Siderosomal ferritin

The missing link between ferritin and haemosiderin?

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A minor electrophoretically fast component was found in ferritin from iron-loaded rat liver in addition to a major electrophoretically slow ferritin similar to that observed in control rats. The electrophoretically fast ferritin showed immunological identity with the slow component, but on electrophoresis in SDS it gave a peptide of 17.3 kDa, in contrast with the electrophoretically slow ferritin, which gave a major band corresponding to the L-subunit (20.7 kDa). Thus the electrophoretically fast ferritin resembles that reported by Massover [(1985) *Biochim. Biophys. Acta* **829**, 377–386] in livers of mice with short-term parenteral iron overload. The electrophoretically fast ferritin had a lower iron content (2000 Fe atoms/molecule) than the electrophoretically slow ferritin (3000 Fe atoms/molecule). Removal and re-incorporation of iron was possible without effect on the electrophoretic mobility of either ferritin species. On subcellular fractionation the electrophoretically fast ferritin was enriched in pellet fractions and was the sole soluble ferritin isolated from iron-laden secondary lysosomes (siderosomes). The amount and relative proportion of the electrophoretically fast species increased with iron loading. Haemosiderin isolated from siderosomes was found to contain a peptide reactive to anti-ferritin serum and corresponding to the 17.3 kDa peptide of the electrophoretically fast ferritin species. Unlike the electrophoretically slow ferritin, the electrophoretically fast ferritin did not become significantly radioactive in a 1 h biosynthetic labelling experiment. We conclude that the minor ferritin is not, as has been suggested for mouse liver ferritin, 'a completely new species of smaller holoferritin that represents a shift in the ferritin phenotype' in response to siderosis, but a precursor of haemosiderin, in agreement with the proposal by Richter [(1984) *Lab. Invest.* **50**, 26–35] concerning siderosomal ferritin.

INTRODUCTION

Iron in excess of that required for production of haemoglobin, myoglobin and iron-containing enzymes is stored in animals in two major forms, ferritin and haemosiderin. Ferritin is a well-characterized protein consisting of 24 polypeptide subunits arranged as a rhombic dodecahedron forming a shell around a central cavity in which up to 4500 Fe atoms may be stored as the mineral ferrihydrite (Ford *et al.*, 1984). Ferritin is normally distributed throughout the cytoplasm, where its iron-cores may be observed under electron microscopy as electron-dense particles of about 6 nm (60 Å) diameter (Iancu, 1983). Haemosiderin forms intracellular iron-containing granules visible under the light microscope in iron-loaded tissues (Wixom *et al.*, 1980) or under the electron microscope as irregular massive clusters of electron-dense particles resembling ferritin iron-cores that suggest an origin in this protein (Iancu, 1983; Richter, 1984). These clusters, which often appear to be membrane-enclosed, are thought to be iron-laden secondary lysosomes, and have been termed 'siderosomes' (Richter, 1984). The electron-dense particles within haemosiderin resemble ferritin iron-cores, and are similar in a number of other properties such as X-ray-diffraction patterns (Fischbach *et al.*, 1971) and Mössbauer spectra (Fischbach *et al.*, 1971; Bell *et al.*, 1984). Peptides can be extracted from rat liver

siderosomes (Richter, 1984) and from purified haemosiderin (Weir *et al.*, 1984) that are immunoreactive with anti-ferritin sera. Such studies have led to the view that ferritin is converted into haemosiderin by degradation of its protein shell, thus accounting for the insolubility of this form of storage iron and its appearance as massive aggregates. Iron-loading causes an increase in both ferritin and haemosiderin, and may give rise to a shift in the distribution of isoferritins. Thus in rat liver ferritin the two different subunits, known as L and H, normally present respectively in about a 15:9 ratio, change relatively so that the proportion of L-subunit is increased and the isoelectric profile shifts towards the basic (Kohgo *et al.*, 1980; Bomford *et al.*, 1981; Treffry *et al.*, 1984a). In the present paper we report a different phenomenon, superimposed upon the shift in phenotypic expression, which gives rise to an electrophoretically fast ferritin molecule.

EXPERIMENTAL

Animals

Female Wistar rats (200 ± 10 g) were iron-loaded by intraperitoneal injection of iron-dextran (Imferon, 50 mg of iron/ml; Fisons, Loughborough, Leics., U.K.). Rats received single doses of 20 mg of iron at intervals of 3–4 days until the total dose reached 20, 60 or 120 mg

Abbreviation used: PAGE, polyacrylamide-gel electrophoresis.

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Scheme 1. Differential subcellular fractionation scheme

This procedure is in accordance with Lahav *et al.* (1982), except for the inclusion of the PII fraction, which we found in preliminary experiments to be more enriched in the electrophoretically fast ferritin than any of the other fractions.

of injected iron. Those rats involved in the isolation of siderosomes received a single dose of 60 mg of iron. Before being killed, rats were kept without food overnight but had access to water.

Subcellular fractionation of rat liver and spleen by differential centrifugation

At various times after the final iron injection, rats were killed, and their livers and spleens were removed, washed, minced and homogenized in 3 vol. of 0.32 M-sucrose in 20 mM-Tris/HCl buffer, pH 7.45, followed by the addition of 4 vol. of 0.25 M-sucrose. Subcellular fractionation was then performed by differential centrifugation based on the procedure of Lahav *et al.* (1982). The final procedure adopted is outlined in Scheme 1.

Partial purification of ferritins from subcellular fractions

Ferritin was purified from the pellet and SIII fractions by heating at 70–75 °C for 10 min followed by rapid cooling to 10 °C, centrifugation at 2000 *g* for 30 min, precipitation of the ferritin in the supernatant with 40% (w/v) $(\text{NH}_4)_2\text{SO}_4$, dialysis of precipitate against phosphate-buffered saline (0.01 M-potassium phosphate buffer, pH 7.2, containing 0.15 M-NaCl) and centrifugation at 2000 *g* for 30 min. Ferritin was then concentrated by ultrafiltration in an Amicon ultrafiltration cell.

Preparative gel electrophoresis of ferritin from the pellet fractions

Ferritin from pellet fractions, partially purified as described above, was subjected to preparative PAGE on slab gels (Arosio *et al.*, 1978) with a 5–15% (w/w) total acrylamide (T)+2.7% (w/w) bisacrylamide/total acrylamide (C) convex gradient. Ferritin was isolated after overnight electrophoresis at a constant 150 V by slicing from the gel those bands that were visible because of their brown colour, homogenizing in phosphate-buffered

saline and allowing the ferritin to extract into buffer overnight. The insoluble acrylamide was then removed by centrifugation (5000 *g* for 15 min), and ferritin in the supernatant was precipitated with 40% (w/v) $(\text{NH}_4)_2\text{SO}_4$. This preparative gel electrophoresis step was performed twice, as recommended by Arosio *et al.* (1978). Ferritin so isolated was finally applied to an ion-exchange column (DEAE-Sephadex A-50) equilibrated with 50 mM-imidazole/HCl buffer, pH 7.1, and was eluted with 0.3 M-NaCl solution in the same buffer, in order to remove remaining polyacrylamide (Brooks & Sander, 1980).

Gel filtration of ferritin from the SIII fraction

Ferritin from the SIII fraction was further purified (after the heating and precipitation steps) by gel-filtration chromatography on Sepharose 6B (column dimensions 75 cm × 5 cm) in phosphate-buffered saline followed by ultracentrifugation at 100 000 *g* for 2 h. The purity of all ferritin samples was checked by PAGE.

Isolation of ferritin and haemosiderin from siderosomes

Rats were injected intraperitoneally with a single dose of iron (60 mg), in the form of iron-dextran. After 14–28 days the livers were removed and siderosomes were purified, as described by Richter (1984). Ferritin was isolated from the siderosomes by subjecting them to osmotic shock with distilled water. It was then purified, as described above, by heat treatment, $(\text{NH}_4)_2\text{SO}_4$ precipitation and gel filtration. Haemosiderin was isolated from the insoluble siderosomal portion, after suspension in water, by repeated freeze-thaw cycles. After each cycle the suspension was centrifuged at 2000 *g* for 15 min and the resulting pellet was resuspended in fresh distilled water. To disaggregate haemosiderin clumps samples were sonicated in water at 15 A for 20 s on ice. The haemosiderin suspension was then centrifuged through 4.1 M-KI, as described by Fineberg & MacKay

(1964), and finally resuspended in distilled water and repelleted by centrifugation at 2000 *g* for 15 min to remove KI.

Measurement of relative proportions of the electrophoretically fast and slow ferritins

Ferritin samples were subjected to native gradient PAGE. After Coomassie Blue staining, ferritin bands were scanned at 550 nm on a Gilford spectrophotometer fitted with a linear scanner. The area under each ferritin peak was measured by cutting out and weighing a tracing of the chart recording of each peak. The total amounts of electrophoretically fast and slow ferritins (referred to below simply as fast and slow ferritins) within each fraction were determined from the relative proportions, obtained as above, coupled with estimations of total ferritin, made by radial immunodiffusion on samples taken before heat treatment and kept frozen at -15°C .

Enzyme assays

The cathepsin D assay employed is based on that described by Anson (1938); 100 μl of sample was added to 0.5 ml of acid-denatured haemoglobin in 0.1 M-sodium acetate buffer, pH 3.6. After incubation at 37°C for 30 min the reaction was stopped with ice-cold 5% (w/v) trichloroacetic acid and the mixture was left on ice for a further 30 min to allow precipitation. The resulting precipitate was centrifuged at 1220 *g* for 20 min at 4°C . The supernatant was then assayed for tyrosine content by Folin determination.

Electrophoretic techniques

SDS/PAGE was performed in 15% T + 2.7% C gels in accordance with Laemmli (1970). Before electrophoresis in SDS, protein samples were dissociated in 0.1 M-Tris/HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 2.3% (w/v) SDS and 5% (v/v) 2-mercaptoethanol by heating in a boiling-water bath for 10 min. Native proteins were electrophoresed in 5–15% T convex-gradient gels or 6% T linear gels in the absence of SDS as described by Laemmli (1970). Analytical isoelectric focusing was performed as described by Vesterberg (1972), in flat beds containing 2% (w/v) Ampholine (pH 4–6 and pH 3.5–10 in a 2:1 ratio), 4.36% (w/v) acrylamide and 0.14% (w/v) bisacrylamide. Isoelectric-focusing gels were stained for protein with Coomassie Blue as described by Vesterberg (1972); other gels were stained for protein with 0.1% Coomassie Blue in 30% (v/v) methanol/7% (v/v) acetic acid and destained in the same solvent. Gels were stained for iron as described by Shinjo & Harrison (1979).

Radiolabelling

L-[4,5- ^3H]Leucine (Amersham International, Amersham, Bucks., U.K.) incorporation was determined on three groups of three rats loaded with 60 mg of iron between 2 and 4 weeks after the final iron injection and one group of three normal rats. Each rat was given 100 μCi of label intraperitoneally 1 h before being killed. Ferritin purified by preparative PAGE was analysed for radioactivity by precipitation with anti-(rat liver ferritin) serum followed by scintillation counting as described by Treffry *et al.* (1984a).

Immunoblotting

Immunoblotting was performed in a Bio-Rad trans-blot apparatus according to the manufacturer's instructions. Immunostaining was performed in accordance with Blake *et al.* (1984). The primary antiserum was raised in New Zealand White rabbits against purified rat liver ferritin as described by Treffry *et al.* (1984b).

Electron microscopy

Siderosome samples were prepared for electron microscopy by fixation in 1.5% (v/v) glutaraldehyde/0.3 M-sucrose/0.1 M-sodium cacodylate buffer, pH 7.4, for 1 h. Samples were post-fixed in OsO_4 and stained with uranyl acetate/lead citrate.

Other methods

Reduction of ferritin to apoferritin and reconstitution of apoferritin to ferritin were performed as described by Treffry & Harrison (1978). Single radial immunodiffusion was carried out as described by Mancini *et al.* (1965). Double immunodiffusion was as described by Hudson & Hay (1980). Protein determination was by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Total iron determination was by the method of Drysdale & Munro (1965).

RESULTS

Immunological identification of the electrophoretically fast component as a ferritin

When partially purified ferritin from homogenates of iron-loaded rat liver was analysed by gradient PAGE, the usual bands representing the 24-subunit 'monomer' and its oligomers (Harrison & Gregory, 1965; Williams & Harrison, 1968) were seen along with an electrophoretically fast minor band (Fig. 1) that also stained for iron. The electrophoretically fast and slow ferritin monomer species, purified from the subcellular fractions as described in the Experimental section, produced fused precipitation lines on Ouchterlony immunodiffusion, indicating the presence of identical antigenic determinants and confirming the fast band as a ferritin. The ratio of immunoreactive ferritin, determined by radial immunodiffusion, to protein, as determined by the method of Lowry *et al.* (1951), was found to be the same for both species, indicating that the minor species is immunologically identical with the major ferritin species.

Subcellular location and effects of iron loading on the electrophoretically fast and slow ferritins

On subcellular fractionation according to the procedure outlined in Scheme 1, followed by partial purification, the electrophoretically fast ferritin was found to be present within the three pellet fractions (PI, PII and PIII) (Fig. 1), but not in the supernatant fraction SIII. Pellet fraction PII usually showed the highest amount of fast ferritin; however, since there was some variation, the amounts in all three pellet fractions are shown combined in Table 1, together with the amounts and percentages found for total ferritin in pellets and the percentage total ferritin in the supernatant. Table 1 shows that, as iron-loading rises, total ferritin per liver is increased nearly 10-fold and the amounts of fast ferritin increase both per liver and as a percentage of total ferritin. Fast ferritin was not detected in any of the

Fig. 1. Native gradient (convex) PAGE (5–15%T) of iron-loaded (60 mg of iron) rat liver ferritin, partially purified from subcellular fractions and stained for protein

Lane I, ferritin from PI fraction; lane II, ferritin from PII fraction; lane III, ferritin from PIII fraction.

fractions from rats that had not been injected with iron. Slow ferritin was found principally in the SIII fraction, not more than 10% of it being associated with the pellets.

The association of fast ferritin with the pellet fractions suggested that it may be present within an organelle. Since we considered that it was most likely to be found in siderosomes, the isolation procedure used by Richter (1984) was adopted. The purified siderosomes were highly

enriched in cathepsin D activity (Table 2) as compared with liver homogenates, as had been shown by Richter (1984) and Glaumann *et al.* (1975). Their purity was checked by electron microscopy. The preparation showed the morphology classically associated with siderosomes: numerous roughly spherical membrane-bound bodies packed with electron-dense particles that resemble ferritin iron-cores. Other cell constituents were only present as minor impurities. Soluble ferritin released from siderosomes was shown to consist solely of the electrophoretically fast species, whereas ferritin from the cytosolic fraction consisted almost entirely of the electrophoretically slow ferritin (Fig. 2). The ratio of Fe atoms to soluble ferritin molecules for the siderosomal fraction was 23000:1, more than 5 times the maximum capacity of rat liver ferritin molecules or about 10 times the iron content found for siderosomal ferritin. Hence much of the siderosomal iron may have been present as haemosiderin rather than ferritin. Iron in purified siderosomes accounted for 4% of total liver iron, a value similar to that found by Richter (1984) (3%). Siderosomes yielded amounts of soluble fast ferritin comparable with that isolated from pellet fractions (Table 1).

Several further characteristics of the electrophoretically fast ferritin are described below. The fast ferritins from siderosomes and pellet fractions gave the same subunit content and mobility on native PAGE, and we therefore conclude that they are identical.

Further characteristics of fast ferritin

The iron content of fast ferritin isolated from the pellet fractions was 2170 ± 450 Fe atoms/molecule, based on a protein molecular mass of 497 kDa [assuming 24 subunits of 20.7 kDa (Leibold *et al.*, 1984)]. This is much lower than the value of 3050 ± 70 Fe atoms/molecule found for the electrophoretically slow ferritin of pellet fractions and of supernatant SIII. Iron could be removed by reduction and re-incorporated without affecting its electrophoretic properties. Fast ferritin was also able to form oligomers, a characteristic of normal ferritin

Table 1. Effect of various degrees of iron-loading on rat liver ferritin

Iron-loading of rats (three per group) was as described in the Experimental section. Rats were killed 14–28 days after the final iron injection. Livers were fractionated as indicated in Scheme 1. Ferritin amounts were determined on crude fractions by radial immunodiffusion. Fast and slow ferritins were determined from the relative proportion of each ferritin within partially purified samples combined with total amounts in the original crude fractions. Data for the three pellet fractions PI, PII and PIII of Scheme 1 are combined in this Table. Abbreviation: N.D., not determined.

	Amount of iron received per rat . . .			
	0 mg	20 mg	60 mg	120 mg
Total ferritin/liver (mg)	4.4 ± 0.5	10.0 ± 1.5	25.5 ± 3.6	39.2 ± 2.4
Total ferritin in combined pellet fractions (μ g)	450 ± 150	940 ± 120	2020 ± 350	1860 ± 120
Fast ferritin/liver from combined pellets (μ g)	0	24 ± 3	270 ± 100	540 ± 36
Fast ferritin/liver (% of total ferritin/liver)	0	0.24 ± 0.03	1.03 ± 0.24	1.38 ± 0.1
Fast ferritin (% of total ferritin in combined pellet fractions)	0	2.55	13.4	28.9
Ferritin in SIII fraction (% of total ferritin)	87.5	88.0	80.7	N.D.
Total ferritin recovery (%)	97.6	97.5	89.0	N.D.

Table 2. Biochemical analysis of fractions obtained during siderosome isolation

These values were obtained on subcellular fractions after the preparation of rat liver siderosomes from three rats that were killed 28 days after receiving a single intraperitoneal injection of 60 mg of iron (iron-dextran). All measurements were made on crude samples. Values for cathepsin D activity are in nmol of tyrosine released/min. Recoveries were obtained from all fractions, including supernatants. Key: P1, pellet formed during centrifugation at 700 *g* for 10 min; Sid, pellet formed during centrifugation at 58 600 *g* for 2.5 h in a discontinuous sucrose density gradient; S2, supernatant formed during centrifugation at 6000 *g* for 8 min.

	Total	P1	Sid	Recovery
Protein (mg/liver)	1430	630 (54.9%)	6.73 (0.47%)	1500 (105%)
Ferritin (mg/liver)	21.0	8.18 (39.0%)	0.27 (1.30%)	18.4 (88.0%)
Ferritin (μ g/mg of protein)	14.7	10.5	40	12.3
Iron (mg/liver)	19.2	10.1 (52.5%)	0.74 (3.86%)	16.6 (85.9%)
Iron (μ g/mg of protein)	13.5	12.9	111	11.0
Cathepsin D activity (nmol/min per liver)	517	290 (56.2%)	60.8 (11.8%)	564 (109%)
Cathepsin D activity (nmol/min per mg of protein)	0.36	0.39	9.03	0.38

preparations. These were clearly seen after elution from the main fast monomer band and re-electrophoresis. They are also electrophoretically fast as compared with those of slow ferritin.

Electrofocusing showed that the slower major ferritin from iron-loaded rats was more basic than that of control rats, as had been found previously (Kohgo *et al.*, 1980), whereas the fast and slow ferritins from iron-loaded animals gave profiles that were virtually indistinguishable (Fig. 3). Therefore, by analogy with other ferritins, a similar subunit content might be expected. However, separation into subunits by SDS/PAGE showed very different subunit compositions (Fig. 4). The electrophoretically slow ferritin from iron-loaded

rats had, as expected, a high content of L-subunits, namely 85% L and 15% H, as compared with ferritin from control rat liver, which gave 69% L and 31% H. Their nominal molecular masses were 20.7 ± 1.2 kDa and 21.3 ± 1.3 kDa respectively. Fast ferritin showed three subunit bands: a major band of 17.3 ± 0.9 kDa (69%), which we here call the 'F-subunit', plus two bands corresponding to the L-subunit (24%) and the H-subunit (7%). Some degradation of ferritin to give bands of about 11 kDa and 8 kDa (but not 17.3 kDa) has previously been reported (Ishitani *et al.*, 1975), and this seems to be prevented or diminished by the addition of a serine proteinase inhibitor such as phenylmethane-sulphonyl fluoride before homogenization. Addition of

Fig. 2. Native gradient (convex) PAGE (5–15%T) of ferritin partially purified from iron-loaded rat liver subcellular fractions obtained in accordance with Richter (1984) and stained for protein

Lane I, ferritin from the siderosomal fraction; lane II, ferritin from the P1 fraction; lane III, ferritin from the S2 fraction (cytosolic). See Table 2 legend for further details on the above fractions. Note that the dimer ferritin band of siderosomal ferritin is of higher mobility than that of cytosolic ferritin.

Fig. 3. Comparison of isoelectric profiles of fast and slow ferritins purified by preparative PAGE from pellet fractions of iron-loaded rat liver

—, Fast ferritin; —, slow ferritin. The profiles were obtained from Coomassie Blue-stained isoelectric-focusing gels by densitometry at 550 nm.

Fig. 4. Subunit composition of rat liver ferritins

SDS/PAGE (15% T) gel, stained for protein. Lane I, slow ferritin purified from control rat livers; lane II, slow ferritin; lane III, fast ferritin. The ferritins for lanes II and III were purified by preparative PAGE from pellet fractions of iron-loaded rat liver.

0.01% phenylmethanesulphonyl fluoride had no effect on the proportion of the minor component in ferritin from pellet fractions from iron-loaded rat liver.

When ferritins were purified from liver fractions from iron-loaded rats that had received an injection of 100 μ Ci of [3 H]leucine 1 h before being killed, the average specific radioactivities of the fast and slow ferritins differed markedly (respectively 11.1 ± 2.5 and 2.0 ± 1.5 d.p.m./ μ g of protein). This experiment was not repeated to check the labelling of fast ferritin within siderosomes because Richter (1984) had found no significant incorporation of radioactivity into siderosomal ferritin.

Fast ferritin in spleen

Subcellular fractionation of spleen from iron-loaded rats also showed the presence of electrophoretically fast ferritin in the pellet fractions, migrating with that from rat liver. It also gave as its major peptide on SDS/PAGE a band of 17.3 kDa, in contrast with the major spleen ferritin, which was predominantly composed of L-subunits. The purified fast spleen ferritin gave an isoelectric-focusing pattern virtually identical with that of the slow spleen ferritin. Since the fast ferritin of spleen was highly enriched in pellet fractions, it is also probably a constituent of siderosomes.

Rat liver haemosiderin

Siderosomal haemosiderin, isolated as described in the Experimental section, was considered to be devoid of soluble ferritin, since the purification procedure selected insoluble components. On SDS/PAGE it gave several bands, one of which migrated to the same position as that of the F-subunit and another to the same position as the H- and L-subunits. Immunoblotting with anti-(rat liver ferritin) serum showed reactivity with these two bands in haemosiderin and fast ferritin and to the H/L-subunit in the slow ferritin species. The other bands gave no significant reaction (Fig. 5).

Fig. 5. SDS/PAGE and immunoblot of ferritin and siderosomal haemosiderin from iron-loaded rat liver

(a) SDS/PAGE (15% T), stained for protein. (b) Western blot of the gel in (a) immunostained with anti-(rat liver ferritin) serum. Lanes I, siderosomal haemosiderin; lane II, siderosomal ferritin; lane III, cytosolic ferritin (S2). Bands corresponding to the H/L- and F-subunits of ferritin stained strongly whereas M_r markers did not stain on immunoblots, indicating that the staining is specific for ferritin and peptides derived from ferritin.

DISCUSSION

Electrophoretically fast ferritin was found to be the sole soluble ferritin of rat liver siderosomes, accounting for just over 1% of the total ferritin per liver. Although we cannot be certain that all the fast ferritin of pellet fractions was within siderosomes, this seems likely. The fast ferritins from pellet fractions and siderosomes were apparently identical, as judged by their electrophoretic mobilities and subunit contents, and similar yields were obtained from the two preparations. It was not found in any of the supernatant fractions in more than trace amounts.

The P1 fraction of Table 2 has an iron/ferritin ratio of 10500 Fe atoms/molecule compared with 4700 Fe atoms/molecule for the cytosolic (S2) fraction. This indicates that the P1 fraction could contain siderosomes. If the iron and ferritin content of the P1 fraction derives from a mixture of cytosolic and siderosomal sources, then about 69% of the P1-fraction iron content is siderosomal in origin. Thus total siderosomal ferritin content measured from the siderosomal fraction may be an underestimate. The recovery after purification of the electrophoretically fast siderosomal ferritin may also be very much lower than that of cytosolic ferritin. This would not be altogether surprising, as fast ferritin may be partially degraded and may be more susceptible to loss during the heating step.

The properties observed for siderosomal ferritin to a large extent match those reported by Richter (1984), namely the similarity in isoelectric-focusing profile and immunological properties to cytosolic ferritin. Richter (1984) did not report the iron content of siderosomal ferritin, nor did he indicate that it had a faster anodal mobility in polyacrylamide gels. He found subunit molecular masses of 17.4–17.8 kDa for one preparation

and 18 and 24 kDa for another as compared with 18 and 21 kDa for cytosolic ferritin, and concluded that these corresponded to L- and H-subunits respectively. In our hands the major subunit (69%) of siderosomal ferritin is clearly smaller (17.3 kDa) than the L-subunit (20.7 kDa, 24%), which was also separated from the H-subunit (21.3 kDa, 7%). These differences are probably due mainly to differences in the resolving powers of the gels. Massover (1985) found a subunit molecular mass of 18 kDa for an electrophoretically fast minor component of siderotic mouse liver with a trace of a 22 kDa subunit, similar to that found in the major ferritin. Although Massover (1985) suggested that the molecule was a new smaller species of ferritin fashioned from smaller subunits synthesized in response to siderosis, it seems to us likely that the fast ferritins of mouse and rat are analogous, although this requires further investigation.

The fast anodal mobility, coupled with the similarity to cytosolic ferritin in its isoelectric-focusing profile, might suggest that fast ferritin is smaller than slow ferritin. However, the apparent immunological identity of the two forms of ferritin together with the precursor-product relationship indicated by the low 1 h incorporation of radioactivity into fast as compared with slow ferritin can be explained if the smaller F-subunits are products of proteolytic or oxidative splitting of the majority of subunits actually within the intact molecule. Cytosolic ferritin is composed largely of L-subunits in iron-loaded rats, and the F-subunit must therefore have arisen to a large extent, or perhaps entirely, from this subunit. The splitting must have caused an increased electrophoretic mobility by some structural change, perhaps in a region of the molecule protruding from its surface. Evidence that siderosomal ferritin does indeed contain such split L-subunits is presented in the following paper (Andrews *et al.*, 1987).

In principle cleavage could occur either in the cytosol, perhaps thus providing a signal for uptake into secondary lysosomes, or on entry into this organelle. The former possibility seems the less likely, given that fast ferritin does not appear as more than a trace constituent of cytosolic ferritin, probably a contaminant due to lysosomal lysis. The absence of the more slowly migrating cytosolic ferritin species within the siderosome suggests that it may have been cleaved rapidly on entry. Siderosomes contain high cathepsin D activity, as shown here and by Richter (1984). However, Richter (1983, 1986) provided evidence that this is not the enzyme responsible for ferritin degradation within lysosomes. It is possible that the high concentration of iron within the lysosome catalyses the production of oxy radicals, which act on ferritin as suggested for other proteins, producing molecules that are more susceptible to hydrolytic attack (Wolff *et al.*, 1986) and giving rise to protein cleavage (Kim *et al.*, 1985).

Electron diffraction and high-resolution electron microscopy (S. Mann, S. C. Andrews, A. Treffry & P. M. Harrison, unpublished work) show that the insoluble siderosomal iron (haemosiderin) is similar in atomic structure to ferritin iron-cores and not to the iron-dextran injected to increase iron-loading. Thus the bulk of the non-ferritin siderosomal iron is not simply engulfed iron-dextran but is probably derived from ferritin. The average iron-core sizes of siderosomal ferritin (indicated by its lower iron/protein ratio) and of purified rat liver siderosomal haemosiderin [shown by unpublished

electron-microscopic observations (S. C. Andrews, S. Mann, J. M. Williams & P. M. Harrison) and by Mössbauer spectroscopy (Williams *et al.*, 1986)] are lower than those of cytosolic ferritin. The lower iron-core particle size of haemosiderin as compared with cytosolic ferritin has been noted previously in other species (Fischbach *et al.*, 1971; Weir *et al.*, 1984). In rat liver it points to siderosomal ferritin as a direct precursor of haemosiderin, although both could have lost iron in parallel. In humans relatively large iron-cores are observed within membranes surrounding siderosomes (Iancu, 1983), even though, when isolated, the haemosiderin particle size is smaller than that of ferritin (Weir *et al.*, 1984). The presence in haemosiderin of peptides reacting in immunoblots with anti-ferritin sera as seen by us and in human haemosiderin by Weir *et al.* (1984) provides further evidence for its derivation from soluble ferritin.

We have also found fast ferritin in subcellular pellet fractions of iron-loaded rat spleen. Although this does not prove its presence within siderosomes, the liver and spleen are both organs known to store iron as haemosiderin as well as ferritin, and may possess the same pathway of lysosomal degradation. We found no fast ferritin and no siderosomes in control rats. This may be because the normal degradation is not lysosomal and the formation of siderosomes is a response only to massive iron-overload. It has been suggested that lysosomal degradation of ferritin to haemosiderin may represent only a minor part of ferritin turnover (Wixom *et al.*, 1980; Richter, 1983).

In summary, our study provides further evidence that haemosiderin, at least within rat liver siderosomes, is derived from ferritin. Richter (1984) suggests that lysosomal degradation follows the sequence:

We agree with this sequence with the addition of an initial cleavage as the first step on uptake into siderosomes, and we would identify 'lysosomal-siderosomal ferritin' with our own 'fast ferritin'. An alternative scheme has been given by Iancu (1983), who observed the presence of 'ferritin clusters' in the cytoplasm of iron-loaded human tissues and suggested that these clusters are taken up intact by lysosomes and ultimately converted into haemosiderin. His scheme is consistent with an increase in the numbers and sizes of ferritin oligomers isolated from iron-loaded tissues (Samarel & Bern, 1978; Hoy & Jacobs, 1981). The extent of oligomerization was not examined in the present study, but it is conceivable that such oligomers are preferentially taken up into rat liver siderosomes and

that the fast ferritin has dissociated from them after initial proteolysis.

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