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Haem binding to horse spleen ferritin and its effect on the rate of iron release

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Horse spleen ferritin is shown to bind haem to generate a haemoprotein, named herein haemoferritin. A total of 14–16 haem molecules are bound per 24 subunits of ferritin. The molecular mass of the non-haem-iron-free haemoferritin has been determined to be 420 ± 40 kDa, indicating that haem binding does not lead to dissociation of the 24 subunits that comprise the ferritin molecule. The functional role of the bound haem has been investigated with respect to the release of iron from the non-haem iron core. The bound haem is shown to increase the rate of iron release in a reductive assay system. In the absence of haem the rate of iron release depends on the redox potential of the reductant, but in the presence of haem the rate is largely independent of the reductant and is faster than the rate for the haem-free ferritin. These data indicate that electron transfer across the protein coat of ferritin is the rate-limiting step of iron release in the absence of haem, but in the presence of haem electron transfer is not rate-limiting.

INTRODUCTION

Ferritin is the iron-storage protein of animals. It consists of a protein shell constructed of 24 subunits, of molecular mass ~ 20 kDa each, that encompasses a cavity, of ~ 8 nm (80 Å) diameter, inside which a non-haem-iron mineral, the iron core, can be formed (Ford *et al.*, 1984; Theil, 1987; Theil & Aisen, 1987; Harrison *et al.*, 1989; Crichton, 1990). The bacterial equivalent of ferritin, bacterioferritin (Stiefel & Watt, 1979), is similar to animal ferritin in its general structure, with the most significant difference being that the bacterial protein is isolated with haem bound into the protein shell, whereas ferritin is isolated in a haem-free form and has generally been regarded as a non-haem protein. However, recently we have shown spectroscopically that horse spleen ferritin can bind ~ 16 haem molecules per 24 subunits in a pre-formed binding site to generate a low-spin haem (Kadir & Moore, 1990*b*). The low-spin nature of the haem indicates that it may function in electron transfer, since the iron is co-ordinatively saturated (Moore & Pettigrew, 1990). The discovery of this haem-bound form of ferritin, which we here name haemoferritin, has prompted us to study the influence of the haem on the rate of iron release from the core. Our results are reported in the present paper together with a procedure for the preparation of haemoferritin.

The mechanism(s) of iron uptake into, and release from, the core of ferritin have been extensively studied and various mechanistic proposals made (Crichton & Roman, 1978; Jones *et al.*, 1978; Clegg *et al.*, 1980; Funk *et al.*, 1985). Most of these assume that the iron enters and leaves the protein as single Fe^{2+} ions via small channels through the shell. Since the core contains iron in the Fe^{3+} oxidation state, such passage of Fe^{2+} requires oxidation and reduction reactions to be coupled with iron uptake and release. It is this aspect of ferritin that is one of the subjects of the present paper.

EXPERIMENTAL

Preparation of haemoferritin

The initial detection of haemoferritin did not involve its isolation with chromatographic procedures (Kadir & Moore, 1990*b*). To achieve this we developed the following protocol. A solution of 1.5 mM-haemin chloride in 0.02 M-NaOH was added to a solution of horse spleen ferritin (Sigma Chemical Co.; ~ 1100 atoms of Fe per molecule; 6 mg of protein/ml in 0.1 M-phosphate buffer, pH 7.4), to give a molar ratio of 24 haem molecules per 24 ferritin subunits. The mixture was left with stirring at 4 °C for 5 h, centrifuged at 5000 *g* for 15 min to remove the small amount of precipitate, and then applied to a Sephadex G25 column equilibrated with 0.1 M-phosphate buffer (pH 7.4). The haemoferritin-containing eluate was dialysed against the phosphate buffer for 36 h and then applied to a DE-52 DEAE-cellulose column equilibrated with the same buffer. The bound haemoferritin was washed with the same buffer and then eluted from the column with the phosphate buffer containing 0.3 M-NaCl. The NaCl was then removed by dialysis against phosphate buffer. Occasionally the haemoferritin eluted from the DEAE column contained excess haemin. This was removed by acetone treatment at pH 7.4 and −19 °C, followed by resuspension of the precipitated haemoferritin and dialysis of the solution (Kadir & Moore, 1990*a*). The haem content of the final preparation was 14–16 haem molecules/24 subunits, as determined by the haemochromogen procedure described by de Duve (1948). This is in good agreement with the spectroscopically determined ratio of 16/24 subunits (Kadir & Moore, 1990*b*).

Apohaemoferritin was prepared by the method described above for the preparation of haemoferritin. Apoferritin was obtained by the dialysis of ferritin against thioglycollic 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.

A Sepharose 6B column was used to determine the molecular mass of apohaemoferritin. The 100 cm × 2 cm column was equilibrated with 50 mM-Tris buffer (pH 7.5) containing 100 mM-KCl, and was run at 4 °C. A Sigma molecular mass marker kit (MW-GF-1000) was used to calibrate the column over the range 29–700 kDa.

Iron release assay

The influence of the bound haem on the rate of iron release was investigated using the assay procedure described by Boyer *et al.* (1988). A reducing reagent and the Fe^{2+} chelator ferrozine or bipyridyl in 0.1 M-acetate buffer (pH 5.6) were mixed in a cuvette, and after 2 min an aliquot of a fully oxidized ferritin or haemoferritin solution in the same buffer was added. The

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formation of $[Fe(ferrozine)_3]^{2+}$ or $[Fe(bipyridyl)_3]^{2+}$ was monitored by recording the increase in absorbance at 562 nm or 520 nm respectively with time. The molar absorption coefficient for the ferrozine complex is 27900 $M^{-1}\cdot cm^{-1}$ (Stookey, 1970), and for the bipyridyl complex it is 8430 $M^{-1}\cdot cm^{-1}$ (Funk *et al.*, 1985). The Fe^{2+} chelators and reductants used in this work were obtained from Sigma Chemical Co.

RESULTS AND DISCUSSION

Preparation and characterization of haemoferritin

The preparation of haemoferritin was monitored spectroscopically (Fig. 1). Prior to the addition of haemin chloride, the ferritin spectrum showed no evidence of the presence of haem (Fig. 1*a*). However, the spectra of the final product were typical of those for protein-bound haem, with two amino acid side chains co-ordinated to the iron (Fig. 1*c*) (Smith & Williams, 1970; Moore & Pettigrew, 1990). These spectra are identical to those described in the original study of haem binding to horse spleen ferritin (Kadir & Moore, 1990*b*). The spectrum shown in Fig. 1(*b*) indicates that at the haem/protein ratio of 24 haems/24 subunits, not all of the haemin was bound to ferritin. The excess haem may be present as isolated or aggregated species associated with the protein, or may be non-specifically associated on the ferritin surface. The important point, however, is that the excess haem can be removed to leave just the haem specifically bound to ferritin.

The molecular mass of horse spleen apohaemoferritin, which lacks a non-haem iron core, was determined by gel chromatography on a Sepharose 6B column. It was found to be 420 ± 40 kDa, the same as normal apoferritin (Ford *et al.*, 1984; Theil, 1987, Theil & Aisen, 1987). Thus haem binding to ferritin does not lead either to the dissociation of the 24 protein subunits or to further association. Therefore it seems probable that the bound haems fit into crevices within the protein shell.

Rate of iron release

The formation of $[Fe(ferrozine)_3]^{2+}$ by the addition of caffeic acid to a ferritin/ferrozine mixture is illustrated in Fig. 2. The linked series of reactions giving rise to this spectrophotometric change is:

$$\text{Core–Fe}_n^{3+} + e^- \xrightarrow{\text{slow}} \text{Core–Fe}_{n-1}^{3+} + Fe^{2+} \quad (1)$$

$$Fe^{2+} + 3\,\text{ferrozine} \xrightarrow{\text{fast}} [Fe(\text{ferrozine})_3]^{2+} \quad (2)$$

The overall rate of reaction is obtained from the increase in absorbance with time, as indicated by Fig. 3. The observed rate corresponds to the rate of reaction (1) (Boyer *et al.*, 1988). Table 1 summarizes the observed rate for the series of reductants studied in the present work with ferritin and haemoferritin.

A series of control experiments was carried out to ensure that the spectrophotometric change measured corresponded to the release of Fe^{2+}. These experiments were particularly important because diphenols chelate Fe^{3+}, and Fe^{3+} chelators such as pyridinones have been shown to remove non-haem Fe^{3+} from ferritin (Brady *et al.*, 1989). The first set of control experiments used identical solution conditions to those employed for the iron release experiment reported in Table 1, except that an Fe^{2+} chelator was not added to the solution. No spectroscopic change over the range 450–750 nm was observed within 2 h of mixing. These experiments were not continued beyond 2 h. Since the spectroscopic changes in the presence of an Fe^{2+} chelator appeared rapidly (Fig. 3), this indicates that the assay reactions occur as indicated by eqns. (1) and (2). Further confirmation of this was obtained from experiments using 4-hydroxycinnamic acid and ferulic acid, neither of which chelates iron in the manner of which diphenols do, as reductants in place of diphenols. These reagents led to the release of iron in an analogous manner to that obtained with diphenols.

Our conclusion that diphenols on their own do not release significant amounts of iron is consistent with the work of Tufano

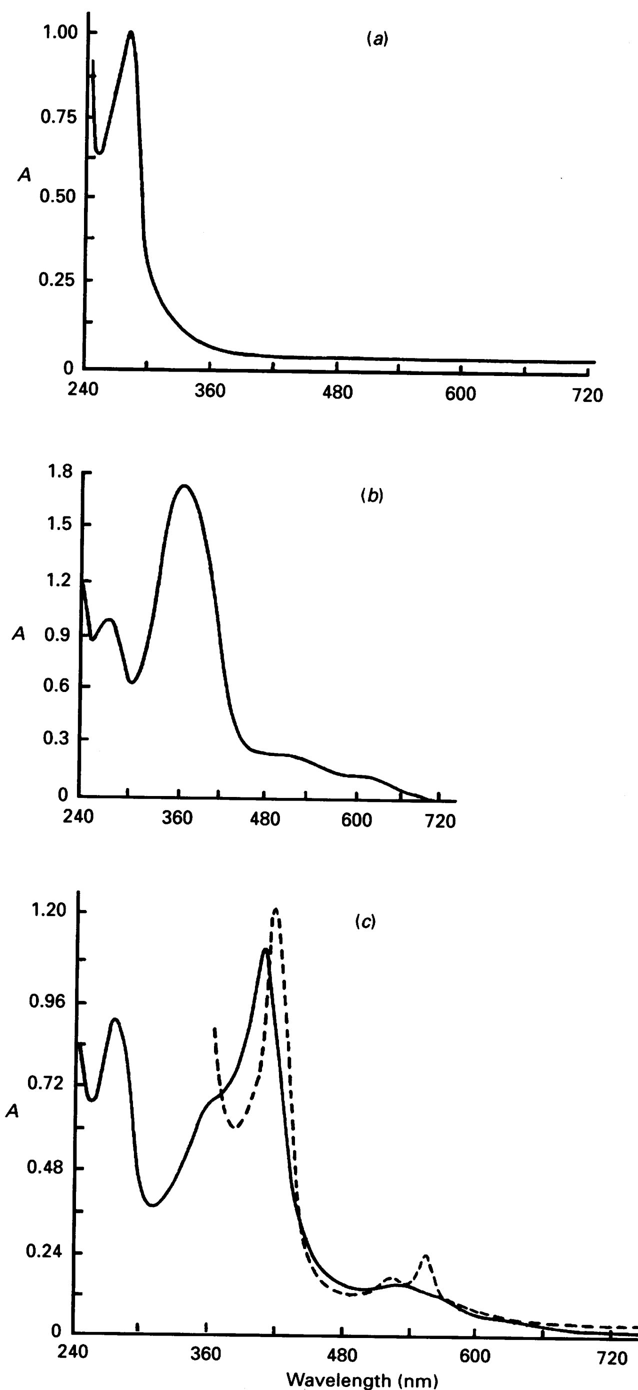

Fig. 1. U.v.–visible absorption spectra of horse spleen apoferritin

(*a*) As prepared. (*b*) The spectrum after addition of haemin chloride to horse spleen apoferritin at a haem/subunit ratio of 1:1. (*c*) The spectra of oxidized (solid line) and reduced (broken line) haemoferritin. The reduced sample was obtained by adding a few crystals of sodium dithionite to the anaerobic oxidized sample. In all cases the protein concentration was 1 $mg\cdot ml^{-1}$ in 0.1 M-sodium phosphate buffer, pH 7.4.

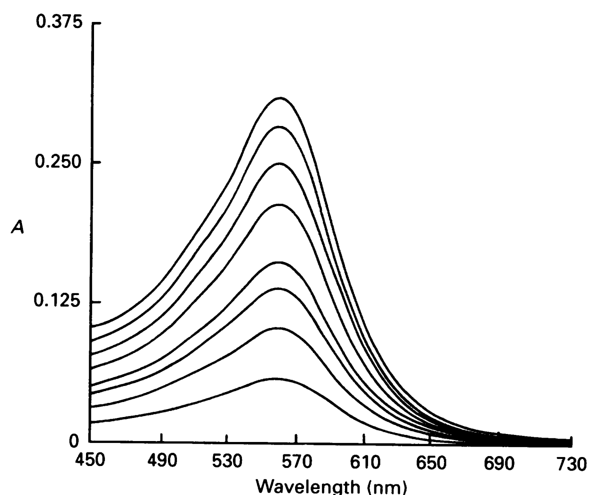

Fig. 2. Visible absorption spectra of the formation of $[Fe(ferrozine)_3]^{2+}$ in the reductive iron assay

The 1 ml cuvette contained 0.5 mg of ferritin·ml^{-1} (containing 1100 atoms of Fe per molecule) and 1.4 mM-ferrozine. Each spectrum corresponds to the successive addition of 0.01 mM-caffeic acid. The spectra were recorded 12 min after each addition.

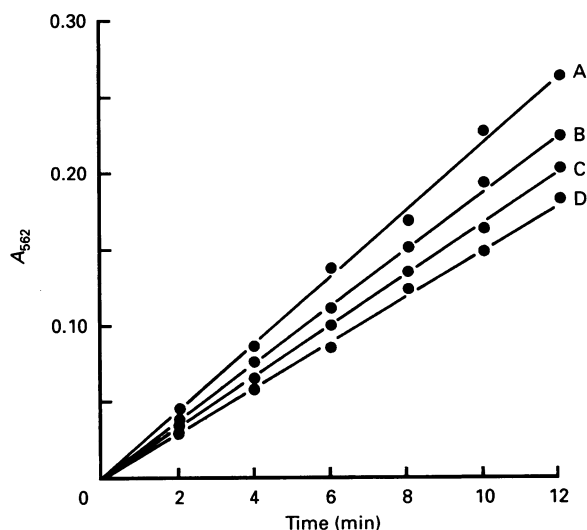

Fig. 3. Initial rate of iron release from horse spleen ferritin resulting from reduction of the iron by diphenols

The 1 ml reaction mixtures consisted of 1.05 μmol of ferritin at pH 5.6 (0.1 M-sodium acetate buffer) and 0.8 μmol of caffeic acid (A), chlorogenic acid (B), 3,4-dihydroxyphenylacetic acid (C) and 3,4-dihydroxybenzoic acid (D).

Table 1. Rates of Fe^{2+} release by various reductants

The redox potentials (E_0) were measured by Felice *et al.* (1976) at pH 4.7 (1 M-acetate). The conditions for the iron release experiments were: pH 5.6 (0.1 M-acetate), 1.4 mM-ferrozine, 0.8 mM-reductant and 1.05 mM-ferritin. Replacement of the ferrozine by 0.4 mM-bipyridyl produced similar results: with ferritin and caffeic acid the rate was 0.91 mM-Fe^{2+}·min^{-1} and with ferritin and chlorogenic acid the rate was 0.79 mM-Fe^{2+}·min^{-1}. Ferulic acid, or 3-methoxycaffeic acid, is a two-electron reductant in which one of the phenol hydroxy groups has been converted to a methoxy group. 4-Hydroxycinnamic acid is a one-electron reductant, and therefore its initial rates are not directly comparable with those of the other reductants.

		Rate of Fe release (nmol of Fe^{2+}·min^{-1})	
Reductant	E_0 (mV)	Ferritin	Haemoferritin
Caffeic acid	570	0.89	1.20
Chlorogenic acid	610	0.75	1.20
3,4-Dihydroxyphenylacetic acid	700	0.67	1.20
3.4-Dihydrobenzoic acid	740	0.62	1.20
Ferulic acid	790	0.58	1.15
4-Hydroxycinnamic acid	950	0.31	0.96

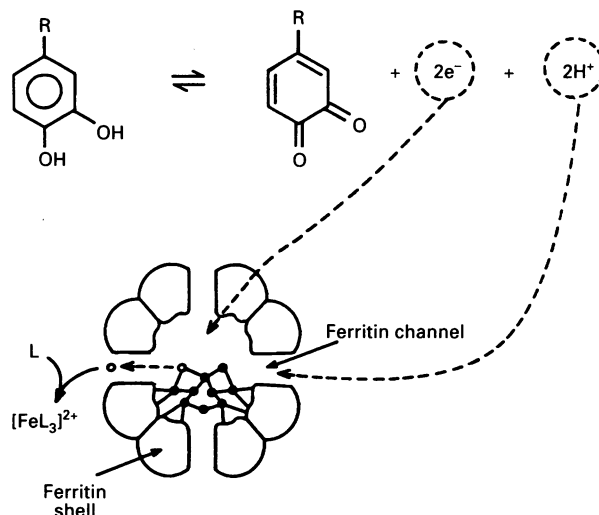

Fig. 4. A possible mechanism for iron release from ferritin

The ferritin molecule is shown in cross-section with four of the channels through the protein shell. The Fe^{3+} ions in the core are represented by ●; the oxide, hydroxide and phosphate in the core are omitted. Oxidation of the diphenol produces electrons and protons which cause the reductive dissolution of the core. Fe^{2+} ions, represented by ○, migrate through the channel and at the mouth of the channel bind to the ferrozine or bipyridyl ligand (L).

et al. (1981). These workers showed that *NN′N″*-tris-(2,3-hydroxybenzoyl)-1,3,5-triaminomethylbenzene and related ligands released < 6% of the non-haem iron from ferritin 6 h after mixing them at pH 7.4.

In the assay employed by us, the diphenols reduce the core Fe^{3+} even though their redox potentials are higher than that of the Fe^{3+} because the formation of the $[Fe(ferrozine)_3]^{2+}$ or $[Fe(bipyridyl)_3]^{2+}$ complex displaces the equilibrium that exists between the reductants and the Fe^{2+}. However, the rate at which these complexes is formed does depend upon the reductants.

Two points are striking about the data in Table 1. Firstly, the rate of iron release from ferritin depends upon the reductant, whereas the rate of release from haemoferritin does not. Secondly, the rate of release from haemoferritin is faster than that from ferritin. These observations indicate that electron transfer is a key step in the release of iron. Fig. 4 illustrates a possible mechanism for iron release.

Oxidation of the diphenol produces electrons and protons which cause reductive dissolution of the core. Fe^{2+} ions migrate through the channel, and at the mouth of the channel bind to ferrozine or bipyridyl. In this model we assume that neither the diphenol reductant nor the ligand enters the ferritin core. In justification of this, we note that although the entry of relatively large molecules into the core has been advocated by some workers (Clegg *et al.*, 1980; Crichton, 1990, and references

therein), various spectroscopic and kinetic studies indicate that this is unlikely (Jacobs *et al.*, 1989; H. Khodr, F. H. A. Kadir & G. R. Moore, unpublished work). In addition, our assumption is consistent with the X-ray structure showing that the maximum diameter of the channels is only 0.4–0.5 nm (4–5 Å) (Ford *et al.*, 1984).

Without entry of the reductant into the core the electrons must pass through to the iron via the protein coat or groups located in the channels. This requires movement of electrons over a distance of up to 2.5 nm (25 Å). Such long-range electron transfer has been shown to occur with reasonable rates in a variety of proteins, with the introduction of hop-centres into the protein increasing the rate (Williams, 1969; Marcus & Sutin, 1985; Mayo *et al.*, 1986; Moore & Pettigrew, 1990). We propose that such electron transfer occurs in our assay system, and that in the absence of haem the rate of electron transfer is the limiting step in iron release. Thus the rate varies with the redox potential of the reductant, with the lower thermodynamic driving force corresponding to a slower rate. Such a relationship is expected from electron transfer theory (Marcus & Sutin, 1985). However, the addition of haem increases the rate of electron transfer to such an extent that it is no longer the limiting step in iron release. Thus there is then no relationship between the driving force of the electron transfer reaction and the rate of iron release.

General discussion

As we have indicated above, haemoferritin is an interesting system for chemical studies of redox-linked iron storage processes. However, whether it has a physiological role is still to be discovered. The possibility that haem is bound to ferritin *in vivo* but is lost on isolation of ferritin, or as the ferritin core is laid down, has been discussed elsewhere (Kadir & Moore, 1990*b*). Recently, however, we have discovered that recombinant human H-chain ferritin isolated from *Escherichia coli* contains bound haem spectroscopically identical to the haem of haemoferritin (F. H. A. Kadir, M. R. Cheesman, A. J. Thomson, A. Treffry, P. M. Harrison & G. R. Moore, unpublished work). Thus the isolation of ferritin from animal tissue needs to be re-investigated. Irrespective of haem binding, the observation that iron release from ferritin is limited by the rate of electron transfer under certain circumstances is important. This directs attention to the possible physiological electron donors to ferritin, and to the properties of the protein coat of ferritin, particularly the possibility that redox active cofactors are present. Both of these topics have been discussed recently by Crichton (1990), who suggests that a redox active cofactor is inactivated during the purification of ferritin.

A further general point relevant to the physiological mechanism of iron release can be made from the data in the present paper. Though the assay system employed reduction to obtain the iron release, the redox potentials of the diphenols (Table 1) are much higher than those of the ferritin core. Watt *et al.* (1985) have determined that the ferritin redox potential at pH 7 is −190 mV. The reason that the ferritin iron is reduced in the assay is that the affinity of ferrozine and bipyridyl for Fe^{2+} is so high that the redox equilibrium is displaced. This indicates that low-potential electron donors are not essential to obtain iron release from ferritin if a high-affinity Fe^{2+} acceptor is present. Therefore, not only should possible physiological reductants of ferritin be identified, but also the iron acceptors.

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