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Ferritin subunits in livers of siderotic mice

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Summary. The major ferritin species of mouse liver has been resolved by SDS-PAGE into two bands similar to the H and L subunits of rat liver ferritin with the L subunit predominating. Amino acid sequencing has confirmed the major, faster-migrating component as L chain. An additional, electrophoretically fast, minor ferritin was isolated from siderosome-containing subcellular fractions. In denaturing gels it gave a single 'F' subunit band of about 17 kDa, significantly smaller than the L and H subunits (about 20 and 21 kDa respectively). A small fragment isolated from the fast ferritin was sequenced. It corresponds to a 19-residue C-terminal peptide cleaved from L subunits in the assembled molecules. The F subunit must be derived from L subunits by loss of this peptide, and is not the expression product of a different gene. 'Fast' ferritins of siderotic mice and rats are thus analogous.

Key words: Ferritin — Siderotic mice — Amino acid sequence — L subunit

Introduction

Ferritin is the ubiquitous iron-storage protein of animals and plants (Ford et al. 1984; Theil 1987). It comprises 24 subunits arranged as a dodecahedral structure with a hollow centre where up to 4500 iron atoms may be sequestered. Mammalian ferritins consist of two subunit types designated H (heavy) and L (light) on the basis of their relative mobilities on sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) (Arosio et al. 1978). These subunit types assemble in various proportions to give a series of 23 heteropolymers and 2 homopolymers.

There is considerable similarity between the amino acid sequences of mammalian ferritin L subunits [84% identity in human and rat (Leibold and Munro 1987)] and H subunits [93% identity in human and rat (Murray et al. 1987) and 97% identity in rat and mouse (Miyazaki et al. 1988)] and also between H and L subunits within any one species (approximately 53% in rat). The human L subunit (M_r 19766) contains 174 amino acid residues whereas the human H subunit (M_r 21099) contains 182 residues (4 extra residues at both the N- and C-termini) (Boyd et al. 1985). In rat, however, the L subunit (182 residues) contains an additional eight residues inserted between positions 157 and 158 of the sequence (aligned according to the human and horse L subunits). Therefore, the size difference between the rat H (M_r 21004) and L subunits (M_r 20700) is relatively small (Murray et al. 1987). A third subunit type, designated F (fast), has recently been noted (Andrews et al. 1987a) as the major subunit of siderosomal ferritin from iron-loaded rat liver. The F subunit (M_r 17300) was shown to have arisen from the L subunit through cleavage between residues 163 and 164 (within the 8-residue insertion). This cleavage, which occurs in assembled molecules on the outside of the shell, gives rise to the higher mobility of siderosomal ferritin as compared to cytosolic ferritin observed on native PAGE (Andrews et al. 1987b).

Reports on mouse liver ferritin suggested that its subunit composition may depart from the normal pattern. Massover (1985) observed the appearance of an additional minor and electrophoretically fast species in native gels of mouse liver ferritin following iron-loading. On SDS-PAGE this fast species gave an 18-kDa subunit whereas

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the normal, major, electrophoretically slow species gave a 22-kDa subunit band. Massover (1985) suggested that the fast species arises through *de novo* synthesis of its 18-kDa subunit in response to iron challenge.

An 18-kDa subunit band has also been observed in ferritin from murine erythroleukaemic (Friend) cells (Peto and Thompson 1986; Beaumont et al. 1987). This subunit appears as a minor component after induction with dimethyl sulphoxide. Both induced and non-induced cells gave either two ferritin subunit bands of 20.5 and 21.5 kDa (Peto and Thompson 1986) or a single band of apparent mass 24.5 kDa (Beaumont et al. 1987) as major components. Beaumont et al. (1987) considered that the 24.5-kDa and 18-kDa bands represent the L and H subunits respectively. From labelling studies, the latter appeared to be a product of post-translational processing of a longer, 22.5-kDa, chain via a 20-kDa intermediate.

The results presented here demonstrate that the 18-kDa subunit observed in the electrophoretically fast ferritin of siderotic mice is neither an H chain nor a new subunit type synthesised in response to iron, but has arisen by post-translational cleavage of L chains. The cleavage, which is found in the ferritin of siderosomal fractions, occurs in intact molecules in a position analogous to that observed in siderosomal rat liver ferritin (Andrews et al. 1987a, b) giving rise, as in rat ferritin, to an electrophoretically fast molecule.

Materials and methods

Animals. Female weanling mice (24–30 g) were each given an intraperitoneal injeciton of 10 mg iron as iron-dextran (0.2 ml Inferon; Fisons, Loughborough, Leics., UK). After 14 days on a normal diet, when their body mass was 30–35 g, the animals were fasted overnight and killed. In a typical experiment, 178 animals were injected and they yielded 300 ± 40 g liver. Control mice, without iron injection, were otherwise maintained as for the iron-loaded mice.

Isolation of ferritin. Ferritin was isolated from the subcellular pellet and supernatant liver fractions essentially as described in Andrews et al. (1987a). Purification from the heat supernatant was by ammonium sulphate precipitation and gel filtration, followed, in the case of ferritins in pellet fractions by preparative gel electrophoresis to separate fast and slow components and ion-exchange chromatography on DEAE-Sephadex A-50 to remove polyacrylamide.

Gel electrophoresis. Native PAGE and SDS-PAGE were carried out as described by Andrews et al. (1987a).

Peparation of apoferritin. Reduction of ferritin to apoferritin was performed as described by Treffry and Harrison (1978) followed by dialysis against 0.9% NaCl to remove the thioglycollic acid and finally against water.

Separation and analysis of apoferritin peptides. Apoferritin was disaggregated into subunits in 70% formic acid and peptides immediately fractionated on Sephadex G-50 as in Andrews et al. (1987b). Amino acid analyses of freeze-dried samples and mild acid cleavage of slow ferritin subunits were performed as described by Andrews et al. (1987b).

CNBr cleavage. After *S*-carboxymethylation essentially according to Hirs (1967), 8-mg samples of carboxymethyl-apoferritin were dissolved in 70% (by vol.) formic acid containing 1 mM dithiothreitol and added to 40 mg CNBr in a stoppered tube. After flushing with nitrogen, the tube was kept in the dark for 20 h, then diluted with 4 ml water and the contents freeze-dried. Fragments were purified by HPLC using an Aquapore RP-300 reverse-phase column and a gradient of 10%–50% solvent B (0.1% trifluoroacetic acid in acetonitrile) in solvent A (0.1% trifluoroacetic acid in H_2O) over 15 min.

Amino acid sequencing. Sequencing was performed using an automated solid-phase sequencer essentially as described by Findlay et al. (1989). In summary, peptides were dissolved in 0.2 M $NaHCO_3$/0.25% SDS, pH 8.5, and coupled at 56° C for 60 min under N_2 to diisothiocyanate glass (17-nm pore size, 200–400 mesh). After washing, the coupled peptides were subjected to automated solid-phase Edman degradation. The derived anilinothiazolinones were converted at 70° C for 20 min in 30% aqueous trifluoroacetic acid and the corresponding phenylthiohydantoin derivatives identified by reverse-phase (C_{18}) HPLC using a gradient of acetonitrile in sodium acetate pH 4.9.

Results

Electrophoretic characteristics of mouse liver ferritin

Cytosolic ferritin prepared from the supernatant fractions of control and siderotic mice was fractionated by native PAGE into a major 24 subunit band and a series of minor oligomeric bands as previously observed for ferritins from other species (Williams and Harrison 1968) (Fig. 1). Ferritin from the pellet (siderosome-containing) fractions of iron-loaded mice gave an additional electrophoretically faster band (Fig. 1, lane 2). Similar observations have been made for ferritin from rat liver siderosomes (Andrews et al. 1987a, b) and siderotic male mouse liver (Massover 1985). Fig. 2A shows the fast and slow components after purification by preparative gel electrophoresis of pellet ferritin. On SDS-PAGE (15%) the fast ferritin showed a single band of 17 kDa (Fig. 2B). Slow ferritin gave a single $\approx$21-kDa band in some gels (Fig. 2B) but in others (Fig. 3) it was clearly separated into two bands of about 20.5 and 21.5 kDa, the major component being the smaller subunit. Slow ferritin from both cytosolic and pellet frac-

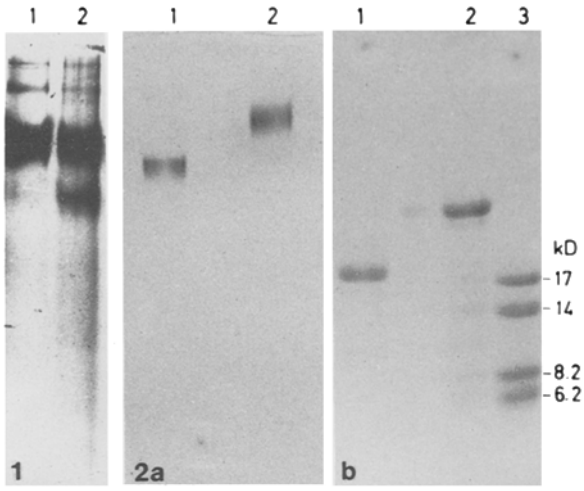


Fig. 1. Native PAGE of mouse liver ferritin. Lane 1, ferritin prepared from the cytosolic fraction of iron-loaded mouse liver, lane 2, ferritin prepared from the pellet fraction of iron-loaded mouse liver

Fig. 2. Native PAGE **(a)** and 15% SDS-PAGE **(b)** of the fast (lane 1) and slow (lane 2) components of mouse liver ferritin prepared from the pellet fraction of iron-loaded mice and purified by preparative gel electrophoresis

tions gave similar subunit patterns. The bands were of similar separation to H and L bands of cytosolic rat liver ferritin, but closer than those of horse spleen or human liver ferritin when run on the same gel.

Analysis of fast and slow ferritins

Fast and slow components purified by preparative PAGE from pellet ferritin were reduced and

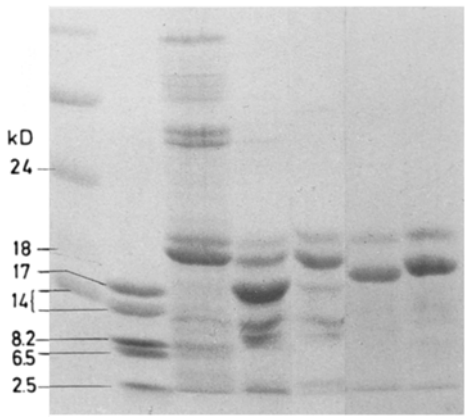


Fig. 3. 15% SDS-PAGE of several ferritins. Lanes 1 and 2, molecular mass markers; lane 3, partially purified cytosolic mouse liver ferritin; lane 4, rat liver siderosomal ferritin; lane 5, cytosolic rat liver ferritin; lane 6, horse spleen ferritin; lane 7, human liver ferritin

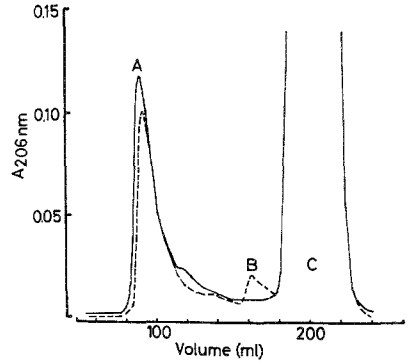


Fig. 4. Gel filtration chromatography of the purified fast (discontinuous line) and slow (continuous line) components of pellet apoferritin using Sephadex G-50 (1.6×90 cm) equilibrated with 10 mM HCl. C represents the salt peak

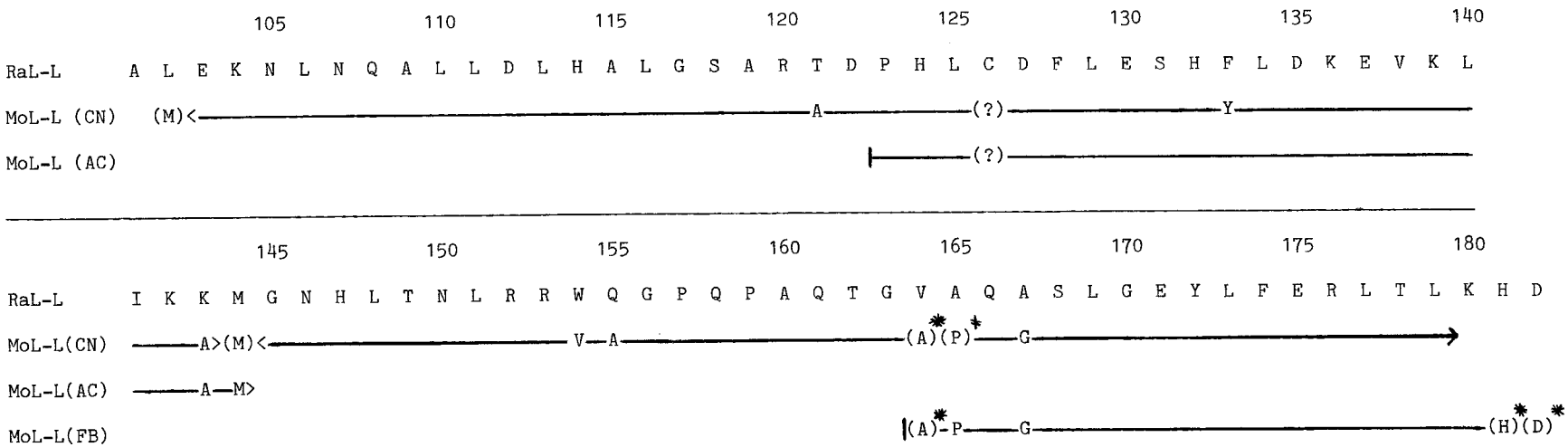
disaggregated into subunits in 70% (by vol.) formic acid and immediately applied to a Sephadex G-50 gel filtration column. Elution profiles (Fig. 4) showed, for the slow component, a single major peak (A) with a slight shoulder, but fast ferritin gave an additional peak (B) eluting just before the salt peak (C). This profile is very similar to that observed for rat fast ferritin under similar conditions (Andrews et al. 1987b). Table 1 shows the amino acid composition of peak B, together with that for the corresponding peak of siderosomal ferritin from rat liver and also their amino acid

Table 1. Amino acid composition and sequence of peak B peptide isolated from fast ferritin

Amino acid	Mouse peak B	Mouse sequence	Rat sequence
Asx	1.14 (1)	(−)	1
Thr	0.90 (1)	1	1
Ser	1.11 (1)	1	1
Glx	2.89 (3)	3	3
Gly	2.06 (2)	2	1
Ala	1.00 (1)	(−)	2
Val	0.12 (0)	0	1
Ile	0.10 (0)	0	0
Leu	3.74 (4)	4	4
Tyr	0.74 (1)	1	1
Phe	0.91 (1)	1	1
His	1.01 (1)	(−)	1
Met	0.20 (0)	0	0
Lys	1.15 (1)	1	1
Arg	0.77 (1)	1	1
Pro	0.92 (1)	1	0

Values for mouse, peak B, are given relative to alanine set at 1.00; total residues = 18.76. The nearest integer values are given in parentheses. The mouse sequence is:
(A)P Q G S L G E Y L F E R L T L K(H) (D); the rat sequence is
V A - A - - - - - - - - - - - - - -

Table 2. Partial amino acid sequence of mouse liver 'slow' ferritin major subunit. Peptides are aligned by homology with the amino acid sequence of rat liver ferritin L chain



RaL-L rat liver L chain (Leibold and Munro 1987), MoL-L mouse liver L chain from homology

MoL-L(CN) cyanogen bromide peptides

MoL-L(AC) mild acid cleavage peptide

MoL-L(FB) small peptide B from fast ferritin

(?) unidentified residue

( )* from composition of FB

( )‡ from sequence of FB

sequences. Both compositions and sequences are very similar. The peak from rat liver siderosomal ferritin has been shown to correspond to the C-terminal 19 residues of rat L chains (Andrews et al. 1987b). Table 2 presents partial amino acid sequence data for the peak B peptide of fast ferritin, and CNBr and acid-cleaved peptides from slow mouse liver ferritin aligned by sequence similarity with the C-terminal sequence (residues 102–182) of the rat liver L chain (Leibold and Munro 1987). The partial sequences, which include the 8-residue insertions, show 89% identity (72 out of 81 residues) and 95% similarity when conservative changes are considered. In contrast, the partial mouse liver L chain sequence is only 49% identical (36 out of 73 equivalenced residues) to that of the mouse macrophage H chain (Miyazaki et al. 1988) and furthermore the H chain contains no counterpart to residues 158–163 (Table 2), which correspond to the '8-residue insertion' region. Thus it is clear that the major component of mouse slow ferritin is an L subunit and that fragment B is from the C-terminal end of the L chain.

Discussion

The sequence data indicate that the predominant, electrophoretically slow species of mouse liver ferritin is composed mainly of L subunits. This suggests that the major, slightly faster, subunit band observed following SDS-PAGE (Fig. 3) corresponds to the L chain and therefore that the minor, slower band corresponds to the H chain. Thus, in contrast to previous reports (Grail et al. 1982; Massover 1985; Beaumont et al. 1987), the subunit composition of the major species of mouse liver ferritin is very much like that of ferritin from the livers of other mammals (Arosio et al. 1978).

The enrichment of siderosome-containing subcellular fractions with electrophoretically fast ferritin indicates that mouse liver fast ferritin is located within siderosomes, as is the case for fast ferritin of iron-loaded rat liver (Andrews et al. 1987a). The 19-residue peptide and the 17-kDa subunit of mouse fast ferritin are apparently derived from the L subunit through cleavage of the peptide bond between residues 163 and 164 (Table 2). The structural similarity between mouse and rat ferritin indicate that this cleavage site lies between helices D and E, within the 8-residue insertion region, on the external surface of the molecule (Rice et al. 1985). The cleavage apparently occurs in most of the L subunits within assembled shells and results in an increased electrophoretic mobility on native PAGE. Thus the fast ferritins of mouse and rat liver are analogous, although the peptide bond cleaved lies between different amino acid residues (glycine and alanine in the mouse L chain and glycine and valine in the rat L chain).

Results presented here and by others (Peto and Thompson 1986) show that mouse ferritin is difficult to resolve into its constituent subunits by SDS-PAGE, indicating that the subunits are of very similar size. This is in accord with a recent report (Beaumont et al. 1988) based on cDNA sequences that the mass of Friend cell ferritin H chains is slightly larger (20.9 kDa) than that of the L chain (20.7 kDa).

The two major bands (20.5 and 21.5 kDa) of Friend cell ferritin reported by Peto and Thompson (1986) may correspond to H and L chains, and the single major band of 24.5 kDa reported by Beaumont et al. (1987) could represent these two subunits unresolved. It is tempting to conclude that the 18-kDa component of induced Friend cell ferritin has arisen by processing of L chains to give a peptide equivalent to the 'F subunit' seen here. It may be, however, that this peptide is H chain processed post-translationally from a larger precursor, as has been suggested (Beaumont et al. 1987, 1988) and that the similarity in size with the F subunit is coincidental. If so, a different processing must be envisaged because the eight-residue insertion in which the L chain cleavage occurs is not present in murine H chains, at least not those of macrophages (Miyozaki et al. 1988). Possibly processing takes place within free H subunits rather than within those of assembled molecules. This clearly needs further investigation.

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