Native and Subunit Molecular Weights of Apoferritin*

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ABSTRACT: The native molecular weight of horse spleen apoferritin was determined to be 440,000–465,000 by sedimentation equilibrium techniques. The subunit molecular weight was investigated by three methods: sedimentation equilibrium in 6 M guanidine hydrochloride, gel chromatography in the same solvent, and electrophoresis in sodium dodecyl sulfate–polyacrylamide gels. A value of 18,500 ± 500 was obtained in each case. These results indicate that apoferritin consists of 23–25 (most likely 24) subunits instead of 20, which has been the commonly accepted number.

Ferritin is the main iron-storage protein of the mammalian body. It has been shown to consist of a protein shell around a central core of iron in the form of a ferric hydroxyphosphate micelle (Farrant, 1954; Harrison, 1964). The protein shell can be prepared by reduction and chelation of the iron and has been termed apoferritin (Granick and Michaelis, 1943). Hofmann and Harrison (1963) have suggested that this high molecular weight protein consists of 20 identical subunits with molecular weights of about 24,000. The subunit molecular weight was not determined in an entirely satisfactory manner by these authors; therefore, we have undertaken a reevaluation of the molecular weights of apoferritin and its subunits with the use of more accurate methods. We find the native molecular weight of apoferritin to be 440,000–465,000 and the molecular weight of its subunits to be 18,000–19,000.

Materials and Methods

Horse spleen ferritin was obtained from Pentex Biochemicals, Kankakee, Ill. (lot 5-2, six-times crystallized, 91 mg/ml and lot 51, twice crystallized, 106 mg/ml), or was prepared by the method developed by Behrens and Taubert (1952). Apoferritin was prepared from this material by both methods described by Granick and Michaelis (1943). Further purification was obtained by sucrose density gradient centrifugation (McCarty *et al.*, 1968). Gradients, isokinetic for a particle density of 1.33, and with an initial sucrose concentration of 5%, were prepared in 30-ml tubes; special enzyme grade sucrose (Mann Research Laboratories, New York, N. Y.) was used. A volume of 0.6–0.8 ml of an apoferritin solution with a concentration of about 20 mg/ml was layered on each gradient. Centrifugation was performed in a swinging-bucket rotor, SW 25.1, in a Spinco Model L ultracentrifuge (Beckman Instruments, Palo Alto, Calif.) for 20 hr at a speed of 24,000 rpm and a temperature of 5°. After the run, the bottom of each tube was pierced with a syringe needle, and the effluent was collected in 1-ml fractions.

Analytical polyacrylamide gel electrophoresis on 5% gels was performed at pH 9.5 according to standard procedures given by Ornstein (1964) and Davis (1964), and at pH 3.3 in 0.9 M acetic acid. No stacking gel was employed in the latter case.

Sedimentation velocity and equilibrium experiments were carried out in a Spinco Model E ultracentrifuge (Beckman Instruments, Palo Alto, Calif.) at 25°.

Molecular weights of apoferritin in dilute salt solutions were determined by sedimentation equilibrium experiments using the short column meniscus depletion method described by Yphantis (1964). Measurements were performed in 0.1 M KCl in 0.05 M buffer. The buffers employed were sodium acetate (pH 4.6 and 5.5), sodium phosphate (pH 7.0), or Tris-HCl (pH 8.0). The initial protein concentration was between 0.2 and 0.5 mg per ml. Protein solutions were dialyzed for 24 hr at 5° against the appropriate solvent before the experiments. A 2.5° double-sector cell and a six-channel cell (Yphantis, 1964), both with sapphire windows, were employed and were filled to a column height of 3 mm. The An-J rotor was used at the low speeds necessary for equilibrium measurements of apoferritin. Equilibrium times were estimated from the semi-empirical equation given by Yphantis (1964) and attainment of equilibrium was checked by measuring the fringe displacements at several radial distances of two successive exposures taken 2–4 hr apart.

Molecular weights in 6 M guanidine hydrochloride (Gdn·HCl;[1] Heico Inc., Delaware Water Gap, Pa.) solutions were obtained with the long column meniscus depletion method described by Chervenka (1970). Advantages of a long column over a short column method are a lower operating speed, which is of importance in experiments with 6 M Gdn·HCl as solvent, and assurance of meniscus depletion even when the sample contains material with a molecular weight lower than that of the main component. The layering technique introduced by Chervenka (1970) markedly reduces the times necessary for attainment of equilibrium in such long columns, and perfect matching of the heights of the solution and solvent columns is also achieved. With Gdn·HCl, it was necessary to dialyze the protein solutions for a minimum of 3 days at 25° prior to ultracentrifugal analysis. The effective partial specific volume of apoferritin in Gdn·HCl was assumed to be the same as its partial specific volume in dilute salt solutions.

Gel chromatography in 6 M Gdn·HCl of fully reduced and carboxymethylated apoferritin was performed as described by Fish *et al.* (1969). In addition, apoferritin labeled with ^{125}I was subjected to gel chromatography in 6 M Gdn·HCl to-

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[1] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate.

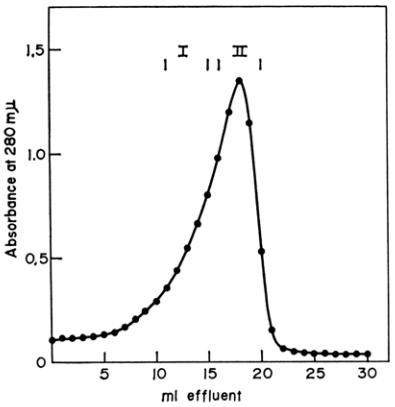

FIGURE 1: Sucrose density gradient centrifugation of apoferritin. A volume of 0.65 ml of a solution of a concentration of about 20 mg/ml was applied to the gradient. Centrifugation was at 24,000 rpm and 5° for 20 hr. The bottom of the tube is to the left.

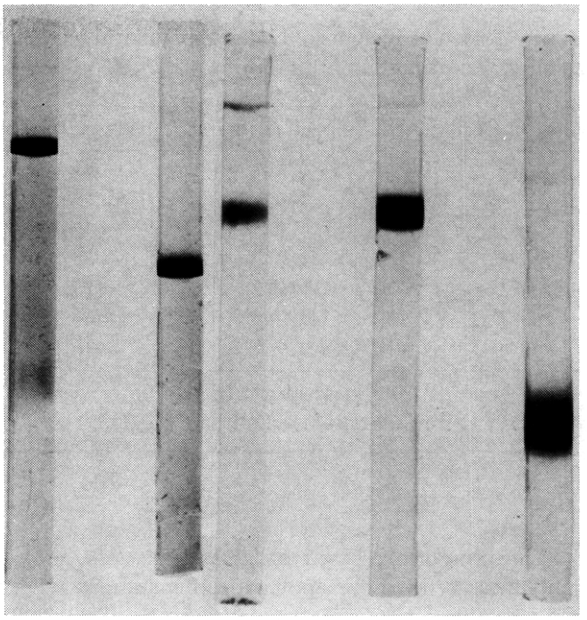

FIGURE 2: Analytical polyacrylamide-gel electrophoresis of apoferritin fractions I and II from sucrose density gradient centrifugation. (a) Fraction II, 25 μg/gel, 60 min (left) and 120 min (right); pH 3.3, 2 mA/gel. The cathode was at the bottom. The apparent band in the lower part of the left-hand gel does not contain protein, but is a brown discoloration of the gel. (b) Fraction I, 15 μg/gel, 60 min (left); fraction II, 50 μg/gel, 60 min (middle)and 120 min (right); pH 9.5, 4 mA/Gel. The anode was at the bottom.

gether with unlabeled myoglobin and α-chymotrypsinogen A as internal standards. The labeling procedure was method A described by Talmage and Claman (1967). Aliquots of selected effluent fractions were dissolved in 20 ml of scintillation fluid, and the precipitate of Gdn·HCl that formed was allowed to settle before the weak β emission of ^{125}I was counted in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument, La Grange, Ill.).

Electrophoresis in the presence of SDS was performed essentially according to the procedure of Shapiro *et al.* (1967), as modified by Weber and Osborn (1969). Proteins were reduced in 1% SDS and 1% mercaptoethanol (pH 7.0) for 16 hr at 37°. Electrophoresis was performed on gels 15 cm in length. After staining and subsequent destaining, the gels were scanned at 650 nm in a Beckman Model DU spectrophotometer (Beckman Instruments, Palo Alto, Calif.) equipped with a Gilford gel scanning device (Gilford Instrument Laboratories, Oberlin, Ohio). Calculations were made as suggested by Weber and Osborn (1969). Carbonic anhydrase, α-chymotrypsinogen A, myoglobin, lysozyme, and cytochrome *c* were used as standards.

Protein concentrations were determined spectrophotometrically in a Cary 15 spectrophotometer (Cary Instruments, Monrovia, Calif.). An $E_{280}^{1\%}$ of 8.6 was used for apoferritin (Hofmann and Harrison, 1963).

Results

All apoferritin samples prepared by the classical procedures had a pale yellow color. This was found to be associated with a minor component which sedimented very rapidly in the ultracentrifuge. In addition to this impurity, two minor fractions with sedimentation coefficients greater than that of apoferritin, the main component, were also found. These probably were the β- and γ-apoferritins suggested by other investigators to be dimers and trimers, respectively, of the α-apoferritin monomer (Richter, 1964; Suran and Tarver, 1965; Harrison and Gregory, 1965; Kopp *et al.*, 1966). Sucrose gradient density centrifugation was employed to separate monomeric apoferritin from the impurities. Figure 1 shows the results of such a separation. The yellow impurity present in the starting material was concentrated at the bottom of the bottom of the tube and was not eluted. The recoveries in all experiments were at least 80% of the applied material, as estimated spectrophotometrically. Since the amount of yellow component was included in the applied but not in the recovered material, the true recovery must be higher. The protein peak was divided into two parts, I and II, which were analyzed separately.

The two fractions were investigated by analytical polyacrylamide gel electrophoresis (Figure 2). The main part of the protein peak, fraction II, was found to contain only a minute amount of a slower migrating impurity (about 0.5% by densitometric measurements) both at pH 9.5 and 3.3, whereas fraction I, which was studied only at pH 9.5, contained 10–20% of this component. In sedimentation velocity experiments, fraction II showed only one sedimenting species, the sedimentation coefficient of which was 16.8 S at a protein concentration of 7 mg/ml. These results indicate that fraction II consists of highly purified α-apoferritin, and this fraction was used for all subsequent analyses.

The molecular weight of native apoferritin was determined by meniscus depletion sedimentation equilibrium experiments under various solution conditions (Table I). No size inhomogeneity was detected under the conditions employed as plots of log fringe displacement *vs.* r^2 were linear from a displacement of at least 80 μ to the bottom of the cell. Two examples of such plots are shown in Figure 3. The value for the partial specific volume of apoferritin, which is necessary for the calculation of the molecular weight, is somewhat in doubt. Rothen (1944) experimentally determined a $\bar{v}$ of 0.747. We have calculated a $\bar{v}$ of 0.733 from the amino acid composition reported by Crichton (1969). Preparations of apoferritin to date have contained various proportions of dimers and higher polymers, and also a yellow contaminant. This most certainly would have affected the pycnometric measurements of Rothen (1944). For these reasons, we feel the value of 0.733 is probably more accurate and have chosen to tabulate our molecular

TABLE I: Molecular Weights of Native Apoferritin by Meniscus Depletion Sedimentation Equilibrium.

Solution Condns[a]	Speed (rpm)	Wt-Av Mol Wt[b]
pH 4.6	9,945	438,000
pH 5.5	7,928	454,000
pH 5.5	9,945	435,000
pH 5.5	9,945	444,000
pH 5.5	11,272	415,000
pH 7.0	9,945	446,000
pH 8.0	9,945	456,000

[a] See Methods for a description of the buffer systems and protein concentrations employed. [b] For $\bar{v} = 0.733$.

TABLE II: z-Average Molecular Weights of Apoferritin in 6 M Gdn·HCl + 0.1 M Mercaptoethanol (pH 7.0) by Long-Column Meniscus Depletion Sedimentation Equilibrium.

Concn (mg/ml)	Speed (rpm)	z-Av Mol Wt $\phi'^{a} = 0.747$	z-Av Mol Wt $\phi' = 0.733$
0.6	42,040	18,800	17,800
0.6	47,660	20,100	19,000
2.2	42,040	19,200	18,300
2.2	47,660	18,400	17,500

[a] ϕ' is the effective partial specific volume of the protein in this solvent.

weight measurements based on this value. The molecular weights measured for our apoferritin preparations ranged between 415,000 and 456,000 with an average of 441,000. An average molecular weight of 456,000 for all determinations was obtained using $\bar{v} = 0.747$. The precision of these measurements, ±5%, is somewhat poorer than generally expected for meniscus depletion sedimentation equilibrium measurements and may be due, in part, to the influence of rotor vibrations which may occur at speeds less than 17,000 rpm (Yphantis, 1964).

The subunit molecular weight of apoferritin was investigated by several methods. Sedimentation equilibrium experiments in 6 M Gdn·HCl without reducing agent and at low protein concentrations (~0.5-mg/ml initial protein concentration) resulted in markedly curved plots which yielded molecular weights in the meniscus region of about 20,000 and in the region close to the bottom of the cell about 40,000. In experiments at the same rather low protein concentration in the presence of 0.1 M mercaptoethanol straight plots from a displacement of about 100 μ to the bottom of the cell were obtained which yielded molecular weights of 18,000–20,000. These results suggest aggregation of the denatured apoferritin subunits in the absence of reducing agent, presumably through disulfide-bond formation or disulfide interchange.

When the protein concentration was increased to about 2 mg/ml in the presence of reducing agent, plots of log fringe displacement *vs.* r^2 exhibited distinct curvature as illustrated in Figure 4. An unequivocal molecular weight of the components cannot be obtained, but the z-average molecular weight of the mixture, *i.e.*, the value obtained by extrapolation of the point weight-average molecular weights to the bottom of the cell, gives an approximation of the higher molecular weight component which is highly favored in this type of average. The M_z values determined in several experiments are given in Table II. It should be noted that the value used for the effective partial specific volume in 6 M Gdn·HCl was the same as was used for $\bar{v}$ in dilute salt solutions. The effective partial specific volume may actually differ from $\bar{v}$ by 0.01–0.02 (Hade and Tanford, 1967; Reisler and Eisenberg, 1969). This fact, together with the discrepancy between the measured and calculated $\bar{v}$ for apoferritin, make the sedimentation equilibrium molecular weight measurements in 6 M Gdn·HCl subject to a rather large inaccuracy. In any event, even when the larger $\bar{v}$ of 0.747 is used for the effective partial specific volume, the apparent subunit molecular weight is still substantially lower than that reported by Hofmann and Harrison (1963).

In order to investigate the relative amounts and sizes of the

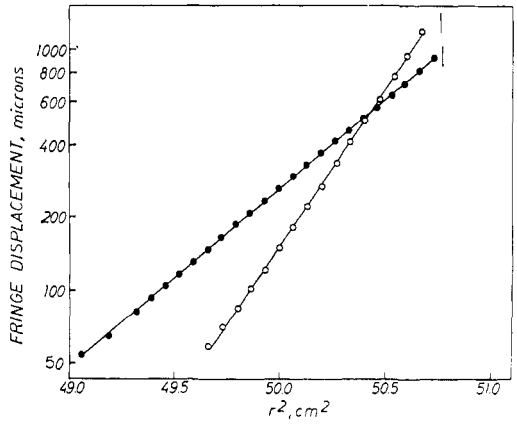

FIGURE 3: Short column meniscus depletion sedimentation equilibrium of apoferritin at 25° in 0.05 M sodium acetate buffer (pH 5.5), containing 0.1 M KCl. Protein concentration 0.2 mg/ml; 7928 rpm (filled circles) and 11,272 rpm (open circles). The vertical lines indicate the cell bottoms.

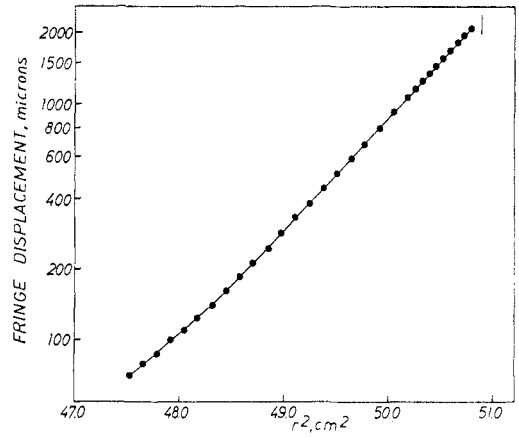

FIGURE 4: Long column meniscus depletion sedimentation equilibrium of apoferritin at 25° in 6 M Gdn·HCl (pH 7.0), containing 0.1 M mercaptoethanol. The protein concentration was 2.2 mg/ml and the speed 42,040 rpm. The vertical line indicates the cell bottom.

components present in the apoferritin subunit preparations, two other methods were used.

Gel chromatography of completely reduced and carboxymethylated apoferritin on agarose in 6 M Gdn·HCl (Fish *et al.*, 1969) gave the elution pattern presented in Figure 5a. One major peak with a molecular weight of 18,000–18,500 and two minor peaks of 13,000–14,000 and less than 5000, respectively, were observed. The values given were obtained in five separate experiments with preparations of apoferritin from different sources. Together, the two small peaks comprise about 10% of the total amount of protein recovered based on absorption measurements at 280 nm. This proportion was approximately the same for all preparations studied. Chromatography of ^{125}I-labeled apoferritin together with unlabelled internal standards yielded a value of 18,200 for the molecular weight of the major component. No attempt was made to similarly determine the molecular weights of the two minor components.

Three components were also detected in polyacrylamide gel electrophoresis experiments of reduced apoferritin in the presence of 0.1% SDS (Figure 5b). In 15 experiments on different preparations of apoferritin an average molecular weight of 19,000 ± 500 was obtained for the major component. Although the accuracy for this method decreases for polypeptides under 15,000 (Fish *et al.*, 1970), the molecular weight of the middle component was estimated to be between 11,000 and 14,000, whereas the size of the smallest component could only be determined to be less than about 10,000. Again, the two minor components together comprised about 10% of the total material.

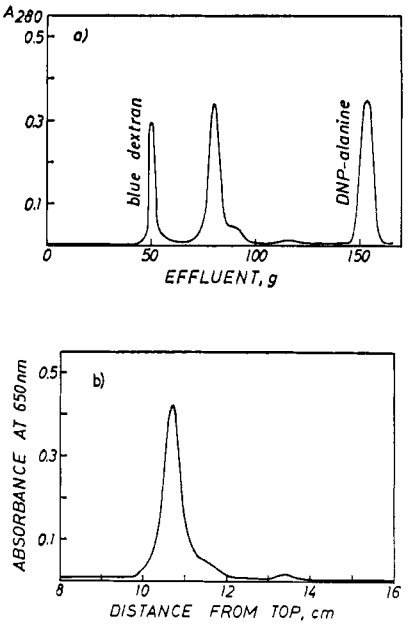

FIGURE 5: (a) Gel chromatography of fully reduced and carboxymethylated apoferritin on a 1.5 × 90 cm column of 6% agarose in 6 M Gdn·HCl. About 3 mg of protein in 0.2 ml of solution was applied to the column. Solute elution positions from the column were determined by weight instead of by volume. (b) Scan of an SDS–acrylamide gel electrophoresis experiment of reduced apoferritin. 25 μg of protein/tube; 450 min at 9 mA/gel.

Discussion

Several native molecular weights of apoferritin have been published in the literature. An early value of 465,000 was obtained by Rothen (1944) by combining extensive sedimentation with rather limited diffusion data. X-Ray diffraction analyses of apoferritin crystals together with measurements of crystal density yielded a value of 480,000 (Harrison, 1963). Light-scattering measurements gave molecular weights between 430,000 and 470,000 (Richter and Walker, 1967). All these analyses, however, were performed on unpurified apoferritin, which contained varying amounts of dimer and higher polymers. We have undertaken a determination of the molecular weight of purified apoferritin monomer (α-apoferritin) by sedimentation equilibrium methods as a first step toward a reevaluation of the number of subunits in the apoferritin molecule. Several experiments under different conditions gave an average value of 440,000 alternatively 465,000, depending on the choice of the value for the partial specific volume of apoferritin. The molecular weight of 440,000 probably is more accurate. The good agreement of these results with the value obtained by Rothen (1944) in the early days of ultracentrifugation is remarkable. Other published molecular weights also agree well with our value. No association of apoferritin molecules in the concentration range 0.1–1 mg/ml was detected; all plots of the logarithm of fringe displacement *vs.* r^2 were linear over this range. This is in conflict with an earlier paper by Richter and Walker (1967), who reported evidence, obtained by light-scattering measurements, for rather pronounced, reversible association of apoferritin monomers to dimers and possibly trimers over the same concentration range. This contradiction will have to be resolved by further work.

The subunit molecular weight of apoferritin has not been thoroughly investigated. Hofmann and Harrison (1963) studied the dissociation of apoferritin by SDS using sedimentation–diffusion and approach-to-equilibrium methods. They reported a value of 25,000–27,000 for the molecular weight of the subunits after subtraction of the estimated amount of SDS bound in the complex. This is, of course, a rather approximate approach. During the course of this investigation, Crichton and Bryce (1970) published an estimate of 18,000 for the subunit molecular weight of apoferritin as determined by SDS–gel electrophoresis. We have studied the size of the subunits of purified α-apoferritin with the use of three different methods, sedimentation equilibrium in 6 M Gdn·HCl, gel chromatography in 6 M Gdn·HCl, and SDS–gel electrophoresis. From a consideration of all results obtained, we suggest that the molecular weight of the apoferritin subunits is 18,500 ± 500. This figure and the native molecular weight of 440,000–460,000 suggests that the number of subunits in the apoferritin molecule is 23–25, instead of 20, as suggested earlier by Hofmann and Harrison (1963). The problem is complicated, however, by the presence in dissociating solvents of a small amount of two lower molecular weight components in all preparations we have studied. These components are not seen in dilute salt solutions either by sedimentation velocity or polyacrylamide gel electrophoresis, and therefore must be associated with the native apoferritin molecule. The two smaller polypeptide chains may be *bona fide* subunits of apoferritin, they may be extraneous proteins that are not an integral part of the apoferritin molecule but are only bound to its surface, or they may be the result of a specific, limited cleavage, either *in vivo* or *in vitro*, of some apoferritin subunits, with the two resulting fragments still associated under native conditions. The latter possibility is supported by the fact that the sum of the molecular weights of the two minor fractions approximates the molecular weight of the main component. So far the most obvious contradiction to this explanation is the fact that the two minor

components were found to be present in a rather constant amount in all preparations of apoferritin studied. These preparations were obtained by two different methods from two commercial lots of ferritin and from several batches of ferritin isolated from horse spleens in our own laboratory. Further work to reveal the nature of the two minor fractions is now in progress. It should be noted that only one component was observed by Crichton and Bryce (1970) in SDS–gel electrophoresis of apoferritin, probably because they stained the gels with Amido Black, a less sensitive dye than the one used in this investigation.

On the basis of X-ray data, Harrison (1963) has suggested apoferritin consists of 20 identical subunits situated at the vertices of a pentagonal dodecahedron. From the evidence presented here, it appears more likely that apoferritin is composed of 24 subunits. These results throw some doubt on Harrison's (1963) interpretation of the X-ray data. In an earlier paper Harrison (1959) has suggested another structure for apoferritin, namely that of 24 subunits situated at the vertices of snub cube. This structure is in better agreement with our data.

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