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ULTRASTRUCTURAL EVIDENCE FOR THE DISSOCIATION OF FREE SUBUNITS FROM FERRITIN UPON DILUTION

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Summary: Previous biophysical and biochemical studies have not detected any free monomeric subunits when multimeric molecules of the metalloprotein, ferritin, were highly diluted with water. Electron microscope examination of diluted ferritin reveals many single objects much smaller than the whole molecules. These small objects are interpreted as being free apoferritin subunits. Since these small objects can be found in samples that originally contain only whole molecules, this result indicates that subunits indeed can dissociate from ferritin upon dilution. The available evidence suggests that the dissociation of subunits normally is balanced by a rapid reassociation into the polymeric state.

Each molecule of the iron-storage protein, apoferritin (MW ca. 450,000), is a polymer believed to be formed by self-assembly from 24 monomeric subunits (1-3). Monomers produced by *in vitro* dissociation can spontaneously polymerize into apoferritin upon removal of the dissociating conditions (4-6). After polymerization, the molecules have the form of a hollow sphere with an outer diameter of 125Å (2,3). The sequestration of iron as a microcrystalline ferric mineral within the central cavity converts apoprotein molecules into the metalloprotein, ferritin.

The protein shell surrounding the iron-rich core of ferritin is extraordinarily stable to very harsh conditions, such as 10M urea, boiling, drying and rehydrating, freezing and thawing, or pH 4 to 9 (6-9). Another aspect of this very high stability is suggested by the studies of Jaenicke and Bartmann (10), and Crichton *et al.* (11), which did not detect any free 17S subunits when ferritin was very highly diluted with water. In the present report, electron microscope examination of diluted ferritin unexpectedly reveals the presence of many objects that are much smaller than the whole molecules. These are interpreted as being free apoferritin subunits which arise by spontaneous dissociation from the polymers.

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Materials and Methods

Pure horse spleen ferritin was purchased from Calbiochem. The peak of molecular monomers and oligomers eluting at the void volume of Sephadex G150 or G200 (Pharmacia) was collected by elution with 0.01M potassium phosphate (pH 7.0); this fractionation removes any free monomeric subunits since these smaller molecules will remain uncollected due to their retarded passage through the gel bed. Aliquots of fractionated or unfractionated ferritin were diluted as needed with deionized doubly-distilled water. Additional fractions were collected at the tail of the void volume peak; these were not diluted further.

For electron microscopy (80-100kV), samples were deposited onto carbon supporting films that were made hydrophilic by glow discharge treatment, and were negatively stained with 2% sodium silicotungstate at pH 7.0 (12,13).

Results and Discussion

After dilution of pure ferritin with water to yield protein concentrations less than 10μg/ml, high resolution electron microscopy with negative staining always reveals the presence of small objects about 30Å in diameter, as well as single and associated ferritin molecules (Fig. 1). The small objects are more numerous than the whole molecules. The small objects mostly have a globular form and present a circular or slightly elongated profile (Figs. 1 and 2). Yet others have a rather irregular and diffuse appearance (Fig. 1), perhaps due to denaturation and unfolding, or to flattening during drying within a too-thin layer of stain. Selected examples of single small objects and of others associated into dimers and tetramers are shown at high magnification in Figure 2. It is important to note that these observations have been made with diluted aliquots of fractionated samples that originally contained only entire ferritin molecules,

Figure 1: Negatively stained ferritin after high dilution with water. In addition to three ferritin shells, many smaller objects also are present. Marker = 200Å.

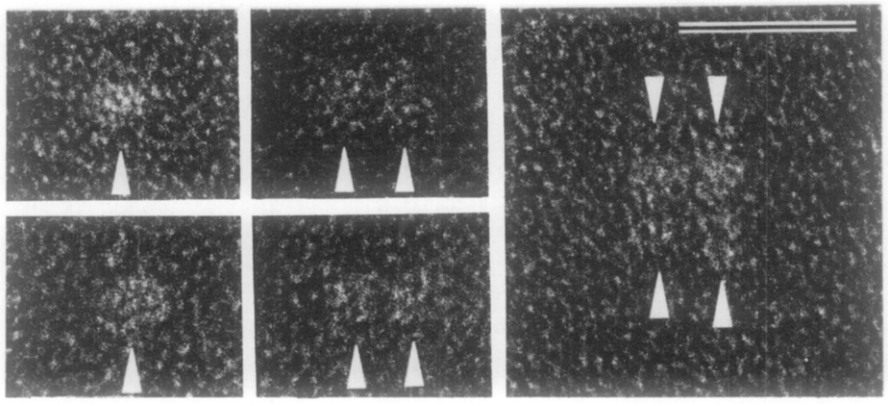

Figure 2: High magnification of monomeric and oligomeric subunits (arrowheads) in preparations of diluted ferritin. Left, subunit monomers; middle, subunit dimers; right, subunit tetramer. Marker = 100Å.

as well as with unfractionated samples. These same preparations also often contain some ferritin molecules having gaps in the profile of their protein shell; incomplete molecules are missing one or more of the apoferritin subunits making up each complete protein shell (7).

The polymerized subunits in horse spleen ferritin recently have been determined to be elongated globules, 55 x 27Å, by x-ray diffraction analysis (14). The small objects now seen in the diluted preparations of ferritin molecules most probably represent single free subunits by virtue of their size, shape, and known origin from pure ferritin. Moreover, the subunits seen in diluted preparations have a size and shape resembling the substructural details recently visualized within the protein shell of ferritin by in-focus high resolution electron microscopy (12). The presence of dimers and tetramers of these small objects provides further indication that they are ferritin subunits, since horse spleen ferritin subunit dimers and tetramers have been reported by Crichton and Bryce (9) using a completely independent means for assay. Since fractionated samples of ferritin which contain only entire polymers have these small objects after dilution, one must conclude that subunits indeed can dissociate from ferritin molecules. The gaps in the protein shells of some ferritin molecules probably are produced by the dissociation of subunits from the polymeric assemblage; whether some other ferritin molecules completely dissociate upon dilution cannot be determined at present.

Several previous investigations have concluded that ferritin does not dissociate upon high dilution with water (10,11). In view of the present findings, these data based upon chromatographic and ultracentrifugal assays must be re-evaluated. It is of fundamental importance to recognize that the reports by Jaenicke and Bartmann (10) and by Crichton et al. (11) only are describing negative results, i.e., the lack of detection of free subunits upon dilution of the polymer. Moreover, neither of these studies describes the use of a positive control (i.e., could free subunits have been detected by the assays used, if they indeed were present?). In contradistinction, the present ultrastructural results show clear positive evidence for the presence of free subunits when ferritin is diluted.

The seemingly contradictory requirements for spontaneous depolymerization and high polymer stability both are compatible with the existence of a dynamic equilibrium between ferritin dissociation and reassociation. If this dynamic situation proceeds very rapidly in both directions, gel chromatography probably would not readily demonstrate the presence of dissociated subunits depending upon the exact conditions of elution and analysis. Jaenicke and Bartmann (10) describe their gel chromatography assay for ferritin subunit dissociation as showing an elution profile at the void volume peak that is "symmetric"; actual inspection of the published profile shows some asymmetry, just as might be expected for a system with dissociation and rapid reassociation. It is possible to test this proposal by using electron microscopy to examine the asymmetric tail of the void volume peak. Myriad smaller objects, presumably representing subunits, are present in undiluted samples of this chromatographic fraction (Fig. 3). Many of these are single or oligomeric, while others are present in irregular associations (Fig. 3). There appear to be many more free subunits here than in diluted samples of the void volume peak. This result supports the proposed existence of an equilibrium between polymers and dissociating subunits.

The most obvious alternative explanations of the present ultrastructural data do not seem to have any validity when critically examined. The multitude of small objects in the diluted samples cannot be contaminating proteins, since the starting material was highly purified ferritin. It seems most

Figure 3: Negatively stained sample of ferritin from the tail of the void volume peak with Sephadex G150. In addition to one ferritin shell, many smaller objects also are present in this undiluted preparation. Marker = 200Å.

unlikely that the chemical agent used for negative staining is causing the depolymerization or breakdown of ferritin molecules, since analogous small objects also can be detected by rotary shadowing of samples that have never been exposed to any stain (12). It should not be considered surprising that the many previous electron microscope studies of negatively stained ferritin (e.g., 15,16) did not report the presence of free subunits. Subunits can be discerned clearly only if they are not covered by the much larger whole molecules, and if there is a sufficiently thin layer of negative stain embedding them; both these conditions necessitate using much lower concentrations of protein than have been utilized by all these other studies.

Electron microscopy should be able to readily reveal even an extremely transient existence of dissociated monomeric subunits since any free monomers moving from one ferritin molecule to another will be stopped in their tracks at the instant of drying. In addition, it is likely that some free subunits are removed from the dynamic equilibrium by their adherence to the supporting film while this still is wet, and therefore are detected. High dilutions with water will lengthen the average distance between polymers, and hence should increase the likelihood of detecting free subunits with an electron microscope assay; this same condition might decrease the likelihood of detecting free subunits by gel chromatography since the effective sensitivity could become inadequate.

The existence of subunit dissociation from ferritin is of biomedical importance and interest with regard to the biogenesis

of hemosiderin. This other iron-storage protein commonly is thought to be generated within lysosomes by the removal of a portion of the protein shell of ferritin, such that an iron-rich insoluble product is formed (3,17,18). Since ferritin is known to be relatively resistant to digestion by trypsin and certain other proteases (19,20), it is not completely obvious how ferritin could be digested to form hemosiderin. In this regard, it is significant that the apoprotein is much more susceptible to catheptic proteolysis under acid conditions (19,20). Any spontaneous dissociation of polypeptide subunits from ferritin would more readily permit their proteolytic breakdown within lysosomes; even if this proteolysis was limited in extent, the clipped subunits probably would not be able to reassociate into ferritin, and thus a deproteinized product would be formed.

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