Mechanisms for the Formation of Ferritin Oligomers†

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ABSTRACT: Ferritin oligomers were isolated and purified by gel chromatography, and the proteins were characterized by electron microscopy, electrophoretic mobilities, and other analytical methods. The dimers and trimers were stable for long periods of time and, in contrast to some earlier reported observations by other authors, no reversible association–dissociation process between monomers and oligomers was discerned. An apoferritin component was not detected in the ferritin oligomer preparations, and, in fact, apoferritin isolated independently by other methods was devoid of oligomers. In general, the oligomers had a higher iron content than did the monomers. Evidence was obtained showing that ferritin was probably aggregated by formation of interchain disulfide bridges; furthermore, this cross-linking process appeared to be mediated by iron.

Purified ferritin preparations are usually heterogeneous with respect to the distribution of iron in their protein shells (Rothen, 1944), and to the state of aggregation of the protein. A native ferritin molecule consists of subunits of 20–24 polypeptide chains, and exhibits an additional capacity to form dimers, trimers, and higher oligomers which are aggregates of whole ferritin molecules. The heterogeneity with respect to the latter state of aggregation is reflected by the appearance of at least three bands of different electrophoretic mobilities on starch or polyacrylamide gels, and the chromatographic resolution of purified ferritin into several fractions (Richter, 1963; Fine and Harris, 1963; Kopp *et al.*, 1963, 1964; Theron *et al.*, 1963; Carnevali and Tecce, 1964). Although multiaggregates have often been observed in various species (Zamiri and Mason, 1968) and in normal and tumor ferritins (Richter and Lee, 1970), the structural basis for this phenomenon was not elucidated. Apoferritin prepared by reduction and removal of the iron from native ferritin is also a heterogeneous mixture containing a main component with a sedimentation coefficient of 17 S and minor components of about 24 and 33 S (Suran and Tarver, 1965; Harrison and Gregory, 1965; Williams *et al.*, 1966). The 24- and 33-S peaks are attributed to dimers and trimers, respectively, and the 17-S component is considered to be the monomer. These components can be identified with specific electrophoretic bands.

The origin and physiological significance of the oligomers of whole ferritin molecules, apparently unbiquitous in ferritin preparations, are presently obscure, but they have been considered possibly to be the first products in the conversion of ferritin to form hemosiderin (Williams and Harrison, 1968). Furthermore, based on the present state of knowledge, one is yet uncertain whether ferritin oligomers are stable or exist in a reversible equilibrium or interchange with monomers. In the present study, ferritin monomers, dimers, and trimers were purified and isolated as stable entities. Requirements for the formation of oligomers and the nature of the interchain linkages are defined.

Experimental Section

Materials. Horse spleen ferritin (6 × crystallized, Cd free) was obtained from Miles Laboratories, Kankakee, Ill., and 2 × crystallized ferritin solutions were obtained from Sigma Corp., St. Louis, Mo. The protein preparations routinely were subjected to chromatography on Sephadex G-200 to remove residual cadmium and other trace impurities and were characterized and compared to earlier preparations by circular dichroism and electrophoretic mobilities. Sucrose and guanidinium chloride were of ultrapure grade obtained from Schwarz/Mann, Orangeburg, N. Y. The 2,4,6-tripyridyl-*s*-triazine, iron-free ammonium acetate, hydroxylammonium chloride, and standard iron solutions of 1.0 and 10.0 μg/ml were obtained from G. Frederick Smith Co., Columbus, Ohio. The polyacrylamide gel reagents were obtained from Eastman, Rochester, N. Y., and the sulfhydryl and alkylating agents were obtained from Calbiochem, La Jolla, Calif. The Sepharose 6B and Sephadex G-200 were obtained from Pharmacia, Inc., Uppsala, Sweden. Deionized doubly distilled water was used to prepare all reagents for the iron determinations and for the preparation, purification, and analysis of the protein solutions.

Methods. Preparation of Ferritin Monomers and Oligomers. The ferritin preparations were fractionated on Sepharose 6B, according to the procedure described previously (Urushizaki *et al.*, 1971). This method enabled us to achieve a much greater resolution of oligomers than corresponding separations on Sepharose 4B (*cf.* Jones and Williams 1972), or on Sephadex G-200. An 85 × 5 cm column (Pharmacia) was used, with ascending flow achieved at a rate of 25 ml/hr using a Sage Model 375 peristaltic pump. Ferritin solutions of 70 mg/ml were applied to the columns and eluted with 0.1 M Tris buffer (pH 7.0). The desired fractions were recycled using Lu-4 type, four-way valves (Pharmacia). After elution the monomer and dimer fractions were reconcentrated to about 50 mg/ml and stored at 4°.

Density Gradient Centrifugation. Ferritin preparations were usually fractionated in linear 30–50% sucrose gradients by centrifugation at 40,000 rpm for 6 hr; this allowed optimum resolution of the apoferritin fractions of the sample.

A Beckman Model L-2-65B preparative ultracentrifuge

* Author to whom correspondence should be addressed. Research Career Development Awardee from the National Institute of General Medical Sciences (5 KO4-GM 19958) National Institutes of Health.

† From the Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461. *Received April 16, 1973.* This work was supported by Grant HL-11511 from The Heart and Lung Institute of the National Institutes of Health.

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with an SW-41 Ti rotor was used for these experiments. A Buchler Auto Densi-Flow apparatus was used to collect the protein in fractions of equal volume from the top of the gradients.

Gel Electrophoresis. The electrophoresis was carried out in 5% polyacrylamide gels in Tris buffer (pH 8.3) according to the procedure of Ornstein (1964). The protein was stained with Amido Schwarz, or Coomassie Brilliant Blue, and the ferric iron by the Prussian blue reaction with acidic ferrocyanide. The excess protein stain was removed in 7% acetic acid, or in 10:25:65 mixtures of acetic acid, ethanol, and water, respectively, and excess ferrocyanide stain was removed by washing with large volumes of distilled water. Protein solutions were concentrated with an Amicon Model 12 ultrafiltration cell fitted with PM-10 diaflo membranes. When comparative studies on oligomer proportions were carried out, equal amounts of protein were applied to tandem gels. A Gilford spectrophotometer with a Model 2410 linear transport was used to scan the gels at several wavelengths between 550 and 650 nm.

Electron Microscopy. The micrographs were obtained using a Siemens 101 electron microscope. The protein specimens were generaly prepared according to the procedure reported previously (Listowsky *et al*, 1972), and a primary magnification of 100,000× was used. The negative staining was achieved using a 1% uranyl acetate solution. Protein concentrations were 0.1 mg/ml.

Chemical Analysis. Protein concentrations were determined by the method of Lowry *et al.* (1951), or by a micro-Kjeldahl procedure. For determination of iron, the protein was first dissolved in 6 N HCl to ensure the complete disruption and solution of the iron micelle. The samples were subsequently diluted to raise the pH above 2.0. Iron was then determined spectrophotometrically based on absorbancy at 593 nm of complexes made with 2,4,6-tripyridyl-*s*-triazine (Fisher and Price, 1964). Atomic absorption measurements using a Perkin-Elmer Model 303 spectrometer were employed to confirm the values obtained colorimetrically.

Results

Several preparations of horse spleen ferritin from different commercial sources all showed at least three bands when examined by polyacrylamide gel electrophoresis. These bands are commonly attributed to ferritin molecules in various states of aggregation, namely monomers, dimers, trimers, and higher oligomers (Suran and Tarver, 1965; Harrison and Gregory, 1965). The ferritins studied here usually contained 75–80% monomer, 15–20% dimer, and less than 5% of the higher oligomers. Horse spleen ferritin prepared with the omission of the heating step (Suran and Tarver, 1965), or freshly prepared ferritins, also have a similar distribution of oligomers.

The oligomeric components of horse spleen ferritin were separated by agarose gel filtration in Sepharose 6B (Urushizaki *et al.*, 1971) and a typical elution pattern is shown in Figure 1. In this range of molecular weight, the resolution afforded by Sepharose 6B appears to be optimum when compared to that obtained with Sephadex G-200 or with other molecular sieve gels tested. By this method, specific fractions of monomer could easily be isolated with less than 2% contamination by the higher oligomers. The dimer or trimer fractions were enriched further by recycling of these specific fractions (identified in Figure 1 by the arrows) through the Sepharose columns, and dimer preparations of greater than 90% purity were routinely obtained. The trimers were more

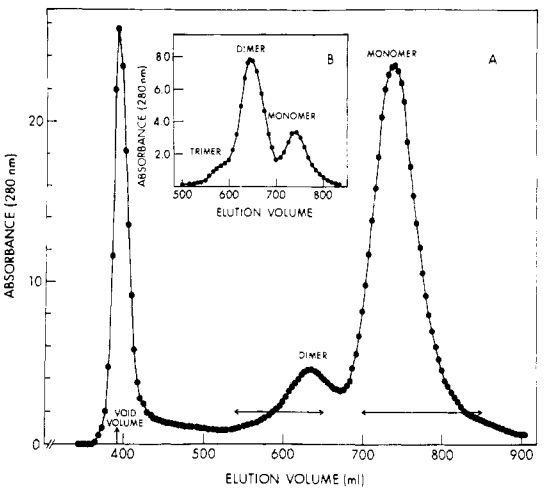

FIGURE 1: Chromatography on Sepharose 6B. The column dimensions were 85 × 5 cm, the flow rate was 25 ml/hr, and fractions of 4 ml each were collected. Ferritin solution (70 mg/ml) (1.0 ml) was applied to the column and eluted with 0.1 M Tris buffer (pH 7.0) in the initial fractionation (A). The monomer and dimer fractions were then recycled separately (recycled portion shown by the double headed arrows), and a typical elution pattern for the recycled dimer is shown in the inset (B). All chromatographic procedures were carried out at room temperature.

difficult to purify and usually were contaminated with about 15–25% of ferritin in the lower states of aggregation.

Once the purification was attained, the composition of each fraction remained constant, and could be recycled as single components. Monomers or oligomers obtained from the gel filtration fractions were checked for purity by electrophoresis on polyacrylamide gels. Electrophoretic patterns of some sample components obtained by these methods are shown in Figure 2. The subsequent studies in this report were carried out using only the gel filtration fractions that were characterized by electrophoretic patterns such as those in Figure 2.

The relative population of monomer or oligomers in all of the fractions remained constant after storage at 4° for over 2 months. This stability over long periods of time was independent of the protein concentration in the range of 0.1–100 mg/ml. In addition to the concentration and dilution occurring in the course of the repeated chromatographic procedures (Figure 1), the monomer solutions were concentrated to 100 mg of protein/ml without any detectable dimer formation. Conversely, the dimer did not dissociate to monomer even at protein concentrations of less than 0.07 mg/ml.

Electron micrographs of dimer and monomer samples are shown in Figure 3. Such representative fields show that at high dilution the dimer molecules tend to cluster in pairs even after drying. The average center-to-center distances as well as the overall appearance of the dimers are similar to those reported by Williams and Harrison (1968). Some of the dried monomer molecules also clustered together since samples were prepared in the absence of spreading agents such as albumin (Williams and Harrison, 1968); but in contrast to the distinct pairing observed with the dimers, irregular combinations occurred in the monomer preparations.

The purified monomer and dimer preparations were fractionated by sedimentation equilibrium in sucrose gradients. After centrifugation for 6 hr in 30–50% sucrose both monomer and dimer exhibited colored bands in the same density range of 1.20–1.31 g/cm^3. However, the dimers contained no detectable apoferritin component above the colored band

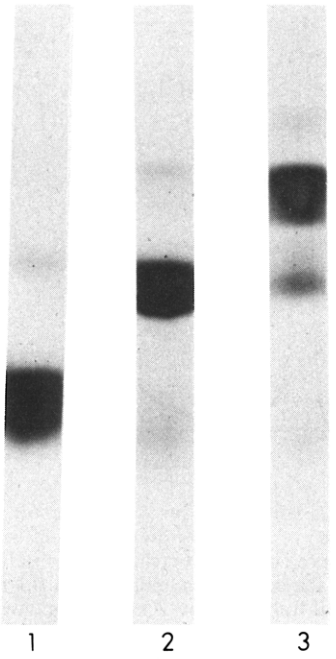

FIGURE 2: Polyacrylamide gel electrophoresis of purified ferritin monomers, dimers and trimers. Gel A represents a purified monomer fraction, containing about 2% dimer as a marker for the other gels. Gel B is an enriched dimer fraction and gel C is the trimer. These purified fractions were used for the studies described in the text and in Tables I and II.

whereas the monomer contained 10–20% apoferritin. Furthermore, the apoferritin component isolated from native ferritin preparations had no dimer or higher oligomers, and exhibited an electrophoretic pattern consistent exclusively with that of the monomer.

Table I shows the iron/protein ratios of the fractions obtained from the sucrose gradients. The iron content of the dimer is consistently higher than that of the corresponding monomer fractions, and the saturated dimer molecules ap-

TABLE I: Iron Distribution in Ferritin Monomers and Dimers.[a]

Iron Content (atoms/mol)	Oligomeric Monomer Total Protein (mg)	Oligomeric Dimer Total Protein (mg)
<100	8.5	0.0
100–500	2.7	1.3
1000–1500	2.8	2.4
1500–2200[b]	17.7	19.8
2200–3000	1.3	9.7

[a] Three tubes each of monomer and dimer (12 mg of protein per tube) were centrifuged in the same rotor head (SW 41Ti) under the conditions outlined in the Experimental Section. Detailed accounts of sucrose gradient centrifugation are given elsewhere (Niitsu and Listowsky, 1973). [b] The predominant component of horse spleen or rat liver ferritin preparations contain about 2000 iron atoms/mol of protein (based on a protein mol wt 450,000, Niitsu and Listowsky (1973)), and this component is considered by us to be the iron saturated species of ferritin. The species containing over 3000 iron atoms represented a very small fraction of the total ferritin. Similar distributions were obtained in CsCl gradients.

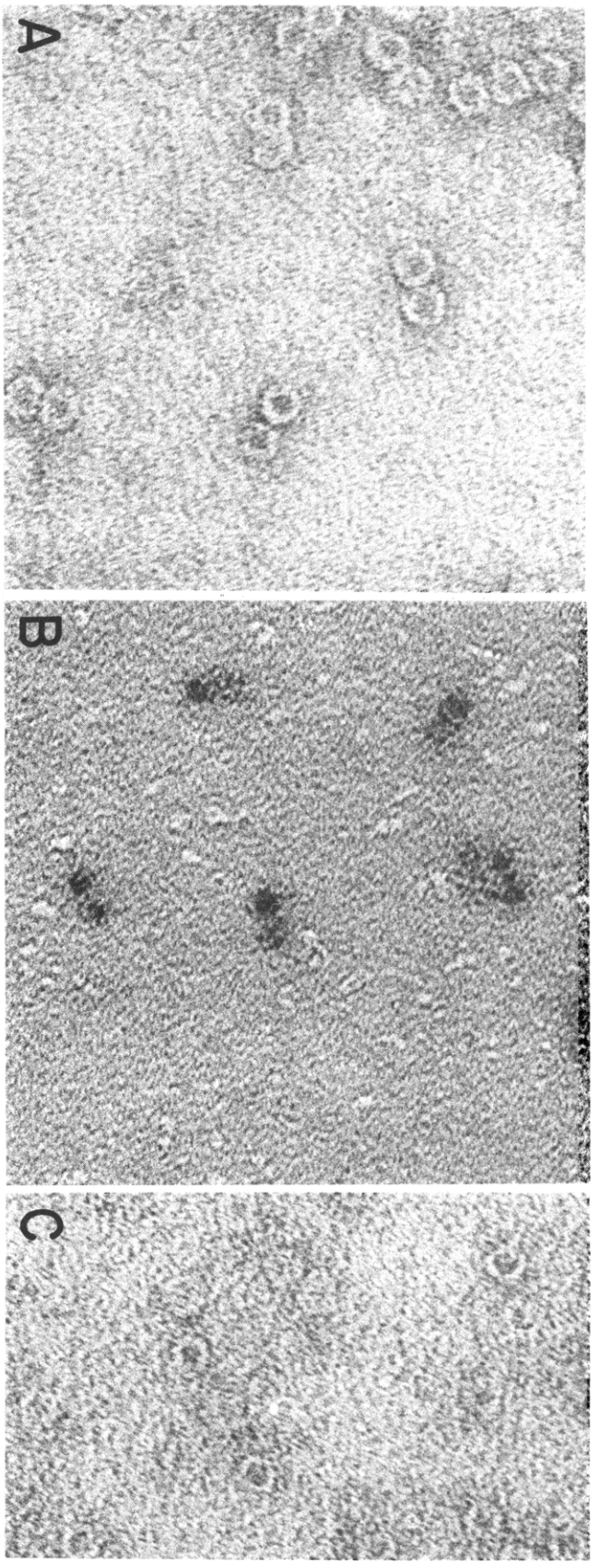

FIGURE 3: Electron micrographs of ferritin monomer and dimer: plate A, negatively stained dimer; plate B, iron cores of the dimer on an unstained grid; and plate C, negatively stained monomers; magnifications × 542,000; see Experimental Section and text for other details of preparation.

pear to contain greater amounts of iron than do the saturated monomers. The overall conformations of the monomer and the dimer were essentially identical, based on their respective circular dichroism spectra (Listowsky *et al.*, 1972) ($[\theta]_{221} = -21{,}000 \pm 2000$ for all fractions).

A number of experimental conditions and reagents were employed to attempt to cleave the dimer to monomer. The relative effectiveness of these substances was monitored by extent of cleavage of dimer as determined by scanning the reaction products after electrophoresis in polyacrylamide gels. The results are summarized in Table II. Evidently 60% sucrose, 80% ethylene glycol, or other organic solvents which have little effect in the overall conformation of the protein (Listowsky *et al.*, 1967) also had no effect on the dimer, nor on the ratio of monomer to dimer. Reduction of the iron of the dimer with sodium hydrosulfite at pH 4.6 and subsequent removal of the iron by dialysis as a bipyridyl complex, the classical method for the preparation of apoferritin, produced an iron-free dimer, but did not induce any substantial increase in the amount of monomer in the dimer preparation. Agents for chelation of iron also had no effect on the dimer. The most effective dissociating agents found were those that cleave disulfide bonds, such as mercaptoethanol and dithiothreitol. The effectiveness of a sulfhydryl reagent was dependent on its concentration, and very high concentrations usually were required to drive the reaction to cause extensive conversion of dimer to monomer. Also, there was no additional increase in the amount of monomer formed by alkylation of the thiol reduced dimers, indicating that once dissociated by mercapoethanol or dithiothreitol the resultant monomers showed no tendency to dimerize.

Oligomeric monomer and dimer, respectively, were treated with 7 M guanidinium chloride (pH 3.5) which induces extensive unfolding of the protein and its concomitant dissociation into subunits (Listowsky *et al.*, 1972). The guanidine was then removed by stepwise dialysis against lower concentrations of guanidinium chloride and finally against water, causing reconstitution of whole apoferritin molecules. The dimer subunits thus could be compared to those of the monomer to ascertain if a specific capacity for pairing is inherent in the subunits of the dimer or if any structural features of the dimer subunits differ from those of the monomer. The results obtained indicated that the predominant reconstitution products from both the monomer and dimer subunits are similar only to ferritin monomers. The structure and conformation, as determined by electron microscopy, and circular dichroism and electrophoretic mobilities (Table II) were that of monomers. The dimer subunits did form small amounts of reconstituted dimer, but these usualy represented less than 5% of the total product. In addition, preliminary analysis on sodium dodecyl sulfate polyacrylamide gels showed that the subunit patterns for ferritin monomers were indistinguishable from those obtained with the oligomeric dimers (Y. Niitsu, K. Ishitani, and I. Listowsky, unpublished observations). A dimerization capacity was observed with the subunits that were not prepared in the presence of thiol reagents, and these dimeric subunits could be eliminated by incubation in the presence of mercaptoethanol.

TABLE II: Estimations of Ferritin Dimer Content by Gel Electrophoresis.

Conditions[a]	% Oligomeric Monomer	% Oligomeric Dimer
Dimer untreated H_2O (pH 7.0)	7	93
Apoferritin of dimer prepared by hydrosulfite reduction under N_2; or 80% ethylene glycol, or 60% sucrose; or 1% sodium dodecyl sulfate; or nitrilotriacetic acid; or EDTA, or dilution to 0.05 mg/ml	No Change	
0.2% Mercaptoethanol	48	52
0.5% Mercaptoethanol	50	50
1.0% Mercaptoethanol[b]	56	44
10.0% Mercaptoethanol[b]	73	27
0.5% Mercaptoethanol, + tenfold excess iodoacetamide	49	51
1% Dithiothreitol[b]	65	35
1% Sodium dodecyl sulfate + 1% mercaptoethanol[b]	52	48
Same as above, carboxymethylated[b]	54 27	45 73
7 M Guanidinium chloride (pH 3.5), protein subunits reconstituted by removal of the guanidinium chloride	96	4
Native monomer, 100 mg/ml	98	2

[a] 100 μg of dimer was used for each experiment. The sample was incubated for at least 1 hr with the indicated reagents. [b] Some release of iron was also attendant to these reactions.

Discussion

Conflicting reports continue to appear in the literature concerning the stability of ferritin oligomers. Thus, Richter and Walker (1967) interpreted their light scattering data in terms of a reversible association–dissociation of the ferritin monomers and oligomers, and Crichton (1971) has proposed that hydrophobic forces may be involved in this process. In a recent study, Jaenicke and Bartmann (1972) indicated that the oligomers were not observed in sedimentation velocity patterns of ferritin mixtures studied at high dilutions, and therefore a reversible dissociation mechanism was also proposed by these authors. In contrast, Williams and Harrison (1968), on the basis of an electron microscopic analysis, suggested that the oligomers of ferritin were stable, and that no monomer–oligomer equilibrium existed. Other workers have also reported that ferritin monomers were stable (Bjork and Fish, 1971; Listowsky *et al.*, 1972). This study presents evidence that the oligomers of ferritin are stable at high dilution for long periods of time, and after exposure to a variety of reagents and environmental conditions. Under the conditions studied the monomer shows no tendency to aggregate nor do the oligomers dissociate to monomer. The system therefore does not follow the expected course of a reversible association–dissociation process.

Several possibilities have been considered in an effort to define the nature of the forces that unite the monomers into oligomers. Although Williams and Harrison (1968) did not determine the mechanism of oligomer aggregation, their data were compatible with an aggregation mechanism similar to that of the subunit association process in the monomer itself. These authors analyzed unstained electron micrographs and reported that in some molecules the average center-to-center

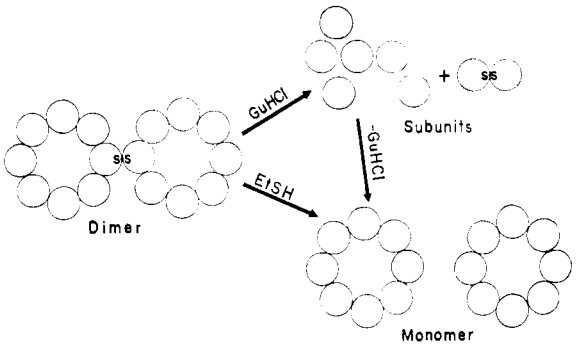

FIGURE 4: Schematic representation of ferritin dissociation. Each monomeric unit probably consists of 20–24 subunits. The subunits marked with an S are those involved in the cross-linking process. EtSH is mercaptoethanol and GuHCl is guanidinium chloride.

distance of the dimer micelles appeared to be substantially less than the expected 120 Å. This observation, together with the data shown here that the dimers have a higher iron content, could support an association mechanism consisting of micelle–micelle interactions of ferritin molecules containing incomplete protein shells that lack several subunits (Pape *et al.*, 1968), thereby exposing some parts of the micelles. A tendency for ferritin micelles to aggregate after denaturation and removal of the protein has been reported earlier (Listowsky *et al.*, 1972). A process involving the aggregation of ferritin cores containing less than a full complement of subunits has also been proposed in a recent consideration of possible aggregation mechanisms (Crichton, 1971). This micelle–micelle mechanism may be ruled out, however, since electron micrographs of negatively stained preparations (Figure 3) show that complete, or very nearly complete, protein shells are interposed between the micelles of the dimers.

Indeed, the results in this study indicate that thiol reagents are the most effective of the substances tested for cleavage of ferritin oligomers. Since much higher concentrations of ethylene glycol and other organic solvents did not affect the dimers, the hydrophobic character of the thiol reagents are apparently not responsible for inducing the dissociation. Based on the known specificity of thiol reagents, the linkages between the oligomeric units are in all likelihood covalent or coordinate covalent, probably involving cysteine residues, and, in fact, probably being interchain disulfide bridges. Also iron would appear to play a role in the formation of the cross-links, since the linkages are not found in apoferritin. Early studies by Mazur and Green showed that some iron is bound to the surface of ferritin molecules (Mazur and Green, 1959), so that one may consider that some iron may be available to participate in the cross-linking process by forming iron–mercaptide bonds or other types of iron–chelate linkages. However, there is no spectral evidence for such structures, and the chelating agents used in these and in earlier studies (Williams and Harrison, 1968) seem to have no effect on the state of aggregation of the ferritin oligomers. Interchain disulfide bridges therefore appear to be the mode of linkage in the oligomers.

Two types of dissociation that ferritin dimers may undergo are shown schematically in Figure 4. Mercaptoethanol or dithiothreitol dissociates the dimer to monomer. Guanidinium chloride induces the formation of ferritin subunits of which only a small proportion are linked *via* disulfide bridges (a minimum of only two of a total of 40–48 subunits need be linked to form dimer). After removal of the guanidinium chloride, the subunits reaggregate and predominantly form monomers. Since the CD results indicate that there are no gross conformational differences between monomers and dimers, modification of the protein prior to oligomer formation must be slight. The electron micrographs shown here also suggest that the morphological appearance of the protein shells of the oligomers is very similar to that of the monomers. Further, the amino acid composition and the peptide maps of ferritin oligomer fractions have been reported to be very similar to those of native ferritin (Suran and Tarver, 1965; Williams and Harrison, 1968). With the exception of the few disulfide linked subunits, therefore, the overwhelming population of subunits of the dimer have properties that are identical with those of ferritin monomers.

The factors that impart oligomer forming capacity to ferritin *in vivo* are presently obscure. It is noteworthy, however, that native apoferritin, the initial product in ferritin biosynthesis (Drysdale and Munro, 1966), contained no oligomers. The stage at which the oligomers are formed in the course of ferritin synthesis has not been elucidated to date. On a statistical basis, it is unlikely that two iron-free molecules link specifically and apoferritin dimers are therefore rarely found. On the other hand, since purified oligomers had almost no apoferritin at all, it is possible that iron loading plays a role or may actually mediate the process leading to oligomer formation.

Acknowledgments

The expert technical work of Mr. Randy Hecht and the generous help of Dr. Guiseppe Rettura are gratefully acknowledged. We are especially indebted to Dr. Cedric Raine for his assistance with the electron microscopy experiments.

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Nuclear Magnetic Resonance Studies of the Interaction of Peptides and Hormones with Bovine Neurophysin†

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ABSTRACT: The interaction of bovine neurophysin-II with lysine-vasopressin and with di- and tripeptide analogs of vasopressin and oxytocin was studied by nuclear magnetic resonance. Slow exchange between free and bound vasopressin at neutral pH allowed little information to be obtained about the hormone–protein reaction by the techniques used. However, fast exchange between free and bound forms of the smaller peptides was demonstrated and gave the following information. The protons on the aromatic ring in position 2 of most bound peptides are differentially broadened by binding, indicating dipolar relaxation of these protons by proximal residues on neurophysin, and confirming participation of this residue in the binding process. Differential line broadening on binding was also demonstrated for protons on the side chains of residues 1 and 3 of the bound peptide, suggesting, in agreement with conclusions drawn elsewhere, that these side chains also participate in binding. The pH dependence of line broadening in Ala-Tyr-PheNH$_2$ was shown to be in agreement with the contention that binding involves a bond between a protonated α-amino of the peptide and an unprotonated side-chain carboxyl of the protein. However, a conformational change in the protein at low pH and an altered form of some of the complexes at low pH are suggested. Nuclear Overhauser effects (NOE) were successfully applied to the study of peptide–neurophysin interaction and indicated that the aromatic ring in position 2 of the bound peptide is in close proximity to the single tyrosine of the protein and to two other classes of protons. The observed NOE effects were negative and an explanation for the direction of the effects as well as for their apparent distribution among both free and bound forms of the peptide are given in an Appendix.

The posterior pituitary gland contains the polypeptide hormones oxytocin and vasopressin in noncovalent association with a group of closely related proteins, the neurophysins (Acher *et al.*, 1955; Sawyer, 1961). Two principal bovine protein components, neurophysin-I[1] and neurophysin-II, which differ in amino acid composition but display similar hormone binding properties have been characterized (Rauch *et al.*, 1969; Breslow *et al.*, 1971; Breslow and Walter, 1972). Binding studies using hormone analogs have led to the conclusion that a primary electrostatic attraction between the protonated α-amino group of the hormones and a carboxylate group on the protein is enhanced by a nonpolar environment created by hydrophobic interactions between residues at positions 2 and 3 of the hormones and unspecified residues on the protein (Stouffer *et al.*, 1963; Ginsburg and Ireland, 1964; Breslow and Abrash, 1966; Hope and Walti, 1971). Breslow *et al.* (1971) have demonstrated that the principal qualitative features of the neurophysin-II–hormone interaction are preserved in the binding of tripeptides containing only the three N-terminal residues of the hormones and have also shown that the side chain in position 1 of the binding peptide is important in binding to neurophysin. With a view toward investigating further the molecular details of the protein–hormone inter-

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[1] Abbreviations used are: NP, bovine neurophysin; *S*-Me-Cys-Phe-IleNH$_2$, *S*-methyl-L-cysteinyl-L-phenylalanyl-L-isoleucinamide; *S*-Me-Cys-Tyr-PheNH$_2$, *S*-methyl-L-cysteinyl-L-tyrosyl-L-phenylalaninamide; Ala-Tyr-PheNH$_2$, L-alanyl-L-tyrosyl-L-phenylalaninamide; Met-TyrNH$_2$ L-methionyl-L-tyrosinamide; NOE, nuclear Overhauser effect; LVP, lysine-vasopressin; indor, internuclear double resonance; SSB, spinning side bands.