Biochemical studies on the isolation and characterization of human spleen haemosiderin

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Haemosiderin was isolated from thalassaemic human spleens by centrifugation through concentrated KI solutions. A method for solubilizing haemosiderin was developed which leaves the iron oxyhydroxide cores and constituent polypeptides intact, facilitating further purification and analysis. Purified haemosiderin contained no detectable haem, trace amounts of carbohydrate, and iron and phosphorus in a molar ratio of 6:1; much of the phosphate may be present as core-adsorbed. Several lipids were present, but it is not certain whether these are contaminants or components of the haemosiderin granules. In all preparations examined, a characteristic group of six to seven peptides of apparent M_r 12900–17800 were found, with a major band at M_r 14500 and, in addition, a minor component of M_r 42000; these peptides co-chromatographed with the cores. Negatively stained electron micrographs suggest that these peptides form an incomplete shell about the cores, consistent with the view that haemosiderin is a proteolytic product of ferritin.

In normal tissues, ferritin is the major mammalian iron-storage protein; it is composed of a protein shell, 8 nm in diameter, of M_r 440000–480000 (Wustefeld & Crichton, 1982), which surrounds and solubilizes a core of iron hydroxide and contains up to 4500 atoms of iron (Clegg *et al.*, 1980). However, in iron-overload conditions such as thalassaemia, haemosiderin becomes the predominant iron-containing material and has been implicated in the pathogenesis of the tissue damage observed in such conditions (Peters *et al.*, 1977). Haemosiderin is poorly characterized, partly because of its insolubility in water, but it is known to contain iron oxyhydroxide cores similar to those of ferritin (Fischbach *et al.*, 1971) and it is known that these cores are present as large, dense, membrane-bound aggregates *in vivo* (Richter, 1978). It is thought that haemosiderin is produced by lysosomal degradation of ferritin (Iancu & Neustein, 1977) or possibly ferritin polymers (Hoy & Jacobs, 1981). Attempts to purify this material

have resulted in products that show considerable variation in composition. All products have high iron/protein weight ratios, although the nature of the protein is unknown. Lipids of various classes, and sometimes haem and carbohydrates, have also been detected (Wixom *et al.*, 1980), as have small amounts (less than 1%, w/w) of ferritin (McKay & Fineberg, 1964a; Van Wyk *et al.*, 1971) in various haemosiderin preparations. In addition, little is known of the trace-metal composition of haemosiderin.

In the present study the biochemical components of human spleen haemosiderin are examined; to assist in this analysis, a method for solubilizing haemosiderin without apparent degradation of individual cores or of the protein shell has been developed.

Experimental

Isolation of haemosiderin and ferritin

Haemosiderin and ferritin were isolated from iron-overloaded spleens removed from patients who had received multiple transfusions for homozygous β -thalassaemia and frozen fresh at -20°C . Haemosiderin was isolated by a modification of the method of McKay & Fineberg (1964b). Spleen

Abbreviation used: SDS, sodium dodecyl sulphate.

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(50–60g) was homogenized on ice in 220ml of 0.15M-NaCl, filtered through gauze and centrifuged at 5000g for 30min; the pellets were pooled, washed by centrifugation in a further 220ml of 0.15M-NaCl and then resuspended in 120ml of KI to a final concentration of 4.1M. The suspension was centrifuged for 120min at 90000g, the pellets resuspended in 60ml of 4.1M-KI and re-centrifuged as above. The pellets were then twice washed in 60ml of distilled deionized water. Ferritin was purified by heat denaturation of spleen homogenate, $(\text{NH}_4)_2\text{SO}_4$ precipitation and gel filtration as described previously (Huebers *et al.*, 1974). Electron microscopy was performed as described by Bell *et al.* (1984).

Analytical methods

Iron concentration was measured by flameless atomic-absorption spectrophotometry with a dual-beam Perkin-Elmer model 703 spectrophotometer with a graphite furnace and an AS1 automatic sampler. The hollow-cathode lamp (248.3nm wavelength) was set at 30mA and 0.2nm slit width (Selden & Peters, 1979). All glassware was washed in 2M- HNO_3 and deionized double-distilled water before use. Amino acid analyses were performed with a Beckman model 121 M analyser. Samples were hydrolysed for 18h at 110°C in 6M-HCl containing norleucine as the internal standard and phenol as antioxidant.

Carbohydrates were determined by g.l.c. of the trimethylsilyl ether derivatives of the methyl glycosides as described by Bhatti *et al.* (1970). Samples were chromatographed on a 17.8cm $\times$ 0.64cm (7in $\times$ 0.25in) Chromosorb WHP column (Chromatography Services, Hoylelake, Merseyside, U.K.), with a gas flow of 35ml/min and a temperature program of 100–230°C at 2°C/min.

Freeze-dried haemosiderin (1–2mg) was dissolved in 0.5ml of 3M-HCl and extracted in the dark with 3 vol. of ethyl acetate. The organic layer was then dried and dissolved in dimethyl sulphoxide/acetonitrile (1:1, v/v) and analysed by high-pressure liquid chromatography (Lim & Chan, 1982). A Varian 5000 liquid chromatograph with a UV-50 variable-wavelength detector set at 400nm was used. Separation was performed in a 10cm $\times$ 5mm ODS-Hypersil column (Shandon Southern Products, Runcorn, Cheshire, U.K.) run isocratically in dimethyl sulphoxide/acetonitrile (1:1, v/v).

Lipid analyses were performed as described by Cairns & Peters (1983). Freeze-dried haemosiderin samples were suspended by sonication in 1.5ml of 0.15M-NaCl containing 500 μg of butylated hydroxytoluene (Sigma) as an antioxidant and 250 μg of cholesterol acetate (Sigma) as an internal

standard. The suspension was extracted for 1h in 9ml of chloroform/methanol (2:1, v/v), centrifuged at 3000g for 5min and the organic layer dried in a stream of N_2 . Lipids were identified by t.l.c. and specific chemical assays.

Solubilization and chromatography of haemosiderin

Lyophilized haemosiderin was suspended in freshly prepared 0.02M-tetramethylammonium hydroxide, pH 12.3, by sonication for 5s at 60W (20 μm amplitude). The mixture was left for 45min at room temperature, with further 5s sonications after 20min and 40min. A cloudy solution formed, which was then centrifuged for 20min at 3000g. The pellet of insoluble haemosiderin, which was washed with 0.02M-tetramethylammonium hydroxide and dried, comprised $5.3 \pm 1.5\%$ (w/w) of the original haemosiderin, and contained roughly the same proportion of iron.

Some precipitate was usually visible in the supernatant if left overnight at 4°C. A more stable solution may be formed by lowering the pH in the presence of detergent. In this procedure, haemosiderin was solubilized in 0.02M-tetramethylammonium hydroxide containing 1% Nonidet P40. After 45min, NH_4HCO_3 (1M) was added dropwise, while the mixture was stirred, to a final concentration of 0.1M with a pH of 8.0. Centrifugation for 20min at 3000g produced a pellet containing 13–16% (w/w) of the original haemosiderin. Again, the iron content in the pellet was similar to that of the solubilized haemosiderin. This preparation was sufficiently stable to be chromatographed overnight without apparent precipitation.

Chromatography of haemosiderin solution was performed on a 2.2cm $\times$ 76cm Sepharose 6B column equilibrated with 100mM- NH_4HCO_3 containing 1% Nonidet, pH 8.0. The flow rate was 15ml/h, and chromatography was performed at 4°C. A 2ml sample of solution was loaded, and fractions (2ml each) were collected.

SDS/polyacrylamide-gel electrophoresis was performed on 10cm $\times$ 10cm $\times$ 0.1cm slab gels as described by Weber & Osborn (1975). Sample gels contained 12.5% (w/v) acrylamide with a 3% (w/v) stacking gel, and were run in a water-cooled Bio-Rad model 220 slab-gel-electrophoresis apparatus.

Column fractions from Sepharose 6B chromatography were precipitated with 0.6M-trichloroacetic acid and the precipitate extracted in diethyl ether/ethanol (2:1, v/v). Pellets were then dissolved in 0.8M-mercaptoacetic acid in 250mM-acetic acid/sodium acetate buffer, pH 5.1. Peptide was then re-precipitated with 0.6M-trichloroacetic acid in the presence of 0.5% Nonidet P40, extracted again in diethyl ether/ethanol and finally dissolved by boiling for 15min in 100 μl of 125mM-

Tris/HCl, pH 6.8, containing 2.5% SDS and 5 μ l of 2-mercaptoethanol (SDS buffer). For electrophoresis of haemosiderin protein, 0.5–1 mg samples of freeze-dried haemosiderin were extracted for 10 min in 100 μ l of 125 mM-Tris/HCl, pH 6.8, containing 2.5% SDS, and centrifuged to remove the insoluble iron oxide; 5 μ l of 2-mercaptoethanol was then added to the supernatant. Gels were stained for protein with Coomassie Brilliant Blue R-250 for 1 h, destained in acetic acid/methanol (Weber & Osborn, 1975) and scanned with a Gelman ACD-18 densitometer set at 600 nm.

Elemental analysis

Samples of haemosiderin and ferritin were dissolved overnight in 3 M-HCl and diluted with distilled deionized water to 1 M-HCl. Cloudy solutions formed, and the insoluble residue was removed by centrifugation at 3000 g for 10 min. Standards of each element were dissolved in 1 M-HCl. Measurements were made with a Bausch and Lomb (ARL) 34000 B inductively coupled plasma atomic-emission spectrophotometer.

Results and discussion

Analysis of haemosiderin

Iron and protein contents of haemosiderin preparations are given in Table 1. The iron content compares closely with the values found for horse spleen (McKay & Fineberg, 1964a) and human liver (Ludewig & Franz, 1970) haemosiderin. Our data indicate iron/protein weight ratios of 1.4–2.0,

compared with 0.1–0.4 for ferritin (Linder & Munro, 1972), in keeping with the generally high iron/protein ratios found in haemosiderin (Wixom *et al.*, 1980).

Galactose (200 μ mol/g), mannose (100 μ mol/g) and a small amount of fucose (20 μ mol/g) were detected in haemosiderin. However, hexosamines and sialic acid were not found (<5 μ mol/g); *N*-acetylgalactosamine and *N*-acetylglucosamine are common constituents of mammalian carbohydrate side chains (Kornfeld & Kornfeld, 1976) and their absence here, together with the generally low levels and variability in the carbohydrate compositions reported previously (McKay & Fineberg, 1964a; Ludewig & Franz, 1970), suggests that these sugars are contaminants.

Haem and porphyrin compounds were not found (<0.1 nmol/mg of haemosiderin), in agreement with Van Leeuwen *et al.* (1977), who found that haemosiderin only contained haem if the tissue was left unfrozen after death; the occurrence of haem in some preparations (Ludewig & Franz, 1970; McKay & Fineberg, 1964b) may be attributable to this.

Lipid analyses (Table 2) reveal the presence of substantial quantities of various lipids in the haemosiderin preparations; similar findings occur with chloroform/methanol extracts of haemosiderin solubilized in tetramethylammonium hydroxide. Phospholipid (Ludewig & Franz, 1970), free fatty acids and small amounts of cholesterol (McKay & Fineberg, 1964b) have previously been detected in haemosiderin. It is possible that this lipid is trapped by the sedimenting haemosiderin granules during isolation, rather than being an intrinsic part of those granules. The phospholipid composition of haemosiderin presented here shows no particular resemblance to lysosomal phospholipids (Henning & Heidrich, 1974) and the low cholesterol level suggests that direct derivation from autophagocytosed erythrocytes is unlikely (Roelfsen *et al.*, 1981).

Solubilization, chromatography and peptide composition of haemosiderin

Solubilization of haemosiderin in 0.02 M-tetramethylammonium hydroxide, pH 12.3, appears to

Table 1. Iron and protein contents of haemosiderin
Iron and protein contents of freeze-dried haemosiderin (means $\pm$ S.E.) isolated from iron-loaded spleens are shown. Protein was determined by amino acid analysis and iron by atomic-absorption spectroscopy. Values in parentheses indicate the number of preparations analysed.

Spleen sample	Content (% , w/w)	
	Iron	Protein
S1	25.4 $\pm$ 3.2 (4)	11.2 $\pm$ 1.3 (3)
S2	22.4 (1)	16.2
S3	24.2 $\pm$ 1.4 (3)	—
S4	24.0 (1)	13.7

Table 2. Major lipid classes in haemosiderin

Individual phospholipid compositions were: sphingomyelin, 5.0 $\pm$ 0.4 (13%); phosphatidylcholine, 5.6 $\pm$ 0.4 (15%); phosphatidylserine + phosphatidylinositol, 15.0 $\pm$ 1.3 (39%); phosphatidylethanolamine, 12.6 $\pm$ 0.6 (33%). Results for three haemosiderin samples are means $\pm$ S.E.M.. The total lipid content was approx. 6.7% (w/w) of haemosiderin.

	Free cholesterol	Cholesteryl esters	Phospholipid	Non-esterified fatty acids	Triacylglycerol
Lipid content (nmol/mg of freeze-dried haemosiderin) . . .	3.8 $\pm$ 2.5	0.4 $\pm$ 0.03	46.4 $\pm$ 2.8	24.9 $\pm$ 9.3	23.9 $\pm$ 2.1
Lipid distribution (%) . . .	4	< 1	47	25	24

disaggregate haemosiderin without causing major disruption of the core, since no dialysable iron complexes were formed and the cores had a similar appearance under the electron microscope before and after solubilization. The electronic spectra of ferritin and haemosiderin solutions are closely similar both in form and absorption coefficient, although these spectra are unfortunately devoid of any diagnostic features (see Fig. 1).

A typical profile obtained from chromatography of haemosiderin solution on Sepharose 6B is shown in Fig. 2; very similar results were obtained for various haemosiderin preparations, with a range of 2.5–10 mg of haemosiderin/ml. It is apparent that about two-thirds of the cores are present as aggregates of apparent M_r greater than about 4000000, equivalent to Stoke's radii of over 10 nm. Recoveries of total iron from the column were 60–70%.

SDS/polyacrylamide-gel electrophoresis of freeze-dried haemosiderin from three spleens (S1, S2 and S3) (Fig. 3) revealed a strikingly similar peptide composition; the main feature is a peptide of apparent M_r 14500, with additional bands at M_r 12900, 14100, 15500, 16600, 17800 and 42000 (calculated from standards). A band running in the same place as, and thus assigned to, ferritin subunit (M_r 20000; Wustefeld & Crichton, 1982) is also present in unpurified haemosiderin. It is not detectable in void fractions from Sepharose 6B chromatography (see Fig. 3), suggesting that the

Fig. 1. Electronic spectra of ferritin and haemosiderin solutions in 100mM- NH_4HCO_3 /1% Nonidet P40. Haemosiderin: A_{240} of a 1mg of Fe/ml solution = 10.3 ± 0.5 (mean $\pm$ S.E.M. for three preparations). Ferritin: A_{420} of a 1mg of Fe/ml solution = 10 (Harrison *et al.*, 1974).

Fig. 2. Sepharose 6B gel chromatography of haemosiderin solution in 100mM- NH_4HCO_3 /1% Nonidet P40, pH 8. Haemosiderin S1 (20mg) was loaded on to the column and 2ml fractions collected; the flow rate was 15ml/h. Calibrants were Blue Dextran for the void volume ($M_r > 4000000$), thyroglobulin (M_r 669000) and ferritin (M_r 440000).

Fig. 3. SDS/polyacrylamide-gel electrophoresis, on 12.5% (w/v) acrylamide reducing gels of ferritin and haemosiderin. Track a, human spleen ferritin; tracks b–d, haemosiderins S2, S3 and S1 respectively. Track e is the void-volume fraction 53 from Sepharose 6B chromatography (Fig. 2). Note the apparent absence of ferritin from this fraction. M_r values were calculated by calibration with standard proteins: phosphorylase b (M_r 9400); bovine serum albumin (M_r 67000); ovalbumin (M_r 43000); carbonic anhydrase (M_r 30000); soya-bean trypsin inhibitor (M_r 20000); and α -lactalbumin (M_r 14400).

ferritin component has been largely separated from the haemosiderin cores. Densitometer scans (Fig. 4) emphasize the similarities in distribution and relative quantities of haemosiderin peptides and reveal the presence of a small shoulder on the low- M_r side of the 14500 band; it is possible that these peptides arise by proteolysis from ferritin. A peptide of M_r about 14000 is commonly present in ferritin preparations and has been shown to be a proteolytic product of ferritin subunit (Arosio *et al.*, 1978). The 42000- M_r peptide could conceivably arise from covalent but non-disulphide coupling of two ferritin subunits; such dimers have been observed in ferritin, particularly in preparations of low iron content, where up to 50% of subunits may be in this form (Mertz & Theil, 1983).

Electron micrographs of negatively stained haemosiderin (Fig. 5) show incomplete, and sometimes absent, rings of non-staining material, probably protein, around the cores, in contrast with ferritin, in which the protein shell appears as

an unbroken ring around the core. This observation supports the biochemical evidence presented above that haemosiderin protein is bound to the cores and is derived from ferritin.

The constancy of the peptide composition of haemosiderin is emphasized by amino acid analyses (Table 3). Comparison of haemosiderin and ferritin compositions shows significantly increased serine and glycine in haemosiderin, whereas leucine, tyrosine and methionine are decreased. Low methionine values may be due to oxidation during hydrolysis, possibly enhanced by iron catalysis. No clear conclusions can be drawn, from comparisons of these data with the ferritin sequence (Wustefeld & Crichton, 1982), as to the possible proteolytic sites involved in haemosiderin formation, since there are seven to eight peptides present in haemosiderin, all of which may be ferritin-derived.

Elemental analysis

A wide range of metals, and also phosphorus, were assayed in purified haemosiderin and in void fractions from Sepharose 6B chromatography (haemosiderin VF) of haemosiderin solutions (see Table 4). Striking amounts of phosphorus are present in haemosiderin; phospholipid can only

Fig. 4. SDS/polyacrylamide-gel electrophoresis of haemosiderin on 12.5% (w/v)-polyacrylamide reducing gels. Gels were stained with Coomassie Blue and densitometer-scanned at 600 nm. Scans a-c, haemosiderins S2, S3 and S1 respectively. Scan d is the void-volume fraction from Sepharose 6B chromatography (viz. Fig. 2). Note the apparent absence of ferritin peptide from this fraction.

Fig. 5. Electron micrograph of negatively stained haemosiderin

Note the variable presence of non-staining material around the cores, often forming an incomplete ring and sometimes apparently absent.

Table 3. *Amino acid analyses of human spleen haemosiderin*

No corrections were made for hydrolytic losses. Cysteine and tryptophan were not determined.

	Amino acid composition (mol/100 mol of recovered residues)				
	Haemosiderins:				
Amino acid	S2	S3	S4	Mean $\pm$ S.E.M.	Ferritin*
Asx	16.7	15.0	16.6	16.3 $\pm$ 0.4	11.0
Thr	4.1	5.6	5.5	5.1 $\pm$ 0.6	4.1
Ser	11.9	8.1	8.7	9.6 $\pm$ 1.4	4.1
Glx	13.3	12.4	13.8	13.2 $\pm$ 0.5	12.8
Pro	4.7	5.4	5.7	5.3 $\pm$ 0.4	2.3
Gly	13.9	11.7	13.1	12.9 $\pm$ 0.8	7.0
Ala	6.6	8.6	9.0	8.1 $\pm$ 0.9	8.7
Val	4.1	5.3	4.9	4.8 $\pm$ 0.4	3.5
Met	0.1	0.1	0.1	0.1 $\pm$ 0.0	2.3
Ile	1.7	3.1	2.7	2.5 $\pm$ 0.5	1.7
Leu	3.0	6.0	5.7	4.9 $\pm$ 1.2	15.7
Tyr	0.8	0.3	1.7	0.9 $\pm$ 0.5	5.8
Phe	2.2	3.2	1.1	2.2 $\pm$ 0.7	4.7
Lys	6.3	7.0	6.0	6.4 $\pm$ 0.4	7.6
His	2.8	2.8	2.7	2.8 $\pm$ 0.0	3.5
Arg	7.9	5.1	2.5	5.2 $\pm$ 1.9	5.2

* Human spleen apoferritin composition calculated from sequence data (Wustefeld & Crichton, 1982).

account for about 7% of this phosphorus, and it seems likely that most of it is present as core-adsorbed phosphate, with approximately six iron atoms per phosphorus atom, compared with a ratio of 8:1 in ferritin (Granick & Hahn, 1944). Phosphorus is also present in haemosiderin VF, supporting this interpretation; the reduced contents in these fractions may in part be due to desorption during solubilization at high pH (Granick & Hahn, 1944).

Potassium, although present in large amounts in haemosiderin, is absent after chromatography, and is thus probably due to residual KI. Calcium levels are also much lower in haemosiderin VF, and may represent contaminating mitochondrial calcium phosphate (Greenawalt *et al.*, 1964). Magnesium, however, does seem to be core-associated, possibly by binding to core phosphate.

Conclusions

It is apparent that the main biochemical components of haemosiderin are iron, lipid and protein, as well as some phosphorus, which may well be present as core-associated phosphate. Peptides are present in remarkably constant composition, as revealed by SDS/polyacrylamide-gel electrophoresis and amino acid analyses, which are very likely bound to the iron oxyhydroxide cores. Circumstantial and electron-microscopic evidence suggests that the major 14500- M_r peptide, and perhaps five to six other peptides between M_r 13000 and 18000, are derived from

ferritin and form a partial shell of protein about the haemosiderin cores. Confirmation of this conclusion awaits purification and structural analysis of individual peptides. If haemosiderin is formed by lysosomal proteolysis of ferritin, then those bonds most susceptible to enzymic attack will be hydrolysed; furthermore, proteolytic peptides that remain bound to the cores may become sterically protected from enzyme action, owing to the formation of aggregates. Thus limited proteolysis of ferritin may lead to haemosiderin with a relatively constant and definable peptide composition.

It is not clear from these experiments whether the lipid found in freeze-dried haemosiderin is a genuine component or whether it is a contaminant. The accumulation of ferritin on lamellar structures before the formation of haemosiderin aggregates in lysosomes (Iancu & Neustein, 1977) implies that lipid may indeed be an intrinsic part of haemosiderin granules.

A possible mechanism by which iron may exert its toxic effects is by the catalysis of free-radical production of iron salts, accompanied by lipid peroxidation (Fong *et al.*, 1973). The presence of large amounts of haemosiderin in lysosomes could result in local elevation of iron salts, since presumably cellular reducing agents must have access to haemosiderin to effect its mobilization during phlebotomy (Shoden *et al.*, 1953). Also, the exposure of the cores of haemosiderin by removal of the protein (see Fig. 5) might facilitate heterogeneous catalysis of free-radical production.

Table 4. Elemental analyses of haemosiderin

Elemental analyses are expressed as mmol of element/mol of Fe of haemosiderin (means $\pm$ s.e.m. for five preparations) and haemosiderin void fractions (VF) from Sepharose 6B chromatography (means $\pm$ s.e.m. for three preparations). Appropriate blanks were subtracted. Abbreviations used: ND, not determined owing to relatively high blanks; UD, less than approx. 0.01 mmol/mol of Fe: Li, Rb, Be, La, Ti, V, Cr, Mn, Co, Ni, Ag, Cd. Trace amounts (0.02–0.04 mmol/mol of Fe) of Ba, Sr and Mo were detected in haemosiderin.

Element	Content (mol/mol of Fe)							
	P	K	Na	Mg	Ca	Cu	Zn	Pb
Haemosiderin	166 $\pm$ 12	103 $\pm$ 19	33.9 $\pm$ 18.4	16.8 $\pm$ 3.6	18.4 $\pm$ 7.2	2.15 $\pm$ 1.53	1.46 $\pm$ 0.36	0.10 $\pm$ 0.03
Haemosiderin VF	67.3 $\pm$ 5.5	UD	ND	13.8 $\pm$ 7.8	4.22 $\pm$ 0.03	ND	ND	UD

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