A protein carboxylate coordinated oxo-centered tri-nuclear iron complex with possible implications for ferritin mineralization

Martin Högbom*1, Pär Nordlund*

Department of Biochemistry and Biophysics, Stockholm University Roslagstullsbacken 15, Albanova University Center, Stockholm SE-106 91, Sweden

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Abstract The crystal structure of an oxo-centered tri-nuclear iron complex formed on a protein surface is presented. The cluster forms when crystals of the class Ib ribonucleotide reductase R2 protein from Corynebacterium ammoniagenes are subjected to iron soaking. The tri-iron-oxo complex is coordinated by protein-derived carboxylate ligands arranged in a motif similar to the one found on the inner surface of ferritins and may mimic an early stage in the mineralization of iron in ferritins. In addition, the structure adds to the very limited data on protein–mineral interfaces.

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1. Introduction

Oxo-centered tri-nuclear iron complexes with a variety of organic ligands have been used to study several physio-chemical phenomena by various spectroscopic methods [1–4]. Several such complexes have been structurally as well as spectroscopically characterized in detail. These are, however, limited to clusters coordinated by relatively small organic ligands. Here, we present, to our knowledge, the first structure of a protein carboxylate coordinated oxo-centered tri-nuclear iron cluster. The cluster is formed on the surface of the ribonucleotide reductase (RNR) R2 protein from Corynebacterium ammoniagenes during soaking of the protein crystals in an aerobic Fe³⁺ containing solution.

Biominalerization processes are the basis for several important functions in biology, for example bone and tooth formation. One such extensively studied process is the formation of the iron core in ferritins, crucial for iron storage, detoxification and mobilization throughout the microbial, plant and animal kingdoms. The canonical ferritins are 24-subunit complexes arranged in 432 symmetry, forming a hollow protein shell with an outer and inner diameter of about 120 and 80 Å, respectively. The individual subunits are four-helix bundles with an additional small fifth helix at about a 60° angle to the bundle axis. The protein stores up to 4500 iron ions inside the shell deposited as hydrous ferric oxide (ferrrihydrite) with varying crystallinity and phosphate content [5–8].

Bacterioferritins are homomultimers and contain a ferrioxidase site within the four-helix bundle. This site is very similar to that found in large diiron-carboxylate oxygen activating proteins like RNR R2, methane monoxygenase and Δ-9 stearoyl-acyl carrier protein desaturase [9,10].

Mammalian ferritins, on the other hand, are hetero multimers composed of two different subunits, the H- and L-chain ferritins. The ratio between the H- and L-chains in the 24-subunit shell varies between different tissues. Although structurally very similar, the H- and L-chains are functionally radically different. The H-chains contain a ferrioxidase site similar to the bacterioferritins, but lack one of the carboxylate iron ligands, whereas the L-chain ferritins contain no such site. The ferrioxidase sites are believed to initiate iron oxidation by binding and oxidizing two ferrous ions that are then deposited on the inside of the protein shell by a poorly understood mechanism. Once the core has started to form, it expands auto catalytically [6]. L-ferritins have, despite the lack of a ferrioxidase site, also been shown to be able to initiate formation of the ferrrihydrite core, at least with large iron increments [6,11,12]. All ferritins contain several conserved carboxylate residues that are clustered in a patch on the inside surface of the ferritin sphere. Mutational studies have been performed on some of these residues and result in diminished core formation. The carboxylates are thus believed to make up nucleation sites for the mineral core, however, the mechanism of action remains unclear [6,7,11–13].

2. Materials and methods

Corynebacterium ammoniagenes class Ib ribonucleotide reductase R2 protein (R2F) was crystallized by the hanging-drop vapor-diffusion method in 24-well cell culture plates. Protein solution containing 10 mg/ml protein in 50 mM Tris–HCl at pH 7.5 was mixed in ratios varying between 1:1 and 3:1 (protein:reservoir) with the reservoir solution consisting of 30% w/v PEG 4K, 200 mM ammonium acetate and 100 mM sodium citrate at pH 6–6.5. Crystals grew after about 20 days. To be able to perform metal soaks, crystals of the apo protein (without iron) were first soaked for one hour in the same solution, but where the citrate buffer had been exchanged for 2-(4-morpholino)-ethane sulfonic acid.
acid (MES) to remove the citrate, which is a metal chelator. Crystals were subsequently soaked in the MES containing mother liquor supplemented with 15% glycerol (for cryo protection) and 10 mM Fe(NH₂)₂(SO₄)₂ under aerobic conditions for 1 h before being flash frozen in liquid nitrogen. Data were collected at beam line ID14-2 at the ESRF, Grenoble, France. The structure was solved using molecular replacement with the refined structure of the C. ammoniagenes R2F protein [14] and refined using the CNS software [15] (see Table 1 for data and refinement statistics). Coordinates and structure factors have been deposited in the pdb (www.rcsb.org/pdb), id 1OQU.

### 3. Results and discussion

In a recent X-ray crystallographic study of the diiron-radical R2F protein of RNR from C. ammoniagenes [14], several metal soaks of the protein crystals were performed under varying conditions to obtain the different oxidation states of the active site iron atoms. While refining the structure from one experiment, we observed an oxo-centered tri-nuclear iron cluster that had formed spontaneously on the surface of the protein (Fig. 1a). The tri-nuclear iron complex (Fig. 1b and c) can be described as an oxo centered cluster with two shorter and one longer Fe coordination to the central oxygen. The core cluster is similar to several previously reported tri-nuclear iron clusters with carboxylate as well as nitrogen dominated organic ligands [2,16–19]. There are six iron-bridging ligands apart from the central oxo atom. Five of these are protein-derived carboxylates and the sixth is assigned as an acetate molecule because of the good fit to the electron density and its presence in the mother liquor at high concentration (200 mM). In addition, there are three terminal ligands assigned as water molecules, completing the octahedral coordination environment of the iron ions.

Table 1

<table>
<thead>
<tr>
<th>Data collection and refinement statistics</th>
<th>C. ammoniagenes R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2₁</td>
</tr>
<tr>
<td>Cell parameters (Å): a; b; c</td>
<td>49.32; 91.24; 136.96</td>
</tr>
<tr>
<td>Unique angle β (°)</td>
<td>91.46</td>
</tr>
<tr>
<td>Resolution (Å) (outer shell)</td>
<td>20.2–2.0 (2.03–2.00)</td>
</tr>
<tr>
<td>No. of observations; unique reflections</td>
<td>185,656; 80,865</td>
</tr>
<tr>
<td>Rcryst (outer shell)</td>
<td>0.073 (0.277)</td>
</tr>
<tr>
<td>I/σ(I) (outer shell)</td>
<td>8.6 (4.0)</td>
</tr>
<tr>
<td>Completeness (%) (outer shell)</td>
<td>98.5 (99.7)</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
</tr>
<tr>
<td>Rcryst (%); Rfree (5% of data) (%)</td>
<td>18.1; 23.9</td>
</tr>
<tr>
<td>RMS dev. bonds (Å); angles (°)</td>
<td>0.014; 1.50</td>
</tr>
</tbody>
</table>

Ramachandran plot, % of residues

| Most favored; allowed; disallowed       | 96.8; 3.2; 0.0    |

*aRcryst = ∑₀|h|(|I₀| - |I₀|)/ ∑₀|h|, where |I₀| is the jth observation of reflection h.

*bRfree = (∑₀|I₀| - |I₀|)/|I₀|, where |I₀| and |I₀| are the observed and calculated structure factor amplitudes, respectively.

Refinement

| Coordinates | 1OQU |

One appealing possibility is that the present cluster is involved in the mechanism of iron incorporation into the R2 protein, however, since the cluster coordinating carboxylates are not conserved among the RNR R2 sequences it seems unlikely that the present cluster is biologically relevant in this system. In contrast, all ferritins contain several conserved carboxylate residues, the L-chains contain five conserved glutamates in helix 2 (E53, E56, E57, E60, and E63 – Homo sapiens numbering). The ExxEExxE motif clusters the carboxylics in a patch on the inside surface of the ferritin sphere. The H-chain ferritins and bacterioferritins have somewhat fewer and less conserved carboxylates in this area [5,6]. L-chains have been shown to be better at promoting core nucleation than H-chains, in addition, mutations of the inner surface carboxylate residues lead to diminished core formation in both H- and L-ferritins. Based on these studies, the carboxylates are believed to make up nucleation sites for the mineral core [6,7,11–13]. Structures of functional inner surface ferritin metal sites are likely to be very hard to obtain as they can be considered to be transition states leading to rapid formation of the large mineral core. For this reason, the interaction between the protein and the mineral core has not been structurally described, indeed, there is generally very little high-resolution information on protein–mineral interfaces [20].

The mechanism of action of the inner surface carboxylates thus remains unclear; do they simply provide a negatively charged microenvironment favoring core formation, as has been discussed, or do they form a specific site for a defined nucleation event? Tri-nuclear Fe-oxy species has been indicated in young ferritin cores by Mössbauer spectroscopy [21]. A carboxylate coordinated tri-iron cluster is an attractive model for such an initial species. The important question whether the previously reported clusters are relevant models has, however, been difficult to answer as they mainly have symmetric coordination environments provided by small ligands, designed to accommodate the geometry imposed by the tri-nuclear iron core. Protein derived ligands, on the other hand, are limited in their conformational space by the protein backbone structure, making it hard to assess the plausibility for formation of such a
cluster in an, inevitably, asymmetric protein coordination environment. The present structure shows not only that it is possible but also that the protein derived carboxylates are, in this case, able to adopt to an almost perfectly octahedral coordination environment in the primary coordination sphere of all three iron ions. The flexibility of the glutamate side chain, or a protein loop structure, is likely an advantage as the potential energetic cost of clustering negatively charged carboxylates before iron binding then can be reduced by separating the charges in space, as observed for E245 in this case.

In conclusion, the conserved carboxylate residues on the inner surface of ferritins are involved in mineral core formation by a poorly understood mechanism, specific or unspecific. The present cluster demonstrates the possibility for these residues to play a specific role, which may be exemplified by the present biomineral core complex. In addition, the present structure opens for the possibility that this type of basic carboxylate-Fe₃ cluster might also exist as a cofactor in other protein systems.

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References