Haemosiderin-like properties of free-radical-modified ferritin

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Conjugated-Schiff's-base-type fluorescence was measured in iron-depleted samples and chloroform extracts of human spleen haemosiderin. Incubation of ferritin with liposomes and ascorbate led to the formation of compounds with similar fluorescence properties. Analysis of protein subunits by SDS/polyacrylamide-gel electrophoresis confirmed that ferritin was damaged in incubations with ascorbate. Since previous studies have shown that intact ferritin is resistant to proteolytic degradation, it is suggested that haemosiderin may be a product of oxidative reactions involving ferritin and lipid.

INTRODUCTION

Iron overload is known to cause tissue damage. Morphological (Iancu & Neustein, 1977) and biochemical (Selden *et al.*, 1980) studies of iron-overloaded tissue have demonstrated that ferritin is increased 5-fold but that most of the excess iron is present as haemosiderin. Both ferritin and haemosiderin have been shown to stimulate lipid peroxidation in liposomes (O'Connell *et al.*, 1985) and production of hydroxyl radicals in the presence of hydrogen peroxide (O'Connell *et al.*, 1986). In both processes ferritin is more effective than haemosiderin, suggesting that haemosiderin formation in iron overload may be a cytoprotective mechanism. The biological mechanisms for haemosiderin formation are, however, uncertain. SDS/polyacrylamide-gel electrophoresis of purified haemosiderin has shown that it is free of the 20 kDa subunits of ferritin, but contains several lower-molecular-mass peptides (Weir *et al.*, 1984).

Ultrastructural studies have demonstrated that haemosiderin occurs within hepatic lysosomes (Iancu & Neustein, 1977), and it has been assumed that, in iron overload, excess ferritin accumulates in these organelles and is partially degraded by cathepsins. However, incubation of ferritin *in vitro* with purified lysosomes does not lead to proteolysis of the protein shell (Coffey & de Duve, 1968) or conversion into haemosiderin, as judged by chromatographic behaviour (Peters & Selden, 1982).

Recently, amino acid analyses have shown that aromatic and thiol residues have low abundance in haemosiderin compared with ferritin (Weir *et al.*, 1984), a finding that is consistent with their oxidation during interconversion. Solubilization of iron from ferritin would be favoured by the acidic intralysosomal pH, and this could lead to stimulation of oxidative free-radical reactions (O'Connell *et al.*, 1985).

Haemosiderin preparations often contain lipid and small amounts of carbohydrate in addition to protein and iron (Weir *et al.*, 1984). Lipofuscin, which is formed in lysosomes of certain aged tissues, has similarities to haemosiderin in that it is composed of partially degraded lipid, protein and other molecules (Reichel, 1968). Lipofuscin is characterized by a yellow fluorescence, which is associated with conjugated Schiff's bases. These are thought to be formed by the reaction of malonaldehyde (a product of peroxidizing lipids) with primary amino groups of proteins and nucleic acids. It is possible that haemosiderin formation is analogous to that of lipofuscin, i.e. it is the result of the accumulation of products of oxidation, primarily of ferritin.

The aim of the present study was to determine whether haemosiderin has Schiff's-base-type fluorescence and whether the exposure of ferritin to free radicals and peroxidizing lipids would convert it into a haemosiderin-like material with respect to fluorescence properties and its peptide composition as shown by polyacrylamide-gel electrophoresis.

MATERIALS AND METHODS

Chloroform and methanol were supplied by Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K., and redistilled before use; other chemicals were of AnalaR grade, supplied by BDH Chemicals, Poole, Dorset, U.K., and used without further purification. Human haemosiderin (Weir *et al.*, 1984) and ferritin (Cham *et al.*, 1985) were isolated from iron-overloaded human spleens that had been removed from patients with β-thalassaemia. Haemosiderin was freeze-dried and stored desiccated at room temperature. Solutions (approx. 10 mM-iron) were prepared in 20 mM-tetramethylammonium hydroxide solution (Weir *et al.*, 1984). Iron (determined with a Perkin–Elmer 2380 electrothermal atomic-absorption spectrophotometer) as a ratio to protein (Bradford, 1976) was 7.3 mg/mg in haemosiderin and 0.2 mg/mg (2500 mol/mol) in ferritin.

Apo-ferritin and apo-haemosiderin were prepared by thioglycollic acid treatment. Solutions of the iron proteins (3 ml) were dialysed against two changes of thioglycollic acid (0.1 mol/l) in sodium acetate (0.1 mol/l) and two changes of NaCl (0.15 mol/l). Each dialysis was for 2 h at room temperature and with continuous bubbling of O_2-free N_2. Dialysed samples contained a precipitate, which was cleared by the addition of tetramethylammonium hydroxide to a final concentration of 20 mmol/l.

Small unilamellar liposomes were prepared from chloroform solutions of egg phosphatidylcholine (Sigma) by using a rotary evaporator to produce a thin film of lipid, which was resuspended in water, with the aid of a few glass beads, to a concentration of 20 mg/ml. After 1 h at room temperature the suspension was sonicated on ice for ten periods of 60 s with intervals of 30 s at 20 μm peak-to-peak, with an MSE 150 W Mark II sonicator fitted with an exponential probe.

Table 1. Fluorescence properties of ferritin and haemosiderin

Units are shown as relative fluorescence-intensity units (RFI) and the results are means for two preparations. Ferritin, haemosiderin and the apoproteins were solubilized in aq. 20 mM-tetramethylammonium hydroxide, with protein concentrations shown in parentheses. Abbreviations: Ex., excitation wavelength; Em., emission wavelength.

Protein	Concn. (μg/ml)	Fluorescence (RFI) U.v. (Ex. 283 nm; Em. 340–357 nm)	Fluorescence (RFI) Visible (Ex. 402 nm; Em. 500 nm)
Ferritin	100	48	2.0
	10	36	1.0
Apo-ferritin	100	643	6.0
Haemosiderin	100	0.5	0.6
	10	1.0	9.9
Apo-haemosiderin	100	250	90.2

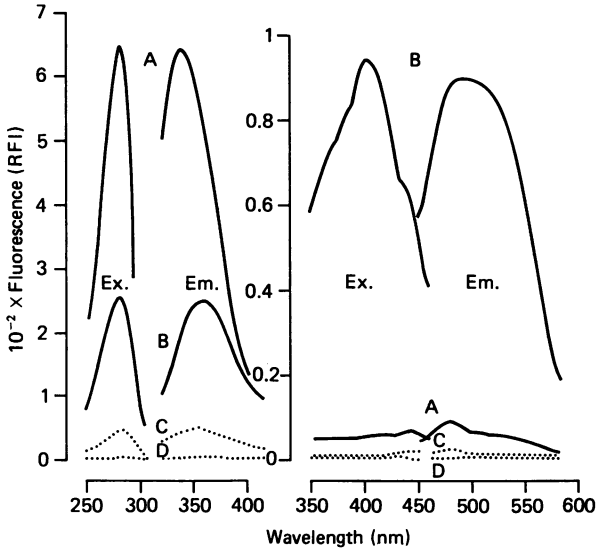

Fig. 1. U.v. and visible fluorescence spectra of apoferritin and apo-haemosiderin

Fluorescence excitation (Ex.) and emission (Em.) spectra of: A, apoferritin; B, apo-haemosiderin; C, ferritin; and D, haemosiderin. The protein concentration was 100 μg/ml in aq. tetramethylammonium hydroxide 20 mmol/l). RFI is defined in Table 1.

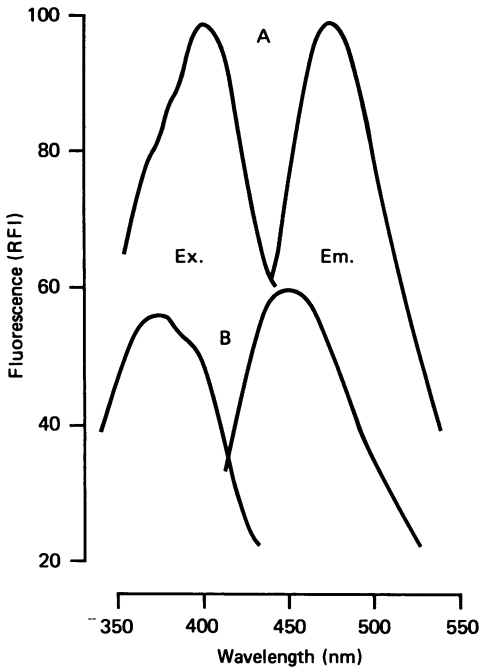

Fig. 2. Chloroform-extractable fluorescence of modified ferritin and haemosiderin

Fluorescence excitation and emission spectra of chloroform extracts: A, ferritin (protein 0.2 mg/ml) pre-incubated for 24 h with ascorbate (500 μmol/l) and liposomes (2 mg/ml); B, haemosiderin (0.6 mg of protein/ml) in the presence of liposomes (2 mg/ml) without pre-incubation. RFI is defined in Table 1.

Ferritin incubations were at 37 °C with additions and concentrations given in the legends to the appropriate Figures. Samples for fluorescence measurements were either aqueous solutions of the iron proteins in tetramethylammonium hydroxide (20 mmol/l) or chloroform extracts from incubations containing ferritin and liposomes. Aliquots (1 ml) were vortex-mixed with 2 ml of chloroform/methanol (2:1, v/v) and centrifuged at 3000 *g* for 5 min. A 1 ml portion of the chloroform layer was added to 0.1 ml of methanol. Fluorescence was measured with a Perkin–Elmer LS-3 fluorescence spectrometer. Emission units were standardized with quinine sulphate [2.68 μmol/l in H_2SO_4 (0.1 mol/l) set to 75 divisions (excitation 402 nm; emission 500 nm).

SDS/polyacrylamide-gel electrophoresis was performed on 10 cm × 10 cm × 0.1 cm slab gels as described by Weber & Osborn (1975). Separating gels contained 15% (w/v) acrylamide with a 3% (w/v) stacking gel, both at pH 8.8, and were run in a water-cooled Bio-Rad model 220 slab-gel-electrophoresis apparatus. Freeze-dried haemosiderin samples (1 mg) were extracted for electrophoresis by heating with 60 μl of 125 mM-Tris/HCl, pH 8.8, containing 2.5% (w/v) SDS at 100 °C for 10 min and centrifuging to remove the insoluble iron oxide. 2-Mercaptoethanol (5μl) were then added to these

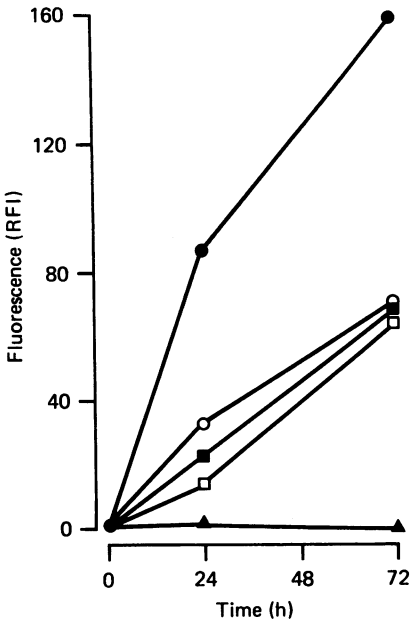

Fig. 3. Changes in the chloroform-extractable fluorescence of ferritin exposed to peroxidizing lipid

Fluorescence (excitation 400 nm; emission 475 nm; mean of two separate experiments) in chloroform extracts from aqueous incubations (37 °C) containing: ●, ferritin (0.2 mg of protein/ml, 1.04 mmol of Fe/l), liposomes (2 mg/ml) and ascorbate (500 μmol/l); ○, ferritin and liposomes; ■, $FeCl_3$ (1.04 mmol/l), liposomes and ascorbate; □, liposomes and ascorbate; ▲, ferritin and ascorbate. RFI is defined in Table 1.

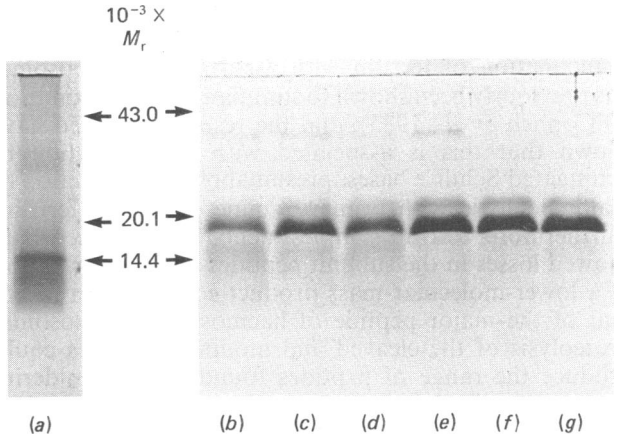

Fig. 4. SDS/polyacrylamide-gel electrophoresis of modified ferritins and haemosiderin

Tracks were loaded with (*a*) haemosiderin, or with ferritin (2 mg of protein/ml) preincubated (37 °C, 24 h) in the presence of: (*b*) 50 mM-sodium acetate, pH 4.5, and 500 μM-ascorbate; (*c*) 50 mM-Tris/HCl, pH 7.4, and 500 μM-ascorbate; (*d*) 500 μM-ascorbate; (*e*) 50 mM-sodium acetate, pH 4.5; (*f*) 50 mM-Tris/HCl, pH 7.4; (*g*) no additions.

supernatants, which were heated at 100 °C for a further 5 min.

Ferritin samples (30 μl) were prepared by adding final concentrations of 125 mM-Tris/HCl buffer (pH 8.8)/2.5% SDS in a total volume of 60 μl, 5 ml of 2-mercaptoethanol and heating at 100 °C for 10 min. Gels were stained for protein with Coomassie Brilliant Blue R-250 overnight and destained in water/acetic acid/methanol (28:7) (Weber & Osborn, 1975).

RESULTS AND DISCUSSION

Aqueous solutions of ferritin had u.v. fluorescence with excitation and emission peaks of 283 and 340 nm respectively (Table 1). This is consistent with the recognized properties of tryptophan-containing proteins (Weber, 1960). The fluorescence intensity was not directly related to ferritin protein concentration, suggesting that the samples were self-quenching. Indeed, u.v. fluorescence was much higher in samples of apo-ferritin than ferritin. Haemosiderin samples prepared from the same spleen, but with a much greater iron/protein ratio, had very little u.v. fluorescence. Again, preparation of the apoprotein dramatically increased the u.v. fluorescence intensity, but the fluorescence of apo-haemosiderin was only 40% of that of protein-matched apo-ferritin (Fig. 1). This is consistent with the lower content of tyrosine and phenylalanine residues in haemosiderin compared with ferritin (Weir *et al.*, 1984).

Aqueous solutions of apo-haemosiderin had substantial fluorescence (Fig. 1) at visible wavelengths (excitation 402 nm; emission 500 nm), consistent with the presence of Schiff's-base adducts of the type found in lipofuscin. Again the fluorescence is largely quenched in intact haemosiderin. Compared with apo-haemosiderin, protein-matched ferritin and apo-ferritin samples from the same spleen had very little 'visible' fluorescence (Table 1).

Chloroform extracts of incubations of ferritin in the presence of liposomes and ascorbate had Schiff's-base-type fluorescence (excitation 400 nm; emission 475 nm; Fig. 2). The fluorescence intensity was substantially greater than that extractable from control incubations in which either ferritin, liposomes or ascorbate was omitted or $FeCl_3$ replaced ferritin at equivalent iron concentrations (Fig. 3). Chloroform extracts of haemosiderin/liposome mixtures were also fluorescent at visible wavelengths (excitation nm; emission; Fig. 2).

As has been reported (Weir *et al.*, 1984), SDS/polyacrylamide-gel electrophoresis of human spleen haemosiderin shows a group of peptides ranging in M_r from 13000 to 18000 (Fig. 4*a*). Human spleen ferritin incubated for 24 h at 37 °C unbuffered, at pH 4.5 or at pH 7.4, with ascorbate (Figs. 4*b*, 4*c* and 4*d*) showed losses of the L-subunit (M_r 19000) and the minor H peptide (M_r 21000) when compared with ferritin incubated under respective conditions of pH in the absence of ascorbate (Figs. 4*e*, 4*f* and 4*g*). Samples incubated in the presence of ascorbate contain a rather diffuse band at M_r 16000. This material was not detectable in the absence of ascorbate and may represent a breakdown product of ferritin. In some samples (Figs. 4*b*, 4*c* and 4*e*) a band is detectable at M_r 40000, which is likely to be ferritin subunit dimer.

CONCLUSIONS

Fluorescence measurements were indicative of a relatively low content of aromatic amino acid residues and high content of conjugated Schiff's bases in haemosiderin as compared with ferritin. This is consistent with the view that haemosiderin is a product of oxidative

reactions occurring intralysosomally in iron-overload syndromes.

Incubation of ferritin with ascorbate and liposomes has previously been shown to stimulate lipid peroxidation (O'Connell *et al.*, 1985), and the results presented have shown that this is associated with the formation of conjugated Schiff's bases, presumably by the reaction of malonaldehyde with primary amine groups of ferritin. Furthermore, ferritin samples incubated with ascorbate showed losses in the subunit peptides and the formation of a lower-molecular-mass product equivalent in M_r to that of the major peptide of haemosiderin. Lysosomal proteolysis of the cleaved and modified peptides could produce the range of peptides found in haemosiderin. Hydroxyl radical is known to cleave lactate dehydrogenase, bovine serum albumin (Schuessler & Schilling, 1984) and yeast glutamine synthetase (Kim *et al.*, 1985). This species could be generated by Fenton chemistry when ferritin iron is reduced and mobilized by ascorbate. Certainly the exposure of ferritin to a free-radical-generating system has given rise to material which is haemosiderin-like with respect to its Schiff's-base content and partially so in its appearance on polyacrylamide-gel electrophoresis.

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