# A Possible Role for the Conserved Trimer Interface of Ferritin in Iron Incorporation†

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ABSTRACT: Ferritin is a large protein, highly conserved among higher eukaryotes, which reversibly stores iron as a mineral of hydrated ferric oxide. Twenty-four polypeptides assemble to form a hollow coat with the mineral inside. Multiple steps occur in iron core formation. First, $Fe^{2+}$ enters the protein. Then, several alternate paths may be followed which include oxidation at site(s) on the protein, oxidation on the core surface, and mineralization. Sequence variations occur among ferritin subunits which are classified as H or L; $Fe^{2+}$ oxidation at sites on the protein appears to be H-subunit-specific or protein-specific. Other steps of ferritin core formation are likely to involve conserved sites in ferritins. Since incorporation of $Fe^{2+}$ into the protein must precede any of the other steps in core formation, it may involve sites conserved among the various ferritin proteins. In this study, accessibility of $Fe^{2+}$ to 1,10-phenanthroline, previously shown to be inaccessible to $Fe^{2+}$ inside ferritin, was used to measure $Fe^{2+}$ incorporation in two different ferritins under various conditions. Horse spleen ferritin (L/H = 10–20:1) and sheep spleen ferritin (L/H = 1:1.6) were compared. The results showed that iron incorporation measured as inaccessibility of $Fe^{2+}$ to 1,10-phenanthroline increased with pH. The effect was the same for both proteins, indicating that a step in iron core formation common among ferritins was being measured. Conserved sites previously proposed for different steps in ferritin core formation are at the interfaces of pairs and trios of subunits. Dinitrophenol cross-links, which modify pairs of subunits and affect iron oxidation, had no effect on $Fe^{2+}$ incorporation. In contrast, zinc, which has two conserved sites at the trimer interface, D131/131′/131″ and E134/134′/134″, inhibited $Fe^{2+}$ incorporation equally in both types of proteins. Thus, the first step of iron core formation in ferritin, the passage of $Fe^{2+}$ into the protein, appears to involve conserved carboxylates at the trimer interface. The results also suggest that the effect of ferritin subunit differences is exerted at a step in core formation after the entry of $Fe^{2+}$ into the protein.

Ferritin is a complex, multisubunit protein which stores iron for normal metabolism as well as for detoxification (Theil, 1987, 1990; Harrison & Lilley, 1990). The iron core of ferritin varies in size (up to 4500 iron atoms), order, and composition (Theil, 1990; Harrison & Lilley, 1990). Formation of the mineral core involves a series of steps, only partly understood, that includes incorporation of $Fe^{2+}$, oxidation on the protein, nucleation, oxidation on the iron core, and mineralization. *The path taken by the $Fe^{2+}$ after passage through the ferritin protein coat can vary.* For example, when the dioxygen/iron ratio concentration is relatively low, $Fe^{2+}$ can be incorporated and then stabilized inside the protein for many hours (Rohrer et al., 1989, 1990). When the dioxygen/iron ratio is high, oxidation is rapid (Bauminger et al., 1989; Hanna et al., 1991a,b; Xu & Chasteen, 1991). Evidence for small numbers of $Fe^{2+}$ and $Fe^{3+}$ bound to the protein has been obtained by EXAFS, Mossbauer, and EPR spectroscopy [e.g., Chasteen and Theil (1982), Yang et al. (1987), Bauminger et al. (1989), and Chasteen et al. (1985)]. In addition, binding sites for a number of other metal ions, e.g., $Tb^{3+}$, $Zn^{2+}$, and $UO_2^{-}$, have been identified in the three-dimensional structures of two types of ferritins (Harrison & Lilley, 1990; Lawson et al., 1991). Among $Fe^{3+}$–oxo species identified during core formation are monomeric dimers and multinuclear forms attached to the protein. $Fe^{2+}$ can also be oxidized on $Fe^{3+}$–oxo multimers and on the bulk mineral. After $Fe^{2+}$ incorporation, no single, exclusive path for $Fe^{2+}$ to the mineral has been established (Rohrer et al., 1987, 1989; Bauminger et al., 1989; Xu & Chasteen, 1991; Chasteen et al., 1986; Frankel et al., 1987; Wade et al., 1991). Moreover, various types of ferritin mineral are produced [reviewed in Theil and Sayers (1990); see also Mann et al. (1987), Rohrer et al. (1990), and Mackle et al. (1991)].

The 24 polypeptide subunits of ferritin, ~20 kDa each, are assembled through noncovalent interactions to form a hollow sphere or protein coat; subunit types fall into two classes H and L.[1] The hydrated ferric oxide mineral forms reversibly inside the protein coat. Sites of primary and quaternary structural conservation include subunit trimer interactions and subunit dimer interactions (Banyard et al. 1978; Harrison & Lilley, 1990). A major function of the protein is orienting the growth of the iron mineral *inside* the protein coat. The protein sites responsible for the several steps in iron core formation are not completely defined, but modification of a $Tb^{3+}$ site on one type of subunit has been associated with changes in oxidation rate on the protein (Lawson et al., 1989). The different types of ferritin subunits are encoded by a multigene family ($n > 2$–3) which is expressed differentially in various cell or tissue types, at least in animals [e.g., Dickey et al. (1987)]. Differential expression of ferritin subunits can be dramatic and altered by medically significant factors. Thus, much attention has been focused on the functional differences

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[1] H (higher or heavy) and L (lower or lighter) sometime relate to the electrophoretic mobility in denaturing gels and to mass, but a number of exceptions exist [reviewed in Theil (1990)]. Sequence comparison provides a more consistent classification.

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between subunit types. $Fe^{2+}$ oxidation can occur on the protein or on the mineral or both. To date, subunit-specific differences relate to oxidation on the protein [e.g., Levi et al. (1988, 1989), and Lawson et al. (1989, 1991)]. However, conserved subunit sites should also be important in various steps of iron core formation.

Among the several steps defined in ferritin iron core formation, incorporation of $Fe^{2+}$ into the protein is the earliest and could be a step that occurs at a site conserved regardless of the subunit heterogeneity of the coat protein. To investigate the possibility that iron incorporation is at a site that is conserved regardless of variation in coat structure and function, inaccessibility of $Fe^{2+}$ to 1,10-phenanthroline was compared in this study for ferritin in protein coats with naturally different subunit composition and from different species; $Fe^{2+}$ inside ferritin was previously shown to be inaccessible to 1,10-phenanthroline (Rohrer et al., 1989). The advantage of using the Fe(II)–1,10-phenanthroline complex is the well-known stoichiometric relationship between absorbance and iron concentration. Earlier studies used changes in the visible absorbance (310–420 nm) that occur when the ferritin iron core forms. However, later it was shown (Treffry & Harrison, 1984) that the absorbance change is not always proportional to iron incorporated. In addition, the rate of absorbance change can vary considerably among different ferritins [e.g., Levi et al. (1988, 1989)] and, thus, involves sites which are not conserved in all ferritins. The proteins studied were from the spleen of sheep and horse. Horse spleen ferritin is predominantly L subunit (H/L = 1:10–20), and sheep spleen ferritin is a 1:1 mixture (H/L = 1:0.6); physiologically, the subunit difference may relate to development or iron content.[2]

## MATERIALS AND METHODS

Sheep spleen ferritin was isolated as previously described (Mertz & Theil, 1983; McKenzie et al., 1989), and horse spleen ferritin was obtained from Boehringer. Iron was removed from ferritin by using thioglycollic acid as previously described (Chasteen & Theil, 1982), and the protein coats were used within a week of preparation. The subunit composition of horse spleen ferritin has been determined from SDS gel electrophoresis and amino acid sequence (Heuterspreute & Crichton, 1981). The subunit composition of sheep spleen ferritin has been analyzed by SDS gel electrophoresis, mRNA hybridization using H and L ferritin subunit probes, and partial peptide sequencing (McKenzie et al., 1989). Intramolecular, covalent cross-linking of pairs of ferritin subunits in sheep spleen ferritin (H/L = 1:0.6) were introduced with 1,5-difluoro-2,4-dinitrobenzene as previously described (Mertz & Theil, 1983; McKenzie et al., 1989); 28–30% of the subunits were linked into pairs. The presence of substantial amounts of H subunits was required to limit cross-linking to subunit pairs, since in horse spleen ferritin (H/L = 1:10–20) the reaction could not be stopped before completion (all subunits cross-linked) (McKenzie et al., 1989).

All reagents were purchased from Fisher Scientific or Sigma with the following exceptions: Hepes (Boehringer-Mannheim); SDS, Chelex 100, and acrylamide (Bio-Rad); Sepharose 6B (Pharmacia); $FeSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$ (Mallinkrodt); Commassie brilliant blue and ammonium sulfate (ICN). Solutions with standard concentrations of iron were from Fisher.

Iron incorporation was determined as the difference between the amount of iron outside the protein and the total iron added. Iron outside the protein was measured by diluting aliquots of the reaction mixture in 0.01 N HCl (to stop oxidation and to release any iron bound to acidic residues on the outer protein surface), adding 1,10-phenanthroline, and measuring the Fe(II)–phenanthroline complex at 510 nm (after adjusting the pH to 5.5). Earlier studies showed that the assembled protein was stable under such conditions and that the $Fe^{2+}$ or $Fe^{3+}$ inside the protein was inaccessible to 1,10-phenanthroline (Rohrer et al., 1989). Iron analysis of ultrafiltrates, from which the protein had been removed, agreed (within 10%) with the values determined by complexation with 1,10-phenanthroline. Measurement of total $Fe^{2+}$ added, determined at $t = 0$, was used as zero incorporation and was always within 10% of that predicted by weight. Moreover, the difference between total Fe (added $Fe^{2+}$ plus $Fe^{3+}$) and $Fe^{2+}$ was less than 3% at $t = 0$. Reactions to measure iron incorporation were initiated by adding freshly prepared solutions of $FeSO_4 \cdot 7H_2O$ to solutions of ferritin protein coats in 0.15 M Hepes·Na at pH = 6.1, 6.5, or 7.0. When used, zinc was added 10 min before the iron. The iron/protein ratio was 480 Fe/molecule (20 Fe/subunit), and the zinc/protein ratio was 240. The reaction volume was 0.5 mL, and the temperature was controlled at 28 °C. The iron concentration was 2.5 mM, and the protein concentration was 0.125 mM in subunits (2.5 mg/mL). Under the conditions used (2.5 mM), dioxygen concentration was not rate limiting, measured indirectly as $\Delta A_{420nm}$; from 0.04 to 2.5 mM, the rate was proportional to iron concentrations. However, at 5 and 10 mM iron, $\Delta A_{420}$ was disproportionately low, suggesting oxygen limitation at the higher iron concentrations. All experiments were repeated 3–7 times, and at least two different independently isolated samples of each type of protein were analyzed.

## RESULTS AND DISCUSSION

*Iron Incorporation into Different Ferritins at Various Values of pH.* During ferritin iron core formation, monoatomic $Fe^{2+}$ in solution outside the protein is converted to solid ferric mineral inside the protein. The path taken by incorporated $Fe^{2+}$ to the mineral can vary [e.g., Rohrer et al. (1987, 1989), Bauminger et al. (1989), Xu and Chasteen (1991), Theil and Sayers (1991), and Wade et al. (1991)]. However, all $Fe^{2+}$ atoms that become part of the mineral core had to be incorporated by the ferritin protein coat, regardless of the path later taken or of the ferritin type. Thus, $Fe^{2+}$ incorporation is likely to represent a step in iron core formation involving conserved sites in ferritin. Inaccessibility of $Fe^{2+}$ to 1,10-phenanthroline was selected as an assay for sites of $Fe^{2+}$ incorporation since it is inaccessible to $Fe^{2+}$ inside the protein coat [see Materials and Methods and Rohrer et al. (1989)]. In addition, the stoichiometry between the iron concentration and absorbance of the complex is well known in contrast to measurements of the absorbance of the Fe–apo-ferritin complex (Treffry & Harrison, 1984).

Comparing $Fe^{2+}$ incorporation for two different ferritin protein coats over a range of pH values provides wide resolution of differential protein effects for the following reasons. First, formation of ferritin iron cores is affected by pH; ~2.5 $H^+$ are released for every Fe atom deposited in the mineral (Spiro & Saltman, 1965). Second, many metal protein interactions are influenced by pH, exemplified by the transferrin–Fe interaction (Harris & Aisen, 1990). Finally, protein-specific

[2] Horse and sheep tissue are usually obtained from animals at different stages of development. Horse tissue is usually from a much older animal than sheep tissue. There is some evidence that L ferritin subunits increase with iron load, which itself increases with age [see discussion in Theil (1990)], which can explain the difference in subunit composition of horse spleen and sheep spleen ferritin.

differences were detected in the past using a range of values of pH and monitoring the change in absorbance at 310–420 nm, an early, indirect measure of oxidation on protein sites [e.g., Lawson et al. (1989, 1991), Levi et al. (1989), and Wade et al. (1991)]. Thus, if $Fe^{2+}$ incorporation (inaccessiblity to chelators) varies with pH, but is independent of protein type, sites common among ferritin protein type are likely to be involved.

$Fe^{2+}$ incorporation was clearly influenced by pH (Figure 1A). For example, after 1 h at pH 7.0, 83–86% of the $Fe^{2+}$ was incorporated into ferritin, whereas only 57–58% was incorporated at pH 6.1. [Preliminary experiments showed that unincorporated $Fe^{2+}$ could be recovered in protein-free ultrafiltrates (see Materials and Methods)]. The more rapid rate of iron incorporation at pH = 7 compared to 6.1 suggests that the driving force for incorporation is oxidation or precipitation/mineralization, or both.

The type of ferritin protein coats [horse spleen ferritin (H/L = 1:10–20) and sheep spleen ferritin (H/L = 1:0)] studied did not change the incorporation of $Fe^{2+}$ (Figure 1), regardless of the value of the pH. No significant differences ($P < 0.01$) were observed in $Fe^{2+}$ incorporation between the two types of ferritin protein coats. In earlier studies, using a less-defined assay (Treffry & Harrison, 1984), ferritin proteins with different subunit composition functioned differently (oxidation on the protein was measured as $\Delta A_{310}$ at pH 6.1), an analysis which is not necessarily related stoichiometrically to the number of iron atoms oxidized or incorporated (Treffry & Harrison, 1984; Rohrer et al., 1987). Ferritins poor in H subunits, e.g., horse spleen ferritin, had a low $\Delta A_{310}$ at pH 6.1 (Levi et al., 1988, 1989; Lawson et al., 1989, 1990; Wade et al., 1991). In the present study, comparison of $\Delta A_{420nm}$ for sheep spleen and horse spleen ferritin protein coats also showed that the protein samples used to measure $Fe^{2+}$ incorporation in Figure 1 do behave differently in the UV–vis absorbance assay ($\Delta A_{420}$). For example, at pH = 6 the initial $\Delta A_{420}$ was 0.186 ± 0.079 for horse spleen ferritin and 0.546 ± 0.161 for sheep spleen ferritin.[3] Although the ferritin protein coat with more H-type subunits (sheep spleen ferritin) had a faster $\Delta A_{420}$ at pH 6.1, Fe incorporation (inaccessibility to 1,10-phenanthroline) was the same as for ferritin rich in L subunits (Figure 1A).

*Inhibition of Iron Incorporation into Different Ferritins by Zinc.* To help characterize the ferritin protein sites that influence passage of $Fe^{2+}$ into the protein, inhibition of iron incorporation by zinc was studied; inaccessibility to 1,10-phenanthroline was used as a measure. Zinc was selected because in horse spleen ferritin two types of zinc sites are formed by amino acids conserved in all ferritin subunits known: D131, D131′, D131″[4] E134, E134′, and E134″; the sites were identified by X-ray diffraction in isomorphous crystals of horse spleen ferritin (Harrison & Lilley, 1990; P. Harrison, personal communication). [A third type of zinc site in horse spleen ferritin is composed of residues that are not conserved in all ferritins (Harrison & Lilley, 1990).] Since zinc inhibits ferritin iron mineralization (core size) in vivo (Coleman & Matrone, 1969), the zinc–protein interaction(s) appear to have physiological relevance. $Fe^{2+}$ incorporation into ferritin was inhibited by zinc at all three values of pH tested (Figure 1B). The extent of inhibition was the same for both protein coats (Figure 1B). For example, the percent incorporation of $Fe^{2+}$ after 1 h at pH 6.1 was 57.9 ± 14% for sheep spleen ferritin without zinc and 31.1 ± 2.8% with zinc.

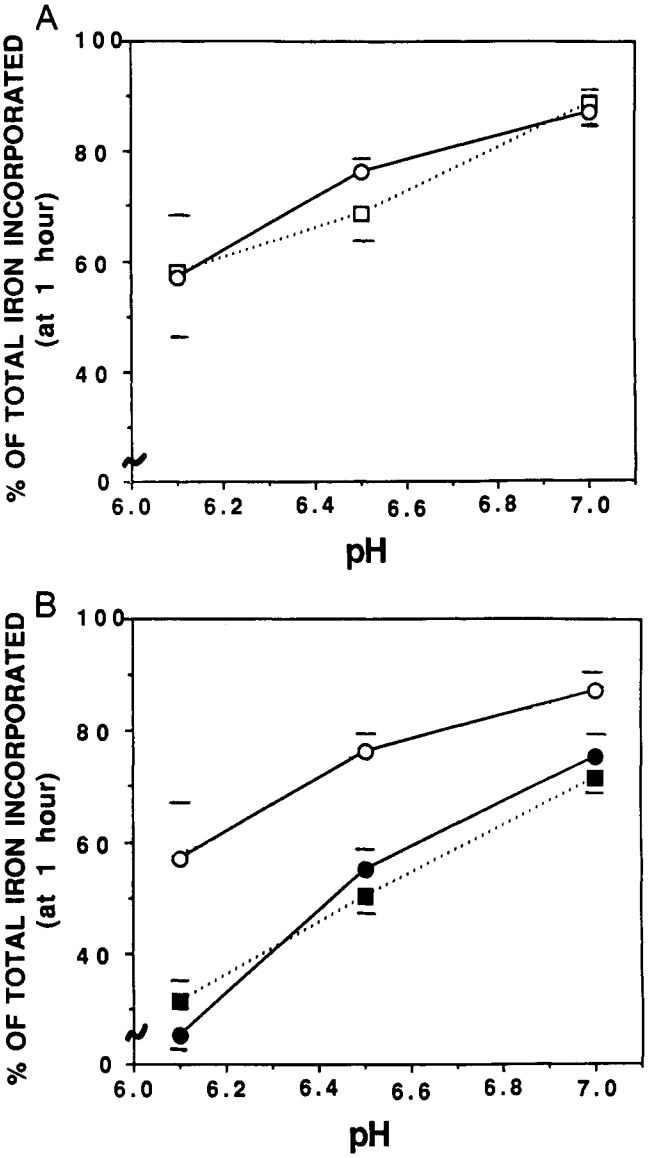


FIGURE 1: (Panel A) Effect of pH on $Fe^{2+}$ incorporation (inaccessibility of $Fe^{2+}$ to 1,10-phenanthroline) in different ferritins. $Fe^{2+}$ incorporation into ferritin protein coats was measured as described under Materials and Methods; the Fe/protein = 480; Fe = 2.5 mM; buffer = 0.15 M Hepes. The similarity of the effect of pH for both ferritins suggests that sites(s) conserved in ferritins are involved in $Fe^{2+}$ incorporation. The results are the average of three independent experiments, and the standard deviation is shown as a horizontal line either above or below each point. Protein coats from horse spleen, L/H = 10–20:1(O–O); protein coats from sheep spleen, L/H = 1:1.6 (□- - -□). (Panel B) Inhibition of $Fe^{2+}$ incorporation by zinc in different ferritins. Zinc was added to 240/protein 10 min before adding iron. (See Materials and Methods.) The results are the average of three independent experiments, and the standard deviation is shown as a horizontal line either above or below each point. Results with zinc are significantly different from those without zinc ($P < 0.01$). (●–●) horse spleen ferritin + zinc; (■- - -■) sheep spleen ferritin + zinc; horse spleen ferritin (no zinc) (O–O) from panel A for comparison. [$Fe^{2+}$ incorporation (no zinc) is the same in both ferritins ($P > 99.99$).]

[3] At pH = 7, the initial $\Delta A_{420}$ was the same for each type of protein: 0.486 ± 0.022 for horse spleen ferritin and 0.556 ± 0.135 for sheep spleen ferritin. Apparently, protein-specific factors predominate in absorbance changes at pH = 6, and generic ferritin features predominate at pH = 7.

[4] The numbering used is that currently favored by most authors and is based on the sequence of the human H subunit [e.g., Levi et al. (1988); Lawson et al. (1989, 1990); and Wade et al. (1991)]. Subtracting four from the numbers will relate them to the older scheme, based on the sequence of horse spleen ferritin (Heuterspreute & Crichton, 1981; Harrison et al., 1987; Harrison & Lilley, 1990). For example, D131 in the current scheme is D127 in the old numbering scheme.

Table I: Modification of Ferritin by Cross-Linking Subunit Pairs Has No Effect on Iron Incorporation (Inaccessibility to 1,10-Phenanthroline), with or without Zinc[a]

| no modification | +XL[b] | +Zn | +Zn + XL[b] |
|---|---|---|---|
| 1.00 | 0.89 ± 0.14 | 0.68 ± 0.03 | 0.65 ± 0.11 |

[a] Values are reported relative to unmodified protein coats from sheep spleen ferritin. Total $Fe^{2+}$ added minus $Fe^{2+}$–1,10-phenanthroline formed after 1 h. For native protein the value was 58.4 ± 12.0%. [b] XL = DNP cross-links between pairs of subunits within the same protein coat (see Materials and Methods). Ferritin protein coats from sheep spleen ferritin, L/H = 1:1.6, with or without DNP cross-links (see Materials and Methods) were reconstituted with 480 Fe/molecule at pH 6.1 without or with zinc (240/molecule). (240 zinc had the same effect as 36 zinc/molecule.) The data are the average of three independent experiments, and the error is presented as the standard deviation. Cross-links decreased $\Delta A_{420}$ by 75%, confirming previous results (Mertz & Theil, 1983; Theil, 1986). In contrast, cross-links had no effect on $Fe^{2+}$ incorporation or the inhibition by zinc emphasizing that $Fe^{2+}$ incorporation is influenced by ferritin at site(s) independent of the subunit dimer interaction.

Similarly, for horse spleen ferritin the percent incorporation was 56.8 ± 11% without zinc and 24.9 ± 5.1% with zinc. Thus, the percent zinc inhibition, calculated from the ratio of $Fe^{2+}$ incorporated with and without zinc, was 44% for horse spleen ferritin and 54% for sheep spleen ferritin at pH 6.1. Such results suggest that a conserved region of the ferritin protein coat which binds zinc (D131 and E134) influences $Fe^{2+}$ incorporation.

D131 and E134 are in a channel through the ferritin protein coat that is formed by the junction of three subunits. The two zinc sites are each formed by three carboxylates; each of the three subunits contributes a carboxylate ligand to each of the two sites (Harrison & Lilley, 1990). Zinc binding in the 3-fold channel could easily impede passage of $Fe^{2+}$.

Conserved carboxylate residues also occur at the dimer interface. To eliminate the possibility that dimers of subunits influenced incorporation of iron into ferritin, interactions between dimers were altered by cross-linking them with 1,5-difluoro-2,4-dinitrophenol. The cross-link had previously been shown to involve peptides at and near the dimer interface (McKenzie et al., 1989). The dinitrophenyl interactions of pairs of cross-links had no effect on iron incorporation (Table I); a later step in iron core formation, measured as $\Delta A_{420nm}$, was changed confirming previous results (Mertz & Theil, 1983; Theil, 1986). Zinc inhibition of $Fe^{2+}$ incorporation was the same whether or not the protein coat was modified by the cross-linking reagent (Table I). The results suggest that modification of the trimer interface (zinc binding) but not of the dimer interaction (DNP cross-links) alters $Fe^{2+}$ incorporation.

Earlier data on the role of the trimer interface in a step in iron core formation has been conflicting. On the one hand, site-directed mutagenesis (D131H,A; E134A; D131H + E134H) in human H-ferritin was first concluded to have no effect (Treffry et al., 1989). On the other hand, chemical modification (alkylation of horse spleen ferritin at C126) was interpreted as showing participation of the trimer channel in oxidation at protein sites or nucleation (Stefanini et al., 1989; Desideri et al., 1991). Both sets of experiments measured ferritin protein function as the rate of change in absorbance. Long-range effects of each type of modification, throughout the large and interdigitating structure of ferritin protein coats, were not fully assessed. In a recent paper, mutations at the trimer interface (D131H and E134A) appeared to affect another step of ferritin core formation measured as core particle size (range and mean) (Wade et al., 1991). In the same paper, the earlier data on iron oxidation (Treffry et al., 1989) were reevaluated to indicate that the altered protein (D131H and E131A) displayed a decreased (50–70%) rate of oxidation.

Two observations (Figure 1, panels A and B) suggest that the trimer interface is indeed involved in $Fe^{2+}$ incorporation, when the analysis of $Fe^{2+}$ incorporation is measured using the Fe(II)–1,10-phenanthroline complex. First, the rate of $Fe^{2+}$ incorporation is independent of the subunit composition of ferritin protein coats [in contrast to $\Delta A_{420nm}$ (Levi et al., 1988, 1989; Lawson et al., 1991)]. Second, zinc, which binds to conserved carboxylate ligands at the trimer interface (Harrison & Lilley, 1990), inhibits $Fe^{2+}$ incorporation (inaccessibility to 1,10-phenanthroline). Although little is known about the three-dimensional structure of the trimer interface in ferritin coats formed from heterologous subunits (X-ray diffraction data are on protein coats of either H or L subunits), the results suggest that three-dimensional structure and function at the trimer interface is conserved and that the structure controls $Fe^{2+}$ entry into the protein. Differences in iron core formation previously observed using other analyses with ferritins with different subunit compositions [e.g., Levi et al. (1988, 1989) and Wade et al. (1991)] appear to depend, therefore, on later steps in iron core formation, beyond the passage of $Fe^{2+}$ into the protein.

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**Registry No.** Fe, 7439-89-6; Zn, 7440-66-6.