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Identification of the ferroxidase centre in ferritin

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Ferroxidase activity in human H-chain ferritin has been studied with the aid of site-directed mutagenesis. A site discovered by X-ray crystallography has now been identified as the ferroxidase centre. This centre is present only in H-chains and is located within the four-helix bundle of the chain fold.

Ferritin; Ferroxidase; Enzyme activity; Ferritin, recombinant

1. INTRODUCTION

Ferritin molecules are tetraeicosameric proteins housing a mineral known as ferrihydrite in a large (7.8 nm diameter) internal chamber [1]. The mechanisms of iron storage in vivo are uncertain, but in vitro iron-core formation in ferritin involves the oxidation of Fe(II) and hydrolytic polymerization of Fe(III) [1-5]. Previous work suggests that the ferritin protein shell provides catalytic centres where Fe(II) oxidation is accelerated [3,7] and nucleation centres which promote the formation of ferrihydrite [5]. However, it has also been shown that Fe(II) can be directly oxidized on the surfaces of mineral particles once nucleation has occurred, thus apparently making the protein ferroxidase centres redundant for iron-core growth [5,7]. On the other hand, if the pH is lowered to 6 or below, iron oxidation and hydrolysis can only proceed in the presence of ferritin [6,7]. Neither oxidation nor nucleation centres have so far been identified. Since Fe(III), catalytically produced in the presence of ferritin at pH 6.0, can be transferred to the protein transferrin, it has been proposed that the ferroxidase centres are on or near the outside surface of the protein shell [6]. However, recent work with ferritins, bearing amino acid sequence changes in the 3-fold inter-subunit channels, suggests that an internal site may be more likely [8].

Most of the earlier work on the mechanism of ferritin formation had been performed with horse spleen ferritin [3,6]. Like most ferritins, it is a heteropolymer of two types of polypeptide chain, known as H and L [9]. Horse spleen ferritin contains mainly L-chains, with only 10-15% H-chains [9]. It has recently been shown with the aid of recombinant ferritins [10] that ferroxidase activity is associated with H-chains and not L [7,11], confirming earlier work [12]. Nevertheless, human L-chain recombinant homopolymers can take up iron slowly at pH 7.0 to form Prussian blue-stainable ferritin even in the absence of ferroxidase centres [11].

Through a combination of X-ray crystallography, production and isolation of site-directed mutant proteins and biochemical studies we are now able to locate the ferroxidase centre at a specific site on human H-chains.

Details of the X-ray analysis of the human ferritin H-chain recombinant, CdM, will be given

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elsewhere. This recombinant bears a single amino acid sequence change (Lys 86 → Gln) to induce crystallization by formation of intermolecular metal bridge contacts. The recombinant was shown to have assembled correctly and to be active in iron uptake [8,13]. Its structure [14] is very similar to that of horse spleen apoferritin [1] and rat liver L-chain recombinant [15], but an electron density map calculated at 2.5 Å resolution shows a new metal-binding site [15]. Its three amino acid ligands are Glu 27, Glu 62 and His 65 (H-chain numbering). These residues are conserved in all known H-chain sequences, but are absent from L-chains (see [2]). We have introduced two additional changes into recombinant CdM, namely Glu 62 → Lys and His 65 → Gly (H to L subunit changes) and show that this altered protein (described as 222) has lost its ferroxidase activity.

2. MATERIALS AND METHODS

The expression and isolation of recombinant human H-chain ferritins, the monitoring of purity and assembly by native and denaturing gel electrophoresis and the addition of iron were all carried out as described in [8]. Iron was also added at 1000 Fe(II) atoms/apoferritin molecule at pH 7.0 and uptake monitored by gel electrophoresis and staining with ferrocyanide as in [7,11]. Ferroxidase activity was assayed via the transferrin reaction as in [6,7].

3. RESULTS AND DISCUSSION

Fig.1 shows the results of electrophoresis in 6% polyacrylamide gels of human H-chain mutant 222, CdM, horse spleen ferritin and recombinant rat liver ferritin. 0.4 mM ferrous ammonium sulphate was added to the proteins in 0.1 M Mops buffer (pH 7.0) to give approx. 1000 Fe atoms/molecule and the samples left for 3 h before electrophoresis. At that time all the samples had the same characteristic brown colour of ferritin. The reconstituted ferritins were stained for protein and iron (fig.1a,b, respectively). Mutant 222 migrated like CdM and had also taken up some iron, although the intensity of the Prussian blue stain was less than that of the other ferritins, including the recombinant rat liver L-chain ferritin. Possibly some non-specific Fe(II) oxidation had occurred outside the protein shell, since in the same experiment, when Fe(II) oxidation was carried out in the presence of bovine serum albumin (BSA) as

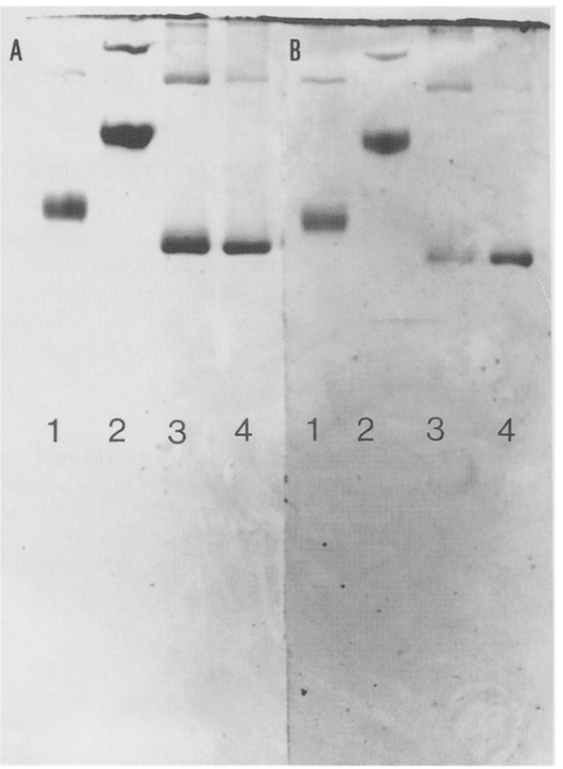

Fig.1. Electrophoresis of ferritin in a 6% polyacrylamide gel; (A) stained for protein with Coomassie blue; (B) stained for iron with potassium ferrocyanide. Tracks: (1) horse spleen ferritin, (2) recombinant rat L-chain, (3) 222, (4) CdM.

a protective colloid, no precipitation of iron oxide occurred, but the BSA did not stain positively for iron. Any iron complex bound to BSA may have dissociated during gel electrophoresis.

The rate at which Fe(II) was oxidized was studied at pH 6.45 and 7.0. Fig.2 shows that, at both pH values, the rate of oxidation of 250 Fe(II) atoms/molecule at a concentration of 50 μM Fe, was negligible for 222 vs that for either CdM or horse spleen apoferritin (measured as increase in absorbance at 310 nm). The rate of formation of the ferric transferrin complex from apotransferrin in the presence of apoferritin was also negligible for 222 vs CdM or horse spleen apoferritin, when 250 Fe(II) atoms/molecule were added at pH 6.0. The formation of an initial Fe(III) complex, which occurred extremely rapidly with CdM, and with mutant proteins bearing changes in the 3-fold channels at pH 6.45 [8,15], was not observed with 222 (not shown).

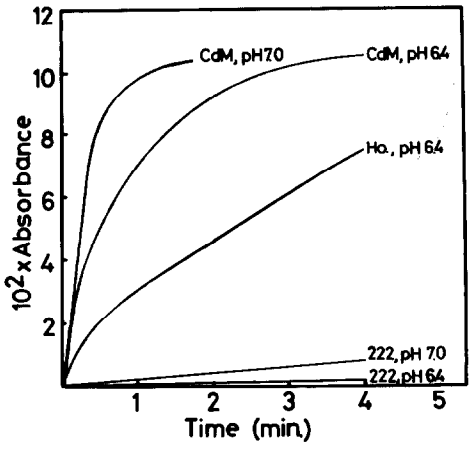

Fig.2. Progress curves of iron uptake by H-chain recombinants and horse spleen apoferritin. Iron uptake was followed at 310 nm. 0.05 M Mes (pH 6.4) or 0.05 M Mops (pH 7.0). 50 μM Fe(II) and 0.1 mg/ml protein.

Crystallization trials have yielded small (<0.1 mm) octahedral crystals of mutant 222. These appear morphologically identical to crystals of CdM and therefore constitute further evidence that correct subunit assembly has taken place.

The results show that although subunit assembly (in mutant 222) had occurred as judged from the gel electrophoresis pattern (fig.1) and by crystallization, ferroxidase activity had been eliminated by the site-directed mutations. Hence, we propose that the metal site identified by X-ray analysis in H-chains is responsible for the catalytic activity. A schematic drawing of this site is shown in fig.3. The iron atom has tetrahedral co-ordination with protein ligands Glu 27, Glu 62 and His 65 and the fourth co-ordination site is occupied by a peak in the electron density map that may be attributed to a water molecule. This could possibly represent the binding site for dioxygen during Fe(II) oxidation. The proposed Fe(II)-oxidation site lies within the subunit about 7–10 Å from the internal surface within the 4-helix bundle (shell thickness about 24 Å). Communication with both outside and inside surfaces of the shell seems to be possible by means of narrow channels through the protein. These may be the routes by which Fe(III) may migrate outwards to form a complex with transferrin (which is too large to enter the apoferritin molecule), or inwards to form core. Evidence that Fe(III) migrates from its initial oxidation site (in horse spleen ferritin) to form core

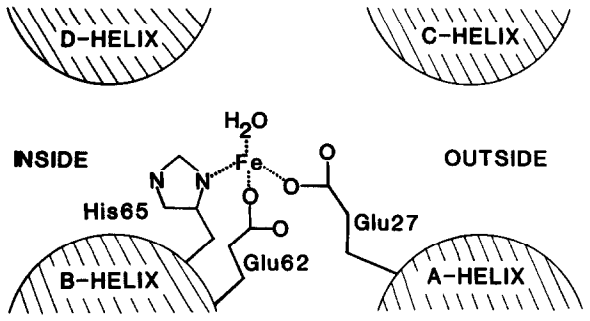

Fig.3. Schematic drawing of proposed ferroxidase centre in recombinant H-chain ferritin.

clusters has been provided by EPR [17] and Mossbauer [18] spectroscopy. This migration could be aided by displacement of further incoming iron as has been suggested previously [19]. However, it is clear that human [11] and rat recombinant L-chain homopolymers and also mutant 222 (fig.1) are capable of taking up iron slowly and forming core at pH 7 even in the absence of the oxidation site. Significant rates of iron incorporation occur, however, only when relatively high iron concentrations are employed and under these conditions the inter-subunit channels may also be used for entry to the cavity. The proposed core nucleation centre could be present in both H- and L-chains, and indeed L-chains may be superior at promoting core formation. However, provided iron can reach the cavity and be oxidized inside or en route, polymerization would be expected even without direct help from the protein, since even a single Fe(III) atom inside the cavity would be at a concentration (approx. 6 mM) far exceeding the solubility of Fe(III) at pH 7 (approx. 10^{-12} M). Autocatalytic Fe(II) oxidation can be significant once an initial core has been established [5,7]. Under cellular conditions, where low Fe/apoferritin may be expected, the ferroxidase activity of H-chains may be needed for core formation to proceed at a significant rate. The iron centre could also perhaps aid the passage of electrons through the shell for core reduction, but this remains to be investigated.

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