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Optical and EPR spectroscopic studies of demetallation of hemin by L-chain apoferritins

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Abstract

Earlier crystallographic and spectroscopic studies had shown that horse spleen apoferritin was capable of removing the metal ion from hemin (Fe(III)-protoporphyrin IX) [G. Précigoux, J. Yariv, B. Gallois, A. Dautant, C. Courseille, B. Langlois d'Estaintot, Acta Cryst. D50 (1994) 739–743; R.R. Crichton, J.A. Soruco, F. Roland, M.A. Michaux, B. Gallois, G. Précigoux, J.-P. Mahy, D. Mansuy, Biochemistry 36 (1997) 15049–15054]. We have carried out a detailed re-analysis of this phenomenon using both horse spleen and recombinant horse L-chain apoferritins, by electron paramagnetic resonance spectroscopy (EPR) to unequivocally distinguish between heme and non-heme iron. On the basis of site-directed mutagenesis and chemical modification of carboxyl residues, our results show that the UV–visible difference spectroscopic method that was used to establish the mechanism of demetallation is not representative of hemin demetallation. EPR spectroscopy does establish, as in the initial crystallographic investigation, that hemin demetallation occurs, but it is much slower. The signal at $g = 4.3$ corresponding to high spin non-heme-iron (III) increases while the signal at $g = 6$ corresponding to heme-iron decreases. Demetallation by the mutant protein, while slower than the wild-type, still occurs, suggesting that the mechanism of demetallation does not only involve the cluster of four glutamate residues (Glu 53, 56, 57, 60), proposed in earlier studies. However, the mutant protein had lost its capacity to incorporate iron, as had the native protein in which the four Glu residues had been chemically modified. Interestingly, a signal at $g = 1.94$ is also observed. This signal most likely corresponds to a mixed-valence Fe(II)–Fe(III) cluster suggesting that a redox reaction may also be involved in the mechanism of demetallation.

Keywords: Apoferritin; Hemin; Demetallation; EPR

1. Introduction

Sequestration of iron in a soluble, bioavailable and non-toxic form within the biosphere is achieved by multimeric iron-storage proteins, ferritins. They are widely distributed throughout most species, and can be divided into two classes, ferritins (FTNs) which contain only non-heme iron and bacterioferritins (BFRs), which contain both heme and non-heme iron. Both classes have a highly conserved 3-dimensional structure [3,4] composed of 24 polypeptide chains. The subunits are related by 432 symmetry (Fig. 1) and form a hollow shell of outside diameter 12–13 nm and inside diameter 7–8 nm, and M_r of about 500,000, delimiting an internal cavity which can store up to 4500 iron atoms in the form of a ferric hydroxyphosphate core with a structure similar to that of the geological mineral ferrihydrite [3,4]. The Dps family of proteins (*D*NA-binding *p*rotein from *s*tarved cells), including the Dps protein of *Listeria innocua*, formerly described as a ferritin [5,6], can also sequester iron in their central cavity to form a mineral core similar to ferritins. They have structural, though

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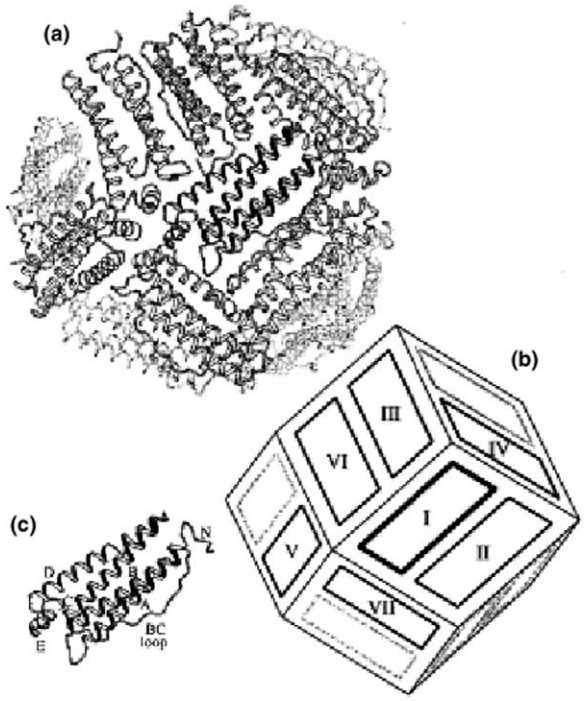

Fig. 1. (a) Overview of a ferritin molecule showing the relative positions and interfaces between symmetry related subunits; (b) labelling scheme of symmetry related subunits; (c) detail of a single ferritin subunit.

not sequence homology with ferritins [7,8] and are dodecameric proteins with 3/2 tetrahedral symmetry.

Bacteria contain both BFRs and FTNs [9]. The first heme-containing BFR was isolated from *A. vinelandii* [10], but from closer examination it became clear [11] that this protein had been previously identified in 1973 as a *b*-type cytochrome [12]. Since then, the presence of bacterioferritins has been established in more than 20 other prokaryotes. The X-ray structures of the bacterioferritins from *Escherichia coli*, *R. capsulatus* and, more recently *D. desulfuricans* have been determined [13–15]. The heme is localised in a hydrophobic pocket situated along the 3-fold symmetry axes, with the iron coordinated axially by two Met residues from symmetry-related subunits, and the porphyrin is clamped in place by numerous van der Waal's contacts with the protein [13]. As expected for a *b*-type heme, the porphyrin was shown to be protoporphyrin IX. The BFR from the anaerobic sulphate reducing bacterium *D. desulfuricans* has been isolated and its X-ray structure determined [15]. As isolated it contains a stable di-iron centre and one heme/subunit dimer. However, quite unexpectedly, it does not contain a *b*-type heme in contrast with all other known BFRs. The heme was unambiguously identified as iron-coproporphyrin III, the first example of such prosthethic group in biological systems [16,17].

The role of heme in bacterioferritins is unknown, although it has been suggested that it could play a role in iron release by mediating electron transfer through the protein shell [9]. However, the removal of heme from *A. vinelandii* holobacterioferritin had no apparent effect on the rate of iron core reduction, at least under the conditions employed [18]. In contrast a kinetic study of iron reduction and release from bacterioferritin has shown that, initially, the heme group is reduced, and then the iron core is reduced by the reduced heme [19] which suggests a role for heme in electron transfer. Mutagenesis studies have shown that the heme group is not necessary for either assembly of the protein or iron uptake [20].

Eucaryotic ferritins have usually been considered to be non-heme proteins. However, in vitro studies showed that it was also possible to incorporate hemin (Fe(III)-PPIX) into horse spleen apoferritin [21]. From electron paramagnetic resonance (EPR), near-infrared magnetic circular dichroism (NIR-MCD) and molecular modelling studies, it was suggested that the heme-binding site was situated around the 3-fold symmetry axes and that the ligands of the heme iron were His 114 and His 124 [22]. However, crystallographic studies of horse spleen apoferritin co-crystallised with Sn(II)-protoporphyrin IX (PPIX) or with Fe(III)-PPIX (hemin), showed that the metal-free PPIX was bound in a hydrophobic pocket along the 2-fold symmetry axes [1,23]. The metal was not found in the crystal structure. When Pt-hematoporphyrin was co-crystallised with horse spleen apoferritin, demetallation also occurred, and the metal was found close to the 3-fold axes [23]. The hydrophobic porphyrin-binding pocket in horse spleen apoferritin is similar to that found in bacterioferritins except that Met is replaced by Arg and that at the entrance of the hydrophobic pocket, there is a highly charged environment, composed of 4 Glu residues (E 53, 56, 57, 60) [1,23].

UV–visible difference spectroscopic studies of incorporation of hemin into horse spleen apoferritin and its demetallation were also carried out; they were based on the observation that the Fe(III)-PPIX-apoferritin complex absorbs at 410 nm and that the PPIX-apoferritin complex absorbs at around 365–370 nm [2]. On the basis of these results and of crystallographic observations indicating that Glu 53, 56, 57, 60 were found to be metal binding sites [24], and to be involved in nucleation of the iron core [25], a mechanism of demetallation of hemin by L-chain apoferritins was proposed [2]. This required acid conditions and involved the cluster of 4 Glu residues situated at the entrance of the hydrophobic pocket. It was proposed that at low pH the carboxyl groups protonate the nitrogens of the PPIX; the ferric iron can then be chelated by the deprotonated carboxylate cluster and finally, the demetallated PPIX can bind in the preformed hydrophobic pocket [2].

This paper reports a UV–visible spectroscopic and EPR study of the demetallation of hemin by L-chain apoferritins. We show, on the basis of results with apoferritins in which chemical modification and site-directed mutagenesis of the cluster of carboxylates has been carried out, that the UV–visible spectroscopic differences that were previously used to propose a mechanism of demetallation do not correspond to demetallation of hemin. However, EPR studies show that demetallation of hemin by L-chain apoferritins does occur, albeit much more slowly than reported in our earlier study. The mechanism, as proposed previously [2],