# Iron (II) oxidation and early intermediates of iron-core formation in recombinant human H-chain ferritin

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The paper describes a study of Fe(II) oxidation and the formation of Fe(III)–apoferritin complexes in recombinant human H-chain ferritin and its variants. The effects of site-directed changes in the conserved residues associated with a proposed ferroxidase centre have been investigated. A change in any of these residues is shown to reduce the rate of Fe(II) oxidation, confirming the importance of the ferroxidase centre in the catalysis of Fe(II) oxidation. Mössbauer and u.v.-difference spectroscopy show that in the wild-type protein Fe(II) oxidation gives rise to Fe(III) monomers, dimers and larger clusters. The formation of Fe(III) $\mu$-oxo-bridged dimers occurs at the ferroxidase centre and is associated with fast oxidation: in three variants in which Fe(II) oxidation is especially slow, no Fe(III) dimers are seen. Within the time scale 0.5–20 min in wild-type human H-chain ferritin, dimer formation precedes that of the monomer and the progression dimer→monomer→cluster is observed, although not to completion. In a preliminary investigation of oxidation intermediates using a stopped-flow instrument, an Fe(III)–tyrosine complex reported by Waldo et al. (1993), is attributed to Tyr-34, a residue at the ferroxidase centre. The Fe(III)–Tyr-34 complex, forms in 0.5 s and then decays, as dimer absorbance increases. The relationship between Fe(III)–tyrosinate and the formation of Fe(III) dimers is uncertain.

## INTRODUCTION

Iron is stored in animals, plants and bacteria as ferritin, a molecule with a large, roughly spherical 8 nm diameter iron-storage cavity encompassed by a protein shell of 2.5 nm thickness and composed of 24 polypeptide chains (Theil, 1990; Harrison et al., 1991). Two distinct types of ferritin have been isolated from *Escherichia coli* (Andrews et al., 1991; Hudson et al., 1994). Both answer to this general description, but one, known as bacterioferritin (BFR) contains haem as well as non-haem iron, whereas the other, FTN, does not. BFR and FTN exhibit only 14% identity of amino-acid sequence (Andrews et al., 1991, 1992). Such distinct types of ferritin molecule have not been found in mammals, but two different subunit types are combined within the same molecule (Arosio et al., 1978). These subunits, known as H and L, show only 55% amino-acid-sequence identity, although members of H- and L-subunit types from different mammals (e.g. humans, rats, mice) show respectively about 90 or 85% identity (see Andrews et al., 1992 for collected ferritin amino-acid sequences). In humans, H-rich ferritins are associated with heart and brain, whereas L-rich ferritins are typical of liver and spleen (Arosio et al., 1978; Dedman et al., 1992). Liver and spleen are major iron-storage tissues and it is suggested that L-rich ferritins are associated with long-term iron storage and H-rich with iron detoxification (Cozzi et al., 1990). Known sequences of ferritins from invertebrates, plants and bacteria are all more similar to mammalian H chains than to L chains (Andrews et al., 1992).

Previously it has been shown that ferritin sequesters iron efficiently only if it is presented as Fe(II) in the presence of oxygen, and that ferritin catalyses Fe(II) oxidation (Niederer, 1970; Macara et al. 1972, 1973; Bryce and Crichton, 1973; Bauminger et al., 1991). This catalysis is associated with H chains (Levi et al., 1988, 1992; Lawson et al., 1989, 1991). Fe(II) added, at pH 7, to L-chain homopolymers (produced by recombinant technology), is oxidized and sequestered as ferrihydrite, but at a rate that is only slightly greater than that of protein-free controls (Levi et al., 1989; Wade et al., 1991). Determination of the site and mechanism of catalysis of Fe(II) oxidation by ferritin H chains and of the functional role of L chains is of great interest. L chains appear to confer greater physical stability on assembled ferritin molecules than do H chains and this is associated, at least in part, with an L-chain-specific salt bridge (Santambrogio et al., 1992). It has also been proposed that L chains are superior in ferrihydrite nucleation and that this can be explained by differences in the distribution of amino-acid residues on their cavity surfaces, especially those with metal-binding potential (e.g. carboxy groups) (Wade et al., 1991; Levi et al., 1992). This paper is concerned with understanding the molecular basis for the 'ferroxidase' activity of H chains and with delineating the steps between the initial Fe(II) oxidation and the nucleation and growth of iron cores.

The conformation of both H and L chains in ferritin molecules is a four-$\alpha$-helix bundle (Ford et al., 1984; Lawson et al., 1991) and these folded subunits assemble into a highly symmetrical shell. H chains, but not L chains, contain a metal-binding site within the bundle (Lawson et al., 1991) and this site has been associated with the observed ferroxidase activity (Lawson et al., 1989; Bauminger et al., 1991; Treffry et al., 1992). Seven residues at the ferroxidase centre of H chains are also highly conserved in H-type ferritins from a wide range of species (Andrews et al., 1992) and ferroxidase activity has been confirmed in several of

Abbreviations used: BFR, bacterioferritin; FTN, ferritin from *E. coli*; CdM, recombinant human H-chain ferritin; HoL, horse L ferritin; $t_f$, freezing time; FeY, FeA and FeB, Fe atom bound at sites Y, A and B respectively.

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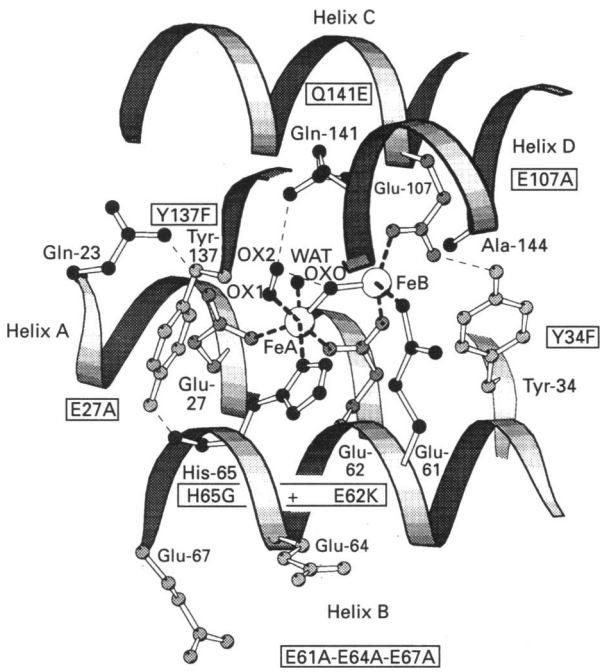


**Figure 1 Schematic diagram of ferroxidase-centre region in human H-chain ferritin**

The ferroxidase centre is modelled with an Fe–O–Fe dimer in place. Ligands of Fe at site A are Glu-27 and His-65 and ligands of Fe at site B are Glu-107 and Glu-61, with Glu-62 acting as a bridging ligand of both Fe atoms. A dioxygen molecule, bound to Fe A, is also modelled. In Treffry et al. (1992) it was suggested that Fe(II) bound at both sites B and A, linked through a hydroxy bridge, may be oxidized by this dioxygen. Glu-64 and Glu-67 are situated on the cavity surface of the apoferritin shell and Glu-61 can take up an alternative position within the cavity. Its movement may thus assist the migration of Fe B into the cavity for iron-core formation. The amino acid substitutions that have been made for this investigation of Fe(II) oxidation and its products are shown boxed. Figures 1 and 10 were prepared using the program 'Molscript' (Kraulis, 1991).

these ferritins. The seven residues are Glu-27, Tyr-34, Glu-61, Glu-62, His-65, Glu-107 and Gln-141. In a previous paper (Bauminger et al., 1991) a human H-chain variant bearing two amino-acid changes, Glu-62→Lys and His-65→Gly was examined by Mössbauer spectroscopy (Lys-62 and Gly-65 are found in L chains). The catalysis of Fe(II) oxidation, characteristic of wild-type H ferritin, was not seen with this variant. Moreover production of an Fe(III) $\mu$-oxo-bridged dimer, seen in both horse spleen ferritin (Bauminger et al., 1989) and in wild-type human H-chain ferritin (Bauminger et al., 1991), occurred neither with this variant nor with protein-free controls. It was concluded, from this work and from examination of u.v.-difference spectra, that Fe(III) dimers are an early product of Fe(II) oxidation, possibly produced by the oxidation of two Fe(II) atoms by a single oxygen molecule (Treffry et al., 1992). A diagram of the ferroxidase centre modelled with an iron dimer in place, essentially as proposed by Treffry et al. (1992), is shown in Figure 1. The drawing displays the conserved seven residues and the immediate neighbours of the proposed centre including two residues, Glu-64 and Glu-67, that lie on the cavity surface. Glu-61, depicted as a ligand of iron at site B in Figure 1, can occupy an alternative position on the cavity surface where, with Glu-64, it can bind a $Tb^{3+}$ ion (Lawson et al., 1991). It has been suggested that these carboxyls, which are found in many ferritins, may participate in ferrihydrite nucleation and that Glu-61 could also assist in the movement of Fe(III) from site B into the cavity (Lawson et al., 1991; Bauminger et al., 1991; Wade et al., 1991; Levi et al., 1992).

In the present study the effects of several sequence changes on the rates of Fe(II) oxidation are determined, and the time-dependence of the distribution of Fe(III) species formed (monomer, dimer, cluster) after oxidation is examined by Mössbauer and u.v.-difference spectroscopy at various pH values and Fe/protein ratios. Amino-acid substitutions have been made mainly in the ferroxidase-centre region as shown in Figure 1. Results are discussed in terms of molecular mechanisms and in relation to other published data.

During the completion of the study of Fe(II) oxidation and Fe(III)-dimer formation with wild-type human H-chain ferritin and its ferroxidase-centre variants, a report of a new Fe(III) complex in bullfrog H-chain ferritin was published (Waldo et al., 1993). This is a transient species absorbing at 550 nm which was assigned as an Fe(III)–tyrosinate. It was observed by adding oxygen to an anaerobic mixture of apoferritin and Fe(II). A preliminary examination of the formation of this complex has now been made with recombinant human H-chain ferritin (CdM) and three of its variants. Here Fe(II) is added aerobically to the apoferritin in a stopped-flow instrument. The relationship between the production of the 550 nm-absorbing species and Fe(III) dimers is discussed.

## MATERIALS AND METHODS

### Ferritins and variants

Site-directed mutagenesis, overexpression in *Escherichia coli* and purification of recombinant human H-chain ferritins were performed as described in Treffry et al. (1989). Iron removal from the recombinant ferritins was done using sodium dithionite according to the method of Bauminger et al. (1991). Recombinant horse L ferritin was a gift from Drs. K. Nagayama and S. Ebina (Nagayama Protein Array Project, Tsukuba, Japan).

The human chain variants used have the following amino-acid changes; Glu-27→Ala (E27A); Tyr-34→Phe (Y34F), Glu-61, -64, -67→Ala (E61A-E64A-E67A); Glu-62→Lys + His-65→Gly (E62K-H65G): Glu-62→Lys + His-65→Gly + Glu-61, -64, -67→Ala (E62K-H65G-E61A-E64A-E67A); Glu-107→Ala (E107A): Glu-134→Ala (E134A); Tyr-137→Phe (Y137F); Gln-141→Glu (Q141E). CdM was used as control. This ferritin has a single amino-acid-residue change on the outside of the molecular surface, namely Lys-86→Gln (K86Q), compared with wild type. This change, which was introduced to enable crystallization (Lawson et al., 1991), is without effect on Fe(II) oxidation. With the exception of the third variant listed above, all the variants have the change K86Q in addition to those described. Single letter codes are used to describe the variants, such that the first letter is the wild-type residue and the last letter represents the new residue that has been introduced at the numbered sequence position or positions. Variant E134A has a single substitution in each subunit such that at the threefold intersubunit channels three Glu-134 metal ion ligands are missing. Horse L ferritin (HoL) has four residues at the ferroxidase centre which differ from those of CdM: Tyr-27, Lys-62, Gly-65 and Glu-141.

### Iron incorporation

Iron incorporation was measured as the disappearance of Fe(II) using a discontinuous assay. Ammonium ferrous sulphate (Aldrich, U.K.; 99.997% pure) at a final concentration of 0.1 mM, was added to 0.2 $\mu$M ferritin in 0.1 M Mops buffer (pH 6.5 or

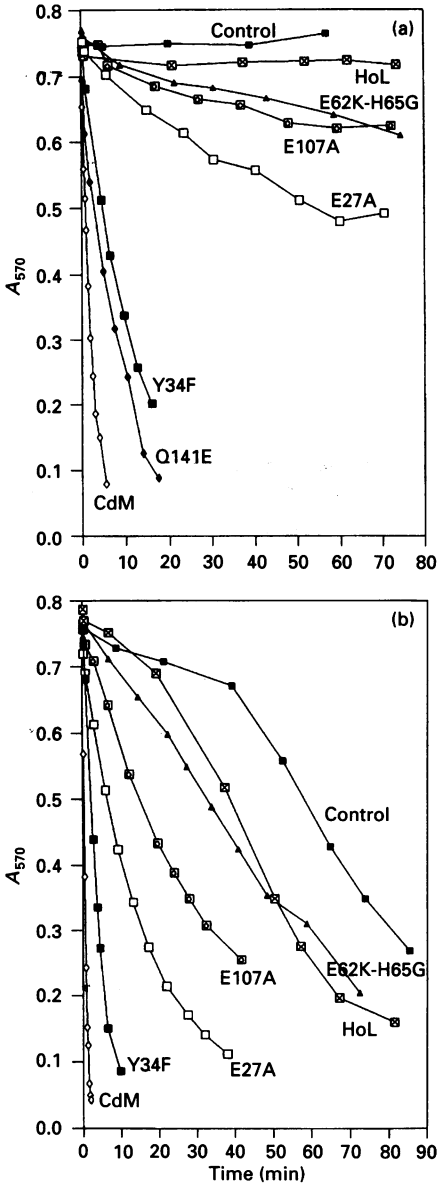


**Figure 2 Iron incorporation into apoferritin variants**

Iron incorporation was followed by a discontinuous assay in which $(NH_4)_2Fe(SO_4)_2$, final concn. 0.1 mM, was added to 0.2 $\mu$M apoferritin (500 Fe atoms/molecule) at either pH 6.5 (**a**), or pH 7.0 (**b**). Fe(II) not incorporated into ferritin was measured as its ferrozine complex. The control contained no protein.

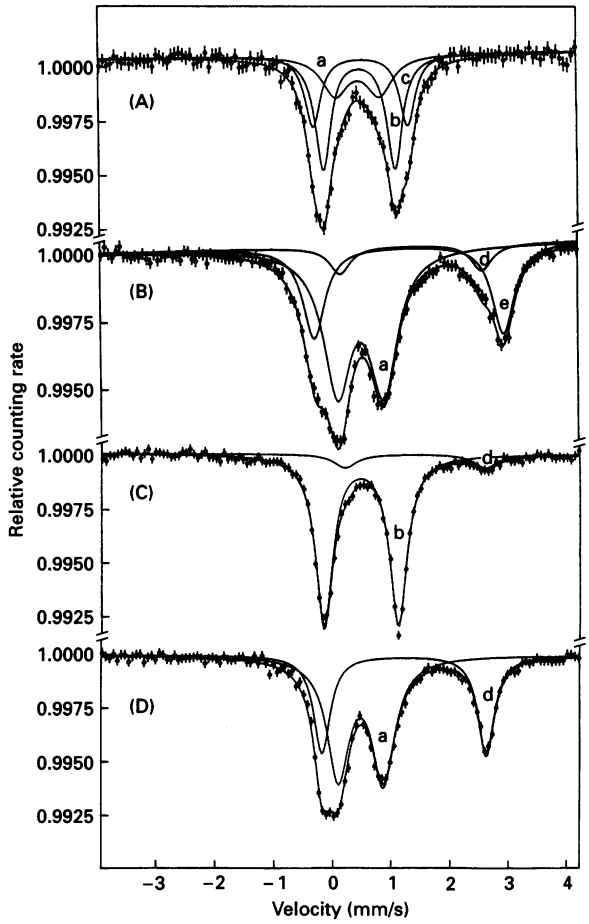


**Figure 3 Mössbauer spectra obtained at 90 K with some recombinant ferritins loaded with 34 $^{57}$Fe atoms/apoferritin molecule at pH 7.0**

Samples A, B and C were frozen at 1 min and sample D at 30 min after Fe(II) addition in air. The various subspectra observed in the computer fits are as follows: (A) (CdM) shows Fe(III) dimers (subspectra b and c) and some Fe(III) clusters (a); (B) (E27A) shows Fe(II) 1 and 2 (d and e) and Fe(III) clusters (a); (C) (Y34F) shows Fe(III) dimer (b) and some Fe(II) (d); (D) (E107A) shows Fe(II) (d) and Fe(III) clusters (a) – note the presence of Fe(II) even after 30 min with this variant. The areas under the various subspectra are proportional to the amount of iron present in that species. The percentages of each species present are shown in Table 2.

7.0). Free Fe(II) was measured as its ferrozine complex by transferring, at intervals, 0.1 ml of the reaction solution into wells of a microtitre plate containing 10 $\mu$l of 5 mM ferrozine (Sigma, Poole, Dorset, U.K.) and 0.1 ml of $H_2O$. The microtitre plate was read at 570 nm using a plate reader (Anthus Reader 2001, Labetech Int., Uckfield, U.K.).

### U.v.-difference spectroscopy

Spectra were recorded using a Cary 1 spectrometer. Spectra were recorded with 40 Fe atoms/apoferritin molecule (6.7 mg/ml protein, 0.536 mM Fe, 0.1 M Mops buffer, pH 7.0) and 12 Fe atoms/apoferritin [11 mg/ml protein, 0.264 mM Fe, in either 0.1 M Mes buffer (pH 5.5), Mops buffer (pH 6.5) or Hepes buffer (pH 7.5)]. A baseline was recorded with protein solutions in 1 mm light-path cuvettes in both sample and blank compartments. $H_2O$ (0.5 $\mu$l) was then added to the blank and 0.5 $\mu$l of Fe(II) solution added to the sample to give the required final concentration. The spectrometer was set to record repeated spectra at 1 or 5 min intervals.

### Mössbauer spectroscopy

Studies were carried out using similar conditions of protein and iron as used for the u.v.-difference spectroscopic studies. $^{57}FeSO_4$ solutions were prepared as described in Bauminger et al. (1989). $^{57}FeSO_4$ was added to apoferritin to give solutions as follows: for 34 Fe atoms/molecule the 0.49 ml samples contained 6.7 mg/ml apoferritin and 0.446 mM $^{57}FeSO_4$ in 0.1 M Mops buffer, pH 7.0; for 10 Fe atoms/molecule the 0.97 ml samples contained 11 mg/ml apoferritin and 0.22 mM $^{57}FeSO_4$ in 0.1 M Mes buffer

**Table 1 Mössbauer parameters for different iron species**

The numbers in parentheses are the errors in the last digits. Abbreviations: LW, full line-width at half maximum of peak height; $H_{eff.}$, effective magnetic field.

| Species | Temperature (K) | LW (mm/s) | Quadrupole splitting (mm/s) | Isomer shift (mm/s) | $H_{eff.}$ (kOe) | Relaxation time (ns) |
|---|---|---|---|---|---|---|
| (a) Fe(III) clusters | 90 | 0.50 (3) | 0.75 (2) | 0.49 (2) | – | – |
| (b) Fe(III) dimer 1 | 90, 4.1 | 0.30 (2) | 1.23 (1) | 0.50 (1) | – | – |
| (c) Fe(III) dimer 2 | 90, 4.1 | 0.30 (2) | 1.61 (2) | 0.53 (1) | – | – |
| (d) Fe(II) 1 | 90, 4.1 | 0.38 (4) | 2.78 (2) | 1.21 (1) | – | – |
| (e) Fe(II) 2 | 90, 4.1 | 0.42 (2) | 3.26 (1) | 1.31 (1) | – | – |
| (f) Fe(III) monomers | 90, 4.1 | 0.5 | – | 0.51 | 550 | 7 |
| (g) Magnetic Fe(III) | 4.1 | 0.44 | – | 0.48 (2) | 460 (5) | – |

**Table 2 Relative amounts of different iron species in recombinant human H-chain ferritin (CdM) and its site-directed variants obtained by Mössbauer spectroscopy after adding 34 $^{57}$Fe(II) atoms/molecule at pH 7.0**

To all samples (6.7 mg/ml apoferritin in 0.1 M Mops buffer, pH 7.0) $^{57}FeSO_4$ was added to give 34 Fe atoms/molecule. Abbreviations: $t_f$, freezing time; n.d., not determined. The freezing time was the time (in min) after Fe addition before samples were frozen. Errors in the percentage monomers are about ±7% and in the other species ±3%. The figures in the last column represent the percentage of cluster iron found as large magnetically ordered clusters.

| Sample | $t_f$ (min) | %Fe(II) species | | %Fe(III) species | | | | Magnetic clusters |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | Dimer 1 | Dimer 2 | Monomer | Clusters | |
| CdM | 1 | – | – | 43 | 25 | 7 | 26 | 13 |
| E27A | 1 | 11 | 28 | – | – | 0 | 61 | 33 |
| E27A | 20 | – | – | – | – | 5 | 95 | 70 |
| Y34F | 1 | 8 | – | 63 | – | 29 | – | n.d. |
| E107A | 30 | 35 | – | – | – | 6 | 59 | 49 |
| E107A | 60 | 30 | – | – | – | 0 | 70 | 58 |
| E107A | 150 | 21 | – | – | – | 9 | 70 | n.d. |
| E62K-H65G-E61A-E64A-E67A | 10 | 15 | 50 | – | – | 3 | 32 | 24 |
| E62K-H65G-E61A-E64A-E67A | 80 | 5 | 8 | – | – | 11 | 76 | n.d. |

(pH 5.5), 0.1 M Mops buffer (pH 6.5), or 0.1 M Hepes buffer (pH 7.5). The solutions were placed in lucite holders and frozen in liquid nitrogen at specified times after iron addition. $^{57}$Fe-Mössbauer spectra were obtained and analysed as described previously (Bauminger et al., 1991).

### Stopped-flow measurements

Reactions were carried out in 0.1 M Mops buffer at pH 6.5 with final concentrations of apoferritin and ammonium ferrous sulphate of 4 $\mu$M and 96 $\mu$M respectively. Measurements were made at fixed wavelengths in an Applied Photophysics (Leatherhead, Surrey, U.K.) stopped-flow instrument.

## RESULTS

### Time course of Fe(II) disappearance measured by colorimetric assay

Figures 2(a) and 2(b) show progress curves for the oxidation of 100 $\mu$M Fe(II) [500 Fe(II) atoms/apoferritin molecule] assayed discontinuously as loss of Fe(II) measured as its ferrozine complex. At the concentration of Fe(II) used [0.1 mM Fe(II)] no significant loss of Fe(II) (or oxidation) occurs in the absence of protein over the first 40 min at pH 6.5 (Figure 2a) and not more than approx. 10% at pH 7.0 (Figure 2b). At pH 7.0 auto-oxidation becomes quite rapid after 40 min. With the exception of HoL, for which little loss of Fe(II) is seen over 70 min at pH 6.5, Fe(II) disappears more rapidly from all the protein samples than from the control. It may be concluded that this is due to the catalysis of Fe(II) oxidation by the protein (which has previously been established) and the sequestering of the Fe(III) as ferrihydrite. With the human H-chain-ferritin variants Q141E, Y34F, E27A, E107A and E62K-H65G the rate of oxidation is reduced, or considerably reduced, compared with that in CdM; Q141E and Y34F being the least affected. Note that, with all of these variants, at least one of the ferroxidase-centre ligands has been altered. In contrast, with variant E134A, in which one of the threefold channel residues has been changed, the rate of oxidation is very little different from that of CdM (Treffry et al., 1993). Substitution of both threefold channel residues D131 and E134 by either histidine or alanine does give rise to a decreased rate of oxidation, but this decrease is considerably less marked than that of E27A or E107A (Treffry et al., 1993). These experiments therefore show that fast oxidation, as measured by Fe(II) loss, is associated with the ferroxidase centre (Figure 1), confirming the conclusion from measurements with a single variant E62K-H65G (Bauminger et al., 1991), and, furthermore, that an intact centre is required for full activity.

### Fe(II) oxidation and Fe(III) intermediates seen by Mössbauer spectroscopy

Figures 3(A)–3(D) show examples of Mössbauer spectra obtained

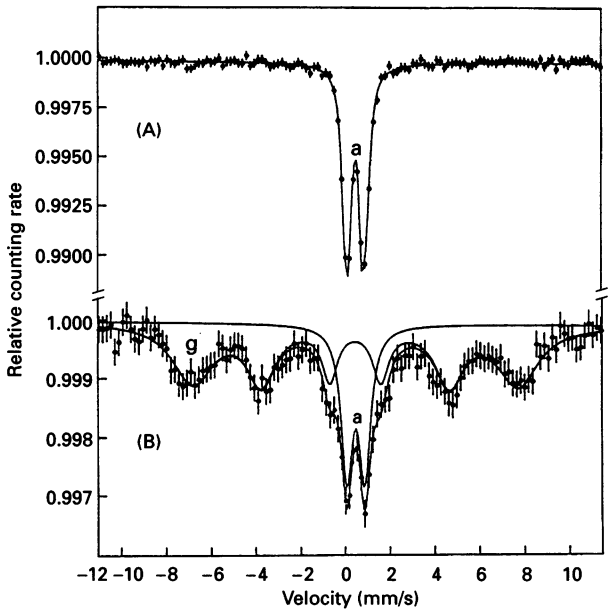


**Figure 4 Mössbauer spectra at two temperatures of variant E27A loaded with 34 $^{57}$Fe atoms/molecule at pH 7.0**

The sample was frozen 20 min after addition of $^{57}FeSO_4$. Conditions are as described in Table 2. Note the expanded velocity scale compared with that of Figure 3. The upper spectrum (A) was obtained at 90 K and the lower spectrum (B) at 4.1 K. The central doublet in A is due to Fe(III) clusters (subspectra a). In B the relative intensity of this doublet is diminished, due to the larger clusters giving a magnetic sextet, g, at 4.1 K.

with some of the ferroxidase-centre variants used in the Fe(II)-loss experiments [but with 34 Fe(II) atoms/molecule], together with computer fitted correlations. Table 1 lists Mössbauer parameters of the Fe(II) and Fe(III) species observed. The

**Table 3 Relative amounts of different iron species in recombinant human H-chain ferritin and its site-directed variants obtained by Mössbauer spectroscopy after adding 10 $^{57}$Fe(II) atoms/molecule at pH 5.5 or 6.5**

All samples were prepared in 0.1 M Mes buffer (pH 5.5) except for E107A, which was in 0.1 M Mops buffer, pH 6.5. Apoferritin concn. was 11 mg/ml. Samples were frozen at $t_f$ min after addition of $^{57}FeSO_4$ (10 Fe atoms/molecule). The numbers in parentheses are the errors in the last digits as obtained from the computer fits to the spectra. Abbreviation: $t_f$, freezing time.

| Sample | $t_f$ (min) | %Fe(II) | %Fe(III) species | | | |
|---|---|---|---|---|---|---|
| | | 1 | Dimer 1 | Dimer 2 | Monomers | Clusters |
| CdM | 30 | – | 15 (3) | 9 (1) | 40 (10) | 36 (4) |
| E27A | 30 | – | – | 9 (2) | 51 (6) | 40 (4) |
| Y34F | 30 | 5 (1) | 17 (3) | – | 63 (5) | 15 (3) |
| E107A | 170 | 37 (4) | – | – | 36 (10) | 27 (4) |

computer fitted correlations indicate the presence of two types of Fe(II) (Table 1, d and e) and two types of Fe(III) dimers (Table 1, b and c), each with slightly different parameters, in some of the recombinant ferritins. Table 2 gives the percentage of each species observed.

Table 2 shows that complete Fe(II) oxidation has occurred within 1 min only with CdM. The relative rates of oxidation are CdM > Y34F > E27A > E62K-H65G-E61A-E64A-E67A > E107A. Oxidation is complete with E27A within 20 min of Fe(II) addition and it is almost complete with E62K-H65G-E61A-E64A-E67A at 80 min. However, even at 150 min 21% of the iron is still unoxidized with E107A. Note that the relative rates of Fe(II) oxidation are similar to those of Fe(II) loss measured colorimetrically, in agreement with the assumption that the latter is due to oxidation to Fe(III). However, in the experiment shown in Table 2 only 34 Fe(II) atoms were added per apoferritin molecule.

The distribution of Fe(III) species is notable. CdM shows the

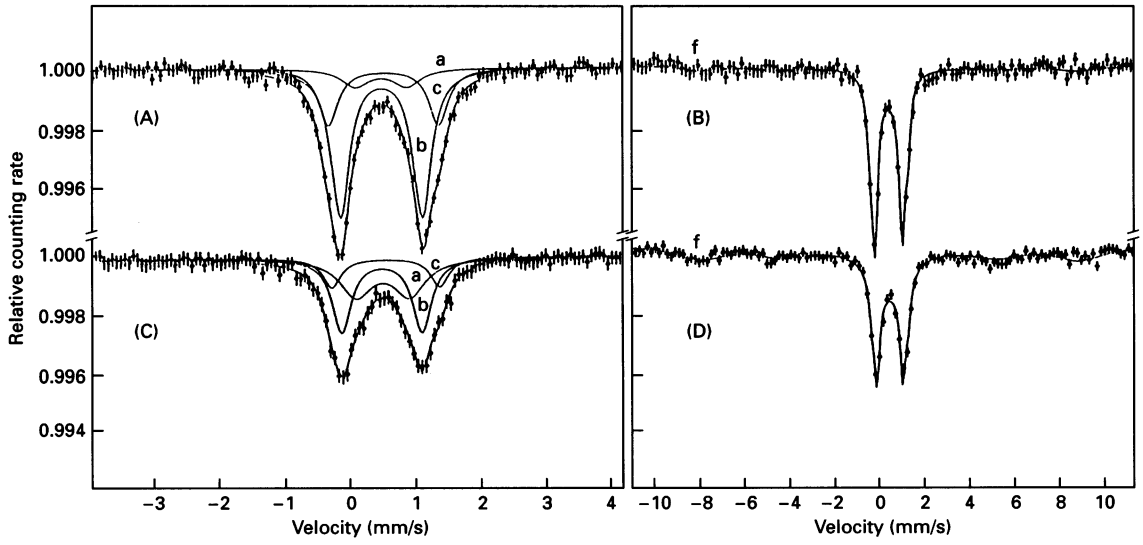


**Figure 5 Mössbauer spectra (at 90 K) of human H-chain ferritin, CdM, loaded with 10 $^{57}$Fe atoms/apoferritin molecule at pH 6.5**

Spectra A and B were obtained with samples frozen at 0.5 min and spectra C and D 10 min after addition of $^{57}FeSO_4$ to apoferritin (11 mg/ml) in 0.1 M Mops buffer, pH 6.5. Note the changes in the relative proportions of the subspectra with time. A and C show Fe(III) clusters (a), and dimers 1 (b) and 2 (c). B and D, which are on an expanded velocity scale, show, in addition to the central doublet spectra, a relaxation sextet due to isolated Fe(III) atoms [Fe(III) monomer, f].

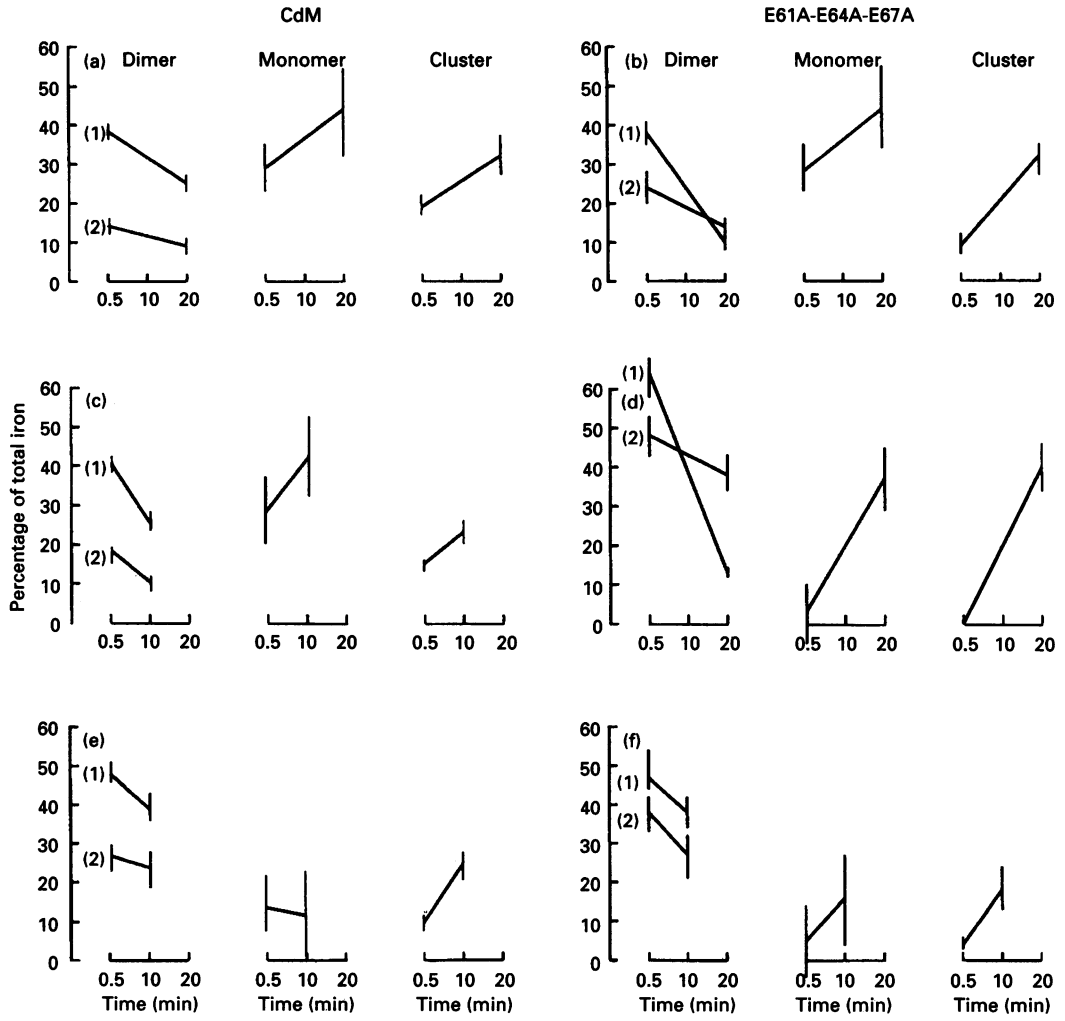


**Figure 6 Changes in percentages of Fe(III) species with time at three pH values**

The percentages of iron as Fe(III) dimer, monomer and cluster were determined by Mössbauer spectroscopy. $^{57}FeSO_4$ (10 $^{57}Fe$ atoms/molecule) was added to apoferritin (11 mg/ml) in 0.1 M Mes buffer, pH 5.5 (**a** and **b**), or 0.1 M Mops buffer, pH 6.5 (**c** and **d**), or Hepes buffer, pH 7.5 (**e** and **f**). Samples were frozen at the times indicated. Samples on left-hand side were CdM, those on right-hand side were E61A-E64A-E67A. Note the decrease in dimers and increase in monomers and clusters with time (up to 20 min).

presence of two Fe(III) dimers with distinct parameters and together these are major species (68%) at 1 min after Fe(II) addition. The three samples with the lowest oxidation rate show no significant $\mu$-oxo-bridged Fe(III) dimer.

Variant Y34F shows only one type of dimer (designated dimer 1) but the total dimer percentage in Y34F and CdM is about the same. Levels of Fe(III) monomer are very low in all samples at the earliest times examined, with the exception of Y34F, in which this monomer corresponds to 29% of the iron. However, in the case of Y34F there is no significant Fe(III) present as clusters at 1 min, whereas, with E27A at 1 min, 61% of the iron is in Fe(III) clusters even though 39% is still as Fe(II). Similarly with E107A at 30 min, 59% of the iron is in Fe(III) clusters although 35% remains unoxidized.

The iron clusters in ferritin show the phenomenon of superparamagnetism. Only clusters which are larger than a critical size, yield a magnetic sextet in Mössbauer spectra at 4.1 K (Mørup et al., 1980). As an example, spectra obtained in one of the samples at 90 K and 4.1 K are shown in Figure 4, where the magnetic sextet 'g' is observed at 4.1 K. Comparison of E27A at 1 min with E107A at 30 min, both of which have about 60% of iron in clusters, suggests that though oxidation is slow and clusters form more slowly in E107A, once they form, they tend to be larger. In E27A, with 60% of the iron in clusters, only about 50% of them yield magnetic spectra, whereas in E107A with 60% of the iron in clusters and 35% still unoxidized, more than 80% of the clusters are magnetic at 4.1 K. By 20 min, however, E27A has about 100% cluster iron, with more than 70% of these as large clusters, indicating that clusters have increased in size over this period at a rate comparable with those forming in E107A.

Mössbauer spectra were also obtained for samples containing only 10 Fe(II) atoms/molecule, either at pH 5.5 (CdM, E27A and Y34F) or pH 6.5 (E107A). The percentages of the various species calculated from computer fitted correlations are given in Table 3. The data show again that Fe(II) oxidation with E107A is extremely slow, and also that at pH 5.5 the percentage oxidation with Y34F is less than with E27A. Under the conditions of Table 3, Fe(III) monomers form a relatively large, and dimers a relatively small, proportion of the product.

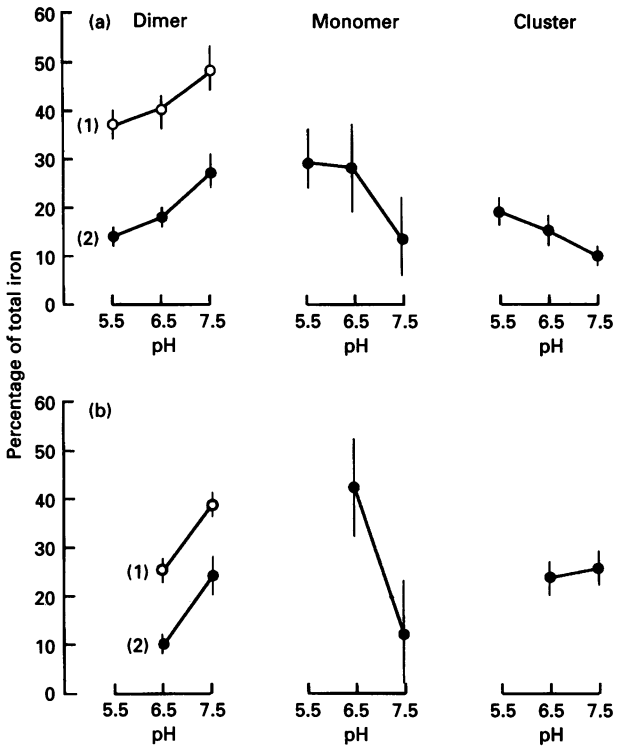


**Figure 7 Effect of pH on the distribution of Fe(III) species in CdM**

The percentages of iron as Fe(III), dimer, monomer and cluster, were determined by Mössbauer spectroscopy. Conditions were as described for Figure 6. Samples were frozen either at 0.5 min (**a**) or 10 min (**b**). Note the increase in levels of dimer and decrease in monomers with increasing pH.

## Mössbauer spectroscopic study of two recombinant ferritins as a function of time and pH

Mössbauer spectra (see examples in Figure 5) were obtained for two recombinant ferritins, CdM and E61A-E64A-E67A at 0.5 min and 10 or 20 min after the addition of 10 Fe(II) atoms/apoferritin molecule at three pH values, 5.5, 6.5 and 7.5. At 0.5 min, CdM samples (Figures 5A–D) all showed 100% oxidation and E61A-E64A-E67A showed 96%. No pH dependence of the percentage oxidation was apparent for this amount of added iron. The distributions of Fe(III) species were both time- and pH-dependent, however.

Figures 6(a) and 6(b) show the percentage of Fe(III) dimers, monomers and clusters plotted as a function of time at the three pH values both for CdM and for the variant E61A-E64A-E67A. The same trends are evident in both recombinant proteins: over the first 10–30 min both types of dimer, (1) and (2), decrease and monomer and cluster levels increase. The results indicate that Fe(III) dimers are the precursors of monomer species over this short time period. In principle, clusters could have gained Fe(III) directly from dimer as well as monomer, although the sequence dimer→monomer→ cluster is indicated by the data. With E61A-E64A-E67A at pH 5.5 and CdM at pH 5.5 and pH 6.5, up to 30% of the iron is in monomer form even at 0.5 min. Thus either the sequence Fe(II)→Fe(III) dimer→Fe(III) monomer is very rapid, or some or all of this monomer is formed independently of dimer. However, in the case of E61A-E64A-E67A at pH 6.5 and pH 7.5, little Fe(III) monomer is visible at 0.5 min, although it increases to sizeable amounts by 10–20 min. This implies that, in this case at least, all or virtually all of the Fe(III) monomer results from dimer dissociation, and it is possible that Fe(III) dimer is the precursor of all the monomeric Fe(III) species seen here. It is not clear from the data whether there is an interconversion of dimer forms before dissociation, or whether Fe(III) monomer arises from both types of dimer independently.

When examined as a function of pH, levels of dimers 1 and 2 increase and monomers decrease with both recombinant proteins, e.g. CdM (Figure 7). The percentage of clusters was relatively less at the higher pH values when measured at 0.5 min, but increased slightly with pH at 10 or 20 min. This suggests a delicate balance between the early intermediates, but with dimers being stabilized at the expense of monomers (their products) at higher pH values.

## Results of u.v.-difference and Mössbauer spectroscopy compared

It has previously been found (Treffry and Harrison, 1984; Treffry et al., 1992) that u.v.-difference spectra, measured after the addition in air of Fe(II) to recombinant apoferritins, change with time. The first spectrum seen at 1 min after addition of 4 Fe(II) to E61A-E64A-E67A or CdM, at pH 6.5, showed shoulders at approx. 310 and 350 nm and was considered to represent mainly Fe(III) dimer (Treffry et al., 1992). At later times, this spectrum diminished, and an absorbance with maximum at about 290 nm appeared, which was assigned as representing or mainly representing an increase in monomeric Fe(III). In contrast, spectra thought to represent Fe(III) clusters are almost featureless (Treffry and Harrison, 1984). These assignments are now confirmed. Figures 8(a)–8(f) show u.v.-difference spectra and the various species obtained by Mössbauer spectroscopy under similar conditions are indicated alongside them. Clearly the spectra are complex but the information obtained from them, although not quantitative, broadly corresponds to that from Mössbauer spectroscopy. Investigated by u.v.-difference spectroscopy only, variant Y137F is indistinguishable from CdM (results not shown) and unlike Y34F with which the Fe(II) oxidation rate is low compared with that in CdM. The positions of Tyr-137 and Tyr-34 can be compared in Figure 1.

## Production of an Fe(III) species absorbing at 550 nm

Figure 9 shows that when Fe(II) is added to CdM in the presence of air [24 Fe(II)/apoferritin molecule] a species absorbing at 550 nm is produced. The absorbance value reaches its maximum at approx. 0.5 s and then decays over approx. 5 s (Figure 9b). During this period, species absorbing at 360 nm continuously increase, reaching a maximum absorbance at approx. 5 s (Figure 9a). In contrast, no species absorbing at 550 nm is observed in Y34F (see Figure 9b).

With this variant the absorbance at 360 nm increases much more slowly than with CdM (Figure 9a). These results strongly suggest that the tyrosinate→Fe(III) charge transfer band (550 nm absorbance) of Waldo et al. (1993) is due to Tyr-34, a ferroxidase-centre residue. However, this absorbing species is also absent from variants E27A and Q141E (results not shown), coupling its formation not just to Tyr-34, but to the presence of an intact ferroxidase centre. The other variants have not yet been examined in a stopped-flow instrument.

The absorbance at 360 nm may have contributions from Fe(III) monomers, dimers and possibly clusters, with dimers likely to be the most important species in CdM and Y34F (see Figure 8). The transient nature of the Fe(III)–tyrosinate absorbance means that it does not contribute significantly to the Fe(III) species seen by

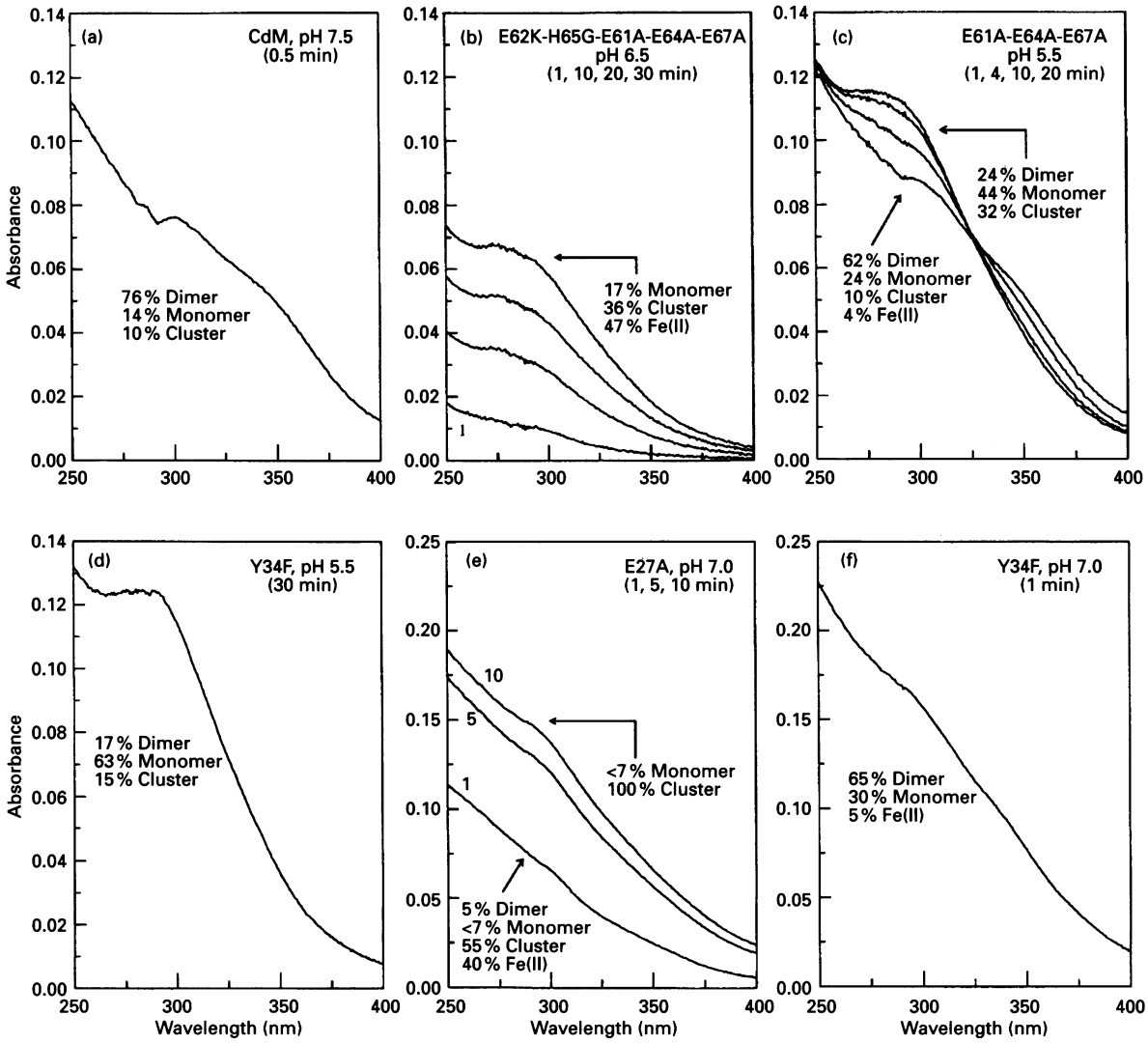


**Figure 8 Comparison of u.v.-difference spectra and iron species found by Mössbauer spectroscopy**

U.v.-difference were recorded and Mössbauer spectra obtained and analysed under similar conditions (see the Materials and methods section) except that in (**a**–**d**) 12 Fe atoms/apoferritin molecule were added to the samples for u.v.-difference spectroscopy and 10 Fe atoms/molecule for the Mössbauer spectra (22 mM apoferritin) and in (**e**) and (**f**) the corresponding figures were 40 Fe and 34 Fe/molecule respectively (13.5 mM apoferritin). U.v.-difference spectra were recorded at the times indicated and the percentages of the various Fe species measured by Mössbauer spectroscopy are indicated.

Mössbauer or u.v.-difference spectroscopy, as these could be observed only at 30 s after addition of Fe(II) to the protein or at later times.

## DISCUSSION

The results described here show that the high Fe(II) oxidation rate associated with ferritin H chains requires the presence of residues Glu-107, Glu-27, Gln-141 and Tyr-34, and they confirm the conclusion from previous work (Bauminger et al., 1991) that Glu-62, His-65 and probably Glu-61 are also required for full activity. These seven residues are situated at the ferroxidase centre as described in Andrews et al. (1991) and Treffry et al. (1992), Figure 1. The association of the catalysis of Fe(II) oxidation with this centre is thus abundantly confirmed and it is also evident that all seven residues are required for full activity, Glu-107 evidently being the most critical. This does not preclude the possible importance of other residues near the active centre. For example residue 144 is always either alanine or serine (i.e. a small side chain) in H chains of eukaryotes. Substitution of this residue by leucine (the residue found in L chains) seems to block both the passageway connecting the ferroxidase centre and the iron-storage cavity that can be seen in the three-dimensional structure of human H-chain ferritin (S. J. Yewdall and P. M. Harrison, unpublished work), and also the proposed Fe–tyrosinate (see below). The effects of changing this residue have not yet been investigated experimentally.

The second point of importance arising from the experimental results presented here is that the conclusion of Bauminger et al. (1991), based on studies with variant E62K-H65G, that fast oxidation is associated with dimer formation (at the ferroxidase centre) is confirmed. With E107A, in which the rate of oxidation is by far the lowest of any variant, no dimeric Fe(III) is found, and again, with E27A, little or no dimer is seen. The relatively

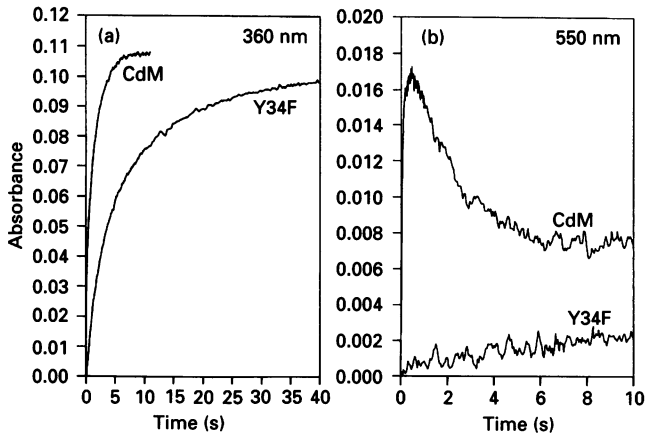


**Figure 9 Changes in absorbance at 360 nm and 550 nm recorded in a stopped-flow instrument after addition of Fe(II) in air to human H-chain apoferritin**

$(NH_4)_2Fe(SO_4)_2$ solution (final concn. 96 $\mu$M) was added to apoferritin (4 $\mu$M) in 0.1 M Mops buffer, pH 6.5 and absorbance measured (**a**) at 360 nm and (**b**) at 550 nm. Note the appearance of a transient species absorbing at 550 nm in CdM, but not in Y34F and the slower increase in 360 nm absorbance in Y34F.

high percentage of Fe(III) monomer in E27A at pH 5.5 (see Table 3), compared with that at pH 7.0 (see Table 2) could, in principle, have arisen either by breakdown of an unstable dimer or independently of dimer formation. Dimer is also absent from E62K-H65G-E61A-E64A-E67A, Table 2, and from recombinant human L-chain ferritin (results not shown), in both of which the rate of Fe(II) oxidation is low. Fe(III) dimer is seen in Y34F (although of only one type, namely type 1). The data of Figures 2 and 3 show that over a time period of 20 min with CdM and E61A-E64A-E67A, levels of Fe(III) dimer decrease and those of monomer increase, implying that dimer is a precursor of monomer as had been deduced by Treffry et al. (1992) purely from u.v.-difference spectra. Levels of both dimers 1 and 2 are slightly enhanced at higher pH values and monomer level diminished, consistent with a precursor–product relationship. However, some monomer can apparently form independently of dimer, e.g. in E107A over a long period of time (150 min).

Clusters are also augmented with time, Figure 2, [and monomers were seen to decrease over longer periods in horse spleen ferritin (Bauminger et al., 1989], so the relationship dimers→monomers→clusters in a period after the first 30 s is indicated for intact ferritin. Evidence is presented elsewhere (Treffry et al., 1993) that some of the monomer Fe(III) migrates to the threefold channels. However, one of the Fe(III) atoms of the dimer may migrate by a different route into the cavity to form the core. It is not clear what the significance is of the two dimer types observed by Mössbauer spectroscopy. They could possibly represent either $\mu$-oxo and $\mu$-hydroxo-bridged forms or correspond to slightly different structural arrangements (or both). It is apparent from the data in Tables 2 and 3 that very little core is formed in Y34F under the conditions shown. Tyr-34 is buried and cannot participate directly in nucleation, however.

When stable iron-core particles have formed, a second mechanism of Fe(II) oxidation is available: direct oxidation on the surface of the inorganic ferrihydrite complex (Macara et al., 1972). Kinetic analysis of Fe(II) oxidation indicates that, with horse spleen ferritin, this mechanism becomes significant when more than 50 Fe(II) atoms/apoferritin molecule are being oxidized (Sun and Chasteen, 1992). In variants E107A, E27A and E62K-H65G-E61A-E64A-E67A at pH 7.0, Table 2, no significant dimer and little, if any, monomer Fe(III) is seen. Nevertheless clusters do form and presumably grow by direct oxidation on core nuclei. Indeed, for the growth of clusters, lack of dimer sites seems to be an advantage. This can be seen for example in the data for E27A and CdM at 1 min, there being nearly three times as much iron in clusters with the variant, even though oxidation is incomplete. Moreover, with E27A at 20 min, 95% of the Fe(III) is in clusters. Under comparable conditions (Bauminger et al., 1991) with CdM, clusters accounted for only 58% of the Fe(III) even at 24 h. Again in contrast with CdM, E62K-H65G-E61A-E64A-E67A showed 76% Fe(III) in clusters at 80 min, Table 2, and recombinant human L-chain ferritin gave 100% clusters at 30 min (E. R. Bauminger, P. M. Harrison, D. Hechel, A. Treffry, S. Levi and P. Arosio, unpublished work). Thus for core formation, in contrast with rapid Fe(II) oxidation, it may be disadvantageous to have a large proportion of H chains, because their dimer centres compete with cavity sites for iron, at least in the early stages of core formation. A relatively low percentage of H chains may be adequate to oxidize any flux of Fe(II) encountered under physiological conditions. Results shown in Figure 6 and Table 2 bear out the conclusion of Bauminger et al. (1991) that the cavity carboxylates Glu-61, Glu-64 and Glu-67 are not essential for core formation [Glu-61 in an alternative position is also a dimer ligand (Lawson et al., 1991; Treffry et al., 1992)]. However, Figure 6 shows a slightly higher percentage of Fe(III) in clusters at 0.5 min in CdM than in E61A-E64A-E67A, suggesting that possession of one or more of these carboxylates (possibly E61) may promote nucleation to some extent.

The data obtained by colorimetric assay with 500 Fe/molecule, Figure 2, show that Fe(II) oxidation with E107A proceeds only slightly faster than in the protein-free controls. In a previous experiment (Bauminger et al., 1991), under conditions similar to those of Table 2 (except for the absence of protein), oxidation in the protein-free control was complete in under an hour. With E107A, oxidation has not gone to completion (about seven Fe(II)/molecule remained out of 34 added) even at 150 min, and with E62K-H62G-E61A-E64A-E67A about three Fe(II)/molecule remained at 80 min. This implies that this iron is somehow protected from oxidation, presumably by binding to apoferritin. There is no indication, however, (from the results of Figure 2) that any of the Fe(II) added to apoferritin variants was unavailable to ferrozine, except as a result of oxidation. This conclusion is also supported by the finding of Watt et al. (1988) and Artymiuk et al. (1991) that Fe(II) within the ferritin molecule was available to both bipyridine and *o*-phenanthroline. In contrast Yablonski and Thiel (1992) and Rohrer et al. (1989) propose that Fe(II) sequestered inside the protein shell becomes inaccessible to *o*-phenanthroline.

The results described above provide a picture of events starting from about 30 s after the addition of Fe(II) to apoferritin and its variants, the experimental 'deadtime' for the aerobic Mössbauer and u.v.-spectroscopy measurements. The mechanism of Fe(II) oxidation and dimer formation within the first 30 s remains to be determined. The results of Figure 9, taken together with those of Figure 2 and Table 2, show that production of the 550 nm-absorbing Fe(III)–tyrosinate species is associated with fast Fe(II) oxidation, as is Fe(III) dimer formation. The Fe(III)–tyrosinate appears rapidly and its decay over the first 5–10 s after Fe(II) addition is paralleled by the rise in the absorbance at 360 nm, probably due mainly, within this short time span, to dimer formation. Results from Figure 9 and Table 2 indicate that although dimer formation is slower in Y34F than in CdM it does occur. However, whereas with CdM both dimers are found at

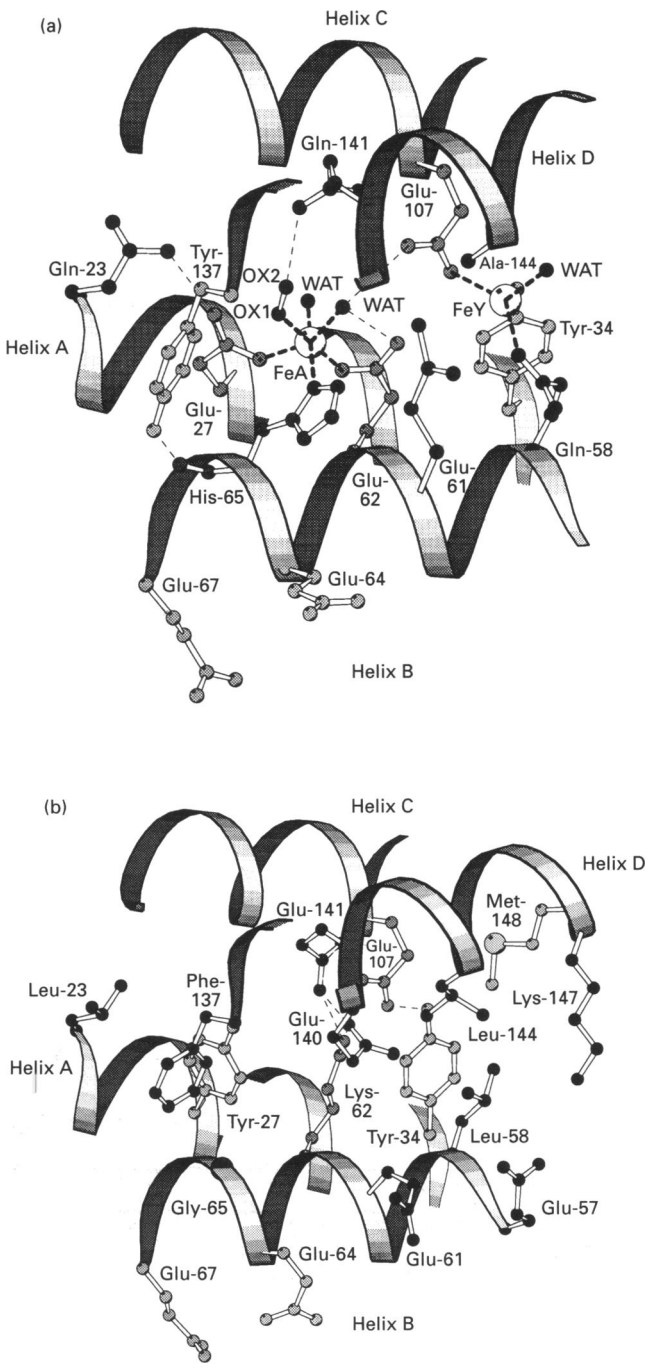


**Figure 10 Schematic diagrams of the ferroxidase-centre region of human H-chain ferritin (a), compared with the equivalent region in horse L-chain ferritin (b)**

Diagram (**a**) shows an Fe atom at site A, linked via a water molecule to Glu-107, as has been found for $Ca^{2+}$ and $Tb^{3+}$ by X-ray analysis (Lawson et al., 1991). It also shows a modelled position of an Fe atom bound to Tyr-34 and Glu-107 (residues conserved in ferritin H chains) with a third potential ligand, Gln-58. One possibility is that an $O_2$ molecule bound to Fe A and hydrogen bonded to Gln-141 (as in Figure 1) might oxidize Fe(II) at Y as well as A. Fe(III) may then migrate from site Y to site B (see Figure 1), so that an Fe(III) $\mu$-oxo-bridged dimer is formed. (**b**) In horse L-chain ferritin sites A and Y are blocked, A and B by a salt bridge and Y by hydrophobic residues.

1 min, only dimer 1 is seen with Y34F, see Table 2. Thus Tyr-34 is not a ligand of Fe(III) in dimer 1, but whether, or not, it is a ligand of dimer 2 is not obvious. One possibility is that production of dimer 2 is dependent on the presence of Tyr-34 and that it is normally a precursor of dimer 1.

Examination of the three-dimensional structure of human H-chain ferritin around Tyr-34 shows that this residue is rather tightly wedged and also that Glu-107 has relatively little room for movement. However, there is space for an iron atom to be bound by Tyr-34 and Glu-107, as shown in Figure 10(a) [FeY (Fe atom bound at site Y)]. A third protein ligand could be Gln-58. This residue is not conserved in all H chains, although it is present in bullfrog ferritin H chain (Andrews et al., 1992). Figure 10(b) provides an explanation of why, although both Tyr-34 and Glu-107 are present in ferritin L chains, no Tyr→Fe(III) charge transfer band is seen (Waldo et al., 1993): site Fe Y is blocked by Leu-144 and Glu-58 is also replaced by a leucine residue. Figure 10(b) also shows the salt bridge between Lys-62 and Glu-107 that prevents iron binding at sites A and B in mammalian L chains and in variant E62K-H65G-E61A-E64A-E67A. In tadpole L ferritin Glu-62 and His-65 are both present, but Glu-27 and Glu-107 are replaced by lysine and glutamine residues respectively (Andrews et al., 1992). The distances between FeY and FeA and FeB in H chains, Figure 10(a) and Figure 1 [the modelled dimer Fe(III) positions] are respectively 0.72 nm (7.2 Å) and 0.42 nm (4.2 Å). Clearly in H chains Fe Y could not form an alternative dimer with FeA [the distance between Fe atoms linked by a $\mu$-oxo-bridge being not more than about 0.36 nm (3.6 Å) (Que and True, 1990)]. Although FeB and FeY might perhaps be brought closer together, examination of the structure suggests that FeA-O-FeB is a more satisfactory dimer. Moreover, E27A gives little or no dimer, consistent with its proposed role as a dimer ligand as in Figure 1 and Figure 10(a). The role of Glu-107 as a ligand of both tyrosinate and dimer Fe(III) (site B) explains its importance for fast Fe(II) oxidation.

An explanation is needed as to why replacement of Glu-27 or Gln-141 causes loss of the Tyr-34→Fe(III) species. One possibility is that these residues are linked, as modelled in Figure 10(a), when two Fe atoms are bound at the centre in sites A and Y. However, other schemes should also be considered and both the mechanism of Fe(II) oxidation and the relationship between the tyrosine–Fe(III) species and the Fe(III) dimers remain to be clarified.

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