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Acta Cryst. (1993). D**49**, 597-600

Location of Haem in Bacterioferritin of *E. coli**

BY FELIX FROLOW [1], A. JOSEPH KALB (GILBOA) [1] AND JOSEPH YARIV [2]

[1] *Department of Structural Biology, The Weizmann Institute of Science, Rehovot* 76100, *Israel*

[2] *Laboratoire de Cristallographie et Physique Cristalline, Université de Bordeaux* I, 33405 *Talence, France*

(*Received* 13 *June* 1993; *accepted* 5 *July* 1993)

Abstract. A low-resolution partial structure of bacterioferritin was solved using a combination of molecular replacement and rigid-body refinement methods. Modification of bacterioferritin crystals by soaking in tetrachloroplatinate results in a phase transition from tetragonal symmetry (space group $P4_22_12$) to a pseudo-cubic one (approximate space group $I432$). Helical parts of human H ferritin structure stripped of side chains beyond the C^β atoms were used as the model. An electron-density map of the refined model revealed a region of extended density which by its shape and position in a pocket between helices was identified as haem. Inclusion of haem in the refinement showed that it can occupy only one of two symmetry-related sites near a twofold axis of the molecule.

Introduction. Similarity of the structures of bacterioferritin of *E. coli* and of eucaryotic ferritin has been amply demonstrated in the first description of this fascinating protein (Yariv *et al.*, 1981). Both structures are those of a hollow protein ball composed of 24 identical subunits arranged according to point symmetry 432. While this symmetry holds strictly for eucaryotic ferritin (Banyard, Stammers & Harrison, 1978), it must break for bacterioferritin because of haem stoichiometry which is maximally that of 12 haems per molecule [Smith, Ford, Harrison, Yariv & Kalb (Gilboa), 1989; Laulhère, Labouré, van Wuytswinkel, Gagnon & Briat, 1992]. Solution of the structure, therefore, must first and foremost answer the question: is the haem located on a twofold symmetry axis of the molecule or is it located in a general position? If the latter is the case, the structure must explain why, of the 24 haem-binding sites present, only 12, at most, can be occupied simultaneously. With these limited objectives in mind, it seemed worthwhile to subject the low-resolution data of a cubic modification of a well diffracting tetragonal crystal of bacterioferritin [Smith, Ford, Harrison, Yariv & Kalb (Gilboa), 1989] to structural analysis. Additional incentives for solving the structure of bacterioferritin even at a low resolution are the recent demonstration that horse spleen ferritin (HSF) binds haem (Kadir & Moore, 1990) and the subsequent solution of the structure of a protoporphyrin IX-HSF complex (Précigoux *et al.*, 1993).

Methods. The cubic modification of tetragonal bacterioferritin crystals was prepared by soaking a tetragonal crystal in 10^{-2} *M* K_2PtCl_4 in the crystallization medium for 24 h, as described [Smith, Ford, Harrison, Yariv & Kalb (Gilboa), 1989]. The crystal was mounted in a sealed thin-walled glass capillary tube containing a drop of the soaking solution at each end. Data were collected by the oscillation method with a Xentronics area detector at a rate of 0.25° per frame per 70 s. The X-ray beam was produced by a Rigaku rotating copper anode operating at 250 mA and 40 kV and was monochromatized by means of a graphite monochromator. An evacuated pinhole collimator (length 30 cm) positioned 3 mm from the crystal was used. The crystal-to-detector distance was 220 mm and the azimuthal offset of the detector was 7°. The data were processed by means of *XDS* (Kabsch, 1988).

The cubic modification of bacterioferritin which we have treated as $I432$ is actually pseudo-cubic. This is suggested by the value of R_{sym} (Table 1) which is considerably higher than one would expect for a good

* This publication is dedicated to the memory of John M. A. Smith whose studies into the structure of bacterioferritin were interrupted by his untimely death.

space-group assignment. Indeed, 73 of the 700 strong reflections used in auto-indexing do not correspond to *I*432 reciprocal lattice points. Statistics for the pseudo-cubic and tetragonal data are summarized in Table 1.

Self-rotation analysis of diffraction data collected between 10 and 4 Å resolution for the parent tetragonal crystal was performed by means of the general and locked rotation function as implemented in the molecular replacement package *REPLACE* (Tong, 1993). Two-dimensional searches in spherical-polar coordinates with κ set at 90, 120 and 180° established the orientations of the non-crystallographic two-, three- and fourfold axes. This was confirmed by means of a locked rotation function where all the local symmetry elements of point group 432 were simultaneously included. Rigid-body refinement was performed by means of *X-PLOR* version 3.1 (Brünger, Kuriyan & Karplus, 1987) implemented on IBM RISC 6000 or Silicon Graphics workstations.

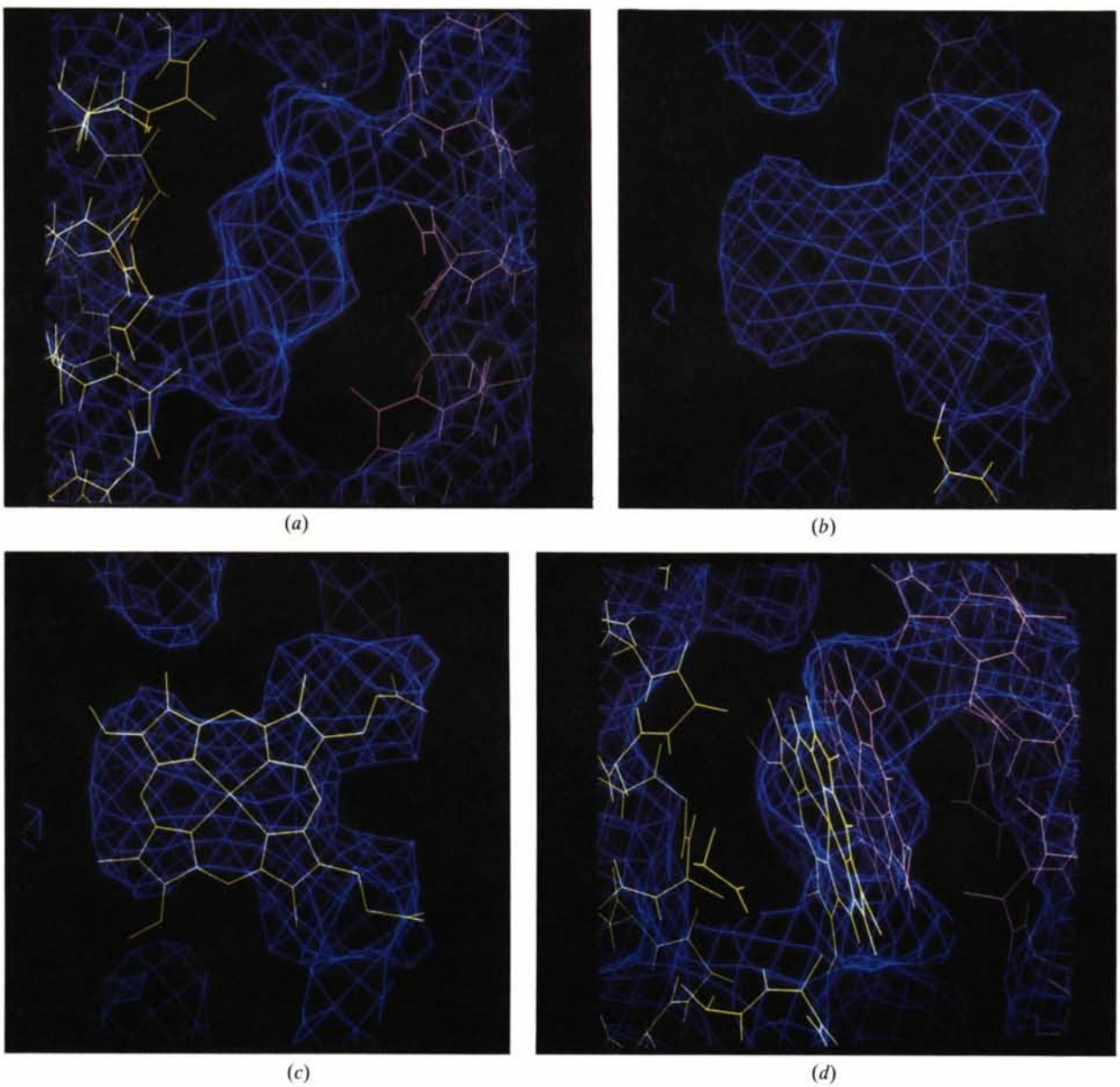

Fig. 1. $2|F_o| - |F_c|$ electron-density maps based on refinement of *A*, *B*, *C* and *D* helices (stage III in Table 1). (*a*) The electron-density map around the twofold axis. The original molecule is yellow, that which is symmetry related is red. Helices *B* (shown) are proximal and *A* are distal (not shown). The twofold axis is perpendicular to the plane. (*b*) The same electron density rotated 90°. The twofold axis is in the plane. (*c*) Model of haem superimposed on the electron density. (*d*) Final haem coordinates (stage IV, Table 1) superimposed on the electron-density map as in (*a*). The distance between best planes of the two symmetry related haem molecules is 1.8 Å.

Table 1. *Crystallographic parameters and data-collection statistics for tetragonal bacterioferritin and its cubic modification*

	Tetragonal	Pseudo-cubic
Space group	$P4_32_12$ (No. 94)	$I432$ (No. 211)
Cell dimensions (Å)	$a = b = 211.1$, $c = 145.2$	$a = b = c = 148.5$
Subunits per asymmetric unit	12	1
Resolution (Å)	3.6	4.5
Reflections		
Measured	153450	13096
Unique	42239	1637
Completeness (%)	94	97
R_{sym} (%)	14	24

Visualization and manipulation of maps and models were carried out with *FRODO* (Jones, 1978) implemented on an Evans & Sutherland Picture System (model PS300).

Results. In contrast to the parent tetragonal crystals, the modified crystal diffracted rather poorly and the data were complete to only 4.5 Å. After 24 h of exposure to the X-ray beam, the observable resolution limit had deteriorated even further and data collection was halted.

The crystal parameters of the cubic modification and those of the parent crystal are compared in Table 1. It has been pointed out that the tetragonal cell is metrically related to the cubic cell, where the pseudo-cubic cell edges correspond to its x-y face diagonals and z axis [Smith, Ford, Harrison, Yariv & Kalb (Gilboa), 1989]. This is borne out by a locked rotation-function analysis (Tong & Rossmann, 1990) of the tetragonal data against a standard 432 point-group Patterson map in which self rotation of 45° about the z axis produced a rotation-function peak approximately 80% as high as the identity peak (data not shown).

The cubic structure was solved by molecular replacement with human H ferritin (Brookhaven Protein Data Bank code 1FHA, Bernstein *et al.*, 1977) stripped of loops and of side chains beyond the C^β atom as the starting model. The coordinates correspond to one of the 24 subunits of human ferritin which generates a full spherical shell centered at the origin of its $F432$ cell. In the cubic modification of bacterioferritin, the asymmetric unit also corresponds to one subunit and the spherical shell is centered at the origin and has a diameter which is nearly equal to that of human ferritin. This close correspondence allowed us to skip the usual cross-rotation and translation searches and the analysis was performed entirely by rigid-body refinement of the model coordinates by means of *X-PLOR* (Brünger, Kuriyan & Karplus, 1987). The starting model included all five helices of human ferritin, *A*, *B*, *C*, *D* and *E*, but this was later modified by elimination of the small helix *E* which appeared to be poorly placed relative to the electron-density maps described below. Rigid-body refinement was carried out in stages and is summarized in Table 2. First, the entire model was refined as a single rigid body. The resulting coordinates were then refined as two bodies corresponding to helix *A* with *B* and helix *C* with *D*, and finally, as four individual helices. At this stage, an electron-density map was calculated with phase angles from the coordinates of the refined model and amplitudes corresponding to $2|F_{obs}| - |F_{calc}|$. In addition to electron density corresponding to the helices of the model structure, the map shows a clearly defined region of electron density corresponding closely to the dimensions of a haem ring positioned on the crystallographic dyad axis (*h* in Wyckoff notation for the space group $I432$) and occupying the space between a pair of twofold-related subunits and sandwiched between the two symmetry-related sets of helices *A* and *B* (Figs. 1*a*, 1*b*). The excellent correspondence between the haem ring and the electron density is strikingly evident in this map (Fig. 1*c*). Another round of rigid-body refinement with haem coordinates included, and in which the haem was refined as an independent rigid body, led to the final structure shown in Fig. 1(*d*) in which the haem is slightly displaced from the twofold axis. Accordingly, the haem is located in a general position. The observed stoichiometry of one haem per pair of subunits is evidently the result of space limitations of the pocket between the subunits which allows only one of the two potential haem-binding sites to be occupied. Since the two sites are identical, they have an equal probability of being occupied.

Table 2. *Rigid-body refinement of the cubic modification of bacterioferritin*

Group definition	Initial R factor	Final R factor	No. of cycles
(I) Entire molecule	0.484	0.435	40
(II) Helix *A* with *B*, helix *C* with *D*,	0.435	0.429	40
(III) Helix *A*, helix *B*, helix *C*, helix *D*	0.429	0.403	40
(IV) Helix *A*, helix *B*, helix *C*, helix *D*, and haem	0.403	0.385	40

Discussion. Results of this study provide experimental evidence that the secondary and tertiary structures of bacterioferritin resemble eucaryotic ferritin, in that it is a bundle of helices. Claims to this effect have been made before but with no experimental backing (Andrews, Smith, Yewdall, Guest & Harrison, 1991; Grossman, Hinton, Minak-Bernero, Slaughter & Stiefel, 1992). These authors also claim that, notwithstanding the lack of homology between bacterioferritin and eucaryotic ferritin, all ferritins have evolved from a common ancestor gene. Perhaps the strongest support for this kind of reasoning comes from the feat of Izuhara, Takamune & Takata (1991) who have found in *E. coli* a new gene (*gen*165) with a sequence 25% homologous to human H ferritin which, when expressed in *E. coli*, was found to be a ferritin (Harrison, 1993). It should be pointed out that this gene is not only distinct from bacterioferritin gene (BFR gene) but that the isolated protein does not contain haem whereas bacterioferritin, besides being an

iron-storage protein, is also a *b*-type cytochrome (Stiefel & Watt, 1979; Yariv, 1983).

Beyond the information about the secondary and tertiary structure of bacterioferritin discussed above and the demonstration that bacterioferritin does contain 24 haem-binding sites but can bind only 12 haems because of steric hindrance, it is not possible to answer many questions of general interest on the basis of the present low-resolution structure. Only at higher resolution will it be possible to identify the ligands to the haem iron in this structure. It has been claimed, on spectroscopic evidence, that the ligands to haem in bacterioferritin are two methionine S atoms (Cheesman, Thomson, Greenwood, Moore & Kadir, 1990; Grossman, Hinton, Minak-Bernero, Slaughter & Stiefel, 1992). In the structure that we have solved the haem iron is near the center of helix *B*, consistent with the possibility that Met52 is the amino acid that contributes the ligand, as suggested. But there is the interesting fact that following this methionine are Lys53 and His54 (Andrews, Harrison & Guest, 1989), all three equally eligible to donate ligands to iron. Then, there is our result that the haem is not in a special position on the twofold axis, as assumed, and that therefore the two ligands, if both are contributed by the protein, are not equidistant from iron because haem iron will always be nearer to one of the two *B* helices in the dimer (Fig. 1*d*). Furthermore, we observe strong electron density, not accounted for by the model, between helix *B* and *A* which suggests that the Met52 S atom is, perhaps, in proximity to the S atom of Met31 on helix *A* (data not shown). If these S atoms carry a free radical (*cf.* Hoffman, Roberts, Kang & Margoliash, 1981), this may suggest a mechanism for iron oxidation by bacterioferritin. If a free radical is present it can also explain the observed very low oxidation potential of bacterioferritin (Watt, Frankel, Papaefthymiou, Spartalian & Stiefel, 1986). All these matters must await solution of the structure of the tetragonal crystal which diffracts to 1.5 Å.

Note added in proof: We have used the low-resolution structure of the pseudo-cubic crystal reported here to solve the structure of the tetragonal crystal on the basis of a single isomorphous heavy-atom derivative at 2.9 Å resolution. Refinement of the structure to 2.5 Å resolution is now in progress and will be reported on completion.

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