Iron release from haemosiderin and ferritin by therapeutic and physiological chelators

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Iron release from both human and horse spleen haemosiderin to desferrioxamine was substantially less than that released from ferritin samples. This finding contradicts a previous report [Kontoghiorges, Chambers & Hoffbrand (1987) *Biochem. J.* **241**, 87–92]. Differences in phosphate content of cores and in core size between haemosiderin and ferritin did not account for the different iron-release rates. Iron released to acetate was found to stimulate lipid peroxidation in liposomes, whereas that released to stronger chelators such as citrate and desferal did not. Absorption spectra and gel-filtration studies suggest that the acetate-solubilized iron was in the form of low-molecular-mass (< 5 kDa) ferrihydrite fragments.

INTRODUCTION

Ferritin is a protein containing individual cores of iron oxyhydroxide enclosed and solubilized. Haemosiderin, by contrast, is an insoluble aggregate of cores and protein (Harrison *et al.*, 1974). In normal tissues, ferritin is the major iron-storage protein; however, in iron overload most of the iron is present as haemosiderin (Selden *et al.*, 1980). It is widely thought that haemosiderin is derived from ferritin by partial degradation of the protein shell (Shoden *et al.*, 1953; Iancu & Neustein, 1977).

Iron release from iron-storage proteins is of importance physiologically in the synthesis of haemoproteins, pathologically in tissue damage associated with iron overload, and therapeutically in chelation treatment. Previous studies have shown that ferritin and haemosiderin will stimulate hydroxyl-radical formation (O'Connell *et al.*, 1986a), lipid peroxidation (O'Connell *et al.*, 1985) and protein modification (O'Connell *et al.*, 1986b), probably by mechanisms involving iron release. In the present study iron release to the therapeutic chelator desferrioxamine and to physiological chelators, acetate and citrate, was investigated.

MATERIALS AND METHODS

Human and horse haemosiderin (Weir *et al.*, 1984) and ferritin (Cham *et al.*, 1985) were isolated from human spleens (removed from patients suffering from thalassaemia major who had received multiple blood transfusions) and from horse spleen. Haemosiderin was freeze-dried and stored at -20°C . On the day of use, solutions (approx. 10 mM-iron) were prepared in 20 mM-tetramethylammonium hydroxide solution (Weir *et al.*, 1984) and diluted in buffer as indicated in legends to appropriate Figures. Apo-ferritin for reconstitution experiments was prepared from cadmium-free horse spleen ferritin (Boehringer Mannheim) by dialysis (2 h per stage) against two changes of 0.1 M-thioglycolic acid in 0.1 M-sodium acetate followed by two changes of 0.15 M-NaCl. All solutions were continuously bubbled with O_2 -free N_2 . Ferritins were reconstituted with $\text{Fe}_2(\text{NH}_4)_2(\text{SO}_4)_3$ and various amounts of KH_2PO_4 (St. Pierre *et al.*, 1988). Ferritin and haemosiderin solutions

were assayed for iron by electrothermal atomic-absorption spectrophotometry (Selden & Peters, 1979), for protein (Bradford, 1976) and for phosphate (Venkatesan & Ormerod, 1976).

Iron release to desferrioxamine (CIBA-GEIGY, Horsham, East Sussex, U.K.) was determined by dialysis (37°C with constant shaking) of 1 ml of ferritin or haemosiderin solution diluted to 1 mM-iron in 50 mM-Tris/HCl buffer, pH 7.4, against 20 ml of 1 mM-desferrioxamine in the same buffer. Samples (1 ml) of dialysate were assayed for iron by electrothermal atomic-absorption spectrophotometry. Iron release to 1,10-phenanthroline was determined essentially as described by Hoy *et al.* (1974). Ferritin or haemosiderin (500 μM -iron) was incubated at 37°C in 50 mM-Tris/HCl buffer, pH 7.4, containing 500 μM -1,10-phenanthroline hydrochloride. Formation of the 1,10-phenanthroline- Fe^{2+} complex was monitored at 510 nm (ϵ $1.15 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Measurement of iron release from haemosiderin to pH 4.5 sodium acetate or sodium citrate buffer depended on the insolubility of haemosiderin at this pH. After incubation for up to 48 h at 37°C , with constant shaking, haemosiderin was pelleted by centrifugation (10000 g for 10 min) and released iron was assayed in the supernatant by electrothermal atomic-absorption spectrophotometry. Where indicated the pellets were resuspended in fresh buffer and further incubated.

Gel-exclusion chromatography was performed with a Sephadex G-25 (superfine grade) (Pharmacia) column (12 mm $\times$ 130 mm), eluted with 50 mM-sodium acetate buffer, pH 4.5. When a Sephadex G-10 column (10 mm $\times$ 100 mm) was used it was eluted with 50 mM- or 200 mM-sodium acetate buffer, pH 4.5. A 300 ng portion of released iron was loaded in each experiment.

Small unilamellar liposomes were prepared by the evaporation of chloroform solutions of egg phosphatidylcholine (Sigma Chemical Co.) to form a thin film of lipid. The lipid was resuspended to a concentration of 20 mg/ml in 50 mM-sodium acetate buffer, pH 4.5. The liposomes were allowed to equilibrate for 1 h and then sonicated on ice for 10 $\times$ 60 s with intervals of 30 s, at 20 μm peak-to-peak with an MSE 150 W mark II sonicator. Lipid-peroxidation experiments were at 37°C in 50 mM-sodium acetate buffer, pH 4.5, with 2 mg of lipo-

somes/ml and iron released from haemosiderin. Malondialdehyde was determined by reaction with thiobarbituric acid (Slater & Sawyer, 1971; $\epsilon_{535} 1.5 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

RESULTS AND DISCUSSION

Comparison of iron release from ferritin and haemosiderin

Human spleen haemosiderin dialysed (at 37 °C at pH 7.4) against excess desferrioxamine released a maximum of 10% of the iron over 48 h (Fig. 1). Iron-matched ferritin (from the same spleen) released 30% of its iron over a similar period, the initial rate of iron release being approx. 3-fold greater than from haemosiderin. This slow release of iron from haemosiderin and relatively rapid release from ferritin was typical of iron preparations from four different human spleens (Table 1). These spleens had iron contents of 0.95–19.33 mg/g of tissue.

Fig. 1. Iron release from human spleen haemosiderin and ferritin to desferrioxamine

Human ferritin (●) and haemosiderin (○) were each diluted to 1 mM-iron in 50 mM-Tris/HCl buffer, pH 7.4. A 1 ml portion of each diluted solution was dialysed at 37 °C against 20 ml of 1 mM-desferrioxamine in the same buffer. Data are the means $\pm$ S.D. for three experiments.

Haemosiderin iron/protein ratios tended to increase with increasing tissue iron content. In each case the iron release from ferritin was substantially greater than that from haemosiderin. The spleens with relatively light iron load yielded only traces of haemosiderin (Table 1). Similar results were obtained with samples prepared from horse spleen (Fig. 2), where 10% and 20% of iron was released from haemosiderin and ferritin respectively. These findings are consistent with greater hydroxyl-radical generation (O'Connell *et al.*, 1986a) and lipid peroxidation (O'Connell *et al.*, 1985) after incubation with ferritin than with haemosiderin. The result is, however, in disagreement with a report by Kontoghiorgis *et al.* (1987), which showed a relatively more rapid release from haemosiderin to various chelators. Their method for haemosiderin preparation seems to be abbreviated from that of Weir *et al.* (1984) and therefore the preparation will be less pure. This is supported by the low iron content of their haemosiderin preparation (7.5%) compared with that obtained in this laboratory (63–88%). It should therefore not be accepted that the

Fig. 2. Iron release from horse spleen haemosiderin and ferritin to desferrioxamine

Horse ferritin (■) and haemosiderin (□) were dialysed as described in Fig. 1. Data are means $\pm$ S.D. for three experiments.

Table 1. Human spleen iron content, haemosiderin and ferritin iron/protein ratios and iron-release rates

Iron release to desferrioxamine was determined as described in the legend to Fig. 1. Data (other than haemosiderin recoveries) are the means for two experiments (with less than 5% variation).

Spleen	Iron (mg/g of tissue)	Haemosiderin recovered (mg/30 g of spleen)	Haemosiderin iron (mg/mg of protein)	Haemosiderin iron release (nmol/24 h)	Ferritin iron (mg/mg of protein)	Ferritin iron release (nmol/24 h)
1	0.25	< 1	—	—	—	—
2	6.58	205	2.2	74	0.13	313
3	0.43	< 1	—	—	—	—
4	0.58	< 1	—	—	—	—
5	0.95	7	1.7	51	0.10	125
6	19.33	600	7.3	48	0.47	190
7	2.57	50	1.7	46	0.14	138

Fig. 3. Iron release from reconstituted horse spleen ferritin to desferrioxamine

Reconstituted ferritins were diluted to 1 mM-iron and 0.44 mM-phosphate (●), 0.36 mM-phosphate (○), 0.34 mM-phosphate (■) or 0.01 mM-phosphate (□) in 50 mM-Tris/HCl buffer, pH 7.4. A 1 ml portion of each diluted solution was dialysed at 37 °C against 20 ml of 100 μ M-desferrioxamine in the same buffer. Data are the means for two experiments.

Fig. 4. Iron release from human spleen ferritin and haemosiderin to 1,10-phenanthroline

Ferritin and haemosiderin were each diluted to 500 μ M-iron and incubated at 37 °C in 50 mM-Tris/HCl buffer, pH 7.4, with 500 μ M-1,10-phenanthroline hydrochloride. Ferritin samples contained 0.14 mg (□) and 0.47 mg (○) of iron/mg of protein. Haemosiderin samples contained 2.2 mg (■), 7.3 mg (●) and 1.7 mg (▲) of iron/mg of protein. Data are the means for two experiments.

formation of haemosiderin favours iron release; indeed, the results from this laboratory have consistently suggested that haemosiderin formation is a protective mechanism in iron overload (O'Connell & Peters, 1987).

Haemosiderin cores have higher phosphate content than those of ferritin (Weir *et al.*, 1984; St. Pierre *et al.*, 1988), and the possibility that this accounts for differences in iron release was investigated. Ferritin was reconstituted

Fig. 5. Iron release from haemosiderin to acetate and citrate

Haemosiderin (500 μ M-iron) was incubated at 37 °C with 50 mM-sodium acetate buffer, pH 4.5 (■), or 10 mM-sodium acetate buffer, pH 4.5 (●), or 50 mM-sodium citrate buffer, pH 4.5 (○). Data are means for two experiments or means $\pm$ S.D. for three experiments.

from apo-ferritin with a range of phosphate concentrations (up to 0.44 mmol/mmol of iron) incorporated into the iron core. The rate of iron release from the reconstituted ferritins to desferrioxamine increased with increasing phosphate content (Fig. 3). This is therefore an important factor in determining the rate of release of iron from ferritin, but does not explain the slow release from haemosiderin described above.

Previous reports (Jones & Johnston, 1967; Hoy *et al.*, 1974) show that iron-matched ferritin samples show decreased rates of iron release to 1,10-phenanthroline with increased iron/protein ratio. Human spleen haemosiderin samples of a wide range of iron/protein ratios were found to release iron to 1,10-phenanthroline 4-fold less rapidly than a lightly loaded human spleen ferritin sample (Fig. 4) and 2-fold less rapidly than a heavily loaded ferritin (core size close to maximum). Thus iron/protein ratio does not fully account for differences in iron-release rates from ferritin and haemosiderin.

These findings leave the possibility of some other difference in the nature of ferritin and haemosiderin cores. Indeed, a difference in the Mössbauer spectra of these iron proteins has been found, suggesting differences in the mineral nature of the iron core (Bell *et al.*, 1984). Direct evidence for such a difference in crystalline structure between ferritin (ferrihydrite) and haemosiderin (goethite) has been obtained (Mann *et al.*, 1988) by high-resolution electron microscopy, and would explain the relatively slow release of iron from haemosiderin.

Iron release from haemosiderin to physiological chelators

Haemosiderin is the predominant iron protein in iron-overloaded tissue (Selden *et al.*, 1980). Therefore even

Fig. 6. Absorption spectrum of acetate-mobilized haemosiderin iron

The Figure shows absorption spectra of iron released by incubating haemosiderin at 37 °C for 24 h in 50 mM-sodium acetate buffer, pH 4.5 (A), iron-matched FeCl_3 (4.1 μM) in the same buffer (B) and buffer alone (C).

relatively slow iron release may be important in the pathogenesis of tissue injury. It was previously shown that haemosiderin will release iron in acetate buffer (O'Connell *et al.*, 1985). It is likely that haemosiderin is exposed to carboxylic acids *in vivo*, and mobilization of iron by these species could therefore be involved in the toxicity of haemosiderin iron. Rates of iron release to acetate and citrate were therefore studied and the properties of the released iron were investigated. A small proportion of haemosiderin iron was released into acetate buffer, pH 4.5 (Fig. 5). The rate of iron release was greater in 50 mM-acetate than in 10 mM-acetate. At equal concentrations citrate buffer, pH 4.5, caused release of iron at an initial rate approx. 20-fold greater than that in acetate buffer. Citrate is known to chelate iron with high affinity, whereas acetate is a weak chelator (Dawson *et al.*, 1986). The results are therefore consistent with release by a chelation mechanism.

Previous studies show that haemosiderin in acetate buffer stimulates lipid peroxidation in liposomes (O'Connell *et al.*, 1985). In the present study haemosiderin (500 μM -iron) was incubated at 37 °C for 4 h with phosphatidylcholine liposomes (2 mg/ml) in 50 mM-sodium citrate buffer, pH 4.5. No thiobarbituric acid-reactive product was detectable, suggesting that this chelator releases iron but inhibits lipid peroxidation. Desferrioxamine and EDTA were found to have similar properties. It is likely that the major role of iron in lipid peroxidation is its reaction with lipid hydroperoxides (Gutteridge & Quinlan, 1983). It is possible that high-affinity chelators block this interaction, but Fe^{3+} bound to low-affinity acetate ligands may well react to generate lipid peroxy radicals. Further work was therefore directed at studying properties of the potentially toxic acetate-released iron.

Fig. 7. Gel filtration of acetate-mobilized haemosiderin iron

The Figure shows elution of ferritin (A_{400}) and acetate-mobilized haemosiderin iron (○) from Sephadex G-25.

Fig. 8. Lipid peroxidation stimulation by acetate-released haemosiderin iron

Phosphatidylcholine liposomes (2 mg/ml) were incubated at 37 °C with iron released after 2 h (■) or 24 h (●) preincubation of haemosiderin (500 μM -iron) in 50 mM-sodium acetate buffer, pH 4.5, or iron-matched FeCl_3 [1.9 μM (□) and 3.1 μM (○) respectively] in 50 mM-sodium acetate buffer, pH 4.5, or native haemosiderin at 3.1 μM -iron (△).

Acetate-released haemosiderin iron has greater u.v. absorbance than iron-matched FeCl_3 in 50 mM-acetate buffer (Fig. 6). This suggests that the released iron is not exclusively monomeric ferric acetate. The size of the released iron particles was investigated by gel filtration. The iron was found to bind to a Sephadex G-10 column, but was eluted in the included volume of a small Sephadex G-25 column (Fig. 7). The particles are therefore likely to have a molecular mass less than 5000 Da.

Iron released from haemosiderin after 2 h or 24 h preincubation in acetate buffer stimulated lipid peroxidation in phosphatidylcholine liposomes (Fig. 8). FeCl_3 in acetate buffer at equivalent iron concentration (1.9 and 3.1 μM) stimulated approx. 30–50 % greater lipid peroxidation, whereas native haemosiderin at these low iron concentrations did not stimulate any lipid per-

oxidation. This again suggests that at least some of the released iron is not present as monomeric ferric acetate but probably occurs as oligomeric ferrihydride solubilized by acetate ligands.

CONCLUSIONS

These and previous results suggest that the deposition of haemosiderin in tissues may afford some protection when compared with the alternative of accumulation of ferritin. However, since most of the iron is in the form of haemosiderin in iron-overloaded tissue, it may play a major role in tissue damage. Toxicity could be mediated by released iron. Most of the released iron seems to be present as oligomeric particles. These could be released directly from haemosiderin cores or formed by the polymerization of monomeric iron.

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