number for HuHF coordinates in Brookhaven Protein Data Base is 1HFA.

human recombinant H-chain ferritin (HuHF) and other ferritins are shown in Figs. 1–8.

3.1.3. Shell assembly, inter-subunit channels and cavity

In the assembled molecule the 24 subunits are related by operation of 4-fold, 3-fold and 2-fold symmetry axes (432 symmetry, Fig. 3). Anti-parallel subunit pairs with their long apolar interfaces around 2-fold symmetry axes are the likely first assembly intermediates (Fig. 3c) [34,88,89]. Subunits pack tightly together except that at 3-fold axes there are narrow channels traversing the shell. Residues around the 3-fold axes are predominantly hydrophilic in the ferritins of higher organisms (Fig. 4). In vertebrate and plant ferritin sequences, side chains lining the narrowest parts of the channels at the cavity end are highly conserved, namely 3 (symmetry-related) aspartates (seq. No. 131) and 3 glutamates (No. 134). These residues bind metals (Cd^{2+}, Zn^{2+}, Tb^{3+} or Ca^{2+} [34,80,82,90])

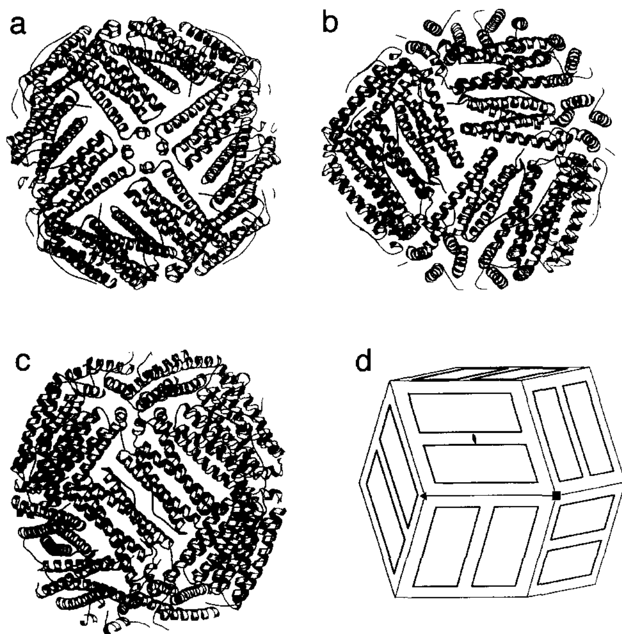


Fig. 3. Subunit packing in molecules of EcBFR. Subunits are depicted as ribbon diagrams. Views are from the outside of the molecule down, (a) 4-fold, (b) 3-fold and (c) 2-fold symmetry axes. For clarity, only the upper subunits are shown. Subunit arrangements in other ferritins are very similar. Note that subunits pack around 2-fold axes as anti-parallel pairs, which can be seen most clearly in (b) and (c). Such pairs are thought to be the first intermediate formed during subunit assembly in horse spleen ferritin [34,88,89]. A schematic general view outlining the positions of subunits in relation to the symmetry axes is shown in (d). Drawings based on structure described in [85] and coordinates in Brookhaven Protein Data Base accession number 1BCF. Note that the 12 haem groups of EcBFR are not shown in this diagram, but they are situated between two subunits at the 2-fold axis interface in positions similar to those depicted for the protoporhyrin IX derivative of horse spleen ferritin in Fig. 7. In EcBFR the haem Fe atoms are bound by two symmetry-related methionines and the haem is tightly wedged between the subunits, with which it makes more than 100 Van der Waals contacts. The propionyl side chains of the haem project into the cavity (as in Fig. 7), enabling them to interact with the mineral of the core.

and the 3-fold channels have been proposed as the main entry route for iron [90–96] and as sites of Fe(II) oxidation [90–92,94]. However, these residues are not both conserved in some invertebrate sequences [37–39]. EcFTN and *H. pylori* FTN (HpFTN, as aligned) have several hydrophobic residues near the 3-fold interface and Asn and Gln in place of Asp 131 and Glu 134 respectively (Fig. 4) [37,66]. No metals are found at the 3-fold interface in EcFTN [86]. EcBFR has a hydrophilic 3-fold interface involving Asp 109, Asp 118 and Arg 117 [85,97] like that predicted from sequence alignment and modelling [98].

The 4-fold axis interface is highly hydrophobic in mammalian L ferritins, being lined by 12 leucine residues. In H-chains there are four histidines at the cavity side and usually four methionines (leucines in HuHF) at the outer surface and BfLF is similar. In invertebrates, plants and bacteria there is more variation, especially in the latter. In pea seed ferritin, as modelled, residues butting on to the 4-fold axes are eight histidines and four arginines. In EcBFR [97] the 4-fold channels are hydrophilic with Asn 148 and Gln 151 pointing towards the symmetry axes. The 4-fold axis regions of HuHF and EcBFR are shown in Fig. 5. In the medium resolution structure of horse spleen ferritin there appeared to be small gaps or channels passing through the shell at the 4-fold axes. However, the refined high resolution structure of HuHF shows very little space around these axes (Fig. 5), as has also been reported for recombinant BfLF [83]. In EcBFR the interface is less tightly packed and there is space for a water molecule to pass through (Fig. 5c).

All ferritin molecules contain an approx. 80 Å diameter iron-storage cavity. Unexpectedly, residues on this cavity (mineralizing) surface are not highly conserved. There are differences on the cavity surface residues of mammalian H- and L-subunits, with L-chains showing a higher preponderance of carboxylates than H-chains [151] (Table 2). Plant ferritins have arrays of carboxyl groups resembling those of L-chains [53,99], but the cavity surfaces of bacterial ferritins seem to be very different (Table 2).

3.1.4. Metal and haem binding

Ferritin subunits fold so that at each end of the 4-helix bundle there is an apolar 'core' [80]. However, all subunits have central hydrophilic regions, Figs. 2 and 6. In the L-subunit of horse (HoLF) and rat this region contains a salt bridge (see Fig. 8) and a similar (modelled) salt bridge in human L-chains contributes stability [82,100]. In BfLF the region contains bound water molecules, but no salt bridge, Fig. 6d, and HuHF has metal-binding sites in the same region [82], Fig. 6a and Fig. 8. Metal ligands and some nearby residues are highly conserved in H-chains of animals and plants and in EcFTN [37,39,53,75] (Fig. 6a,b and Fig. 8, and Table 1) and HpFTN [66] the metal ligands are the same as in HuHF except for the substitution of Glu61 by Glu144. Direct confirmation that these sites bind iron has recently been obtained for EcFTN [86] (Fig. 6c),

and a third iron has also been identified at a nearby position. In the haem-containing BFRs five of the residues are conserved and are now known to bind metal. Although the actual metal has not been identified, it is likely to be Mn^{2+} which is present in the crystallising solution [85,97] (Fig. 8). This highly important functional site is associated with ferroxidase activity (see below).

BFRs, as isolated, contain iron-protoporphyrin IX with unique bis-methionine co-ordination [101,102]. Modelling of haem-binding sites [98] tested by mutagenesis [103] suggested that it is bound at an inter-subunit site near to the cavity surface at 2-fold axis positions with two Met 52 residues of neighbouring subunits as haem iron ligands. The recently reported 2.9 Å resolution structure of EcBFR confirms the similarity of BFR to mammalian ferritins and places the haem group at the 2-fold axis interface as predicted [85,97]. An intriguing new observation is that protoporphyrin IX can also bind to HoSF at the same interface and in a similar inter-subunit cleft (Fig. 7) [104]. HoSF was co-crystallised with Sn-protoporphyrin IX, but the Sn was lost on binding to the protein. Previously a spectroscopic analysis of haem bound to horse spleen apoferritin indicated bis-histidine ligation of the haem iron and a model with haem bound near to 3-fold axis positions was proposed [105]. Possibly the haem was bound with bis-arginine ligation in the 2-fold cleft. Binding of protoporphyrin in this position raises the question as to how it reaches this site. Is the shell sufficiently flexible to allow passage of the flat ring between subunits through the hydrophobic interface or does the shell dissociate? Whether the binding of protoporphyrin IX to vertebrate ferritins has any physiological significance remains another interesting but unanswered question. It may be noted that Arg 63 of the binding pocket, which lies against the flat protoporphyrin ring, is conserved in all known sequences of vertebrate ferritins with one exception, namely BfLF [36,37]. Arg 63 is also present in ferritins of invertebrates and plants, but not in the non-haem ferritins of bacteria [37,66]. EcFTN, but not HpFTN, has Met 52 equivalent to the haem-iron ligand of EcBFR. None of these ferritins contains haem as isolated, however. The presence of haem seems to facilitate reductive iron release from both vertebrate [106] and bacterial [107] ferritins, but it is without major effect on Fe(II) oxidation rates in EcBFR [103] (see below). The overexpressed haem-free variant EcBFR-M52H contains more iron, as isolated, than the equivalent

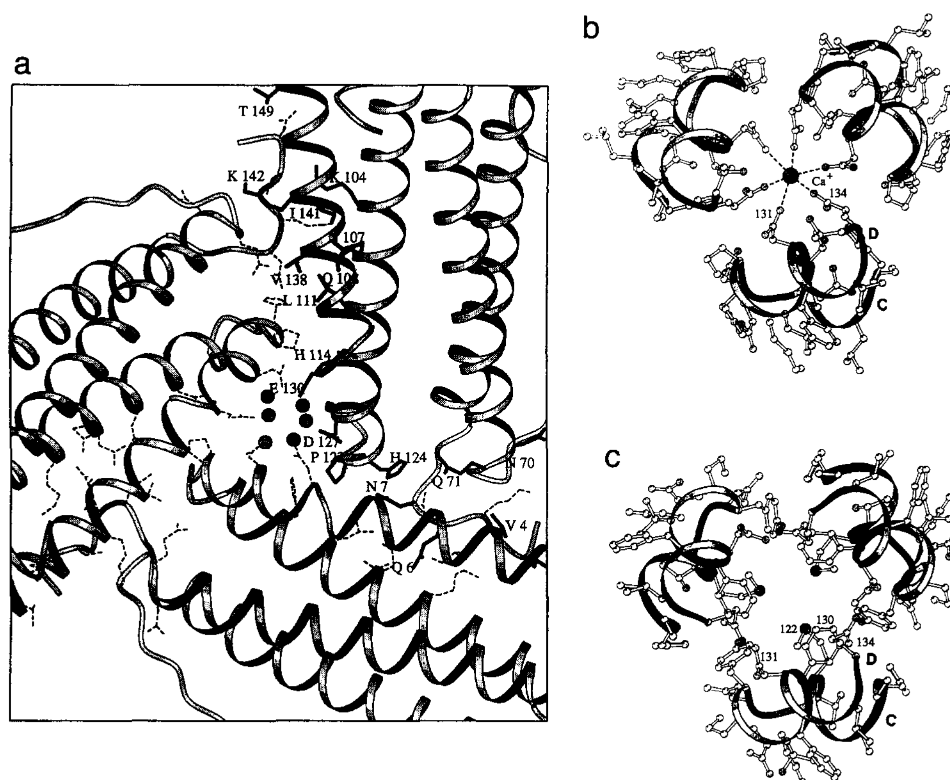
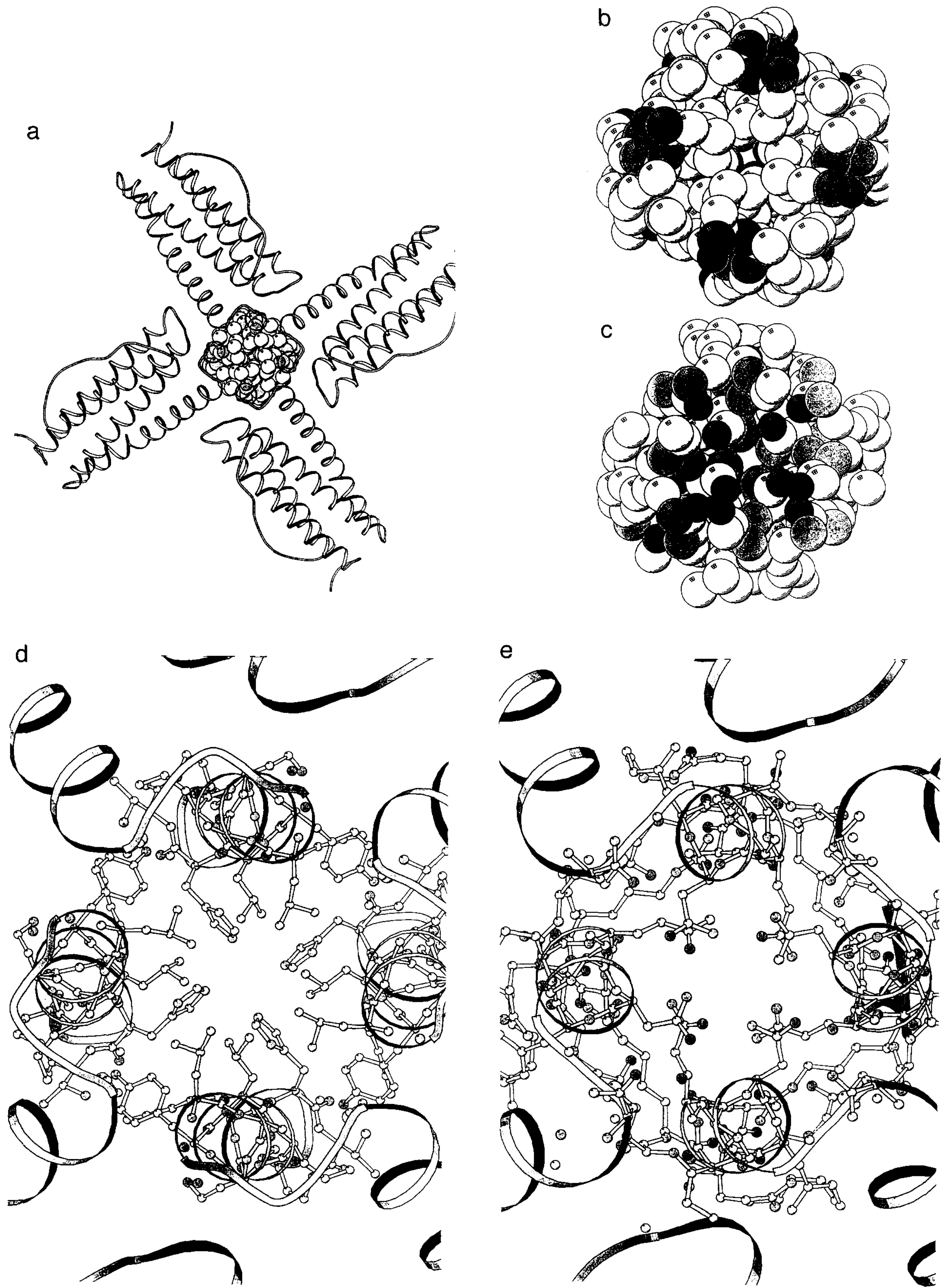


Fig. 4. Intersubunit interface around the 3-fold axis in various ferritins viewed from inside the molecule. (a) BfLF; (b) HuHF; (c) EcFTN. In (a) solvent molecules are in dark grey and side chains of one subunit are labelled. In (b) Asp131 and Glu134 are in positions equivalent to those labelled D127 and E130 in (a). Note the central Ca^{2+} ion which is bound by the six carboxylates. HuHF was crystallised from $CaCL_2$. In (c) the interface is very different with no metal ions or water molecules obvious. Asn and Gln replace the Asp127 and Glu130 and Asp131 and Glu134 of (a) and (b) respectively and point away from the 3-fold axis, whereas, in contrast, Met122 and Phe130 point toward the axis. The residue numbers used for EcFTN are based on its sequence alignment to HuHF, Table 1. Those for BfLF are actual numbers (add 4 to align with HuHF). We thank N.M. Allewell for (a) and P.D. Hempstead for (b) and (c).


a
b
c
d
e

wild-type EcBFR, possibly indicating an effect of haem on iron release in vivo [103].

3.2. Ferritin iron-cores

Unlike the highly conserved ferritin protein shell, the structure of ferritin iron-cores is quite variable due, in part, to differences in composition, notably the content of inorganic phosphate [99,108–111]. Iron-core structures (see Ref. [112] for review) have been studied principally by X-ray or electron diffraction [109,111,113,114], electron microscopy [41,99,109,115–117] and Mössbauer spectroscopy [41,44,99,110,111,117–124] and, to a less extent, by EXAFS [125–127]. Native cores in horse [113,114], human [109] and rat [111] ferritins are ferrihydrite (5 $Fe_2O_3 \cdot 9H_2O$ [128]), with varying degrees of crystallinity, as judged by the above techniques. Each Fe(III) atom is surrounded by approx. 6 oxygen atoms at a distance of 1.93 A [125–127]. Most native ferritins contain some inorganic phosphate, while giving ferrihydrite diffraction lines, e.g., horse spleen which has on average about 1 P per 10 Fe atoms [108]. The iron-cores of *Pseudomonas aeruginosa* and *A. vinelandii* BFRs are essentially hydrated ferric phosphate with P:Fe ratios of between 1:1 and 1:2 [109,110]. In pea seed (*Pisum sativum*) ferritin the ratio is 1:2.8 [99]. In all three cases, the iron cores are amorphous. On the other hand, ferritins isolated from iron-loaded rat livers [111], from the haemolymph of the limpet *Patella laticostata* [41], and from the chiton *Acanthopleura hirtosa* [41] have iron-cores of limited crystallinity, even though they contain very little phosphate (P:Fe respectively for rat, limpet and chiton being 1:40, 1:44 and 1:36). It is likely that core crystallinity is influenced by the rate of iron deposition as well as by the composition of the medium. Also, ferritins which had amorphous iron-cores as isolated (e.g., from *A. vinelandii* or *Pisum sativum*) can give crystalline ferrihydrite cores on reconstitution [99,117]. The bulk mineral structure seems to show no preferred orientation with respect to the protein shell [113], even though the groups on its cavity surface are thought to be important for its nucleation (Ref. [83] and see below). As isolated, ferritins may contain just a few Fe atoms or up to about 4500. Ways in which the presence of inorganic phosphate may affect core development in vitro are discussed below. Phosphate appears to bind to the surface of the growing core and then some of it to become incorporated as more iron is added. A novel non-ferrihydrite small cluster of unknown structure, which contains no phosphate, has recently been observed by Mössbauer spectroscopy at an early stage of core reconstitution in EcFTN [124].

4. Iron storage processes

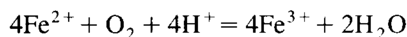
A part of a dose of ^{59}Fe injected into a rat as labelled ferric ammonium citrate or iron dextran will later be found in the liver as soluble cytosolic ferritin [129–133]. The steps by which this iron is processed have not been fully elucidated. Some of the iron delivered by transferrin also finds its way into cell-sap ferritin. Transferrin is taken up into endocytic vesicles by receptor-mediated endocytosis [19,134,135]. Release of its iron within the endosome is probably mediated by protons and facilitated by binding the receptor [136–138]. Neither the chemical form in which iron is delivered to ferritin molecules in vivo nor its intracellular donor is known, but storage involves gradual accumulation by binding of iron to core particle surfaces [130,139]. Except for vertebrate L-chains, all ferritins sequenced contain conserved residues that have been associated with ferroxidase activity, as described below. This has led to the tacit assumption that iron uptake into ferritin is an oxidative process starting from Fe(II) and that the oxidant is dioxygen, although other physiological oxidants have been considered.

4.1. Iron-loading of apoferritin shells – the catalysis of Fe(II)-oxidation by apoferritin in vitro

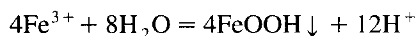
Although ferric citrate is probably one of the low molecular weight iron complexes of the cell sap, attempts to load apoferritin shells with ferric citrate (or with other Fe(III) compounds) in vitro have failed [140] unless a reductant like ascorbate is present [141]. Iron supplied as an Fe(II) salt under oxidising conditions is taken up by apoferritin in vitro, and is oxidised and converted into ferrihydrite in its cavity to give a product closely resembling natural ferritin [142]. Consequently most studies of ferritin functionality have been based on its capacity to oxidise Fe(II) and incorporate it aerobically at neutral pH in the absence of natural iron binding molecules such as citrate, phosphate or amino acids. Under these largely

Fig. 5. Intersubunit interface around a 4-fold axis in HuHF and EcBFR viewed from outside the molecule. (a): 4 HuHF subunits around a 4-fold symmetry axis with the atoms in the vicinity of the axis shown as spheres drawn with Van der Waals radii. Thus residues are in close contact with only a very narrow 'channel' around the axis. (b): enlargement of central region of (a); white spheres represent carbons and grey spheres oxygen and nitrogens; (c): equivalent regions of EcBFR; (d): detail of 4-fold interface of HuHF showing three residues of each subunit (two leucines and a histidine) pointing toward the 4-fold symmetry axis; (e): detail of 4-fold interface of EcBFR. The character of this interface differs from that of (d). Only two residues of each subunit (asparagine and glutamine) point toward the axis. The 4-fold interface in EcBFR is less tightly packed and more hydrophilic than that of HuHF. (Compare (b) and (c) and (d) and (e)). It can also be seen by comparing d and e and Fig. 2a) and Fig. 3a) that the E helices, which pack around the 4-fold axes, are much shorter in EcBFR than in HuHF.

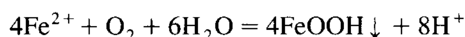
unphysiological conditions Fe(II) oxidation is unfavourable (in the absence of ferritin), but it is driven forward by the hydrolysis of the Fe(III) and its precipitation as insoluble ferric oxyhydroxide:



$$4Fe^{2+} + O_2 + 4H^+ = 4Fe^{3+} + 2H_2O$$



$$4Fe^{3+} + 8H_2O = 4FeOOH\downarrow + 12H^+$$



$$4Fe^{2+} + O_2 + 6H_2O = 4FeOOH\downarrow + 8H^+$$

Iron-core formation within ferritin is much faster than formation of ferric oxyhydroxide under protein-free conditions [142–147]. This is due, in the first instance, to catalysis of Fe(II) oxidation by apoferritin [96,143–149], but binding to residues within the cavity may lower the activation energy of nucleation of the oxidised product [142,150]. The protein also influences the form of oxyhydroxide produced. Thus the mineral formed within ferritin is ferrihydrite, whereas either α- or γ-FeOOH (goethite or lepidocrocite) is produced in the absence of the ferritin shell under otherwise identical conditions [114]. Although considerable variation in cavity surface residues is tolerated (Table 2), the efficiency of ferrihydrite nucleation probably depends on the distribution of cavity surface charges [150,151]. In EcFTN, the early non-ferrihydrite

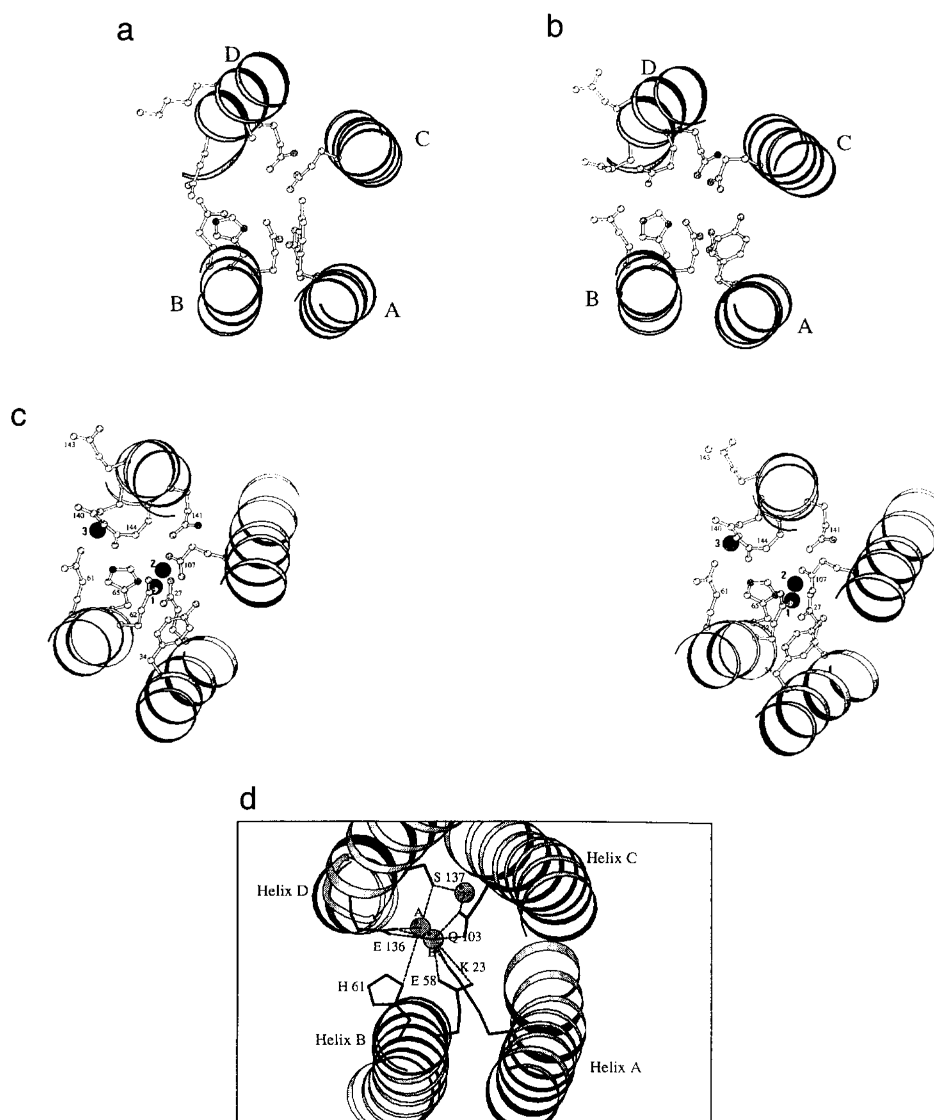


Fig. 6. Central hydrophilic region of ferritin subunits. (a) HuHF and (b) EcFTN, viewed from similar directions, showing the conserved residues (Glu 27, Tyr 34, Glu 61, Glu 62, His 65, Glu 107 and Gln 141) in the central region of the 4-helix bundle (see (c)) for residue numbers); (c): stereodiagram of EcFTN derivative obtained by soaking a crystal with $(NH_4)_2$ $Fe(SO_4)_2$. Three Fe atoms (grey spheres) are found in each subunit, a pair (Fe1 and Fe2, separation 3.8 Å) in the central region and a third Fe atom (Fe3) on the inside surface bound by Glu 61, Glu 140 and Glu 144 (numbers as aligned to HuHF). Note that residue 144 is alanine in HuHF. In a different orientation, Glu 144 can ligate the upper Fe atom of the diiron pair. Data from Ref. [86]; (d) BfLF. Many of the residues of HuHF and EcFTN are now changed (numbers as in L-ferritins – add 4 to equivalence to HuHF). The grey spheres represent water molecules. Courtesy of N.M. Allewell.

Table 2
Acidic and basic residues on the inner surface of mammalian ferritins compared with equivalent residues on some other ferritins

Res. No.	Mam H	Mam L	S.ma1 [a]	PS [b]	EcFTN	EcBFR
50	N	G	G	G	G	R
52	A	C/G/S	Y	A	A	N
53	K	H	K	K	A	D
54	Y	F	F	F	F	V
57	H	E	N	E	R	H
58	Q	L	E	S	H	E
61	E	E	E	E	E	D
64	E	E	Q	E	T	K
68	K	R	K	K	R	R
136	H	H/Y	E	E	–	–
140	E	E/K	I	E	E	D
147	E	K	K	K	L	W
171	D	E	D	D	D	A
176	G	K	H	H	T	E

The residues of columns 2 and 3 are conserved unless otherwise indicated. C-terminal residues not included. The residues in the last two columns are according to the alignment of Table 1.
[a] *S.mansoni* ferritin type 1.
[b] Pea seed ferritin aligned according to Ref. [53].

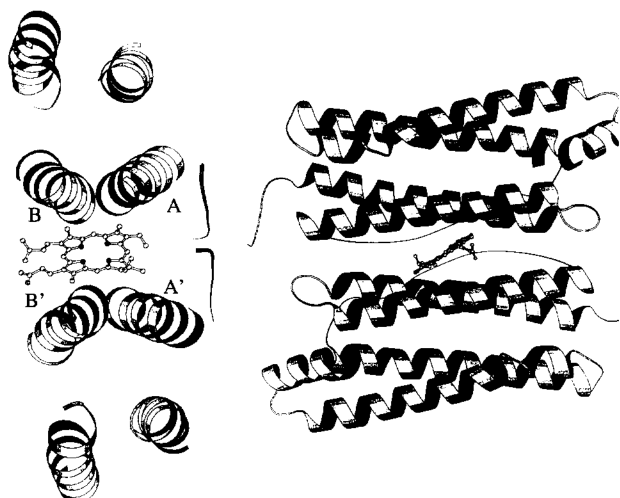


Fig. 7. Inter-subunit interface around 2-fold axis in protoporphyrin IX derivative of HoSF. (a) Part of shell with inside surface on the left and outside surface on the right; (b) subunit dimer showing protoporphyrin IX on 2-fold axis, viewed from the inside of the molecule. Based on coordinates in Brookhaven Protein Data Base, accession number 1 HRS and Ref. [104].

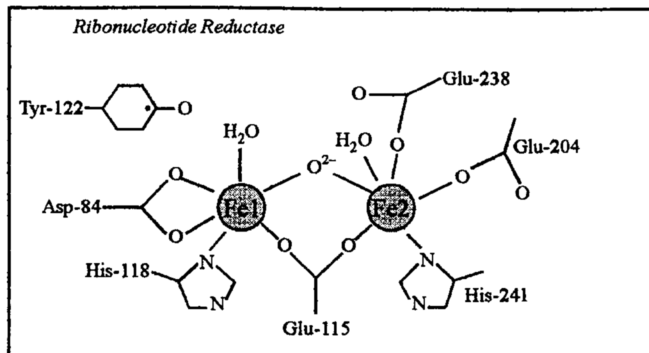
product of iron core reconstitution, found by Mössbauer spectroscopy, may reflect a unique distribution of amino acid residues, although this novel cluster accounts for only a small part of the iron (the bulk is ferrihydrite) [123,124].

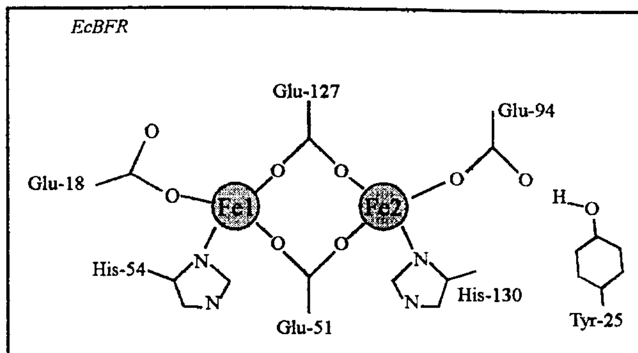
A large amount of work aimed at understanding iron-storage mechanisms has been carried out over the last 20 years, mainly in vitro. Experimental results are often difficult to interpret because of the many binding sites competing for iron within the protein subunit, on the cavity surface and at intersubunit interfaces. Traversal of the protein shell, Fe(II) oxidation, Fe(III) migration, ferrihydrite nucleation and iron-core growth can occur simultaneously at different sites in the same or different molecules and this leads to both intra- and inter-molecular competition for iron. A tendency towards 'all-or-none' distribution was noticed in early reconstitution experiments and a 'crystal growth' model was formulated [142]. As originally proposed, this involved the following stages [142,144]: first Fe(II) is bound and oxidised at catalytic centres on the protein, next Fe(III) clusters form in the cavity which yield stable nuclei of crystalline mineral (ferrihydrite) and, lastly, these nuclei provide a second (growing) surface onto which Fe(II) can be deposited and oxidized. Thus molecules that had nucleated would take up iron faster than those which had not, providing an important new driving force for iron sequestration. An alternative proposal was that *all* Fe(II) entering the ferritin molecule is catalytically oxidised by the protein before reaching the cavity [145,152].

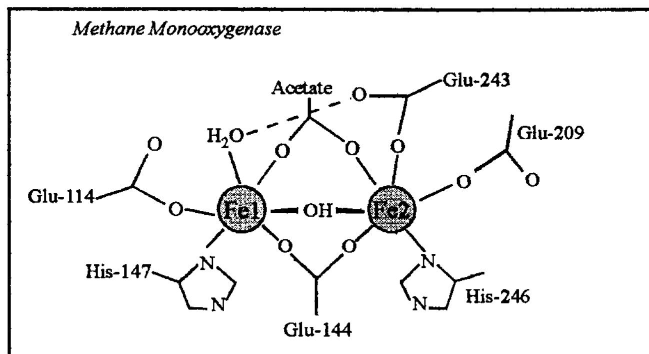
Recent studies of Fe(II) oxidation kinetics with horse spleen apoferritin and with HuHF which involved measurements of oxygen consumption [149,153–156] imply that the latter situation prevails at low Fe(II)/protein ratios (<50 Fe atoms/molecule) when oxidation on the protein shell is rate-limiting. Under other conditions, however, there is evidence that once a core is formed, Fe(II) can be bound and oxidised on its surface. In summary: (1) apoferritin loses its initial specificity for O_2 as oxidant when an iron-core is present [155]; (2) many more Fe(II) binding sites are present in ferritin than in apoferritin [120,157]; (3) the stoichiometry of Fe(II) oxidation increases from about 2 Fe(II)/O_2 at low Fe(II)/apoferritin up to about 4 Fe(II)/O_2 at higher ratios or when Fe(II) is added to ferritin [149,156,158–160]; (4) a mixed valence Fe^{3+}-Fe^{2+} species giving a $g = 1.87$ EPR signal is generated by the addition of 120 Fe^{2+} to 18 Fe^{3+}-ferritin or by the partial oxidation of 120 Fe^{2+} [161,162]; (5) when 4 ^{57}Fe(II)/molecule is oxidised by ferritin, 77% of the Fe(III) is attached to the core at 1 min and only 23% to the protein, but when the Fe(II) is added to apoferritin most of the iron is found on the protein and very little in iron-core clusters at early times, [163]; (6) after the early production of small Fe(III) clusters, the rate of Fe(III) deposition increases [142,147]. Kinetic analysis of Fe(II) oxidation in horse spleen ferritin and in HuHF indicates that the relative contributions of sites on the protein shell and on the iron-core depend on the amount of Fe(II)/molecule added, the amount of iron already present and the number of catalytic sites (H-chains)/molecule [124,149–156]. Observations (5) and (6) could also be explained if the stable Fe(III) clusters exerted a 'pull' on protein-bound Fe(III) by virtue of the greater thermodynamic and kinetic stability of cluster Fe(III). Thus the crystal growth hypothesis can be re-formulated to include the proposal that stable ferrihydrite nuclei in the ferritin cavity can provide surfaces for deposition of iron both as Fe(III) and Fe(II). In vivo it could be important for ferritin to accept iron as Fe(III).

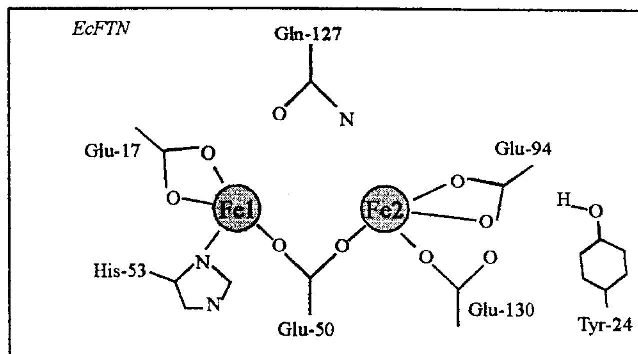
Since the determination of apoferritin's three-dimen-

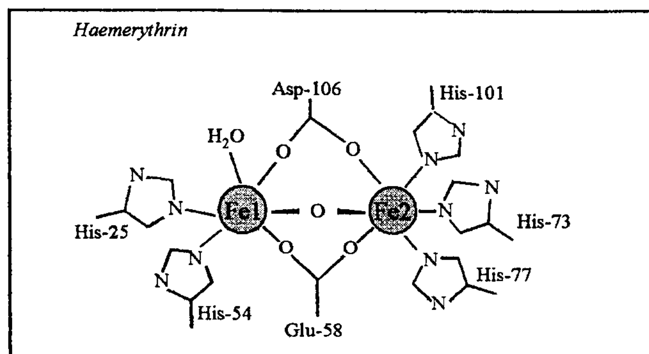
sional structure [34,80–82], work has focused on (a) the location of the catalytic sites, (b) the mechanism of catalysis of Fe(II) oxidation and (c) subsequent steps leading to the clustering of Fe(III) atoms and the generation of ferrihydrite. It was first proposed that Fe(II) was bound and oxidised in the 3-fold inter-subunit channels [90–94]. The stoichiometry of binding of several metals (e.g., Cd^{2+}, Tb^{3+}, Mn^{2+} and Fe^{2+}) of about 0.3 (or, in some cases, 0.67) per subunit and the competition by Fe^{2+} and Fe^{3+} for the other metal sites seemed to support such a hypothesis [34,35,80,90,91,95,120] and led to the conclusion that Fe^{3+} must migrate from such sites. Study of the effects of modifying a cysteine residue (no. 130) close to the 3-fold channels on Fe^{3+} EPR signals [92], or of iron on a

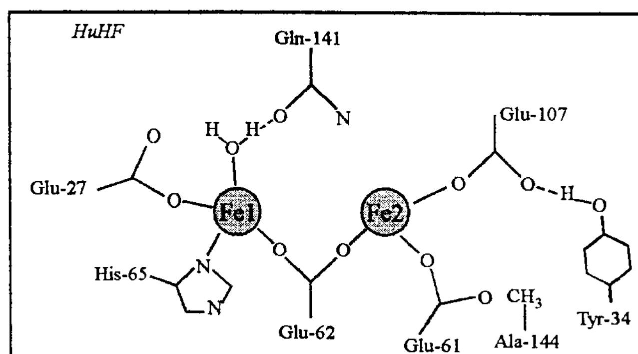


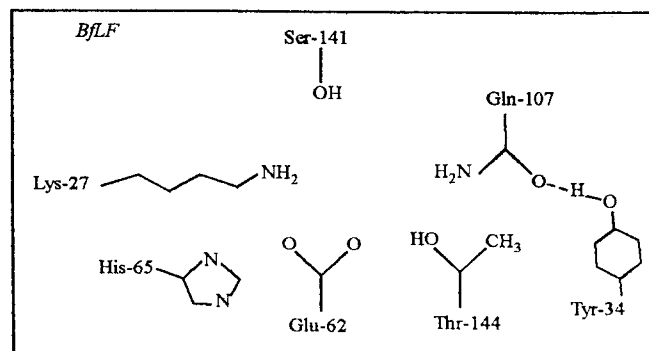


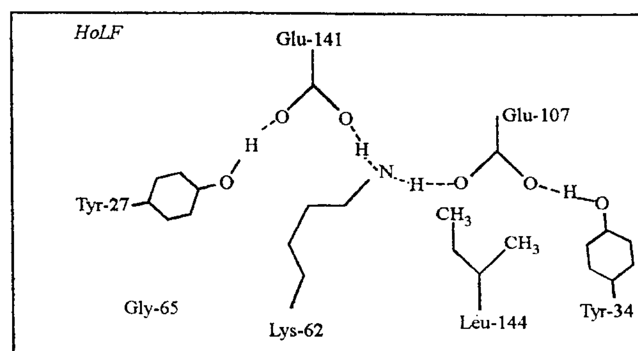












spin-label attached to this residue [94] were interpreted as implicating these channels as iron-binding entry and oxidation sites. These experiments were carried out with horse spleen apoferritin, which contains 85–90% L-chains. Electron-nuclear double resonance [164] and multifrequency electron spin-echo envelope modulation [165] measurements reveal the presence in horse spleen ferritins of a nitrogenous ligand (probably histidine) as well as carboxyls and a water molecule bound to VO^{2+}/Fe^{2+}. However, when it became evident that H-chains, not L-, are responsible for the catalysis of Fe(II) oxidation, the likelihood of direct 3-fold channel involvement diminished, since the channel residues are largely conserved in both H- and L-subunit types of vertebrates. Moreover, although EcFTN also catalyses Fe(II) oxidation, its 3-fold channels do not bind metals [86,124], Fig. 4c.

4.2. The catalytic sites on H-chains: the ferroxidase centres

Much of the recent work leading to new understanding of Fe(II) oxidation by apoferritin has been on recombinant ferritins containing a single subunit type. First, the catalytic activity has been associated, not with L-chains, but with H-chains, in human ferritin [96,124,146–148,150,151,156,166–168], in bullfrog ferritin [154,169] and in the H-type chains of pea seed [79] and both *E. coli* ferritins [124,170]. Second, a metal centre identified by X-ray analysis of human H-chain homopolymers, but absent from L-ferritin [35,82] (Figs. 6 and 8) has been implicated by site-directed mutagenesis. Thus the substitution of residues at this centre (E27A, Y34F, E62K + H65G, E107A and Q141E) leads to a diminution of ferroxidase activity [37,96,124,146–148,150,151,167,171,172]. Third, a μ-oxo-bridged Fe(III) dimer found to be an early oxidation intermediate in horse spleen apoferritin by Mossbauer spectroscopy is now known to be H-chain-specific, to be associated with ferroxidase activity and to require ferroxidase centre ligands for its formation [124,146,148,163,171] (Table 3). An important feature of the data in Table 3 is that even with only 10 Fe(II) added, up to 80% of the iron is in μ-oxo-bridged Fe(III) dimers at 1 min after Fe(II) addition, indicating that dimer formation is a highly cooperative process. The μ-oxo-bridged Fe(III) dimers can also be observed by UV difference spectroscopy [171]. Within under 30 s of adding Fe(II) to apoferritin in air an absorption spectrum appears (maxima about 305 and 345 nm) that is similar to those of the Fe(III) μ-oxo-bridged dimers of ribonucleotide reductase and met-haemerythrin as well as several model complexes [173].

In ferritin, an important property of the dimers for the iron-storage process is their relative lability. Both Mössbauer [96,124,148] and UV spectroscopic data [171] show that some of the dimers dissociate within about an hour of their formation, although it is uncertain how this dissociation is brought about. Dimers are partially replaced by monomeric Fe(III) and later by Fe(III) clusters and the distribution of Fe(III) amongst these species depends on pH, time and numbers of Fe added [96,124,148]. Some of the monomeric Fe(III) so produced has been located in the 3-fold channels [96], and it may be guided from the ferroxidase centres to these channels by groups on the protein shell [96]. This implies that there is communication between the channels and the ferroxidase centres in vertebrate ferritins. However, this does not apply to EcFTN, which binds Fe(III) atoms at the putative ferroxidase centre Fig. 6 but none in the 3-fold channels, which lack suitable ligands [86] (Fig. 4c).

Evidence for a three phase mechanism corresponding Fe(II) binding, catalytic Fe(II) oxidation and core formation has been derived for EcBFR from EPR and visible spectroscopy and stopped-flow kinetic data [174]. Fe(II) was added aerobically and its binding to EcBFR was monitored as a perturbation of the 380–425 nm region of the haem absorption spectrum. Maximum absorbance change was found with the binding of 50 Fe atoms/molecule (2 Fe(II)/subunit), which occurred without concomitant haem reduction. This was the fastest observed phase with $t_{1/2} \sim 50$ ms. Non-haem Fe(II) oxidation, which was monitored at 340 nm, showed two distinct phases. The first (phase 2 of the overall process) was complete within a few seconds and also saturated after addition of 50 Fe(II) atoms/BFR. Phase 2 was interpreted as oxidation of two Fe(II) atoms at the ferroxidase centres. This conclusion

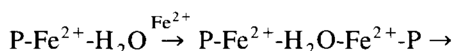
Fig. 8. Schematic drawings of the dinuclear metal sites of three H-type ferritins compared with similar sites in ribonucleotide reductase, methane monoxygenase and haemerythrin and with the equivalent regions of two L-type ferritins. The dinuclear iron sites of ribonucleotide reductase, methane monoxygenase and haemerythrin are from their crystal structures (respectively Refs. [81,182,185,183,184]). The different disposition of ligands in haemerythrin with respect to the 4-helix bundle places it in a separate class of diiron proteins (class 1) from that of ribonucleotide reductase and methane monoxygenase (class 2). The ligands and ligand positions of the dinuclear metal site in EcBFR clearly place this ferritin in class 2. The diiron site of EcBFR is based on the crystal structure in which the metal ions are likely to be Mn(II) [85]. Hence this arrangement, with double carboxylate bridging, is expected for the diferrous derivative of EcBFR. In contrast, the dinuclear site shown for ribonucleotide reductase, which has one carboxylate and one -oxo-bridge corresponds to the diferric form [182]. The diiron site for EcFTN is based on the crystal structure of an iron-containing derivative [86] (see Fig. 5c)). Note that Glu 127 of EcBFR is replaced by Gln 127 in EcFTN and His 130 is replaced by Glu130, thus maintaining four carboxylate ligands. HuHF also has Gln 141 (equivalent to Gln 127 of Ec FTN), but Ala 144 replaces Glu 130 and another carboxylate, Glu 61, can act as an alternative ligand of Fe2. In BfLF and HoLF (residues numbered as in HuHF) there is further divergence in sequence and these ferritins do not form the dinuclear sites characteristic of the H-ferritins. Instead, in BfLF, there is a network of hydrogen bonds involving the residues shown and three water molecules (not shown here, but see Fig. 6d), and in HoLF, Lys 62 forms a salt bridge. Ala144 of HuHF is replaced in BfLF by threonine and in HoLF by leucine.

was supported by the observation of EPR signals ascribed to 22 [Fe(II) Fe(II) NO] dimers per BFR molecule, when 50 Fe(II) was added to apo-BFR anaerobically in the presence of ascorbate followed by NO [170] and by the low oxidation rates observed with ferroxidase centre variants E17A, E51A and E94A (see Fig. 8) [175]. The third, slowest, kinetic phase, which corresponds to growth of the iron core, took up to 80 min to complete and was considered to represent Fe(II) oxidation on iron-core surfaces. Recent studies with haem-free variants indicate that the rate of Fe(II) oxidation (phase 2) is not diminished in the absence of haem, but is slightly enhanced [103].

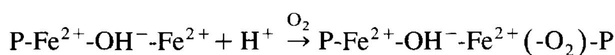
4.3. The mechanism of Fe(II) oxidation at ferroxidase centres

Two alternative schemes for Fe(II) oxidation have been considered. In the first, (a), two Fe^{2+} atoms are bound to the protein (P) in close proximity allowing the formation of an OH^- bridged Fe^{2+} dimer. O_2 binds to one of the Fe^{2+} atoms allowing oxidation of both of them with concomitant formation of H_2O_2 [171].

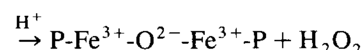
(a) 2-electron oxidation



$$\text{P-Fe}^{2+}\text{-H}_2\text{O} \xrightarrow{Fe^{2+}} \text{P-Fe}^{2+}\text{-H}_2\text{O-Fe}^{2+}\text{-P} \rightarrow$$



$$\text{P-Fe}^{2+}\text{-OH}^-\text{-Fe}^{2+} + \text{H}^+ \xrightarrow{O_2} \text{P-Fe}^{2+}\text{-OH}^-\text{-Fe}^{2+}(\text{-O}_2)\text{-P}$$

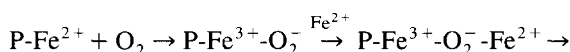


$$\xrightarrow{H^+} \text{P-Fe}^{3+}\text{-O}^{2-}\text{-Fe}^{3+}\text{-P} + \text{H}_2\text{O}_2$$

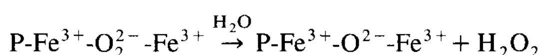
Variants of this scheme may be envisaged in which oxidation of the two Fe^{2+} occurs without prior formation of an OH^- bridge between them, but with bridging either through a water molecule or through dioxygen itself. The cooperativity seen for dimer formation (Table 3) could result from two-electron oxidation in an Fe^{2+} pair. Pairwise oxidation has also been indicated by recent stopped-flow kinetic data [172].

In an alternative oxidation scheme [149,156], (b), a single Fe^{2+} atom is bound to the protein (P). It is then oxidised without the dissociation of the superoxide so produced. A second Fe^{2+} is then bound and oxidised with the net result that peroxide is produced (as in the first scheme):

(b) two successive 1-electron oxidations.



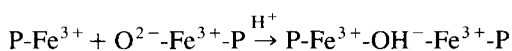
$$\text{P-Fe}^{2+} + \text{O}_2 \rightarrow \text{P-Fe}^{3+}\text{-O}_2^- \xrightarrow{Fe^{2+}} \text{P-Fe}^{3+}\text{-O}_2^-\text{-Fe}^{2+} \rightarrow$$



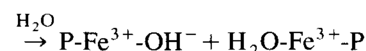
$$\text{P-Fe}^{3+}\text{-O}_2^{2-}\text{-Fe}^{3+} \xrightarrow{H_2O} \text{P-Fe}^{3+}\text{-O}^{2-}\text{-Fe}^{3+} + \text{H}_2\text{O}_2$$

This scheme was based on kinetic studies of horse spleen ferritin [149] and of HuHF [156] which indicated that the reaction was first order in both iron and oxygen. However, rate measurements made with an oxygen electrode may be misleading because of its relatively slow response compared with rates of Fe(II) disappearance measured in a stopped-flow spectrometer [172].

The oxo bridge derives from water in the first scheme and from dioxygen in the second. No evidence has yet been obtained for the origin of this oxygen in ferritin. Recent Resonance Raman experiments using $^{18}O_2$ indicate that in ribonucleotide reductase, but not in stearoyl-ACP desaturase, the bridging O is derived from dioxygen [176,177]. After oxidation in ferritin (by either scheme) splitting of some of the dimers is envisaged, e.g.,



$$\text{P-Fe}^{3+} + \text{O}^{2-}\text{-Fe}^{3+}\text{-P} \xrightarrow{H^+} \text{P-Fe}^{3+}\text{-OH}^-\text{-Fe}^{3+}\text{-P}$$



$$\xrightarrow{H_2O} \text{P-Fe}^{3+}\text{-OH}^- + \text{H}_2\text{O-Fe}^{3+}\text{-P}$$

Table 3
Mössbauer analysis of products of aerobic oxidation in presence of various ferritins

Sample	Fe/mol [a]	tf [b]	%Fe(II)	% Fe(III) species			
				Monomer	Dimer	Cluster	Magnetic cluster
apo-rHuHF	10	1 min	-	6	78	16	
	10	3 h	-	36	35	29	19
	12	1 min	-	26	64	10	-
	12	24 h	-	20	50	30	10
apo-HoSF	12	1 min	-	19	25	56	28
	12	24 h	-	-	-	100	92
apo-rHuHF	34	1 min	-	6	78	16	n.d.
apo-EcFTN	34	1 min	-	22	30	48	n.d.
apo-rHuLF	34	3 min	40	10	-	50	n.d.
apo-rHuLF	34	30 min	-	-	-	100	n.d.
apo-rHuHF (E27A)	34	1 min	39	-	-	61	n.d.
apo-rHuHF (E107A)	34	30 min	35	6	-	49	n.d.

Data obtained in 0.1 M Mops buffer (pH 7.0), see Refs. [124,146,148] for details.
[a] Numbers of Fe(II) atoms/mol added.
[b] Time of freezing after aerobic Fe(II) addition.
Note: (1) that HuLF and HuHF variants oxidizing iron slowly (E107A and E27A) show no dimers; (2) that HuLF and HoSF (85% L) show relatively large percentages of clusters (including large, magnetic clusters) compared with HuHF; (3) that Fe (III) dimer formation in HuHF shows co-operativity (e.g., 8 Fe atoms out of 10 added in dimers at 1 min).

followed by migration of one or both atoms from the ferroxidase centre to the iron-storage cavity and to the 3-fold channels. If only one Fe^{3+} left the ferroxidase centre and was replaced by Fe^{2+} then one-electron oxidation might follow.

The O atoms *of the core* come from water and not from dioxygen, since when $^{18}O_2$ was used as an oxidant, no evidence was found for the presence of the heavy isotope in the resulting iron core [178]. It is interesting to recall an earlier oxidation scheme [36] which was not dissimilar to that of Scheme (a) except that O_2 was bound between two Fe(II) on neighbouring subunits and both oxygen atoms were subsequently incorporated into the core.

4.4. Fe(III)-tyrosinate and tyrosine radicals

Very recent work suggests that Fe(II) oxidation in ferritin is more complex than any of these mechanisms implies. When O_2 was admitted to an anaerobic mixture of apo-BfHF and $FeSO_4$ (1 mM in subunits, 24 Fe/molecule [pH 7.0], 0.45 M Cl^-), or when FeSO4 was added to an aerobic solution of the apoferritin, a purple colour appeared, which was replaced within a few minutes by the amber colour characteristic of ferritin iron cores [169]. The purple species (absorbance maximum 550 nm) was identified by Resonance Raman spectroscopy as a tyrosinate → Fe(III) charge transfer complex [169]. A species absorbing in the same region appeared very rapidly when Fe(II) was added to apo-BfHF in a stopped-flow spectrometer under aerobic conditions [154]. It was estimated to account for about 20% of the Fe(II) oxidised. Aerobic addition of Fe(II) to HuHF (34 Fe/molecule, pH 7.0) also led to an increase in absorbance at 550 nm. This absorbance reached a maximum in under 0.5 s and then decayed over about 5 s (although not to zero) [148]. Under these conditions the absorbance was found to extend broadly over the range 400–800 nm with a maximum at 650 nm [172] and the presence of Fe(III)-tyrosinate in HuHF has not yet been verified. Possibly another species (e.g., diferric-peroxo), or more than one absorbing species, contributes to the wide band in HuHF [172]. Absorbance in the near UV (300–400 nm) also increased in HuHF and continued to do so during the period in which the visible absorbance declined [148,172]. A kinetic study of the putative Fe(III)-tyrosinate in BfHF showed that after its initial rapid formation and decay, it was not completely regenerated by a second Fe(II) addition until after a lapse of 12 h [154]. In HuHF the initial coloured species is likely to have already decayed by the time (30 s) when the major species (the Fe(III) μ-oxo-bridged dimer) was observed by conventional Mössbauer [96,124,148,163,172] and UV-difference [148,171] spectroscopy. The identity of the initial species and its mechanistic relationship to the Fe(III) dimers is uncertain, and remains a problem for research. The Fe(III) dimers fail to form in ferroxidase centre variants E27A and E107A of HuHF in which Fe(II) oxidation is very slow [148]. In the variants examined so far, the visible absorbance also fails to appear in E27A, Y34F and Q141E (E107A not examined), showing that its formation depends on an intact ferroxidase centre [148,168]. However, its appearance seems not to be essential for dimer formation, for catalysis of Fe(II) oxidation, or for the subsequent steps leading to mineralization, since, in both EcBFR (in which residue 141 is glutamate, not glutamine) and in HuHF variants Q141E and Y34F, and also in EcFTN, these processes occur without detection of the coloured species [148,124,172,174]. However, it is conceivable that in these ferritins the coloured species forms and decays within the dead time of the stopped-flow spectrometer. Tyr 34, a conserved residue at the ferroxidase centre, has been proposed as the tyrosinate Fe(III) ligand [148]. However, Waldo et al. [169] consider this is unlikely since it is present in L-ferritins, which do not give the Fe(III)-tyrosinate species. They proposed that the ligand is Tyr 29, a residue on the outer surface of the molecule [169], but no reduction in absorbance at 550 nm is found in HuHF-Y29F (or in Y32F or Y137F) [172]. It is intriguing, nevertheless, that the blue species is not seen in several ferritins (including EcBFR and EcFTN) which do contain Tyr 34, but in which iron ligands or charge balance at the ferroxidase centre are altered [148,172,174]. Indeed a blue absorbance has been generated in an EcFTN variant E130A (E144A using H-chain numbering) see Figs. 6 and 8 [172].

Another interesting recent observation (by EPR spectroscopy) is that of a tyrosine radical. This radical is associated with Fe(II) oxidation in HuHF, although it is seen only after oxidation is complete and decays within a few minutes [179]. Again Tyr 34 seems to be the residue concerned. However, only one tyrosine radical is observed per 300 Fe(II) atoms oxidised and its significance is unclear. A different EPR signal attributed to an amino acid radical but not to tyrosine or tryptophan is also seen in rapidly frozen samples within about 20 ms after addition of Fe(II) to horse spleen apoferritin [180]. Understanding the mechanism of Fe(II) oxidation in H-ferritin and the subsequent steps leading to mineralisation is one of the most challenging and exciting aspects of current research.

4.5. The dinuclear Fe centres of ferritins and other proteins compared

The dinuclear metal centres observed or proposed for the three ferritins described above (HuHF, EcFTN and EcBFR) which have been associated with catalytic Fe(II) oxidation are shown schematically in Fig. 8, where they are also compared with the di-iron centres of ribonucleotide reductase (RNR, R2 subunit) [181,182], methane monoxygenase (MMO) hydroxylase (subunit) [183] and haemerythrin [184]. The di-iron centres of these proteins are found, like those derived for the ferritins, at the centres of helical bundles. Based on the relative arrangements of iron ligands with respect to their positions along the

helices the dinuclear iron proteins fall into the two classes: haemerthyrins in class I, RNR R2 and MMO and stearoyl-ACP desaturase in class 2 [177]. The similarities between the six centres are striking, especially between the dinuclear centre of EcBFR and those proposed for the diferrous forms of ribonucleotide reductase (based on the di-Mn(II) derivative [181,185]) and also stearoyl-ACP desaturase [177] and the acetate derivative of methane monoxygenase [182]. It is clear that the ferritins fall into the class 2 group. In EcBFR each Fe atom is ligated by one histidine residue and the two Fe atoms are bridged by two carboxy groups as in the di-Mn(II) (and presumably di-Fe(II)) form of R2. It may be speculated that the structure of the dinuclear centre shown for EcBFR corresponds to the diferrous form and that breakage of the Glu 127 bridge accompanies Fe(II) oxidation, as proposed for RNR R2 [181,175]. In R2, formation of the dinuclear Fe(III) centre is accompanied by the production of a stable tyrosine radical (Tyr 122), Fig. 8, at about 5 Å from Fe1 and this radical is implicated as a source of reducing potential in the activity of the enzyme complex [182]. In HuHF, Tyr 34 lies at about 5 Å from Fe2. This tyrosine is conserved in all known ferritin sequences, including those of L-subunits and it may have a structural rather than a functional role. An organic radical giving a $g' = 2$ EPR signal has been reported for EcBFR, but it has not been identified [98].

Another point of great interest is the non-identity of the dinuclear centres of the three ferritins in Fig. 8. EcFTN and HuHF contain only one histidine ligand (of Fe1), but have glutamine instead of the second bridging glutamate (Glu 127) of EcBFR. Also, the glutamate ligands of Fe2 in EcFTN and HuHF, replacing His 130 in EcBFR, are at different sequence positions. Glu 130 of EcFTN corresponds to Ala 144 in HuHF, whereas Glu 49 (Glu 61 using H-chain numbering and a proposed ligand of Fe2 in HuHF) is a ligand of Fe3 (not shown in Fig. 8, see Fig. 6c and Table 1). Glu 130 is at an equivalent position to His 130 of EcBFR (in helix C) but Glu 49 (61), in helix B, is at a different position not only to those of His and Glu 130, but to the equivalent ligand of the other class 2 di-iron proteins (see Fig. 8). The significance of the differences in the three iron sites of these ferritins remains to be determined. Glu 130 (or its sequence equivalent) is known only in the two bacterial non-haem ferritins (EcFTN and HpPFR) [63,66] (Table 1).

4.6. Role of third iron site?

Evidence has been summarised above implicating the dinuclear iron sites of Figs. 6 and 8 in the initial oxidative steps leading to iron storage in HuHF, EcFTN and EcBFR. In HuHF a third metal site has been observed on the inner surface of the protein shell [82]. Glu 61 and Glu 64 are the ligands binding Tb^{3+} at this site, which was proposed to be a nucleation centre for the iron core. Movement of Glu 61 (also a ligand of Fe2 in Fig. 8) was proposed to assist in the transfer of iron from site 2 to the putative nucleation centre. However, substitution of Glu 61 and Glu 64 by alanine produced no dramatic perturbation of core formation or of Fe(II) oxidation [146]. Moreover the observed initial stoichiometry of 2Fe(II) oxidised per O_2 suggests that only the dinuclear iron is involved in the first oxidative step [149,156,158–161,172].

The situation in EcFTN may be different. Here there are clearly three Fe^{3+}-binding positions (Fig. 6c). The third site (ligands Glu 140 and 144 (H-chain numbers)) again lies on the cavity surface (at 7–8 Å from the dinuclear iron). However, its position and ligands are unique to EcFTN (residue 144 is Ala in HuHF). Moreover, the initial stoichiometry of about 3 Fe(II) oxidised/O_2 has been observed using an oxygen electrode (Treffry, Zhao, Quail, Guest and Harrison, unpublished work). Thus oxidation of the three Fe^{2+} of Fig. 6c by a single dioxygen molecule is a possibility. It is particularly interesting to note that, in ribonucleotide reductase R2 subunit, three Fe^{2+} may be oxidised by one dioxygen molecule during the formation of the diferric site, together with the nearby tyrosine radical and the 4-electron reduction of dioxygen. In this case, no third Fe binding site has been observed [186,187]. In EcBFR evidence for formation of a mononuclear iron site has been provided by observation of EPR signals ($g = 2.04$, 2.015, 2.00) attributed to $Fe(NO)_2$ (LeBrun, Andrews, Moore and Thomson, submitted). Neither the position of this iron in the protein shell nor its relationship to the dinuclear centre is known, but a site on the cavity surface is suggested.

4.7. Why heteropolymers? – the role of L-chains and the disadvantage of too many H-chains

Another intriguing structure-function problem is the role of L-chains of vertebrate ferritins that lack the intra-subunit ferroxidase centres of H-chains. Whereas horse spleen ferritin has a relatively high iron content (averaging 2700 Fe atoms/molecule) and high average percentage L-chain (ca. 85%), the L-chain fraction extracted from it is devoid of iron [77]. In addition, recombinant human L-polymers bind less than 10 Fe atoms/molecule during expression in *E. coli*, whereas the H-homopolymer accumulates up to 200–300 Fe atoms/molecule under the same conditions [166]. Although ferritin molecules containing only L-chains do not seem to take up a significant amount of iron in vivo, the L-chain clearly has an important role in association with H-chains. In vitro studies of its functionality together with those of H-chain homopolymers, and reassembled H/L copolymers, have provided vital information concerning the complementarity of the two types of ferritin chain. Recombinant human L-chain homopolymers are able to build iron-cores starting from Fe(II) at pH 7.0, although more slowly than human H-chain apoferritin and native horse spleen apoferritin (which has about 3 H-chains/molecule [93,96,146–148,150,151,156,

166,167,172]). It has been postulated [165] that L-chains possess an alternative Fe(II) oxidation site (probed by VO^{2+}-binding) and that this could be a metal site with His 136 and Asp 139 as ligands identified by X-ray crystallography and situated near the 3-fold channel on the cavity side [188]. However, in BfLF His 136 and Asp 139 are replaced by proline and serine respectively [36,189] and oxidation must occur elsewhere in this ferritin, probably at nucleation sites involving carboxy ligands [83]. Recent data show that the substitution of carboxyl groups lining the 3-fold channels or exposed on the cavity surface of HuHF do reduce the rate of core formation possibly due to interference with iron entry and nucleation respectively [190]. It has also been found that recombinant HuLF will accept Fe(III) produced by HuHF and use it for core formation [151]. In contrast, H ferritins are relatively poor core-formers [146,191,192]. It is also noted that iron-cores in recombinant L-ferritins and on native heteropolymers are slightly larger and more regular than those in H-chain ferritin as viewed by electron microscopy [150]. These experimental findings have led to the view that L-chains may be better at ferrihydrite nucleation [150,151]. A possible explanation is that the cavity faces of L-chains have more carboxy ligands (or negative charges) than those of H-chains (Table 2). This explanation is supported by the finding, in HuLF, that the substitution by histidine of Glu 57 and Glu 60 (residues exposed on the cavity surface) reduced the efficiency of core formation [190].

Although the H-chain is essential for rapid Fe(II) oxidation [37,147], too great a proportion of H-chains leads to protein aggregation in vitro, probably due to Fe(III) loss and hydrolysis outside the molecule [191]. Iron newly oxidized in H-ferritin may also be lost to other molecules: in vitro it can be donated to transferrin [193] as well as to other ferritin molecules [192]. Production of H_2O_2 in Fe(II) oxidation catalysed by H-chains [159,171] could also lead to toxicity, if the peroxide is not disposed of, but the rapid provision of alternative oxidation sites on iron core particles avoids this problem. Thus it seems to be advantageous for ferritin molecules to contain both H- and L-chains. In recent experiments with H/L heteropolymers reassembled in specific proportions, iron was incorporated optimally in molecules containing between 5 and 8 H-chains [194]. In Mössbauer experiments relatively high proportions of iron atoms are found in iron core clusters in native ferritins (horse spleen) [146] or in HuHF and HuLF mixtures [124] than in H-homopolymers (Table 3). Studies aimed at constructing a ferroxidase centre in the human L-chain showed that the presence of the metal ligand residues, especially Glu 62, strongly interfered with L-chain folding. However, when the variants were induced to fold by co-assembly with wild-type L-chains, they showed ferroxidase activity comparable to that of the H-chain [195].

As far as is known, plant ferritins are homopolymers of a single subunit type and this also applies to bacterial

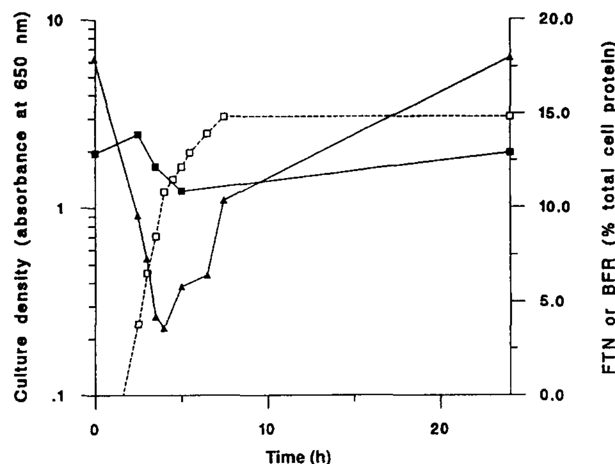


Fig. 9. Overproduction of EcFTN and EcBFR during the growth cycle of *E. coli*. □ A_{650}; ■ EcFTN; △, EcBFR. Reproduced with permission from [123].

ferritins. Plant ferritins, so far sequenced, combine a pattern of carboxylate residues on their cavity surfaces like that of mammalian L-chains [99,151] with conserved ferroxidase centre residues typical of H-chains (except that in one of two known pea seed ferritin sequences residue 62 is histidine, not glutamate [37,53]). Recombinant pea seed ferritin (containing the seven ferroxidase centre residues) has recently been shown to exhibit ferroxidase activity [79]. EcFTN has all seven ferroxidase centre residues conserved (Fig. 6) and also catalyses Fe(II) oxidation [123,124] but, as indicated above, it displays some differences in its iron centre (Figs. 6 and 8) and also shows a different distribution of cavity residues from those in either H- or L-chains of mammals (Table 2). In BFRs of *E. coli* [62,75], Fig. 8, and *A. vinelandii* [64], five of the putative ferroxidase centre residues are conserved. EcBFR is known to catalyse Fe(II) oxidation although at a slower rate than EcFTN or HuHF [37,75,123] and this catalysis is greatly impaired by substitution of each of three carboxylates (made one at a time) at the proposed ferroxidase centre [175]. It is not clear why two different ferritin molecules have evolved in *E. coli*, although it has been postulated that EcFTN, which remains at a constant level throughout bacterial growth, is responsible for iron homeostasis, whereas EcBFR, which is produced in maximal amount during stationary phase (Fig. 9) is more comparable to the L-ferritins of vertebrates [123,196]. However, in studies on *bfr* and *ftn* single and on *bfr, ftn* double mutants of *E. coli*, no physiological function was identified for EcBFR, whereas EcFTN was shown to play a role both in iron-storage and in the efficient utilisation of iron (Andrews, personal communication).

4.8. The route of Fe(II) entry into ferritin protein shells

The most direct route (about 12 Å) from the outside of the molecule to the ferroxidase centre appears to be a

small intra-subunit 'channel' in the H-chain [82]. In L-chains the ferroxidase centre is blocked by a salt bridge, and the channel by a leucine residue. It is not known whether Fe(II) uses the channel in H-chains. However, it is known that Fe(II) can reach the cavity of L-chain ferritin homopolymers.

Various studies have implicated the 3-fold inter-subunit channels directly or indirectly in iron entry as discussed in Section 4.1. Several metal ions, (Cd^{2+}, Ca^{2+}, Zn^{2+} and Tb^{3+}) have been shown by X-ray analysis to bind in the 3-fold channels of both H- and L-homopolymers and native heteropolymers of ferritin [34,36,82] (see Fig. 4 for an example). Inhibition of iron incorporation by zinc or terbium indicated that these metals have common sites both in horse spleen ferritin [90,197], and in recombinant human H-chain ferritins [96], although this does not definitely or exclusively implicate the channel sites. However, metal binding and competition studies (e.g., Ref. [90]) and use of horse spleen ferritin chemically modified in the outer regions of the 3-fold channels [92,94] show that these regions bind Fe(II). EPR spectroscopic measurements on Fe^{3+}. NO complexes of recombinant human H-chain ferritin specifically pinpointed His128, Cys130 and probably His118 as iron ligands which are situated in the outer channels [198]. Again recombinants with residue substitutions in the narrowest parts of the channels (Asp131 and Glu134 changed to alanine or histidine) incorporated iron more slowly than the wild type [96]. However, if the threefold channels are used for the passage of iron a specific mechanism for passing Fe(II) along them does not seem to be required in all ferritins, since channel residues are not highly conserved in bacterial and invertebrate ferritins (Table 1). Alternative routes may be available, such as the intra-subunit channel.

4.9. Iron oxidation in the absence of dioxygen

Although, in the presence of O_2, ferritin can utilise its ferroxidase centres, under anaerobic conditions, Fe(II) added to apoferritin, or produced in situ by core reduction (see below), can also be oxidized either by ferricyanide or by oxidized cytochrome *c*, cytochrome *c*-551, cytochrome *b*5, metmyoglobin or plastocyanin giving apparently normal cores [199,200]. These cores must form by Fe(III) hydrolysis without incorporation of atoms of molecular oxygen. Rates of haem reduction by reduced horse ferritin were found to be $< 0.2\ M^{-1} \cdot s^{-1}$ for metmyoglobin, but $1.1 \times 10^2\ M^{-1} \cdot s^{-1}$ for horse ferri-cytochrome *b*5 and $1.1 \times 10^3\ M^{-1} \cdot s^{-1}$ for horse ferri cytochrome *c* [200]. These results imply that the redox mechanism in the absence of O_2 involves long range electron transfer through the ferritin protein shell to iron within the cavity. Cytochromes, which are located in the mitrochrondia, are unlikely to be potential physiological redox partners for ferritin, however. An alternative to long range electron transfer from an external donor apparently resides in the protein shell itself. Microcoulometric titrations of (oxidised) horse spleen apoferritin performed by Watt and colleagues indicated that the protein can acquire up to six electrons [201,202]. These authors concluded that the protein shell itself must contain redox centres. When ferricyamide was added anaerobically to the protein these centres were apparently re-oxidised, since Fe(II) added to the treated protein was converted to Fe(III) (as measured by EPR spectroscopy). It is uncertain whether the redox properties are intrinsic to the protein shell or whether they reside in a 'cofactor' or redox active substance attached to the protein, but the numbers suggest the possibility that they could be associated either with H-chains or with the 3-fold or 4-fold inter-subunit interfaces. Watt et al. [202] suggest the involvement of tyrosine residues near the 4-fold interfaces. Anaerobic oxidation by nitric oxide of protein bound Fe(II) has been observed in both mammalian ferritin [198] and EcBFR ([170] and LeBrun, Andrews, Moore, Thomson, submitted). The physiological significance, if any, of this observation needs to be assessed.

4.10. The effect of phosphate on iron-core formation

Native ferritin iron-cores usually contain some inorganic phosphate [99,108–112] but most in vitro iron-loading experiments have been carried out with iron alone. When phosphate is added with iron, smaller, less regular cores are formed [121,123,125]. About 60% of the phosphate present in horse spleen ferritin is released in the initial stages of iron release and is probably superficial [203,204]. Ferritin iron-cores reconstituted without phosphate will absorb this and other anions [203]. Such experiments, together with the relatively low intracellular concentration of iron compared to phosphate, point to some degree of compartmentalisation of these components in animal cells. The higher P:Fe ratios and the disorder observed by electron diffraction and EXAFS in iron-cores of plant [99] and bacterial ferritins [120,123] may reflect higher phosphate concentrations in the media from which these ferritin acquire their iron, e.g., 12 mM in plastids [99]. The amount of phosphate in these ferritins is, indeed, so high (e.g., 640 P/molecule in pea seed ferritin [99]) that they may be considered to be stores of phosphorus as well as of iron.

Recent experiments on the kinetics of Fe(II) oxidation by horse spleen apoferritin in the presence of phosphate (12 Fe(II) added per apoferritin molecule with equimolar phosphate [205]) have shown two effects. The rate at which Fe(II) is oxidised, measured by Mössbauer spectroscopy, is significantly increased in the presence of phosphate and the rate of loss of monomer Fe(III) ($g' = 4.3$ EPR signal) due to the formation of EPR-silent core is also accelerated. The 2-fold acceleration of Fe(II) loss is interpreted as an effect of anion binding on the redox potential of the ferroxidase centre (making it more negative), al-

though an alternative explanation may be that fewer ferroxidase centres are occupied by Fe(III) owing to the greater affinity of the phosphate-containing iron-core. There is no evidence that phosphate binds to apoferritin in the absence of iron. At variance with this result was the finding of no acceleration by phosphate on rates of Fe(II) oxidation measured as O_2 consumption [149]. The effect of phosphate on the anaerobic binding of Fe(II) has also been investigated [206,207]. The presence of phosphate markedly increased Fe(II) binding, but only 8 Fe(II) per molecule were retained after gel filtration of either horse spleen apoferritin or ferritin containing a phosphate-free reconstituted core. The iron was assumed to be bound in the 3-fold channels and this result was taken as evidence against the hypothesis that (in the absence of phosphate) Fe(II) is bound and oxidised at iron core surfaces in the second stage of ferritin formation. However, this conclusion is not necessarily correct since the Fe(II) atoms need only to be bound transiently before oxidation. Apo-AvBFR binds more Fe(II) than horse spleen apoferritin, namely 25 Fe(II)/molecule at pH 5.5 and 150 at pH 9.0, with proton release increasing to up to 2 per Fe(II) at the higher pH [206]. The larger number of Fe(II) atoms bound by holo-BFR than by HoSF is attributed to its greater phosphate content [206]. The effect of phosphate on iron loading in EcBFR has recently been investigated [170]. Aerobic dialysis with phosphate leads to the loss of EPR signals due to non-haem iron, probably due to the shepherding by phosphate of iron from the ferroxidase centre into the cavity with the formation of a ferric phosphate core. Anaerobic dialysis leads to Fe(III) reduction and the transfer of Fe(II) from the core to the non-haem sites on the protein.

4.11. Can ferritin store iron as Fe(II)?

Given the low redox potentials of ferritin (− 190 mV at pH 7) and BFR (−420 mV) iron cores and given the intracellular presence of a variety of molecules that may act as oxidants, it seems probable that ferritin iron is normally Fe(III). However, it has been proposed that under oxygen limitation ferritin can retain or store iron in the reduced state [110,120,208,209]. If ferritin or BFR iron-cores are reduced stoichiometrically in the absence of chelators in vitro, most of the Fe(II) is retained by the protein shell during anaerobic gel filtration [110,120]. Addition of chelators causes this Fe(II) to be released. In other experiments, 20 mM Fe(II) was added to apoferritin (480 Fe(II)/molecule, pH 7.0) in air and it was shown by EXAFS that Fe(II) oxidation took several hours to complete [208]. The Fe(II) was judged to be inside the protein shell because of its failure to react with o-phenanthroline. It is difficult to assess the validity of this conclusion, however, because Fe(II) seems to be readily released to chelators in other experiments [110,120,199,210]. The fractions retained may have been oxidised to Fe(III). In other work, significant binding and retention of Fe(II) atoms has been found under anaerobic conditions only if a phosphate-containing core was present or if Fe(II) and phosphate were added together [207]. However, recently a 'magneto-ferritin' has been produced under controlled reconstitution conditions (including slow oxidation) [211]. This ferritin is black and contains magnetite-maghemite (Fe_3O_4-γFe_2O_3) [212]. It is presently unknown whether any iron is stored as Fe(II) in vivo since ferritin has always been isolated in the presence of air. In this connection, it is interesting that a ferritin (of low iron content) has been isolated from an obligate anaerobe, *Bacteroides fragilis* [72]. One hypothesis is that in the absence of superoxide dismutase or catalase, this ferritin's function is to protect against free radical damage in the event of sudden exposure to oxygen, rather than to act as a store of Fe(II) under anaerobiosis [72].

4.12. Can ferritin bind Fe(III)? The transfer of Fe(III) between ferritin molecules

An iron storage mechanism based on Fe(II) oxidation may have evolved in ferritin because of the extremely limited solubility of Fe(III). Although, in principle, Fe(III) could be delivered as its soluble complex with a small chelate molecule, e.g., citrate or ATP, problems could arise with respect to the traversal of the ferritin shell by such a complex, and to the competition between protein ligands and chelate molecules for the Fe(III). In in vitro experiments only limited amounts of ^{59}Fe(III), presented as its citrate complex, were bound to ferritin cores and none to the protein shell [213]. Significant uptake occurred only in the presence of ascorbate [141]. However, it is noteworthy that iron oxidised by one ferritin molecule can be transferred to another, where it is bound by the iron-core [151,124,192]. Two types of experiment have indicated such transfer: (1) ^{57}Fe(III), mainly or entirely in the form of monomeric species, has been found by Mössbauer spectroscopy to move to molecules containing Mössbauer silent ^{56}Fe cores [124,192]. (2) Ferritin molecules, that, on their own, are unable to oxidise Fe(II) and develop iron cores at pH 5.5 (L-chain ferritin or HuHF with altered ferroxidase centres), are enabled to incorporate iron by the presence of molecules with fully active ferroxidase centres [124]. These findings are consistent with the observation that part of the iron newly oxidised by ferritin can be donated to iron binding macromolecules like transferrin [147,193]. How the iron is released from the ferroxidase centre is unknown, but there is evidence that, in addition to movement into the cavity, some of the oxidised iron can transfer to the 3-fold channels, where it is observed as a mononuclear species [148]. The iron probably moves from the channels of the donating species to those of the acceptor ferritin or into its own cavity and the direction of movement is determined by the relative capacities of the ferritins to mineralise. It is unclear whether transfer requires the close association of donor and acceptor. The

possibility that ferritin can act as an Fe(III) donor in vivo is intriguing, particularly if it can donate its iron to other (non-ferritin) molecules. Perhaps of more relevance is the evidence that ferritin can take up Fe(III) if presented in a suitable form.

A number of in vitro experiments have been reported, which have led to the conclusion that caeruloplasmin, acting as a ferroxidase, can act as a source of Fe(III) for storage within ferritin [214–216]. This conclusion was based on the assumption that any increase in absorption at 310 or 380 nm in apoferritin- caeruloplasmin-Fe(II)-O_2 mixtures was due to ferritin core formation. However, more recent work has shown that, far from increasing iron incorporation into ferritin, caeruloplasmin competes with it for iron, with which it forms soluble Fe(III) polymer complexes [217]. The proposal [218] that iron oxidised by xanthine oxidase can be incorporated into ferritin should now be re-examined.

4.13. Model complexes for ferritin iron-cores

Several synthetic oligomeric iron-oxo complexes have been produced which contain two, three, four, six, eight, eleven Fe(III) [219–221] or sixteen Fe atoms together with a single Co or Mn [222]. The Fe(III) atoms are linked by oxo or hydroxo bridges with carboxylate or phenolate ligands attached to the outermost irons of the cluster. Analysis of these complexes (e.g., Mössbauer parameters and magnetic properties as a function of size) may provide insights into ferritin core formation. One of the more interesting of the synthetic analogues, particularly in relation to the magnetoferritin [211,212] referred to above, is a mixed valence polymer produced under low oxygen concentration containing 4 Fe(III) and 8 Fe(II) atoms at interstitial positions of a cubic close packed oxygen lattice [223]. Most of these complexes have been formed in non-aqueous solutions. However, controlled polymerisation in aqueous media has given defined, water-soluble complexes which contain 17 or 19 Fe(III) [221,224], as shown in Fig. 10.

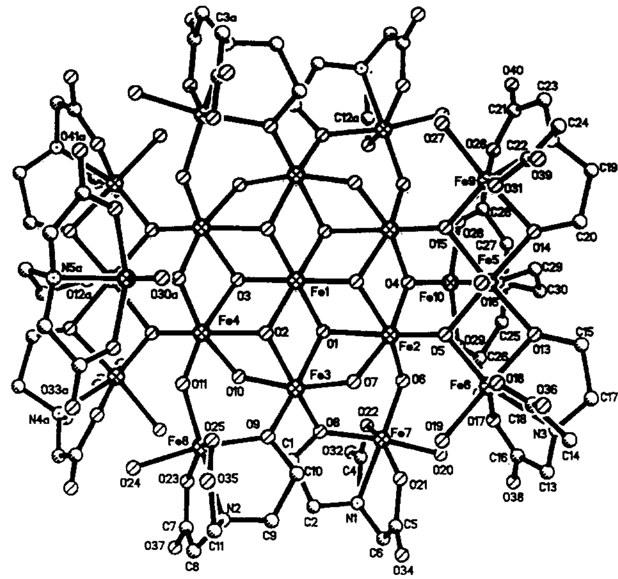


Fig. 10. Structure of water-soluble Fe(III) polymer containing 19 Fe(III) atoms. In this hydroxo(oxo) iron cluster the close-packed cluster core is enclosed in a shell of 'heidi' ligands (heidi = $N(CH_2COOH)_2(CH_2CH_2OH)$, which are complexed to further iron atoms. The cluster core unit is $[Fe_7(\mu_3\text{-}OH)_6(\mu_2\text{-}OH)_4\{(\mu_3\text{-}O)Fe\}_2]^{13+}$ and this is surrounded by ten iron heidi units linked to the core by μ_3-O, μ_2-OH and alkoxo bridge units from the heidi ligands. The formula of the whole complex is: $[Fe_{19}(\mu_3\text{-}O)_6(\mu_3\text{-}OH)_6(\mu_2\text{-}OH)_8(\text{heidi})_{10}(H_2O)_{12}]^{+}$. Diagram by courtesy of A.K. Powell.

5. Haemosiderin

Normal cells store iron mainly in ferritin molecules, but under conditions of iron excess some of it is shunted into another storage form known as haemosiderin [225]. The name 'haemosiderin' reflected its presumed origin, but its iron is non-haem. Under the electron microscope haemosiderin is recognised as irregular, massive clusters of electron-dense particles, most of which are membrane-enclosed [226]. These are thought to be iron-laden secondary lysosomes and have been named 'siderosomes' [227]. Clusters, visible under the light microscope, are typical of iron-loaded tissues, including liver, spleen, bone marrow, brain, heart and many other tissues, including some which are malignant [225–227]. Haemosiderin is typically insoluble, as isolated, in contrast to the soluble ferritin, but it can be solubilized by treatment with alkali and detergents [228]. Ultrastructural immuno-gold staining showed that siderosome granules are recognised by anti-ferritin antibodies, but their immunoreactivity is significantly lower than that of the cytosolic ferritin [229]. These data support the hypothesis that haemosiderin is a degradation product of ferritin [225,227]. This has been confirmed for phytosiderin, an insoluble iron-containing product isolated from pea seed, which was found to contain a peptide derived by radical mediated cleavage of 20 N-terminal residues from the ferritin subunit [230]. Siderosomes isolated from iron-overloaded rats were found to contain an electrophoretically fast (F) type of ferritin containing a subunit of 17.3 kDa, as opposed to the cytosolic ferritin subunit of 20.7 kDa [231]. The shorter subunit was shown to originate by the specific cleavage of a 19 amino acid C-terminal peptide from the L-subunit [232]. Electron diffraction and Mössbauer analyses showed that siderosomal ferritin and haemosiderin iron cores are similar and have ferrihydrite structures. Their particle sizes are smaller than those of cytosolic ferritin cores [111]. These findings indicated that ferritin is taken up by lysosomes, and that subsequent processing involved a partial dissolution of the core, and degradation to siderosomal ferritin and to the insoluble haemosiderin. The enzymes causing the cleavage have not been identified, but earlier studies indicated that

cathepsin D is probably not involved, although this proteolytic enzyme is highly represented in siderosomes [227]. It is not unlikely that a partial reductive dissolution of the iron core catalyses production of free radicals, which damage the protein and facilitate its proteolytic attack [233]. The haemosiderins from various iron-loaded animals were consistently found to have ferrihydrite-like iron cores similar to those of ferritin [234], but unexpected and important differences were observed in haemosiderins from some pathological tissues. The livers of patients with primary (genetic) haemochromatosis treated by venesection were found to contain haemosiderin with amorphous ferric oxide cores [127,235], while spleens and livers of patients with secondary iron overload (due to treatment of thalassemia by repeated blood transfusion) contained a haemosiderin with crystalline goethite-like iron 'cores', and this was unrelated to treatment by chelation therapy [127,236,237]. These differences were indicated by Mössbauer spectroscopy, [236], EXAFS [127] and electron diffraction [235], and were found only in haemosiderins and not ferritins, which invariably had ferrihydrite cores. The haemosiderin from primary haemochromatosis was found to contain a major peptide of 20 kDa, and to release iron to oxalate 3-fold faster than that from secondary iron overload, which contained a major peptide of 15 kDa [238]. Recently, it was found that prolonged centrifugation in potassium iodide precipitated a non-ferritin, non-haemosiderin fraction which was termed pre-haemosiderin. It contained peptides of 17 and 21 kDa, the origins of which are unknown, and the structure of its iron cores resembled that of the corresponding haemosiderin: goethite-like in secondary haemochromatosis and ferrihydrite in the other conditions [237]. These findings imply that there are multiple mechanisms of haemosiderin formation, and that the intrinsic toxicity of haemosiderin iron may vary with its origin. The structural differences between haemosiderin and ferritin iron cores in the two types of iron overload could not be accounted for by a simple conversion of crystal structure, and, in these pathologies, ferritin may not be an immediate precursor of haemosiderin. It has been postulated that, in secondary haemochromatosis, ferritin iron may be partially solubilized in the lysosomes and then allowed to reprecipitate to form the more stable, and less toxic goethite-like structure [112]. In primary haemochromatosis, fast iron incorporation might induce a fast intracellular iron precipitation (possibly outside ferritin) with the formation of poorly-ordered cores [111].

6. Ferritin as a source of iron

Mobilization of storage iron may occur under a variety of circumstances. In the embryonic red cell iron deposited in ferritin is rapidly utilized for haemoglobin production [239]. Blood loss, e.g., phlebotomy used in the treatment of iron-overload in humans [240], causes iron to be drawn from storage sites in the liver and spleen and used for haemoglobin synthesis in new red cells in the erythroid marrow. Experimentally, storage iron can be mobilized in perfused liver [241] or cultured rat hepatocytes [242] by the action of chelators, e.g., desferrioxamine, which are also administered in humans for removal of excessive iron stores. Iron can also be removed from both ferritin and haemosiderin in vitro [111,243]. Theil [36] has drawn attention to differences in the physiological roles of storage iron in different cell types. Probably all cells utilize iron stores for 'housekeeping' purposes, i.e., to enable synthesis of cytochromes and iron-containing enzymes within the same cell, while certain cells (hepatocytes, embryonic red cells and macrophages) store iron for use by other cells, e.g., in the recycling of iron by senescent red cells, iron is temporarily stored in the reticuloendothelial cells of spleen and liver. Release mechanisms could be related to different demands for iron within different cell types. Although it is usually assumed that iron can be made available from intact ferritin molecules, it must also be reutilised from degraded molecules, since large iron-core deposits do not normally build up. In vitro, the relative availability of haemosiderin and ferritin iron depends in the origin of the haemosiderin deposits: in general, iron is less easily mobilised from ferritin than from the haemosiderin of primary overload, but more easily mobilised from ferritin than from the crystalline goethite deposits of secondary iron overload [111,243].

6.1. *In vitro studies of iron release from ferritin and bacterioferritin and their biological relevance*

Ferritin and haemosiderin iron can readily be released by small reductants, assisted by Fe(II) chelators such as 2,2′-bipyridine [120,244], bathophenanthroline sulphonate [245] or ferrozine [148,246]. Reductants commonly used in the preparation of apoferritin include dithionite [120,244] and thioglycollate [201,247], and a variety of 'physiological' reducing agents have also found to be effective or partially effective in iron release, e.g., reduced flavins [248,249], superoxide [245,250,251], dihydrolipoate and related sulfydryls [252] and ascorbate or the mono-dehydroascorbate radical [253]. None of these is clearly implicated in mobilisation of ferritin iron in vivo, however. Cellular concentrations of flavins are very low, but it has been proposed that an NAD(P)H-dependent ferrireductase promotes the mobilization of ferritin iron by means of a reduced flavin shuttle [254]. This enzyme, which has been partially purified, will reductively release iron from transferrin and lactoferrin [254]. Ferritin iron can also be removed by photoreduction [255]. Ferritin iron has been reported to be mobilised by nitric oxide [256], but this has been reinvestigated and no significant release by NO generated from 5-nitrosothiols was found in the absence of a strong Fe(II) chelating agent [257] which may have perturbed the earlier results [256].

In the crystalline state pores through the apoferritin shell are only a few Å wide. Nevertheless dynamic structural fluctuations may allow some of the small reductants to enter the protein shell fast enough to interact directly with the core and remove its surface iron [249]. That this is possible is suggested by the finding that protoporphyrin IX binds at the inner surface of the horse spleen apoferritin shell, although a very long incubation period was allowed before crystallisation for structure determination [104]. An Fe(II) chelator (which may be the reductant itself) will help Fe(II) to find its way out of the molecule. Small Fe(III) chelators which mobilize Fe(III) from ferritin over hours or days (e.g., hydroxypyridinones) may also enter the molecule and leave bearing iron as the Fe(III)-chelate complex [258]. Low angle neutron scattering and NMR studies of horse spleen ferritin show that molecules of comparable size, e.g., sucrose and maltose, reach the cavity within a few seconds [259,260]. A recent kinetic and spectroscopic investigation of iron release to four chelators indicates that they can acquire iron from the dinuclear sites within the shell, possibly, by formation of ternary complexes [261]. However, rates of release measured with a series of organic reductants correlate better with redox potential than with molecular size, an observation interpreted to imply that they do not penetrate the shell at a significant rate [262,263]. In the presence of ferrozine, a chelator with much higher affinity for Fe(II) than for Fe(III), reductants with redox potential higher than that of the ferritin core are able to release ferritin iron [263]. Dihydroflavodoxin, a protein of M_r about 30 000 is also capable of reducing core iron in both horse spleen ferritin and AvBFR under anaerobic conditions [199]. This probably results from long range tunnelling of electrons mediated by aromatic groups within the protein [199]. Such long-range effects are potentially of great importance, since they obviate the necessity for small molecule reductants under physiological conditions.

A speculative scheme for in vivo ferritin iron mobilization [263] is shown in Fig. 11. In this model, iron is normally in the Fe(III) state, but when reduced, it remains within the protein unless removed by an Fe(II) acceptor. A low molecular weight (5600 Da) protein has been identified in human reticulocytes which seems to participate in the intracellular transport of iron for haem synthesis [264] and this has been suggested as a possible acceptor for ferritin iron [263]. The scheme is attractive because it proposes different mechanisms for iron uptake and iron release (this also applies to release after degradation within lysosomes). Since ferritin itself can act as a ferroxidase during iron uptake, no external oxidase is needed provided O_2 is available.

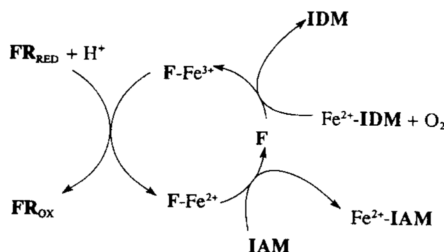


Fig. 11. Speculative scheme for physiological iron uptake and release by ferritin. Iron enters ferritin (F) as Fe^{2+} and is oxidised in the protein by O_2. The Fe^{2+} is acquired from an iron donor molecule (IDM). A ferrireductase (FR) is required to release iron as Fe^{3+}, which becomes attached to an iron acceptor molecule (IAM). Reproduced from [263] with permission.

Electrochemical titrations of horse spleen ferritin and AvBFR show that the iron core is more difficult to reduce in the latter (e.g., at pH 7, $E_{1/2} = -420$ mV for BFR compared with -190 mV for horse spleen ferritin [110,120,107]). This can be attributed to the phosphate-rich composition of the cores in native BFR, since reconstitution in the absence of phosphate yields cores which exhibit a more positive potential [204]. The presence of iron core also influences the reduction of the haem in BFR. Spectroelectrochemical measurements show that the haem reduction potential becomes more negative by about 250 mV in the presence of core (-475 mV for holo- and -225 mV for apo-BFR, where holo- and apo- refer to the core) [108,248]. The question arises, does the presence of haem affect the reductive release of iron from the core? Although the measured potentials suggest that haem is more difficult to reduce in BFR than the core, nevertheless Watt et al. [239] propose that reduction of the latter is mediated by haem. They found that, although neither core nor haem reduction potentials show pH dependence when measured in the pH range 7 to 9, holo BFR takes up 1.8 ± 0.2 protons per iron atom reduced [107] (a value similar to that for horse spleen ferritin, which shows reduction potentials varying from -190 mV at pH 7 to -416 mV at pH 9 [120]). This result could be explained if the electrons are taken up first by haem, and are then passed irreversibly to the core at higher potential [106]. The binding of haem to horse spleen ferritin was also found to accelerate iron release from the core [106]. The interaction between haem and core is consistent with the location of the latter between subunits on the inside surface of the protein shell as shown for EcBFR [85].

6.2. Ferritin as a donor of iron to hepatocytes: its involvement in their cycling of red cell iron

When ^{59}Fe-labelled rat liver ferritin was injected into a rat much of the label appeared in liver parenchymal cells [129,130]. Rat hepatocytes contain specific ferritin receptors [265,266] and a scavenger function is suggested [266]. Studies of the binding of radio-labelled rat liver ferritin to primary cultures of rat hepatocytes gave 4×10^4 binding sites per cell with an affinity constant of 1×10^9 M^{-1} at 4°C [267]. At 37°C maximal uptake from these sites was 1.3×10^5 ferritin molecules/cell per hour. This corre-

sponds to 5×10^6 Fe atoms/cell/min for ferritin molecules bearing 2400 Fe atoms. Some further iron could have been taken up by binding of ferritin at low affinity sites. Ascorbate enhanced the cellular uptake of ferritin iron and apotransferrin inhibited it [268]. The finding that chloroquine, which is thought to impair receptor recycling, depressed both ferritin and iron uptake suggests that receptor-bound ferritin is delivered to the lysosomes for degradation and iron release [268]. The iron from phagocytosed erythrocytes is converted into and released as ferritin. When rat Kupffer cells were cultured with hepatocytes, the released ferritin was taken up by the hepatocytes [23], where it then seemed to be processed by degradation within the lysosomes and some of its iron was released to the hepatocyte cytosol ferritin and also incorporated into mitochondria.

The hepatocyte receptor has been isolated from humans [269], rat [265] and pig [270]. Its major component is a protein of M_r 53 000. Consistent with its proposed scavenger function the receptor was found to have a broad specificity both with respect to H- and L-subunit composition and even across species [271].

Although this degradative pathway for release of iron from ferritin derived from other cells seems quite well established, and although cytosolic ferritin from the same cell can also be taken up and degraded [132], it cannot be ruled out that ferritin iron can also be made available from intact molecules in vivo.

7. Regulation of iron storage

7.1. Ferritin biosynthesis

Ferritins from bacteria, plants and animals show a remarkable conservation in structural features and they all interact and incorporate iron in vitro in similar ways [37]. However, their expression is regulated with strikingly different mechanisms in the three kingdoms, probably as a consequence of major differences in iron handling.

The two types of iron storage protein found in bacteria (the heme containing BFR, found in *E. coli*, *A. vinelandii* and *P. aeruginosa* [75], and the non-heme FTN found in *E. coli*, *B. fragilis* and *H. pylori* [63,66,72]), do not co-assemble to form heteropolymers [123]. The isolated ferritins and bacterioferritins appear to contain relatively low amounts of iron, a few hundred atoms per molecule, compared with an average of 1000 to 3000 atoms found in ferritin isolated from human liver or horse spleen. In particular the ferritin from the anaerobe *B. fragilis* was found to be essentially iron-free [72]. In *E. coli* the two proteins are regulated independently: the BFR content increases in the stationary phase of growth, while FTN remains constant throughout the growth cycle [72]. The study of the mechanism of the regulation of their expression may give important insights into their functional roles. Induction by iron has been found for the BFRs of *Rhodobacter sphaeroides* [272] and *E. coli* (Grogan, Thackray, Andrews and Guest, unpublished work) but not for that in *Synechosystis* [71]. In *E. coli*, iron induction of *bfr* expression is dependent on the ferric uptake regulator protein (Fur), although not on direct interaction of Fur with the *bfr* gene (Grogan et al., unpublished work).

Plant and animal ferritins differ in the cytological localization and the control of their synthesis in response to iron. Plant ferritins are found in chloroplasts and plastids [49]. In soybean, ferritin subunits are synthesised as 32 kDa precursors with an N-terminal transit peptide. The subunits are taken up by chloroplasts and processed to give the 28 kDa subunit found in the isolated protein [54,78]. An additional 26 kDa subunit has been found, but it originates from processing of the 28 kDa peptide [230]. The purified ferritins appear to be composed of a single type of subunit, although in some species (e.g., cowpea and maize) more than one functional gene has been identified [273]. Ferritin expression is regulated by development and by iron, and the latter has been studied in detail. Iron salts increase plant ferritin protein up to 60-fold, with a parallel increase in ferritin mRNA [56,78]. Iron acts only at a transcriptional level, with no evident effect on translation. Some light on the mechanisms of iron-induced ferritin expression came with the observation that iron induces the accumulation of abscisic acid, and that abscisic acid itself stimulates ferritin synthesis, albeit not at the same levels as does iron [274]. Thus abscisic acid and iron pathways are linked but probably not identical. Indeed it has been found recently that in maize only one of the two transcripts (FM2) accumulates in response to abscisic acid and, although both ferritin mRNA subclasses FM1 and FM2 respond to iron, FM1 accumulates before FM2 [275]. In de-rooted plantlets abscisic acid is not involved in the induction of ferritin synthesis due to iron overload [276]. Hydrogen peroxide treatment was found to induce an accumulation of ferritin mRNA at low iron concentrations and it was concluded that in plants ferritin plays an important role in the response to oxidative stress [276]. Thus ferritin may have more complex functions in plants than the simple storage of iron.

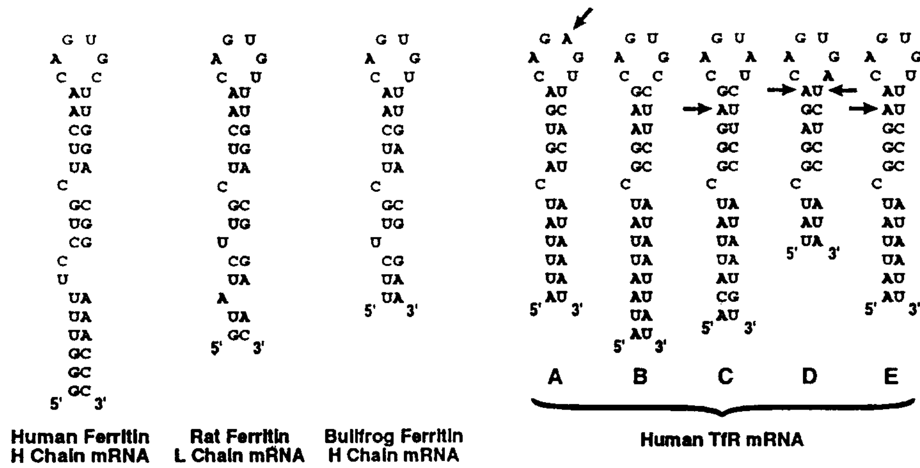
In animal cells, ferritin is invariably found in the cell sap and in lysosomes. Trace amounts of ferritins are also found in serum and body fluids of human and other mammals, but in some invertebrates, particularly insects and molluscs, the secreted ferritins are present in much larger proportion. An electron microscopic analysis of the larvae of the insect *Calpodes ethlius* showed more ferritin in the vascular system than in the cell sap [48]. Iron induced the synthesis of this ferritin also by increasing mRNA levels, and when the transcripts were added to a cell-free translation system, the encoded subunits were taken up and processed by dog pancreatic microsomes, a demonstration of the secretory nature of this type of ferritin [45]. The fresh water snail *Lymnaea stagnalis*

contains two types of ferritin, an intracellular form in the soma, and a secretory form in the yolk [38,277]. The yolk ferritin is secreted by the mid-gut gland and released in the blood lymph to be taken by the tissues. This secretory ferritin seems to act as a source of tissue iron. Recent cloning and sequencing of both types of *L. stagnalis* ferritin showed that the yolk ferritin has a canonical 18 amino acid leader sequence for protein export. Yolk ferritin expression is under developmental control, while the soma ferritin is regulated by iron at the translational level [33]. In rat liver a significant proportion of H- and L-chain mRNA is associated with membrane bound polysomes and it increases during experimental inflammation [278,279]. An accurate in vitro study indicated that these mRNAs are translated, but are not taken up and processed by dog pancreatic microsomes, indicating that they do not encode for the secreted and glycosylated form of ferritin, the origin of which remains uncertain [280]. Probably the distinctive feature of vertebrate ferritin is the presence of L-chains (the only ferritin subunits without ferroxidase activity) which normally associate with the H-chains to produce heteropolymers with the ability to catalyse Fe(II) oxidation.

7.2. Iron induction of ferritin synthesis in animals

Induction of ferritin synthesis in response to iron administration has been well documented in rats [129,131–133] and in cell cultures [281–283], since it was first observed by Granick as an increase in ferritin in the gastrointestinal mucosa of guinea pigs after iron feeding [284]. The response to iron is rapid, e.g., in rats there was a maximal increase in synthesis of labelled ferritin at 6 h after an intraperitoneal injection of iron [129], and this may reflect

Examples of IREs



Consensus IRE

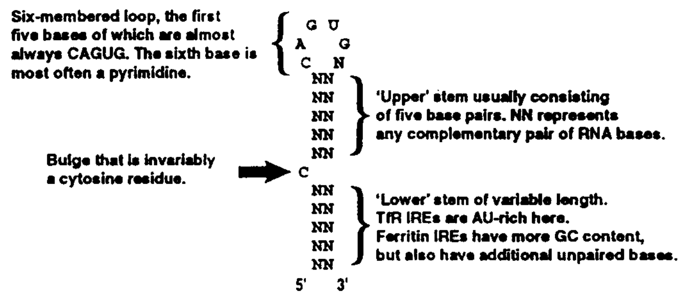


Fig. 12. Sequence and hypothetical structure of IREs. The upper panels shows sequences of IREs from the 5′UTRs of mRNAs for human ferritin H-chain, rat L-chain, and bullfrog H-chain, along with IREs from the 3′UTR of the mRNA for human transferritin receptor. The arrows indicate substitutions with the corresponding IREs of chicken transferrin receptor mRNA. The lower panel shows the consensus IRE motif deduced from the sequences of all ferritin and transferrin receptors mRNAs, along with the characteristics of the IRE consensus. Reproduced with permission from [370].

the need to provide an adequate means of iron disposal while at the same time avoiding cell damage. The injected iron is taken up into both pre-existing and newly synthesized molecules [129–133].

The mechanisms by which iron regulates ferritin biosynthesis have been intensively studied. The major control mechanism is largely post-transcriptional although a minor increase in ferritin mRNA has also frequently been observed [285–287]. Iron administration to rats induced a 2-fold increase in L-chain mRNA, leaving H-chain mRNA unaffected, the result of a specific transcriptional activation of the L-gene [287]. Similar findings have been reported for iron-loaded patients [288]. Long-term iron overload seems to up-regulate L-chain translation [289]. Both mechanisms explain the selective up-regulation of L-chain in iron overload. The relative increase of L-chain mRNA may be peculiar to the liver, since various cultured cells, including HeLa cells [290], human myoblasts [291], activated T-cells, monocytes [292] and human hepatocytes [293] respond to iron with a 2-to 5-fold increase of both H- and L-chain mRNAs. Also cells from *Xenopus leavis* respond to iron and cadmium with a 5-fold increase of ferritin mRNA transcription [294]. A recent study showed that the iron-induced transcriptional up-regulation of H-chain mRNAs varies greatly in different cell lines [295]. Significant differences were observed also between different erythroid cell lines: in human K562 cells the heme iron-induced increase of ferritin mRNAs was ascribed to stabilization of the transcripts [296], while in the mouse Friend erythroleukemia cells, heme and protoporphyrin IX similarly, and selectively, up-regulated H-chain transcription [297]. The stimulatory effect was attributed to the H-chain promoter.

7.3. Translational regulation of ferritin by iron

A cytoplasmic translational control mechanism was suggested by the observation that rats, which had received injections of actinomycin D or cordycepin prior to ferric ammonium citrate, gave the same two-fold increase in the amount of ferritin mRNA in liver polyribosomal fractions as rats receiving iron only [298]. The increase in polysome-associated ferritin mRNA was not accounted for by an increase in the overall amount of mRNA since it was paralleled by a decrease in the cytoplasmic post-ribosomal ferritin mRNA. Therefore the increase in ferritin must have been due, not to new messages, but to mobilization from a store of inactive cytoplasmic mRNA. It was first supposed that this cytoplasmic mRNA might be inactivated by attachment of a ferritin subunit, acting as a repressor, and that iron caused derepression by promoting assembly of subunits into ferritin 24-mers which were then able to incorporate iron [283,298]. Although subsequent work has confirmed such a proposal, the repressor protein is not a ferritin subunit, but a much larger molecule of $M_r \sim 90\,000$ [299].

In rats, both H- and L-ferritin messengers have been shown to respond to iron to the same extent and in the same way: a shift from an inactive ribonucleoprotein fraction to polysomes [299]. Similar effects were observed in the bullfrog embryonic red cell without an overall increase in total mRNA [300–302]. Sequencing of H- and L-rat ferritin mRNAs showed them to have unusually long 5′-untranslated regions (UTRs), respectively of 210 and 168 nucleotides [299]. Within the first 75 nucleotides stem-loop structures are predicted by computer analysis [299–303]. A sequence of 28 nucleotides has been found to be highly conserved within putative stem-loops in the 5′-UTRs of H- and L-ferritin mRNAs of human [304–306], bullfrog [301], chicken [307], rabbit [308] and the somal ferritin of the snail *Lymnaea stagnalis* [38] as well as in rat [299,303]. The properties and functions of this family of mRNA non-coding sequences have recently been reviewed [309]. Fig. 12. shows selected stem loop structures and a consensus sequence for the 28 nucleotides. Such a sequence has been demonstrated to be essential (and sufficient) for the translational response to iron: by deleting it from the 5′-UTR, by altering its sequence and by producing iron induction of a reporter protein (chloramphenicol acetyltransferase (CAT) or human growth hormone) by construction of chimaeras in which the 5′- or 5′- and 3′-UTR regions of a ferritin mRNA are fused to its reading frame [303,308,310–314]. Its position in the 5′-UTR is also important – if it is shifted to a position 67 or more nucleotides downstream from the 5′-terminus cap structure, iron-dependent translational regulation is lost [315]. The 28-nucleotide sequence is now known as the iron-responsive element (IRE). Its 6-nucleotide loop sequence CAGUGN (where N is any nucleotide other than G), and, in particular, its 5′ C residue are highly critical for its function [312,314–319].

Also important is a conserved cytosine within a 3-nucleotide bulge predicted to occur between the upper and lower stems [317–319]. The actual nucleotide sequence of the stem regions themselves is less important than the conservation of Watson-Crick base pairing [317,319,320]. In a recent study, random substitutions were made in the 6-base loop and unpaired bulge cytosine positions, and sequences binding the iron regulatory protein (IRP) were selected from the 16384 IRE molecules so produced [318]. Surprisingly loops with sequence 5′-UAGUAN bound with high affinity as well as the expected 5′-CAGUGN and it was therefore proposed that base-pairing occurs between positions 1 and 5 of the loop.

Another factor, essential for iron-dependent translational regulation, is the presence of a cytosolic protein of $M_r \sim 90$ kDa, which binds specifically to the IRE with high affinity ($K_d = 10^{-10}$ to 10^{-11} M). This has been shown by gel retardation assays in which the radio-labelled RNA is incubated with cytoplasmic protein extracts [321], by RNA-protein cross-linking induced by UV radiation [299] and by competition studies with variant RNA sequences [322]. This protein has been known variously as

the IRE-binding protein (IRE-BP), iron regulatory factor (IRF), ferritin repressor protein (FRP), P-90 or iron regulatory protein (IRP). IRP will be used here in accordance with general agreement. Under normal iron levels, three pools of IRP are present in liver cytosol: the high affinity protein, the protein-mRNA complex and a low affinity protein [321–325]. The latter can be activated by treatment with thiols (2-mercaptoethanol or dithiothiothreitol) in vitro [321,323] or, to some extent, with the iron-chelator desferrioxamine [322]. A protein with high IRE-binding activity is present in cells cultured with desferrioxamine [322]. Conversely, the presence of iron salts or haemin in cell culture media causes binding activity to be diminished 50- to 100-fold, as does the addition of ferrous salts to the purified IRP [322–328]. Thus iron regulation of ferritin mRNA translation involves a protein able to bind iron reversibly; it acts as a repressor in unbound form and is dissociated from the messenger when it binds iron. A high degree of evolutionary conservation is indicated by the occurrence of proteins with virtually identical properties in rat liver extract [299], human placenta (a doublet of $M_r \sim$ 97 and 100 kDa) [329], rabbit liver [327] and in other vertebrates, insects and annelids [330]. Based on cDNA sequences these proteins also show a high conservation (> 90%) of amino acid sequence [331,332].

The IRP has been purified from human [333] and rabbit [327] liver and from human placenta [334]. Use of IRE-affinity chromatography [334] ensured the isolation of a protein with high binding affinity for the IRE. cDNA clones encoding human IRP have also been isolated and expressed and both the cDNA and the protein have been sequenced [335–338]. Its gene has been localised to chromosome 9 [335]. A second protein (IRP2 or IRF_B) which also binds IREs with high affinity has been isolated from humans [335] and rodents [299,339]. The human protein has a sequence closely similar to IRP1 and was localised to chromosome 15. The protein (IRP2) isolated from rodent cells appears to be structurally related to IRP, although it is larger (about 105 kDa), it gives distinct peptide patterns and it does not react with IRP1 antibodies [339]. Its tissue distribution is also different, giving highest IRE-binding activity in intestine and brain, whereas that of IRP is greatest in liver, intestine and kidney. The significance of the two IRPs is not clear. However, very recent data show that rat IRP2 differs from IRP1 in significant respects: unlike IRP1, IRP2 is down-regulated by iron (due to increased turnover) and it exhibits no aconitase activity and is not activated by treatment with thiols or desferrioxamine [340,341]. IRP1 contains 889 amino acids as does the protein of human placenta, for which an M_r of 98 398 is calculated [338]. Murine and rabbit IRP1 have highly homologous sequences [342]. The presence in IRP of a sequence CXXC, commonly found in Fe-S cluster-containing proteins, indicated that it may contain such a cluster. The next major development in ideas concerning its iron-sensing mechanism followed from the observation that IRP sequences aligned well (with 30% overall identity and 57% similarity) to the amino acid sequence of the Krebs cycle enzyme aconitase from porcine mitochondria [319,332,336–343]. Moreover, the gene for cytoplasmic

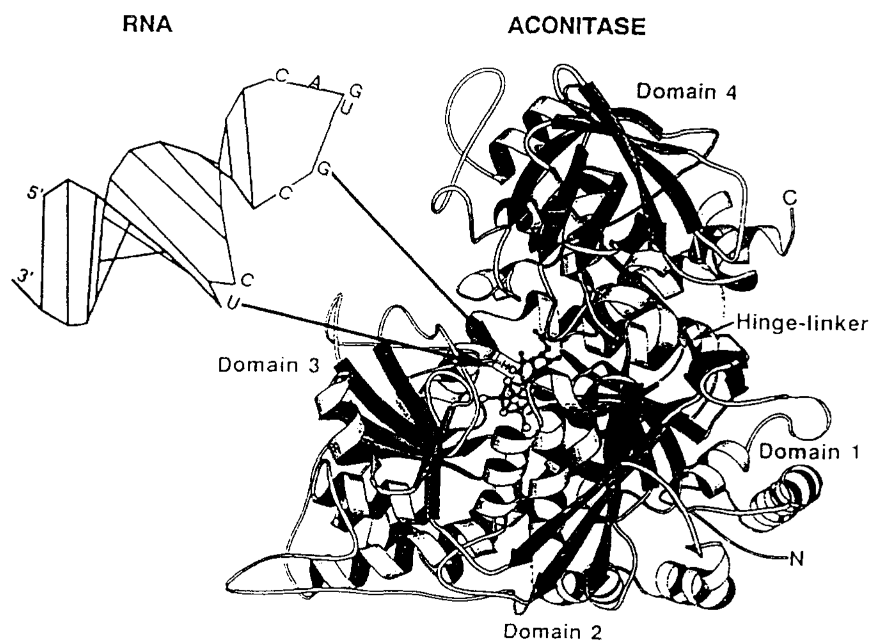


Fig. 13. Model of an IRE-like stem-loop and aconitase. Mitochondrial aconitase is used as a model for the IRP structure. Domain 4 of the protein is connected to domains 1–3 by the hinge linker. Opening of the cleft between these regions probably allows loss of the [Fe-S] cluster and its replacement by the IRE. The arrows indicate the RNA and protein regions that are probably implicated in their interaction at the IRE binding site. Reproduced with permission from [352].

aconitase is, like that of IRP1, located on chromosome 9. Aconitase is known to occur in two forms, an active enzyme containing a [4Fe-4S] cluster and an inactive form with a [3Fe-4S] cluster [344]. The inactive form can be activated in vitro by introduction of Fe^{2+} under reducing conditions, which parallel those which enable the IRP to bind to the IRE and act as a translational repressor. Comparison of the amino acid sequences of IRPs, with porcine, mitochondrial and *E. coli* aconitases shows striking conservation of cluster ligands and residues known to be involved in aconitase substrate binding and catalysis [342,343]. This suggested that IRP could be a functional aconitase. This was confirmed [345] and reciprocal RNA binding and aconitase activity of IRP demonstrated both in vitro [329] and in mouse fibroblasts [346] and a mouse erythroleukaemic cell line expressing human IRP [347]. There is now good evidence that IRP and cytosolic aconitase are one and the same: the amino acid composition and partial sequence data of purified beef liver cytosolic aconitase are virtually identical to those derived from the cDNA sequence of human IRP [348].

The known three-dimensional structure of bovine mitochondrial aconitase has three closely integrated domains and a fourth domain connected by a hinge linker [349,350]. The [4Fe-4S] cluster is sited in a cleft between domains 4 and domains 1–3. It is proposed there are two conformations of the IRP, a 'closed' form that is similar to the known aconitase structure, and an 'open' form produced by movement of domain 4 and loss of iron such that it is enabled to recognise and bind the IRE [344,347]. It was first proposed that the change in activity resulted from alteration of the aconitase/IRP protein between [4Fe-4S] and [3Fe-4S] forms. However, the activity switch is now thought to be between the [4Fe-4S] form and the apoprotein, since only the latter binds the IRE with high affinity comparable to that of the IRP in vivo [323,344,347,351,352]. The reversibility of activation/inactivation of aconitase/IRE binding activity of IRP(I) by iron salts in the presence of cysteine or iron chelator shows that chelatable iron does not cause degradation [324]. A major contact site between the IRE and IRP has recently been mapped with the aid of cross-linking induced by UV-irradiation followed by protease digestion of the complex [352]. Comparison of the isolated peptide with the predicted structure (based on that of mitochondrial aconitase [349,350]) placed the site of the IRE attachment deep within the aconitase active site cleft (Fig. 13). In this model the closed conformation containing the [4Fe-4S] cluster would be inaccessible to the RNA and cleft-opening must occur to allow IRE-binding. A similar conclusion has been reached by mutagenesis of three cysteines (seq. nos. 437, 503 and 506), which are predicted to bind the [4Fe-4S] cluster The three Cys → Ser variants showed no aconitase activity but, although binding was not regulated by cellular iron levels, they were able to bind IRE in the presence of β-mercaptoethanol. Thus the IRE is able to bind near to Cys 437 in the absence of cluster [328–356]. Substitution of active site cleft arginine residues also greatly reduced IRE binding affinity in retardation assays [353,355].

7.4. Haemin as the ferritin inducer: an alternative model of regulation

One question relating to the mechanism of ferritin induction that has been particularly controversial is: in what form does haemin exert its regulatory effect?

Since haemin (iron protoporphyrin IX), as well as iron salts, is capable of inducing ferritin mRNA translation in tissues, cell cultures and cell-free systems (e.g., Refs. [129,283,297,311,326,357–361]) the question arises: does haemin induction require the prior degradation of the protoporphyrin, so as to release its iron, or does it have a direct effect, e.g., by binding to and modulating the structure of the IRP? Conflicting answers have been given by different experimenters. Eisenstein et al. [362] concluded from studies in rat fibroblasts that release of iron by haemoxygenase is essential for its induction of ferritin synthesis. Swenson et al. [322] found that addition of haemin led to rapid breakdown of IRP complexes and proposed the presence of a specific haemin binding site. Lin et al. [360,363,364] found that haemin formed a stable cross-linked complex with the IRP and suggested that this was the principle step in the process of derepression leading to its dissociation from the IRE. These workers later concluded [357] that haem may act by triggering degradation of the repressor. They suggested that there may be two mechanisms, a reversible one involving the binding of an [4Fe-4S] centre by the IRP and a degradative and irreversible mechanism leading to decreased level of repressor protein. However, recent experiments with cultured mouse fibroblasts and rabbit cells grown with haemin or desferrioxamine showed no coupling between IRE-binding activity measured by gel-retardation assay with radiolabelled IRE-containing mRNA and IRP levels obtained from Western blots [360]. Also no change in IRP levels pulse labelled with [^{35}S]methionine could be found in cells treated with haemin or chelator. Tang et al. [365] concluded that iron must be released from haemin to exert its effect on ferritin biosynthesis in agreement with the earlier studies [362].

7.5. The untranslated regions of ferritin on RNA and the repression of translation: the involvement of IRE flanking regions?

The stem-loop structure formed by the 28 nucleotides of the IRE was originally predicted by computer analysis. Experimental evidence supporting such secondary structure is provided by examining the action of both protein (RNases) and chemical (Fe EDTA and 1,10-phenanthroline) nucleases on ferritin repressor-mRNA complexes

in in vitro systems [366,367]. 'Footprints' and 'toe-prints' showed that repressor protein binding was confined to the stem-loop, but that this binding caused increased base-stacking in regions flanking the stem-loop. It was proposed that the mRNA structure required for regulation included the flanking region (some 55 nucleotides in all). This proposal has been disputed as a result of examining the iron-regulated expression of human growth hormone in transfected mouse fibroblasts containing plasmids in which cDNAs, with IREs in various positions and with various flanking sequences, had been inserted upstream from the human growth hormone coding region [318,368]. The conclusion from these experiments is that the most stringent requirement for regulation is the position (near the 5′ end of the mRNA) of the IRE and, that provided this requirement is met, the sequences flanking either end of the IRE are unimportant. Considerable variation in IRE sequence is tolerated, provided its secondary structure is preserved but alterations, e.g., in the size of the loop, may reduce the affinity of the binding protein [317]. However, recent studies of the effects of mutation in the flanking regions indicated that disruption of a base-pair triplet led to diminished regulation, which could be regained by a second mutation to restore base pairing [369]. High conservation of the base-pair triplet was found in 9 vertebrate sequences (GAG/CUC in H-ferritin and GGG/CCC in L-ferritin) and it was suggested that the flanking regions may enhance the stem-loop-protein interaction [369].

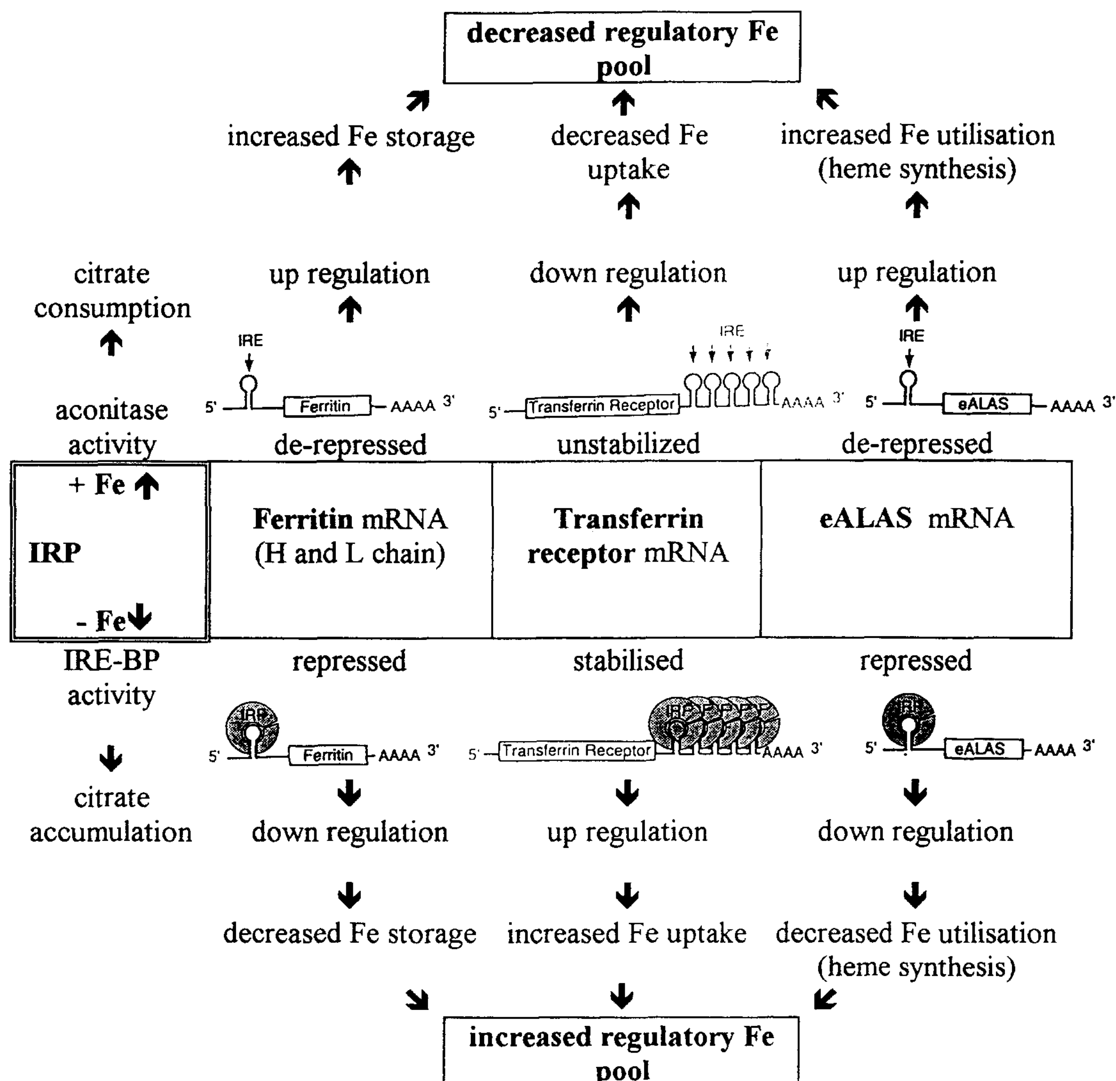


Fig. 14. Scheme of cellular iron homeostasis in vertebrates. Cellular iron homeostasis is regulated post-transcriptionally through iron-dependent conformational changes of the IRP. In iron deficiency IRP has IRE-binding activity and represses the translation of ferritin H- and L-chains, and, in erythrocytes, also of eALAS, the major regulatory enzyme of heme synthesis. In addition binding of the IRP stabilises transferrin receptor mRNA, thus leading to increased protein expression. Under iron excess, IRP has low affinity for the IRE and instead has aconitase activity. This leads to derepression of ferritin and eALAS synthesis, to destabilization of TfR mRNA, and possibly to faster aconitase turnover. This leads to a decrease in the size of the regulatory iron pool. IRP systems may regulate translation of other IRE-containing mRNAs (e.g., that of mitochondrial aconitase).

Like the 5′-UTRs, the 3′-UTRs of ferritin L-subunit mRNAs show extensive sequence conservation across species as do those of H-subunit mRNAs, although H- and L-mRNAs show little homology [358,373]. It has been suggested that these regions may also play a role in translational control through interactions with the IRP which prevent nuclease binding and stabilize the mRNA [358,373].

7.6. Co-ordinated regulation of iron metabolism

The above discussion has focused on the translational control of ferritin synthesis. However, a very important feature of this regulation is that IREs are not confined to ferritin mRNA. While the latter has one IRE in its 5′-UTR, the 3′-UTR of transferrin receptor mRNA has *five* putative hairpin structures, closely related to the ferritin IRE in sequence [313,319,336,342,371–374]. They bind the same IRP as binds to ferritin mRNA and at least four are able to bind simultaneously in 1:1 stoichiometry [329,375]. Deletion of the entire 3′-UTR leads to constitutively high expression of TfR mRNA [376], whereas simultaneous mutagenesis of all five IREs gives a non-regulated, rapidly degrading mRNA [316]. Iron modulation of TfR is opposite to that of ferritin: high iron levels lead to low TfR expression and vice versa [377–380]. Binding of IRP is thought to protect TfR mRNA against degradation [316,329].

Thus when iron is needed, more TfRs are synthesized enabling more iron to be taken up, and, when cells are iron-replete, more ferritin is synthesized to protect against its toxicity. The co-ordinated regulation of these two processes is summarised in Fig. 14.

A single IRE which binds specific IRPs has recently also been found in the 5′-UTRs of both murine and human erythoid-specific δ-aminolaevulinic acid synthase (eALAS) mRNA [378,379] and iron regulation of eALAS synthesis has been demonstrated [380,381]. As with ferritin, IRP binding blocks initiation of translation of eALAS mRNA [380,381]. Large amounts of haemoglobin are produced during terminal stages of red cell differentiation. ALAS catalyses the first, and probably rate-limiting, steps of the haem biosynthetic pathway. Thus iron availability regulates haem biosynthesis during development of erythroid precursor cells in co-ordination with cellular iron uptake and storage [371,374]. The mRNA of porcine mitochondrial aconitase also contains one IRE in its 5′-UTR, which binds human IRP (although not the mitochondrial enzyme), so aconitase synthesis itself may be regulated by iron [345,380]. When iron is limiting, the aconitase activity of IRP, and possibly the mitochrondrial enzyme, increases, with a consequent increase in citrate consumption. The reverse occurs in conditions of iron-excess, with the probable increase in cellular citrate accumulation. The apparent direct coordination of citrate and iron levels may be physiologically important considering that citrate is one of the major cellular iron-binding molecules [336,380].

Recently implicated in the regulation of iron metabolism is the gas radical molecule, nitric oxide (NO). NO has many physiological functions including blood pressure regulation, immunomodulation, and neurotransmission [382,383]. It has been shown to modulate the activity of iron-containing enzymes including those with [4Fe-4S] clusters. It has now been found to be an affector molecule regulating the aconitase and IRE-binding activity of IRP in murine macrophages and fibroblasts and in human K562 cells and thus to provide a link between iron metabolism and NO pathways [384–386]. It is postulated that NO interacts with the [4Fe-4S] cluster of the cytoplasmic aconitase causing conformational rearrangement that enables it to bind the IRE. Hence NO causes repression of ferritin mRNA translation and stabilises the transferrin receptor in RNA against degradation. Alternatively, or additionally, it may interact directly with the iron regulatory pool reducing its size [384,385,387]. In K562 cells iron mobilised by NO from intracellular iron compounds seems to be reincorporated into ferritin [388].

Exposure of cells to H_2O_2 also triggers a reduction in ferritin synthesis and an increase in synthesis of the transferrin receptor [389]. Again H_2O_2 appears to lead to the disassembly of the [4Fe-4S] clusters of aconitase leading to an increase in IRE binding, but it may not do so by direct action on the clusters [389].

7.7. Non-iron mediated induction of ferritin synthesis

Ferritin synthesis is stimulated at various levels during development, cellular differentiation and inflammation and by some hormones and cytokines, with mechanisms which may be partially related to decompartmentalization of cellular iron.

Early studies showed that chemically induced inflammation in rat models increased ferritin synthesis in liver in two waves: the first peaked at 6 h and the second at about 24 h [390]. More recent studies indicated that total ferritin mRNAs remained constant [278], but a slight decrease of L-chain mRNA has been observed in the liver, but not in the spleen at 24 and 48 h of inflammation [283]. Thus the up-regulation of ferritin synthesis appears to be mainly at a post-transcriptional level, in agreement with the observations that a short-term incubation of hepatoma Hep G2 cells with the inflammatory cytokine IL-1-induced ferritin expression by a translational mechanism, similar, but not identical, to that used by iron [391]. Sequences on the 5′ untranslated region of H-ferritin mRNA appear to be responsible for the IL-1 translational up-regulation, and they are distinct from the IRE consensus sequence [392]. Also other inflammatory cytokines such as IL-6 were found to stimulate ferritin synthesis, probably as a secondary response to an increase of iron uptake [393]. Other studies showed that tumour necrosis factor (TNF) induces a selec-

tive accumulation of H-chain mRNA in mouse adipocytes, human muscle cells and in the human monocytic cell line U937 [291,394] by an iron-independent mechanism acting at a transcriptional level [291]. Also IL-1 was found selectively to stimulate H-chain transcription in muscle cells, and the stimulation was additive to that of TNF [395].

Various, probably most, differentiation processes are associated with increases in ferritin mRNA levels, mainly of the H-chain. They include differentiation of the human promyelocytic cell line HL-60 to neutrophils and macrophages [391], differentiation of mouse leukaemia [396], and rat erythroid cells [397,398], differentiation of mouse TA1 cell to adipocytes [399], differentiation of the non-fusing myogenic cell lines BC3H1s [400], and in vitro differentiation of monocytes to macrophages [292]. The more tumorigenic human colon carcinoma cell lines express higher amounts of H-chain mRNA due to a specific increase in transcriptional rate, possibility stimulated by *c-myc* proteins [401]. In myogenic cell lines the increase was specific to H-chain mRNA and induced by cAMP [400].

Rat thyroid cell respond to the thyroid-stimulating hormone TSH with an increase of H-chain mRNA [402] probably mediated by cAMP. The response is mediated by a product which acts in *trans* on the ferritin promoter [402]. Insulin stimulated steady-state levels of H- and L-chain mRNAs in cultured rat glioma cells at a transcriptional level [403]. Incubation of rat pancreatic insulin cells with glucose induced a dramatic increase of H-chain and a decrease of L-chain mRNAs levels, under conditions that change the level of insulin mRNA and cellular capacity to release insulin [404]. Ferritin synthesis in the liver of *Xenopus leavis* is stimulated by estrogens [405]. Some cell types appear to respond to alterations of the redox status with an up-regulation of ferritin synthesis at a post-transcriptional level: this has been observed in oligodendrocytes cultured in hypoxic conditions [406] and in macrophages exposed to sulphydryl-reactive agents under hypoxic conditions [407]. Arsenite was reported to up-regulate ferritin H-chain mRNA in HeLa cells, possibly due to an increased level of oxygen radicals [408]. Treatments of rats with phorone, a glutatione depleting drug, was found to greatly stimulate ferritin expression in the liver, and the effect was attributed to iron decompartimentalization caused by the oxidative stress [409].

Hydroxyurea-resistant cells were found to have an up-regulated synthesis of M1 and M2 subunits of ribonucleotide reductase and of both ferritin subunits [410]. These cells had higher levels of ferritin mRNAs partially caused by gene amplification [410].

Other mechanisms that regulate ferritin expression seem to involve the modulation of transcript stability. Mattia et al. [411] showed that heme induces an increased expression of ferritin in human erytholeukemic K562 cells by an increase of mRNA half-life. This was suppressed by the inhibition of protein synthesis, suggesting that it was protein-mediated. Other studies showed that the different steady-state levels of H- and L-chain mRNAs in human hepatoma cell lines at different stages of differentiation were caused mainly by differences in the stability of the transcripts and not by transcription rates [296].

8. The ferritin genes

Multiple copies of both H- and L-ferritin genes have been found in humans and other animals, most of them being intron-less pseudogenes. Man has about 16 copies of the H-gene [304] and 5 copies of the L-gene [305]. The rat genome contains about 20 copies of L-gene [306] and 4 copies of the H-gene [303], mouse has 3 H-genes and 10-14 L-genes [412]. In contrast chicken contains a single copy of the H-gene [307]. In each case only a single active gene has been identified. Some curiosity was stimulated by the location of H-genes on human chromosome 6, which is also the locus of genetic haemochromatosis. Two of these have been identified, but they are inactive pseudogenes [413]. The functional human H- and L-genes have been located on chromosome 11q23 and 19 respectively [414,415], and the mouse H-gene on the B-region of chromosome 19 [416]. The structures of genomic H-genes of human [304,417] mouse [418,419], rat [303] and chicken H-chain [307] and of the L-genes of human [305] and rat [306] have been reported. They all contain 3 introns of different lengths which splice the sequence in constant positions [420]. The 5′ flanking regions of H- and L-chain genes bear no similarities, while a high degree of conservation has been found among the chains of different species.

Functional analyses of a relatively short portion of the 5′ flanking regions (about 500 bp) has been reported for the human H-gene. The genomic sequences were fused to the CAT reporter gene and used in transient expression experiments and DNA-protein interactions assays. Three regulatory regions were identified. One, named A region, has been located between nt -132 and -109 upstream of the transcription initiation site. It is recognised by the RNA polymerase II transcription factor SP1 [421], and is responsible for about 50% of the activation of gene expression in various cell lines. A second regulatory sequence (B box) located from -62 to -42 nt is recognised by an uncharacterized *cis*-acting element, which enhances transcription and is more abundant in cells treated with the antibiotic geneticin [422]. A third regulatory sequence is between nt -272 and -291, and, being G-rich, has been named G-fer [423]. It binds a nuclear factor present in different cell types which has properties very similar to the inhibitory factor-1 (IF-1). This is an ubiquitous factor that interacts with similar G-rich elements of the mouse type-1 collagen genes. Alteration of the G-fer site increased promoter activity by about 8-fold [423]. A 400 bp H-chain promoter was sufficient to confer TSH-dependent regulation to an heterologous CAT gene [402]. The cloning of a

longer sequence of the mouse ferritin H-chain gene allowed identification of regulatory regions 4–5 kilobases upstream of the transcription start site. One region of 37 bp, named FER-1, at 4.1 Kb 5′ of the transcription initiation site, acts as an enhancer and as a target of the adenovirus oncogene EIA-mediated repression [424]. The second region identified, named FER-2, which is of 40 bp is located further upstream (4.8 kb form transcription start site) and contains a perfect match to the consensus binding motif to the erythroid transcription factor NF-k2, and a second sequence that may bind a different member of the NK-kB/Rel family [425]. FER-2 constitutes the major regulatory TNF-responsive element of the H-gene, and may be important in the induction of ferritin synthesis caused by oxidative stress, mediated by NF-kB transcription factors.

A study on ferritin in fetal brain identified an unusually long H-chain transcript arising from an alternative polyadenylation site. It contained an additional 279 nt at the 3′ untranslated region [426]. The transcript was found in various tissues, with the highest relative concentration in brain, kidney and lung [427].

9. Other biological functions associated with ferritins

There are indications that ferritins may have other functions in addition to the well assessed role in storing intracellular iron in a non-toxic and bioavailable form. Ferritins are found in unicellular organisms where the needs for iron storage are not obvious [30]; also they are present in high concentrations in some cells, which are iron-poor such as insulin-secreting cells [404] and the ferritins found in human body fluids clearly do not store iron [428]. The additional functions attributed to ferritins found inside and outside the cell are summarised below.

9.1. Ferritin and oxidative damage

It has often been suggested that ferritins detoxify intracellular iron [428], but how they perform this protective role remained unclear. Iron toxicity is mainly related to its capacity to promote the formation of reactive oxygen species (ROS) which are the major effectors of oxidative damage [429]. This is associated with a 'free' iron or non-heme iron pool consisting of low molecular iron complexes, the concentration of which is extremely low and probably regulated by ferritins. In most cells, ferritin is the most abundant and most concentrated form of iron. Stored in ferritin this iron is relatively inert as a promoter of ROS formation, but it becomes active when released by reducing agents [430]. The physiological reductant (and toxicant) oxygen superoxide produced by xanthine-xanthine oxidase [431] or by stimulated polymorphonuclear leukocytes [432] can release a minor proportion of ferritin iron [433], which is sufficient to promote lipid peroxidation [434]. Also the toxicant, redox cycling, xenobiotics, including Paraquat, adriamycin and alloxan, mobilize ferritin iron. They can produce free radicals with reduction potentials enabling them to stimulate ferritin iron release and lipid peroxidation [430,435–437]. Nitric oxide binds to ferritin [200] and it was reported to mobilise ferritin iron and thus to promote lipid peroxidation [258], but its role as a pro-oxidant has been questioned [438,259]. Adryamicin-induced iron release was greater in ferritins with higher L-chain content [439]. These in vitro results lead to the suggestion that an unregulated release of iron from ferritin caused by ischemia, inflammation or xenobiotics may be sufficient to catalyse oxidative damage [430]. However, direct evidence that ferritin has a pro-oxidant activity in vivo is still missing.

Iron-induced ROS formation is linked to the presence of ferrous iron [429] and enzymatic systems that remove ferrous ions are expected to reduce iron toxicity. This has been demonstrated for ceruloplasmin [440] and for ferritin H-homopolymers [441] both having ferroxidase activity. In contrast, ferritin L-homopolymers did not reduce iron-induced lipid peroxidation. This suggested that ferritin may have an anti-oxidant function, that is related to its subunit composition. Additional evidence that ferritin has a protective role in oxidative damage came from studies on cellular systems. Balla and collaborators [442] showed that the cytotoxicity of oxidative agents on endothelial cells was strongly reduced by pre-incubating the cells with heme iron. The reduction of the activity was parallel to the increase of endogenous ferritin expression. Also exogenous ferritins which were taken up by the cells showed protective effects, provided that they had ferroxidase activity [442]. The increase in ferritin synthesis was apparently mediated by heme oxygenase, an enzyme induced by oxidative stress which liberates iron from heme [443,444]. The released iron stimulates ferritin synthesis through the IRE-IRP machinery. It was proposed that the newly synthesised ferritin reduced the amount of iron available for ROS production and cellular damage. This proposal is consistent with the observations that the toxicity of oxidative agents [445] on various tumour cell lines decreased with the increase of intracellular ferritin content. Similar observation was made with TNF, the cytotoxic effect of which may be linked to oxidative damage [446]. A specific induction of H-chain synthesis induced by TNF [291] or by transfection [447] was found to reduce the expression of L-chain, probably due to an activation of IRP caused by iron deprivation. This provides indirect evidence that H-chain ferroxidase activity directly regulates the size of an 'intracellular transit iron pool', which may participate in oxidative damage.

Thus, recent data support the hypothesis that ferritin has iron-detoxifying functions. However, under particular patho-physiological conditions, iron release from ferritin may be unregulated and ferritins may act as pro-oxidant agents.

9.2. Regulators of protein synthesis?

Studies directed to the analysis of non-ribosome-bound RNP structures in duck erythroblasts identified a prosome-like particle with a strongly inhibitory effect on in vitro translation of globin and non-globin mRNAs [448]. Later, the particle was identified as a ferritin molecule, based on its amino acid sequence and chemico-physical and immunological properties [449]. More recently, the characterisation of the U-particles which accumulate in mouse L-cells following infection with mengovirus revealed them to be mouse ferritin molecules [450]. The U-particles were found to inhibit mRNA translation in vitro [450]. Additional evidence that ferritins may have a role in the regulation of protein synthesis came from a report by Wu and Noguci [451]. They co-transfected HeLa cells with cDNAs of H- and L-ferritin chains together with globin promoters fused to reporter genes. The expression of the reporter genes was up-regulated by both H- and L-ferritin chains, (with L having a stronger effect), suggesting that ferritin may have a direct role in globin gene expression. Presently it is unclear whether these putative effects of ferritin on protein synthesis are related to iron.

9.3. Extracellular ferritins

Some insects and molluscs secrete a large amount of iron-containing ferritin which may function as an iron transport molecule. These ferritins have a leader sequence for membrane targeting and processing [33,40]. It remains to be studied where and how they incorporate iron in the secretary pathway. Largely obscure are the origin and mechanism of production of the extracellular ferritins found in mammals for which, so far, no transcripts with appropriate signal sequences have been identified. Part of the circulating tissue ferritin probably arises from tissue damage, but the presence of specifically glycosylated subunits and its tight regulation in response to iron and to various inflammatory conditions indicate that most of it is actively secreted. The extracellular ferritins found in serum and body fluids account for a minor proportion of total ferritin. They are typically iron-poor and their structures vary from having no detectable H-chain in serum [452] to a high proportion of H-chain in milk [453]. There is no direct evidence that the ferritins in body fluids have functional roles, and the indication that the secreted ferritins are functionally active is based on studies with recombinant and extracted intracellular ferritins and on the identification of specific receptors on various cellular membranes. Such receptors have been described in liver cells [267,271,272], on human lymphocytes and erythroblasts [454,455], on adipocytes [456] and on various cell lines [457–459]. It is presently unclear how many ferritin receptor types exist, but a major difference between the ones found on liver cells and on the other cell types is evident. The hepatic receptors have a broad specificity with respect to H and L composition and even across species [460], while those on lymphocytes and cells lines are specific for the H-chain [454–457,460]. They seem to have distinct roles in the transfer of iron among different cells and in the regulation of cellular proliferation and function.

9.4. Ferritin and the regulation of cellular proliferation

Broxmeyer and collaborators in 1981 proposed that ferritins have a regulatory role in the production of granulocytes and macrophages [461]. The hypothesis that a sub-population of ferritins has suppressive activity on bone marrow myeloid progenitor cells was later confirmed by the use of recombinant homopolymers of H- and L-chains in vitro and in vivo [462,463]. This activity was found to be associated not only with the H-chain, but also with its ferroxidase activity. It could be reversed either by the supplementation of heme iron to the progenitors cells, or by an excess of an H-chain variant with an inactivated ferroxidase centre [462]. A current hypothesis is that the suppressive activity is exerted via the binding to the specific H-chain receptors and by the interference of the ferroxidase activity with cellular iron uptake. Similar suppressive activity has been found on the proliferation of stimulated lymphocytes [454] and K562 cells [455]. Cells from patients with haematological disorders are less sensitive to the myelosuppressive activity of H-ferritin, thus favouring the progression of the disease [464]. More recently it has been shown that a sub-population of mononuclear phagocyte progenitor cells is sensitive to ferritin suppression [465]. This sub-population consists of pre-lineage commitment progenitors, which need bacterial lipopolysaccharide to respond to the specific growth factor M-CSF. This suppressive activity appears to be mediated by a cytokine produced by adherent macrophages responding to H-ferritin [466]. Also in this case the H-ferritin with an inactivated ferroxidase centre was not effective in suppressing cellular proliferation. The mechanism of this action is far from clear.

Other studies showed that the proliferation of cells in serum-free medium is stimulated, to some extent, by ferritin. This was found in neuroblastoma cell lines, the growth of which was stimulated 1.4-fold by the addition of ferritin [467]. Human erythroleukemia K562 cells [468] and the human lung cancer T3M-30 cells [469] grown in protein-free media were found to release into the medium a ferritin which stimulated growth of various cells, including leukaemia HL60 and melanoma SK-28. Whether the stimulus was due to iron associated with the molecule was not studied. Recently it has also been shown that natural ferritins rich in H- and L-chains suppress antibody production in B-cells [470]. In Epstein-Barr-virus transformed human B-lymphoblastoid cell lines ferritin inhibited the expression of mRNA. This is additional evidence of the suggested immunosuppressive activity of ferritin.

9.5. Genetic hyperferritinaemia

Of particular interest is the recent description of a genetic, autosomal and dominant form of hyperferritinaemia. Three families have been reported in Italy and France [471–473]. All the affected subjects show high serum ferritin levels (about 10 times normal), and congenital cataract. Those who were treated by phlebotomy readily became iron-deficient but their serum ferritin levels remained unchanged. The identification of a point mutation in the IRE loop sequence of L-chain mRNA explained both the dominant transmission and the lack of response of the hyperferritinaemia to iron deprivation [472,473]. The mutations identified in the Italian and French families were different, but both led to reduced IRE affinity for IRP. Analyses of an immortalised cell line obtained from one patient showed a normal level of L-chain mRNA and abnormally high levels of L-type ferritin which are not modulated by iron [473]. This is consistent with L-chain expression which is not under the control of the iron-IRP machinery, and is constitutively expressed. Data from the few tissues analysed indicate that the intracellular L-ferritin levels, like those of serum ferritin, are several-fold higher in the patients than in normal subjects. This is strong evidence that the same (defective) gene encodes for both intracellular and secreted serum ferritin. Probably, the most surprising finding was that despite the abnormal high levels of L-ferritin in all tissues, the subjects show no evident abnormalities except for neonatal cataract, the severity of which varies in the different families. Thus, crystalline lens may be the only tissue which is sensitive to L-chain overexpression, for unknown reasons. The erythron and other tissues strongly dependent on iron are apparently unaffected, and body iron metabolism and absorption are normal. The apparent passive role of L-chains is probably due to their lack of ferroxidase activity, implying that this activity is necessary for the full functionality of ferritin. In fact cell lines overexpressing H-ferritin show an iron-deficient phenotype [447], consistent with an expanded iron storage compartment, whereas cells lines overexpressing L-chain do not [473].

10. Conclusions and perspectives

The ubiquitous distribution of ferritin in all iron-containing organisms implies that this protein is essential for the handling of cellular iron. It is clear that the structural architecture of this molecule is remarkably well conserved and so is its capacity to incorporate iron. Its unique structure is designed to keep polynuclear iron in a soluble and available form, which has a limited crystallinity. Most, and probably all, ferritins (except perhaps serum ferritin) have ferroxidase activity, i.e., catalyse the conversion of the toxic Fe(II) into the less dangerous Fe(III). This is important not only for facilitating iron sequestration, but probably also in regulating intracellular iron speciation and chemical reactivity. The essentiality of ferritin, even for cells with no obvious need for iron storage may be due to these dual roles: to regulate iron toxicity and to avoid formation of polynuclear forms from which iron bioavailability is not easily controlled. Recent findings of goethite-like structures in some pathological iron-overloaded tissues indicate that conditions may exist in which the cell's ability to store iron in ferritin is either bypassed or saturated. The mechanism of ferritin iron incorporation, although not fully understood, appears to follow conserved pathways from bacteria to mammals: iron is taken up through pores in the protein shell, is oxidised at specific sites (at least initially), and laid down as inorganic material in a preformed cavity. Ferritins from different origins have sequence differences, some of which may reflect a response to a cellular need. It is difficult presently to assess the biological significance of these differences, due to limitations in the tests for ferritin functionality, which are mainly based on largely non-physiological in vitro systems. Although unravelling iron storage mechanisms based on in vitro experimentation is exciting and important, still more challenging, and ultimately of even greater importance, is how and from what pool, ferritin acquires its iron in vivo. Little information is currently available either on the types of iron accessible to ferritin in vivo or on the effects of cellular components on ferritin iron uptake and release. Novel and more physiological systems are now needed to analyse the various biological roles of the ferritins, including one of the least well understood aspects of iron metabolism: how iron moves from one molecule to another. The discovery of the dual activity of IRP and the recognition that routes of iron utilisation (e.g., heme synthesis), iron absorption and storage are under tight co-ordination have clarified relationships between cellular iron uptake, utilisation and sequestration. Citrate is also regulated by IRP, and this has led to the speculation that this potential iron chelator may have a role in intracellular iron exchange, but its effects on ferritin activity have not been studied in detail. IRP is the sensor of cellular iron status in vertebrates, and ferritin is probably the molecule which actively regulates intracellular iron levels. Such regulation is likely to be both dynamic and complex, involving modulation in rates of iron sequestration and release, alteration in the redox status of iron and interaction with many as yet unidentified cellular components. The understanding of the biological functions of the iron storage compartment is a major challenge which is relevant not only to normal physiology but to many diseases associated with perturbations of iron metabolism.

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