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Local Conformational Changes in Ferritins with Variable Iron Content

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Abstract—Conformational changes were induced in human spleen ferritin by partial or complete removal of iron, and the immunoreactivity of the ferritin samples with variable iron content was analyzed. We established that a decrease in iron content resulted in bimodal changes in immunoreactivity of the epitopes recognized by the monoclonal antibodies G10 and F11. Immunoreactivity demonstrated a 3-6-fold decrease on lowering iron content from 800 to 40 atoms per protein molecule, followed by a sharp (4-14-fold) increase that was observed when low-iron ferritin was converted to iron-free apoferritin. These bimodal changes suggest the presence of more than two conformational states of ferritin with local alterations of the epitopes recognized by the monoclonal antibodies. The global conformation of ferritin, however, remained essentially unaltered, as demonstrated by ferritin interaction with polyclonal antibodies. Together, the results indicate that local conformational changes in the ferritin protein shell occur on progressive iron removal that results in low-iron and iron-free forms of ferritin. These changes are most clearly seen in apoferritin when compared to low-iron ferritin.

Key words: ferritin, apoferritin, monoclonal antibodies, immunoreactivity, epitope conformation

Ferritin, a protein with M_r of approximately 500 kD, plays a key role in iron homeostasis in a variety of organisms and constitutes a natural reservoir for metabolic iron [1-3]. Ferritins are found in microorganisms, plants, and animals and allow the intracellular iron concentration to be more than 10^{11} as high as the iron solubility. The ferritin molecule constitutes a protein shell that is made of 24 subunits with the 432 symmetry; the shell forms a cavity with the internal diameter of 8 nm [2]. Multiple ferritin isoforms also called isoferritins are formed in various tissues of mammals due to the presence of three types of subunits, the L (light, 19 kD, composed of 174 amino acids), H (heavy, 21 kD, 182 amino acids), and G subunit (glycosylated polypeptide with net molecular weight of 23 kD) [4]. Tissue ferritins are composed of various proportions of the H and L subunits. In contrast, serum ferritin is mostly composed of the two immunologically related L and G subunits. The H and L subunits are encoded in the 11th and 19th chromosomes, respectively, and demonstrate significantly different immunological properties. The G subunit found only in extracellular ferritins seems to result from post-translational modification of the L chain [4, 5].

Iron is stored within the ferritin shell in the form of a mineral ferrohydroxide core. The protein shell is pierced by the eight hydrophilic and six hydrophobic channels that provide access to the external space and confer the protein properties of an open system [6, 7]. It is generally believed that the hydrophilic channels provide uptake and release of iron and other molecules involved in the iron storage and mobilization processes [8-12]. The process of ferritin iron uptake comprises Fe(II) and Fe(III) translocation, Fe(II) oxidation to form Fe(III), and intracapsular mineralization of Fe(III) [2, 3]. The Fe(III) to Fe(II) reduction and subsequent release from the mineral core is a slow and poorly understood process [13-15] that is critical for biosynthesis of iron proteins. The latter proteins are involved in major functions of a living cell, such as respiration, photosynthesis, nitrogen fixation, and cell growth and division [2]. It was recently shown that the rate of the ferritin iron release can be accelerated by local unfolding of structures involved in a cooperative interaction of the ferritin subunits [16]. These data suggest that local unfolding that was either induced or enhanced by unidentified cytoplasmic processes might underlie the mechanism of regulation of iron release *in vivo*.

The iron mobilization from the ferritin mineral core *in vitro* generally involves the reduction of Fe(III)

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deposited in the core and subsequent chelation of extracapsular Fe(II) [10, 13, 17-20]. In the present work, we induced conformational changes within human spleen ferritin by incubating the protein with the chelators, 2,2′-bipyridyl and EDTA, in the presence of thioglycolic acid, a procedure that resulted in complete or partial iron removal. Using mono- and polyclonal antiferritin antibodies, we analyzed the immunoreactivity of ferritin samples differing with regard to the iron contents. The data demonstrate that local conformational changes occur in the ferritin protein shell when the iron content decreased from moderate to low values, and especially when apoferritin was compared with low-iron ferritin.

MATERIALS AND METHODS

Reagents. The following reagents were used in the study: Toyopearl HW-55f (Toyo Soda, Japan); caprylic acid and thioglycolic acid (Fluka, Switzerland); 1,10-phenanthroline, biotin N-hydroxysuccinimide amidocaproate, bovine serum albumin (BSA), streptavidin–horseradish peroxidase conjugate, and *o*-phenylenediamine (Sigma, USA); protein molecular mass standards (Serva, Germany). Other reagents were chemically pure domestically produced products.

Preparation of ferritin. Human spleen ferritin was obtained with the procedure that was previously described [21], with some modifications. The spleen tissue was homogenized in 0.05 M sodium borate buffer, pH 8.5, using one volume of fresh tissue per two volumes of buffer, and centrifuged for 10 min at 4500*g*. The supernatant was heated for 15 min at 68 ± 1°C on a water bath, then cooled to the room temperature, and the sediment comprising thermally unstable proteins was removed by centrifugation for 15 min at 4500*g*. The supernatant was further fractionated using 30 and 60% saturation of ammonium sulfate. The ferritin-comprising fraction obtained after two precipitations with 60% ammonium sulfate was resuspended in a minimal volume of 0.1 M sodium borate, pH 8.5, and loaded on a Toyopearl HW-55f column (2.5 × 100 cm). The fractions comprising ferritin were pooled and reloaded onto the same Toyopearl HW-55f column. After the second chromatographic run, the purity of ferritin estimated by SDS-PAGE was at least 97%.

Ferritin samples with variable iron content. The samples were obtained by three consecutive dialyses. Three milliliters of the ferritin solution (2.6 mg/ml) were dialyzed against 400 ml of 50 mM NaH_2PO_4, pH 3.5, containing 1.5% thioglycolic acid, 5 mM EDTA, and 5 mM 2,2′-bipyridyl. After 10, 20, and 60 min of dialysis, 0.5-ml samples were taken and further dialyzed for 4 h against 50 mM NaH_2PO_4, pH 3.4, containing 1% thioglycolic acid and 5 mM EDTA. Finally, all the samples were dialyzed against 50 mM sodium phosphate buffer, pH 7.4.

Determination of iron content. Iron content was determined by the *o*-phenanthroline method [22]. As calibration standards, water solutions of $FeCl_3$ with concentrations varying from 0-20 μg/ml were used. To 1 ml of $FeCl_3$ standard solution or a ferritin solution in water, 0.5 ml of 1% $KMnO_4$ in 0.6 M HCl were added, followed by incubation for 2 h at 50°C. Then 0.1 ml of saturated solution of 1,10-phenanthroline containing 1 M $NH_2OH{\cdot}HCl$ and 4 M ammonium acetate was added. The mixture was agitated and incubated for 30 min at 50°C. The samples were brought to room temperature, and optical density at 510 nm was measured. The number of iron atoms per ferritin molecule in samples was determined using the equation:

$$N = Q_f/Q_p \cdot 8035.7,$$

where N is the iron content expressed as the number of atoms per protein molecule, Q_f is iron concentration (μg/ml) determined in the assay, Q_p is protein concentration (μg/ml), and 8035.7 is the ratio of molecular mass of ferritin and atomic weight of iron.

Antibodies. Mouse monoclonal antibodies G10, F11 (IgG2a subclass), and C5 (IgG1) directed to human spleen ferritin were obtained by fusing immune splenocytes of mouse BALB/c line with mouse myeloma cells P3X63Ag 8.6.5.3, as previously described [23]. The antibodies were purified from ascitic fluids using consecutive precipitation with caprylic acid and ammonium sulfate [24]. The antibody preparations were at least 97% pure, as determined by SDS-PAGE in the presence of 2-mercaptoethanol, and comprised the two band of the immunoglobulin heavy and light chains with molecular weight of about 25 and 50 kD.

Preparation of biotinylated ferritin and monoclonal antibodies. To 1 ml of a ferritin (1.2 mg/ml) or monoclonal antibody (2 mg/ml) solution in 0.05 M sodium borate buffer, pH 8.5, 0.36 mg of biotin N-hydroxysuccinimide amidocaproate dissolved in 0.12 ml of dimethyl formamide was added. After 4 h at room temperature, the mixture was dialyzed against 0.1 M sodium phosphate, pH 7.4. To the solution of a biotinylated protein, glycerol was added to a final concentration of 50%, and the protein was stored at –20°C.

Enzyme immunoassay. Binding of the G10, F11, and C5 antibodies to ferritin was studied with a two-site immunoassay. Three micrograms of each antibody were adsorbed onto the inner surface of polystyrene tubes by overnight incubation in 0.25 ml of sodium borate, pH 8.5. The tubes were washed with distilled water, and remaining binding sites onto the polystyrene surface were blocked by 1-h incubation with 0.25 ml of a solution containing 1% bovine serum albumin (BSA) and 0.1 M sodium phosphate, pH 7.4 (PBS-BSA). After washing of the tubes, increasing amounts of ferritin were added to each tube. After another 1.5 h and washing, 250 ng of the

biotin–antibody conjugate were added. The tubes were incubated for 1.5 h and washed, followed by addition of 250 ng horseradish peroxidase–streptavidin conjugate and incubation for 30 min. The enzyme activity was determined by addition of 0.6 ml of the substrate solution containing 0.02 M *o*-phenylenediamine and 0.02 M H_2O_2 in 1% sodium citrate, pH 5.0. After 10 min, the reaction was stopped by adding 0.6 ml of 10% sulfuric acid, and extinction was measured at 492 nm.

Binding of ferritin to rabbit polyclonal antibodies was studied by a competition assay. Polyclonal antibodies (0.25 ml, 12 μg/ml in 0.05 M sodium borate, pH 8.5) were introduced into polystyrene tubes, incubated overnight, and washed with distilled water, followed by 1-h incubation with 0.25 ml of PBS-BSA. After washing with distilled water, 25 ng of the ferritin–biotin conjugate was added together with increasing amounts of ferritin in 0.25 ml of PBS-BSA. Following 2.5-h incubation and two washings, 250 ng of streptavidin conjugated to horseradish peroxidase was added into each tube, and after 40 min the peroxidase activity was determined as described above.

Binding of recombinant L apoferritin and human spleen ferritin to monoclonal antibodies was analyzed in a two-site immunoassay using polystyrene plates. For adsorption, 2 μg of monoclonal antibody G10 or C5 were incubated overnight in the wells of plates in 0.2 ml of 0.05 M sodium borate, pH 8.5. The wells were washed and incubated for 1 h with 0.2 ml PBS-BSA solution. After two washes, increasing amounts (from 0-50 ng) of ferritin in 0.05 ml and 0.15 ml PBS-BSA were placed into wells, and plates were incubated for 1.5 h with subsequent two washes. The conjugate of biotin with the antibody G10 or C5 (200 ng) was added to each well in 0.2 ml of PBS-BSA, and after 1.5-h incubation and washing, 200 ng of streptavidin–horseradish peroxidase was added in 0.2 ml PBS-BSA. After another 1-h incubation and washing, 0.2 ml of the solution comprising 0.02 M *o*-phenylenediamine and 0.02 M H_2O_2 in 0.1 M sodium citrate buffer, pH 5.0, was added for 5-8 min, then the reaction was stopped by addition of 0.05 ml 10% sulfuric acid the enzyme activity was determined as above.

All the binding assays were performed at room temperature in triplicates. For a graphic presentation, average mean values were employed. Association constants, K_a, were determined from double reciprocal plots. Variations of the K_a measurements were within 15%.

Other methods. The purity of the proteins was estimated by SDS-PAGE in 5% stacking gel and 17% separation gel with the buffer system described by Laemmli [25]. Protein samples were reduced with 2% 2-mercaptoethanol for 15 min in a boiling water bath. The gels were stained with Coomassie R-250. The concentration of ferritin was determined by Lowry assay [26] with bovine serum albumin employed as a standard. The antibody concentration was determined spectrophotometrically from the extinction coefficient $A^{1\%}_{1cm,280}$ of 16.2.

RESULTS

In the present work, we obtained and analyzed five samples of human spleen ferritin with different iron content. The stock sample, native spleen holoferritin, contained some 800 iron atoms that were retained by the protein after the completion of the isolation procedure. Other samples with reduced iron content were obtained by iron chelation with 2,2′-bipyridyl and EDTA in the presence of thioglycolic acid and contained 200, 50, 40, and less than 2 (apoferritin) iron atoms per protein molecule. We analyzed the binding of ferritin to rabbit polyclonal and the three monoclonal antibodies, G10 and F11 (mouse IgG2a subclass) and C5 (mouse IgG1). According to our previous data [27, 28], all these monoclonal antibodies recognize conformational epitopes on ferritin.

Ferritin binding to polyclonal antibodies. Binding of holo- and apoferritin to rabbit polyclonal antibodies were analyzed in a competition assay. The design of the assay avoided conformational changes of ferritin that would be induced by the direct adsorption onto polystyrene and/or chemical modification of ferritin. In our assay, the ferritin samples competed with biotinylated ferritin for binding to immobilized antibodies (Fig. 1). We employed biotinylated holoferritin as a label taking into account our previous observation that biotinylation does not alter the immunoreactivity of ferritin [23]. The binding constants were found to be $1.0 \cdot 10^9$ and $1.3 \cdot 10^9$ M^{-1}, respectively, for apoferritin and holoferritin containing 800 iron atoms per

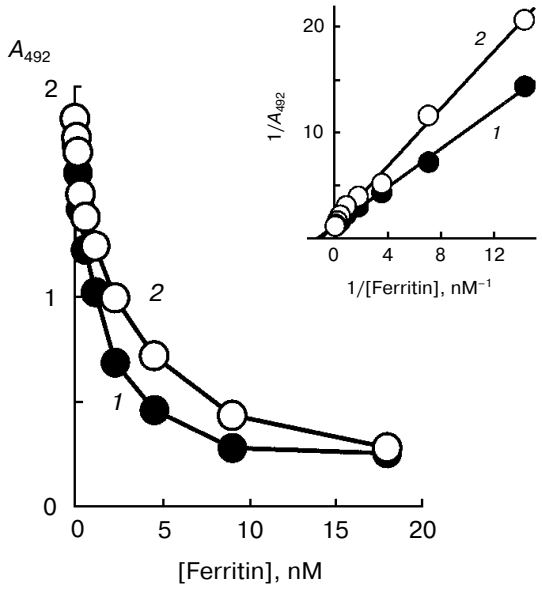

Fig. 1. Competition analysis for binding of human spleen holoferritin (*1*) and apoferritin (*2*) with rabbit polyclonal antibodies. Insert: determination of binding constants for ferritin (*1*) and apoferritin (*2*) from double reciprocal plot.

molecule (Fig. 1, insert). These results obtained with polyclonal antibodies demonstrate that the epitopes of apoferritin remain essentially unaltered, thus suggesting the lack of significant alterations in global conformation of the two forms of ferritin.

Ferritin binding to monoclonal antibodies F11, G10, and C5. The binding affinity of holoferritin and the ferritin samples with the complete or partial removal of iron was studied by a two-site "sandwich" assay that is applicable due to the multisubunit nature and the presence on the ferritin molecule of repetitive epitopes for each monoclonal antibody. In this assay, ferritin interacted with both the polystyrene-adsorbed "capture" antibody and soluble biotinylated antibody. The interaction was quantified using streptavidin–horseradish peroxidase conjugate.

Interaction with the F11 antibody. The association constant, K_a, for the F11 antibody binding to holoferritin comprising 800 iron atoms per protein molecule was $2.3 \cdot 10^9$ M^{-1}. After the partial removal of iron down to 200-40 atoms per ferritin molecule, the association constants were from 2.8-4.5 times lower and constituted $(0.5\text{-}0.8) \cdot 10^9$ M^{-1}. Following the complete removal of iron, K_a increased to $3.2 \cdot 10^9$ M^{-1}. It is noteworthy that the association constant for apoferritin is only slightly (1.5-fold) higher than the holoferritin constant; however, the K_a value for iron-free apoferritin is 4.0-6.4 times higher than the constants observed for ferritins with residual iron (Fig. 2, right ordinate axis).

Thus, the F11 monoclonal antibody demonstrates only small conformational changes in the F11-reactive epitopes of holoferritin versus apoferritin. These apparently small changes, however, resulted from the biphasic changes that we observed for the ferritin samples with the variable iron content. In the first phase, we observed a decrease in the affinity of the F11 antibody binding to ferritins with lowering iron content, with the maximal changes found for the ferritin samples containing 40-50 iron atoms per molecule. After subsequent complete removal of iron in the second phase resulting in apoferritin, a significant enhancement of binding affinity occurred. These data suggest significant conformational changes that occurred in the ferritin molecule on the progressive iron removal from holoferritin and eventual formation of apoferritin.

Binding to the G10 antibody. The constant of the G10 antibody bonding to holoferritin was $3.4 \cdot 10^{10}$ M^{-1}. The progressive iron removal down to 200-40 atoms per molecule resulted in a 3.8-5.7-fold decrease in the binding constants that were as low as $(0.6\text{-}0.9) \cdot 10^{10}$ M^{-1}. On the complete removal of iron, we observed a 2.5-fold increase in the binding constants versus that of holoferritin and a 9-14-fold increase versus the ferritin samples with residual iron (Fig. 2, left ordinate).

From these data, we conclude that, in comparison with the F11-reactive epitope, the G10-reactive epitope possesses one order of magnitude higher affinity toward both ferritin and apoferritin. Furthermore, the G10-reactive epitope is less conformationally stable and more sensitive to changes induced by the iron removal and generation of apoferritin.

Binding to the C5 antibody. The association constants for the C5 antibody binding to holo- and apoferritin measured in the two-site assay were close, with the values being $3.8 \cdot 10^9$ and $4.6 \cdot 10^9$ M^{-1}, respectively (Fig. 3).

Together, probing with monoclonal antibodies suggests that ferritin epitopes demonstrate variable stability toward the removal of iron. The G10-reactive epitopes demonstrate the least conformational stability, and the G10 antibody therefore distinguishes between the conformational states of ferritin with moderate iron content (some 800 atoms per molecule), low content (40-50 atoms), and iron-free state of apoferritin. It is worth note that the G10 antibody provides the most reliable distinction of the conformational state of ferritin with moderate iron content and apoferritin. The F11-reactive epitopes possess different conformation in ferritins with moderate and low iron content as well as in low-iron ferritin and apoferritin; however, these epitopes adopt virtually the same conformation in apoferritin and ferritin with moderate iron content. In this context, the C5-reactive epitopes seem to have the most conformational stability and cannot be distinguished in apoferritin and ferritin with moderate iron content.

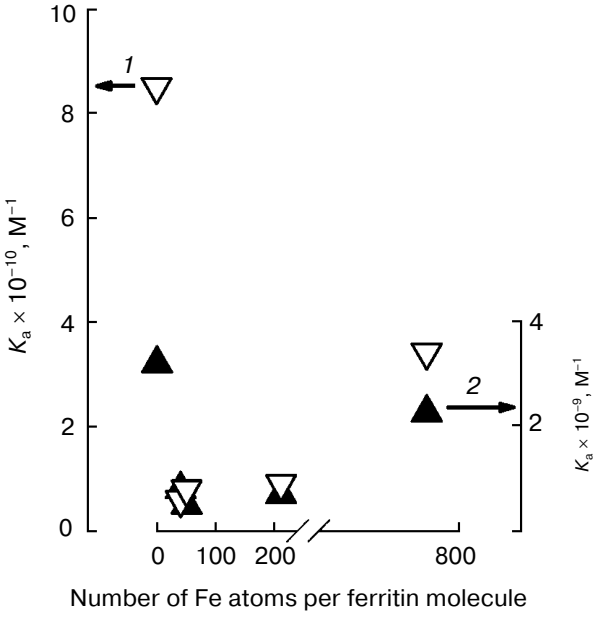

Fig. 2. Two-site assay for binding of ferritins with variable amount of iron to mouse monoclonal antibodies G10 (*1*) and F11 (*2*). Ferritin samples were allowed to react first with polystyrene-adsorbed "capture" antibody, then with biotinylated antibody. The interaction was estimated with the streptavidin–horseradish peroxidase conjugate.

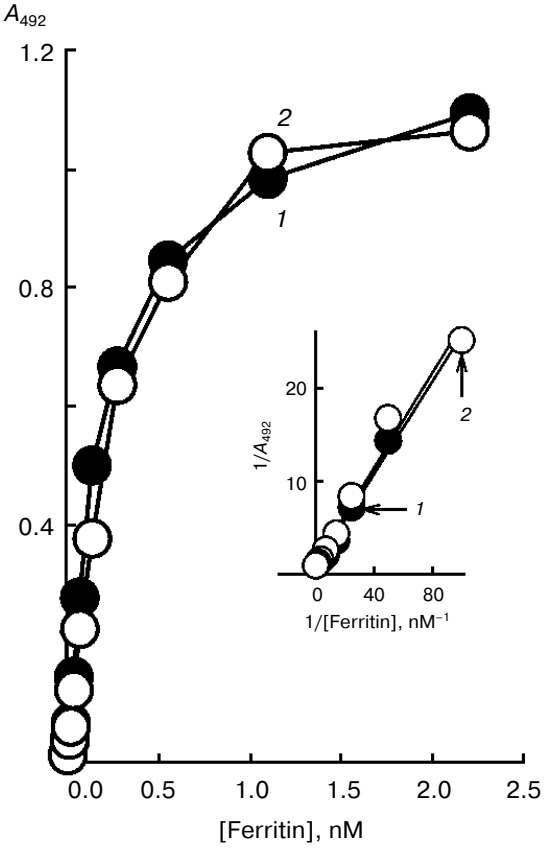

Fig. 3. Two-site assay for binding of holo- (*1*) and apoferritin (*2*) to mouse monoclonal antibody C5. For the design of the assay, see legend to Fig. 2. Insert: double reciprocal plot for determining binding constants.

Stability of subunit contacts in holo- and apoferritin. To estimate stability of quaternary contacts in subunit interfaces in holo- and apoferritin, the proteins were incubated in 10% SDS for 1 h at 60°C, followed by the PAGE analysis. Under electrophoresis in reducing conditions, the purity of both holo- and apoferritin samples is more than 98%, with the minor H subunit (~21 kD) having the same mobility as the major L subunit. Electrophoresis without both 2-mercaptoethanol and boiling revealed a larger amount of relatively unstable subunit contacts in holoferritin versus apoferritin (Fig. 4a). From densitometric scans (Fig. 4b), the percentage of these destabilized contacts does not exceed 15-20% even under our conditions that intentionally provoke the subunit dissociation. These destabilized subunit contacts in holoferritin seem to be at least partially involved into the G10-reactive epitopes, which results in the lower immunoreactivity of these epitopes in ferritin versus apoferritin. Furthermore, the band attributable to a subunit dimer (38-40 kD) was observed in ferritin but not in apoferritin, which suggests that mechanisms of subunit dissociation differ in ferritin containing iron and iron-free apoferritin.

Immunochemical identity of spleen ferritin and ferritin standard of the World Health Organization (WHO). The above results are significant for designing enzyme immunoassays to be employed for serum ferritin determination in clinical diagnostics. Most of the contemporary enzyme immunoassays follow a two-site "sandwich" design in which ferritin of calibration standards or serum samples binds initially to the "capture" antibody adsorbed onto the inner surface of wells in polystyrene plates, followed by binding to the labeled soluble anti-

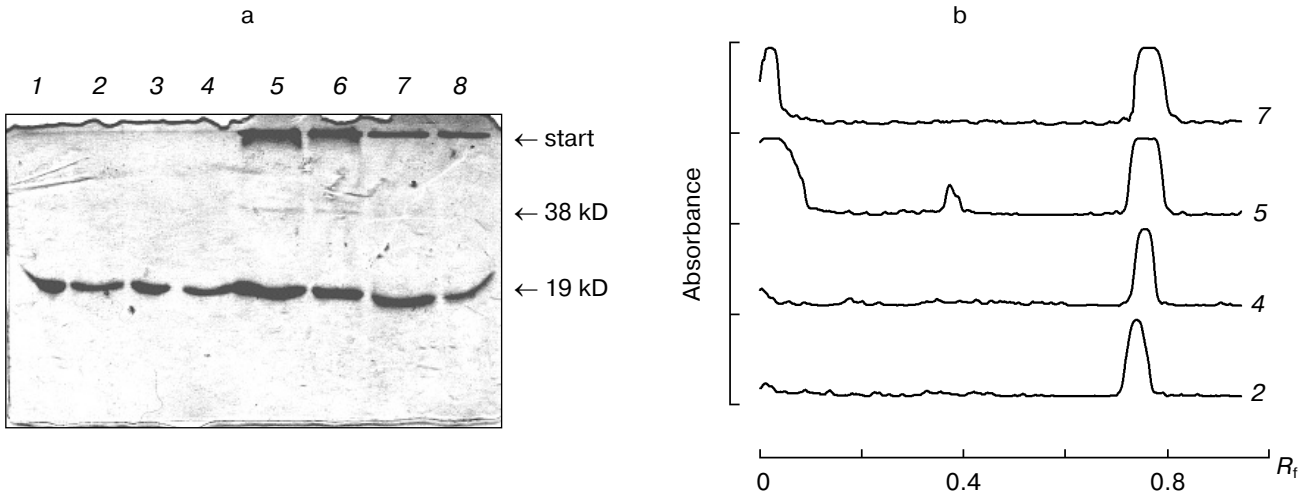

Fig. 4. Electrophoretic pattern (a) and its densitometric scan (b) for holo- and apoferritin: *1*, *2*) holoferritin under the reducing conditions containing 2-mercaptoethanol; *3*, *4*) apoferritin under the same conditions; *5*, *6*) holoferritin in 10% SDS; *7*, *8*) apoferritin in 10% SDS. The amount of the loaded protein was 10 (*1*, *3*), 5 (*2*, *4*), 40 (*5*, *7*), and 20 μg (*6*, *8*).

body. The prerequisite for the development of immunoassay kits is the immunochemical identity of an antigen present in calibration standards and in serum samples; this identity is critical for large multisubunit proteins like ferritin that have a great number of allowed conformations. Therefore, the commercial immunoassay kits employ as a calibration standard the protein antigen approved by WHO.

In our immunoassay, the two monoclonal antibodies, G10 and C5, were labeled by biotin, and the amount of polystyrene-bound label was compared using streptavidin–horseradish peroxidase conjugate (Fig. 5). The G10 antibody possesses the high antigen binding constant (Fig. 2) and retains its binding parameters after adsorption onto plastic surfaces and after conjugation with biotin or horseradish peroxidase. However, the ability of the G10 antibody to discriminate between ferritins with the variable iron content resulted in immunochemical non-identity of ferritin involved in our calibration probes (human spleen ferritin) and the WHO standard (3rd International Recombinant Standard, NIBSC code 94/572) (Fig. 5, insert). For variable concentrations of antigen samples that meet the requirement of immunochemical identity, the plot of the expected (calculated) versus the really determined (recovered) concentration should display a straight line that either coincides with or is parallel to the theoretical line (Fig. 5, line *3* in the insert). With the G10 antibody employed in our immunoassay both as the "capture" and the labeled antibody, the plot of the recovered versus expected concentration of the WHO ferritin standard displays a straight line that is not, however, parallel to the theoretical line (Fig. 5, insert, line *1*). At high ferritin concentrations, this non-identity resulted in a 2.5-fold overestimation of the ferritin concentration versus the expected concentration. At lower ferritin concentrations (~15 ng/ml), the recovered concentration is close to the expected one and exceeds the latter by as little as 30% (Fig. 5, insert). Given that the currently approved WHO standard represents recombinant human L-apoferritin [29], the above results could be expected from our data summarized in Fig. 2. When the C5 antibody was used as a "capture" antibody instead of the G10 antibody, spleen ferritin displayed immunochemical identity with the WHO standard (Fig. 5, line *2* in the insert), as judged by the magnitudes of recovered versus expected ferritin concentrations that lie within the experimental error. Furthermore, the 2-fold dilution of the ferritin samples resulted in a 2-fold decrease in the recovered concentration.

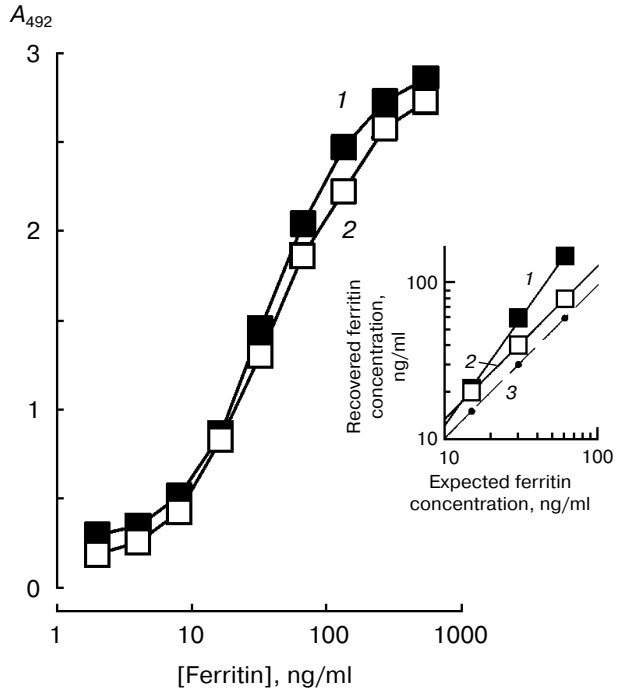

Fig. 5. Analysis of immunochemical identity of human spleen ferritin and recombinant L ferritin. To generate dose–response curves, ferritin was allowed to react first with polystyrene-adsorbed antibody G10 (*1*) or C5 (*2*), then with biotinylated antibody G10. Insert: recovered (experimentally determined) concentration of recombinant L ferritin (ordinate axis) plotted versus expected (calculated) concentration (abscissa axis). Antibody employed for generating dose–response curves: *1*) G10; *2*) G10 and C5; *3*) expected (calculated) curve.

These results confirm our finding that the conformation of the C5-reactive epitopes is apparently unaltered by variations in the amount of ferritin iron, nor does it depend on the presence or absence of the H subunit. In contrast, the conformation of the G10-reactive epitopes differs in recombinant purely L apoferritin and spleen ferritin comprising ~15% H subunit.

DISCUSSION

In this study, we report the two major observations that are related to local conformational changes in ferritin induced by a progressive loss of iron, the process that involves native ferritin, low-iron ferritins, and iron-free apoferritin. As a first observation, we showed that the conformation of the major epitopes depends on the amount of iron entrapped in the mineral core within the protein shell. Second, we established biphasic changes of the constants of monoclonal antibody binding to apoferritin and ferritins with varying amount of iron (Fig. 2). Immunoreactivity in terms of the binding constants decreases from 3-6-fold as the iron content decreases from 800 to 40 atoms per protein molecule, followed by a sharp increase in binding affinity after complete iron removal and formation of apoferritin (Fig. 2). From these data, we conclude that at least three iron-related conformational states of ferritin are present and can be discriminated with monoclonal antibodies. These con-

formational states involve apoferritin and ferritins with low and moderate iron content. The conformation of apoferritin appeared to have the highest immunoreactivity of antigenic epitopes as demonstrated by the highest constant of binding to monoclonal antibodies. It is, however, noteworthy that iron-related conformational states of ferritin possess conformational alterations that can be attributed to local, not global, conformational changes, as judged from the data obtained with polyclonal antibodies (Fig. 1).

In the context of the second observation, our results do not provide sufficient experimental explanation for mechanisms underlying a decrease in ferritin immunoreactivity after partial iron removal from 800 to 40 atoms per protein molecule (Fig. 2). It was, however, shown that remarkably high stability of the ferritin molecule is provided by the highly cooperative network of intra- and intersubunit interactions [30, 31]. This stabilization mechanism might be responsible for partial unfolding of subunits induced by the dissociation of the ferritin shell [31]. Given that we employed a procedure for iron removal that involves a partial dissociation of ferritin [32], one can presume that a decrease in immunoreactivity resulted from local unfolding that might occur in subunit interfaces. This presumed local unfolding seems to be irreversible on incomplete iron removal. As an indirect evidence for this assumption, Takagi et al. (1998) in their crystallographic studies of frog recombinant ferritin demonstrated that local unfolding in subunit interfaces, although did not alter the reassembly/refolding of the quaternary structure and did not result in global conformational changes, did result in a marked increase of the iron release [16]. In our studies, complete iron removal as a final step of the procedure seems to result in local refolding and an increase in immunoreactivity of apoferritin in comparison with low-iron ferritin samples.

Our data together with previous findings provide a conceivable explanation for higher immunoreactivity of apoferritin versus holoferritin (Fig. 2). It was previously shown that the maximal amount of iron incorporated into ferritin core is 4500 atoms per protein molecule [33]; however, for spleen and liver ferritins the amount of iron was found to vary in a broad range, from 300 to 2000 atoms per molecule [34]. Spleen ferritin analyzed in our study comprised 800 atoms per molecule. It seems reasonable to assume that the moderate amount of iron in ferritin is associated with optimal subunit interactions but suboptimal immunoreactivity of immunodominant epitopes that reaches the optimal level in the iron-free state of apoferritin. Accordingly, immunoreactivity of apoferritin in our studies appeared higher when probed with the antibody G10 and, to a lower extent, with F11 antibody. It was previously shown that a fraction of H ferritin-oxidized iron might be transferred to other proteins such as L-ferritins and transferrin [35, 36]. It was also demonstrated that Fe(III) exchange occurs among ferritin molecules when an acceptor molecule contains a lower amount of iron than the donor [35, 37]. This iron exchange presumably results in local unfolding of subunit interfaces, thus reducing immunoreactivity. The electrophoretic pattern obtained with holo- and apoferritin after incubation in 10% SDS at 60°C provides experimental support for this assumption (Fig. 4). In these electrophoretic studies, the percentage of dissociated subunits was by 15-20% higher for holoferritin.

In summary, we probed with monoclonal antibodies human spleen ferritin samples with variable iron content and established that the progressive loss of iron resulted in local conformational changes of the ferritin protein shell, with the global conformation remaining essentially unaltered. In terms of binding constants, these local changes were maximal when untreated native ferritin was compared with low-iron ferritin obtained after partial iron removal. The presence of residual iron resulted in a decrease of immunoreactivity of the G10- and F11-reactive epitopes in comparison with both untreated holoferritin and iron-free apoferritin. Given the enhanced affinity of apoferritin binding to monoclonal antibodies G10 and F11, a presumed optimal conformation adopted by ferritin epitopes is not a single consequence of complete iron removal. Taking into account numerous previous indications that specific ligands and/or cofactors generally stabilize proteins, one can presume that an increase in the conformational flexibility might additionally contribute to enhanced immunoreactivity of apoferritin.

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