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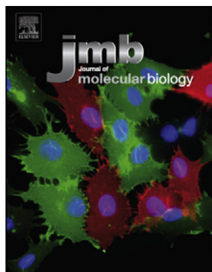

Structural Basis of Novel Iron-Uptake Route and Reaction Intermediates in Ferritins from Gram-Negative Bacteria

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Abstract

Iron and oxygen chemistry is mediated by iron proteins for many biological functions. Carboxylate-bridged diiron enzymes including ferritin have the common mechanism of oxygen activation via peroxodiferric intermediates. However, the route for iron uptake and the structural identification of intermediates still remain incomplete. The 4-fold symmetry channel of *Helicobacter pylori* ferritin was previously proposed as the iron-uptake route in eubacteria, but the amino acid residues at the 4-fold channel are not highly conserved. Here, we show evidence for a short path for iron uptake from His93 on the surface to the ferroxidase center in *H. pylori* ferritin and *Escherichia coli* ferritin. The amino acid residues along this path are highly conserved in Gram-negative bacteria and some archaea, and the mutants containing S20A and H93L showed significantly decreased iron oxidation. Surprisingly, the *E. coli* ferritin S20A crystal structure showed oxygen binding and side-on, symmetric μ-η^2:η^2 peroxodiferric and oxodiferric intermediates. The results provide the structural basis for understanding the chemical nature of intermediates in iron oxidation in bacteria and some of archaea.
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Introduction

Iron has a dual character in biology, a crucial element for a wide range of redox reactions but a threat to living organisms due to high toxicity [1–3]. Iron and oxygen chemistry is mainly mediated by iron proteins for a variety of biological functions including biomineralization [4,5]. Although iron oxidation is an ancient form of energy metabolism, dissimilar iron oxidation pathways have evolved independently after the rise of oxygen in the atmosphere [6]. Ferritin, a spherical protein shell of 24 highly conserved subunits, is an iron storage and detoxification protein found in all living organisms, including bacteria and archaea. Up to 4500 irons can be sequestered inside the protein shell from the intracellular labile pool, avoiding the deleterious effects from iron toxicity. Each subunit forms a four-helix bundle, in which a carboxylate-bridged dinuclear iron center catalyzes the oxidation of ferrous ions into a ferric oxide core. The ferroxidase center shares structural similarities with the active site of carboxylate-bridged diiron enzymes such as ribonucleotide reductase R2 (RNR R2) and bacterial multicomponent monooxygenase including methane monooxygenase, toluene monooxygenases and phenol hydroxylase, Δ^9-stearoyl-acyl carrier protein desaturase, *N*-oxygenase AurF, and aldehyde decarbonylase [7–11]. The ferritin and carboxylate-bridged diiron enzymes share common intermediates generated in the reaction of iron oxidation and transient peroxo- and oxodiferric complexes [12–16]. Among the peroxodiferric model complexes characterized by spectroscopic studies, a μ-1,2-peroxodiferric structure was suggested as one of the favorable configurations [8]. The peroxodiferric complex has been studied in vertebrate ferritins [12,17,18], but the structural identification of the intermediates remains incomplete.

We previously showed that the 4-fold symmetry channel of *Helicobacter pylori* ferritin (Hpf) can serve as the iron-uptake pathway in eubacteria, whereas the 3-fold symmetry channel is the likely route of iron entry in human and insect ferritins [19,20]. Mammalian

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ferritins have shown different pathways for mineral core formation: a protein-catalyzed ferroxidation and a mineral surface oxidation [21]. The protein-catalyzed ferroxidation was suggested to initiate the core nucleation within the protein shell. We proposed that the 4-fold channel can be the earliest path for iron uptake in prokaryotes and some archaea, prior to the emergence of eukaryotic ferritins [19]. However, the amino acid residues at the 4-fold channel are not highly conserved in prokaryotic ferritins. During the process of identification of the ferroxidase center, we observed a peak on anomalous difference Fourier maps derived from crystals soaked in Cd^{+2}, which is close to His93 on the surface of Hpf. His93 is only 6 Å away from the ferroxidase center. In this study, we provide evidence that the path from His93 to the ferroxidase center can be a major route for iron entry into the ferroxidase center in Hpf. We tested this path in both Hpf and *Escherichia coli* ferritin (Ecf) mutants at the corresponding sites, which showed significantly reduced ferroxidation activities. Furthermore, one of the mutant crystal structures, Ecf S20A, surprisingly revealed peroxo- and oxodiferric intermediates in different subunits.

Results and Discussion

Novel iron-uptake paths in Hpf and Ecf

Although the 4-fold symmetry channel of Hpf was proposed previously as the iron-uptake pathway in eubacteria [19], the amino acid residues including His149 at the 4-fold channel of Hpf are not highly conserved in prokaryotes (Fig. S1). In vertebrates, the amino acid residues are rather hydrophobic. In this regard, observation of the peak on anomalous difference Fourier maps close to His93 on the surface of Hpf prompted us to pursue mutational studies along the path from His93 to the ferroxidase center. The distance between His93 and the ferroxidase center is only 6 Å, which is much shorter than the distance from the pore entrance of the 4-fold symmetry channel to the ferroxidase center (~30 Å). Notably, the amino acid residues along the short path from His93 to the center were found to be highly conserved in Gram-negative bacteria and some archaea: His93 and Ser20 (Figs. 1 and S1). However, they are not conserved in Gram-positive and eukaryotes. In order to identify a potential entry site for iron uptake, we prepared Hpf mutants along the path, including H149L at the 4-fold channel: S20A, H93L, H149L, S20A/H93L, H93L/H149L, and S20A/H93L/H149L [19], and determined their crystal structures at 1.3–3.3 Å resolutions at low-iron-bound state (S20A/H93L diffracts to low resolution of ~4 Å) (Tables 1 and S1).

The $2|F_o|$-$|F_c|$ electron density maps in the native and H149L showed a strong peak corresponding to the anomalous peak near His93 (Figs. 1 and S2). A solvent molecule (WAT) formed hydrogen bonds with Glu50 and Ser20 that adopted alternative conformations. In contrast, the peak close to His93 was absent in mutants S20A, H93L, H93L/H149L, and S20A/H93L/H149L. In particular, the mutants, H93L and H93L/H149L, showed alternative conformations of Glu50 with a water molecule at Fe_A (Fig. S2), which appeared to adopt a half native and a half S20A mutant character. As noted, these amino acid residues are well conserved across the Gram-negative bacteria and some archaea (Fig. S1). Structures of ferritins from *E. coli*, *Vibrio cholera*, and *Pyrococcus furiosus* indeed showed very similar conformations of His93 and Ser20 to those of Hpf, which are replaced by alanine and glutamine or leucine in Gram-positive bacteria and human ferritins, respectively (Fig. S3).

The short path from His93 to the ferroxidase center for iron uptake was previously predicted for subunits with the ferroxidase center by simulation studies [20]. We also performed molecular dynamics (MD) simulations and found that Fe^{2+} was able to enter the path to the ferroxidase center within 2.5 and 3.6 ns in the native Hpf and Ecf proteins, respectively (Figs. S4 and S5). In contrast, the rate of iron uptake was four times slower in Hpf H93L compared to the native and was not even observed within the 10-ns simulation in Hpf S20A/H93L (Fig. S6). In the case of Ecf mutants, iron uptake in S20A showed comparable rate to the native, but it was much slower in other mutants. The extent of iron uptake in the S20A, H93L, and S20A/H93L mutants, defined by the ratio (%) of the number of Fe ions at the ferroxidase center between the native and mutant proteins, was as low as 20–80% and 43–71%, compared to the native Hpf and Ecf, respectively. Thus, the rate and extent of iron uptake to the ferroxidase center were much higher in the native than those in the mutants.

Direct observation of intermediates during iron oxidation

To examine the underlying structural properties of this potential novel iron entry route via His93 and Ser20, three mutants of Ecf, S20A, H93L, and S20A/H93L, were constructed, expressed, and purified to homogeneity. The crystal structures of the mutants were determined at 1.8–2.5 Å resolutions at low-iron-bound state, with six subunits in the asymmetric unit (Table 1). In the Ecf native structure, a strong peak close to His93 was observed, but not in the mutants (Fig. S7). In two of these mutant structures, S20A (subunits A, B, and E) and H93L (all six subunits), extra electron density in the $|F_o|$-$|F_c|$ difference Fourier maps was found at the ferroxidase center for which two Fe^{3+} ions were modeled. A single oxygen atom was modeled at the strong positive peak between the two Fe ions to coordinate to Fe_A and Fe_B. It was confirmed by the $|F_o|$-$|F_c|$ omit electron density maps, with the

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