The Monomers and Oligomers of Ferritin and Apoferritin: Association and Dissociation†

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ABSTRACT: We have reinvestigated the association and dissociation of ferritin and apoferritin in phosphate buffer (pH 7.2, I = 0.05). When oligomer-enriched solutions of horse spleen ferritin were mixed with more concentrated, but unenriched solutions of horse spleen apoferritin, there was dissociation of the ferritin oligomers, as determined by polyacrylamide gel electrophoresis and from iron/protein ratios. Some evidence was also obtained for association of monomers in the mixture of ferritin and apoferritin after pelleting and redissolution of pellets in minimal volumes of the phosphate buffer. Monomer-enriched, biosynthetically labeled rat liver ferritin was pelleted, redissolved in minimal volumes of phosphate buffer, and separated by polyacrylamide gel electrophoresis; the fractions were isolated and counted. The results revealed that an association of monomers of the rat liver ferritin had taken place which doubled the concentration of dimers. However, our results also indicate that association by concentration was limited to a fraction of monomers.

The existence of oligomeric forms of ferritin and apoferritin has been repeatedly demonstrated (references in reviews of Crichton, 1973; Harrison et al., 1974). The three major fractions of horse spleen ferritin and apoferritin identified by gel electrophoresis and electron microscopy correspond to the three discrete components of horse spleen apoferritin found by analytical ultracentrifugation (Suran and Tarver, 1965; Harrison and Gregory, 1965; Kopp et al., 1966). These three fractions represent monomers, dimers, and trimers of ferritin molecules (Suran and Tarver, 1965; Harrison and Gregory, 1965; Kopp et al., 1966; Richter and Walker, 1967; Williams and Harrison, 1968). The nature of the force that binds monomeric units into dimers or trimers has remained controversial. The hypothesis that, in the oligomers, monomer units are firmly and stably linked by covalent bonds (Harrison and Gregory, 1965) was abandoned because a variety of reagents that attack disulfide, peptide, or ester linkages were found not to affect the distribution of monomers and oligomers of horse spleen ferritin or apoferritin in aqueous solutions (Williams and Harrison, 1968). Concentrations of dioxane, up to 50%, also failed to dissociate oligomers of horse spleen ferritin into monomers, so that the role of hydrophobic interactions, if any, is uncertain (Williams and Harrison, 1968). Hydrogen bonding has seemed unlikely because of the failure of 7 M urea, 6 M guanidine hydrochloride, or high concentrations of various salts to cause dissociation of the oligomers (Williams and Harrison, 1968). Richter and Walker (1967) calculated the association constants of horse spleen apoferritin at 25°C from light-scattering data, which indicated the presence of intermolecular ligands weaker than covalent bonds, but stronger than hydrogen bonds or van der Waals' forces. The values were consistent with reversible association involving noncovalent bonds. Jaenicke and Bartmann (1972) noted concentration-dependent dissociation of apoferritin oligomers by analytical ultracentrifugation while Björk (1973) did not detect this.

If covalently bonded oligomers of ferritin are present in situ in cells, one could expect to find an intracellular bonding mechanism, and this in turn would have significant biological implications. Thus, Williams and Harrison (1968) have suggested that stably linked oligomers may be involved in the transition from ferritin to hemosiderin. In any case, there must be reasons why the concentration of oligomers in preparations of ferritin from various tissues is usually between 10 and 15%.

In this paper, we report results of further investigations of the state of aggregation of ferritin and apoferritin molecules in solutions in vitro. These results indicate concentration-dependent dissociation and association of horse spleen ferritin and apoferritin molecules as well as concentration-dependent association of rat liver ferritin.

Experimental Procedure

General Plan. In one set of experiments, mixtures were prepared that contained highly diluted "oligomer-enriched horse spleen ferritin" and unfractionated horse spleen apoferritin in different proportions. Thus, in each original mixture, there were at least four species, viz., iron-containing ferritin oligomers, iron-containing ferritin monomers, apoferritin oligomers, and apoferritin monomers. If no dissociation–association takes place, the Fe/Pr[1] ratio of the oligomer fraction and of the monomer fraction obtained by preparative electrophoresis should be constant because ferritin and apoferritin have identical electrophoretic mobilities (Richter, 1963). However, if dissociation or association takes place in the oligomer–monomer system, then the Fe/Pr ratios would change. Thus, we used iron as a marker to study the dissociation–association behavior of horse spleen ferritin. It is known that under nonreducing and nonchelating conditions, iron in ferritin is very stable.

In another set of experiments, biosynthetically labeled ferritin was obtained from rat livers. The monomer fraction was separated. The radioactive, labeled monomer was then mixed with cold rat liver ferritin and ultracentrifuged to obtain a pellet. The pellet was redissolved in buffer, then

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[1] Abbreviation used is: Fe/Pr, iron to protein ratio.

subjected to disc gel electrophoresis. Radioactivity of various fractions was counted and compared with densitometric readings, which indicated the positions of monomers and oligomers.

Horse Spleen Ferritin. Horse spleen ferritin, two times crystallized, was purchased from Miles Laboratories Inc. (Kankakee, Ill.) and was further purified by passing through a 0.5m Bio-Gel A column (100–200 mesh, 75 cm long and 2.5 cm in diameter) and subsequent elution with phosphate buffer (0.02 *M*, pH 7.2). This procedure revealed impurities in the excluded volume that came off ahead of ferritin, and also impurities that were retained in the column after elution of ferritin. The purified horse spleen ferritin was precipitated with 30% (w/v) ammonium sulfate, redissolved in 0.02 *M* phosphate buffer (pH 7.2), and again passed through the 0.5m Bio-Gel A column. Afterwards, the ferritin was concentrated by precipitation in 30% ammonium sulfate and redissolved in distilled water. It was then dialyzed against the phosphate buffer. The purity was checked by disc electrophoresis in 6.5% polyacrylamide gel, using lutidine–glycine buffer as described below.

Horse Spleen Apoferritin. Apoferritin was prepared by reducing and simultaneously chelating iron from purified horse spleen ferritin with dithionite and α,α'-bipyridyl as described by Bielig and Bayer (1960). In this procedure the pH remained between 5.5 and 5.2 and no colloidal sulfur was formed. Purity of apoferritin was checked by polyacrylamide gel electrophoresis.

Oligomer-Enriched Horse Spleen Ferritin. Purified horse spleen ferritin was passed through a 5m Bio-Gel A (100–200 mesh) column (30 cm × 1.5 cm), and collected with a fraction collector (3 ml/tube). The material in each third tube was checked by polyacrylamide gel electrophoresis. Gels were stained for protein and those tubes which contained mainly oligomers (dimers and trimers) were pooled and concentrated and were called "oligomer-enriched horse spleen ferritin". The proportions of dimers to monomers were determined by densitometry of gels stained for protein.

Mixing of Horse Spleen Apoferritin and Oligomer-Enriched Horse Spleen Ferritin. Different amounts of oligomer-enriched horse spleen ferritin were first diluted in the phosphate buffer (0.02 *M*, pH 7.2) and allowed to stand at room temperature for 0.5 hr, then appropriate volumes of predetermined horse spleen apoferritin solutions were added so as to make final volumes of 5 ml. After mixing, the solutions were centrifuged for 5 hr at 40000 rpm in a Beckman SW 50.1 rotor (150000*g*). Supernatants were discarded, and buttons dissolved in 0.5 ml of phosphate buffer.

The recovery of ferritin and apoferritin was calculated and considered to be complete for the following reasons. The sedimentation coefficient of the lightest and smallest component of the system (apoferritin monomer) is ~17.6 S (Rothen, 1944). The *K* factor under conditions we used (40000 rpm, 150000*g*, for 5 hr in Beckman SW 50.1 rotor) was calculated as

$$K = \frac{\ln r_{max}/r_{min}}{\omega^2} \frac{10^{13}}{3600} = 65.96$$

Clearing time (t) = K/S = 3.75 hr. Hence, all components in the system should be pelleted in 5 hr.

Polyacrylamide disc gel electrophoresis was done, using 12 tubes. Duplicate gels were stained for protein, and the monomer–dimer ratios were determined by densitometry. Dimer and monomer bands were separated by cutting from the remaining 10 tubes of gel and the protein was eluted with the 0.02 *M* phosphate buffer at pH 7.2. Protein solutions thus obtained were dialyzed exhaustively against the phosphate buffer and their protein and iron content was determined.

Rat Liver Ferritin. Male Sprague-Dawley rats weighing 250–300 g were loaded with iron by injecting 1 ml of iron–dextran (50 mg of Fe/ml) intraperitoneally twice a week for 4 weeks prior to sacrifice (total: 400 mg of iron). Rats were killed by ether anesthesia and their livers excised. Ferritin was extracted by established methods (Drysdale and Munro, 1965). The ferritin was purified by gel filtration as was horse spleen ferritin (see above), and its purity was checked by polyacrylamide disc gel electrophoresis.

Monomer-Enriched Radioactive Rat Liver Ferritin. Male Sprague-Dawley rats weighing 250–300 g were loaded with iron as described in the last paragraph. Ferric ammonium citrate (400 μg of Fe/100 g body weight) was given intraperitoneally 5 hr prior to sacrifice. Two hours before sacrifice, uniformly ^{14}C-labeled L-amino acid mixture (New England Nuclear, Boston, Mass.) was injected intraperitoneally at a dose of 5 μCi/100 g body weight. Rats were killed by ether anesthesia and the livers excised. Ferritin was prepared and purified as described in the last paragraph, then passed through a 5m Bio-Gel A column (100–200 mesh) to separate monomers from oligomers.

Monomer-enriched radioactive rat liver ferritin was concentrated by centrifugation at 40000 rpm in a Beckman SW 50.1 rotor for 5 hr (150000*g*).

Polyacrylamide Disc Gel Electrophoresis. The method for analysis of ferritin was modified from Ornstein's (1964) original method (Richter, 1964), the electrophoresis apparatus being similar to that described by Davis (1964). Stock solution A was made up of 4 ml of 0.5 *N* KOH, 9.5 g of DL-glycine, 0.035 ml of *N,N,N',N'*-tetramethylethylenediamine, and water (to make 50 ml). Stock solution B was made up of 0.14 g of ammonium persulfate and water (to make 25 ml). Stock solution C was made up of 26 g of acrylamide, 0.8 g of bis(*N,N'*-methylenebisacrylamide), 15 mg of $K_3Fe(CN)_6$, and water (to make 50 ml). Buffer was made up of 13.7 g of DL-glycine, 32.8 ml of 2,6-lutidine, and water (to make 1000 ml). The pH of the buffer was adjusted to 6.6. Polyacrylamide gel (6.5%) was formed by adding six volumes of solution A to one volume of each, solution B and solution C, at room temperature. Polymerization was effected with a 100-W tungsten lamp at a distance of about 30 cm from the tops of the tubes during 1.5–2 hr. Appropriate amounts of sample were gently layered on top of each gel. Lutidine–glycine buffer was then carefully run down along the inside of each tube on top of the sample to fill up the tubes, which were then positioned in the electrophoresis apparatus. The two buffer chambers were filled up with the same lutidine–glycine buffer and an initial current of 8.3 mA/tube was maintained for 3 min, after which the current was changed to 4.2 mA/tube for an appropriate time interval. After the run was completed, the gels were stained for protein in a 0.5% solution of Amido Black 10B (Naphthol Blue Black) in 7% acetic acid for 4 hr, or, if stained for iron, the gels were immersed in equal volumes of 2% HCl and 2% $K_4Fe(CN)_6$ until a good Prussian blue reaction had developed. For destaining of Amido Black, the gels were placed in a rapid gel destaining apparatus (Metaloglass, Inc., Boston, Mass.), using a buffer of 7% acetic acid with a current of 250 mA passing across the gels. Destaining took 1 hr. The destained gels were immersed in 7.5% acetic acid and subjected to comparative densitometry

in a Canalco densitometer. According to Kruski and Narayan (1974), there is a linear relationship between optical density and concentration of ovalbumin stained with Amido Black 10B up to about 40 μg of ovalbumin. The quantities of ferritin or apoferritin protein we used did not exceed 30 μg/gel. For extraction of protein from the gels after the run, the desired gel segments were cut and ground in a mortar, then phosphate buffer (pH 7.2) was added and stirred. After extraction, the gels were removed by centrifugation at 25000g for 1 hr and the supernatants were exhaustively dialyzed against 0.02 M phosphate buffer (pH 7.2) before further analysis.

Gel Electrophoresis for Liquid Scintillation Counting. The radioactive ferritin was subjected to electrophoresis in 6.5% polyacrylamide gel in the lutidine–glycine buffer (see above). For each run of six tubes, approximately 20 μg of protein in a volume of 10 μl was layered on top of each gel. One of the gels was stained for protein. The others were fixed overnight with 10% trichloroacetic acid, then washed several times with 10% Cl_3CCOOH, and cut into 2-mm segments in a gel slicer. The gel slices were made to swell by adding NCS tissue solubilizer (Amersham/Searle Co., Arlington Heights, Ill.) and heated at 50°C in a tightly capped vial for 2 hr; 5 ml of Aquasol (New England Nuclear, Boston, Mass.) was then added to the vials, which were counted in a liquid scintillation counter. Quenching curves were determined for ferritin; counting efficiency was determined by the external standard method.

Determinations of Protein and Iron. Protein nitrogen was determined by the micro-Kjeldahl method, iron by atomic absorption spectrophotometry.

Results

As shown in Table I, horse spleen ferritin oligomers dissociate into monomers at very low concentration. The concentration of oligomer-enriched ferritin solution was 2.76 mg/ml, that of apoferritin solution was 0.81 mg/ml. The oligomer-enriched ferritin was first diluted, then mixed with apoferritin as shown in Table I. The resulting solutions were concentrated by ultracentrifugation at 150000g for 5 hr. The theoretical monomer to dimer ratios of each mixture were calculated from the known monomer–dimer composition of the apoferritin and ferritin used to make the mixture, and compared to the actual monomer–dimer ratios, determined by densitometry. Five determinations were made in each experiment and the mean and standard deviations calculated. In all experiments, the actual monomer–dimer ratios were higher than the estimated values, which indicates dissociation. The differences between estimated and actual monomer–dimer ratios were significant (t test). Percentages of dissociation of oligomers were calculated from the estimated and actual monomer–dimer ratios. They revealed increase of the monomer fraction upon dilution, hence dissociation of oligomers, although whether ferritin or apoferritin or both contributed to the dissociation cannot be established from the data in Table I. Because of the relatively small amounts of oligomeric apoferritin in experiments 3 and 4, we may assume that it was mainly ferritin that dissociated in these two experiments.

The iron to protein ratio of the monomer fraction in ferritin was 1:4.19; it was 1:4.01 in the dimer fraction. The apoferritin contained no detectable iron (sensitivity of atomic absorption method 0.5 ppm). As shown in Table II, the possibility that horse spleen ferritin monomers associated into oligomers was tested by determining iron to protein ratios of various mixtures of monomers and dimers (experiments 3 and 4). If one assumes that no dissociation or reassociation ever occurred in the system, then Fe/Pr in experiment 3 should have been 1:25.1, but was 1:14.8, a highly significant difference; in experiment 4, it should have been 1:8.65, but was 1:5.7, again a highly significant difference. If, instead, one assumes dissociation of ferritin oligomers (33% in experiment 3, 20.4% in experiment 4) without reassociation (assumption 2, Table II), then Fe/Pr in experiment 3 should have been 1:15.8; actually, it was 1:14.8, an insignificant difference. By contrast, in experiment 4, the theoretical Fe/Pr was 1:7.04, the actual one 1:5.7, which is a highly significant difference. The discrepancy between the results of experiments 3 and 4 will be considered in detail in the Discussion.

It should be noted that the horse spleen ferritin and apoferritin used in these experiments were found, by disc electrophoresis in polyacrylamide gels, to contain no other proteins.

Figure 1 shows the distribution of radioactivity of monomer-enriched rat liver ferritin after polyacrylamide gel electrophoresis. There was a large peak that corresponded to monomer (gel slices 10–20) and a long radioactive tail (gel slices 5–9), situated between the monomer and dimer peaks (gel slices 1–6). The nature of the tail was not clear, but it seems likely that this represented a product of radiolysis of the ferritin. We have found that solutions of ^{14}C-labeled rat liver ferritin (0.2%) undergo marked radiolysis upon storage at 4°C during 7 days. The analysis shown in Figure 1 was done on the day of preparation, in order to minimize radiolysis.

Since we had no direct evidence that the radioactive tail behind the monomer fraction noted after electrophoresis represented products of radiolysis, the possibility that it was due to an isoferritin was explored.

From a pool of 30 Sprague-Dawley rat livers, nonradioactive ferritin was prepared and subjected to gel electrophoresis as before. Upon protein staining, the monomer and oligomer fractions were the same as those found in the radioactive ferritin, containing the classical α (monomer), β (dimer), and γ (trimer) bands as well as traces of δ and ϵ bands that are normally present in rat liver ferritin (Richter and Lee, 1969).

Figure 2 shows the distribution of radioactivity in *concentrated,* monomer-enriched rat liver ferritin after disc electrophoresis in polyacrylamide gel. Ratios of monomers to oligomers were calculated by adding counts under each peak. The results show that the percentage of oligomers in samples of monomer-enriched rat liver ferritin increased upon concentrating the fraction by pelleting (150000g for 5 hr) and redissolving the pellets to make a final concentration of 2% protein. Table III gives the calculated results of six experiments. The differences between percentages of oligomers present before and after concentration were tested by t and F tests and proved to be significant at $p < 0.001$.

Discussion

The results of our experiments show (1) that, in oligomer-enriched solutions of ferritin, a significant fraction of oligomers dissociates into monomers upon dilution; and (2) that, upon concentration by ultracentrifugation, a significant fraction of monomers associates into oligomers. The processes of dissociation and association appear to be dependent upon concentration and not to involve oxidation or reduction of ferritin or the action of a chaotropic agent.

Table I: Dissociation of Horse Spleen Ferritin Oligomers upon Dilution.

	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Apoferritin[a]	Oligomer-Enriched Ferritin[a]	Apoferritin[a]	Oligomer-Enriched Ferritin[a]	Apoferritin[a]	Oligomer-Enriched Ferritin[a]	Apoferritin[a]	Oligomer-Enriched Ferritin[a]
1. Amount of protein added to mixture (mg)	0.726	0.0691	0.645	0.138	0.484	0.442	0.161	0.69
2. Amount of monomer Added to mixture (mg)	0.629 ± 0.071	0.013 ± 0.00027	0.559 ± 0.062	0.026 ± 0.001	0.42 ± 0.047	0.082 ± 0.002	0.140 ± 0.016	0.128 ± 0.003
3. Amount of dimer added to mixture (mg)	0.097 ± 0.011	0.036 ± 0.00078	0.086 ± 0.010	0.072 ± 0.002	0.064 ± 0.007	0.230 ± 0.005	0.021 ± 0.002	0.359 ± 0.008
4. Dilution of oligomer-enriched ferritin solution[b]	200×		100×		30×		20×	
5. Total monomer added (mg)	0.642 ± 0.071		0.585 ± 0.063		0.502 ± 0.049		0.268 ± 0.019	
6. Theoretical final concentration of monomer (mg/ml)[c]	1.284		1.170		1.004		0.536	
7. Total dimer added (mg)	0.133 ± 0.012		0.158 ± 0.012		0.294 ± 0.012		0.380 ± 0.010	
8. Theoretical final concentration of dimer (mg/ml)	0.266		0.316		0.588		0.76	
9. Monomer–dimer ratio (theoretical)	4.85 ± 0.6:1		3.68 ± 0.44:1		1.60 ± 0.27:1		0.71 ± 0.05:1	
10. Monomer–dimer ratio after mixing (densitometry tracing)[d]	6.6 ± 0:1		6.38 ± 0.1:1		3.07 ± 0.04:1		1.14 ± 0.03:1	
11. Significance of difference (lines 9 and 10)[e]	$p < 0.01$		$p < 0.001$		$p < 0.001$		$p < 0.001$	
12. % dissociation estimated[f]	23.3		36.3		33		20.4	

[a] Apoferritin solution contained 86.7 ± 1% monomer and 13.3 ± 0.3% dimer; oligomer-enriched ferritin solution contained 18.7 ± 0.5% monomer and 52 ± 0.8% dimer. Both were determined by densitometry. [b] Obtained by the ratio between initial protein concentration and resulting protein concentration of oligomer-enriched ferritin after dilution to 5 ml. [c] After sedimentation at 150000g (average) for 5 hr and redissolving of pellet in 0.5 ml of phosphate buffer (pH 7.2). [d] Five determinations. [e] t test. [f] Calculated from the theoretical and actual monomer-dimer ratio.

Table II: Association of Horse Spleen Ferritin and Apoferritin Monomers upon Concentration.

	Experiment 3		Experiment 4	
	Horse Spleen Apoferritin	Oligomer-Enriched Horse Spleen Ferritin	Horse Spleen Apoferritin	Oligomer-Enriched Horse Spleen Ferritin
Monomer protein added (mg)	0.42 ± 0.047	0.082 ± 0.002	0.14 ± 0.016	0.128 ± 0.003
Dimer protein added (mg)	0.064 ± 0.007	0.230 ± 0.005	0.021 ± 0.002	0.359 ± 0.008
Total monomer protein added (mg)	0.502 ± 0.049		0.268 ± 0.019	
Total dimer protein added (mg)	0.294 ± 0.012		0.380 ± 0.010	
Total monomer iron added (mg)	0.02 ± 0.0005		0.031 ± 0.0009	
Total dimer iron added (mg)	0.056 ± 0.001		0.0875 ± 0.002	
	Monomer	Dimer	Monomer	Dimer
Assumption 1 (no dissociation–association occurred) Estimated iron–protein ratio	1:25.1 ± 1.3	1:5.26 ± 0.2	1:8.65 ± 0.3	1:4.34 ± 0.19
Assumption 2 (dissociation of ferritin oligomers, no reassociation) Estimated iron–protein ratio	1:15.8 ± 1.26	1:5.25 ± 0.22	1:7.04 ± 0.39	1:4.31 ± 0.13
Iron–protein ratio calculated from resultant mixture	1:14.8 ± 1.56	1:5.8 ± 0.98	1:5.7 ± 0.63	1:4.75 ± 0.60
t test of significance between assumption 2 and actual results	$0.20 < p < 0.50$ insignificant[a]	$0.20 < p < 0.50$ insignificant	$p < 0.001$ significant	$0.02 < p < 0.05$
t test of significance between assumption 1 and actual results	$p < 0.001$ significant	$0.20 < p < 0.50$ insignificant	$p < 0.001$ significant	$0.02 < p < 0.05$

[a] See Discussion.

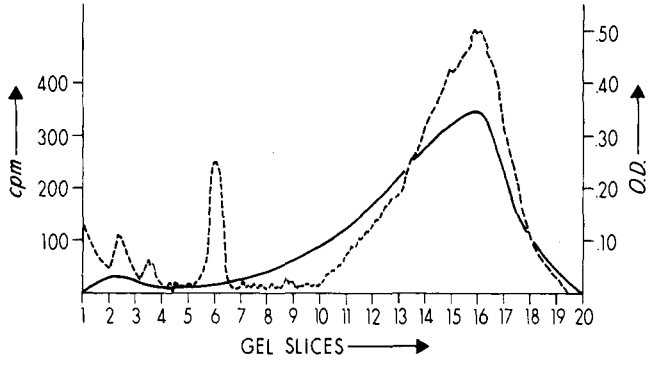

FIGURE 1: Distribution of radioactive monomer-enriched rat liver ferritin after polyacrylamide gel electrophoresis before concentration by ultracentrifugation (—) and densitometric tracing of carrier rat liver ferritin in same gel stained to show positions of monomers, dimers, and higher oligomers (- - -).

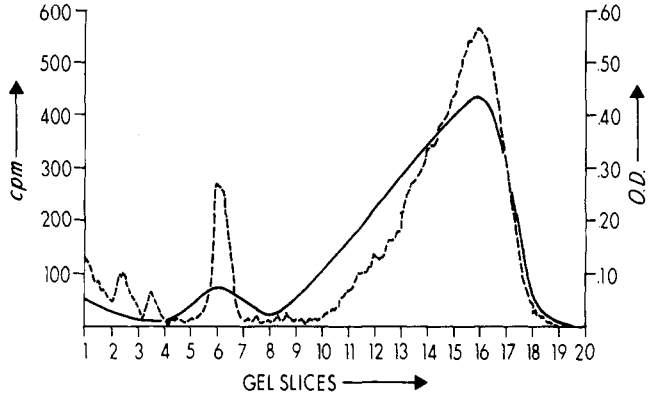

FIGURE 2: Distribution of radioactivity of *concentrated*, monomer-enriched rat liver ferritin after polyacrylamide gel electrophoresis (—) and densitometric tracing of carrier rat liver ferritin in same gel stained for protein (- - -).

The results of the first set of experiments demonstrate that the horse spleen ferritin monomer fraction increased upon dilution (Table I), and that the extent of dissociation was dependent upon concentration. The dissociation reached 20.4% when oligomer-enriched horse spleen ferritin with an initial protein concentration of 0.14 mg/ml was mixed with a solution containing 0.03 mg of horse spleen apoferritin/ml, but reached 36.3% when oligomer-enriched ferritin with an initial concentration of 0.0276 mg/ml was mixed with a solution containing 0.13 mg of apoferritin/ml. The degree of dissociation of the oligomers was less than expected at the lowest concentrations tested (0.014 mg/ml). However, the final monomer–dimer ratio (6.6) at the lowest concentrations tested indicates that dimers amounted to about 13%, which is close to the natural dimer content of horse spleen ferritin (10–15%).

The iron to protein ratios (Table II) gave insufficient information about the association of monomers into oligomers upon concentration. This might have been due to insufficient sensitivity of the methods used, but these ratios do indicate dissociation of oligomers into the monomers upon dilution. In experiment 3 (Table II), the iron/protein ratio in the final mixture did not, however, differ significantly from that expected if one assumes dissociation without reassociation. By contrast, the iron/protein ratio of the final mixture obtained in experiment 4 (Table II) indicates that

Table III: Percentage of Oligomers in Samples of Monomer-Enriched Rat Liver Ferritin before and after Concentration

Expt	Before Concentration	After Concentration
1	3.16	6.62
2	2.45	6.41
3	2.50	8.47
4	4.00	6.58
5	3.91	5.75
6	4.33	6.67
Av	3.39 ± 0.81	6.74 ± 0.91

association as well as dissociation had taken place. To account for the discrepancy between results of experiments 3 and 4, the following explanation is offered. If in experiment 3 (Table II) not only some oligomers of ferritin, but also some of apoferritin dissociated, the theoretical iron/protein ratio in the monomers would be significantly lower than 1: 15.8 (assumption 2, Table II). Since the amount of apoferritin used in experiment 3 was three times that used in experiment 4, the participation of apoferritin dimers in the dissociation process in experiment 3 probably should not be neglected, as it is under assumption 2 in Table II.

In the second set of experiments, with ^{14}C-labeled rat liver ferritin, reassociation of monomers into oligomers upon concentration was clearly evident. Sedimentation into a pellet produced a significant association of ferritin monomers into dimers and higher oligomers, which remained upon subsequent dilution to 2 mg/ml. Again, in these experiments, no reagents were used that would have cleaved covalent bonds.

The force that binds the molecules remains uncertain. Conflicting reports on this point have been cited in the introduction. Although Niitsu and Listowsky (1973) postulated, on the basis of experiments with thiols, that a disulfide linkage is responsible for the formation of dimers from monomers, they had reported earlier (Listowsky et al., 1972) that trimers of horse spleen ferritin dissociated on standing, in the absence of thiol reagents. They also found (Niitsu and Listowsky, 1973) that, after treatment with sodium dodecyl sulfate, the electrophoretic properties of subunits of ferritin monomers were indistinguishable from those of subunits obtained from dimers of ferritin. If the dimers had been constituted of monomers in disulfide linkage, a fraction of the subunit preparation ($\frac{1}{24}$ by weight) should have differed from subunits obtained through dissociation of ferritin monomers, i.e., there should have been a fraction of subunit dimers since treatment with dodecyl sulfate does not break disulfide linkages.

Richter and Walker (1967) concluded from light-scattering data that apoferritin monomers prepared from horse spleen ferritin by extraction of iron can reversibly associate in a concentration-dependent manner. Jaenicke and Bartmann (1972) reached a similar conclusion from sedimentation velocity studies, formation of dimers of horse spleen apoferritin being favored at concentrations of 0.1 mg/ml or higher, and formation of trimers and higher orders of aggregates at $C > 0.4$ mg/ml. On the other hand, Björk (1973) reported that in sedimentation equilibrium, light-scattering, and sedimentation velocity studies, he obtained no evidence for a reversible association of the monomer of horse spleen apoferritin, nor for dissociation of dimers. These contradictory findings may have resulted from the use of different methods.

As here reported, when polydisperse solutions of horse spleen apoferritin were mixed with solutions of ferritin that had been prepared by diluting oligomer-enriched solutions of ferritin, no increase in the oligomeric content of the final mixture resulted. While this outcome might have been only apparent, i.e., due to insufficient sensitivity of the analytical methods used, it could have been due to differences between properties of ferritin and apoferritin. Williams and Harrison (1968) found that a fraction of horse spleen ferritin which originally contained close to 100% monomer had, after reduction by dithionite in sodium acetate buffer at pH 5.0, dimers and trimers in proportions close to those in unfractionated preparations of ferritin. Similar observations were reported by Suran and Tarver (1965), who thought that conversion of ferritin to apoferritin by reduction and removal of iron resulted in changes in the "degree of aggregation" (i.e., in intermolecular association or associability). They also found that freeze-drying and storage of ferritin as a precipitate in 30% ammonium sulfate appeared to increase the proportion of oligomeric forms of ferritin in solutions prepared later from the precipitate. In our experience (Table III), pelleting of monomer-enriched rat liver ferritin from solution also increased the oligomeric fraction.

Although the experiments here reported indicate that ferritin monomers do associate in vitro and that ferritin oligomers dissociate in a concentration-dependent manner, concentration per se is unlikely to be the only factor affecting these processes. Structural heterogeneity in populations of ferritin molecules (e.g., small conformational differences, presence of deaminated sites, spurious binding of cations or anions in a fraction of molecules) may importantly influence the degree to which association or dissociation can take place.

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