Novel Properties of L-type Polypeptide Subunits in Mouse Ferritin Molecules*

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Properties of the L- and H-type polypeptide subunits forming ferritin 24-mer molecules in mice were investigated, using the products of in vitro transcription and translation from the two cloned genes, and recombinant ferritin molecules (H$_{24}$L$_0$ or H$_{0}$L$_{24}$) produced by transformation in Escherichia coli. Several different conditions for analytical electrophoresis reproducibly show that the relative migration position of the two mouse ferritin subunits is reversed from that reported for ferritin H- and L-subunits in all other mammals; since mouse and human H-polypeptides almost co-migrate, this unusual relative mobility is due largely to novel properties of the murine L-subunit.

This unusual electrophoretic property of the mouse L-subunit has led to conflicting reports about the subunit composition of natural mouse ferritin. Here, we show that the single major electrophoretic band given by liver ferritin purified from mice having a short-term iron overload matches that produced by the genetically defined L-polypeptide and that some bona fide H-subunits are also detected.

In conclusion, it is reasonable to assume that, when mouse ferritin samples will be analyzed under the same conditions as those described here, the slower species will correspond to the L-type subunit. However, when dealing with ferritin from species other than human or mouse, it should be kept in mind that upon electrophoretic analysis of ferritin polypeptide, the designation of an electrophoretic band as being H- or L-type subunits will be very uncertain without corroborative data from genetic, immunological, or amino acid sequencing.

Regulated gene expression of several different highly specialized proteins enables eukaryotic cells to achieve homeostasis of their iron content and iron metabolism (1). The metalloprotein, ferritin, is the major binder of non-heme iron within the cytoplasm of cells in humans, other vertebrates, invertebrates, fungi, and plants, as well as many different bacteria (see Refs. 2-5 for reviews). The ability of ferritin to keep unused intracellular iron in a soluble and relatively safe form balances the essential cellular requirement for this metal with the potential danger of its producing oxidative stress by catalyzing the formation of oxygen free radicals.

Each ferritin molecule in mammals can bind up to several thousand atoms of ferric iron as microcrystallites of the biomimetic, ferrihydrite (2, 3). The special quaternary structure of this large metalloprotein (diameter = ~130 Å (13 nm)) determines that the bound iron is surrounded by an outer shell made from 24 associated polypeptide subunits (2-5). The two kinds of ferritin subunits in mammals, H (heavy)-type (molecular mass = ~21 kDa) and L (light)-type polypeptides (molecular mass = ~19 kDa), have over 50% amino acid sequence homology (see Refs. 4-6 for reviews). Both subunits fold into similar globular units at the secondary and tertiary levels of protein structure (2) and readily co-assemble in various proportions to form a family of mostly heteropolymeric molecules (e.g. H$_{12}$L$_2$, H$_{14}$L$_{10}$, H$_{5}$L$_{19}$, etc.) (7). Although these two subunits share many structural features, they now are known to have some different functional properties (e.g. see Refs. 8 and 9).

The two major kinds of polypeptide subunits in mammalian ferritin and apoferritin molecules have been defined rigorously at the levels of amino acid sequence, nucleotide sequence of their genes or cDNAs, and reactivity with subunit-specific monoclonal antibodies (6, 10, 11). However, it is a very common practice with electrophoretic analysis of ferritin to designate the more anodal band in the pair of resolved polypeptides as L-type subunits, and the more cathodal band as H-type subunits (7, 10). Many current research studies are using SDS-PAGE$^*$ analysis of ferritin expression in mouse cells to investigate eukaryotic gene regulation and cellular iron physiology. Although the products of in vitro transcription and translation from cDNAs for mouse ferritin H- and L-polypeptides revealed that their electrophoretic migration positions were the opposite of those reported for all other mammalian species (12), the bands of murine ferritin subunits resolved with SDS-PAGE continue to be misidentified in many current studies. This necessarily has resulted in some mistaken and misleading conclusions.

Genes and cDNAs encoding both types of mouse ferritin subunits have been cloned and sequenced in several laboratories (12-16). To further analyze the properties of bona fide murine ferritin H- and L-type subunits, the present study has used polypeptides encoded by these cDNAs; the genetically defined polypeptides also have been compared directly to the natural subunits of purified mouse liver ferritin. Our results show that migration of the mouse L-polypeptide is reproducibly slower than that of the mouse H-polypeptide under several different conditions for electrophoresis; the observed inversion of the usual relative positions for ferritin H- and L-subunits

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¶ The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
with SDS-PAGE is caused by the anomalous properties of the murine L-subunit. Thus, it is risky to assign a given SDS-PAGE band to ferritin H- or L-type subunits in other species without having a confirmation from either genetic, immunological, or amino acid sequence data.

**MATERIALS AND METHODS**

Proteins—Liver ferritin from inbred mice with a short-term parenteral siderosis was purified as described previously (17, 18). Anti-mouse ferritin polyclonal antiserum was produced in rabbits by conventional immunization protocols as published previously (19); the antiserum was processed to remove any antibodies reacting or cross-reacting with proteins in murine serum by exposure to pooled normal mouse serum covalently coupled to solid phase beads. Rabbit anti-human ferritin polyclonal antiserum was purchased from Dako. Polypeptide standards with known mass were purchased from Sigma and Amersham Corp.

Cell-free Transcription and Translation from Mouse H- and L-ferritin cDNAs—Mouse ferritin H- and L-type subunits were synthesized in vitro as described previously (12). Briefly, sense mRNA was produced from CDNA clones for mouse L-subunits (pMLF27) (12) or H-subunits (pMHF) (13). Large amounts of transcripts were obtained using Sp6 or T7 polymerase, and these mRNAs were translated in vitro with a reticulocyte lysate kit (Promega) in the presence of [35S]methionine (400 Ci/mM). The polypeptide products were analyzed directly by SDS-PAGE (see below).

Metabolic Labeling and Immunoprecipitation of Ferritin— Cultured mouse erythroblasts or mouse lymphoblastoid cells established from peripheral blood lymphocytes after Epstein-Barr virus transformation (20) were harvested (10^8 cells), washed twice, resuspended in 5 ml of methionine-free minimal essential medium supplemented with 10% fetal calf serum, and incubated for 2 h at 37°C. 75 μCi of [35S]methionine were added to each culture, followed by further incubation for 2 h; the cells were then washed twice in ice-cold phosphate-buffered saline, followed by lysis with three freeze-thaw cycles in immunoprecipitation buffer (pH 7.4; 50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, and 1% SDS). After centrifugation, the supernatant, or amino acid sequence data.

The first protocol for SDS-PAGE is based upon the classic conditions developed by Laemmli (23) and used a separating slab gel with 12 or 15% polyacrylamide run at constant current (12). A second protocol using 10% polyacrylamide run at constant voltage (24). A third protocol used a separating slab gel containing 20% polyacrylamide and 10% glycerol (13, 14), run at constant current as described by Reiser et al. (24). A fourth protocol used a separating slab gel with an exponential gradient of 15–30% polyacrylamide, run overnight at constant voltage as described previously (25). For bulk samples of natural or recombinant proteins, bands of polypeptides were revealed by autoradiography using direct exposure of the dried slab gel to x-ray film (Kodak type X-AR).

**RESULTS**

Analysis of Ferritin Polypeptide Subunits Synthesized in Vitro from Cloned cDNAs and Produced by Mouse or Human Cells in Culture—When [35S]-labeled ferritin polypeptides immunoprecipitated from either mouse erythroblasts or human lymphoblastoid cells are run on the same SDS-PAGE gel, a pair of labeled bands of ferritin subunits is observed for both species (Fig. 1, lanes 1 and 4). Published studies uniformly indicate that the more anodal band of human ferritin polypeptides corresponds to the L-subunit and the more cathodal band is the H-subunit (e.g. see Refs. 7, 10, and 11). However, when polypeptides generated by consecutive cell-free transcription and translation from the cloned cDNAs encoding mouse L- or H-type ferritin subunits are resolved by analytical SDS-PAGE, the L-type subunit is the slower migrating species (Fig. 1, lanes 5 and 6). This migration pattern has been repeatedly observed when the electrophoretic analyses were performed in any of the three participant laboratories. Direct comparison of the subunits in immunoprecipitates from the cultured cells with those produced in vitro from mouse cDNAs clearly demonstrates that the H-subunits for both species almost co-migrate (Fig. 1, lanes 1, 4, and 5), whereas the mouse L-subunit migrates much less far toward the anode than does its human counterpart. The relative migration positions of the two ferritin subunits thus are reversed in mice as compared to humans.

Analysis of Subunits in Recombinant Mouse Ferritin Molecules—Transformation of E. coli with cDNA encoding either the mouse H- or L-type ferritin subunit results in a large production of stable 24-mer homopolymeric molecules (i.e. H24L0 or H0L24). Each kind of purified recombinant 24-mer ferritin molecule exclusively gives only one major band of polypeptide subunits with SDS-PAGE (Fig. 2), as expected for a homopolymer. Three different systems for analytical electrophoresis all show that the genetically defined mouse L-polypeptide (Fig. 2, lane 3) migrates notably less far toward the anode than does the murine H-polypeptide (Fig. 2, lane 1). Some minor bands also can be resolved and are believed to represent degradation products of the full-length polypeptides. No SDS-PAGE bands are detected at migration positions corresponding to subunit dimers (data not shown).

Comparison of Subunits in Natural Mouse Liver Ferritin to Subunits in Homopolymeric Recombinant Mouse Ferritin—SDS-PAGE analysis of natural ferritin purified from livers of mice with a short-term parenteral iron overload has been described previously as resolving one major band of polypeptide subunits (molecular mass = 22 kDa) and two closely associated minor bands (17). Due to this unusual pattern with three bands, no assignment of any band could be made to the H- or

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**Figure 1. Comparison of mouse and human ferritin polypeptides resolved by SDS-PAGE.** Specific immunoprecipitates of newly synthesized ferritin from human lymphoblastoid cells (lanes 1 and 2; lane 1 has twice the sample amount as lane 2) and from mouse erythroblasts (lanes 4) were resolved on a 12% SDS-PAGE slab. Lane 3 is a blank lane, containing nonradioactive standards for molecular mass. Products of in vitro transcription and translation from plasmids encoding the mouse ferritin H-subunit (lane 5) or L-subunit (lanes 6) were run on a similar gel and aligned with the in vivo synthesized mouse subunits. Respective positions of human (left) and mouse (right) H- and L-subunits are indicated. Anode (+) is below.
L-types of ferritin subunits. Use of genetically defined ferritin polypeptides now shows that the single major and one of the minor bands resolved in this natural ferritin co-migrate with bands coming from the recombinant L-polypeptide (Fig. 3, lanes 1, 4, and 5). When a sufficiently large amount of natural liver ferritin is analyzed, a minor band co-migrating with the major band of recombinant H-polypeptides is revealed (Fig. 3); this indicates that L-rich liver ferritin molecules from mice with siderosis actually do contain some H-type subunits.

**DISCUSSION**

Use of genetically defined polypeptides permits a rigorous identification of the H- and L-type subunits forming ferritin and apoferritin molecules. The present results clearly show that the more cathodal migration position of the mouse ferritin L-subunit is observed reproducibly with several different systems for SDS-PAGE and therefore is not likely to be some technical artifact; moreover, the unusual migration position of the mouse L-polypeptide is observed whether this is synthesized in vitro from cloned genes, in vivo within cultured cells, or within E. coli as recombinant proteins. The pattern of relative migration of the two ferritin subunits in mice is the opposite of that reported for ferritin subunits in all other mammalian species examined to date, where the L-subunit always migrates to the anodal side of the H-subunit (e.g. humans, horses, and rats (7, 10); rabbits (25)). Since mouse H- and human H-subunits almost co-migrate, the reversed relative positions of H- and L-subunits in mice must be due to novel properties of the murine L-polypeptide.

What causes the unusual migration of the mouse ferritin L-subunit is not clear. Amino acid sequence and composition derived from cloned cDNAs for the two mouse ferritin polypeptides (12–15) are quite similar to the consensus for each ferritin subunit in humans and other mammals (5, 6, 10, 26–28); despite this similarity, the mouse L-subunit uniquely has an anomalous migration with SDS-PAGE. Although some differences in content of certain residues are recognized for mouse versus human ferritin L-subunits (Table I), none can be expected to cause the observed change in SDS-PAGE migration since these nonglycosylated polypeptides are reduced and denatured for this analysis. Differences in the primary sequence are expected to influence the SDS-PAGE migration only if the denaturing conditions do not completely remove all elements of secondary structure; such causation for anomalous SDS-PAGE migration of certain polypeptides has been postulated previously for other proteins (e.g. see Ref. 29).

Each ferritin subunit consists of a bundle of four antiparallel α-helices (A to D) with a fifth helix (E) lying at a right angle to the main axis (2). An additional octapeptide is inserted into the turn between the D and E helices of ferritin L-subunits in mice and rats (12, 27) and causes both nominal mass and chain length of the H- and L-polypeptides to be nearly equal (Table I). However, here we demonstrate that the electrophoretic mobility of mouse H- and L-polypeptides are clearly different. This

**FIG. 2.** Identification of polypeptide subunits in recombinant homopolymeric 24-mer mouse ferritin molecules, using three different protocols for SDS-PAGE. Details about analytical electrophoresis are given under “Materials and Methods.” All gels were stained to reveal total protein content. Anode (++) is below. A, SDS-PAGE with separating gel of 15% polyacrylamide, B, SDS-PAGE with separating gel containing 20% polyacrylamide and 10% glycerol. C, SDS-PAGE with separating gel having 15–30% exponential gradient of polyacrylamide. Samples were 24-mer H-homopolymers (lanes 1), a mixture of H-homopolymers and L-homopolymers (lanes 2), and 24-mer L-homopolymers (lanes 3). Relative separation of the two types of ferritin subunits, bandwidth, and resolution of minor bands all vary according to the characteristics of each SDS-PAGE protocol.

**FIG. 3.** Comparison of polypeptide bands resolved by SDS-PAGE in recombinant homopolymeric mouse ferritin molecules, with those in pure natural liver ferritin molecules from mice having iron overload. SDS-PAGE with 15–30% exponential gradient of polyacrylamide, stained to reveal total protein content. Anode (++) is below. Samples are a mixture of pure recombinant H- and L-homopolymers (lane 1), pure mouse liver ferritin (lanes 2 and 4), each from a different gel, molecular mass standards (lane 3), and pure recombinant L-homopolymer (lane 5). The bands shown for the molecular mass standards are: carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa. Positions of H- and L-subunits are indicated together with the H band in pure liver ferritin (arrowheads) and a slower band common to pure liver ferritin and the recombinant L-homopolymer (arrow).

**TABLE I**

Selected characteristics of apoferritin polypeptide subunits from mice, rats and humans, based upon amino acid sequences derived from published reports about cloned genes

|                | Mouse<sup>a</sup> | H<sup>b</sup> | L<sup>b</sup> | Rat<sup>c</sup> | H | L | Human<sup>d</sup> | H | L |
|----------------|-------------------|--------------|--------------|-----------------|---|---|-------------------|---|---|
| No. of amino acids | 181              | 182          | 181          | 182             | 182 | 174          |                  |    |    |
| Inserted octamer   | No               | Yes          | No           | Yes             | No  | No           |                  |    |    |
| Molecular weight (M<sub>r</sub>)  | 21,066          | 20,802       | 20,995       | 20,805          | 21,225  | 20,019       |                  |    |    |
| Mol % of positive charges<sup>e</sup> | 17.0        | 15.8         | 18.2         | 16.4            | 16.4  | 16.6         |                  |    |    |
| Mol % of negative charges<sup>f</sup> | 25.7        | 26.9         | 27.1         | 25.1            | 29.0  | 24.0         |                  |    |    |

<sup>a</sup>See Refs. 12–16.

<sup>b</sup>Subunit type.

<sup>c</sup>See Refs. 27 and 28.

<sup>d</sup>See Ref. 26.

<sup>e</sup>Counted as H, K, and R residues by PEPTIDE-SORT program of the Genetics Computer Group (Madison, WI. 53706).

<sup>f</sup>Counted as D, E, N, and Q residues.
condition is mostly due to the unusual mobility of the L-subunit, which is intermediate between the 20.1- and the 24-kDa molecular mass markers (see Fig. 3) and slower than would be expected from its calculated molecular weight (M_r = 20,802). It is possible that the extra octapeptide, although not altering the three-dimensional structure of the ferritin L-subunit (30), might affect its electrophoretic mobility.

It is difficult to evaluate from the available literature whether the rat L-subunit presents the same unusual electrophoretic mobility. There is a 92% homology between the amino acid sequence for the ferritin L-subunits of rats (27) and mice (12). However, the additional octapeptide specific of rodent L-subunits differs at three positions between rat (QTGVAQAS) and mice (QTgapQGS) and might not alter the electrophoretic properties of each L-polypeptide to the same extent. Therefore it seems worthwhile to reevaluate the electrophoretic properties of the polypeptide products encoded by the cloned genes for rat ferritin subunits.

The subunit composition of natural mouse ferritin molecules has remained quite controversial (17, 31) due to the unusual reversal of the traditional positions for H- and L-subunits with SDS-PAGE. The present analysis of genetically defined polypeptides now demonstrates that most subunits in liver ferritin molecules from mice with parenteral iron overload are L-type polypeptides. Two minor bands originally were resolved with SDS-PAGE of this natural ferritin (17). One of these minor bands (Fig. 3, arrow) migrates to the cathodal side of the single major band and commonly would be identified as an "H-subunit"; our present results clearly show that it corresponds only to the L-subunit. However, bona fide H-type subunits now also have been detected in small amounts within this natural liver ferritin, indicating that these mouse ferritin molecules indeed are at least partly heteropolymeric (e.g. H_2L_23).

An increasing number of research investigations using mouse cells to study gene regulation and iron metabolism are reporting the designation of H- or L-type subunits for SDS-PAGE bands derived from anti-ferritin immunoprecipitates. Based upon our results, many of these published identifications are incorrect and the deduced conclusions therefore are misleading. We hope that the present data will prevent further mistaken identifications from being made.

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Note Added in Proof—We have just become aware that the genetically-defined rat ferritin L-polypeptide has been shown to migrate to the anodal side of the rat H-polypeptide (32). This result indicates that 1) the anomalous relative migration of ferritin L-subunits from mice is not found with those from the other rodent, and 2) the novel migration of the mouse L-subunit is not due simply to the presence of eight additional amino acids (since an octamer peptide also is inserted into rat L-subunits).

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