Haem binding to horse spleen ferritin

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Horse spleen ferritin, a spherical protein shell of 24 subunits, contains no haem when extracted. This contrasts with ferritins isolated from bacterial sources which have the capacity to bind up to 24 haem groups [(1990) FEBS Lett. 271, 141-143] via two methionine residues [(1990) Nature 341, 771]. Here it is shown that horse spleen ferritin can bind between 15 and 17 haems per 24 subunits with an apparent association constant of 2.2-3.2 $\times 10^4$ M^{-1}. The strength of haem binding appears to be unaffected either by the presence of the core or by the oxidation state of the haem. The demonstration of the ability of animal ferritin to bind haem strengthens the similarity between it and bacterioferritin and could have major consequences for its mechanism of action in physiological iron uptake and release processes.

Haem binding; Horse ferritin; Bacterioferritin

1. INTRODUCTION

The iron-storage protein of animals, ferritin, and its bacterial equivalent, bacterioferritin or bacfer, resemble each other in consisting of 24 subunits, of molecular mass 18 000-24 000, that pack together to form a roughly spherical protein shell of ~20 Å thickness surrounding a cavity of ~80 Å diameter [1,2]. The non-haem iron-storage mineral is contained in the central cavity. A major difference between the two proteins as isolated is that bacfer contains non-covalently bound haem-*b* whilst animal ferritin does not [1-3]. This is the origin of the alternative names for bacfer of cytochrome b_1 and cytochroma b_{557} [3-5]. The haem content of bacfer as isolated is variable: for example, *Azotobacter vinelandii* bacfer contains 12 haems per 24 subunits [3], whereas *Pseudomonas aeruginosa* bacfer contains 5-9 haems per 24 subunits ([5], and unpublished data). Recently it has been shown that the addition of haemin chloride to *Pseudomonas aeruginosa* bacfer results in the haem content of this protein rising to 24 haems per 24 subunits [6]. Stimulated by this result we have investigated the binding of haem to horse spleen ferritin and in the present paper we report that this protein can accommodate 15-17 haems per 24 subunits.

2. MATERIALS AND METHODS

Horse spleen ferritin prepared by the normal procedure involving heat treatment and ultracentrifugation [7] was obtained as a solution from Sigma Chemical Co. (Poole, Dorset, UK). Its average content of Fe^{3+} determined by atomic absorption spectroscopy was 0.125 mg metal/mg protein, or ~1100 Fe^{3+} ions per ferritin molecule. Haemin chloride and sodium dithionite were obtained from Sigma Chemical Co. All spectra were measured with an Hitachi 557 double-beam spectrophotometer or Hitachi U-2000 spectrophotometer. Protein concentrations were determined by the method of Lowry et al. [8].

Two forms of horse ferritin have been studied: the protein as purchased (apo-holo horse ferritin) containing the non-haem iron core, and protein (apo-apo horse ferritin) from which the non-haem iron core had been removed. The latter was prepared by dialysis of the apo-holo protein against 0.12 M thioglycollic acid at pH 4.25 and 4°C, followed by dialysis against 0.025 M sodium phosphate buffer containing 0.15 M sodium chloride, pH 7.4, to remove excess thioglycollic acid and the Fe^{2+} containing species.

The experimental procedure [6] used to monitor incorporation of haem into ferritin involved the measurement of difference visible spectra. The sample cuvette contained 0.7 ml of a solution of ~6×10^{-6} M ferritin in the required buffer and the reference cuvette contained only buffer. Aliquots of a freshly prepared 1.5 $\times$ 10^{-3} M solution of haemin chloride in water were added to both the cuvettes and the absorbance measured over the wavelength range 350-650 nm 15 min after mixing. The final solutions from each titration were kept for up to 7 days and their absorbance spectra recorded with buffer only in the reference cuvette.

3. RESULTS AND DISCUSSION

A typical set of spectra for the addition of haemin chloride to anaerobic apoferritin at pH 8.5 and in the presence of sodium dithionite is shown in Fig. 1A. With increasing concentration of haemin chloride positive bands appear in the difference spectrum at wavelengths of 424 nm, 528 nm and 557 nm. These bands arise from protein-bound haem. A plot of the absorbance at 424 nm versus the haem/ferritin molar concentration ratio is hyperbolic (Fig. 1A; inset) and shows that ~16 haems are bound to 24 subunits with an apparent association constant (K_a) of 2.6×10^4 M^{-1}. Similar results were ob-

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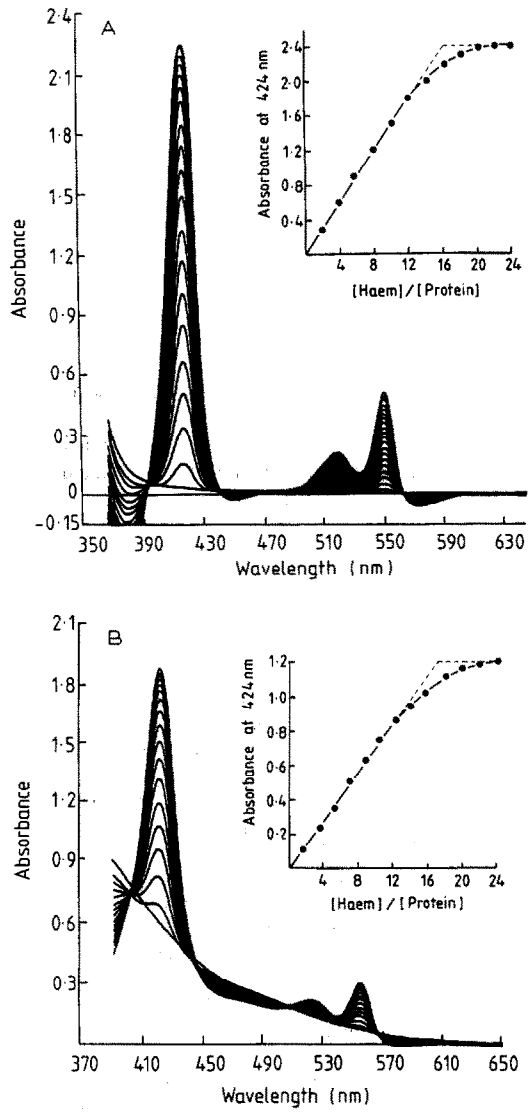

Fig. 1. UV-visible difference spectra for the interaction of haemin chloride with 6.25×10^{-6} M horse spleen apoferritin (A) and 3.25×10^{-6} M horse spleen holoferritin (~1100 atoms Fe per molecule) (B) in the presence of sodium dithionite. Both experiments were at pH 8.5 (0.1 M phosphate), and carried out in a cuvette sealed under N_2 with a rubber bung. Aliquots of a freshly prepared 1.5×10^{-3} M solution of haemin chloride were added with a syringe. The difference spectra correspond to successive 5×10^{-3} ml additions of the haemin chloride solution. The inset graphs show the change in absorbance at 424 nm with increasing [haem]/[protein] for successive 10×10^{-3} ml additions of the haemin chloride solution. The maximum absorbance corresponds to full binding of haem to ferritin. The stoichiometry of the binding is indicated by the intersection of the broken lines. The values for K_a and stoichiometry given in the text were obtained from Hill plots. The rising baseline in (B) is due to absorbance by the non-haem iron core.

tained with anaerobic holoferritin in the presence of sodium dithionite at pH 8.5 (Fig. 1B); the haem/subunit ratio was 17:24 with a K_a of 2.3×10^4 M^{-1}. Analogous results were obtained with apoferritin and holoferritin under reducing condition at pH 6.1.

Corresponding experiments with apoferritin (Fig. 2) and holoferritin under oxidising conditions yielded

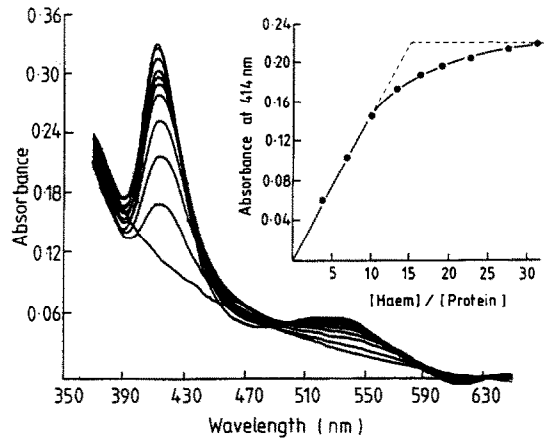

Fig. 2. UV-visible difference spectra and binding curve for the interaction of haemin chloride with 1.5×10^{-6} M horse spleen apoferritin under air at pH 6.1 (0.1 M phosphate). Each difference spectrum corresponds to successive 2.5×10^{-3} ml additions of the 1.5×10^{-3} M haemin chloride solution (other details as given in the caption to Fig. 1). The rising baseline in the spectra is a result of slight turbidity of the apoferritin solution.

essentially the same results as described above, though the appearance of the spectra was different because the haem iron was in the ferric state. Haem was bound by ferritin in a haem/subunit ratio of 15–17:24 and with a K_a of $2.2\text{–}3.2 \times 10^4$ M^{-1}. Thus the strength of haem binding appears to be unaffected by the presence of the core and by the oxidation state of the haem.

The UV-visible spectrum of the haem-loaded apoferritin with buffer in the reference cuvette is shown in Fig. 3. The absorbance bands have wavelength maxima of 414 nm, ~530 nm and ~640 nm (oxidised), and 424 nm, 528 nm and 557 nm (reduced). The spectrum of the oxidised protein shows that there is some high-spin character, the band at ~640 nm is diagnostic of this [9], but the appearance of the spectrum of the reduced protein indicates that, in this state, the haem iron is low-spin, and therefore has two strong-field axial ligands. The identity of these ligands cannot be obtained from the experiments described in the present paper. However, since there are only four amino acids with side chains that have ligand field strengths sufficient to generate the low-spin rate of haem [10], namely, histidine, lysine, methionine and cysteine, we suggest that the two axial ligands are from this group. Magneto-optical spectra of the type used to show the haem of bacfer is coordinated by two methionines are needed for the ligand assignment [10].

The haem binding by ferritin illustrated by Figs 1 and 2 is not stoichiometric with the number of subunits. This is most probably a result of subunit heterogeneity with only 15–17 subunits having a haem binding site. SDS-PAGE gels of the ferritin used in this work revealed only one band, consistent with previous work showing that horse spleen ferritin is composed largely of L-

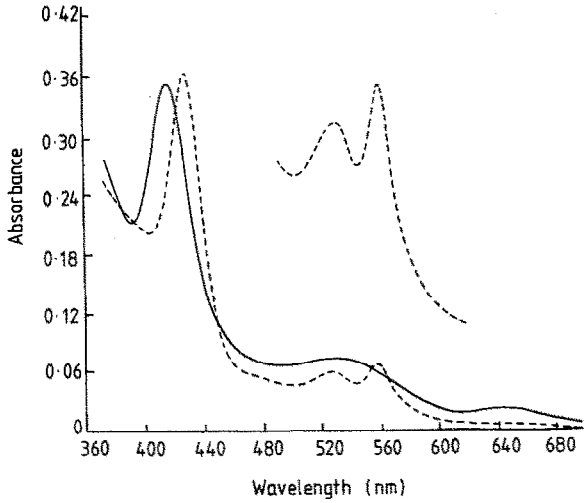

Fig. 3. UV-visible spectra of haem-loaded 0.2×10^{-6} M horse spleen apoferritin at pH 6.1 (0.1 M phosphate) and at a [haem]/[protein subunit] ratio of 16:24. The solid line is the spectrum of the oxidized form and the broken line the spectrum of the dithionite-reduced form.

subunits [11]. However, different L-subunits are known to exist and these are not separated by SDS-PAGE [2,12]. Also, a small proportion of H-subunits may not have been resolved by our gels. Therefore we suggest that it is normal L-subunits that bind haem.

The apparent association constants measured in Figs 1 and 2 are comparable to those for haem binding to bacfer: at $0.7\text{–}1.4 \times 10^4$ M^{-1} the bacfer data [6] show that haem binding in this protein is slightly weaker than in the horse ferritin. This, together with the fact that haem binding to horse ferritin occurs relatively rapidly to generate a bis-ligated haem, indicates that there is a preformed haem binding site in the protein coat of ferritin. Thus we propose that ferritin contains a specific haem binding site. We discount the alternative explanation of adventitious binding of haem since we know of no other protein which binds haem non-specifically to generate the kind of protein-bound haem observed in the present work. For example, haem bound to hemopexin is low-spin whilst haem bound to serum albumin is high-spin [13]. In the former protein a site providing for bis-histidine axial ligation exists on the protein whilst in the latter case the iron does not appear to be ligated by the protein [14]. The physiological function of haem binding to these proteins has been investigated. Hemopexin aids haem transport in blood and receptor-mediated uptake of haem into cells [15,16], whilst albumin binds haem less strongly than does hemopexin [17] and appears to act as an adventitious haem scavenger in blood. Haem is readily transferred from albumin to hemopexin [17].

If ferritin contained haem in vivo the similarity between it and bacfer would be strengthened. In addition, the presence of haem could have major consequences for the iron uptake and release processes. These involve oxidation and reduction reactions though the nature of these is unclear at present [2,12]. However, there is a major problem with assigning a physiologically significant role to ferritin-bound haem; namely, animal ferritin as isolated is haem-free. The general view has been that it is haem-free because it does not contain haem in situ but it may be that haem is lost during isolation. Alternatively, haem may be present when the core is laid down but lost as the holoferritin ages. In this context it may be important that the standard methods of ferritin isolation employ ultracentrifuge steps that select for holoferritin in preference to apoferritin [7]. Also, as Theil [18] points out, commercially available horse spleen ferritin may represent the late stages of iron overload. Spleen ferritin from young animals has a lower iron content and, in some cases, a different subunit composition. Thus, the haem binding characteristics of a range of ferritins should be explored. These points need investigating. Indications that haem may be lost on the storage and isolation of ferritin come from several sources. The haem content of *Pseudomonas* bacfer as isolated is variable at 5–9 haems per 24 subunits [5], even though the protein can accomodate 24 haems per 24 subunits [6], and *Saccharomyces cerevisiae* ferritin is reported to contain haem when purified by a centrifugation and chromatographic procedure, but to be haem-free when isolated using an ammonium sulphate precipitation step and heat treatment to denature contaminating proteins [19]. This latter procedure is widely used to isolate ferritin and bacfer.

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Note added in proof

Since submission of the manuscript we have investigated the binding of ferrihaem chloride to 0.4 μM horse spleen apoferritin in an analogous manner to that described in the paper for solutions of 6.25 μM ferritin, and observed the same haem/protein stoichiometry of 16 haems per 24 subunits reported above. Similarly, with 0.4 μM *Pseudomonas aeruginosa* bacterioferritin the stoichiometry is 24 haems per 24 subunits as previously reported by us (FEBS Lett. (1990) 271, 141–143).