Spectroscopic identification of the haem axial ligands of haemoferritin and location of possible haem-binding sites in ferritin by molecular modelling

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Horse spleen ferritin will bind up to 16 protoporphyrin IX haem groups per 24 subunits *in vitro* [Kadir & Moore (1990) FEBS Lett. 276, 81–84] at a site that causes the haem to be low spin for both ferric and ferrous states. E.p.r. spectra at 10 K of the oxidized form of the resulting haemoferritin gives *g* values of 2.93, 2.26 and 1.55, characteristic of low-spin haem. The near-i.r. magnetic circular dichroism spectrum shows a porphyrin-to-ferric charge-transfer band at 1590 nm. The spectroscopic parameters indicate that the haem group is probably bound by two histidine ligands. Molecular modelling studies reveal one type of potential haem-binding site in horse L-chain ferritin with bis-histidine co-ordination. This is an intersubunit site which lies in a pocket within the ferritin protein shell in the region of the 3-fold channel. The ligands are His-114 and His-124 in horse L-chain. A second possible set of sites in human H-chain ferritin involves His-60 residues in the pockets between pairs of subunits. These are considered less likely sites of haem occupancy. There are three of the intersubunit sites in horse L-chain ferritin at each of the eight 3-fold channels. We propose that conformational crowding between haem-binding sites at a given channel prevents more than two haems per channel being bound.

INTRODUCTION

Ferritin, the iron-storage protein of animals, has a central role in iron homeostasis [see Harrison *et al.* (1991) and Crichton (1991) and references therein]. It consists of a shell formed from 24 protein subunits surrounding a central cavity in which the non-haem iron is stored (Ford *et al.*, 1984; Crichton, 1991; Harrison *et al.*, 1991). Iron uptake into the store, and iron release from the store, occurs with the passage of iron across the protein shell.

Bacterioferritin (BFR) is a structurally similar bacterial protein (Stiefel & Watt, 1979); it too consists of a protein shell composed of 24 subunits surrounding a non-haem iron mineral phase. A major difference between ferritin and BFR is that, as isolated, BFR contains haem and ferritin does not. However, recent optical spectroscopic studies of horse spleen ferritin have shown that *in vitro* it binds the protoporphyrin IX haem group to generate haemoferritin (Kadir & Moore, 1990; Kadir *et al.*, 1992). Approx. 16 haems are bound per 24 subunits at a site that causes the haem to be predominantly low-spin in both the ferric and ferrous states. This indicates that the haem iron is bound by two strong-field ligands of the proteins (Moore & Pettigrew, 1990). In the present paper we report e.p.r. and magnetic circular dichroism (m.c.d.) spectra of the oxidized form of haemoferritin, which allows the axial ligands of the haem to be identified as the side chains of two histidine residues, and a molecular modelling study, using the X-ray structures of horse spleen ferritin and human liver H-chain ferritins, which identifies possible haem-binding sites and suggests an explanation for the non-stoichiometric nature of the binding.

EXPERIMENTAL

Horse spleen ferritin was obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.) and the non-haem iron removed by dialysis against thioglycolic acid, at pH 4.25 and 4 °C for 5 days followed by dialysis against 25 mM-phosphate buffer at pH 7 for 2 days. Haemoferritin was prepared from the resulting apoferritin by the procedure by Kadir *et al.* (1992).

Spectroscopic measurements

Electronic absorption spectra were recorded on a Hitachi U-2000 or Hitachi 557 spectrophotometer.

M.c.d. spectra were recorded on a Jasco J-500 D spectropolarimeter, for the wavelength range 300–1100 nm, and on a home-built circular dichrograph in the range 800–3000 nm. The sample was loaded into a split-coil superconducting solenoid (Oxford Instruments plc, Osney Mead, Oxford, U.K.; SM-4) capable of generating a maximum magnetic field of 5 T and giving temperature control at the sample between 1.5 K and 300 K. To ensure that good optical-quality glasses were formed on freezing, glycerol was added to samples to 50 % (v/v). These additions left the absorption and e.p.r. spectra unchanged.

E.p.r. spectra were recorded on an X-band spectrometer (Bruker ER200D with an ESP1600 computer system) fitted with a liquid-helium flow cryostat (Oxford Instruments plc; ESR9). Concentrations of the low-spin ferric haem of ferritin were obtained by double integration of the e.p.r. spectrum and comparison with the integrated e.p.r. spectrum of a known

Abbreviations used: BFR, bacterioferritin; m.c.d., magnetic circular dichroism; CT, charge transfer.

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concentration of an aqueous copper(II)–EDTA complex using the method of Aasa & Vänngård (1975).

Molecular modelling

The molecular modelling package FRODO (Jones, 1978) implemented on an Evans and Sutherland PS390 graphics system was used to identify possible haem-binding sites within the ferritin quarternary structure. The X-ray co-ordinates of horse spleen L-chain ferritin (0.26 nm resolution) and human liver H-chain ferritin (0.19 nm resolution) were used in conjunction with their amino acid sequences to search the ferritin molecule systematically for suitable ligand positions. The possible ligands examined included all histidine, arginine, lysine and methionine amino acid positions. By considering a computer-generated model of a central subunit surrounded by its six nearest symmetry-related neighbours, all possible combinatorial ligations, both intra- and inter-subunit within the ferritin molecule were highlighted and then analysed. Putative ligation partners were eliminated on the basis of either their separation distances within the protein shell, after consideration of side chain rotations, or the unfavourable geometry required to form a haem-binding site from those ligands.

Independent of the geometric-search approach, the Connolly surface program (Connolly, 1983) was used to generate a solvent-accessible surface, with a probe radius 0.14 nm, of the horse L-chain and human H-chain ferritin co-ordinates. Any significant cleft or internal pocket within the molecular surface was noted for its depth, ease of accessibility and position.

RESULTS AND DISCUSSION

E.p.r. and m.c.d. spectra of haemoferritin

Identification of the axial ligands of low-spin Fe(III) proto-porphyrin IX can be achieved by observing the energy of a characteristic porphyrin-to-iron(III) charge-transfer (CT) transition in the optical spectrum between 800 and 3000 nm, and by measurements of the g -values using e.p.r. spectroscopy (Gadsby & Thomson, 1990; Cheesman *et al.*, 1991). The energy of the CT transition and the g values depend sensitively on the d-orbital energies of Fe(III), which in turn are governed by the ligand-field strength of the axial ligands to the iron. By study of the e.p.r. and m.c.d. spectra of a wide variety of haemoproteins, and model

Fe(III) porphyrins with known axial ligation, a spectroscopic scale has been constructed to enable axial ligand diagnosis to be made with confidence. The CT band is more readily located by means of the m.c.d. spectrum simply because the region of the optical absorption spectrum at wavelengths longer than approx. 1400 nm is partially obscured by the vibrational overtone absorption. Vibrational transitions have m.c.d. signals which are several orders of magnitude weaker than those of electronic transitions, especially of paramagnetic species.

The m.c.d. spectrum at 4.2 K and 5 T magnetic field of horse spleen haemoferritin, which contains approx. 16 haems/24 subunits, shows a CT band peaking at 1590 nm, with a shape typical of low-spin Fe(III) haem (Fig. 1). The spectrum contains no other bands between 1800 and 3000 nm. In addition, the m.c.d. spectrum is free from additional bands between 650 and 1000 nm.

The e.p.r. spectrum of the same sample at 10 K has g values of 2.93, 2.26 and 1.55 (Fig. 2). These signals are broadened beyond detection when the sample temperature is raised to 100 K. These features are typical of low-spin Fe(III) haem and are similar to the spectra of bis-histidine-co-ordinated protohaem IX (Gadsby & Thomson, 1990). The spectrum also contains pronounced signals at g approx. 2.45 and 2.05 which persist above 100 K. These appear to arise from copper(II) ions and are invariably detected in ferritin after addition of haem (M. R. Cheesman, N. Le Brun, G. R. Moore & A. J. Thomson, unpublished work). The source of the copper(II) ion is not the added haemin.

A set of g values of 2.93, 2.25 and 1.55 together with a CT band peaking at 1590 nm are typical of bis-histidine haem ligation. Extensive studies of the e.p.r. and near-i.r. m.c.d. spectra of low-spin Fe(III) haemoproteins with known ligation have enabled a protocol for axial ligand identification to be established (Gadsby & Thomson, 1990). Histidine–histidine ligation leads to a CT band lying between 1500 and 1630 nm. The $g_{\max}$ value of the low-spin rhombic trio of e.p.r. signals of bis-histidine-co-ordinated haems can vary widely between 2.90 and 3.77 (Thomson & Gadsby, 1990; Berry *et al.*, 1990). Co-ordination of protohaem IX by lysine–histidine leads to a CT band between 1480 and 1500 nm, although there are only three well-characterized examples. The $g_{\max}$ values in these cases lie between 3.33 and 3.55 (Gadsby & Thomson, 1990). Hence distinction between bis-histidine and histidine–lysine co-ordination is possible only when e.p.r. and near-i.r. m.c.d. data are available and provided that the $g_{\max}$ value falls in the region of g approx. 3.0. This is the case for haemoferritin and is the basis for the conclusion that the axial ligation type is bis-histidine. We did give consideration to the possibility that a lysine–histidine ligation site is present which generates untypical e.p.r. $g_{\max}$ values. However, no such site could be found by molecular modelling whereas a plausible bis-histidine site is apparent. For these reasons we propose binding by two histidine ligands.

One further piece of information can be derived from the data, namely, the relative orientation of the two histidine ligands. It has been shown that the intensity of the m.c.d. peak of the CT band of low-spin Fe(III) haem is dependent on the equivalence of the x and y axes in the porphyrin plane brought about by the orientation of the histidine planes above and below the iron (Thomson & Gadsby, 1990). The g values are also sensitive to this angle. The spectra of haemoferritin exclude an angle close to 90° between the planes of the histidine rings and are consistent with an angle much closer to 0° or 180°.

Molecular modelling studies

The molecular modelling studies revealed two types of potential haem-binding sites in human H-chain ferritin, and one type of potential haem-binding site in horse L-chain ferritin, with bis-

Fig. 1. Near-i.r. m.c.d. spectrum of horse spleen haemoferritin

The sample was 290 μ M in low-spin ferric haem and dissolved in 25 mM-Hepes (pD 7.8)/glycerol (1:1, v/v). Spectra were recorded with a magnetic field of 5 T and at the following temperatures: 1.6 K (—) and 4.2 K (---).

Fig. 2. X-band e.p.r. spectrum of horse spleen haemoferritin recorded at a temperature of 10 K

Sample details as in legend to Fig. 1. The instrumental parameters were as follows: microwave frequency, 9.04 GHz; microwave power, 2.02 mW; and modulation amplitude, 2 mT.

Fig. 4. Solvent-accessible surface showing a modelled cleft near the 3-fold channel in which a protohaem IX group is bound

For clarity the dotted surface surrounding the haem is contoured at 50% of the normal van der Waals radii of its atoms. Haem ligands His-114 and His-124 are from neighbouring chains.

Fig. 3. Three protohaem IX groups near a 3-fold channel of horse spleen ferritin

The view is from the outside of the molecule and directly down the channel axis. Haem axial ligands are two histidines: His-114 from the C-helix of one subunit and His-124 from the CD turn of another (see Fig. 4). Each subunit participates in the binding of two haems.

histidine co-ordination. The horse L-chain ferritin sites are the same as one of the sets of human H-chain ferritin sites. These are intersubunit sites which occur in a pocket within the ferritin protein shell in the region of the 3-fold channels (Fig. 3). The proposed ligands are: His-114 and His-124 (horse L-chain) or His-118 and His-128 (human H-chain). These pairs of histidines are reorientated by a combination of side-chain rotations and minor main-chain displacements to come into juxtaposition to

form the bis-histidine co-ordination site. The main-chain displacement affected residues 107–131 in horse L-chains (111–135 in human H-chains) and occurred with acceptable main-chain torsional angles for all amino acids. Less reorganization was necessary for horse L-chains than for human H-chains because the side chains in that region are smaller for horse L than for human H-chains. The ring plane angle for the histidines co-ordinated to the same haem is 180° and the separation between their $N_{\epsilon 2}$ atoms is 0.418 nm (Fig. 4). Haem binding at these sites would not entail the haem burrowing through the shell into the protein cavity because the binding pocket is accessible from the outside of the molecule. Some 90–95% of the haem bound at these sites would be shielded from bulk solvent according to the Connolly surface calculations. Although located between subunits the binding stoichiometry of these sites is one per subunit.

The second possible set of sites in human H-chain ferritin are also intersubunit sites. They are located between the symmetry-related His-60 residues found in the pockets between pairs of subunits around 2-fold axes. However, haem binding at these sites seems unlikely since in order to reach them the haem would have to burrow through the protein shell. Moreover, owing to the limited depth of the pocket, approx. 40% of each bound haem would be exposed to solvent in the central cavity. Also, since this site does not exist in horse L-chain ferritin, residues at an equivalent positions being glutamates not histidines, we do not believe it is relevant to our spectroscopic studies.

Comparison of model with previous studies

Previous studies have shown that the maximum haem loading of haemoferritin is approx. 16 haems per 24 subunits and this ratio can be accounted for by the model presented in Fig. 3. There are eight 3-fold channels and hence a possible maximum of 24 such haem-binding sites in the 24-mer ferritin molecule. However, the haems at each 3-fold channel are close together: the shortest interhaem distance for any pair of haems is approx. 0.5 nm. If the occupancy of one (or two) haem site(s) at any 3-fold channel interfered with haem binding at a second (or third) site,

only 8 or 16 haems would be bound rather than 24. Thus we propose that conformational crowding prevents complete haem loading at the sites. The relative speed with which haem binding occurs suggests that no major structural change is required (Kadir & Moore, 1990), and is consistent with a location at the external surface of ferritin of the haem-binding pockets, as suggested by the sites in Fig. 3.

We should point out that, although the histidines proposed as haem ligands are present in both L- and H-chains of mammalian ferritin, they are not conserved in ferritins from invertebrates and plants [see Andrews *et al.* (1991, 1992) for amino acid sequence compilations]. The ability to bind haem may therefore have been acquired relatively recently in evolution.

The studies reported in the present paper do not address the question of whether bound haem has a role to play in the physiological function of ferritin. However, it may be relevant to note that the bound haem does influence the rate of release of iron from haemoferritin in '*in vitro*' assays (Kadir *et al.*, 1992; Moore *et al.*, 1992). Also, Zn^{2+} , which is known to bind at two sites in the 3-fold channels (Harrison *et al.*, 1989), decreases the incorporation of iron into the non-haem iron core (Treffry & Harrison, 1984; Harrison & Lilley, 1989). This could mean that iron uptake occurs via the 3-fold channel and, if so, haem bound at these channels may influence its rate. However, substitution of Asp-127 and Glu-131, which are the Zn^{2+} ligands, in recombinant H-chain ferritin (Harrison *et al.*, 1989) produces only a small decrease in the rate of iron incorporation (Treffry *et al.*, 1989), and an alternative explanation for the effect of Zn^{2+} on iron uptake is that the Zn^{2+} competes with incoming Fe^{2+} for the ferroxidase ligands on the H-chains (Lawson *et al.*, 1991). Studies of zinc binding and iron uptake by haemoferritin may shed some light on this.

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