Ferritins are nearly ubiquitous iron storage proteins playing a fundamental role in iron metabolism. They are composed of 24 subunits forming a spherical protein shell encompassing a central iron storage cavity. The iron storage mechanism involves the initial binding and subsequent O₂-dependent oxidation of two Fe²⁺ ions located at sites A and B within the highly conserved dinuclear “ferroxidase center” in individual subunits. Unlike animal ferritins and the heme-containing bacterioferritin, the Escherichia coli ferritin possesses an additional iron-binding site (site C) located on the inner surface of the protein shell close to the ferroxidase center. We report the structures of five E. coli ferritin variants and their Fe³⁺ and Zn²⁺ (a redox-stable alternative for Fe⁴⁺) derivatives. Single carboxyl ligand replacements in sites A, B, and C gave unique effects on metal binding, which explain the observed changes in Fe²⁺ ions located at sites A, B, and C within the highly conserved dinuclear “ferroxidase center” in individual subunits. Unlike animal ferritins and the heme-containing bacterioferritin, the E. coli ferritin possesses an additional iron-binding site (site C) located on the inner surface of the protein shell close to the ferroxidase center. We report the structures of five E. coli ferritin variants and their Fe³⁺ and Zn²⁺ (a redox-stable alternative for Fe⁴⁺) derivatives. Single carboxyl ligand replacements in sites A, B, and C gave unique effects on metal binding, which explain the observed changes in Fe²⁺ oxidation rates. Binding of Fe²⁺ at both A and B sites is clearly essential for rapid Fe²⁺ oxidation, and the linking of Fe₃⁺ to FeC enables the oxidation of three Fe²⁺ ions. The transient binding of Fe²⁺ at one of three newly observed Zn²⁺ sites may allow the oxidation of four Fe²⁺ by one dioxygen molecule.

Living organisms take advantage of the catalytic and redox properties of metals to drive a wide range of vital biological processes. This exploitation of metals is mainly enabled by incorporating them into proteins in various forms. An important class of metal proteins are those that contain dinuclear metal (or dimetal) centers at their active sites (1–4). A common feature of this functionally diverse family is that each dimetal center is embedded in a bundle of four or more helices in a way that enables the binding of dioxygen and/or hydrogen peroxide. With the exception of the dioxygen carrier hemerythrin, in which the metal ligands are found in the motifs HXXXE and HXXXXD, known dimetal centers have either one or two ligand sets within the motif EXXH. Each EXXH motif is located in a separate helix with the Glu residue acting as a bridging ligand between iron atoms, often together with either a second Glu (within a second EXXH motif) or one or more H₂O, OH⁻, or O₂⁻-bridging molecules. This organization enables the metals to form a close pair (3–4 Å apart) (3–8). Variation within this general construction and in the surrounding protein has given rise to molecules with a variety of functions.

Most of the known examples of this class of protein have two iron atoms at their dimetal center. Examples include the R2 protein of ribonucleotide reductase (5), the hydroxylase component of the methane monooxygenase (MMO) (6), and a homodimeric protein (ruberythrin) isolated from anaerobic bacteria, which has a di-iron center in its N-terminal domain and an Fe-Cys₄ cluster in its C-terminal domain (8). Examples of dimanganese proteins include the dimanganese catalase of some lactobacilli and thermophilic bacteria (3) and a manganese derivative of the R2 protein (9). Binding at the di-iron centers of MMO or R2 leads to oxygen activation and results in either the insertion of an oxygen atom into methane (MMO) (6) or the production of a stable catalytically involved tyrosyl radical (R2) (5). In contrast, hemerythrin binds dioxygen reversibly, whereas the binding of H₂O₂ to dimanganese catalase results in its catalytic disproportionation to H₂O and O₂ (3).

The ferritins are a special class of di-iron proteins that have roles both in iron housekeeping and in iron detoxification (1, 10). Iron is an essential element for almost all organisms. Its ability to form complexes such as heme and the iron-sulfur clusters as well as the di-iron enzymes mentioned above endows cells with a variety of functions. On the other hand, free iron is toxic, due, principally, to its part in generating highly reactive oxy-radicals that can damage cell constituents. To provide a “safe haven,” ferritins store iron as a relatively inert mineral (ferrihydrite-phosphate or hydrous ferric phosphate) “core,” which can be utilized as a source of iron for biosynthesis (1, 10). Ferritins are widespread in nature, being found in eubacteria and archaeabacteria as well as in eukaryotes (1). Their molecules are assemblies of 24 structurally equivalent protein chains or subunits related in 432 symmetry and forming a nearly spherical shell (Fig. 1A) with an 80-Å diameter iron storage cavity (7, 12–17). The protein subunits are folded as four helix bundles about 27 Å long with a fifth shorter helix lying at about 60° to the bundle (Fig. 1B). The di-iron centers are situated in the middle of the ferritin subunits (Fig. 1B) and are often known as “ferroxidase centers” because of their ability.
to confer ferroxidase activity (however, some types of ferritin subunit lack the ability to form di-iron centers) (17). Although ferritins characterized by this overall design are found throughout the living kingdom, the sequence identity between members from diverse species may be as low as 12%, and functional variations have arisen from such differences (1, 7).

It is striking that bacteria possess more than one type of ferritin (1). Many bacteria contain “bacterioferritins,” which differ from classical ferritins in that, in addition to the 24 di-iron centers, they also have up to 12 heme groups per molecule (located on the dyad axes between subunit pairs) (18).

The function of the heme groups is uncertain, but they may modulate the reductive release of iron from the “core” (19). *Escherichia coli* has both a bacterioferritin (EcBfr) and a ferritin (EcFtnA). Both are fully functional iron storage proteins with 24 di-iron centers. *E. coli* also encodes an uncharacterized ferritin-like protein (EcFtnB) with good sequence identity (33%) to EcFtnA (1, 7). However, its dinuclear center ligands are poorly conserved, suggesting that it probably does not function as a normal ferritin.

Several ferritins have been shown to contain more than one type of ferritin chain. In humans, for example, two types of chain, known as H and L, are found to coassemble in proportions that vary with the tissue of origin and body-iron status (10). The H chains contain di-iron centers, but the L chains do not (10–12).

The three-dimensional structures of native or recombinant ferritins from human (12, 17), horse (12, 16), rat (17), mouse (20), frog (14, 15), and *E. coli* (13) have been determined, but most of these are L ferritins, and relatively little high resolution information has been obtained on the structures of the di-iron centers of H-type ferritins. Moreover, the iron centers in the animal H ferritins (e.g. from horse and human) are relatively labile and, upon oxidation, the di-iron tends to move into the iron storage cavity, where it becomes “invisible” to X-rays because the mineral-iron core is complexed in a form that is not specifically orientated to the protein structure (11, 21, 23).

In *E. coli* (13), the iron is less labile, allowing the structure of the iron-containing protein to be compared with the metal-free and Zn$^{2+}$ complex forms (zinc being used as a redox-stable alternative to Fe$^{3+}$). The corresponding high resolution structures reveal the ligand geometry of the di-iron centers and of a novel, nearby “third” site (Fig. 1C) (13, 23). Structures of the Mn$^{2+}$ derivatives of EcBfr (18) and the Ca$^{2+}$ derivative of recombinant human H chain ferritin (HuHF) (17) have been determined at medium and high resolution, respectively. In the present study, the structures of five variant EcFtnA molecules and their Fe(III) and Zn(II) derivatives are analyzed. Each variant bears a single substitution (Ala for Glu) in one of the metal ligands. The results provide new insights into the functional behavior of the metal sites in EcFtnA and their relationships to those in other ferritins. Fig. 2 displays schematic representations of the metal centers of EcFtnA, EcBfr, and HuHF. It can be seen that the metal centers of the two *E. coli* ferritins differ in several respects. First, the di-iron atoms in EcFtnA are bridged by a single carboxylate (Glu$^{60}$), whereas EcBfr has two bridging carboxylates (Glu$^{61}$ and Glu$^{127}$). Second, Glu$^{130}$, which is a ligand of Fe$_B$ in EcFtnA, is replaced in EcBfr by His$^{130}$. Third and most dramatically, EcFtnA has an additional iron site, Fe$_C$, not present in EcBfr. Fig. 2 also shows that the di-iron center in HuHF differs from those in both EcFtnA and EcBfr. Like EcFtnA, it has a single bridging carboxyl (Glu$^{62}$) with a Gln (Gln$^{127}$) at a position equivalent to that of Gln$^{127}$ in EcFtnA and the bridging Glu$^{127}$ of EcBfr. Another difference is the site B ligand, Glu$^{61}$, which occupies a sequence position differing from those of the Glu$^{130}$ ligand of EcFtnA or His$^{130}$ of EcBfr. Note that Glu$^{61}$ is equivalent in sequence position to one of the site C ligands in EcFtnA (Glu$^{69}$),
but HuHF has no metal in this position because of changes in the carboxylate ligands (Glu$^{129}$ and Glu$^{130}$ to Lys$^{143}$ and Ala$^{144}$, respectively).

Differences in behavior of these three types of ferritin iron center have been described and must reflect the observed variation in structure. The most studied of the mammalian ferritins are horse spleen ferritin, which is a heteropolymer co-assembled from both H and L chains in various proportions, and the single recombinant H or L homopolymers from human, horse, or mouse which have been expressed and assembled separately. When a pair of Fe$^{2+}$ atoms at the dinuclear centers of HuHF or horse spleen ferritin binds a dioxygen molecule, the two Fe$^{3+}$ atoms are oxidized to two Fe$^{3+}$ with the production of H$_2$O$_2$ and the formation of μ-oxo-bridged diferric centers (22, 24–26).

Surprisingly, the diferric centers are quite labile. The ferric atoms can migrate into the storage cavity of the same or even another molecule, where they form ferriferricyanide by hydrolytic polymerization (11, 22, 25, 27). When this happens, two processes are possible: the original dinuclear centers can be reutilized, or iron can be oxidized directly on the growing core surface by a 4Fe$^{3+}$/O$_2$ reaction (11, 22, 25). The function of the L subunit seems to provide a site that favors the electron reduction of dioxygen. This site consists of clusters of Glu residues located on the L-subunit inner surface and acts as a nucleation center for the formation of mineral, thus stimulating its deposition within the inner cavity (7, 16). EcBfr and EcFtnA each consist of just one type of subunit with features more reminiscent of H subunits than L subunits (1, 13, 17, 18). Nevertheless, measurements of Fe(II) oxidation stoichiometry give values of 3.5–4.0 Fe(II)/O$_2$ for both proteins (28–30). In the case of EcBfr, this stoichiometry seems to result from a combination of two processes: the reduction of O$_2$ to H$_2$O$_2$ in an initial di-Fe(II) oxidation, followed by the rapid consumption of the H$_2$O$_2$ thus produced in another di-Fe(II) oxidation, with both reactions giving Fe(III)-O-Fe(III) dimers as their products (29). Interestingly, the ferric dimers produced by EcBfr are much less mobile (more stable) than those formed in HuHF.

The behavior of EcFtnA differs from that of EcBfr or HuHF. Both kinetic and Mössbauer spectroscopic measurements indicate that Fe(II) atoms at the unique C site are oxidized along with those at the A and B sites, which, as in both EcBfr and HuHF, are linked by μ-oxo-bridges (30–33). New evidence is presented here for additional metal (Zn$^{2+}$) sites, which might enable the concerted oxidation of four Fe(II) by one O$_2$ molecule. The structural data also indicate that the presence of Fe$^{3+}$ at site C stabilizes Fe$^{3+}$ at the A and B sites such that the tendency of Fe$^{3+}$ at these sites to migrate into the cavity is decreased. Thus, inactivation of site C converts EcFtnA into a more HuHF-like ferritin.

**EXPERIMENTAL PROCEDURES**

**Protein Samples**—Site-directed mutagenesis by the pALTER system (Promega, Southampton, UK) and the overproduction and purification of variant EcFtnA derivatives were as described previously (34). Protein samples were dialyzed against EDTA to remove endogeneous iron. Protein concentrations were determined with the Bio-Rad reagent.

**Crystallization and Data Collection**—Crystals of all five EcFtnA variants were grown at 20 °C by microdialysis against 20 mM piperazine-HCl buffer (pH 5.2) containing 1 mM EDTA and 0.1–0.6 M NaCl. Crystals grew after 1 week, and x-ray analysis showed that they all belonged to the space group I4 with cell parameters $a = b = 130.0 \pm 0.7$ Å, $c = 173.0 \pm 0.7$ Å (for individual cell dimensions, see Tables I and II) and with six subunits in the asymmetric unit. For the metal-soaking experiments, crystals were transferred into identical solutions to those in which they had grown except that EDTA was omitted and either ammonium ferrous sulfate or zinc chloride was added to a concentration of 10 mM. Metal soaks were carried out for 3 h before crystal mounting and data collection.

X-ray data were typically collected to a resolution of 2.2 Å from a single crystal at room temperature. All data sets were obtained using a rotating anode with a copper target and recorded on a MAR image plate detector. They were processed using DENZ0 (35) and merged and scaled using ROTAVATA and AGROVATA (36). Most data sets were more than 95% complete, with a multiplicity of at least 2.0 and with more than 70% of the reflection intensities greater than 3 $\sigma$. However, for some of the metal-soaked crystals, the diffraction limit was slightly less, as indicated in Tables I and II along with individual data processing statistics.

**Structure Solution and Refinement**—The coordinates of the wild-type EcFtnA soaked in iron (13) were used as the starting model for solving the structures of each variant. In each case, the model was edited to
Metal Binding to Five Site-directed Variants of Ferritin

### RESULTS

The Metal-free Proteins—The iron-binding regions of metal-free wild-type EcPtnA and the five variants (E17A, E49A, E50A, E94A, and E130A) are shown in Fig. 3. The structures of all other regions of the variants are essentially the same as in the wild-type protein.

A notable feature of the wild-type protein shown in Fig. 3A is the hydrogen bond network linking glutamates 17, 50, and 94, Tyr24, His53, Gln127, and two water molecules W1 and W2, with hydrogen bond distances lying between 2.48 and 3.07 Å. Inevitably, this network is disrupted by the glutamate substitutions, which also lead to loss of one or both of the water molecules, although the Tyr24OH–Glu94OE1 and Gln127NH2 bonds are retained in all except E94A and E17A (where Glu94 has moved). Tyr24, which lies in a hydrophobic pocket, remains in a similar conformation throughout. Some of the glutamate side chains, in contrast, exhibit a considerable degree of flexibility, especially Glu130 (the MB-MC bridging ligand) and the three site-directed variants E50A, E94A, and E130A (where Glu50 has moved). Tyr24, which lies in a hydrophobic pocket, remains in a similar conformation throughout. Some of the glutamate side chains, in contrast, exhibit a considerable degree of flexibility, especially Glu130 (the MB-MC bridging ligand) and the three site-directed variants E50A, E94A, and E130A (where Glu50 has moved). The geometry of each final model was checked using PROCHECK (39), and the individual refinement statistics are given in Tables I and II.

### Table I

Crystallographic data processing

Shown are cell dimensions, resolution (Res), number of unique reflections (Refl), internal agreement, completeness (Comp), multiplicity (Mult), and intensity of the 18 data sets analyzed. $R_{\text{merge}} = \sum |I_i - \langle I \rangle| / \langle I \rangle$. WT, wild type.

| Structure | Cell (Å, Å) | Res $^a$ | Refl $^b$ | $R_{\text{merge}}$ | Comp/Mult | $I > 3\sigma$ |
|-----------|-------------|---------|----------|-------------------|------------|-------------|
| WT        | 129.92, 173.25 | 2.0     | 87,413   | 5.8               | 97.7/2.3   | 77.7        |
| WT (Fe)   | 130.38, 174.01 | 2.0     | 96,599   | 4.6               | 99.0/3.3   | 85.4        |
| WT (Zn)   | 130.21, 173.76 | 2.2     | 69,434   | 4.0               | 96.3/1.9   | 86.4        |
| E17A      | 130.04, 173.36 | 2.3     | 60,723   | 6.2               | 95.4/2.0   | 77.7        |
| E17A (Fe) | 129.95, 173.26 | 2.3     | 61,765   | 8.8               | 98.3/2.5   | 70.0        |
| E17A (Zn) | 130.13, 173.77 | 2.4     | 53,226   | 7.8               | 96.3/2.3   | 72.3        |
| E49A      | 129.80, 172.80 | 2.2     | 68,562   | 7.0               | 96.3/2.0   | 80.3        |
| E49A (Fe) | 130.04, 173.56 | 2.2     | 69,124   | 5.2               | 96.3/2.0   | 78.2        |
| E49A (Zn) | 130.03, 173.80 | 2.2     | 67,755   | 5.5               | 96.3/2.0   | 79.8        |
| E50A      | 129.98, 172.70 | 2.3     | 65,178   | 4.9               | 95.9/1.9   | 83.8        |
| E50A (Fe) | 130.14, 173.53 | 2.2     | 68,974   | 8.2               | 95.5/2.0   | 76.4        |
| E50A (Zn) | 129.88, 172.46 | 2.8     | 33,502   | 5.9               | 98.3/2.0   | 76.3        |
| E94A      | 129.44, 172.53 | 2.3     | 61,658   | 5.1               | 96.8/1.9   | 81.6        |
| E94A (Fe) | 129.73, 173.18 | 2.2     | 69,006   | 5.9               | 96.8/1.9   | 81.6        |
| E94A (Zn) | 129.73, 173.28 | 2.3     | 62,080   | 5.1               | 96.8/1.9   | 81.6        |
| E130A     | 129.78, 173.01 | 2.2     | 68,562   | 6.4               | 96.2/2.2   | 75.1        |
| E130A (Fe)| 129.97, 172.99 | 2.6     | 41,425   | 6.1               | 94.3/1.7   | 78.2        |
| E130A (Zn)| 130.13, 173.68 | 2.2     | 69,804   | 6.5               | 97.0/2.4   | 74.4        |

### Table II

Refinement statistics

Agreement of each refined model to the data, number of atoms, and waters in each model, average temperature factor for main chain atoms, and root mean square (r.m.s.) deviation from ideality for bond lengths and angles. $R = \sum | F_o - | F_c | / \langle | F_c | \rangle$. WT, wild type.

| Structure | $R$ | Atoms (waters) | $B$ | r.m.s. deviations |
|-----------|-----|----------------|-----|-------------------|
| WT        | 0.188 | 8381 (377) | 34.3 | 0.007 0.692       |
| WT (Fe)   | 0.179 | 8756 (734) | 29.9 | 0.009 0.766       |
| WT (Zn)   | 0.168 | 8410 (394) | 28.8 | 0.008 0.692       |
| E17A      | 0.207 | 8136 (156) | 42.5 | 0.006 1.198       |
| E17A (Fe) | 0.203 | 8117 (131) | 43.6 | 0.006 1.234       |
| E17A (Zn) | 0.185 | 8282 (241) | 32.6 | 0.008 1.408       |
| E49A      | 0.208 | 8252 (272) | 43.5 | 0.006 1.142       |
| E49A (Fe) | 0.193 | 8213 (209) | 36.8 | 0.006 1.199       |
| E49A (Zn) | 0.186 | 8264 (266) | 34.5 | 0.007 1.388       |
| E50A      | 0.199 | 8458 (474) | 40.3 | 0.007 1.295       |
| E50A (Fe) | 0.171 | 8613 (621) | 35.0 | 0.007 1.217       |
| E50A (Zn) | 0.192 | 8219 (239) | 44.4 | 0.008 1.477       |
| E94A      | 0.194 | 8525 (545) | 36.8 | 0.007 1.251       |
| E94A (Fe) | 0.172 | 8554 (562) | 34.7 | 0.006 1.207       |
| E94A (Zn) | 0.174 | 8615 (623) | 31.5 | 0.007 1.335       |
| E130A     | 0.184 | 8525 (553) | 33.7 | 0.007 1.286       |
| E130A (Fe)| 0.190 | 8524 (552) | 36.8 | 0.006 1.207       |
| E130A (Zn)| 0.195 | 8300 (310) | 37.0 | 0.007 1.258       |
FIG. 3. Metal-free wild-type (A) and variant (B–F) structures. The electron density in the region around the ferroxidase center of EcFtnA (A) and the five variants E17A, E49A, E50A, E94A, and E130A in the absence of metal (B–F) are shown as stereo diagrams produced by BOBSCRIPT (46). The electron density is shown at a 1.0 \( \sigma \) contour level, and the hydrogen bonds (indicated by dashed lines) are those that fulfill the criteria of Kabsch and Sander (47).
FIG. 4. Wild-type (A) and variant (B–F) structures in the presence of zinc. The electron density in the region of the ferroxidase center of EcFtnA (A) and the five variants E17A, E49A, E50A, E94A, and E130A (B–F) in the presence of zinc is shown as stereo diagrams produced by BOBSCRIPT (46). The electron density is shown at two contour levels, corresponding to $\sigma$ values of 1.0 and 6.0 for the lighter and darker shades, respectively. The three metal sites are labeled A, B, and C. Hydrogen bonds are assigned as in Fig. 2.
bonding between Gln\textsuperscript{127} and Glu\textsuperscript{94}. Hence, in these structures, Gln\textsuperscript{127} must have its amino group directed toward OE1 of Glu\textsuperscript{94}. If Gln\textsuperscript{127} is orientated in the same way in the wild type metal-free structure of Fig. 3A, then it follows that Glu\textsuperscript{94} is deprotonated here too. To account for the remainder of the hydrogen-bonded network observed in Fig. 3A it further follows that both Glu\textsuperscript{17} and Glu\textsuperscript{50} are protonated, leaving a single negative charge on the carboxylate cluster (the side chain of Glu\textsuperscript{130} being turned away). His\textsuperscript{53}ND1 must also be protonated, and if it is also positively charged, then the negative charge on Glu\textsuperscript{94} would be balanced. In the alternative arrangement of Gln\textsuperscript{127} with Glu\textsuperscript{94} protonated, then either Glu\textsuperscript{17} or Glu\textsuperscript{50} must be protonated, and the other must be negatively charged. His\textsuperscript{53}ND1 would receive a proton from W2, and the cluster would have a net charge of 1\textsuperscript{-}. The first suggested arrangement is probably to be preferred.

Loss of a site M\textsubscript{A} or M\textsubscript{B} ligand in all of the variants except E49A would interfere with this charge balance as well as lowering the affinity of one or both of the metal sites. In EcFtnA, W2 is not fully present, and this water is absent from E49A so that the network shown in Fig. 3A is incomplete in these proteins. Not shown in this figure for clarity is residue His\textsuperscript{46}, which can occupy alternative positions in which it is hydrogen-bonded either to Glu\textsuperscript{94} or to Glu\textsuperscript{130} (13).

Zn\textsuperscript{2+} Derivatives of EcFtnA and Its Variants—To obtain metal derivatives, the crystals were soaked in either zinc chloride or ferrous ammonium sulfate. Zn\textsuperscript{2+}, being a known inhibitor of Fe\textsuperscript{2+} binding and oxidation (40), was used as a probe for Fe\textsuperscript{2+} because of the ease of Fe\textsuperscript{2+} oxidation. For the same reason, the iron was assumed to be Fe\textsuperscript{2+} after the 3-h aerobic soak in the ferrous salt solution. Indeed, iron oxidation was confirmed in some variants by the development of a brown color due to iron core ferricydrite formation.

Zn\textsuperscript{2+} Binding Sites—As described previously (13), the Zn\textsuperscript{2+} derivative of wild-type EcFtnA contained only two bound Zn\textsuperscript{2+} atoms, situated 3.43 Å apart at sites M\textsubscript{A} and M\textsubscript{B} of the dinuclear center. There was no Zn\textsuperscript{2+} at site M\textsubscript{C} (Fig. 4A). Three of the variants (E17A, E49A, and E130A) showed Zn\textsuperscript{2+} with high occupancy at sites M\textsubscript{A} and M\textsubscript{B}, and two variants (E17A and E130A) had significant density near the site M\textsubscript{C} identified in the wild-type Fe\textsuperscript{3+} derivative (13) (Fig. 4, B–F). On refinement, the occupancy of site M\textsubscript{C} in both variants was only 0.4 relative to that of either M\textsubscript{A} or M\textsubscript{B}. Unlike wild-type EcFtnA, three additional low occupancy Zn\textsuperscript{2+} sites M\textsubscript{D}, M\textsubscript{E}, and M\textsubscript{F} were observed in variants E17A, E49A, and E130A and these are displayed in Fig. 5. Zn\textsuperscript{2+} ligand distances are listed in Table III, together with Zn\textsubscript{A}–Zn\textsubscript{B} distances and Zn\textsuperscript{2+} occupancies.

The stereochemistries of Zn\textsubscript{A}\textsuperscript{2+} and Zn\textsubscript{B}\textsuperscript{2+} in variants E17A, E49A, and E130A are very similar to those of wild-type (Fig. 4B, C, F, and A, respectively, and Table III) with a notable swinging inwards of Glu\textsuperscript{130} to ligate Zn\textsuperscript{2+} in both the E49A and wild-type structures and with Glu\textsuperscript{50} forming a syn-syn bridge in all four structures. His\textsuperscript{53} is a Zn\textsuperscript{2+} ligand in the four proteins. In the position occupied by Glu\textsuperscript{17} in the other proteins, E17A has an electron density peak at a distance of ~2.17 Å from Zn\textsubscript{A}\textsuperscript{2+}, which was modeled as a chloride ion, and in E130A the missing site M\textsubscript{B} ligand, Glu\textsuperscript{110}, is replaced by a water molecule. Common to the Zn\textsuperscript{2+} derivatives of all three variants as well as of wild type is the presence of electron density between Zn\textsubscript{A} and Zn\textsubscript{B}, which is interpreted as a water molecule, W1, that acts as a second bridging ligand. Thus, the ligand geometries in

![Fig. 5. Additional zinc sites in variant E17A.](http://www.jbc.org/Downloadedfrom)
the four di-Zn\(^{2+}\) structures are roughly tetrahedral for both Zn\(^{2+}\) atoms, although wild-type Zn\(^{2+}\) has an additional weak ligand, water W2, at an average distance of 2.67 Å, whereas the second glutamate oxygen, Glu\(^{80}\), is 2.70 Å from Zn\(^{2+}\)\(^2\). The Zn\(^{2+}\)–Zn\(^{2+}\) separations are 3.40 Å in variant E49A and 3.43 Å in E130A, with the distance in E17A being reduced to 3.32 Å (Table III). Another feature of the four structures is the maintenance of Tyr\(^{34}\)OH–Glu\(^{84}\)O1 and Gln\(^{112}\)NH\(_2\)–Glu\(^{84}\)O2 hydrogen bonds.

Unlike the four di-Zn\(^{2+}\) structures, variant E50A shows no bound Zn\(^{2+}\), presumably because the absence of the Zn\(^{2+}\)–Zn\(^{2+}\) bridging ligand destabilizes both dinuclear sites for di-valent metal binding (Fig. 4D). There was no density either for the water replacing Glu\(^{50}\) or for two water molecules seen in metal-free wild type (Fig. 3A), and the hydrogen bond network was further disrupted by side chain movement. In contrast to E50A, site M\(_A\) is well occupied in E49A, although the occupancy of site M\(_B\) is low. The refined occupancies for sites M\(_A\) and M\(_B\) are 0.88 and 0.49, respectively. The coordination around Zn\(^{2+}\)\(^2\) is identical to that in wild type, but in the absence of Glu\(^{50}\) a new electron density peak was seen. This was refined as a water molecule.

Three new minor Zn\(^{2+}\) positions not seen in wild-type EcFtnA were found in E17A, E49A, and E130A although not in the other two variants. These sites are shown for E17A in Fig. 5. The first site M\(_B\) (occupancy 0.63), lies toward the inside of the protein shell on the interface between subunits related by a 3-fold axis. The ligation of Zn\(^{2+}\) by Asp\(^{63}\) of one subunit and by His\(^{28}\) of its symmetry-related neighbor replaces an ion-pair interaction between the same two amino acid side chains, and two other electron density peaks are assigned to Cl\(^{-}\) ions (Fig. 5A). This Zn\(^{2+}\) lies roughly equidistant from the dinuclear center of the two subunits (direct distances of 15.1 and 17.8 Å). The second Zn\(^{2+}\) (site M\(_C\), occupancy 0.48) is attached to His\(^{93}\) toward the outside of the shell with two Cl\(^{-}\) ions nearby (Fig. 5B) and lies at about 11 Å from the dinuclear center. The third Zn\(^{2+}\) (site M\(_B\), occupancy 0.56), which is present on only 8 of the 24 subunits, is located at intermolecular crystal contacts and involves Glu\(^{14}\) of one molecule and His\(^{106}\) of another as ligands, with Cl\(^{-}\) as a third ligand (Fig. 5C).

**Fe\(^{3+}\) Binding Sites**—The three Fe\(^{3+}\) sites located in wild-type EcFtnA are displayed in Fig. 6A, and the equivalent regions of the ferrous ammonium sulfate-soaked variants can be seen in Fig. 6, B–F. As discussed previously (13), iron is bound at both sites M\(_A\) and M\(_B\) in the wild-type structure, but with the M\(_B\) site occupancy only 0.68-fold that of M\(_A\). Another notable observation is the strong occupation (0.91) of site M\(_B\) by Fe\(^{3+}\). The Fe\(^{3+}\) is linked to Fe\(^{3+}\) by a long (5.79 Å) Glu\(^{130}\) bridge in an anti-anti conformation (Fig. 6A).

Variant E17A showed low occupancy (0.42) for site M\(_B\) (Fig. 6B), with no metal present in either M\(_A\) or M\(_B\). Thus, the affinity of site M\(_B\) for Fe\(^{3+}\) is evidently low in the absence of any Fe\(^{3+}\) in site M\(_A\). No iron core electron density was seen, but iron core formation was evident from the brown color of the crystals, which showed that iron oxidation and hydrolysis had taken place. To examine the possibility that iron could have been present in sites M\(_A\) and M\(_B\) at earlier times, shorter crystal soaks of 1 and 10 min were investigated. However, no iron was seen in the dinuclear sites at either time, and the occupancy of site M\(_B\) was even lower than after 3 h. Only one of the variants (E49A) that exhibited dinuclear Zn\(^{2+}\) binding showed Fe\(^{3+}\) occupation of the M\(_A\) and M\(_B\) sites, and the occupancy of M\(_B\) by Fe\(^{3+}\) was greater (0.95) than in wild type. In E49A, only a site M\(_C\) ligand had been removed, with the consequence that there was no iron binding at M\(_B\), and that the M\(_B\)-M\(_C\) bridging ligand Glu\(^{130}\) had changed its orientation to ligate only Fe\(^{3+}\) (Fig. 6C).

In the Fe\(^{3+}\) derivative of E50A (Fig. 6D), which showed no Zn\(^{2+}\) binding, a strong peak of electron density was found at the M\(_B\) site, giving an occupancy of 0.79 on refinement. Very weak density was also found at M\(_C\) (0.32). Another difference between Zn\(^{2+}\) and Fe\(^{3+}\) binding was observed with variant E94A. Here Zn\(^{2+}\) was bound strongly at site M\(_A\) (0.88), less strongly at M\(_B\) (0.49), and not at M\(_C\) (Fig. 4E), whereas Fe\(^{3+}\) was found at M\(_A\) and M\(_B\) in E94A with low occupancies (0.39 and 0.55, respectively) but not at M\(_C\) (Fig. 6E). No bound Fe\(^{3+}\) was observed at any of the specific protein sites in variant E130A, although the presence of Fe\(^{3+}\) in the iron core was again evidenced by the brown color of the crystals. None of the six Fe\(^{3+}\) derivatives examined showed metal density in the minor Zn\(^{2+}\) binding sites M\(_D\), M\(_E\), or M\(_F\) or at any other non-specific site.

In all three sites (M\(_A\), M\(_B\), and M\(_C\)) of wild-type EcFtnA, the Fe\(^{3+}\) has approximately octahedral coordination except that water W3 (not seen in the Zn\(^{2+}\) derivative) was rather far from the iron atoms (3.05 and 2.87 Å from Fe\(^{3+}\) and Fe\(^{3+}\), respectively, indicating very weak coordination). Fe\(^{3+}\) is ligated by Glu\(^{17}\), His\(^{34}\), and another water (W2), which, like W2 of the metal-free proteins, is hydrogen-bonded to Gln\(^{127}\)O1. Glu\(^{50}\) bridges Fe\(^{3+}\) and Fe\(^{3+}\) in a syn-syn-...
FIG. 6. Wild-type (A) and variant (B–F) structures in the presence of iron. The electron density in the region of the ferroxidase center of EcFtnA (A) and the five variants E17A, E49A, E50A, E94A, and E130A (B–F) in the presence of iron produced as in Fig. 3 is shown.
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Ranges over all six subunits are given below in parentheses. Mutated residues are highlighted by X, and those distances not determined due to nonbinding or weak binding of metal are marked with n or w, respectively. Occupancies (Occ) for each iron atom are also given, calculated by fixing individual B-factors at 25 Å², with ranges again in parentheses.

| WT | E17A | E49A | E50A | E94A | E130A |
|----|------|------|------|------|-------|
| Fe₇ (Occ) | 1.0 (1.00-1.00) | 1.0 (0.99-1.00) | 0.96 (0.93-1.00) | 0.79 (0.69-0.88) | 0.55 (0.48-0.62) |
| Glu130OE1 | 2.09 (2.04-2.16) | 2.01 (1.95-2.08) | 1.96 (1.92-2.08) | 2.06 (1.94-2.14) | n |
| Glu130OE2 | 2.10 (2.07-2.16) | 2.14 (2.07-2.25) | X | 2.07 (1.94-2.14) | n |
| His53ND1 | 2.02 (1.92-2.05) | 2.12 (1.93-2.41) | 2.24 (2.13-2.33) | 2.25 (2.10-2.45) | n |
| W1O | 1.98 (1.80-2.16) | 1.97 (1.86-2.06) | 2.02 (1.85-2.15) | n |
| W3O | 2.25 (2.17-2.33) | 2.08 (1.93-2.30) | 2.61 (2.43-2.96) | n |
| W3O | 3.05 (2.71-2.30) | 2.48 (2.34-2.66) | n |
| Fe₇ (Occ) | 0.68 (0.64-0.74) | 0.95 (0.89-1.0) | n |
| Glu130OE2 | 2.15 (2.10-2.20) | 1.79 (1.77-1.85) | X | n |
| Glu130OE1 | 2.15 (2.08-2.29) | 2.06 (1.80-2.29) | n |
| Glu130OE2 | 2.52 (2.48-2.59) | 2.40 (2.30-2.50) | X | n |
| Glu130OE2 | 2.38 (2.29-2.46) | 2.00 (1.93-2.08) | n |
| W1O | 1.98 (1.92-2.14) | 1.85 (1.72-2.00) | n |
| W3O | 2.87 (2.55-3.09) | n |
| Fe₇ (Occ) | 0.91 (0.88-0.94) | 0.32 (0.27-0.35) | 0.32 (0.28-0.35) | 0.39 (0.34-0.44) | n |
| Glu130OE2 | 2.06 (1.97-2.18) | w | X | w | 1.86 (1.80-1.97) |
| Glu130OE2 | 2.04 (2.00-2.09) | w | n | w | 1.83 (1.72-1.96) |
| Glu130OE2 | 2.12 (2.02-2.23) | n | w | w | 1.86 (1.73-2.05) |
| Glu130OE1 | 1.89 (1.86-2.19) | n | w | w | 2.24 (1.90-2.43) |
| W4O | 2.02 (1.89-2.19) | n | w |
| W5O | 2.27 (2.15-2.35) | n |
| Fe₇ - Fe₉ | 3.24 (3.18-3.27) | n | 3.48 (3.43-3.54) | n |
| Fe₇ - Fe₉ | 5.79 (5.73-5.84) | n | n | n |

Hydrogen bonding through an OH⁻ proton. This would give the cluster a net charge of −1. Although the hydrogen bonds linking Tyr73, Glu49, and Gln127 remain intact, the hydrogen atoms in the remainder of the network in Fig. 3A are lost from Glu72, His53, and Glu51 as a consequence of metal binding.

There are some notable differences between the coordination of Fe₇³⁺ and Fe₈⁺ in variant E49A compared with wild type (Fig. 6, C and A, respectively). The two iron atoms are further apart (3.43 Å instead of 3.24 Å as in wild-type) (Table IV), and W3 is missing. In the absence of Fe₇⁺, Gln127 moves closer to Fe₈⁺ such that the average bond length of Glu130OE1-Fe₈⁺ (3.03 Å) is much shorter than in wild-type (3.28 Å), and this, combined with the shortening of Glu130OE2-Fe₈⁺ (1.79 instead of 2.15 Å) may explain the increased occupancy of site M_B (Table IV). The binding of Fe₈⁺ by Glu130 involves considerable side chain movement, and the coordination of Fe₈⁺ in wild type (and in E94A) also necessitates realignment of the carboxyl ligands Glu286, Glu285, and Glu284 to a lesser extent. In E94A and in wild type, Fe₈⁺ has similar geometry with two water ligands (W4 and W5) in addition to monodentate ligation by the two glutamates. The coordination of Fe₈⁺ in E50A (Fig. 6D) is unusual compared with that in wild type or E49A. In addition to the usual protein ligands, Glu37 and His27, there are two well ordered water molecules, W1 (at 2.02 Å) and W2 (at 2.08 Å), and these are hydrogen-bonded to both OE1 and OE2 of E94 in a syn-syn conformation and also to S31 and N141, respectively (not shown). A small movement of Glu37 is required to give the arrangement described, and this is accompanied by a small movement of Tyr34 to maintain its hydrogen binding. In E50A, as in E94A in which there is no Fe₇⁺, Glu130 binds only Fe₇⁺ along with its two other carboxyl ligands Glu86 and Glu286. Glu129 is not a ligand in E50A, presumably because only three glutamate ligands are required to neutralize the Fe⁺⁺ charge, and its side chain points away from the metal site. In E94A, the side chain of Glu129 has alternative positions, although in one position it would seem partially to ligate Fe₈⁺.

**DISCUSSION**

**Structures of the EcFtnA Proteins**—The five glutamate/alanine substitutions produced few changes in the structures of the ferritins except for some side chain movements in the dinuclear center and, in some variants, the replacement of the missing carboxylate by a water molecule. Likewise, upon metal binding, there was some side chain reorientation to allow metal chelation but little indication of any significant change in conformation of the subunit or arrangement of subunits in the protein shell. Hence, the spectroscopic (31, 40) and kinetic (30, 34) effects of the amino acid changes on metal binding are localized to the metal binding sites and their immediate environment.

**Importance of the Dinuclear Center**—Not only do the x-ray data presented here confirm the localization of ferroxidase activity to the dinuclear sites, M_A and M_B, but the observed functional effects of the individual substitutions are explicable by the changes in metal binding. Thus, the most drastic alterations in the binding of both Zn²⁺ and Fe³⁺ produced by the substitutions E50A and E94A also give the largest reduction in the rates of Fe²⁺ oxidation (30, 34). Hence the x-ray analysis supports the conclusion that fast oxidation depends on the presence of a pair of Fe²⁺ atoms enabling the transfer of two electrons to dioxygen. The observed oxidation rate with E49A is only slightly less than that with wild-type (30), and the binding of both divalent and trivalent metals is similar in the two proteins.

Thus, substitution of the site MC ligand, Glu49, leads greatly diminishing the rate of oxidation (30). Hence, the presence of iron in site M_C is not essential for fast oxidation, and in this regard it may be noted that human H chain ferritin, which has no site M_C, oxidizes iron as efficiently as EcFtnA (30). Although no Fe²⁺ was observed in sites M_A or M_B of variants E17A or E130A (Fig. 6), both sites were fully occupied by Zn²⁺ (Fig. 4). This, together with the known ability of Zn²⁺ to displace both Fe²⁺ and Fe³⁺ from these sites, supports the conclusion that Fe²⁺ oxidation takes place pairwise in these variants. In the iron-containing derivatives, it is inferred that
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oxidation must have occurred before x-ray data were collected but that the loss of $M_A$ or $M_B$ site ligands, respectively, from these variants had greatly reduced their affinity for Fe$^{3+}$ and enabled Fe$^{3+}$ to leave these sites for core formation in the protein shell. Kinetic studies indicated that the Fe$^{2+}$ oxidation rate was diminished with E17A and (to a small extent) with E130A compared with wild-type, but such losses in oxidative efficiency were not comparable with those found for variants E50A or E94A.

**Coupled Dimetal Pairs**—A feature common to wild type and variants in which both the $M_A$ and $M_B$ sites are occupied by Zn$^{2+}$ or Fe$^{3+}$ is the presence of bridging electron density coupling the metal ions. This is likely to be a water molecule in the charge-neutral complex formed by two Zn$^{2+}$ and four carboxylate ligands. However, Mössbauer spectroscopic data indicate that a $\mu$-oxy-bridged Fe$^{4+}$ dimer is formed immediately after Fe$^{2+}$ oxidation and that, with time, this is transformed into a species having different parameters, which may be a $\mu$-hydroxo-coupled Fe$^{3+}$ pair (32, 33). The x-ray data indicate the presence of such a bridge in both E49A and wild type without clearly distinguishing the type of coupling between the Fe$^{3+}$ atoms. Indeed, a mixture of $O^-$ and OH$^-$ bridges is a possibility.

**The Role of Iron in Site C**—Although it has been inferred that Fe$^{2+}$ must bind at site $M_C$ in wild-type prior to its oxidation along with Fe$^{2+}$ at $M_A$ and $M_B$ (32, 33), the first direct evidence for the binding of a divalent metal at this position is given in Fig. 4, B and F. With ligation to the protein only by Glu$^{49}$ and Glu$^{126}$, the binding is weak in the variants E17A and E130A, and no Zn$_{\infty}^{2+}$ was observed in wild-type or any of the other variants. It has been noted previously (13) that a strong electron density peak appears at site $M_C$ in the wild-type Fe$^{3+}$ derivative (Fig. 6A). Weaker binding is now also observed in E94A, E50A, and E17A (Fig. 6). The absence of Fe$_{\infty}^{3+}$ from E49A and E130A emphasizes the importance of the ligands that have been changed. The electron density for Glu$^{126}$ is always well ordered and correctly orientated for metal binding, indicating that it is also an essential ligand, whereas Glu$^{129}$, which is often disordered, is only brought into play when required to form a stable, charge-neutral complex as in wild type, where Glu$^{130}$ bridges both Fe$_{\infty}^{3+}$ and Fe$_{\infty}^{3+}$.

The Zn$^{2+}$ and Fe$^{3+}$ binding data displayed in Figs. 4–6 provide structural underpinning for several other observations. Mössbauer spectroscopic data indicate that Zn$^{2+}$, which binds only weakly, if at all, at site C, is unable to displace monomeric (site $M_C$) Fe$^{3+}$, although it is able to compete with both Fe$^{2+}$ and Fe$^{3+}$ for at least one of the dinuclear sites (33). Of greater functional relevance is the finding that the blue peroxo derivative (Fig. 6), observed here for the first time, may enable dioxygen to acquire a fourth electron, leading to its oxidation. The finding of Fe$_{\infty}^{3+}$ in the first time, may enable the oxidation of four Fe$_{\infty}^{2+}$ out of its oxidation site into the iron storage cavity for ferricyanide reduction. However, this suggestion needs further investigation, since kinetic measurements (performed in solution) have indicated that the dinuclear site of E49A is more readily vacated than that of the wild-type protein (30). On the other hand, the crystallographic data for EcFtnA indicate that, when Glu$^{130}$ is absent, the dinuclear center no longer provides stable binding for Fe$_{\infty}^{3+}$, and the iron now forms hydrolytic polymers that turn the crystals brown. The ease of core formation shown here for E130A and in solution for other site $M_C$ variants (30), imply that site $M_C$ is not an iron core nucleation center. It is not clear whether any other residues promote ferricyanide nucleation in EcFtnA, but electron micrographs and the iron staining of electropherograms show that the mineral forms within the protein shell (1). The fact that no electron density attributable to mineral was detected does not mean that the cavity was empty but must mean that the microcrystalline structure of the mineral of EcFtnA, as in other ferritins (21), is not specifically orientated with respect to that of the protein.

In conclusion, genetically engineered substitutions of dinuclear center ligands show drastic effects on iron or zinc binding and negligible effects on the structure as a whole. The structural analysis, which is the first such study for any ferritin, now provides a sound structural basis for the conclusion, based previously on solution studies, that binding of Fe$_{\infty}^{2+}$ pairs at these centers is responsible for their rapid oxidation. The binding of iron at a third site ($M_{\infty}$) near the di-iron pair ($M_A$ and $M_B$), which is observed only in bacterial ferritin, could allow the coupled oxidation of a third Fe$_{\infty}^{2+}$. Moreover, iron binding at one of these even more remote sites ($M_{\infty}$–$M_{\infty}$), observed here for the first time, may enable the oxidation of four Fe$_{\infty}^{2+}$ by one dioxygen molecule with the production of water.

**Relationship with Other Ferritins and Metalloproteins**—The results given here provide an interesting comparison with the other ferritins of Fig. 2. Although comparable structural data are not available for HuHF or EcBfr variants, Glu substitutions equivalent to E17A and E94A have been made in these proteins (E27A and E107A, respectively, in HuHF and E18A and E94A in EcBfr). In all three ferritins, the site B ligand substitution enormously increases the $t_{1/2}$ of oxidation as does the site A ligand change in HuHF (24, 28) and EcBfr (28, 42). Although considerable, the effect of the E17A substitution in EcFtnA is less marked than the site A change in the other two ferritins. Interestingly, the equivalent substitutions Q127E in EcFtnA and Q141E in HuHF had little observed effect on the $t_{1/2}$ of oxidation, whereas the converse substitution in EcBfr, E127Q, drastically reduced the oxidation rate (28). It seems that both carboxyl bridges are essential for the stability of the center in this ferritin. Of further interest is the comparison of the effects of the E130A and E49A...
substitutions in EcFtnA with HuHF (which has Ala144 at the Glu140 position) and with the HuHF-E61A variant (equivalent to E49A in EcFtnA). The first results to note are that neither E49A in EcFtnA nor E61A in HuHF, site C and site B ligands, respectively, gave a large diminution in the Fe(II) oxidation rates, and the same is true of the site B ligand change E130A in EcFtnA (28, 30). Of further interest is the finding that in the Zn2+ derivative of the EcFtnA variant E130A, the site C ligand, Glu130 moves closer to Fe3+ at site B (Fig. 6F and Table IV). In HuHF, Glu161 was observed in two orientations in crystals grown from solutions containing CaCl2, one directed toward site B and a second toward the inner surface of the protein, where it ligated a Ca2+ (17) together with residue Glu64 (at a weak “C” site different from site C in EcFtnA). If this Fe(II) is bound at site C, then it is not oxidized along with the Fe(II) at sites A and B, but it was suggested that movement of Glu61 might shepherd Fe3+ from site B to site C in the cavity for ferrhydrite nucleation (17). This evidently does not happen in EcFtnA, since Glu94 is not a site B ligand (Fig. 1C), and although Glu94 is a site C ligand, site C is not part of a ferrhydrite nucleation site in this ferritin (30). Although divalent Zn2+ ions are found at sites A and B in the EcFtnA variant bearing the substitution E130A (Fig. 4F), this change leads to the loss of Fe3+ from all three sites (Fig. 6F) and hence to increased ferrhydrite core formation. In this respect, the EcFtnA variant more closely resembles HuHF than does wild type EcFtnA, and again, the observed change in oxidation stoichiometry from 3.5−4.0 to 1.8 Fe(II)/O2 given by the E130A substitution shows another HuHF-like property (30). Thus, although when Fe3+ is present at site C the lengthening of the Glu190−Fe94−Fe3+ bond may lead to the loss of some Fe3+ from site B, the use of Glu190 instead of Glu94 at site B seems to stabilize iron at the di-iron site. The sequence differences between HuHF and EcFtnA may result from a greater and more immediate demand for iron storage in the mammalian ferritin, although initially this occurs at the expense of the production of hydrogen peroxide when its center is used for Fe(II) oxidation.

A further common feature of some of the dinuclear centers is the presence of an outer sphere tyrosine residue (Figs. 2, 6, and 10). A tyrosine similar in position to those depicted in Fig. 2 is found in both ruberythrin (8) and dimanganese catalase (3), where it is a conserved feature, and in both proteins the tyrosine is hydrogen-bonded to a site B carboxyl ligand as in the ferritins (e.g. Tyr24−Glu94 in EcFtnA) (Fig. 2). In dimanganese catalase, it is suggested that this tyrosine may function as a “safety valve,” allowing the enzyme to form a relatively reversible free radical following hyperoxidation of the cluster, thus protecting the active site and preventing permanent oxidative damage to the protein. In the R2 protein, a conserved tyrosine, which is hydrogen-bonded to a site A ligand (usually Asp) forms a nuxyl free radical when the reduced center is oxidized by dioxygen and participates in an electron transfer chain leading to the reduction of ribonucleotide (4, 5). In both EcFtnA and HuHF, the Y24F/Y34F change leads to a 4.5-fold increase in the t1/2 of Fe(II) oxidation (28), but the conservation of this residue in the PtnB of E. coli (1) and in mammalian L chains (10) could mean that its role is largely structural. However, tyrosyl radical production occurs in HuHF but not in recombinant horse L ferritin, and it is further associated with ferroxidase activity through the finding that a variant (E62K and H65G) lacking ferroxidase activity did not give the EPR radical signal (43). The tyrosyl radical signal was also absent from the HuHF-Y34F variant but not from those bearing change Y137P, Y29F, or Y32F, clearly implicating Tyr24 as its source (43). However, the low level of tyrosyl radical production (about one per 300 Fe2+) oxidized implies that it is not of critical importance to the protein-catalyzed mechanism of iron oxidation in this ferritin.
Insights into the Effects on Metal Binding of the Systematic Substitution of Five Key Glutamate Ligands in the Ferritin of *Escherichia coli*

Timothy J. Stillman, Paul P. Connolly, Charlotte L. Latimer, Andrew F. Morland, Michael A. Quail, Simon C. Andrews, Amyra Treffry, John R. Guest, Peter J. Artymiuk, and Pauline M. Harrison

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