Iron-oxo clusters biomineralizing on protein surfaces: Structural analysis of *Halobacterium salinarum* DpsA in its low- and high-iron states

Kornelius Zeth*†, Stefanie Offermann*‡, Lars-Oliver Essen§, and Dieter Oesterhelt*¶

*Department of Membrane Biochemistry, Max Planck Institute for Biochemistry, Am Klopferspitz 18, D-82152 Martinsried, Germany; and §Department of Chemistry, Phillips University, Hans-Meerwein-Strasse, D-35032 Marburg, Germany

Edited by William N. Lipscomb, Harvard University, Cambridge, MA, and approved June 28, 2004 (received for review March 15, 2004)

The crystal structure of the Dps-like (Dps, DNA-protecting protein during starvation) ferritin protein DpsA from the halophile *Halobacterium salinarum* was determined with low endogenous iron content at 1.6-Å resolution. The mechanism of iron uptake and storage was analyzed in this noncanonical ferritin by three high-resolution structures at successively increasing iron contents. In the high-iron state of the DpsA protein, up to 110 iron atoms were localized in the dodecameric protein complex. For ultimate iron storage, the archaeal ferritin shell comprises iron-binding sites for iron translocation, oxidation, and nucleation. Initial iron–protein interactions occur through acidic residues exposed along the outer surface in proximity to the iron entry pore. This narrow pore permits translocation of ions toward the ferroxidase centers via two discrete steps. Iron oxidation proceeds by transient formation of tri-iron ferroxidase centers. Iron storage by biomineralization inside the ferritin shell occurs at two iron nucleation centers. Here, a single iron atom provides a structural seed for iron-oxide cluster formation. The clusters with up to five iron atoms adopt a geometry that is different from natural biominerals like magnetite but resembles iron clusters so far known only from bioinorganic model compounds.

Iron not only is an essential cofactor of many enzymes but also has been a major threat to cellular life since the advent of atmospheric oxygen 2.8 billion years ago. Despite the abundance of iron, its cellular uptake is compromised, because this element occurs as Fe$^{3+}$ in a mostly insoluble form under physiological pH and oxidizing conditions. Furthermore, free Fe$^{3+}$ ions generate highly reactive hydroxyl radicals by Fenton reactions with biogenic oxygen species like superoxide anions or hydrogen peroxide (1). Consequently, the iron present in the cytosol has to be tightly sequestered by complexation to proteins. The protein family of ferritins evolved as such a specialized iron storage container, which incorporates intracellular iron in a bioavailable and nontoxic form. Here, iron is taken up into oligomeric ferritin shells as Fe$^{3+}$, oxidized therein to Fe$^{4+}$ by ferroxidase centers, and deposited in the large cavities of the ferritin oligomers as crystalline or amorphous ferrhydrite-like cores (2). Such polymerization processes often occur in nature and usually rely on preorganized bioinorganic templates, as provided by proteinaceous surfaces. Several other examples of biological iron oxidation and polymerization processes exist as well, such as the formation of magnetosomes by the magnetic bacterium *Magneto-spirillum gyphiswaldense* or the presence of magnetite-like crystals in unicellular and multicellular organisms such as algae, salmon, pigeons, and humans (ref. 3 and references therein).

Two major subfamilies of ferritins are known that differ in their resolution structures at successively increasing iron contents. In the high-iron state of the DpsA protein, up to 110 iron atoms were localized in the dodecameric protein complex. For ultimate iron storage, the archaeal ferritin shell comprises iron-binding sites for iron translocation, oxidation, and nucleation. Initial iron–protein interactions occur through acidic residues exposed along the outer surface in proximity to the iron entry pore. This narrow pore permits translocation of ions toward the ferroxidase centers via two discrete steps. Iron oxidation proceeds by transient formation of tri-iron ferroxidase centers. Iron storage by biomineralization inside the ferritin shell occurs at two iron nucleation centers. Here, a single iron atom provides a structural seed for iron-oxide cluster formation. The clusters with up to five iron atoms adopt a geometry that is different from natural biominerals like magnetite but resembles iron clusters so far known only from bioinorganic model compounds.

Iron is much more diverse, with many members promoting iron incorporation and others acting as immunogens, neutrophile activators (9), cold-shock proteins, or constituents of fine-tangled pili (10). Another mode of protection against reactive oxygen species implies the preferential consumption of hydrogen peroxide instead of oxygen during biomineralization (11).

Due to the absence of the C-terminal fifth helix of 24-mer ferritins, members of the Dps subfamily assemble only to dodecameric protein shells that have cubic 23 symmetry and outer and inner diameters of 9 and 4.5 nm. Consequently, the iron-storage capacity of these proteins is smaller, and ferritin dodecamers from *Helicobacter pylori* and *Listeria innocua* were reported to oxidize and sequester up to 500 iron atoms inside their cavity (9, 12, 13). Hereby, the main entry of iron and other ionic species into these members of the Dps subfamily is postulated to occur along pores that penetrate the protein shell at the threefold axes of symmetry (9, 13), and that were also identified as entrance sites in the 24-mer ferritins (2). In the crystal structures of Dps proteins from *E. coli* (14), *L. innocua* (13), and *Bacillus anthracis* (15), these access channels are conserved, besides an interfacial iron-binding site that was assigned in these Dps-like ferritins as a di-iron ferroxidase center for catalytic iron oxidation.

So far, structures of the ferritin family have been described from two of the three domains of life, eukaryotes and eubacteria, but not from archaea (16). Iron uptake and storage by these enzymes were extensively studied (2), but neither the uptake route nor the redox...
pathway could be analyzed in atomic detail. The lack of x-ray crystallographic data about biominalerization steps in ferritins might be caused by the partial lack of order in their iron cores (2). Here, we report crystal structures of an archaeal Dps-like ferritin from the euryarchaeote H. salinarum at a low- and two high-iron states. This ferritin functionally resembles its euarchaeal homologues but exhibits unique structural features for iron uptake and storage pathways. By x-ray crystallography, the sites of initial iron binding, translocation, oxidation, nucleation, and storage in a bioavailable form were delineated at high resolution. Like other processes in nature, the iron storage process here described can be understood on the basis of matrix-controlled biominalerization.

Experimental Procedures

Crystallization, Iron Complexation, and X-Ray Data Collection. The purification of H. salinarum DpsA and its crystallization in crystal forms A–D are described elsewhere (unpublished results). A single nontwinned low-resolution dataset was collected from a form A crystal (space group P321, a = b = 91 Å, c = 223 Å) at beamline ID14-2 of the European Synchrotron Radiation Facility (ESRF), Grenoble, France, at 2.8 Å (FELR). Later, a low-iron-high-resolution dataset (FEHR) was collected at 1.6 Å from crystal form B (space group P321, a = b = 91.11 Å, c = 150.04 Å) as 1° oscillation frames at 100 K on ESRF beamline ID29 by using a charge-coupled device detector (ADSC) quantum detector at λ = 1.0052 Å.

DpsA crystals initiating biominalerization were prepared under transient anaerobic conditions. Form A crystals were taken from a single drop and transferred into a 2-μl drop containing the precipitant solution plus 20 mM FeCl3 and 2 mM sodium diethionate. The crystals were incubated for 30 (FE30) or 120 min (FE120) against the reservoir solution under aerobic conditions to permit slow reoxidation of iron. As reference served a crystal from the same batch without soaking (FE0). Single anomalous dispersion difference peaks at 100 K on ESRF beamline ID29 by using a charge-coupled device detector (ADSC) quantum detector at λ = 1.0052 Å.

The initial dataset was further refined by alternative cycles of model rebuilding in O (20) and automatic refinement using CNS and REFMAC with noncrystallographic symmetry restraints (18, 21). When the refinement converged at an R factor/Rfree of 0.206/0.260 for data between 25 and 2.8 Å, the refinement was continued at 1.6 Å with the high-resolution dataset FEHR. The final structure with six Fe3+ atoms bound has a R factor/Rfree of 0.164/0.210 for data between 30 and 1.6 Å (Table 2).

For the FE0, FE30, or FE120 datasets, model phases were generated for the calculation of anomalous difference Fourier maps after initial refinement against the FEHR structure as a starting model. Additional iron atoms were included in the further refinement process of the FE30 and FE120 structures, if anomalous difference peaks >3 I/σ(I) were found at noncrystallographic symmetry-equivalent positions. A few additional iron were added if waters that were originally placed into densities showed remarkably lower B factors than their environment. All magnesium ions from the FEHR structure with its low iron content were replaced by iron in the FE30 and FE120 structures. The final models of the FE30/FE120 datasets consisted of the same number of protein residues, sulfates, and Na+ ions as the FEHR structure but comprised 38 Fe3+/30 Fe3+ and 40/312 waters, respectively (see Table 1).

Results and Discussion

Crystallization and Structural Analysis of H. salinarum DpsA Protein. The crystallization of soluble proteins from halophilic organisms is often hampered by the exceedingly high solubilities these proteins exhibit at saturating salt concentrations (22). Consequently, we pursued an systematic approach to identify crystallizable halophilic proteins to expand the structural repertoire. The cytosol of the halophile H. salinarum was therefore fractionated by conventional chromatography techniques, and concentrated fractions were subjected to an adapted sparse-matrix screen. Crystallized proteins were identified by N-terminal Edman degradation by using single protein crystals and by comparison of these sequences with the genome sequence (see www.halolex.mpg.de). One of them was the archaeal ferritin DpsA protein (ref. 23; 182 aa, M, = 20,100), which was crystallized in the trigonal crystal forms P321 (crystal form A) and P321 (crystal form B) by using polyethylene glycol 400 as a precipitant.

The structure of DpsA from H. salinarum was solved from crystal form B by self-rotation functions and molecular replacement using the structure of E. coli Dps as a search model (Protein Data Bank ID code 1DPS, residues 25–167; for details, see Experimental Procedures). The final structure was refined at 1.6-Å resolution (FEHR low-iron dataset) and comprised four ferritin molecules with 705 protein residues, 6 Fe3+ ions, 4 Mg2+ sites, 2 Na+ ions, 2 sulfates, and 796 water molecules in the asymmetric unit (see Fig. 1A for representation of ions and Table 1 for definition of datasets and iron sites). The traces of the polypeptide chains were clearly defined by electron density from Ala-7 to Leu-181. In addition, the N terminus of chain A in crystal form B extends to Ser-2, because

Table 1. Definition of iron sites

<table>
<thead>
<tr>
<th>Dataset</th>
<th>FEHR</th>
<th>FE0</th>
<th>FE30</th>
<th>FE120</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of irons in TSS</td>
<td>Low (0 min)</td>
<td>Low (0 min)</td>
<td>High (30 min)</td>
<td>High (120 min)</td>
</tr>
<tr>
<td>No. of irons in FOC</td>
<td>1 (F1)</td>
<td>1 (F1)</td>
<td>3 (F1–F3)</td>
<td>1 (F1)</td>
</tr>
<tr>
<td>No. of irons in NIT</td>
<td>0</td>
<td>0</td>
<td>3 (N11–N13)</td>
<td>3 (N11–N13)</td>
</tr>
</tbody>
</table>

Sequence variations were given in parentheses.

Subsequently performed by molrep (19). The best solution matched a correlation coefficient of 0.164 and an R factor of 0.54. The model was further refined by alternative cycles of model rebuilding in o (20) and automatic refinement using CNS and REFMAC with noncrystallographic symmetry restraints (18, 21).
this N-terminal stretch makes crystal contacts with chain A of a symmetry-related dodecamer.

X-ray fluorescence spectra collected from a native crystal independently proved the presence of iron (data not shown). Accordingly, anomalous difference Fourier syntheses that were calculated at 2.2-Å resolution from a single anomalous dispersion dataset of the same crystal (FE0) verified the six iron positions of the FEHR dataset with $I/\sigma(I)$ ratios $>4$. However, the occupancies of singly occupied ferroxidase centers were subsequently reduced to 0.5 to match the anomalous peak heights and the $B$ factors of the protein environment.

**Structural Comparison of DpsA with 24-Mer Ferritins and Dps-Like Orthologues.** The archaeal DpsA monomer folds into a four-helix bundle that is commonly found in the 24-mer ferritins and members of the Dps subfamily. One hundred eighteen of 182 residues (65%) belong to $\alpha$ helices (Fig. 1B). Helices 2 and 5 ($\alpha2$ and $\alpha5$ in Fig. 2B) form the inner wall of the protein shell, whereas helices $\alpha1$ and $\alpha4$ line the cytoplasmically exposed outer protein surface (Figs. 1B and 2B). Helices 2 and 4 are connected by a long irregular stretch that is intersected in its middle by the short helical segment 3 ($\alpha3$, residues Pro-96-Ala-103). A superposition of the *H. salinarum* monomer on the subunits of the Dps-like ferritin of *L. innocua* and Dps of *E. coli* yields root-mean-square deviations (rmsd) of 1.34 and 1.27 Å for 158 and 149 Cα positions, respectively (13, 14). Structural differences to the eubacterial orthologs are mainly found for the $\alpha4$$\rightarrow$$\alpha5$ loop region, which comprises three additional residues in the haloarchaeal ferritin. Furthermore, the halobacterial ferritin comprises an elongated N-terminal tail enriched in negatively charged residues and a C-terminal “sickle-like” region (Asp-172-Leu-181). This sickle region is clearly different from the C-terminal region (helix $\alpha5$) in the structurally related 24-mer ferritins of eukaryotes, e.g., horse spleen L-chain (rmsd of 1.68 Å for 169 Cα positions), where helix 5 was found to stabilize the 24-mer particle along fourfold symmetric contacts (6, 24).

Accordingly, the *H. salinarum* ferritin assembles to a 23-symmetric homododecamer. Helix 5 faces the inner compartment of the dodecamer like helix 2, but the latter resides at almost identical relative locations in the ferritin dodecamers from *E. coli*, *H. salinarum*, and *L. innocua*. The functional role of helix 2 is underlined by at least one iron-binding site [ferroxidase center (FOC) site, Fig. 2A] that is conserved among eubacterial Dps homologues and a cluster of carboxylate residues [nucleation site 1 (NI) center, Fig. 2B] that resembles the iron core nucleation site of mammalian ferritin L chains (1, 25).

**Iron-Binding Sites in Low- and High-Iron State DpsA Complexes.** The crystallized halobacterial ferritin DpsA was purified as an intact and iron-comprising complex from the cytosol of *H. salinarum*. In the low-iron-state crystals FEHR (1.6 Å resolution, low iron) and FE0 (2.2-Å resolution, single anomalous dispersion, low iron), the presence of 16 iron-binding sites per protein dodecamer was clearly confirmed by anomalous difference Fourier maps. All of these sites were attached along the interior protein surface, of which 12 iron atoms were placed at the dimeric FOC and four at the trimeric

---

Fig. 1. Iron-binding sites of low- (FEHR/FE0 data) and high-iron (FE30) content DpsA protein are compared. (A and C) Isolated iron-binding sites of the DpsA ferritin displayed without the protein backbone. The FOC, NI, and NII centers are encircled or boxed. The iron- and sulfate-binding sites only of the *H. salinarum* ferritin are displayed; iron sites are rendered in red, magnesium in yellow, sodium in blue, and sulfate in yellow/magenta. (B) Ribbon model of the quaternary high-iron structure (FE30) of the ferritin dodecamer as viewed down the threefold axis. Each of the four trimers related by twofold symmetry is in a different color. Figs. 1–3 were made with MOLSCRIPT (33), RASTERED (34), and DINO (http://cobra.mih.unibas.ch/dino/intro.php).

Fig. 2. Overview on the iron-binding sites in *H. salinarum* ferritin based on the FE30 data set. (Left) The general environment of the iron atoms; (Right) detailed analysis of the residues involved and some distances following the iron entry to the final storage center are given. (A) Iron translocation over the DpsA protein shell from the outside (out) to the inner cavity (in). A surface representation of the outer and inner surface is shown in cyan. Side view of the iron translocation channel (from the FE30 dataset) with the initial iron-binding site T1 and the successive iron-binding sites T2 and T3, which occur in the iron translocation channel. The important side chains guiding the iron atoms are shown. (B) Two ferroxidase centers of symmetry-related protein molecules (colored in blue and red) are viewed along the twofold axis. The helices $\alpha1$$\rightarrow$$\alpha5$, important residues, and the three iron-binding subsites F1–F3 of the FOC and nucleation center NI are depicted. Nucleation center NI, including the two symmetry-related iron atoms N11 and N13 and a third iron atom as well as the symmetry-related liganding residues Glu-72 and Glu-75, is shown. (C) View almost perpendicular to the threefold molecular axis. Overview of the ferroxidase and the two nucleation centers NI and NII. Possible routes for iron atom transfers from the FOC to NI and NII, respectively, are marked. Distances among iron centers and possible storage possibilities are indicated.
subunit interfaces, nucleation site 2 (NII) (see Fig. 1A for all sites and Fig. 3d for the anomalous density of NII).

Recently, DpsA of *H. salinarum* was shown to complex up to 100 iron atoms per dodecamer, which are at least partly constituents of large ferric iron clusters according to EPR spectroscopy (23). To locate iron-binding sites still missing in the native structure of *H. salinarum* DpsA, we incubated crystals for 30 (FE30) and 120 min (FE120) with Fe2+ under conditions that may promote biomimeralization. In the FE30 crystals, a total number of 110 irons per dodecameric complex could be identified (Fig. 1C), whereas in the FE120 crystals, the number of irons decreased to 86 iron, because only the F1-binding sites of the 12 ferroxidase centers were occupied (data not shown). Overall, the iron sites can be subdivided into four classes according to their locations and presumptive function in the DpsA complex (see also Table 1): (i) iron access route (three subsites T1–T3 in FE30 and FE120); (ii) the ferroxidase center FOC (three subsites F1–F3 in FE30, F1 in FE120); (iii) nucleation center NI (three subsites N1–N3 in FE30 and FE120; N11 and N13 are symmetry related); (iv) nucleation center NII (five subsites, N21–N25, in FE30 and FE120, N22, N23, and N24 are related by symmetry). It is noteworthy that the overall numbers agree quite well with the amount of bound iron determined in isolated samples of DpsA (23).

**Translocation Sites T1–T3: An Iron Access Route.** Significant structural differences between the meso- and halophilic homologues of the Dps subfamily are observed along the threefold axes of the dodecamers. In the *Listeria* ferritin and Dps from *E. coli*, a hydrophilic pore with a diameter of ~8 Å runs between the a4a5 loops of three symmetry-related subunits and is exclusively formed by acidic residues. Because these features are well conserved among 12- and 24-mer ferritins, it was suggested that the pore region around this threefold axis represents the major entrance site for Fe2+ into the ferritin protein shell (4, 13, 26). In the halophilic ferritin dodecamer, this pore is plugged by a row of four residues, Glu-141, His-150, Arg-153, and Glu-154 (not shown). Only the threefold related Glu-154 residues serve as ligands of the NII nucleation center (see Figs. 2C and 3A and B). Moreover, a sulfate ion that may be introduced during the purification process is observed 6.7 Å apart from the N21 iron atom in the midst of the plugged pore, where it makes numerous hydrogen bonds with the threefold symmetry-related His-150 and Arg-153 residues (Fig. 2C, side chains of liganding residues are not shown). Hence in the *H. salinarum* ferritin, the passage of ions along this pathway would be feasible only if major conformational changes transiently open the channel.

In contrast to other structurally characterized 12- and 24-mer ferritins, the DpsA structure reveals a pathway for iron binding and translocation. At the outer protein periphery, an initial translocation site (T1) is found ~20 Å distant from the catalytic FOC (Fig. 2A). The T1 site is close to the entrance of a water-filled translocation pore leading to the inner compartment of the ferritin shell. The iron of the T1 site is coordinated by Glu-171 and Asp-172. The T1 site is placed on top of a shallow depression, which is surrounded by N- (Arg-8-Asp-18) and C-terminal (Glu-167-Asp-173) stretches derived from two different monomers. These stretches contribute to a cluster of six acidic residues (Glu-13, Glu-15, Asp-18, Glu-167, Glu-171, and Asp-173), which might together act as an electrostatic guide for incoming Fe2+. An analogous electrostatic arrangement was also observed around the threefold axis of the *L. innocua* ferritin (13). A second binding site (T2) is buried inside the pore, 13 Å away from the FOC. The iron in the T2 site is bound to the residues Glu-56, Gln-86, and His-168, which are recruited from two adjacent monomers. It is noteworthy that in the FEHR and FE0 structures, a magnesium ion replaces the T2 site iron of the FE30 and FE120 structures. Between the T2 site and the FOC, a third binding site (T3) is about halfway between these. Here, weak interactions are formed to residues His-164 and Gln-86. The distances between the four iron sites are all in the range of 5–9 Å (T1–T2, 9 Å; T2–T3, 5 Å; T3–F1, 7 Å, see Fig. 2A and C).

From the structural data, one might conclude that a hydrated
Fe$^{2+}$ atom is initially bound to the outer surface of DpsA and progressively guided through the pore by hopping in discrete steps from the initial binding site T1 via T2 and T3 to the ferridoxin site. This pathway is obviously unique to the haloharchael ferritin. For example, in the *Listeria* ferritin, two bulky aromatic residues tightly plug the corresponding entrance channel (13). Only the minimal pore diameter in the DpsA complex of ~5 Å and the distance between the pores and the catalytic FOCs resembles the pores of mesophilic ferritins, which are supposed to be exclusively formed along the threefold symmetry axes (2). Instead, the diversion from the threefold symmetric pores of mesophilic ferritins might be dictated by the hypersaline environment. First, due to the usage of a non-symmetric site, the *H. salinarium* ferritin provides entrance pores for iron to the inner compartment pores instead of only four, as in the mesophilic Dps-like ferritins. The increased permeability of the DpsA protein shell might circumvent the problem that electrostatic guidance of iron ions as described for other ferritins is not feasible for a halophilic ferritin ortholog, because long-range electrostatic interactions are efficiently shielded by the almost saturating salt content of the haloarchaeal cytosol (≈5 M KCl).

Second, the participation of histidine residues in the iron translocation pathway (His-164, His-168, and His-172) differs significantly from other mesophilic Dps orthologs. Coordination of Fe$^{3+}$ cation pathway (His-164, His-168, and His-172) differs significantly from the Fe-H-Fe distance of the canonical distance of Fe-O-Fe-Fe$^{3+}$/H$^{+}$26. The averaged distance calculated for the four protein molecules (averaged value of four symmetry-related monomers) apart from residues Glu-83 (A) and the His-64 (B) of the adjacent monomer.

The Ferroxidase Center FOC. The ligands of the ferroxidase center are conserved throughout the dodecameric ferritins, including ferritin from *L. innocua* and Dps from *E. coli*. In the low-ion state structures FE0 and FEHR, only a single iron atom (F1 subsite) is bound to the FOC. The iron is coordinated in a hexagonally distorted manner to two water molecules and the conserved residues Asp-79 (A), Glu-83 (A), His-64 (B) from two monomers, which are related by a twofold symmetry axis (data not shown). Such a geometry is similar to the FOCs found in the structures from *L. innocua*, *B. anthracis*, and *E. coli* (13–15).

In the high-ion state dataset FE30, two additional subsites, F2 and F3, were detected at the FOC (Fig. 2B). The F2 subsite is 3.2 Å (averaged value of four symmetry-related monomers) apart from F1 and F2 and almost exactly replaces a water molecule, which was observed in the low-ion-state structures. The ion is coordinated to residues Glu-83 (A) and the His-64 (B) of the adjacent monomer. The averaged distance calculated for the four protein molecules related by non-crystallographic symmetry of 3.2 Å between F1 and F2 reflects the canonical distance of a Fe-O-Fe μ-oxo-bond. Surprisingly, a third iron (F3) was found at an averaged distance of 3.9 Å to F2 and 4.9 Å to F1. This F3 ion is coordinated to Glu-80 and Glu-83 of the same monomer. Interestingly, the F2 and F3 subsites are apparently transiently occupied only during the iron charging of DpsA crystals, because these sites were missing in the FE0/FEHR and FE120 and other DpsA structures where crystals were soaked longer than 120 min (data not shown). Such a loose coordination of iron in the F2 subsite was also noted for the *Bacillus brevis* Dps structure, where a water ligand bridges the F2 iron to the histidine ligand (26). Consequently, the F2 and F3 subsites might be occupied only by Fe$^{2+}$, which is continuously oxidized to Fe$^{3+}$ during the prolonged incubations in the presence of oxygen. However, due to the low resolution of the FE30 dataset, we cannot yet prove the water and oxygen coordination geometries of the F2 and F3 subsites. Although the F2 site participates in the oxidation of hydrogen peroxide, the functional role of the F3 subsite is still elusive. Interestingly, such a third iron-binding site was also located in the *E. coli* ferritin (EcfFtNa) next to the nonhomologous di-iron ferroxidase center (27). In the *E. coli* ferritin, the third iron site assists in the complete reduction of O$_2$ to water, which avoids the generation of reactive oxygen species. Accordingly, Dps-like ferritins are highly efficient with hydrogen peroxide as oxidant but may also accept O$_2$ with much lower turnover (11). Furthermore, the third site of the *E. coli* ferritin was also reported to stabilize the di-iron FOC, such that the proportion of protein-bound iron was larger than in ferritins without this site (28). Such a slowed incorporation of iron into iron cores was suggested to allow better bioavailability to the microorganism compared to the highly bio-moneralizing mammalian ferritins.

In any case, if the oxidation of F2 and F3 site ions leads to Fe$^{3+}$ oxidation from the di-iron or tri-iron FOC, these ions would have to migrate ~12–13 Å to the closest N1 or NII nucleation sites (Fig. 2C). The distance between the F3 subsite and the NII nucleation center might be overcome by intervening residues like Glu-158 and Glu-161, which are both in 6-Å distance to subsite F3 and the nucleation center NII, respectively.

**Nucleation Center NII.** Two acidic amino acids, which reside next to the twofold symmetry axis of the dodecamer, were supposed to form the iron core nucleation site along helix 2 (α2 in Fig. 2B) in the ferritin from *L. innocua* (13). It was suggested that, after oxidation at the ferroxidase site, one Fe$^{3+}$ is displaced by Fe$^{2+}$ and migrates to the proposed helix 2 iron nucleation site. However, the acidic residues Glu-44 and Asp-47 of *L. innocua* ferritin (*E. coli*, Glu-64, Asp-67; *B. subtilis*, Glu-47, Glu-50) are replaced by leucine and glycine in *H. salinarum* DpsA (Leu-65, Gly-68). As an alternative position along the dimeric interface, which are related by twofold symmetry. Three iron peaks (N11–N13) are observed there in a different structure map, two of which are protein-bound (N11 and N13). A short distance of 2.2 Å between two peaks (N11 and N13) can be explained only by their proximity to the twofold axis and the exclusive occupancy of this site by a single iron atom. Because the iron ligands Glu-72 and Glu-75 are not conserved among Dps-like ferritins and a further polynuclear nucleation center (NII) was identified, the mechanism of Ilari et al. (13) for iron accumulation in *Listeria* ferritin (12, 13) is not directly applicable to the halophilic DpsA.

**Nucleation Center NII: An Iron-Oxide Nanocluster.** The anomalous difference maps of FE0, FE30, and FE120 revealed a new nucleation center (NII) for the deposition of iron oxides (Fig. 3A and B). In DpsA in its low-ion state (FEHR and FE0), a single iron atom (subsite N21) is bound with full occupancy along the threefold axis and seems to serve there as a seed for iron cluster formation (Fig. 3A). In the FEHR structure, the Fe$^{5+}$ of this N21 subsite is primitively coordinated to three symmetry-related Glu-154 (Fe-OE1, 2.35 Å) residues and three symmetry-related water molecules (Fe-W1/2/3, 2.1 Å). At the N21 subsite, strong ordering of the solvent environment is observed, because three water molecules form an unusual three-layered crown around the central Fe$^{3+}$ ion (see Fig. 3A). In the FE30 and FE120 structures, some of these water molecules are replaced by threefold symmetry-related iron or oxygen atoms (Fig. 3B; subsites N22, N23, N24, O1, O2, and Q3), which ligand N21 subsite to Glu-154 (Fe-OE2, 2.1 Å). Together with subsite N21, these subsites form a distorted tetrahedron with a distance of 2.9 Å to N21, 2.15 to a bridging oxygen, and distances of 3.5 Å between each other (Fig. 3B and schematically presented in C). In the better-resolved FE120 structure, three water-derived ligands, presumably OH$^-$ or O$^{2-}$, were observed as triply bridging ligands between N21 and pairs of the other subsites.

The structure of the [4Fe-3O] cluster in the NII nucleation center is clearly different from the cubane-like [4Fe-4S] clusters found in many redox-active iron–sulfur proteins with typical
iron–iron distances of ~2.8 Å. The 4-iron-3-oxo cluster resembles a site-differentiated cubane with \( C_{3v} \) symmetry, although additional OH\(^{-}\)/O\(^{2-}\) ligands could not be resolved as missing corners between subunits N22 and N24 of the FE120 structure. Such a geometry with widened iron–iron interatomic distances between subunits N22 and N24 is not found among the 588 entries of the protein database (as of December, 2003), which comprise [4Fe-4S] clusters. Likewise, a search in the Inorganic Crystal Structure Database (http://icsd.ill.fr/icsd) demonstrated that the [4Fe-3O] cluster is not observed as a substructure of other iron–oxide/hydroxide minerals such as ferrihydrite, magnetite, or lepidocrocite. Therefore, the unique arrangement of the iron atoms in the four subunits N21–N24 appears to be enforced by the unique threefold symmetric protein environment.

Recently, a synthetic model compound of a singly bridged double cubane was described [Inorganic Crystal Structure Database entry PUWTTEG (29)], in which a [4Fe-4S] cluster adopts almost exactly the same geometric arrangement as the [4Fe-3O] cluster of the nucleation site (Fig. 3D). The cluster of this model compound is linked to a [2Fe-2Mo-4S] cluster via the fourth sulfido ligand, whose oxygen analog is missing in the FE120 structure. Consequently, one might infer that the observed cluster in the NII site of \( H. salinarum \) DpsA might serve as a center for the further outgrowth of larger iron-oxo clusters. Indeed, the FE30 and FE120 structures show a fifth iron-binding site (N25) with low occupancy that protrudes further into the inner compartment of the DpsA protein shell. This subsite is equidistant from subunits N22-N24 (3.0 Å) and lacks any interactions with the protein. From this study, it is not yet clear whether the observed NII cluster further increases in size on prolonged iron incubations as larger iron-oxo clusters or even as nanocrystals, which may generally be disordered inside the protein shell and hence not trackable by x-ray crystallography (30).

**Biological Function.** So far, DpsA from \( H. salinarum \) is the only known example of an archaeal ferritin. Because the haloarchaeon \( H. salinarum \) is an aerobic chemoorganotrophically growing organism (31), the function of DpsA, iron storage and protection against reactive oxygen species, presumably resembles its eubacterial relatives (32). The improved mobility of iron is also a demand of the halophilic environment. Because larger crystalline iron-oxide assemblies were not expected, but clusters of three iron atoms were demonstrated for a bacterial iron-trafficking protein (32). Iron reduction and release should proceed faster from such small iron-oxide clusters than from larger crystalline assemblies due to their higher surface to volume ratios. Due to its unique occurrence, one might currently speculate that the improved mobility of iron is also a demand of the halophilic environment. Because larger crystalline iron-oxide assemblies were not yet described among the 12-mer ferritins, it is feasible to assume that nature created alternative ways of iron storage by using an increased number of small iron clusters.

We are very grateful to J. Kellermann for N-terminal sequencing and S. Monaco, S. Arzt (European Synchrotron Radiation Facility), and C. Schulze-Briese (Swiss Light Source) for beamline assistance.