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Expression, purification, crystallization and preliminary X-ray diffraction results from *Campylobacter jejuni* ferritin

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The prokaryotic ferritin gene of *Campylobacter jejuni* was overexpressed in *Escherichia coli* under control of the bacteriophage T7 promoter and the protein (Cj-FTN) purified. Preliminary crystallization experiments have been performed using the hanging-drop vapour-diffusion method with ammonium sulfate as the precipitant. Diffraction studies show the crystals belong to the *I*432 space group (a = 151.52 Å). Structure solution by molecular replacement is in progress while crystal quality improvement is carried out.

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1. Introduction

Iron-storage proteins are the subject of extensive study (for a review, see Harrison & Arosio, 1996, and references therein). They are present in most living organisms (animals, plants, fungi and bacteria) in which they are known to store excess iron in a non-toxic form available for cellular metabolism. These proteins are of two types: ferritins and bacterioferritins (BFRs). The former, mainly encountered in eukaryotic tissues, are non-haem iron proteins, whereas the latter, mainly isolated from prokaryotes, are haemoproteins. A common feature of this protein superfamily is a well conserved three-dimensional structure, although their amino-acid sequence identities can be as low as 14% (Andrews *et al.*, 1992). The molecular entity is a hollow shell composed of 24 structurally equivalent subunits, arranged in 432 symmetry, in which up to 4500 Fe atoms can be sequestered as an inorganic complex. The protein subunit, a four-helix bundle with a fifth short terminal helix, belongs to one of two types known as H and L, depending on the amino-acid sequence. The high structural similarity of BFRs and eukaryotic ferritins has recently been confirmed with the three-dimensional structures of two different crystal forms of Ec-BFR (Frolow *et al.*, 1994; Dautant *et al.*, 1998) which, furthermore, clearly place the location of the haem groups in a pocket at the pseudo-twofold axis interface between subunits. The axial haem-iron ligands are found to be the S atoms of two equivalent Met residues (Met52 and Met52′), which are highly conserved in most bacterioferritin sequences.

In the last few years, evidence of the presence of not only bacterioferritins but also ferritins has been demonstrated in prokaryotes. Izuhara *et al.* (1991) cloned an *E. coli* gene (*gen*-165 or *rsgA*) which encodes a protein (Ec-FTN) sharing 21% amino-acid sequence identity (38% similarity) with the eukaryotic ferritins and 17% identity (32% similarity) with Ec-BFR (Hudson *et al.*, 1993). Other prokaryotic ferritins have been isolated and characterized in *Bacteroides fragilis* (Rocha *et al.*, 1992), *Helicobacter pylori* (Frazier *et al.*, 1993; Doig *et al.*, 1993) and, more recently, in *C. jejuni* (Wai *et al.*, 1995). Finally, two such proteins have been identified in *Haemophilus influenzae* (Fleischmann *et al.*, 1995) and presumably a new one in a 460 kbp DNA sequence of the *E. coli* K-12 genome (Itoh *et al.*, 1996).

Little structural information is available on prokaryotic ferritins. The structure determination of Ec-FTN is in progress (Harrison & Arosio, 1996). Even though atomic coordinates are not yet available, some main structural features have already been discussed, such as the location of a di-iron site embedded in the four-helix bundle and of another iron site lying on the inner surface of the protein shell (Hempstead *et al.*, 1994).

Nevertheless, several structural aspects still remain undetermined. For instance, it is premature to assert that a pocket similar to that found in bacterioferritins at the haem-binding site exists in prokaryotic ferritins. A first clue will be given with the structural description of Ec-FTN, the sequence of which presents a Met residue at position 52, as in most bacterioferritin sequences. A more complete answer will be given in the case where no methionine lies at that position. This is one reason why we have overexpressed, purified and initiated a crystallization study of *C. jejuni* ferritin (Cj-FTN).[1] This protein is composed of a single chain of 167 residues sharing 46% amino-acid sequence identity

[1] In order to adopt a common nomenclature for the prokaryotic ferritin family, the *C. jejuni* ferritin is designated as Cj-FTN rather than CFT as previously quoted.

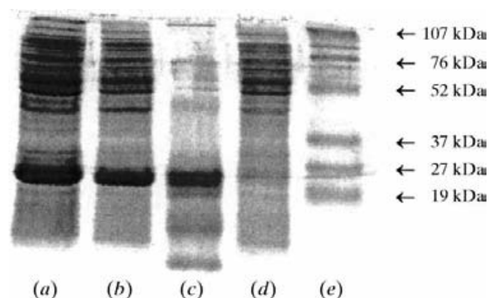

Figure 1
12% SDS–PAGE gel, Coomassie blue staining. Lanes (*a*) and (*b*), total proteins from induced cells (grown at 310 K and induced for 3 h with 40 µ*M* IPTG). Lane (*c*), horse spleen apoferritin. Lane (*d*), total proteins of uninduced cells. Lane (*e*), Bio-Rad low-range molecular-weight markers (107–19 kDa).

(66% similarity) with Ec-FTN. It is noteworthy that Met52 is replaced by a serine in Cj-FTN. In this paper, we present the methods used for expression and purification, followed by the preliminary crystallization and X-ray diffraction results obtained at 3.8 Å resolution.

2. Experimental procedure

2.1. Expression and purification

As previously described (Wai *et al.*, 1997) the pET3b vector from Novagen Inc. (Madison, USA) was used for construction of a Cj-FTN over-expressing plasmid (pET3b-CFT), and *E. coli* strain BL21(DE3) was the host for Cj-FTN overexpression. The *Cj-FTN* gene in the *E. coli* strain was expressed under control of the bacteriophage T7 promoter. The cells were inoculated in 2 × 400 ml LB medium containing 50 µl ml^{-1} ampicillin. Expression was induced for 3 h at 310 K by addition of 0.4 m*M* isopropyl-β-D-thiogalactoside (IPTG) to the medium. The suspension was cooled to 273 K then centrifuged (4000*g*, 10 min, 277 K). The resulting pellet was resuspended in PBS buffer (1 ml per 2 ml of initial growth medium), centrifuged and resuspended again in 250 µl TET buffer [0.1 *M* Tris–HCl, pH 8, 1%(*w*/*v*) TWEEN 20]. The proteins were released from the cells by sonication (4 × 2 min, 277 K) and separated from the bacterial debris by centrifugation (10 000*g*, 20 min, 277 K). The supernatants were collected and subjected to a thermal denaturation step at 348 K for 10 min in a shaking water bath, in order to eliminate the contaminating proteins sensitive to temperature. The protein solution was precipitated by addition of 70% ammonium sulfate (1 h, 277 K). After centrifugation (10 000*g*, 20 min, 277 K), the pellet was resuspended in 20 m*M* Tris–HCl buffer pH 7.5, dialysed against the same buffer and 27 ml of protein solution were obtained. The presence of the Cj-FTN protein was checked (Fig. 1) on 12% SDS–PAGE according to the method of Laemmli (1970), and the protein concentration (3.0 mg ml^{-1}) was estimated by the Bio-Rad R250 reagent at 595 nm.

The Cj-FTN solution was applied to a MonoQ HR 10 × 100 mm anion-exchange column (Protein Waters). The system was equilibrated with 20 m*M* Tris–HCl buffer pH 7.5 and the protein was eluted with a linear gradient from 0 to 1 *M* NaCl at a flow rate of 1 ml min^{-1}. The elution of the protein was monitored by UV spectroscopy at 280 nm and corresponds to 0.45 *M* NaCl concentration (Fig. 2), a value very close to the one reported by Wai *et al.* (1995). The eluted peaks were checked by SDS–PAGE for their molecular weight. Figs. 2(*a*) and 2(*b*) show the elution profiles for crude and purified Cj-FTN, respectively. The fractions containing the protein were extensively dialysed against 20 m*M* Tris–HCl pH 7.5 and concentrated in Slide-A-Lyser 0.5–3.0 ml cassettes, M_W cutoff = 16 kDa (Pierce/Biotech Company, Rockford, USA) to yield 300 µl of Cj-FTN at a final concentration of 3 mg ml^{-1}.

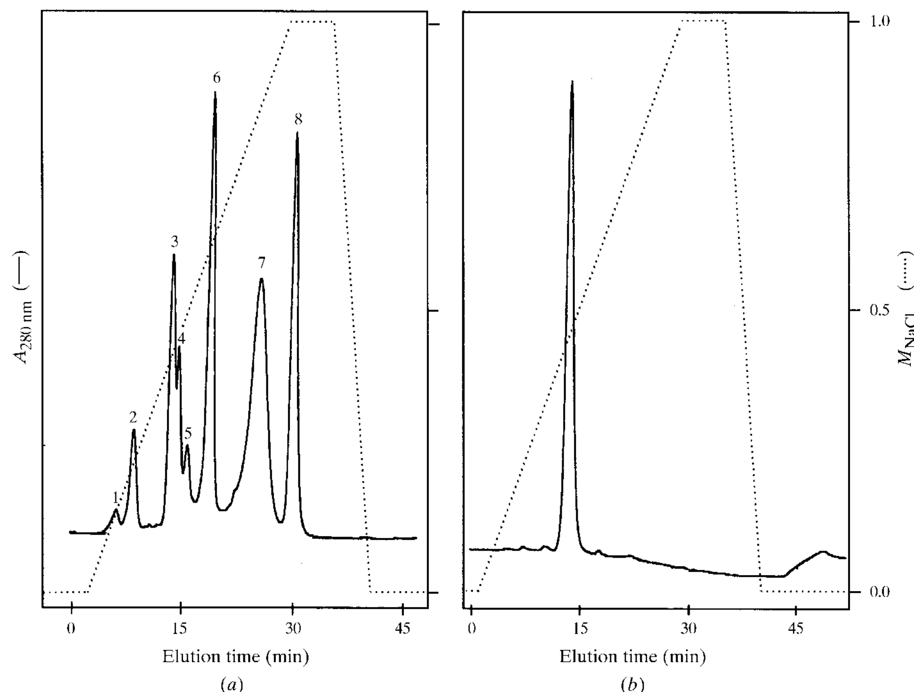

Figure 2
Elution profiles of Cj-FTN solution from an anion-exchange MonoQ column. (*a*) Crude chromatogram; the Cj-FTN protein is eluted in peak 3 (elution time: 13.7 min) corresponding to 0.45 *M* NaCl. (*b*) Final chromatogram after purification.

2.2. Crystallization

Colourless Cj-FTN crystals were obtained by the hanging-drop vapour-diffusion method at room temperature. Initial screening for the crystallization conditions of Cj-FTN (3 mg ml^{-1}) was carried out using those conditions which have previously been found to yield apoferritin crystals (ammonium sulfate in presence of cadmium sulfate; Granier *et al.*, 1997; Langlois d'Estaintot *et al.*, 1996). These experiments yielded only amorphous precipitates. Therefore, the Grid Screen Ammonium Sulfate Kit (Hampton Research, Laguna Hills, CA, USA) was tried. Some very small single crystals were obtained with 2.4 and 3.2 *M* ammonium sulfate in 0.1 *M* Bicine buffer pH 9. After optimization of the crystallization conditions, drops containing 2 µl of protein solution (3 mg ml^{-1} in 20 m*M* Tris–HCl pH 7.5) and 2 µl of reservoir solution (2.3–2.7 *M* ammonium sulfate in 0.1 *M* Bicine buffer pH 8.25–8.75) were equilibrated against 1 ml of reservoir solution at 293 K. Crystals appeared within a week and grew to their final dimensions (0.25 × 0.25 × 0.25 mm) in the following two weeks (Fig. 3).

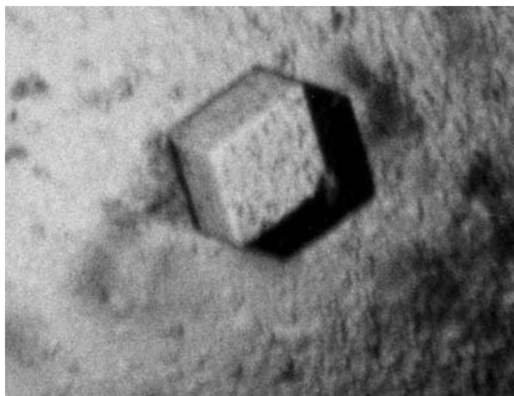

Figure 3
Photograph of Cj-FTN crystal obtained by the hanging-drop vapour-diffusion method. The dimensions are 0.25 × 0.25 × 0.25 mm.

3. Preliminary results and discussion

X-ray diffraction took place at 293 K using a Cu $K\alpha$ rotating-anode generator and a 300 mm MAR Research image-plate detector (crystal-to-detector distance set to 350 mm). Data from 19 frames of 3.0° crystal oscillation with 40 min exposure time were collected to 3.8 Å resolution. All images were processed using the *MOSFLM* software (Leslie *et al.*, 1996). The crystal belongs to the cubic space group $I432$ with unit-cell parameters $a = b = c = 151.52$ Å. This leads to a calculated V_m value of 3.94 Å^3 Da^{-1} and a solvent content of 68.6%, assuming one subunit in the asymetric unit (Matthews, 1968). Further data analysis was performed with the *ROTAVATA* and *AGROVATA* programs from the *CCP4* package (Collaborative Computational Project, Number 4, 1994). The whole recorded data set from 21.0 to 3.8 Å gives a total number of 31737 reflections, which is reduced to 2667 unique reflections with an $R_{\rm sym}$ value of 0.28. This unsuitably high value of $R_{\rm sym}$ drops to 0.16 when reflections belonging to the 4.5–3.8 Å outer resolution range, which have low intensity [mean value of $I/\sigma(I)$ = 1.6] are excluded.

The final data set (from 21 to 4.5 Å), which will be used for an early structure determination, is 97.6% complete. It includes a total number of 22218 reflections reduced to 1887 unique reflections (multiplicity 11.7), 86.3% of which have $I > 3\sigma(I)$. The structure solution is in progress using molecular replacement with an apoferritin molecule built from the 1FHA entry of the Brookhaven Protein Data Bank as a starting model.

At this point in our study, we are aware of the limited diffraction quality of this first batch of crystals. With such a low resolution (4.5 Å), many aspects of the structure and function may remain unanswered. Further crystallization trials are under active investigation.

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