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Correlations between Subunit Distribution, Microheterogeneity, and Iron Content of Human Liver Ferritin†

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ABSTRACT: Subunit heterogeneity of human liver ferritin was investigated by two-dimensional electrophoretic methods. The protein which ordinarily remains assembled in 10 M urea solution was dissociated into subunits in acid–urea or sodium dodecyl sulfate solutions. In agreement with earlier studies, the subunits migrated as two bands in sodium dodecyl sulfate or acid–urea gel electrophoresis systems or in two-dimensional combinations of these systems. Isoelectric focusing methods, however, resolved four major subunit bands and three to five minor bands. Each of these components migrated as either a 22 000 or a 19 000 molecular weight component in sodium dodecyl sulfate gel electrophoresis in the second dimension. The multiple subunit model, which is contrary to currently accepted representations of ferritin structure, is compatible with certain inherent properties of the protein. Thus, ferritin was fractionated on the basis of iron content to show that the relative amounts of individual subunit types were directly dependent upon the iron composition of the protein. Iron-loaded molecules were deficient in the most basic subunit types, and apoferritin was enriched in these components. Aspects of microheterogeneity of assembled ferritin molecules were correlated to subunit heterogeneity, and discrete differences in subunit populations among purified isoferritin components were demonstrated.

Ferritin, an iron-containing protein found in most mammalian tissues and widely distributed in nature, plays a prominent role in iron metabolism of eukaryotic cells (Granick, 1946; Crichton, 1973; Linder and Munro, 1973; Harrison and Hoy, 1973; Harrison et al., 1974; Harrison, 1977; Drysdale et al., 1977; Fagard and Saddi, 1977; Marcus et al., 1978). The protein subunits are arranged in the form of a hollow sphere that is uniquely suited to envelop large amounts of iron in its central cavity (Haggis, 1965; Harrison et al., 1967; Fischbach et al., 1969; Spiro and Saltman, 1969; Massover et al., 1973; Webb and Gray, 1974; Harrison et al., 1974). With improved technical developments for resolving closely related proteins, ferritins from most sources were shown to consist of a family of proteins that have been referred to as "isoferritins" (Drysdale, 1970; Urushizaki et al. 1971; Drysdale, 1974; Powell et al., 1975; Massover, 1978). It was suggested that this microheterogeneity originates from different subunit types that are present in different combinations or proportions in assembled molecules (Vulimiri et al., 1975; Adelman et al., 1975; Ishitani et al., 1975a,b; Lavoie et al., 1977; Drysdale, 1977). Ferritin undergoes a number of posttranslational structure modifications, including glycosylation (Shinjyo et al., 1975; Lavoie et al., 1977; Cynkin and Knowlton, 1977) and proteolytic processing (Niitsu et al., 1973; Ishitani et al., 1975c), and possible correlations of these factors to aspects of its microheterogeneity have been discussed in recent reviews (Marcus et al., 1978; Drysdale, 1977).

In the present report, heterogeneity of human liver ferritin is probed by two-dimensional gel electrophoretic methods. Conditions for dissociation and fractionation of the protein are defined, and a systematic relationship between subunit heterogeneity and microheterogeneity of assembled molecules is considered.

Experimental Procedures

Materials

Urea was ultrapure grade from Schwarz/Mann. Acrylamide and other electrophoretic reagents were obtained from

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Bio-Rad Laboratories. Sodium dodecyl sulfate ($NaDodSO_4$) was obtained from Pierce Chemicals and recrystallized from hot ethanol. The nonionic detergent nonidet P-40 was obtained from Particle Data Laboratories, and ampholines were from LKB or Brinkmann. All other chemicals were reagent grade.

Methods

Preparation of Ferritin. The protocol used for the isolation of human liver ferritin was similar to that of Marcus and Zinberg (1975). A fresh human liver obtained at the time of post-mortem was rinsed free of blood, trimmed into small cubes, washed with cold phosphate-buffered saline (PBS) [0.02 M phosphate buffer (pH 7.3), 0.9% NaCl, containing 0.1 mM phenylmethanesulfonyl fluoride] and homogenized in the buffer using a Sorvall OmniMixer at 4 °C. The extract was then centrifuged at 13 000*g* at 4 °C for 18 h. The supernatant was applied to an immunoadsorbent column of an IgG fraction of goat antiserum to human liver ferritin coupled to Sepharose 4B by the cyanogen bromide method. The column was washed with buffer until the effluent was free of protein. The bound protein was then eluted from the column with 2.0 M potassium thiocyanate solution. Since human serum IgG protein contaminants are also found in the eluant, the antigen was passed through an immunoadsorbent column coupled to antibodies to human serum.

To remove ferritin aggregates, the preparation from the second immunoadsorbent column was passed through a column of Sepharose 6B equilibrated with the above buffer. The column dimensions were 90 × 5 cm with a flow rate of 25 mL/h. Ferritin solution (70 mg/mL) was applied (1 mL) to the column and eluted in 5-mL fractions. The ferritin obtained in this manner did not react with anti-human serum and appeared as a single major band on electrophoresis on 3.5–7.5% acrylamide gels which stained either for iron or protein.

Human liver ferritin was also prepared by traditional procedures that involved heating the homogenate to 70 °C for 10 min, centrifugation, precipitation of protein in the supernatant with 50% (w/v) ammonium sulfate, and gel filtration on Sepharose 6B columns (Niitsu and Listowsky, 1973a,b). Purified ferritins also exhibited a single band on polyacrylamide gel electrophoresis in 5% gels that were stained for protein or iron. The preparations were routinely characterized by ellipticity values (Listowsky et al., 1972) that were virtually unaffected by 10 M urea.

Fractionation of Ferritin. An LKB 2117 Multiphor apparatus was used for preparative flat-bed isoelectric focusing. Beds were formed on glass trays supplied in an LKB 2117-501 Ampholine electrofocusing kit for granulated gels. Ultrodex (LKB) for flat-bed electrofocusing and Pevikon (Mercer Chemical Corp., New York, N.Y.) were mixed together in a ratio of 12.0 g of Pevikon to 0.56 g of Sephadex, and the slurry was poured as described by Otavsky et al. (1977). Beds were electrofocused under constant power with the total applied power limited to 8 W.

The gel trays were placed on the Multiphor apparatus and maintained at about 5 °C. Efficient heat transfer was ensured by moistening the tray and glass cooling block with 0.1% Triton X-100. Protein application was made with a sample applicator midway between the electrodes to avoid extremes of pH (LKB application note 198). Samples up to 3 mL were applied in a zone 2 × 10 cm. The sample contained the bed ampholyte solution to avoid changing the conductivity of the region. After voltage and current had stabilized, the bed was fractionated with a metal grid and the pH measured directly. The fractions were then washed with PBS and centrifuged until all of the protein was in the supernatant fraction.

Dissociation in $NaDodSO_4$ solution was carried out by heating ferritin samples at 80 °C for 3 min in the presence of 1% $NaDodSO_4$. Detailed accounts of changes in the three-dimensional structure of the protein attendant to these procedures have been described earlier (Ishitani et al., 1975c). The solutions were cooled to 25 °C and nonidet P-40 was added to a final concentration of 2%. These samples were used for gel electrofocusing in urea.

Gel Electrofocusing in Urea. Urea solutions were always freshly prepared, exposed to ion-exchange resins, and treated with charcoal to remove isocyanide and other potential contaminants. Solutions were usually kept at 4 °C and electrophoresis procedures were carried out the same day that the gels were prepared.

Electrofocusing gels were prerun for 1 h to remove any urea decomposition products and excess ammonium persulfate. Tris buffer (1 mM, pH 7.5) was incorporated into some electrophoretic systems to react with any isocyanate that may have survived the above procedures. In spite of these precautions, it was noted that if the protein was heated or incubated for long periods of time in urea additional electrofocusing bands appeared. The new bands were usually present in small amounts and were more acidic than the ordinary subunit constituents.

Dissociated ferritin samples were equilibrated in 9 M urea solution containing 1:1 mixtures of pH 4–6 and 5–7 ampholine (2% final concentration) and 2% nonidet P-40 detergent. For protein dissociated in $NaDodSO_4$, electrofocusing solutions contained 8% NP-40 (Ames and Nikaido, 1976). Electrofocusing was carried out for 14 h. A Clifford Densicomp instrument Model 445 (Clifford Instruments, Natick, Mass.) with an automatic integrator was used for densitometric scans of the gels.

Dissociation of Ferritin in Urea. Ferritin (0.5–2.0 mg/mL) was dissolved in aqueous 9 M urea. The pH of the urea solution was adjusted to 2.5 by the addition of small amounts of 1 N HCl, and the protein was incubated at 25 °C in 9 M urea at pH 2.5 for 10 min. The solution was then readjusted to pH 7.5 and centrifuged at 57 000*g* for 2 h, and the clear supernatant was collected. The essentially protein-free pellet of aggregated inorganic micelles of iron was discarded (Listowsky et al., 1972). Nitrogen was bubbled through and maintained as a low stream above the solution. The solution of subunits in 9 M urea was reduced by the addition of a 50-fold molar excess of dithiothreitol and alkylated with a 57-fold excess of iodoacetamide. The latter procedures, to prevent formation of disulfide bridges, did not affect the overall appearance of the gel electrofocusing patterns, but some minor bands that were occasionally evident were eliminated by these procedures. These samples were utilized for either circular dichroism or gel electrophoresis studies.

Two-Dimensional Electrophoresis. The cylindrical electrofocusing gels were applied to a slab gel containing 0.1% $NaDodSO_4$ and 8.5% acrylamide according to the procedure of O'Farrell (1975). Electrophoresis in the second dimension was carried out for 8 h. The gels were stained with Coomassie brilliant blue R-250 in methanol–acetic acid–water (5:1:4) and destained in methanol–acetic acid–water (3:1:6).

Fractionation of Ferritin on the Basis of Iron Content. Human liver ferritin was fractionated by density gradient centrifugation in 30–50% sucrose gradients according to procedures described previously (Niitsu and Listowsky 1973a). High-density ferritin fractions contained about 2500 iron atoms/mol of protein, and apoferritin fractions obtained from

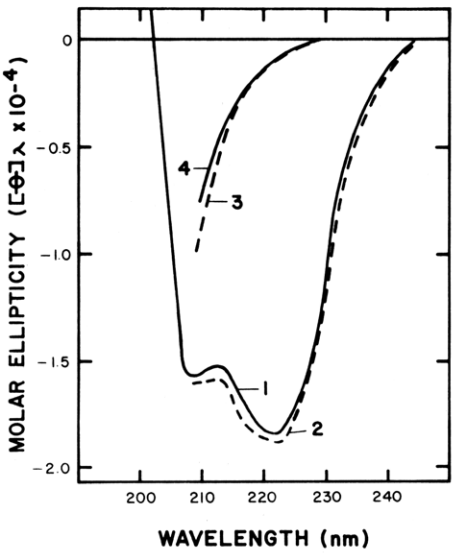

FIGURE 1: Circular dichroism spectra of human liver ferritin. Curve 1 (solid line) is human liver ferritin in aqueous solution (pH 7.5). Curve 2 (dotted line) is ferritin dissolved in 9 M urea solution (pH 7.5). Curve 3 (dotted line) is the ferritin in 9 M urea (pH 2.5). Curve 4 (solid line) is ferritin incubated in 9 M urea (pH 2.5) for 10 min, and then the pH was adjusted to 7.5, before measurements were made. Protein concentrations were 50 μg/mL and 1-cm path-length cells were used for measurements at wavelengths above 218 nm and 1-mm path-length cells for measurements below 218 nm. Molar ellipticities in deg·cm^2/dmol were calculated using a mean residue weight of 109 for ferritin (Listowsky et al., 1967).

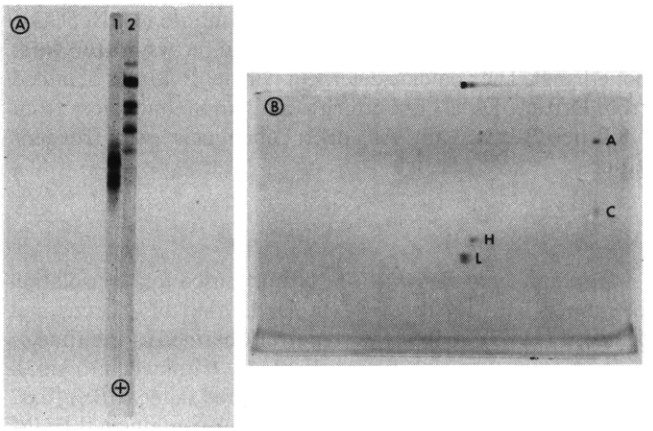

FIGURE 2: (A) Isoelectric focusing of human liver ferritin in urea. The sample in gel 1 was treated in 9 M urea solution (pH 7.5) and applied directly to the gel. This represents electrofocusing of an assembled ferritin preparation. The sample in gel 2 was incubated in 9 M urea solution (pH 2.5) for 10 min, and the pH was readjusted to 7.5. These are ferritin subunits. The gels contained 2% carrier ampholytes (1:1 mixtures of pH 4–6 and 5–7 ampholine) and 2% nonidet P-40 solution. Other electrofocusing conditions are described under Experimental Procedures. (B) Electrophoretic analysis of human liver ferritin in acid–urea and sodium dodecyl sulfate gels. Human liver ferritin was dissociated in 9 M urea (pH 2.5), and electrophoresis in the first dimension was carried out according to Panyim and Chalkley (1969) in 5% acrylamide gels. A stained gel that was run tandem to the one applied to the second-dimension slab gel is shown at the top of the figure. Electrophoresis in the sodium dodecyl sulfate–gel of the second dimension was carried out under conditions used in Figure 3. The molecular weight markers: (A) bovine serum albumin (67 000); (C) chymotrypsinogen (26 000). The H and L subunits had mobilities corresponding to molecular weights of 22 000 and 19 000, respectively.

the top of the gradients were essentially devoid of iron (less than ten iron atoms/mol). Electrofocusing of apoferritin was described in an earlier report (Lavoie et al., 1977).

Circular Dichroism. A Cary Model 60 spectropolarimeter with 6001 CD attachment was used for the circular dichroism measurements. Cells with 1 cm and 1 mm path lengths were used, and absorbancies of solutions were less than 2.0 at all wavelengths recorded. The temperature of the cell compartment was 26 °C. Protein concentrations were estimated by the method of Lowry et al. (1951), previously standardized for ferritin (Niitsu and Listowsky, 1973b).

Results

Dissociation into Subunits. Ferritin and apoferritin are exceptionally stable proteins, and 8–10 M urea solutions at neutral pH have no effect on the conformation or subunit structure of ferritins (Listowsky et al., 1972). Human liver ferritin was, therefore, incubated in 9 M urea solution at pH 2.5 for 10 min at room temperature to promote its dissociation into subunits. According to the circular dichroic data shown in Figure 1, the protein is denatured and unfolded under these conditions. Sedimentation velocity patterns are characterized by a 2.1S component exclusively (Listowsky et al., 1972), and no faster sedimenting aggregated forms are detected in the ultracentrifuge even at high protein concentration. After dissociation and unfolding at pH 2.5 in urea, the subunits do not reaggregate, nor is the native conformation restored if the pH of the 9 M urea solution is readjusted to 7.0–8.0 (Figure 1). The protein may thus be obtained in two distinct conformational states in neutral 9 M urea solutions: (1) an assembled multimeric ferritin molecule with a conformation almost identical to that of the native protein, and (2) unfolded subunits, if the protein is preexposed to the low pH conditions.

The undissociated and acid–urea dissociated forms of the protein were compared by gel isoelectric focusing in 8 M urea (Figure 2A). The isoelectric points of all of the ferritin subunits are more basic than any individual assembled isoferritins. These data imply that unfolding and attendant dissociation into subunits occur with a characteristic change in the surface charge of the protein, and no fully assembled molecules remain after acid–urea treatment. Of greater significance, however, is the extensive subunit heterogeneity evident in electrofocusing patterns of the dissociated ferritin. These data contradict the current working models which assume that the heterogeneity of isoferritins stems from different proportions of only two different subunit types (Drysdale, 1977). In comparison to isoelectric focusing of the subunits, which resolves additional components that may be distinguished on the basis of subtle charge or mobility differences (Figure 2A), electrophoretic mobilities on sodium dodecyl sulfate gels, which resolve polypeptides on the basis of size, and electrophoresis in acid–urea gels, which combine size and charge properties to resolve the subunits, reveal only two different components (Figure 2B). It appears that resolution in the acid–urea system depends primarily on polypeptide size differences even in high porosity gels (4–5%).

In spite of the precautions taken to prevent carbamylation in urea (Experimental Procedures), dissociation or prolonged incubations in urea remain suspect. Ferritin was, therefore, also converted into subunits by heating in sodium dodecyl sulfate as an independent means to dissociate the protein. The Na-DodSO$_4$ containing subunits are amenable to electrofocusing procedures in the presence of urea and the nonionic detergent nonidet P-40 (NP-40) (Ames and Nikaido, 1976). Samples treated in this way exhibited the same pattern of heterogeneity as those dissociated in acid–urea solution, demonstrating that the multiple components were not generated by artifacts produced by dissociating the protein in urea.

Subunit Patterns. Two-dimensional gel electrophoretic methods were applied to this ferritin system for a more scru-

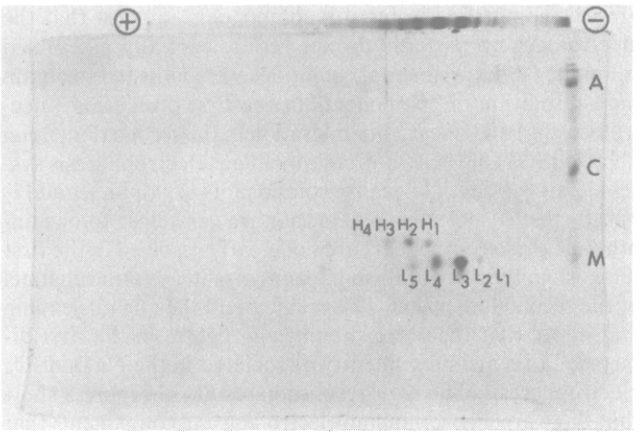

FIGURE 3: Two-dimensional electrophoresis of human liver ferritin. Electrofocusing in the first dimension was under the same conditions of gel 2 in Figure 2A. A stained electrofocusing gel run in tandem with the sample applied to the second dimension is superimposed on the top of the figure. The protein in this procedure was treated to remove iron, reduced, and alkylated according to the procedures described under Experimental Procedures. The second-dimension slab gel was 8.5% acrylamide and the 0.1% sodium dodecyl sulfate system described in the text. Components designated as H had mobilities corresponding to a molecular weight of about 22 000, and bands designated L migrated as 19 000 molecular weight components. The molecular weight standards shown are (A) bovine albumin (67 000), (C) carboxypeptidase (34 000), and (M) myoglobin (17 000). Densitometric tracings of the stained gel gave the following proportions of subunits: L_3, 0.37; L_4, 0.24; H_2, 0.13; H_1, 0.10; L_5, 0.05; L_2, 0.04; H_3, 0.03; H_4, 0.02; L_1, 0.02.

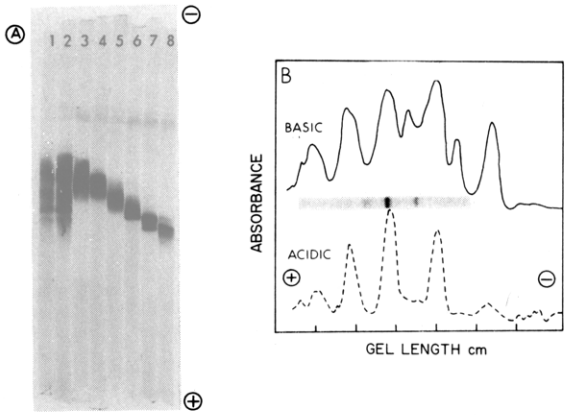

FIGURE 4: Isoelectric focusing of components purified by preparative electrofocusing procedures. The gels shown in A were undissociated components, and gels 1 and 2 are two preparations of human liver ferritin. Gels 3–8 are components purified by preparative flat-bed electrofocusing procedures described under Experimental Procedures. The patterns shown in part B of the figure are densitometric scans of subunit electrofocusing gels of the most basic isoferritin (component shown in gel 3 of part A) and the most acid component (gel 8 of part A). Proteins were denatured in acid–urea solution under conditions described in the text and examined by electrofocusing in urea according to methods in Figure 2. Components L_1, L_2, and H_1, are missing in the most acidic isoferritin.

pulous inspection of the urea-electrofocusing subunits. Human liver ferritin was dissociated into subunits by the acid–urea method described above, the pH was readjusted to 7.5, iron was removed by centrifugation, and the protein was reduced with dithiothreitol and alkylated with iodoacetamide (cf., Experimental Procedures for details of these methods). These precautions were taken to minimize potential complications that could arise from random formation of disulfide bridged structures. Almost identical patterns were obtained with samples that were not reduced and alkylated.

Disc gel isoelectric focusing in 8 M urea (plus 2% NP-40 detergent) was carried out in the first dimension, and these gels were applied to a sodium dodecyl sulfate–slab gel in the second dimension. Results of this analysis are shown in Figure 3. Four components with electrophoretic mobilities corresponding to molecular weights of 22 000 and five components of 19 000 molecular weight were resolved in this procedure. The 22 000 molecular weight peptides have been designated as H (H_1, H_2, H_3, H_4) components and the 19 000 molecular weight components as L (L_1, L_2, L_3, L_4, L_5) to conform with the nomenclature proposed by Drysdale and co-workers (1977). The most acidic 19 000 molecular weight component (L_5) is more acidic than the most basic of the 22 000 molecular weight components (H_1). Subunit L_4 also has a p*I* very close to that of H_1.

If nonidet-P-40 is omitted from the procedure or if the acid–urea dissociation process is not carried to completion, various aggregates could be observed in the electrofocusing system. The patterns in Figure 3, however, show no overlap of components due to aggregation in urea and, in all likelihood, are monomeric subunits.

The relative proportions of the different subunit types were estimated from densitometric scans of the two-dimensional gels and of the urea focusing gels, and the data are summarized in the figure. Components L_3, L_4, H_1, and H_2 account for over 80% of the total subunit population.

Subunit Composition of Purified Isoferritins. To study subunit composition of the assembled protein, individual isoferritins were purified by preparative flat-bed isoelectric focusing procedures (cf. Experimental Procedures). The limited heterogeneity of each component as compared with the native protein is evident from the analytical gel electrofocusing of the fractionated isoferritins shown in Figure 4A. Individual isoferritins were degraded into subunits by acid–urea treatments described above and exposed to urea–gel electrofocusing procedures. A gradation in population of the different H and L subunit types was evident among the isoferritins. Subunit electrofocusing gels of the purified isoferritins and the relative amounts of each subunit type for most acidic and most basic isoferritin fractions are shown in Figure 4B.

Subunit Distribution and Iron Content. Ferritin was fractionated on the basis of iron content by density gradient centrifugation methods (Niitsu and Listowsky, 1973). Subunits of the low-density apoferritin fraction, a low iron content fraction, and a high-density iron-loaded fraction of ferritin were compared by electrofocusing methods (Figure 5). With assembled molecules in nondenaturing media, apoferritin is enriched in the most basic isoferritin moieties, and the iron-loaded molecules are devoid of the most basic constituents and are enriched in the more acidic components (see gel scan in Figure 5A). Subunit gel electrofocusing patterns shown in Figure 5B demonstrate a direct correlation between iron content and subunit distribution among the isoferritins. Thus, the iron-loaded molecules are completely devoid of the most basic L type subunits and enriched in L_4 and H_2 subunits, whereas apoferritin is enriched in the more basic subunits.

Discussion

Models of ferritin subunit structure have evolved from considerations of a single subunit type (Hofmann and Harrison, 1963), to a two subunit system (Drysdale et al., 1977), and finally to the multiple subunits described in the present study. It is likely that subunit heterogeneity accounts for the microheterogeneity of assembled ferritin molecules, which in turn has a direct relationship to tissue and species specificity and variations observed in developing and neoplastic cells (Alpert et al., 1973; Linder et al., 1975; Munro et al., 1975; Richter

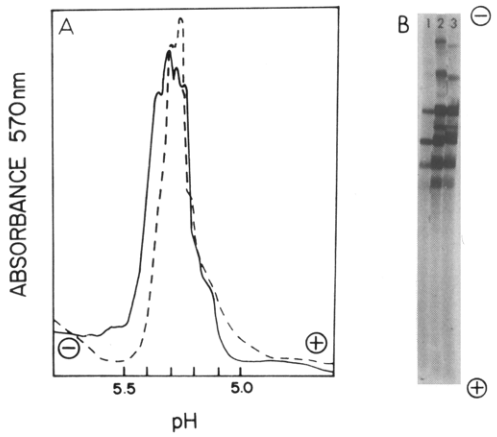

FIGURE 5: Subunit distribution and iron content of ferritin. (A) Assembled components. Densitometric scans of electrofocusing gels of high iron content ferritin component (dotted line) and unfractionated human liver ferritin (solid line). These are assembled components. Note that the most basic component (presumably apoferritin) is missing in the high iron content ferritin. (B) Subunit gels. Gel 1 is a high iron content (about 2500 Fe atoms/mol of protein) fraction obtained by density gradient centrifugation of human liver ferritin. Gel 2 is natural apoferritin and gel 3 is a low iron content fraction (less than 200 Fe atoms/mol of protein) obtained from the density gradient. The protein was dissociated in 9 M urea and pH 2.5, and electrofocusing conditions for subunits in urea were the same as those described in Figures 2–4.

and Lee, 1976; Bomford et al., 1977). The data presented here may be interpreted in terms of subclasses or modified subunit types that stem from two main polypeptide chains (H and L subunits).

In any study that proposes the existence of multiple forms of a single protein it is important to minimize methodological artifacts that may potentially generate additional electrophoretic components. The ferritin system, in particular, has a number of inherent problems associated with it. For example, it has been suggested that carrier ampholytes tend to distribute in a stepwise gradient near pH 5 and, thus, could generate some of the sharp electrofocusing bands observed with ferritin and other proteins (Russell et al., 1978). The current observations which establish direct correlations between subunit banding patterns and other factors, such as iron content, or microheterogeneity of assembled shells of purified isoferritins, appear to preclude spurious phenomena associated with gel-electrofocusing procedures.

Assembled ferritin molecules are exceptionally stable even in the presence of denaturants (Listowsky et al., 1972). It is therefore essential to ensure that dissociation into subunits is complete, to rule out the possibility that the multiple electrofocusing components represent different combinations of partially aggregated subunits. The evidence presented in this study is contrary to any involvement of incomplete dissociation or partial reassembly of subunits, for the following reasons: (1) Circular dichroic data show that after treatment in acid–urea solution the protein remains in a random-coil structure in urea. Once the energy barrier for the conversion of native to denatured ferritin is overcome at the low pH, the denatured or unfolded state of the protein is sustained by the urea solution. It is unlikely that a completely unfolded protein would retain the proper geometry for intersubunit recognition or interactions to occur. (2) Samples dissociated by heating in $NaDodSO_4$, a totally different denaturing system, exhibit the same subunit electrophoretic patterns as those treated in acid–urea solution. (3) Reproducible differences in the subunit distribution between iron-free apoferritin, a component containing little iron, and the iron-loaded ferritin fractions, as well as differences among the purified isoferritin components, suggest that the electrofocusing patterns do not reflect partially assembled subunits. (4) Experimental conditions were adjusted to obtain high-resolution of components in the first dimension (electrofocusing) and linearity of migration in the second dimension ($NaDodSO_4$) of the two-dimensional gel electrophoresis system. This is essential because components H_1 and L_4, and H_2 and L_5, have isoelectric points that are very close to one another. If these components are not clearly resolved in the first dimension, they would form linear arrays in the same channel of the second dimension. This result could then be misleading and imply that there are subunit aggregates in the first dimension that are subsequently dissociated in the $NaDodSO_4$ electrophoresis. The high-resolution results in Figure 3 show that there are no overlapping electrofocusing components that contain both H and L type subunits. If subunit aggregates or partially assembled molecules are invoked to explain the multiple electrofocusing components, there is no a priori reason to assume that heterologous aggregates (H with L) would be precluded. On the basis of these results, it appears to be indisputable that the subclasses of the H and L subunits are monomeric units.

The use of urea to dissociate the protein into subunits requires the consideration of possible carbamylation reactions involving isocyanate contaminations occasionally found in urea solution (Stark et al., 1960). Some precautions taken to prevent such carbamylation reactions were outlined under Experimental Procedures above. In addition, the results obtained appear to preclude fortuitous protein carbamylation reactions for the following reasons: (1) Electrofocusing results were reproducible in a series of experiments for a given ferritin preparation. (2) Ferritins dissociated in $NaDodSO_4$ exhibited subunit patterns that were identical to those obtained with acid–urea dissociated proteins. (3) Native isoferritins are all more acidic than their corresponding subunits dissociated after treatment with urea (Figure 2). If carbamylation occurred during dissociation in urea, urea-treated components would be expected to be more acidic than the untreated or assembled ferritins. (4) Isoferritin components that were obtained by fractionation of ferritin in the absence of urea (Figure 4) showed a discrete gradation in the population of subgroups of the H and L type subunits. (5) Ferritins from different human tissues have different populations of subclasses of the subunits (Lavoie and Listowsky, unpublished results). (6) If random carbamylation reactions occurred, a pattern consisting of two major unmodified components (H and L) and a series of more acidic carbamylated components would be observed. The observed pattern, with two components more basic than the preponderant L_3 component and one component more basic than the major H_2 component, is incompatible with random carbamylation reactions, since the unmodified or most basic subunit would be the expected major component. Individually, the above factors are not conclusive, but collectively they are compelling grounds to rule out carbamylation as a determinant of the subclasses of ferritin subunits.

Subunit electrofocusing of reduced and alkylated ferritin indicates that the subunit microheterogeneity is not attributed to intrachain (or interchain) disulfide bridges or differences in the disposition of cysteine residues. In earlier studies it was shown that ferritins are susceptible to specific proteolytic cleavage (Lavoie et al., 1977) that may occur at a site between two helical domains in the protein (Banyard et al., 1978). Incorporation of protease inhibitors such as phenylmethanesulfonyl fluoride in the preparation, however, did not alter subunit patterns, but small peptide differences due to proteolytic processing have not been ruled out in this work. Studies

on iron-free apoferritin exclude the influence of iron or iron-catalyzed reactions on observed subunit patterns. Prevention of other adventitious occurrences that could give rise to subunit heterogeneity are discussed under Experimental Procedures.

In spite of extensive homologies in primary structure (Lavoie, unpublished observation; Drysdale, personal communication), the possibility that the multiple subunits represent different gene products has not been ruled out. Furthermore, any of a large number of posttranslational modifications of ferritin subunits could account for the electrophoretic subclasses. The exact nature and origin of these structural differences are presently obscure, but it is clear that they account for microheterogeneity of assembled ferritin molecules. The subtle structural modifications of subunits that generate subunit subclasses of both the L and H classes may have no appreciable affect on the overall three-dimensional structure of the protein and may not be detected in crystallographic studies on ferritin (Harrison, 1963, Banyard et al., 1978). Furthermore, it is difficult to rationalize the observed microheterogeneity of assembled ferritins on the basis of the two subunit (H and L) models alone. Thus, horse spleen ferritin exhibits a characteristic pattern of microheterogeneity (Ishitani et al., 1975a), in spite of the fact that it is almost exclusively composed of the L type subunit (Collet-Cassart and Crichton, 1976). A systematic interrelationship between individual purified "isoferritins" and populations of different subunit classes may be derived from the data in Figure 4. In comparative studies, it would not suffice to compare proportions of H and L subunits, to distinguish between isoferritins. It is essential to establish the distribution of subclasses of H and L, to properly characterize a ferritin preparation.

The number of different subunit types found and their electrophoretic properties were consistent for several different human livers. Relative populations of H and L type subunits do vary among different human liver ferritin preparations. Preliminary evidence shows that differing amounts of certain ferritin subunit types may be related to the iron load or metabolic state of individuals (Niitsu, Otsuka, and Listowsky, unpublished observations), and the subunit population may, thus, impart important functional properties to the protein. The data in Figure 5 explicitly indicate that subunit distributions are contingent upon the iron content of individual ferritins. The gel electrofocusing of subunits, thus, defines discrete pattern changes that are related to the iron load of the protein. These differences were not readily discerned on the basis of Na-DodSO_4 or acid–urea gel electrophoretic methods which only show two subunit bands. Furthermore, the subunit distribution in iron-loaded components is similar to that of the purified acidic isoferritin, and the iron deficient components have subunit patterns similar to those of basic isoferritins. These results are consistent with earlier observations that natural apoferritin of human liver consists primarily of the most basic components (Drysdale et al., 1977; Lavoie et al., 1977).

The general mechanisms by which ferritin subunits are processed and structural relationships between the multiple subunit types are presently unknown. It is also not clear if individual subunit types undergo specific interconversion to one another as the metabolic state of the protein is modulated. The data presented here show that iron-containing molecules probably undergo structural changes attendant to, or after, iron loading. It is possible that the structural modifications are required to predispose the molecule or facilitate the iron-sequestering process or, conversely, that the structural changes may occur as a consequence of the iron-sequestering process; iron loading may be facilitated by specific subunit populations. In this context, it is noteworthy that iron replenishment occurs subsequent to protein synthesis, and iron-saturated protein molecules usually have been present in the cell for longer periods of time than apoferritin (Drysdale, 1977). Structural modifications resulting in the disappearance of specific subunit types could occur during this time. In addition, the intracellular localization of the protein may influence processing such as glycosylation and other reactions.

In contrast to the continuous distribution of assembled components (see Figures 4A and 5A, for example), the subunit patterns are characterized by a discrete periodicity in p*I* values of about 0.1–0.15 pH unit. In preliminary studies, we have observed extensive homologies in proteolytic patterns of the different subunits. It is, therefore, conceivable that a simple charge-altering phenomenon, such as deamidation of selective glutamine or asparagine residues, could account for the different electrophoretic components. These charge modifications attend iron loading, influence the secondary structure of the protein (Listowsky, unpublished observation), and, in all likelihood, account for the well-defined microheterogeneity of assembled ferritins.

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