In Vitro Reconstitution of Ferritin*

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ABSTRACT: Examination of "ferritin" formed by oxidative incubation of Fe^{2+} with apoferritin, a procedure widely used for reconstitution, reveals a proteinized iron complex whose size is different from native ferritin. Incubation of noncrystalline ferritin and apoferritin at neutral pH and 25° resulted in the reconstitution of crystalline protein whose structure and chemical behavior was identical with ferritin. Noncrystalline ferritin was characterized by chemical and electron microscopic techniques and found to be identical with ferritin, except that there is a partial removal of the protein subunits which surround the iron micelle. Reconstitution appears to result from a transfer of protein subunits from apoprotein to noncrystalline ferritin, and not by the movement of iron from the noncrystalline fraction to apoferritin. A model is proposed for the synthesis of ferritin in which oxidation and reduction phenomena are not required. In this model, the iron micelle is formed by the polymerization of low molecular weight iron complexes within the cell.

Following the formation of the polynuclear iron core, protein subunits of apoferritin interact with the micelle by noncovalent forces to form ferritin. The protein subunits are stabilized by their arrangement around the iron.

Ferritin, the principal iron-containing protein in liver, spleen, and other tissues, was originally characterized by Laufberger (1937). Since its isolation, this protein has been the subject of extensive physical and chemical studies which have defined its structure as well as characterized its role in iron metabolism. Ferritin is a conjugated macromolecule consisting of a spherical electron dense core or micelle approximately 70 Å in diameter surrounded by a shell of 20 identical protein subunits. The over-all external diameter of the molecule is about 120 Å. The micelle is a ferric hydroxyphosphate polymer with a probable composition of $(FeOOH)_8$. $(FeO:PO_2H_3)$ (Michaelis *et al.*, 1943; Granick and Hahn, 1944). Some investigators have suggested that the micelle is divided into subunits organized into a regular tetragonal geometric array (Farrant, 1954; Richter, 1959). However, these findings are ambiguous and recent X-ray diffraction measurements (Harrison, 1963; Fishback and Anderegg, 1965) suggest that the micelles are spheres, 74 Å in diameter. The probable conformation of the protein shell is a pentagonal dodecahedron with a small central space in each pentagonal face (Harrison, 1960, 1963).

The magnetic susceptibility of the iron micelle is 3.8 Bohr magnetons (Michaelis *et al.*, 1943). Investigations of the Mössbauer spectra of ferritin at 77°K (Boas and Window, 1966) and at 70°K (Blaise *et al.*, 1965) reveal an isomer shift of 0.47–0.50 mm/sec and a quadrapole splitting of 0.60–0.74 mm/sec.

Although it is well known that ferritin plays a key role in the storage and metabolism of iron in mammalian systems, three principal questions concerning the chemistry and biochemistry of iron in this molecule remain: (1) the mechanism by which the iron polymerizes to form uniformly spherical micelles; (2) the manner in which the iron micelle is incorporated into the protein shell; and (3) the chemical reactions involved in mobilizing iron from the micelle.

Recent experiments concerning the limited hydrolysis of Fe^{3+} in aqueous solution (Spiro *et al.*, 1966; Allerton *et al.*, 1966) show that uniformly sized spherical ferric hydroxide nitrate micelles, 70 Å in diameter, are formed spontaneously. Their magnetic and Mössbauer properties are essentially identical with those of ferritin (Dr. G. Brady, private communication). Extension of these findings to the hydrolysis of ferric citrate (Spiro *et al.*, 1967a,b) and ferric fructose (Aasa *et al.*, 1964; J. Renner, unpublished results) also revealed that polynuclear, spherical micelles that are stable under physiological conditions were formed. The average diameter of the ferric citrate micelle is 75 Å. The chelating agents are complexed at the surface of the particles and prevent micelle–micelle interaction and precipitation. The visible and ultraviolet spectra of these polymers are quite similar to ferritin. The close relationship of these polynuclear iron systems to ferritin led us to reexamine the mechanism by which the iron micelle is incorporated into this protein.

Previous efforts to elucidate the mechanisms operative during iron incorporation and mobilization in ferritin have been based on the assumption that an oxidation–reduction system is required to facilitate iron solubilization and mobilization. This redox hy-

* From the Division of Biology, University of California at San Diego, La Jolla, California 92037. *Received June 14, 1967.* This work was supported by grants from the John A Hartford Foundation, the National Institutes of Health (AM05484), and the California Division of the American Cancer Society.

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pothesis followed from earlier studies (Bielig and Bayer, 1955; Loewus and Fineberg, 1957) that ferrous iron in an oxidizing environment could be bound, *in vitro*, to apoferritin in reasonable quantities to yield a product which resembled ferritin. Attempts to introduce ferric iron into the protein have been largely unsuccessful.

It seemed reasonable to reconsider the possibility that the ferric core of ferritin was synthesized first, and that the protein subunits then formed a hydrophylic shell to impart stability and solubility to the molecule. In his early chemical and physical characterization of ferritin, Granick (1942) observed a small but consistent amount of iron protein which resembled ferritin in all of its properties except its ability to crystallize in the presence of 5% $CdSO_4$. This noncrystalline fraction was present in the supernatant after repeated crystallizations of ferritin. Further, Granick commented on the formation of crystalline ferritin when the noncrystalline material was incubated with apoferritin. Of particular interest to us was the apparent absence of any redox system to mediate the reconstitution observed.

We reinvestigated this phenomenon to more clearly define the chemical and physical properties of noncrystalline ferritin and to elucidate the mechanisms by which ferritin is synthesized. At the same time we compared ferritin reconstituted from Fe^{2+} and apoferritin under an oxidizing condition with the protein formed from noncrystalline ferritin and apoferritin. Significant differences were observed in the size of the iron micelle as well as the surrounding protein shell. We propose a mechanism involving initial synthesis of a polynuclear iron hydroxide micelle and subsequent association of protein subunits around the micelle primarily directed by short-range forces. The participation of an oxidation–reduction reaction is not required.

Experimental Procedures

Preparation of Ferritin and Noncrystalline Ferritin. Both crystalline and noncrystalline ferritin were prepared from horse spleen using the general techniques described by Granick (1942). Following maceration and heat extraction at 80° in distilled water, the homogenate was filtered while hot. The clear filtrate was adjusted to pH 4.6 and centrifuged at 1280 rpm for 30 min. The precipitate was discarded and ferritin was crystallized from the clear supernatant by adding $CdSO_4$ to a final concentration of 5% by weight. Crystals were separated by centrifugation, dissolved in 2% $(NH_4)_2SO_4$, and recrystallized with $CdSO_4$. The supernatant from each crystallization was retained as the noncrystalline fraction. Solubilization and recrystallization of ferritin can be repeated several times to obtain noncrystalline protein. Both the ferritin and noncrystalline ferritin were dialyzed for several days against distilled water. After dialysis, the preparations were adjusted to pH 7.4 and centrifuged to remove any sediment.

Preparation of Apoferritin and Noncrystalline Apoferritin. Apoferritin was prepared in a manner similar to that reported by Granick (1943). A 1.5% aqueous solution of twice-crystallized ferritin was adjusted to pH 4.6 with acetic acid and dialyzed against 1 M acetate buffer (pH 4.6) which had been freed of oxygen by bubbling with nitrogen and made 4% by weight with sodium dithionite. The buffer was changed after 24 hr and following an additional 24-hr dialysis, bipyridine was added to the ferritin solution (300 mg/100 ml) which was made 0.1 M in cysteine. The solution was dialyzed against acetate–dithionite for 24 hr and then against water for 48 hr. The apoferritin was dialyzed another 8 hr against a solution 0.1 M in both 1,2-diaminocyclohexanetetraacetic acid (Chel-CD, Geigy Chemical Corp.) and nitrilotriacetic acid (pH 7.5). Removal of all chelating and reducing agents was achieved by dialyzing against distilled water.

To obtain noncrystalline protein, apoferritin solutions were made 2% in $(NH_4)_2SO_4$ and recrystallized with 5% $CdSO_4$. The soluble supernatant was separated from the crystalline apoferritin and dialyzed to remove salts. Both crystalline and noncrystalline apoferritin solutions were adjusted to pH 7.4 and centrifuged to remove any insoluble material prior to use.

Determination of Iron and Nitrogen Content. Samples were hydrolyzed under reflux with an equal volume of a 1:1 mixture of concentrated HNO_3–H_2SO_4. Aliquots of the digest were analyzed for iron with the bathophenanthroline technique described by Bothwell and Mallet (1955). Nitrogen content of aliquots was measured using the micro-Kjeldahl procedure developed for the AutoTechnicon total nitrogen analyzer.

Reconstitution of Ferritin from Fe^{2+} Apoferritin. Ferrous ammonium sulfate and apoferritin were used to reconstitute ferritin by a technique similar to that of Bielig and Bayer (1955). Ferrous ammonium sulfate was added to 10 ml of a 1% apoferritin solution in 20 mM $NaHCO_3$ (pH 7.4) so that the weight fraction of Fe:N was 2:1. The solution was incubated at 0° with agitation using a stream of oxygen. The initial colorless solution turned red-brown as the Fe^{2+} was oxidized. After 1 hr the solution was made 5% by weight with $CdSO_4$ and stored overnight at 4°. The crystals formed were removed by centrifugation and redissolved in 2% $(NH_4)_2SO_4$. The intensely colored solution was centrifuged to remove insoluble material and the supernatant was dialyzed against distilled water to remove salt. Centrifugation removed insoluble precipitates. The "reconstituted" ferritin solution was subjected to electron microscopic examination. Iron and nitrogen determinations were also made.

Reconstitution of Ferritin from Noncrystalline Ferritin and Apoferritin. A 1.5-ml aliquot of a 1% apoferritin solution was incubated with 8.5 ml of noncrystalline ferritin containing 600 mg of iron/ml. The final solution was 20 mM in $NaHCO_3$ (pH 7.4) and had an Fe:N weight ratio of 2:1. The mixture was incubated at 25° with agitation for 1 hr and then made 5% with $CdSO_4$. The precipitate obtained after standing overnight at 4° was removed by centrifugation and dissolved in 2% $(NH_4)_2SO_4$. The soluble protein solution was

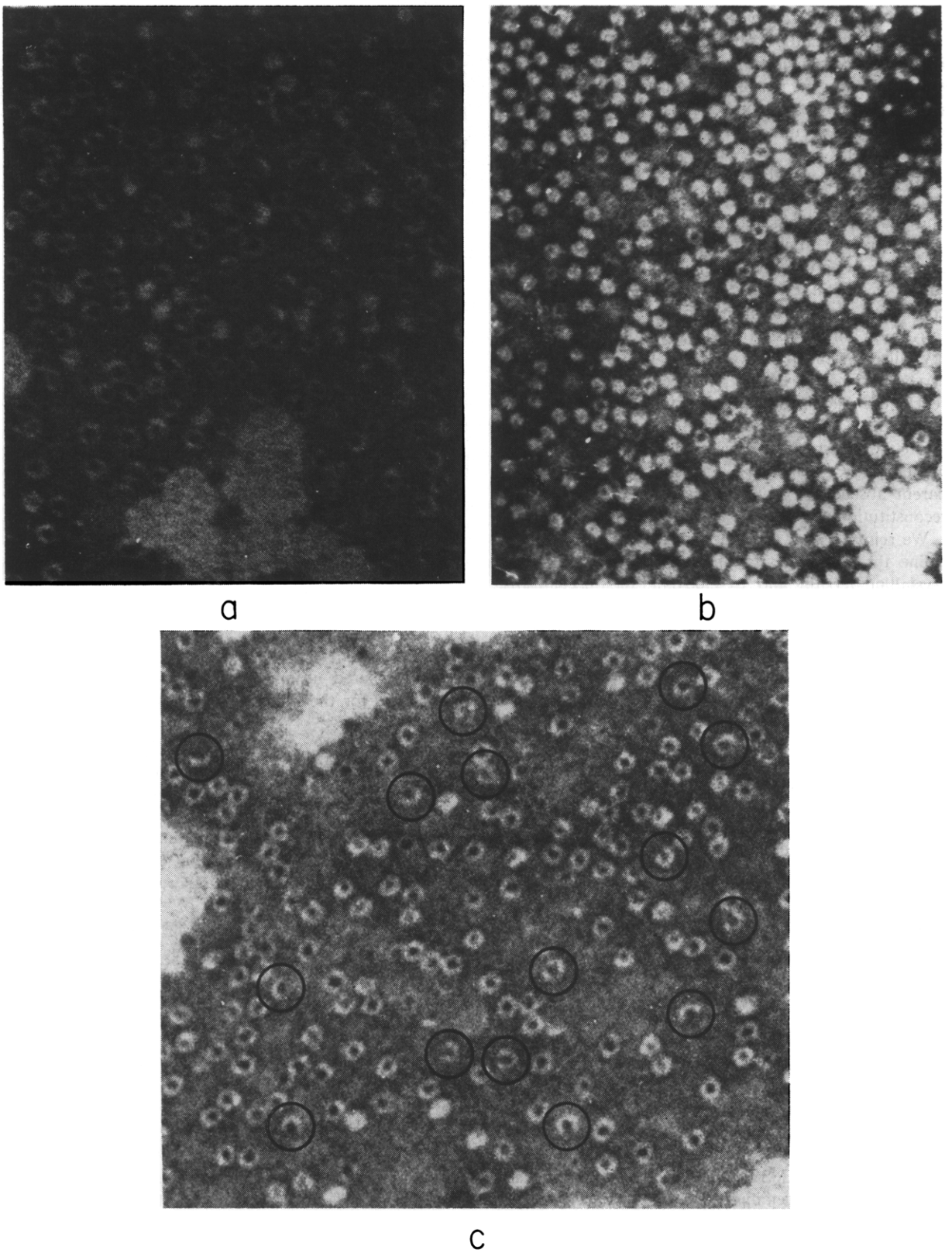

FIGURE 1: Electron micrographs of preparation stained with 2% phosphotungstate (1:1) (pH 7.2). Magnification approximately $3 \times 10^5\times$. (a) Crystalline ferritin, (b) apoferritin, and (c) noncrystalline ferritin. Circled molecules in c show iron micelles with partial protein shells.

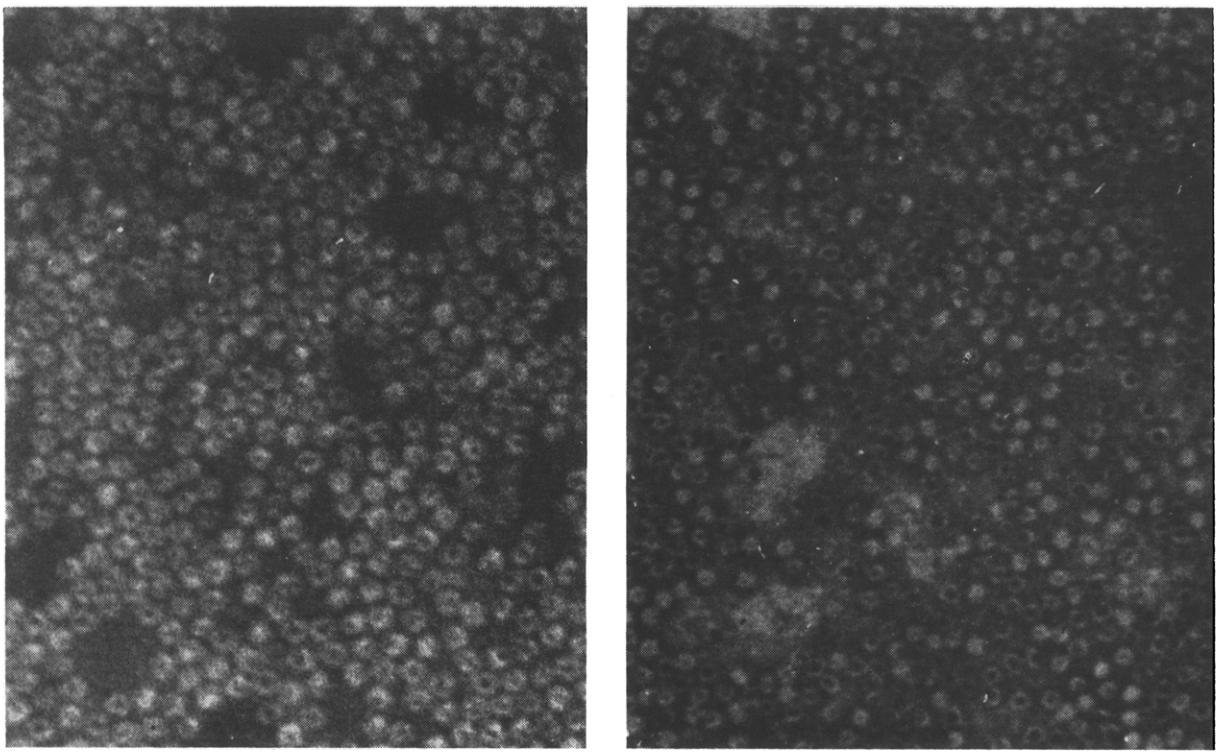

FIGURE 2: Electron micrographs of reconstituted ferritin prepared as described in Figure 1. (a) Using ferrous ammonium sulfate and apoferritin; (b) using noncrystalline ferritin and apoferritin.

dialyzed against distilled water. A duplicate sample of noncrystalline ferritin was carried through an identical procedure except that apoferritin was omitted as a control. Following incubation, the control was processed in the same manner as described above. There was a small but detectable $CdSO_4$-precipitable material present in the control. The amount of iron in the control precipitate was measured and subtracted from that obtained in the presence of apoferritin.

Electron Microscopy. Negatively stained preparations of crystalline and noncrystalline ferritin as well as ferritin reconstituted by the two procedures described above were prepared by combining samples of each preparation with 2% phosphotungstic acid (pH 7.2) in a 1:1 volume ratio. Polystyrene latex balls, 1800 Å in diameter, were added to the solutions and the material was deposited on Formvar-covered, carbon-stabilized, 200-mesh electroplate specimen grids by either drop or high-velocity spray techniques. The grids were air dried and then observed in an RCA 3MU-G electron microscope using a 50-kV accelerating potential. Measurements of particle size were made using a Nikon optical comparator.

Results

Electron micrographs of crystalline ferritin and apoferritin are shown in Figure 1a,b. The characteristic central electron dense micelle surrounded by the unstained protein shell is seen in the ferritin. Some of the apoprotein molecules show an electron dense central region. This is due both to the influx of phosphotungstate into the cavity from which iron was removed and to a limited retention of iron. Noncrystalline ferritin is shown in Figure 1c. Its appearance is quite similar to that of ferritin except for the significantly increased number of crescent shaped structures (shown circled). It appears as if there is a loss or rearrangement of the protein subunits with a consequent exposure of the surface of the iron micelle.

A comparison of the structures of ferritin reconstituted from ferrous ammonium sulfate plus apoferritin (FAS–APO)[1] and noncrystalline ferritin plus apoferritin (NCF–APO) are presented in Figure 2a,b. The average dimensions of crystalline ferritin and the reconstituted ferritins were determined (Table I). The electron dense center of the ferritin reconstituted from FAS–APO was consistently smaller with an average diameter of 48 Å. The reconstituted ferritin from NCF–APO had an electron dense core whose diameter was 70 Å. Furthermore, the outer diameter of the protein shell is considerably reduced in the FAS–APO experiments.

[1] Abbreviations used: FAS–APO, ferrous ammonium sulfate plus apoferritin; NCF–APO, noncrystalline ferritin plus apoferritin.

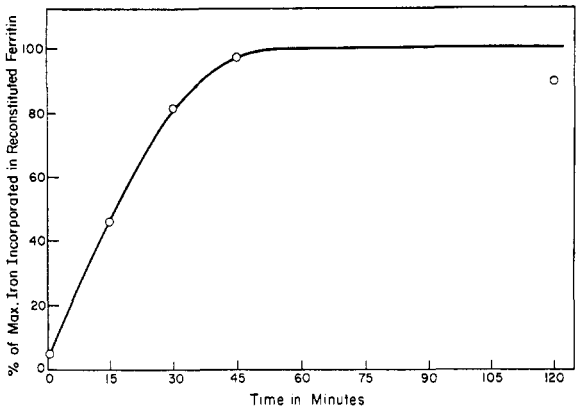

FIGURE 3: Reconstitution of ferritin from noncrystalline ferritin and apoprotein as a function of time.

It appears as if the over-all dimension is determined by the diameter of the micelle. The thickness of the protein shell is unaltered in both experiments. The diameter of the NCF–APO is the same as for crystalline protein.

Loewus and Fineberg (1957) have reported a 60–80% reconstitution of "ferritin" from the FAS–APO system. In these studies the criterion for reconstitution was an increase in optical absorbance at 400 mμ. We have confirmed these results, but were disturbed by the lack of specificity in the method. It has been shown that other polynuclear species of Fe(III) exhibit similar absorbance (Charley *et al.*, 1963). Furthermore, the variation in size of the FAS–APO-reconstituted molecule from the native ferritin revealed by electron microscopy as well as the reported decrease in the biological activity (Bielig and Bayer, 1955) of this molecule suggested that spectral absorbance was not a sufficiently rigorous measure of reconstitution.

In order to assess the amount of reconstituted ferritin formed in the NCF–APO system, we monitored the reconstituted material for size and shape in the electron microscope and measured the iron content in the crystallizable material directly. The rate of reconstitution was investigated by incubating noncrystalline ferritin and apoferritin as previously described. At intervals, aliquots of the mixture were removed and all proteins were precipitated with an equal volume of saturated $(NH_4)_2SO_4$. The solution was centrifuged and the protein was dissolved in water. Addition of $CdSO_4$ to a final concentration of 5% caused the formation of a crystalline ferritin. The amount of reconstitution at each time interval was determined by measuring the iron content in the precipitate (Figure 3). The reaction appears to reach equilibrium under these conditions within 60 min. In a series of ten experiments, the amount of iron incorporated into the reconstituted ferritin from the iron initially present in solution ranged from 40 to 90% within a mean value of 67%.

TABLE I: Molecluar Dimensions of the Diameter of Native and Reconstituted Ferritin Measured from Electron Micrograph of Negatively Stained Preparations.

Sample	Over-All Protein (Å)	Micelle (Å)
Crystalline ferritin	120	71
Apoferritin	92	
Reconstituted ferritin (FSA–APO)	102	48
Reconstituted ferritin (NCF–APO)	115	70

Discussion

Reconstitution of crystallizable ferritin in significant quantities occurs when noncrystalline ferritin is incubated with apoferritin at neutral pH and 25°. This process does not involve an oxidation and reduction system. The structure of the reconstituted ferritin is identical in chemical behavior and microscopic morphology with the parent ferritin.

Reconstituted ferritin obtained from oxidation of ferrous ammonium sulfate in the presence of apoferritin, on the other hand, produces a "ferritin" whose morphology is quite different from that of the native protein. The reduced dimension of the iron micelle, 48 Å instead of 70 Å, and the over-all diameter, 102 Å instead of 120 Å, suggest that the configuration of the ferritin molecule and perhaps its biological activity are sensitive to the structure of the micelle. That the iron core influences the over-all dimension of the protein shell is confirmed by measurement of apoferritin, whose average over-all diameter is 92 Å.

It is pertinent to examine the nature of noncrystalline ferritin prior to proposing a mechanism by which it participates in the reconstitution of ferritin. Electron micrographs show that the iron micelle of noncrystalline ferritin is identical in size and shape with those found in ferritin. Many of the protein molecules exhibit incomplete protein shells. In some of them, there is evidence for the stripping away of protein from the surface of the iron micelle (Figure 1c). Many of the noncrystalline ferritin molecules appear intact. However, it is well to remember that only when the defect in the protein morphology appears on the two-dimensional perimeter of the molecule is it recorded by the techniques of negative staining and electron microscopy.

There is a consistent fraction of crystalline ferritin which fails to recrystallize no matter how many times the cycle of recrystallization is repeated. Further, the protein:iron ratios in the NCF supernatant fraction are always equal to those in the ferritin from which it is formed. This leads us to propose that there is an equilibrium (eq 1). If the equilibrium is perturbed by

$$\text{ferritin} \rightleftarrows \text{noncrystalline ferritin} + \text{apoprotein subunits} \quad (1)$$

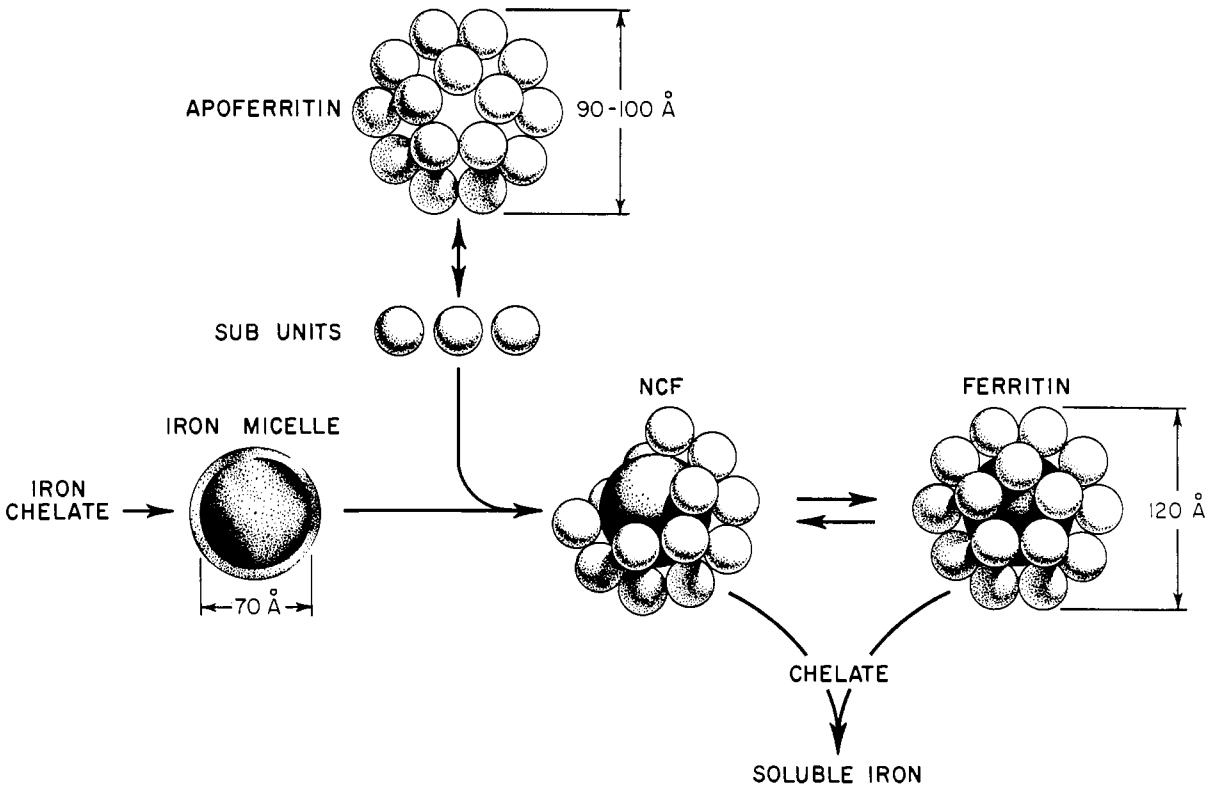

FIGURE 4: Model proposed for ferritin synthesis and mobilization.

the addition of large amounts of purified apoferritin, the formation of ferritin will be favored. Preliminary experiments from our laboratory using ^{14}C-labeled noncrystalline ferritin indicate that incomplete protein shells can be completed by addition of subunits from added apoferritin.

The ability of apoferritin to crystallize with $CdSO_4$ and the maintenance of the spherical shell of apoprotein even when the iron micelle is removed suggest that rather strong protein subunit interactions are operative. The stability of this structure to 8 M urea and pH 11 is also noteworthy (Hofmann and Harrison, 1963). The iron micelle also must exert a stabilizing force and directly influence structure of ferritin.

It has been difficult to conceptualize how the ferric hydroxide micelle could be laid down within a preformed apoferritin molecule by mechanisms previously proposed. It has now been demonstrated that controlled hydrolysis of Fe^{3+} and of several ferric chelates leads to the formation of polynuclear iron spheres, 60–100 Å in diameter. In the absence of chelating agents, the spheres are metastable and as pH is increased above 1.8, insoluble ferric hydroxide precipitates. In the presence of chelators such as citrate and fructose, it was shown (Spiro *et al.*, 1967a) that the organic ligands complex with the polynuclear iron probably at the surface and maintain the polymer in a soluble state at neutral or alkaline pH.

We therefore propose the over-all scheme shown in Figure 4 to account for the synthesis of ferritin in the absence of a redox mechanism. The polynuclear iron micelle is initially formed from the low molecular weight soluble chelates, which facilitate the entry of this metal into the cell. Protein subunits either present in the cell or synthesized in response to the presence of iron interact by noncovalent bonding with the micelle and are stabilized as ferritin. The size of the iron micelle is determined primarily by the solution chemistry of the iron chelates.

In the following paper (Pape *et al.*, 1968) we will demonstrate that iron can be effectively mobilized both from NCF and ferritin by low molecular weight chelating agents also in the absence of a redox system. The model we propose is compatible with observations of both iron storage and mobilization *in vivo*. The presence of excess iron accumulated over extended periods of time leads to the deposition of large amounts of both ferritin and hemosiderin (Granick and Hahn, 1944). Since hemosiderin contains a higher iron to protein ratio than does ferritin, it is possible that the exposure of a large portion of the micellar surface leads to micelle–micelle interaction. Aggregates of a stainable iron hydroxide are deposited within the cell. Our data confirm and extend the hypothesis advanced by Drysdale and Munro (1966) that iron-induced ferritin synthesis by the intestine or liver is a direct function of the stabilization of the protein subunits against proteolytic attack.

The role of chelation in iron chemistry has been recently reviewed (Saltman, 1965). Of particular

importance are the recent discoveries which demonstrate that iron chelates are mandatory participants in the transmembrane mobilization and utilization of iron. The tissue sites of deposition can also be controlled by the chemical nature of the chelate (Helbock and Saltman, 1967). The biological efficacy of an iron chelate is related to its solubility, its tendency to polymerize, and the rate at which it exchanges its metal with specific binding sites in macromolecules within the cells or tissues. Conversely the rate at which a chelator attacks and solubilizes the iron micelles of ferritin is also dependent upon steric factors as well as stability constants of the chelator for the metal.

The redox hypothesis is currently the most widely accepted mechanism for the mobilization of iron into and out of ferritin. Two lines of experimental evidence have made this a very appealing theory. First, it appeared that only by reduction could iron be mobilized from ferritin at a rate compatible with *in vivo* experiments. Second, only by incubation of apoferritin with ferrous iron under oxidizing conditions could "ferritin" be reconstituted. The first premise with respect to mobilization of ferritin iron will be examined in detail in the following paper (Pape *et al.*, 1968). The reconstitution experiments of Bielig and Bayer (1955) and by Loewus and Fineberg (1957) serve as the experimental basis for the second premise. Our results demonstrate that the "ferritin" formed in these experiments is distinctly different from native protein.

The results presented here have confirmed and extended the initial observations of Granick (1942) that redox reactions are not required and indicate that an effective reconstitution of ferritin both qualitatively and quantitatively is accomplished by incubation of noncrystallizable ferritin with apoprotein.

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