Acta Cryst. (1994). D**50**, 739–743

A Crystallographic Study of Haem Binding to Ferritin

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(*Received* 1 *July* 1993; *accepted* 24 *March* 1994)

Abstract

Ferritin, the iron-storage protein, binds porphyrins, metalloporphyrins and the fluorescent dyes ANS (8-anilino-1-naphthalenesulfonic acid) and TNS (2-*p*-toluidinyl-6-naphthalenesulfonic acid), similarly to apo-myoglobin. Octahedral crystals of horse-spleen apo-ferritin (HSF; 174 amino acids) complexes prepared by the addition of haem, hematoporphyrin or Sn-protoporphyrin IX to a solution of apo-ferritin crystallize in space group $F432$ with cell parameter a = 184.0 Å. X-ray crystallographic analysis of single crystals prepared from a mixture containing haem or Sn-protoporphyrin IX shows that the haem-binding sites in these crystals are occupied by protoporphyrin IX, which is free of metal, rather than by the original metalloporphyrin. The present paper describes the structure of horse-spleen apo-ferritin cocrystallized with Sn-protoporphyrin IX. The 6797 reflections up to 2.6 Å resolution used in the refinement were obtained from a data set recorded on a Nicolet/Xentronics area detector with Cu $K\alpha$ radiation from a Rigaku RU 200 rotating anode. The final structure comprises 1613 non-H atoms, two Cd atoms and 170 solvent molecules. Four residues are described as disordered. The root-mean-square deviations from ideal bond lengths and angles are 0.013 Å and 2.88°, respectively. Protoporphyrins are observed in special positions on the twofold axes of the ferritin molecule with a stoichiometry of 0.4 per subunit.

Introduction

The universal iron-storage protein ferritin, when isolated from animals and plants, was known as a non-haem iron protein (Granick, 1942; Granick & Michaelis, 1943; Fankuchen, 1943; Crichton, 1973; Theil, 1987), while bacterioferritin, the ferritin of prokaryotes [Smith, Ford, Harrison, Yariv & Kalb (Gilboa), 1989; Laulhére, Laboré, Van Wuytswinkel, Gagnon & Briat, 1992], contains haem as shown by electronic absorption spectra characteristic of a *b*-type cytochrome (Yariv, 1983).

Nevertheless, using spectroscopic techniques, Kadir & Moore (1990*a*) showed more recently that HSF is able to bind haem. This result is of importance since haem is also known to act as an inducer in the expression of ferritin in vertebrates (Lin *et al.*, 1990). Therefore, haem may be an integral part of eucaryote ferritins and not only of bacterioferritins. This refocused interest on the two proteins.

Not only the location of both the haem-binding site and the ferroxidase centre, but also the stoichiometry of haem binding to HSF and to bacterioferritin have been discussed in many recent publications (Smith *et al.*, 1989; Kadir & Moore, 1990*b*; Grossman, Hinton, Minak-Bernero, Slaughter & Stiefel, 1992; Moore *et al.*, 1992; Lawson *et al.*, 1989, 1991). Comparable binding properties were found in apo-myoglobin suggesting that the haem-binding site is rather non-specific (Gibson & Antonini, 1966). Other metalloporphyrins, as well as porphyrins and fluorescent dyes bind to apo-myoglobin (Stryer, 1965; Boxer, Kuki, Wright, Katz & Xoung, 1982).

We prepared a number of crystals of ferritin complexes. Some of them were obtained by co-crystallization with the ligand and others were prepared by soaking apo-ferritin crystals in the ligand solution, always in the presence of cadmium sulfate. Detailed descriptions of crystal preparation, data collection and structure solution, as well as the comparison of the apo-ferritin structures with its protoporphyrin IX complexes will be reported in a forthcoming publication, now in preparation.

The three-dimensional structure of horse-spleen ferritin (HSF) has been solved (Clegg, Stansfield, Bourne & Harrison, 1980). It consists of 24 identical subunits arranged in a cubic 432 symmetry around a hollow core of 80 Å diameter, into which iron is deposited as ferrihydrite (Banyard, Stammers & Harrison, 1978; Ford *et al.*, 1984). The mechanism by which iron is deposited in and mobilized from ferritin remains unknown, however.

Here we present the outstanding features of the apo-HSF protoporphyrin IX complex. This is the first report of a crystallographic study of the haem-binding site in ferritin, even if, during the writing of this paper, the low-resolution structure of bacterioferritin has been solved providing evidence of a nearly similar location of the haem molecule in this structurally related protein (Yariv, 1993). Besides these results, the structure of mammalian ferritin offers no indication of a haem-binding site (Moore *et al.*, 1992).

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ISSN 0907-4449 © 1994

Coordinates of the present structure have been deposited in the Brookhaven Protein Data Bank.*

Data collection

Apo-ferritin (Sigma) complexed to Sn-protoporphyrin IX (Porphyrin) crystallized in the cubic space group $F432$ with a = 184.0 (1) Å. The cell parameters were fitted by least squares to θ values of 25 high-angle reflections measured on an Enraf–Nonius CAD-4 diffractometer operating with an Enraf–Nonius GX21 rotating anode. The data set was recorded to 2.6 Å resolution on a Nicolet/Xentronics area detector, with a Rigaku RU 200 rotating-anode source at 40 kV, 90 mA. The crystal-to-detector distance was set to 13.17 cm. A total of 37 452 intensities were measured, leading to 7426 independent reflections. Details of the individual and merged data sets are summarized in Table 1.

* Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1HRS, R1HRSSF). Free copies may be obtained through The Managing Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England (Reference: GR0267).

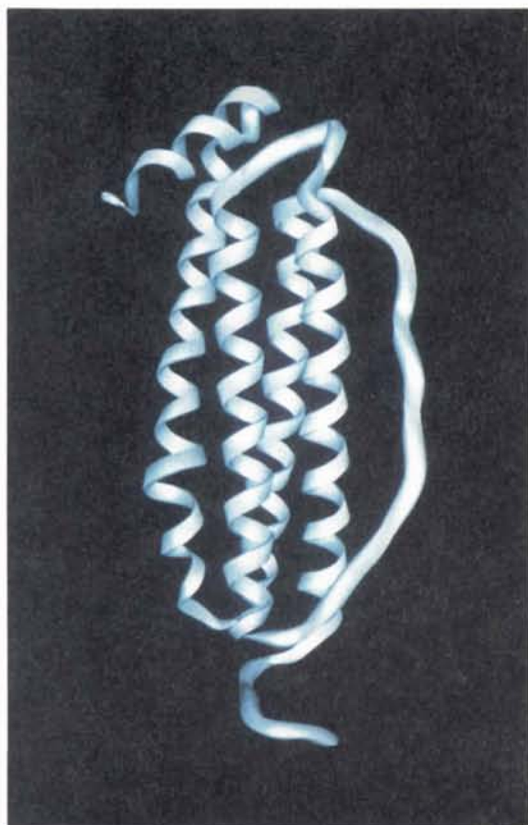

Fig. 1. Ribbon representation of the Cα backbone of a HSF subunit. The four longer α-helices A, B, C and D are antiparallel and the fifth short helix (labelled E in the text) lies at the C-terminal part of the subunit.

Structure solution and refinement

The data set used included 85% of the measurable reflections to a resolution of 2.6 Å, the outermost shell of data, $2.7 \geq d \geq 2.6$ Å, being 50% complete. To solve the structure, we used a 'quasi-molecular' replacement method starting initially with a model of horse-spleen apo-ferritin (Bourne, Harrison, Rice, Smith & Stansfield, 1982). The model was built using the Rossmann program (from the Protein Data Bank) and the Cα stereoview of a dimer published by Bourne *et al.* (1982). A few portions of the four longer helices (A, B, C, D) were regularized and the main-chain atoms C, N, O, Cα and Cβ were

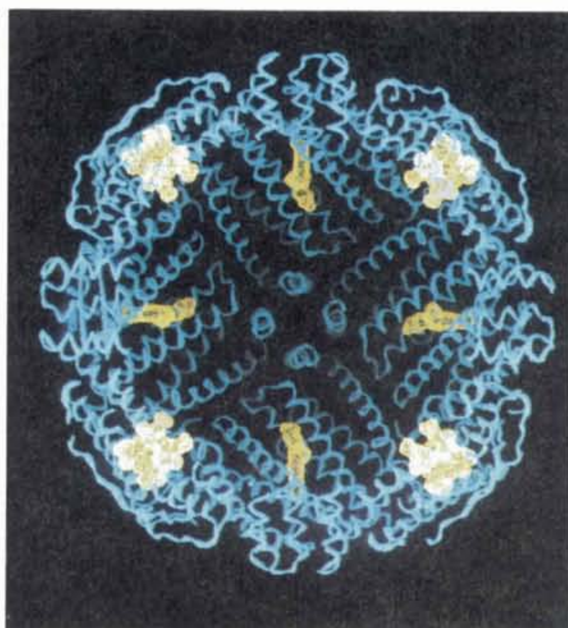

Fig. 2. Inside view of the ferritin core along the fourfold axis. The porphyrin molecules are schematically represented by their van der Waals contours. The binding site is clearly inside the shell with the propionate groups pointing towards the cavity.

Fig. 3. Stereoview of the porphyrin ($2F_o - F_c$) density map contoured at the 1.2 r.m.s. level.

Table 1. *Data collection and data processing*

Cell parameter determination	
Method for cell dimensions	Least-squares fit to measured θ values
θ data for cell dimensions	25 reflections, $21 \leq 2\theta \leq 39°$
Diffractometer	Enraf–Nonius CAD-4
X-ray source	GX21 Enraf–Nonius rotating anode
Operating conditions (kV, mA)	45, 90
Data collection	
X-ray source	Rigaku RU 200 rotating anode
Detector	Nicolet/Xentronics area detector
Measurement total	37 452
Measurement independent	7426
Measurement $I \geq 2\sigma(I)$	6797
Resolution (Å)	10–2.6

constructed using *TURBO-FRODO* (Roussel, Fontecilla-Camps & Cambillau, 1990). Several three-dimensional rigid-body minimizations of the four helices and of each helix independently using *X-PLOR* (Brünger, Kuriyan & Karplus, 1987), in the 10.0–5.0 Å resolution range, led to a starting solution. First the solution was improved by locating a Cd atom, as observed in the apo-ferritin structure (Bourne *et al.*, 1982), the Cα atoms in the neighbourhood of the twofold axis, the short fifth helix *E* and the loop between helices *D* and *E*. Visualization of the resulting crystal packing on a graphics display, with the program *TURBO-FRODO* implemented on a Silicon Graphics 4D system, showed reasonably good contacts between symmetry-related units.

Many steps of refinement were carried out using the program *X-PLOR*. Increasing the resolution range allowed us to gradually locate the side-chain atoms, leading to an *R* factor of 0.24. An overall temperature factor of 10 Å^2 was assigned to all non-H atoms. Simulated-annealing refinement was carried out with *X-PLOR*. A maximum temperature of 3000 K was slowly decreased by steps of 25 K in the 10–2.6 Å resolution range. The *R* factor decreased from 0.24 to 0.21. At this stage of the refinement, both electron-density maps with coefficients $(2F_o - F_c)$ and difference maps calculated from the protein clearly enhanced not only solvent sites but also a substantial electron density which was assigned to the protoporphyrin IX molecule. A total of 170 water molecules were gradually included in the refinement with the criteria that they displayed well defined $(F_o - F_c)$ density peaks (amplitude ≥ 2 e.s.d.'s of the electron-

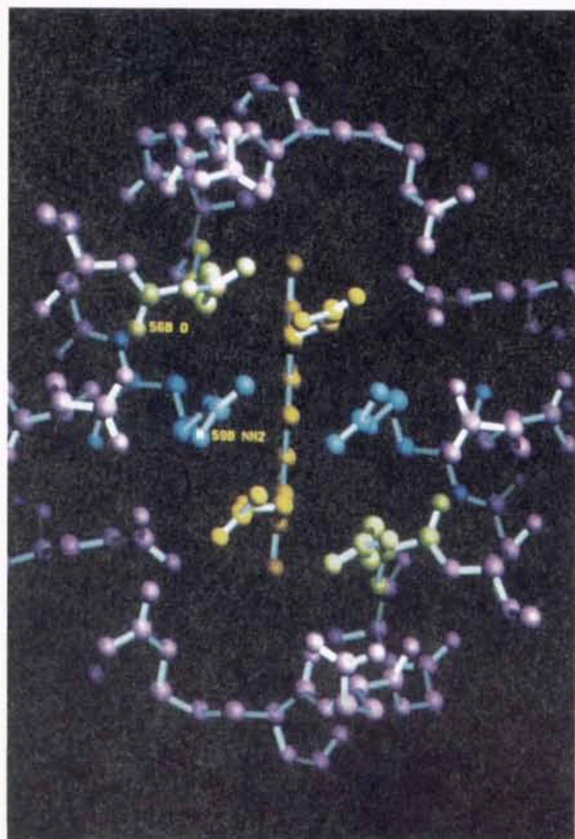

Fig. 4. Ball-and-stick representation of both the protoporphyrin IX and the surrounding amino acids of the dimer showing the relative orientations of Glu56 and Arg59.

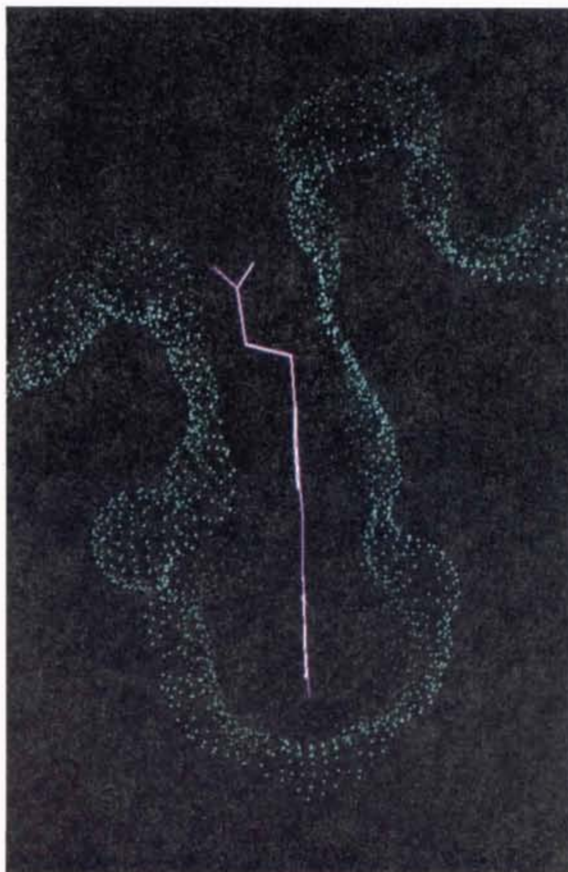

Fig. 5. Details of the Connolly surface of a dimer at the close vicinity of the porphyrin.

density difference) and a reasonable hydrogen-bonding geometry. In the final cycles of the refinement, the occupancy factor of the protoporphyrin molecule was found to be 0.4 leading to an R factor of 0.18. At this point it was not possible to establish the nature of the heavy atom (Sn or Cd) with any confidence because of the slight difference in their molecular weights.

Results

As shown in Fig. 1, the molecule consists of five α-helical regions. According to hydrogen-bonding patterns and main-chain dihedral angles, those amino-acid residues found in α-helical conformations include residues 10–38, 45–72, 92–119, 123–156 and 159–170. There is only one short β-strand from residue 74 to residue 85. An inter-helical salt bridge is observed between the lysine 58 Nξ moiety and the carboxyl group of glutamic acid 103 which is also hydrogen bonded to Tyr30 and Glu137. Such a salt bridge should increase the stability of the four-helix *A–D* bundle as described by Santambrogio *et al.* (1992). In Fig. 2, 12 subunits of one HSF molecule are represented by Cα tracings in order to emphasize the positions of protoporphyrin on the internal face and between two subunits. The view shows the inner cavity of the molecule down the fourfold axis. Fig. 3 obviously shows no evidence of electron density for the metal in the centre of the non-protein object, a result which seems to be confirmed by the very similar electron-density maps obtained during the refinement of the haem–apoferritin complex.

Fig. 4 represents one orientation of the protoporphyrin in its site between two subunits, located at a statistical position on the twofold axis. The local environment of the protoporphyrin in the pocket is mainly hydrophobic. The protoporphyrin is surrounded by Leu24, Tyr28, Leu31 and Leu81; the only hydrophilic residue is Ser27. One of the protoporphyrin propionate groups is hydrogen bonded to the guanidinium groups of Arg52 (3.10 Å) and Arg59 (2.80 Å), and to the carboxyl group of the symmetry-related Glu56 (2.86 Å). The second propionate is hydrogen bonded to the carboxyl group of Glu56 (3.03 and 3.06 Å).

Discussion

Since the structure has been solved, there is no doubt that the electron density observed in crystals of HSF co-crystallized with Sn-protoporphyrin IX or with haemin is the signature of a porphyrin and not of a metalloporphyrin. We suspect that the metal may be lost when the metalloporphyrin enters or binds to the site. In such a case, the process could well be of physiological significance.

The ligand stoichiometry in the crystal state of the complexed HSF, namely 12 protoporphyrins per molecule, is not much different from that which Kadir & Moore (1990*a*) found by spectroscopic titration in solution, *i.e.* 14–17 haem groups per molecule. Even more satisfying is the correspondence between 12 protoporphyrins per molecule of HSF and the maximal haem content found in isolated bacterioferritins (Smith *et al.*, 1989; Laulhére *et al.*, 1992). When the haem content found in extracted bacterioferritins was reported to be significantly lower, the reason could well have been due to the isolation procedures employed. The only reported discrepancy appears to be the claim that bacterioferritin can bind 24 haem groups, albeit with two kinds of kinetics (Kadir & Moore, 1990*b*).

Previous spectroscopic and molecular modelling results (Moore *et al.*, 1992) suggested the haem group is probably bound by two histidine ligands, in the region of the threefold axis. Nothing in the structure of the complex reported here suggests that there are two kinds of haem-binding sites in a ferritin molecule.

The present study describes a well defined cleft in the complex (Fig. 5). This cleft is located at the inner side of the protein and can accommodate a protoporphyrin IX in a site corresponding to the postulated haem-binding site in bacterioferritin (Grossman *et al.*, 1992), with Arg59 replaced by a methionine residue. The Cβ atoms of two Arg59 residues occupy symmetry-related positions in the intersubunit between two *B* helices and are well positioned to allow methionine S atoms to be the fifth and sixth iron ligands. Such a result allows us to conclude that ferritins and bacterioferritins are probably evolutionarily related proteins.

The authors wish to thank their colleagues from Marseille and Strasbourg for use of their diffractometer facilities.

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