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Ferritin: Design and Formation of an Iron-Storage Molecule

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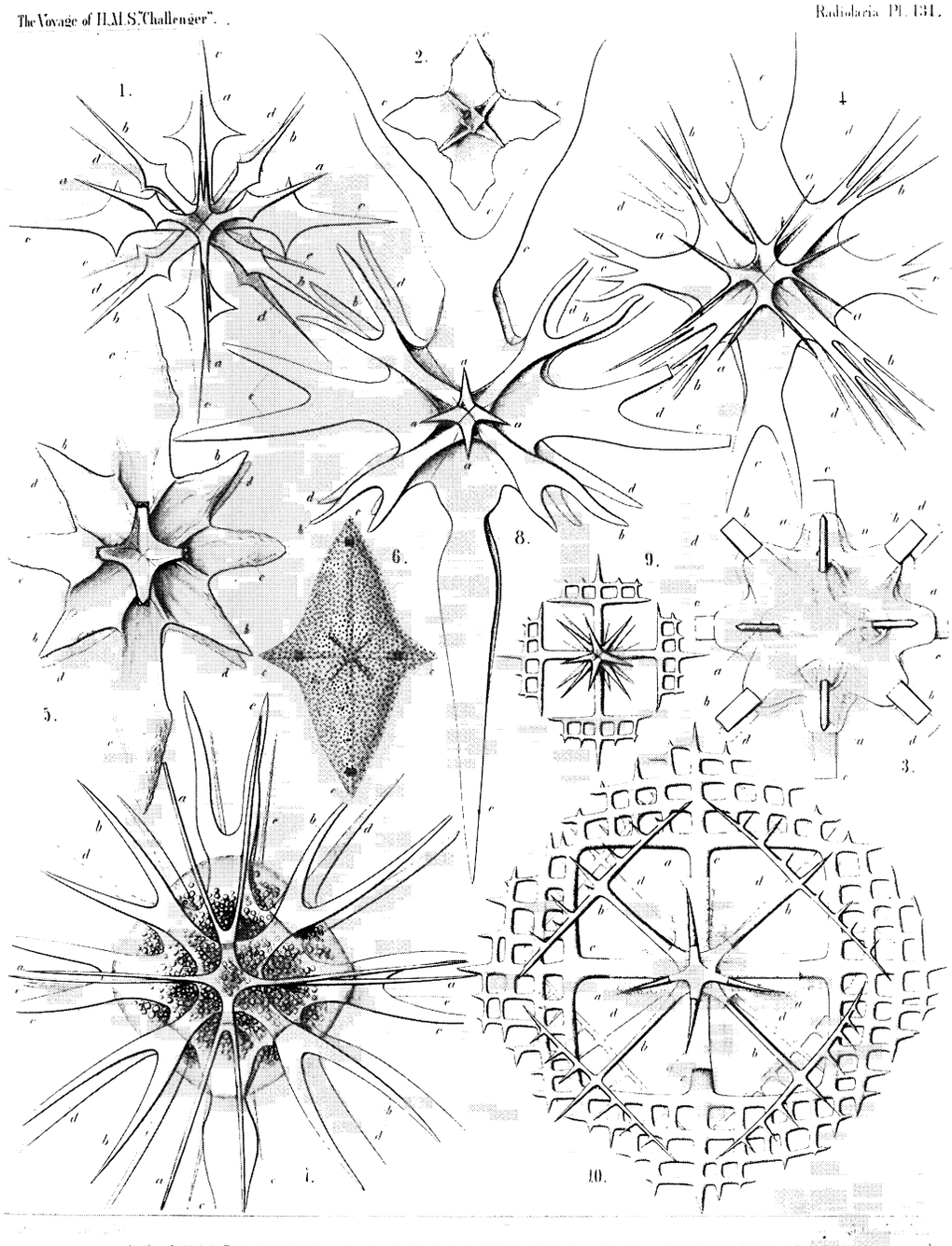

1 3. QUADRILONCHE. 4 6. BELONOSTAURUS. 7. 8. LONCHOSTAURUS.
9 10 LITHOPTERA.

IRON OXIDES, ICE

Class Agantharia. Plate of various species from the report on the Radiolaria collected by H.M.S. Challenger during the years 1873–1876 (Haeckel, E. 1887 Rept. Sci. Res. of the Voy. of H.M.S. Challenger. *Zoologist* **18**).

These common planktonic radiolarian-like animals have skeletons made of celestite, each spine consisting of a single rhomboidal crystal. Sea water is undersaturated with respect to $SrSO_4$ and the skeletons dissolve rapidly after the death of the animal. This is an example of a less usual mineral, not described in the symposium.

(*Facing p.* 551)

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Ferritin: design and formation of an iron-storage molecule

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Although essential for most forms of life, too much iron is harmful. To cope with these antagonistic phenomena an iron-storage molecule, ferritin, has evolved. The structure of horse spleen apoferritin, which has recently been refined, consists of 24 symmetrically related subunits forming a near-spherical hollow shell. In ferritin the central cavity is occupied by an iron core of 'ferrihydrite', a geologically ephemeral mineral found in hot or cold springs and in mine workings, or produced in the laboratory by heating solutions of ferric salts. Ferritin itself forms most readily from apoferritin, in the presence of dioxygen, from Fe^{II}, not Fe^{III}. Access to its interior is through small intersubunit channels, and the protein influences both the rate of Fe^{II}-oxidation and the form of oxide produced.

Introduction

Ferritin and haemosiderin form the major pool of non-haem iron in animals and plants (Harrison *et al.* 1974*a*, 1980; Munro & Linder 1978; Clegg *et al.* 1980; Iancu 1983). In normal humans these iron-storage forms account for nearly a quarter of the total body iron, although their levels are sensitive to changes in the flux of iron entering and leaving the body. Ferritin is a protein with a characteristically high, although variable, iron content. Typically, as isolated from liver or spleen, iron accounts for 10–20% of its molecular mass, although it can reach over 30%. Its iron is present in small crystalline particles that can be readily imaged by electron microscopy (Massover & Cowley 1973). These particles form the 'iron core' of a roughly spherical molecule of 125 Å† diameter. This diameter and many other molecular characteristics, which include electrophoretic mobility, solubility and antigenicity are properties of the protein shell. This shell also imposes a maximum diameter on ferritin's iron core of about 80Å, but since, chemically, it is an inorganic 'hydrous ferric oxide-phosphate' complex that contains nearly 60% iron by mass, as many as 4500 iron atoms can be stored within a single molecule.

Like ferritin, haemosiderin has a high, but variable, iron content (Wixom *et al.* 1980; Iancu 1983). Unlike ferritin it is isolated as insoluble granules, which appear to be derived from secondary lysosomes, and it is thought to originate from breakdown of ferritin. The absolute and relative amounts of iron stored as ferritin and haemosiderin vary with iron loading and cell type. Both forms act as reserves, which are augmented when iron levels increase and which can be mobilized to meet demand. Haemosiderin iron, which is less accessible, exceeds ferritin iron when iron levels are high. The greater accessibility of ferritin iron may result from its location in the cytoplasm and from its molecular architecture, although the intracellular mechanism or mechanisms by which its iron is made available are still uncertain. However, in ferritin, sequestration of iron within a well defined protein molecule provides a means by which storage of this metal may be controlled. Such control can occur in at least two ways.

† $1 Å = 10^{-10}$ m.

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The stimulation by iron of apoferritin biosynthesis (Drysdale & Munro 1966) provides a form of coarse control. The formation of ferritin from apoferritin is self-catalysing. These mechanisms are of considerable importance because of the toxicity of free iron, and this is underlined by the ubiquity of ferritin in all types of eukaryotic cell. Ferritin may also be responsible for the intracellular delivery of iron for haem synthesis (Speyer & Fielding 1979; Ulvik & Romslo 1978). So it is not merely a passive store but a reserve of iron and a means by which homeostasis of this element is maintained. We shall not consider haemosiderin further except to point out that the iron mineral present in both storage forms is the same or closely similar (Fischbach *et al.* 1971). This mineral was originally thought to be unique, because it differs from all the well known iron Fe^{III} oxides and oxyhydroxides. However, comparatively recently, a mineral 'ferrihydrite' has been described (Towe & Bradley 1967; Chukrov *et al.* 1973) whose properties closely resemble those of the ferritin iron core mineral (Towe & Bradley 1967; Harrison *et al.* 1967). An atomic structure proposed for 'ferrihydrite' is shown in figure 9. Mineralization in ferritin is unusual in that it occurs within the confines of a protein shell that limits its potential for crystal growth. The protein also has to provide a chemical environment favourable to its formation, and a knowledge of the protein structure is therefore essential for an understanding of how its iron core is formed. The uniqueness of the ferritin structure derives from the manner in which 24 protein subunits are assembled in cubic 4 3 2 point symmetry, which allows the formation of its central cavity (Harrison 1959; Banyard *et al.* 1978). This symmetry has not been widely observed in other proteins. One example is the E2 component, dihydrolipoyl transsuccinylase, of the 2-oxoglutarate dehydrogenase complex of *Escherichia coli* (Derosier *et al.* 1971). The other, more immediately relevant to iron mineralization, is that of cytochrome b_1 of *E. coli* (Yariv *et al.* 1980; Yariv 1983). Other similar molecules include a type b cytochrome of *Azotobacter vinelandii* (Stiefel & Watt 1979; Li *et al.* 1980) and of *Azotobacter chroococcum* (Chen & Crichton 1980). These cytochromes which have also been given the name 'bacterioferritin', are similar to ferritin not only in shape, size and manner of subunit assembly, but also in that they can harbour an iron mineral in the cavity of the molecule.

X-ray analysis has now provided us with a detailed description of the three-dimensional structure of horse spleen apoferritin (Rice *et al.* 1983*a*). We shall show how knowledge of this structure provides insights into how its subunits are assembled, and into the manner in which iron is deposited. The constancy of ferritin structure in various species implies that its structure and function are well matched.

The structure of horse spleen apoferritin

Apoferritins and ferritins from spleen and liver of human, horse, dog, rat, ferret and mouse (Hoare *et al.* 1975; Rice *et al.*, unpublished results) form isomorphous octahedral crystals in the cubic space group F432 with unit cell, $a \approx 185$Å. X-ray diffraction patterns are isomorphous and clearly indicate structural similarities in molecules of all these species. The 24 structurally equivalent subunits pack in 4 3 2 symmetry as shown schematically in figure 1.

Refinement of the structure of horse spleen apoferritin at 2.8Å resolution with incorporation of amino acid sequence information (Heuterspreute & Crichton 1981), shows that 129 of its 174 residues are found in five α-helices. As shown schematically in figure 2, the subunit consists of a bundle of 4 long helices, A (residues 10–39), B (45–72), C (92–120) and D (124–155) with a fifth, shorter helix, E (residues 160–169). Intramolecular contacts between the four helices

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extend over a length of about 35Å. The longest helix, D, protrudes beyond this main interhelical contact region and the chain folds sharply back so that helix E lies at an acute angle to the main axis of the subunit. Another feature of the subunit is a long loop, L (residues 73–91), joining the C-terminus of helix B to the N-terminus of helix C. The view of the subunit given in figure 2 is a slice through the shell such that helices B and D each have one face towards the inside of the shell, and A and C each have one face towards the outside of the molecule. The loop L is also on the outermost surface.

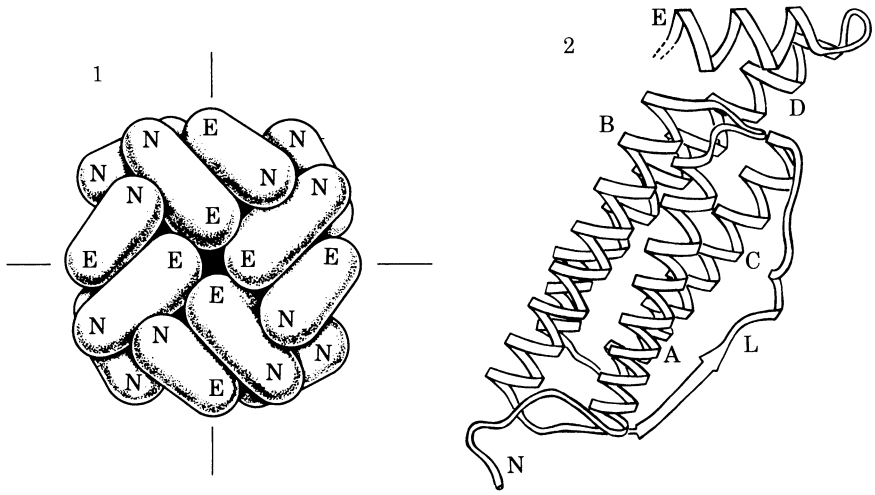

FIGURE 1. Schematic representation of the ferritin molecule viewed down a molecular four-fold axis illustrating 4 3 2 symmetry. Each subunit is represented by a sausage-shaped building brick with ends labelled N and E. The N-terminal region of the polypeptide chain lies close to the end labelled N and the E-helix close to that labelled E.

FIGURE 2. Ribbon diagram of the α-carbon backbone of an apoferritin subunit. The main body of the subunit is a bundle of four long helices A, B, C and D, with a short helix, E, lying at an acute angle to the bundle axis. The N-terminus, N, lies at the other end of the subunit to E. The loop, L, joins helices B and C and together with its two-fold related counterpart, L′, forms a section of antiparallel β-sheet within the dimer. The final two residues in the C-terminal tail are ill-defined in the current electron density map and are not included in this diagram.

Side chain interactions within the subunit form distinctive regions. At either end of the helical bundle the interactions between the protein side chains form tightly packed hydrophobic cores. In contrast, in the centre of the bundle, the subunit interior changes in character and includes several buried hydrophilic or polar residues, Tyr 23, Lys 58, Glu 103 and Glu 137. These interact to form a network of hydrogen bonds with a salt bridge between Lys 58 and Glu 103.

Helix E interacts with the subunit through hydrophobic residues Phe 166, Leu 171 and with specific hydrogen bonds involving Glu 163.

SELF ASSEMBLY OF APOFERRITIN

The apoferritin shell is remarkably stable: it withstands heating to 80 °C for 10 min, and incubation in 10 M urea or 10 g l^{-1} sodium dodecylsulphate at room temperature. There is little evidence for dissociation even in extremely dilute solutions at neutrality in the absence of denaturants, and marked dissociation occurs only as the pH is lowered below 3.0. Dissociation is accompanied by subunit unfolding (Listowsky *et al.* 1967) and dissociation–reassociation equilibria have been followed by u.v. difference spectroscopy (Crichton & Bryce 1973) and ^{1}H-n.m.r. spectroscopy (Imai *et al.* 1981). Transient dimers and tetramers (Crichton 1972) or

hexamers (Stefanini *et al.* 1979) may form as assembly intermediates, but in situations approaching physiological conditions the equilibrium lies well on the side of whole shells.

In apoferritin the subunits are very tightly packed to give a roughly spherical molecule with rhombic dodecahedral geometry. There are a large number of inter-subunit interactions that form regions of marked hydrophobicity and other regions where polar interactions predominate.

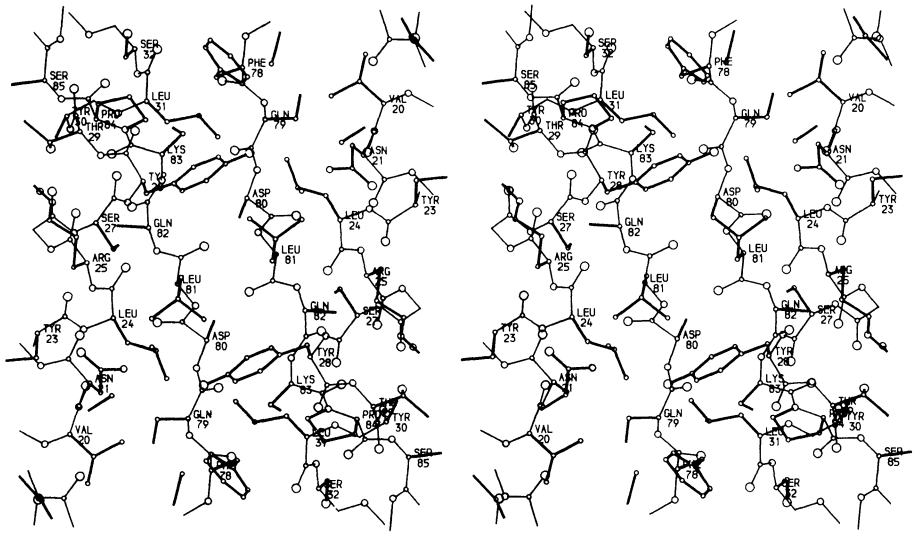

FIGURE 3. Stereo view down a twofold axis of the apoferritin molecule showing the hydrophobic nature of the interface between subunits of the dimer. Helices A and the symmetry related A′ lie towards the exterior surface of the protein shell and a section of β-sheet is formed by loops L and L′. Side chains from L and L′ interdigitate between those from A and A′, thereby causing these helices to be separated by about 15Å.

One face of the apoferritin subunit contains hydrophobic residues from helix A (Val 20, Leu 24, Tyr 28, Leu 31) and the loop L (Phe 78, Leu 81, Pro 84). By interaction with an equivalent region of a second subunit this hydrophobic patch of some 22Å length is buried from solvent and a twofold symmetry axis is generated. This is shown in figure 3.

Contact between helices A and the dyad related A′ is prevented in dimer formation by the interdigitation of the hydrophobic side chains from loops L and L′, shown schematically as a section through the dimer in figure 4. This situation leads to a separation of helices B and B′ across the dimer interface by some 15Å and direct interaction of side chains from these helices cannot occur over this distance. This is observed in our electron density maps as partial disorder for several of the side chains in helix B.

Another hydrophobic region on the subunit surface is that comprising one face of helix E (Leu 161, Tyr 164, Leu 165, Leu 169 and Leu 154 near the C-terminus of helix D), which can be seen in figure 5. If these hydrophobic residues are to be partially buried from solvent then further assembly of dimers must occur. Although there may be several routes by which shell assembly from dimers may proceed, the schematic diagrams in figure 6 illustrate how the dimer structure, with its perpendicular E helices, is ideally suited to the generation of a molecule with 4 3 2 symmetry. A ribbon diagram showing the positions of the hydrophobic residues in the region of contact around the four-fold axis is given in figure 7.

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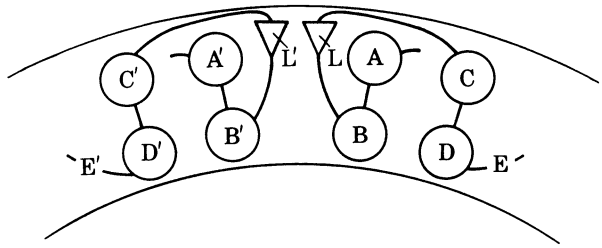

FIGURE 4. Schematic diagram of a section through the apoferritin shell showing a dimer. The view is orthogonal to that in figure 3. Interactions involving side chains from loops L and L′ and helices A and A′, shown in figure 3, keep A and A′ apart and cause the formation of a groove between helices B and B′ on the inside surface of the molecule.

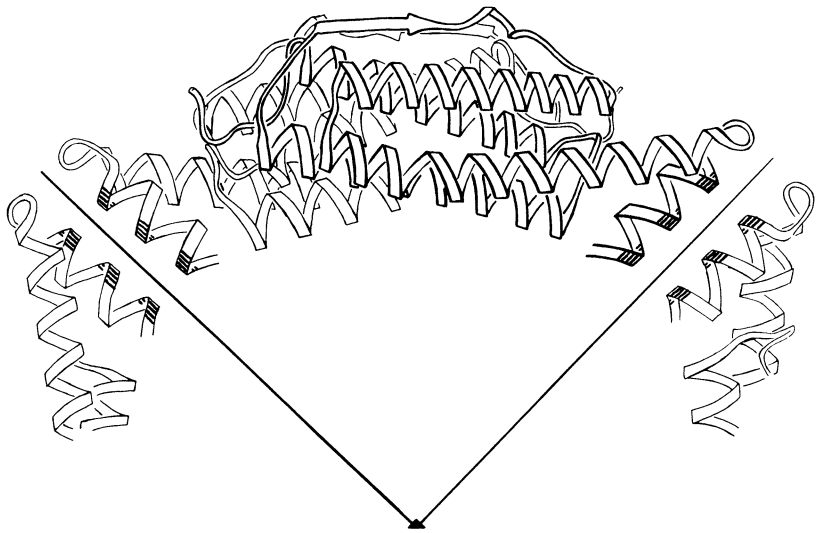

FIGURE 5. Ribbon diagram of the apoferritin dimer and parts of two other subunits. The hydrophobic face of helix E is shaded.

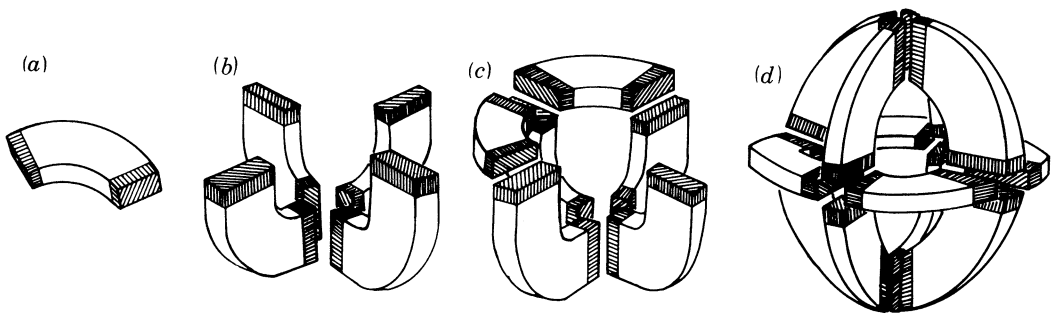

FIGURE 6. Schematic series of diagrams depicting the need for the further aggregation of dimers to bury hydrophobic patches from solvent. It is proposed that dimers are the first intermediates to form, but the precise order of subsequent condensation is not known.

(*a*) Formation of a dimer buries the largest hydrophobic surface, but leaves the E-helix hydrophobic residues, indicated by shading, exposed to solvent. The pair of E-helices of the dimer are oriented at 90° to each other and are therefore ideally suited to the 4 3 2 symmetry of the final molecule.

(*b*) Formation of an octamer from four dimers buries half their exposed hydrophobic surfaces and creates a fourfold channel.

(*c*) Additional dimers will readily orientate themselves by bringing hydrophobic patches together, thereby creating a threefold channel.

(*d*) Additional dimers combine to generate the entire molecule, an icosatetramer (24-mer), where all hydrophobic patches are buried.

Note that in this representation the threefold channels have been greatly exaggerated for ease of viewing.

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Features of the assembled apoferritin shell important to its accumulation and release of iron and its iron-storage function will be discussed after consideration of the structure and formation of the iron core.

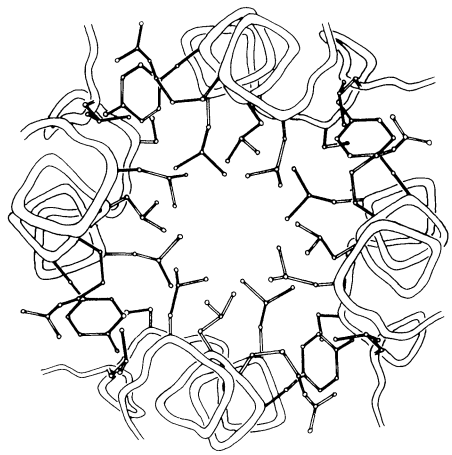

FIGURE 7. Illustration of the hydrophobic nature of the fourfold channel. Each of the four E-helices, represented by ribbons, provides three leucine side chains to line the channel.

FERRIHYDRITE: THE IRON CORE MINERAL OF FERRITIN

Amorphous or colloidal hydrous iron oxides are the first products of the hydrolysis and precipitation of dissolved iron from solution. Synthetic specimens prepared by the addition of ammonia (van der Giessen 1966) or sodium bicarbonate (Brady *et al.* 1968) to ferric nitrate are microcrystals of diameter 30–70Å and compositions $Fe_2O_3 \cdot (1.2H_2O)$ and $Fe_5HO_8 \cdot 4H_2O$, respectively. The heating of ferric nitrate solutions to 90 °C produces similar particles (Towe & Bradley 1967). The natural mineral, with composition $5Fe_2O_3 \cdot 9H_2O$, has been named ferrihydrite. It is found as a relatively new deposit on the walls of old mine workings and is considered to be a geologically ephemeral material (Chukrov *et al.* 1973). Ferrihydrite is also found in association with iron oxidizing bacteria in hot water springs such as those emanating from volcanoes. It also occurs as a deposit on an open-mesh organic matrix in immature radular teeth of the mollusc *Cryptochiton stelleri* although the mature teeth contain Fe_3O_4 deposits (Towe & Lowenstam 1967). Ferrihydrite ages to α-Fe_2O_3 on heating to 40 °C for 10–14 d or to α-FeOOH in 0.02 M $FeSO_4$ at pH 6 or in 1 M KOH. The X-ray or electron diffraction patterns of ferrihydrite show lines at 2.52, 2.25, 1.97, 1.72, and 1.48Å and this pattern distinguishes it from other oxides and hydroxides of iron, such as α-, β-, γ-, δ-, ε- FeOOH, α-, β-, γ- Fe_2O_3, Fe_3O_4, FeO, $Fe(OH)_2$, and green rusts (Murray 1979; Towe 1981). The electron and X-ray diffraction patterns of ferritin iron cores (Haggis 1965; Harrison *et al.* 1967) and of haemosiderin (Fischbach *et al.* 1971) also show these five characteristic lines.

Although formed inside the protein shell, ferritin iron cores remain intact when the subunits are removed. The exposed cores clump together and give precipitates resembling haemosiderin (Matioli & Baker 1963; Fischbach *et al.* 1971). With or without their covering protein the cores give the same diffraction pattern (Harrison *et al.* 1967) and the finding that at high angles ferritin single crystals give X-ray powder patterns characteristic of the mineral indicates that apoferritin has little or no orientational influence on the mature iron cores (Fischbach *et al.* 1969). Removal of the protein requires drastic treatment such as 1 M NaOH (Granick 1942).

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During this treatment about 80 % of the inorganic phoshate known to be associated with ferritin iron cores is lost (Granick 1942). Its loss has little influence on the diffraction patterns. Hence it may be concluded that this phosphate is largely adventitious. This is also suggested by the observation that much of the phosphate is lost during the early stages of iron release, when a 'last-in-first-out' principle is obeyed (Treffry & Harrison 1978), and by the ability to reconstitute ferritin in the absence of phosphate (Harrison *et al.* 1967; Macara *et al.* 1972).

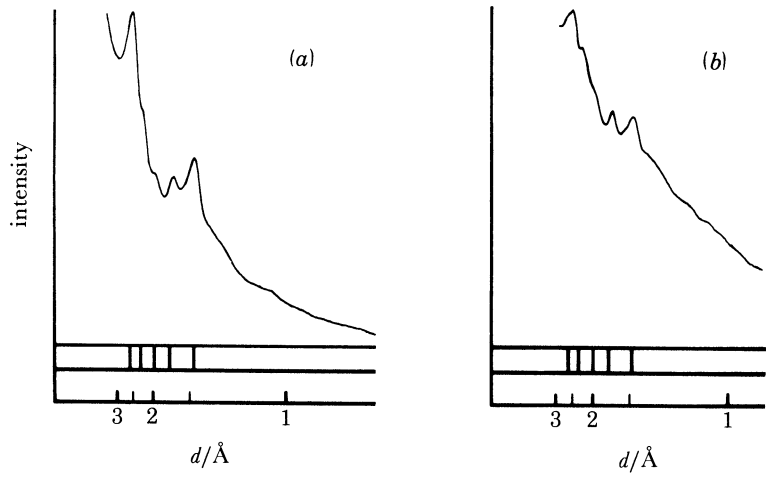

FIGURE 8. Powder diffraction patterns of (*a*) air-dried iron cores from horse ferritin and (*b*) moist human haemosiderin (sample by courtesy of M. Weir and T. J. Peters). These data were collected on film by using monochromated Mo X-radiation. The five diffraction maxima characteristic of 'ferrihydrite' are at 2.52, 2.25, 1.97, 1.72 and 1.48 Å, and are marked in each plot.

The X-ray or electron diffraction patterns of ferritin iron cores and of ferrihydrite exhibit five characteristic lines and several weaker bands at higher resolution. The lines are broad and their width corresponds to a crystallite size of about 50Å or less (Harrison *et al.* 1967; Towe & Bradley 1967). The diffraction lines are weak, diffuse and often overlap, and it is extremely difficult to obtain accurate intensity measurements. Densitometer traces of typical diffraction photographs of ferritin and of haemosiderin are shown in figure 8. The limited data set is barely sufficient to define unambiguously the atomic structure of this mineral.

The diffraction pattern of ferrihydrite has been indexed on either a hexagonal unit cell with $a = 5.08$Å and $c = 9.4$Å (Towe & Bradley 1967), or a cubic cell of side $a = 8.37$Å (van der Giessen 1966), and that of ferritin on a cubic unit cell with $a = 4.43$Å (Haggis 1965) or two alternative hexagonal cells of $a = 11.79$Å and $c = 9.90$Å (Girardet & Lawrence 1968) or $a = 2.94$Å and $c = 9.40$Å (Harrison *et al.* 1967). These different unit cells reflect different arrangements of close-packed oxygen layers and of interstitial iron atoms. Only two specific structures have been proposed, taking into account the X-ray data. These are a structure with four oxygen layers and iron in both octahedral and tetrahedral sites (Harrison *et al.* 1967) related to arrangements then proposed for δ-FeOOH and green rust II (Francombe & Rooksby 1959; Bernal *et al.* 1959) and a second structure also with four oxygen layers but with iron atoms in octahedral coordination only related to the structure of α-Fe_2O_3 (Towe & Bradley 1967). Both structures give reasonably good agreement between observed and calculated diffraction lines. Electronic absorption spectra show a band near to 900 nm typical of octahedrally coordinated Fe^{III}, whereas no bands have been found in the region 400–700 nm,

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which could be assigned to transitions of Fe^{III} in tetrahedral sites (Webb & Gray 1974). The model of Towe & Bradley (1967), which is shown in figure 9, is now generally accepted (Chukrov *et al.* 1973). Although related to the structure of haematite, α-Fe_2O_3, its unit cell contains four oxygen layers, not six, and its Fe^{III} sites are underpopulated such that the overall stoichiometry is 5 Fe:12 O instead of 2 Fe:3 O as in haematite, and some of the O^{2-} atoms are replaced by water molecules or by OH^-. Two other structures have been proposed for ferritin iron cores, although neither is clearly defined. Both are folded ribbons of either bridged tetrahedra (Brady *et al.* 1968) or octahedra (Heald *et al.* 1979), derived respectively from radial X-ray scattering or EXAFS data. The former can be ruled out because of the absence of octahedral iron and the latter is incompatible with the X-ray or electron diffraction patterns. The EXAFS measurements have, however, provided confirmation of the presence of Fe^{III} with six (6.4 ± 0.6) nearest oxygen neighbours, although this does not distinguish ferritin iron from that of most of the iron oxyminerals.

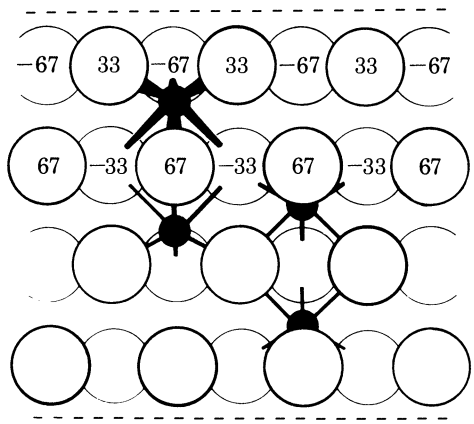

FIGURE 9. The Towe & Bradley (1967) model for 'ferrihydrite' showing four oxygen (○) layers with iron (●) atoms octahedrally coordinated. The dotted lines indicate levels at which lateral displacements are optional.

Except for the ribbon structure of Heald *et al.* (1979), in which phosphate is assumed to terminate the oxygen sheets, phosphate ions have not been placed in the ferritin iron core or ferrihydrite structures. However, they may well replace some of the surface hydroxyls, as has been suggested for goethite treated with H_3PO_4 or NaH_2PO_4 (Russell *et al.* 1974). *In vivo* the phosphate of ferritin iron cores appears to be in dynamic equilibrium with cell phosphate (Van Kreel *et al.* 1972) and *in vitro* it can be replaced by dissolved phosphate ions or by other anions, as shown by use of radioactive probes (Treffry & Harrison 1978) or by ^{1}H-n.m.r. measurements (Imai *et al.* 1978).

FORMATION OF FERRITIN FROM APOFERRITIN

The structure of ferritin as a giant clathrate compound or cage of protein surrounding an inorganic polymer poses problems for its formation. Since its protein shell is compact except for pores only 3–4Å across, it is inconceivable that preformed ferrihydrite particles of 50Å diameter could enter a fully assembled apoferritin shell. Since the biosynthesis of apoferritin precedes that of ferritin (Drysdale & Munro 1966), assembly of apoferritin subunits around iron cores can be ruled out. Hence Nature must have devised an alternative means of producing ferritin from apoferritin.

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In principle, iron might be available in the form of low molecular mass complexes of Fe^{III}, Fe^{II}, or both, and of free Fe^{II}. (At pH 7 the concentration of free Fe^{III} would be exceedingly small.) There are many experiments that show that a ferritin-like molecule can be reconstituted from apoferritin and Fe^{II} in the presence of either O_2 (Beilig & Bayer 1955; Loewus & Fineburg 1957; Harrison *et al.* 1967; Bryce & Crichton 1973) or of KIO_3 as oxidant (Harrison *et al.* 1967; Niederer 1970; Macara *et al.* 1972; Goldner *et al.* 1983). It has also been shown that iron injected as ^{59}Fe-ferric ammonium citrate is rapidly acquired by rat liver ferritin (Hoy & Harrison 1976). However, it is unknown whether or not this iron has to be reduced before its incorporation. *In vitro* experiments to form ferritin by starting from apoferritin and Fe^{III} complexes have so far proved unsuccessful, although in the presence of a small iron core some iron (a net gain over loss) was taken up from ferric citrate (Treffry & Harrison 1979). *In vitro* experiments suggest that if ferritin is formed intracellularly from Fe^{II} by an oxidative mechanism, this process is probably 'compartmentalized' to avoid interference from cytosolic inorganic phosphate (Harrison *et al.* 1977; Treffry & Harrison 1978).

The autoxidation of Fe^{II} by O_2 may formally be described by the equation

$$4\,Fe^{II}\,(aq) + O_2 + 4H^+ \rightleftharpoons 4\,Fe^{III}\,(aq) + 2H_2O.$$

The standard redox potential for this reaction is $+0.459$ V and the equation indicates that the forward direction is favoured by low pH. However, at physiological pH the situation is changed by the almost complete removal of Fe^{III} (aq), owing to hydrolysis, for example

$$[Fe(H_2O)_6]^{3+} \rightleftharpoons [Fe(H_2O)_5(OH)]^{2+} + H^+ \rightleftharpoons [Fe(H_2O)_4(OH)_2]^+ + H^+,$$

$$2[Fe(H_2O)_5(OH)]^{2+} \rightleftharpoons [(H_2O)_4Fe(OH)_2Fe(H_2O)_4]^{4+} + 2H_2O \rightleftharpoons [(H_2O)_4Fe(O)(OH)Fe(H_2O)_4]^{3+} + H^+,$$

and the further condensation to form polynuclear species. 'Uncontrolled' Fe^{III} would therefore precipitate. The ferritin molecule has been designed so these processes proceed in a controlled manner within the apoferritin shell. However, the overall equations do not necessarily represent the actual sequence of events. So oxidation might proceed by successive one- or two-electron steps, and partial hydrolysis to $Fe(OH)^+$ (aq) or $Fe(OH)_2$ (aq) may precede oxidation.

We can list seven processes that may be associated with a mechanism for incorporation of iron into ferritin. Various models may then be proposed from combination of these features.

(1) Fe^{II} enters the central cavity through a channel in the protein shell and is unaltered in the process.

(2) Fe^{II} passes through the shell undergoing oxidation to Fe^{III} while in the channel.

(3) The protein possesses an oxidation site or sites somewhere on the inside surface.

(4) Oxidation of Fe^{II} to Fe^{III} occurs on the developing mineral core.

(5) There is a site on the inside surface of the protein that acts initially to oxidise Fe^{II} as in model (3), but this site also forms the nucleation site for the iron core. It is therefore buried as the core develops, with oxidation subsequently taking place as in model (4).

(6) There are specific nucleation sites on the inner surface of the shell, which bind Fe^{III} after oxidation elsewhere.

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(7) Autonucleation, in which Fe^{III}, once formed inside the protein shell, precipitates spontaneously forming a mineral core with no specific attachment to the protein.

Processes (1) and (2) represent mechanisms for iron transport into the protein cavity, (2)–(5) mechanisms of iron oxidation, and (5)–(7) different modes of iron core formation. The situation in ferritin is complicated further by the fact that there may well be several of these mechanisms operating at the same time.

Although we do not yet know its mechanism, studies of ferritin reconstitution *in vitro* provide some clues. There is evidence that apoferritin binds iron in both its oxidation states (Chasteen & Theil 1982; Rosenberg & Chasteen 1982). Under appropriate conditions, apoferritin acts catalytically so that the iron core forms faster than FeOOH can be precipitated from solution (Niederer 1970; Macara *et al.* 1972; Bryce & Crichton 1973), which indicates a role for the protein in Fe^{II} oxidation. Also, apoferritin influences the product, such that ferritin (containing 'ferrihydrite') is formed even under conditions in which, in the absence of apoferritin, either α- or γ-FeOOH would be formed (Harrison *et al.* 1967). It may be relevant to note that finely divided silica or alumina catalyze the formation of 'ferrihydrite' from Fe^{II} and that rapid oxidation is a prerequisite for the production of this mineral form in preference to α- or γ-FeOOH (Chukrov 1973). It appears therefore that the protein either 'guides' the formation of the 'ferrihydrite' by providing a nucleation site or alternatively 'ferrihydrite' formation results automatically by the rapid oxidation of Fe^{II} to Fe^{III}.

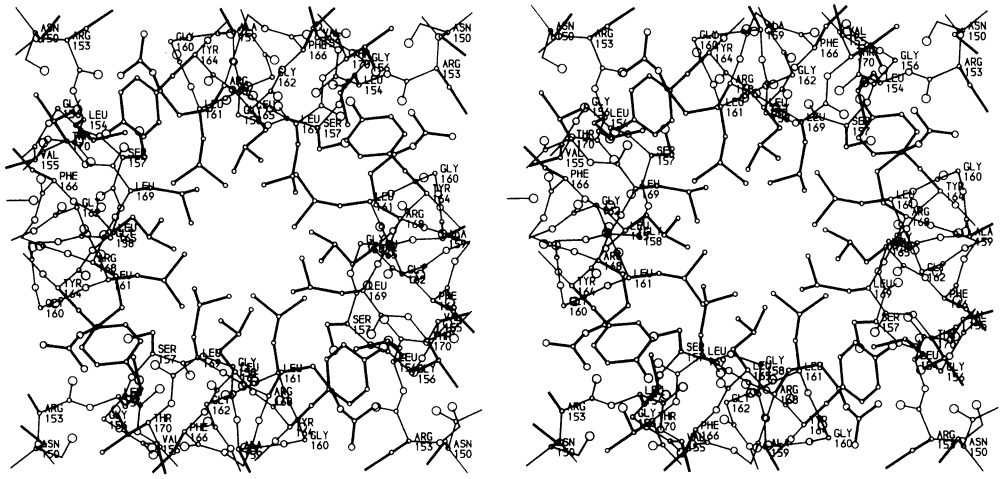

FIGURE 10. A stereo view along the fourfold axis from the interior cavity showing details of a hydrophobic channel.

Another salient feature of ferritin reconstitution is that its kinetics are complex (Macara *et al.* 1972). Under conditions favouring fast iron incorporation, progress curves are sigmoidal (implying autocatalysis) and the 'ferrihydrite' produced has a relatively large crystallite size and nearly 'all-or-none' distribution within ferritin molecules. The kinetic study also indicated that whereas the initial slow phase of iron deposition was catalysed by apoferritin, the subsequent fast accumulation of iron appeared to be governed by the amount of 'ferrihydrite' present. This was further emphasized by analysis of the initial rates of iron uptake into ferritin fractions having increasing numbers of iron atoms per molecule. The complex, bell-shaped, dependence observed can be explained if these rates were governed by the availability of crystal surface of particles growing in a confined space (Macara *et al.* 1972; Harrison *et al.* 1974*b*;

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Clegg *et al.* 1980). This evidence strongly suggests that oxidation can take place on the surface of the developing iron core. The presence of an oxidation site on the protein surface and the involvement of the iron core in iron oxidation is also indicated by the finding that although O_2 is required for the initial oxidation of Fe^{II} on apoferritin, once iron core formation has started Fe^{II} oxidation can proceed equally easily with KIO_3 as oxidant in the absence of O_2 (Treffry *et al.* 1979; Goldner *et al.* 1983). It has been observed (Treffry *et al.* 1978) that the number of Fe^{II} atoms oxidised per O_2 molecule increases from one to four as more iron is added to ferritin (and also in the absence or presence of apoferritin as Fe^{II} concentrations are increased). This variable stoichiometry is hard to understand if Fe^{II} oxidation occurs only at specific catalytic sites on apoferritin. It could be explained if oxidation occurs on the iron core surface since, as this grows, there are more available sites for iron, with the possibility that O_2 could be completely reduced to $2H_2O$ by acquisition of four protons and four electrons in rapid succession from four neighbouring Fe^{II} atoms. With regard to mechanisms by which iron enters the central cavity, there are two types of channel through the apoferritin shell and they are of strikingly different character. There are six equivalent hydrophobic channels, each lined by twelve leucine residues, three on each of four short helices surrounding the four-fold symmetry

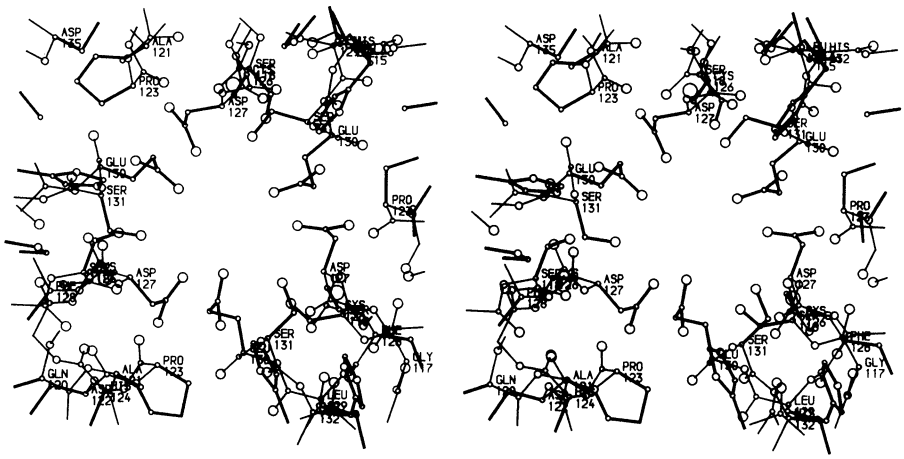

FIGURE 11. A stereo view of the apoferritin shell around the threefold axis viewed from the exterior of the molecule. This channel, lined with aspartate, glutamate and serine residues, is markedly hydrophilic.

axes. These channels are about 12Å long and 3–4Å wide and Fe^{II} might enter the cavity through them. In contrast the eight three-fold axis channels (figure 11) are hydrophilic, being lined by three aspartate residues (on the cavity side of the shell) and three glutamate residues (towards the outside of the molecule). These groups of residues are only about 5 Å apart along the channel, which is also about 3–4Å wide. However, this channel is funnel-shaped, broadening out towards the outside surface to give a wide hydrophilic region. Evidence, summarized in Harrison *et al.* (1980), indicates that a variety of small neutral molcules, cations and anions, can penetrate into the cavity. The different types of channel may exhibit some selectivity towards these molecules and ions. In apoferritin crystals grown from $CdSO_4$ solutions the three-fold axis channels are occupied by two peaks of electron density attributed to Cd^{II} ions (Rice *et al.* 1983*b*). These channels could represent the entry ports for iron and possibly also sites of Fe^{II} oxidation or simply a means of funnelling Fe^{II} ions to the inside for oxidation there. They could also be used by iron leaving the molecule.

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In vitro, iron can be removed from ferritin by $FMNH_2$ and the release of ferritin iron for haem synthesis in mitochondria, a process mediated by $FMNH_2$, has been described (Ulvik & Romslo 1978; Ulvik *et al.* 1981). While small reductants and chelators, which can release ferritin iron, probably enter the molecule and attack the iron core directly, the question of whether $FMNH_2$ (a molecule of about 13Å diameter) can enter is more difficult. One possibility is that it might not penetrate the shell, but passes electrons through the three-fold channels by way of two iron atoms sitting in them. It may be noted that the rate at which iron is released, as Fe^{III}, from ferritin in the presence of transferrin, which clearly cannot enter the molecule, is about a million times slower than that at which it is released, as Fe^{II}, by reduced flavin (Harrison *et al.* 1980). This and other results (Jones *et al.* 1978) have been cited in favour of penetration, but the evidence is not clear cut.

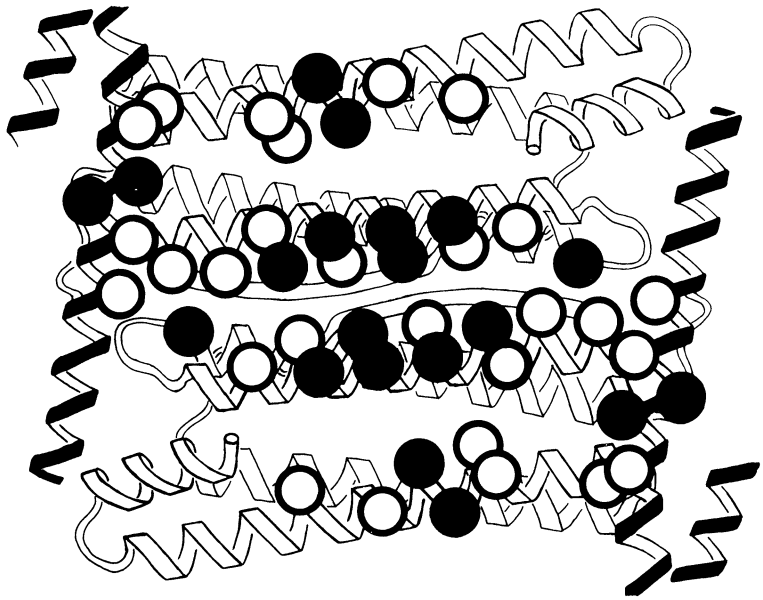

FIGURE 12. Schematic diagram of part of the inside surface of the apoferritin shell viewed down a twofold axis. Acidic side chains are represented by solid circles and basic, hydroxylic and amino residues as open circles. This illustrates the large number of charged groups available for possible interaction with the iron core, the majority of which are disordered in the apoferritin structure.

The inner surface of the cavity is lined with 'loose' hydrophilic residues. In striking contrast to the rest of the molecule where nearly all of the polar side chains are involved in salt bridges or networks of hydrogen bonds, many of the side chains facing the inner cavity are either partially or totally disordered (His 49, Arg 52, Glu 53, Glu 56, Glu 57, Arg 59, Glu 60, Arg 64 and Lys 67 all on B, Glu 136, Lys 139 and Lys 142 on D, as well as three residues at the C-terminus Lys 172, His 173 and Asp 174.) The cause of some of this side chain disorder has been alluded to earlier. Since the residues involved are primarily glutamate, lysine or arginine it may well be that some of these serve to nucleate the iron core and hence are disordered in apoferritin. A schematic diagram of part of the inside surface is shown in figure 12. The high concentration of glutamate residues on the two B helices of the dimer interface is interesting, especially since chemical modification of carboxyl groups is known to prevent iron incorporation (Wetz & Crichton 1976; Treffry *et al.* 1977) and iron is known to protect carboxyl groups (including Glu 53, Glu 56 and Glu 57) from modification (Wetz & Crichton 1976; Crichton *et al.* 1982).

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In studying ferritin we are not looking at a molecule with a single catalytic site where a single reaction takes place. Apoferritin contains 24 subunits related by 4 3 2 symmetry, which implies that there could be 24 or a multiple or submultiple of 24 oxidation and nucleation sites, and there is not necessarily the same number of each if these processes are separated. However, it is most unlikely that, say, twelve iron core particles would start simultaneously and grow at the same rate. Indeed there is much evidence to suggest that this is not so. Electron microscopy shows that a significant proportion of molecules contain single crystals that are large enough to fill the entire space within the apoferritin shell (Massover & Cowley 1973). The tendency to all-or-none distribution of iron on reconstitution also implies competition for iron between molecules (Macara *et al.* 1972). Most recent studies, by various spectroscopic methods (electron spin resonance (Chasteen & Theil 1982; Rosenberg & Chasteen 1982), optical absorption (Treffry & Harrison 1982)), which have included the use of a variety of metal probes, have provided strong evidence for metal clustering. After deposition of an average of no more than two or three iron atoms per apoferritin molecule, clustering is apparent and addition of further iron to these nuclei begins to be preferred to saturation of equivalent binding sites on the protein.

Concluding remarks

Neither the elucidation of the structure of horse spleen apoferritin nor the kinetic and binding data on this protein have yet led to a clear description of the mechanism of iron storage and mobilization. However, examination of the structure has served to highlight key regions on the molecular surface that may be involved in its catalytic role in iron oxidation and hydrolysis, the passage of iron through the protein shell, and the nucleation of the iron core. These regions are close to the molecular two-fold, three-fold and four-fold symmetry axes, and it is significant that the recently determined amino acid sequence of human and rat ferritin (Addison *et al.* 1983) show strong homology in these areas. The difficulty of determining the mechanism of iron uptake and release may well be due to the simultaneous operation of chemically distinct mechanisms. The availability of the molecular structure now enables us to design specific experiments for the solution of this problem.

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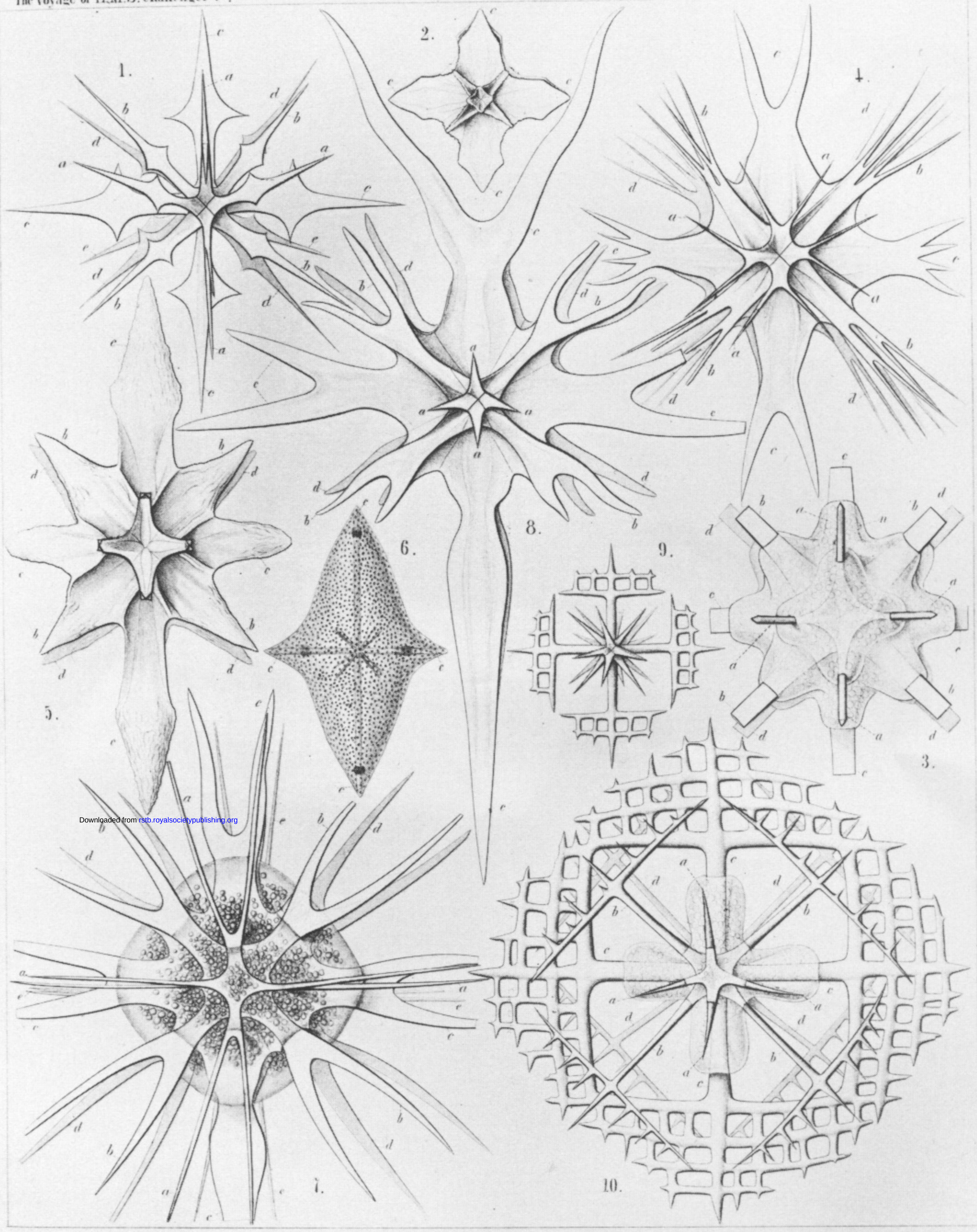

1–3. QUADRILONCHE. 4–6. BELONOSTAURUS. 7. 8. LONCHOSTAURUS.
9. 10. LITHOPTERA.

IRON OXIDES, ICE

Class Agantharia. Plate of various species from the report on the Radiolaria collected by H.M.S. Challenger during the years 1873–1876 (Haeckel, E. 1887 Rept. Sci. Res. of the Voy. of H.M.S. Challenger. *Zoologist* **18**).

These common planktonic radiolarian-like animals have skeletons made of celestite, each spine consisting of a single rhomboidal crystal. Sea water is undersaturated with respect to $SrSO_4$ and the skeletons dissolve rapidly after the death of the animal. This is an example of a less usual mineral, not described in the symposium.