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A new form of ferritin heterogeneity explained

Isolation and identification of a nineteen-amino-acid-residue fragment from siderosomal ferritin of rat liver

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Ferritin present within siderosomes of iron-loaded rats has a faster anodal mobility than that of cytosolic ferritin from the same rats. A 19-amino-acid-residue peptide was isolated from this fast ferritin and shown to be derived from the *C*-terminal end of its L-subunit. A 17.3 kDa peptide seen on electrophoresis in denaturing gels of this ferritin accounts for the major portion of the original 182-residue subunit. The two peptides arise from cleavage within the 'insertion region' of the L-subunit sequence that occurs between the D and E helices and lies on the outside of the assembled molecule. This cleavage is present in about 80% of the L-subunits of siderosomal ferritin but nevertheless leaves the molecular structure otherwise intact. It gives rise to an apparent decrease in molecular size, accounting for the faster anodal mobility on native gels. Hence a new form of heterogeneity in ferritin preparations has been explained.

INTRODUCTION

The iron-storage protein ferritin plays an important part in iron metabolism. Ferritin is a large roughly spherical molecule consisting of 24 subunits arranged as a protein shell about a central cavity. Within this cavity up to 4500 Fe atoms are stored as microcrystalline hydrated ferric oxide. X-ray diffraction of horse spleen apoferritin combined with amino acid sequence data has enabled its detailed three-dimensional structure to be determined at high resolution (Rice *et al.*, 1983; Ford *et al.*, 1984), and rat liver ferritin has been shown to have a similar structure (Harrison *et al.*, 1985). One of the major problems in the study of ferritin is the heterogeneity of this protein. Ferritins vary in charge, size, immunological properties, iron content, subunit content and rate of iron accumulation. Such variations may be observed from tissue to tissue, within tissues, and according to iron status and pathological condition. It is now apparent that ferritin is made up from at least two subunit types, the H-subunit (about 21 kDa) predominant in heart tissue, and the L-subunit (about 20 kDa) predominant in liver tissue. The H- and L-subunit types are derived from different genes and give only about 55% identity in amino acid sequence (Costanzo *et al.*, 1984, 1986; Leibold *et al.*, 1984). Differing proportions of these two subunits and post-translational modifications such as glycosylation (Cragg *et al.*, 1981), intersubunit cross-linking (Mertz & Theil, 1983) and phosphorylation (Ihara *et al.*, 1984) are believed to underlie the heterogeneous nature of ferritin. Multiple gene copies that may exhibit microheterogeneity of sequence have been found for both H- and L-subunits in rat (Leibold *et al.*, 1984) and in humans (Jain *et al.*, 1985; Costanzo *et al.*, 1986), although most of these may represent pseudogenes (Costanzo *et al.*, 1986; Santoro *et al.*, 1986), at least in the case of human ferritin.

Our previous studies (Andrews *et al.*, 1987) have revealed a new form of ferritin heterogeneity, found in rat liver only after iron-loading. This novel ferritin, which was distinguished from cytosolic ferritin by its faster anodal mobility during PAGE, was the only form of ferritin found in siderosomes and may have been derived from cytosolic ferritin within this organelle (Andrews *et al.*, 1987). Its faster mobility, coupled with the similarity in its isoelectric profile, indicated that its apparent molecular size is smaller than that of cytosolic ferritin. Electrophoresis under denaturing conditions showed that the major subunit of siderosomal ferritin has a nominal molecular mass of about 17.3 kDa. A small proportion of L-subunit together with a trace of H-subunit were also shown to be present. These and other data, taken together with the resemblance between the two forms in their immunoreactivities and abilities to take up and release iron, have suggested that siderosomal ferritin is derived from cytosolic ferritin by cleavage of a proportion of L- and/or H-subunits within the intact molecule (Andrews *et al.*, 1987). In the present paper we provide direct evidence for such a cleavage, based on the isolation and characterization of a 19-amino-acid-residue peptide from siderosomal ferritin, which we show corresponds to the *C*-terminal end of the L-subunit.

METHODS

Isolation of ferritins

Siderosomal ferritin was either isolated from siderosomes and further purified by gel-permeation chromatography on Sepharose 6B (column dimensions 5 cm × 75 cm) in phosphate-buffered saline (0.01 M-sodium phosphate buffer, pH 7.2, containing 0.15 M-NaCl) or isolated from membrane fractions by preparative PAGE as described in the preceding paper (Andrews *et al.*, 1987). Cytosolic ferritin was purified from either supernatant or pellet fractions of iron-loaded rat liver as described in the preceding paper (Andrews *et al.*, 1987).

Abbreviation used: PAGE, polyacrylamide-gel electrophoresis.

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Amino acid analysis

Freeze-dried apoferritin samples were dissolved in 300 μl of 6 M-HCl (AristaR) containing phenol (10 nmol/ml), and the tubes were sealed under vacuum. The sealed tubes were incubated at 110 °C for 24 h. After hydrolysis, amino acid composition was determined on a single-column amino acid analyser (Locarte Scientific Instruments) or an LKB 4400 system.

N-Terminal amino acid determination

N-Terminal amino acids were identified by dansylation (Gray & Hartley, 1963) followed by chromatography of the dansyl derivatives on polyamide thin-layer plates in accordance with Woods & Wang (1967).

Amino acid sequencing

Automated amino acid sequencing was kindly performed by Dr. J. Findlay (Department of Biochemistry, University of Leeds), on a solid-phase Sequenator. The peptide was coupled to *p*-phenylene di-isothiocyanate–glass (Wachter *et al.*, 1973) and sequenced by automated solid-phase Edman degradation (Laursen, 1971). The amino acid anilinothiazolinone derivatives were converted into the corresponding phenylthiohydantoin derivatives and identified by reverse-phase h.p.l.c. (Zimmerman *et al.*, 1977). The amino acid phenylthiohydantoin derivatives were quantified at 265 nm, and serine and threonine were confirmed by the detection of their dehydro derivatives at 313 nm.

Acid cleavage

Mild acid cleavage of rat liver apoferritin was performed by using one of the reaction conditions suggested by Landon (1977). Samples of apoferritin (2.5–7.5 mg/ml) in 70% (v/v) formic acid containing 5 mM-2-mercaptoethanol were flushed with N_2, sealed and incubated at 37 °C for 60 h. The formic acid was blown off under a stream of N_2 and the digested protein was freeze-dried. A sample of each of the digests was boiled for 10 min in 0.1 M-Tris/HCl buffer, pH 6.8, containing 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol and 2.3% (w/v) SDS. These samples were then subjected to SDS/PAGE as described by Laemmli (1970).

Separation of peptides from siderosomal and cytosolic ferritins

A 1 mg portion of apoferritin was freeze-dried, taken up in 70% (v/v) formic acid to disaggregate the ferritin molecule and immediately applied to a Sephadex G-50 (fine grade) column (1.6 cm × 90 cm) that had been equilibrated in 10 mM-HCl. This experiment was performed twice for cytosolic ferritin and three times for siderosomal ferritin. The elution peaks were collected in 2–3 ml fractions, pooled and freeze-dried. Amino acid analysis and *N*-terminal determination were carried out on each elution peak.

All other methods were as described in the preceding paper (Andrews *et al.*, 1987).

RESULTS

Table 1 displays the amino acid compositions of siderosomal and cytosolic ferritins. The two compositions are similar, differences being within experimental error. They are also close to those obtained from cDNA sequences of L-subunits (Leibold *et al.*, 1984). This is consistent with results (Andrews *et al.*, 1987) indicating that cytosolic ferritin contained 85% L-subunits.

Table 1. Amino acid compositions of siderosomal and cytosolic ferritins

The amino acid compositions are expressed as residues per subunit. Each subunit is assumed to consist of 179 amino acid residues (excluding cysteine and tryptophan, which were not determined). The results were obtained from two amino acid analyses for siderosomal ferritin and three for cytosolic ferritin, and are expressed as means ± S.D. The amino acid composition in the last column was based on the cDNA sequence reported for the rat liver ferritin L-subunit by Leibold *et al.* (1984).

	Composition (residues/subunit)		
Amino acid	Siderosomal ferritin	Cytosolic ferritin	cDNA sequence
Asx	19.72 ± 0.15	18.66 ± 0.84	18
Thr	6.73 ± 0.25	7.12 ± 0.38	7
Ser	8.34 ± 1.66	9.03 ± 0.49	8
Glx	28.39 ± 0.89	27.68 ± 2.33	28
Pro	3.98 ± 1.48	4.16 ± 1.27	4
Gly	11.42 ± 0.86	13.11 ± 1.70	12
Ala	17.00 ± 0.60	17.07 ± 0.51	18
Val	10.37 ± 1.86	9.88 ± 0.80	8
Ile	2.85 ± 0.59	3.20 ± 0.27	2
Leu	26.33 ± 1.06	26.05 ± 0.76	28
Tyr	4.29 ± 0.11	3.81 ± 0.80	4
Phe	8.56 ± 0.22	8.01 ± 1.27	9
His	6.87 ± 0.76	7.17 ± 0.38	7
Lys	10.50 ± 0.26	10.47 ± 0.22	12
Met	2.35 ± 0.06	2.13 ± 0.00	2
Arg	11.33 ± 2.90	12.44 ± 0.19	12
Total	179	179	179

No *N*-terminal amino acid was detected for cytosolic ferritin, but an *N*-terminal valine residue was found for siderosomal ferritin. The L-subunit of rat liver ferritin contains an *N*-terminal threonine residue, and this is known to be blocked through acetylation (Huberman & Barahona, 1978; Leibold *et al.*, 1984). Hence under normal circumstances, the L-subunit of rat liver ferritin does not possess a free *N*-terminus available for dansylation. The finding of an *N*-terminal valine in siderosomal ferritin suggests that the molecule has been cleaved.

The rat liver ferritin L-subunit sequence (Leibold *et al.*, 1984) shows the presence of a single Asp–Pro bond at amino acid residues 122–123. A mild acid cleavage at this susceptible bond (Piskiewicz *et al.*, 1970) would be expected to split the L-subunit into two fragments, an *N*-terminal fragment (1–122) and a *C*-terminal fragment (123–182) of 60 amino acid residues. These fragments should be separable on SDS/PAGE and identifiable by their relative mobilities. However, should the L-subunit possess a nick at some other point, the size of either the C-terminal or *N*-terminal fragment would be decreased by an extent dependent on the exact position of the initial cleavage.

After digestion in dilute acid, cytosolic ferritin produced two major bands on SDS/PAGE of sizes corresponding to the *N*-terminal and *C*-terminal frag-

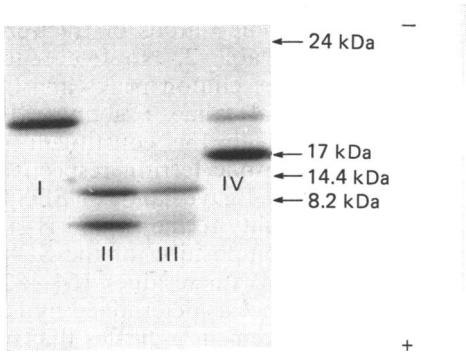

Fig. 1. Mild acid cleavage of siderosomal and cytosolic ferritins

Proteins were separated by SDS/PAGE (15%T) and stained for protein. Lane 1, cytosolic ferritin; lane 2, cytosolic ferritin after mild acid cleavage; lane 3, siderosomal ferritin after mild acid cleavage; lane 4, siderosomal ferritin.

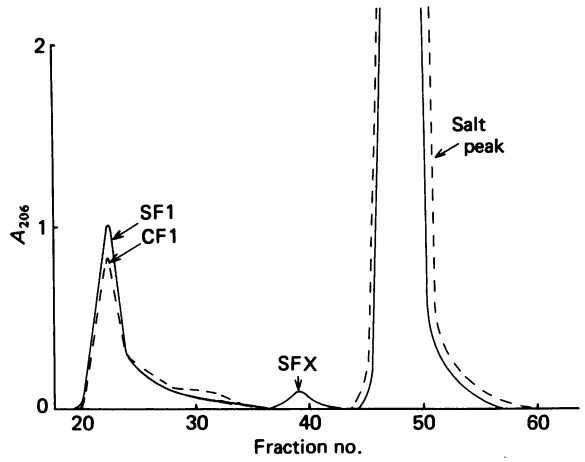

Fig. 2. Gel filtration of siderosomal ferritin (SF, ——) and cytosolic ferritin (CF, ----) under denaturing conditions

Gel filtration was performed as in the Methods section, in 10 mM-HCl on a Sephadex G-50 (fine grade) column (1.6 cm × 90 cm).

ments expected for the L-subunit (Fig. 1). However, the siderosomal ferritin digest gave only one major band, which corresponds in size to the larger, *N*-terminal, fragment, and only a trace of a smaller band representing the *C*-terminal fragment (residues 123–182) is visible in the gel. This could be explained if siderosomal ferritin possessed a nick towards its *C*-terminus. The expected very-low-molecular-mass bands representing the *C*-terminal cleavage products of siderosomal ferritin were not visible, possibly owing to poor fixation to the gel.

The finding of *N*-terminal valine residues allows us to position the point of cleavage either at Glu–Val (137–138) or at Gly–Val (163–164), since there are only two valine residues in the *C*-terminal region 123–182. Of these two sites the Gly–Val bond seems the more likely in relation to the three-dimensional structure of the molecule. This bond lies in an exposed interhelical region between the D and E helices, in contrast with Glu–Val (137–138), which is buried within the subunit in a region near the internal cavity of the protein shell (see below). Furthermore, cleavage at Gly–Val (163–164) would produce an *N*-terminal fragment of about 18 kDa, close to that observed (17.3 kDa), whereas splitting at Glu–Val

Table 2. Amino acid compositions and comparison of the SFX and SF1 peptides

Cysteine and tryptophan were not determined. Sequence (1) was obtained from amino acid residues 1–163 of the rat liver ferritin L-subunit sequence, sequence (2) was from amino acid residues 164–182 of that sequence and sequence (3) was from the entire 182-residue sequence excluding the two tryptophan and single cysteine residues (Leibold *et al.*, 1984). The amino acid composition for peptide SF1 is expressed as residues per 160 residues, the composition for peptide SFX is expressed as residues per 19 residues and the sum of the compositions for peptides SF1 and SFX is expressed as residues per 179 residues. Results were from single determinations.

	Composition (residues/subunit)					
Amino acid	Peptide SF1	Sequence (1)	Peptide SFX	Sequence (2)	Peptides SF1+SFX	Sequence (3)
Asx	17.8	17	1.12	1	18.9	18
Thr	5.80	6	1.00	1	6.81	7
Ser	6.97	7	1.03	1	7.99	8
Glx	25.8	25	3.48	3	29.4	28
Pro	4.95	4	0.0	0	4.95	4
Gly	10.9	11	1.16	1	12.0	12
Ala	14.9	16	1.96	2	16.9	18
Val	7.46	7	0.92	1	8.38	8
Ile	2.79	2	0.05	0	2.84	2
Leu	23.2	24	3.70	4	26.9	28
Tyr	3.05	3	0.68	1	3.73	4
Phe	6.94	8	0.96	1	7.90	9
His	6.42	6	0.92	1	7.34	7
Lys	9.38	11	0.97	1	10.4	12
Met	2.30	2	0.0	0	2.30	2
Arg	11.4	11	1.05	1	12.4	12
Total	160	160	19	19	179	179

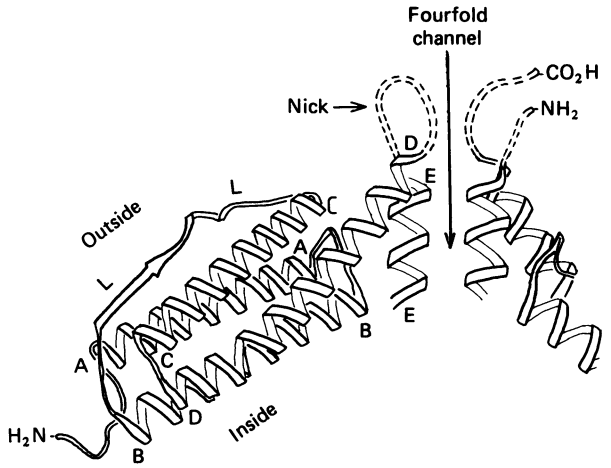

Fig. 3. Ribbon diagram of the α-carbon backbone of a rat liver ferritin L-subunit, with part of a second subunit

The region drawn in broken lines represents the 12 amino acid residues between helices D and E, for which secondary structure remains undetermined. This diagram shows the approximate position of the Gly–Val (163–164) cleavage giving rise to the SFX peptide found in siderosomal ferritin. The position of the subunit with respect to the inside and outside surfaces of the 24-mer protein shell is indicated.

(137–138) would give an *N*-terminal peptide of only 15.6 kDa.

The finding of free *N*-terminal valine in siderosomal ferritin implies that the *C*-terminal fragments are not dissociated when the subunits are nicked. Hence it should be possible to isolate and identify these fragments. This was done by disaggregation in 70% (v/v) formic acid followed by gel filtration as described in the Methods section. Siderosomal ferritin shows an elution peak (SFX) not present in cytosolic ferritin, eluted just before the salt peak (Fig. 2). Fractions corresponding to each elution peak were pooled and frozen-dried.

The amino acid compositions of fractions SFX and SF1 are shown in Table 2. No free *N*-termini were detected in any of the elution peaks apart from SFX, which gave *N*-terminal valine. Fractions SF1 and CF1 probably correspond to the constituent subunits of siderosomal and cytosolic ferritin respectively. Indeed, samples of fraction CF1 gave bands on SDS/PAGE that corresponded in mobility to the L- and H-subunits.

The amino acid composition of the SFX peptide is compared in Table 2 with residues 164–182 of the rat liver L-subunit sequence as determined by Leibold *et al.* (1984). The good agreement indicates that peptide SFX is indeed the 19-residue *C*-terminal fragment derived from the proposed Gly–Val cleavage. The amino acid sequence of peptide SFX, kindly determined for us by Dr. J. Findlay, and shown in Fig. 3, confirms that this is the case.

The amino acid composition of peptide SF1 (Table 2) agrees well with that calculated for residues 1–163 of the L-subunit sequence. The sum of the amino acid residues in peptides SF1 and SFX give reasonable agreement with that of the intact subunits.

DISCUSSION

We have now unambiguously identified the presence in siderosomal ferritin of a peptide corresponding to a cleavage point 19 residues from the *C*-terminus of the L-subunit. We have no experimental data indicating that cleavage of the H-subunit has occurred. The amino acid composition and sequence data of the SFX peptide are accounted for very well by the *C*-terminal residues of the L-subunit alone. Although the complete sequence of the rat liver H-subunit is not yet available, a partial cDNA sequence thought to represent the 24 *C*-terminal residues of the H-chain shows several sequence changes in this region as compared with the L-subunit (see Fig. 3). However, since the H-subunit represents only a small fraction of cytosolic ferritin, any splitting that may have occurred might have gone undetected.

It is clear from the data we have presented that a subunit cleavage has occurred in otherwise intact

Table 3. Alignment and comparison of the SFX peptide and rat liver ferritin amino acid sequences

RaL-L represents rat liver ferritin L-subunit (cDNA), RaL-Hp represents rat liver ferritin H-subunit (partial sequence) (cDNA), and RaL-SFX represents SFX peptide from rat liver siderosomal ferritin (protein). Both the RaL-L and RaL-H sequences are from Leibold *et al.* (1984). Stretches of sequence that form helices in the assembled molecule are shown as boxed regions and are positioned according to alignment with horse spleen ferritin (Rice *et al.*, 1983). The *N*-terminus of peptide SFX was identified from dansylation, and the last three residues in parentheses were based on the amino acid composition. The amino acid sequence (to residue 16) was determined by Dr. J. Findlay. A 2.5 nmol portion of peptide was taken for coupling and the initial sequencing yield was 800 pmol. The average repetitive yield was 97%.

	D helix	Insertion region	E helix	
	154	160	170	180
RaL-L	-W Q G P	Q P A Q T G V A	Q A S L G E Y L F E R L	T L K H D
RaL-SFX		V A Q A	S L G E Y L F E R L	T L(K H D)
RaL-Hp	-M G A P	E S	G M A G Y L F D K H	T L G H G D E E

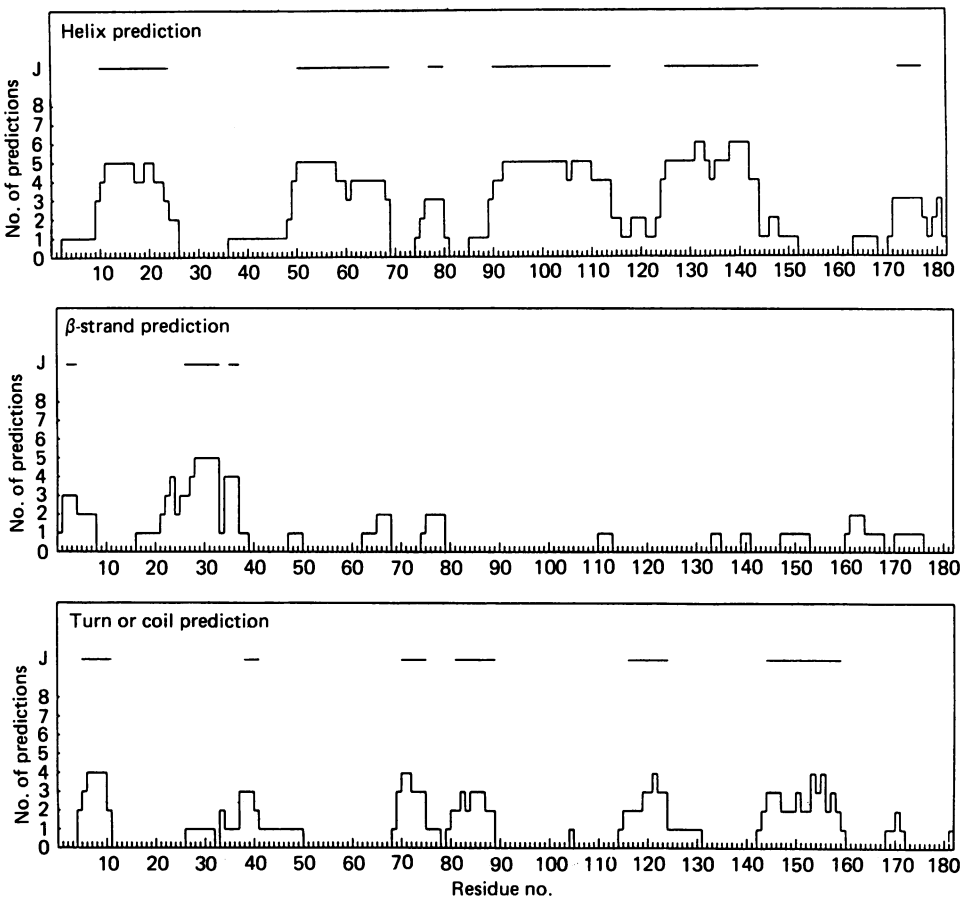

Fig. 4. Secondary-structure prediction for the rat liver ferritin L-subunit

The amino acid sequence is derived from the cDNA sequence obtained by Leibold *et al.* (1984). Note the low values predicted in the region 156–167.

siderosomal molecules that is not found in cytosolic ferritin. In Fig. 3 we show the position of the cleavage site in relation to the three-dimensional structure of the assembled molecule. Rat liver apoferritin must have a conformation close to that of horse spleen apoferritin, since their electron-density difference maps are almost featureless (Harrison *et al.*, 1985). However, compared with the L-subunit sequence of horse spleen apoferritin, that of the rat liver L-subunit contains an eight-residue insertion between residues 157 and 158 numbered according to the horse spleen apoferritin sequence (Addison *et al.*, 1983) (Table 3). The horse spleen subunit contains a bundle of four long helices A, B, C and D comprising respectively residues 10–39, 45–72, 92–120 and 124–155, and a short helix E, comprising residues 160–169. Thus the D–E inter-helical region contains four residues, 156–159 inclusive. In rat liver apoferritin the insertion extends this region to 12 residues, 156–167, and the E-helix is now numbered 168–177. Thus the cleavage site that we have identified as between 163 and 164 is in this extended region towards the *C*-terminus. No electron density for the insertion was seen in the rat liver apoferritin electron-density map, and hence we concluded that it has disordered conformation (Harrison *et al.*, 1985). This is supported by a recent secondary-structure prediction [kindly performed by Dr. J. L. White using the combined prediction program of Eliopoulos *et al.* (1982)] that predicts the helical regions quite well and indicates a low probability of secondary structure within the region of interest (Fig. 4). We note that two out of the 12 residues are proline (see Table 3). Hence we have drawn this region as an extended loop in Fig. 3. Cleavage in this loop could occur without effect on the conformation of the subunits within the shell or their inter-subunit contacts.

Siderosomal apoferritin has been crystallized from solutions containing $CdSO_4$ as octahedra of very similar appearance to those of cytosolic apoferritin obtained under similar conditions. Although the crystals were too small for X-ray-diffraction results to be obtained, it seems likely that the crystals from the two apoferritins are isomorphous and hence that the molecules are structurally similar. Cleavage in the region shown in Fig. 3 would not be expected to affect crystallization from $CdSO_4$ solution, since there are no intermolecular contacts involving this region. Intermolecular contacts are known to occur via double Cd^{2+} bridges using Asp-80 and Gln-82 as ligands (Ford *et al.*, 1984). These occur in the long loop, L, between helices B and C, remote from the D–E turn region (Fig. 3).

Although the cleavage point that we have found relates satisfactorily to its position in the three-dimensional structure, it is not clear why it causes the markedly increased electrophoretic mobility of siderosomal ferritin molecules observed by PAGE. Presumably, in the unmodified protein, the protruding D–E inter-

helical residues 156–167 diminish mobility by causing drag and thus increasing the effective molecular size disproportionately to their actual volume. In siderosomal ferritin the inter-helical loop may thus have become transformed into two 'loose ends', which would be more mobile than the original conformation, leading, perhaps, to a more stream-lined ferritin molecule. Although attempts to measure the *C*-terminal residues of peptide SF1 gave inconclusive results owing to low yields, amino acid composition data in Tables 1 and 2, which show identical threonine contents in siderosomal and cytosolic ferritin, suggest that not more than one residue may have been removed. Loss of any of the eight residues 156–163 would leave molecular charge unchanged.

It is uncertain whether the specific cleavage of rat liver ferritin has a functional significance. Since it probably occurs actually within the siderosome, it may represent the first step in the degradation of soluble ferritin to give insoluble haemosiderin, perhaps making the protein more susceptible to further proteolysis or to denaturation. There is no evidence that this lesion occurs in ferritins of iron-loaded tissues from other species, however, with the possible exception of mouse liver. Massover (1985) has observed in siderotic mouse liver ferritin a minor fast component with subunits of molecular mass 18 kDa, which he interprets as a smaller molecule assembled *de novo* from these small subunits. It may well be that this ferritin is similar to the siderosomal ferritin of rat. The 'small' mouse liver ferritin, however, contains virtually no subunits corresponding to unmodified H- or L-subunits, such as we find in the siderosomal ferritin of rat liver. This may be a consequence of increased iron-loading or represent a difference in the relative efficiency of the modification process in the two species. An electrophoretically fast component has not been reported for iron-loaded human liver ferritin. In human ferritin H- and L-subunits, where the D–E turn comprises only four amino acid residues, this region may be less susceptible to cleavage by proteolysis or oxidative attack either because it is less prominent, or less flexible, or because its sequence is different. Thus in the rat the observed lesion could be a fortuitous event. Alternatively it could represent an adaptive response to iron-overload in those animals in which the D–E insertion occurs. In rat liver ferritin the eight-residue insertion seems to be present only within L-subunits and not in H-subunits (Leibold *et al.*, 1984). If its cleavage does facilitate the conversion into haemosiderin, the differentially increased synthesis of L-subunits giving increased proportions of L-subunit-rich molecules in response to iron-loading (Kohgo *et al.*, 1980; Treffry *et al.*, 1984) could represent a physiological response.

No enzyme causing the cleavage between glycine and valine residues has been identified, but it is unlikely to be cathepsin D, even though this enzyme is present in high activity in siderosomes (Richter, 1984; Andrews *et al.*, 1987). Richter (1983) has also concluded from the pH-dependence of early ferritin siderosomal proteolysis that cathepsin D is probably not involved. Possibly the cleavage may have been non-enzymically produced by reactive oxygen species generated in the presence of reduced iron, as found for glutamate synthetase (Kim *et al.*, 1985).

There has been much controversy over the nature of isoferritins. Thus, although the finding of at least two sequences in human ferritins (Costanzo *et al.*, 1984; Boyd *et al.*, 1985) provides strong support for the 'heteropolymer' hypothesis of Drysdale (1977), it has been pointed out that association of two subunit types in differing proportions could not alone explain the observed heterogeneity (Bomford *et al.*, 1981; Treffry *et al.*, 1984) and that post-translational (probably post-assembly) modification must also occur (Treffry *et al.*, 1984). We have now identified one such modification and its position in the three-dimensional structure of the molecule.

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