studies referred to above (Holme *et al.*, 1971) and the stoichiometry of these reactions are in accord with this mechanism.

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Denaturation of Horse Spleen Ferritin in Aqueous Guanidinium Chloride Solutions†

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ABSTRACT: The denaturation of ferritin and apoferritin in aqueous medium at room temperature has been described in terms of certain structural aspects of the protein. In aqueous solution, the circular dichroism spectra of native apoferritin and of ferritins with varying iron content were indistinguishable. The shape of the curves and magnitude of the far-ultraviolet ellipticity bands suggested that these proteins have substantial helical contents, and that almost 90% of their polypeptide chains may be in ordered structures. Sedimentation velocity studies showed that the iron-containing ferritin protein molecules were dissociated into subunits in 7.0 M aqueous guanidinium chloride solution of pH 7.5, but apoferritin remained in an aggregated state under these conditions. At pH 4.5, however, guanidinium chloride did induce the formation of apoferritin subunits. Circular dichroism studies indicated that extensive disruption of ordered structures accompanied subunit formation. The dissociation process was reversed significantly by removal of the guanidine. The reaggregated product exhibited electrophoretic and sedimentation properties similar to those of native apoferritin and its morphological appearance as viewed by the electron microscope, also resembled native apoferritin. Circular dichroism data obtained with the reassembled apoferritin suggested that most of its helical structure had been restored.

Ferritin is a well-characterized iron storage protein found in spleen, liver, and other mammalian tissues (Granick, 1942; Harrison, 1964; Crichton, 1971). The iron occurs as a hydrated ferric oxide–phosphate micelle, about 70 Å in diameter (Farrant, 1954; Fischbach and Anderegg; 1965; Spiro and Saltman, 1969) contained in the central cavity of a protein shell resembling a uniform hollow sphere with a diameter of approximately 120 Å (Harrison, 1963). Mechanisms of incorporation, retention and subsequent release of the iron are not fully understood. The molecular weight of the protein has been estimated to be 430,000–480,000 (Rothen, 1944: Richter and Walker, 1967), and for many years it was thought to consist of 20 subunits situated at the vertices of a pentagonal dodecahedron (Harrison, 1963; Hofmann and Harrison, 1963; Easterbook, 1970). Recent evidence, however, favors a structure containing 24 subunits (Bjork and Fish, 1971; Bryce and Crichton, 1971). Horse spleen ferritin preparations, considered to be highly purified, are heterogeneous with respect to iron content, and show three or more protein components on gel electrophoresis (Richter, 1963; Harrison and Gregory, 1965). The latter are considered to be stable aggregates of ferritin corresponding to dimers, trimers, and higher oligomers (Williams and Harrison, 1968).

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Ferritin is exceptionally stable and is not easily dissociated into subunits or denatured either at elevated temperatures or in the presence of urea (Hofmann and Harrison, 1963). In the present report, characteristics of the denaturation processes involving ferritin and apoferritin in the presence of aqueous guanidinium chloride are described. Subunit formation, as determined by sedimentation velocity, are correlated to conformational changes observed by means of circular dichroism (CD).

Experimental Section

Materials. Horse spleen ferritin preparations were obtained as the six-times-crystallized cadmium removed, or twice-crystallized cadmium-free samples, from Pentex-Miles Laboratories, Kanakakee, Ill. CD spectra and electrophoretic behavior on polyacrylamide gels of these commercial materials were identical with the respective properties exhibited by ferritin prepared from horse spleen in this laboratory using the methods of Granick (1942). Guanidinium chloride sucrose and urea used were of Ultra Pure grade obtained from Schwarz-Mann, Orangeburg, N. Y.

Methods. Linear gradients of 30–50% sucrose were prepared to fractionate ferritin on a density gradient. A Beckman-Spinco Model L-2 ultracentrifuge with a swinging-bucket SW 25.1 rotor was used for these experiments. After centrifugation at 25,000 rpm for 3 hr the contents of the tubes were fractionated to provide eight protein-containing bands (fractions I–VIII). The sucrose was removed either by ultrafiltration using Diaflo type UM-10 membranes (Amicon Corp., Lexington, Mass.), or by dialysis, and each protein fraction was characterized by its iron to nitrogen ratio (mg of Fe:mg of N). A colorless low-density fraction (fraction I) was obtained and found to contain less than 0.01 Fe:N ratio. Fraction II had a relatively low iron content with a Fe:N ratio of less than 0.1. Fractions III–VIII had increasingly greater iron contents, fraction VIII having a Fe:N ratio of 2.3. Protein concentrations were determined by the micro-Kjeldahl or Lowry (Lowry *et al.*, 1951) methods, and iron by the method of Wong (1928). The protein solutions were prepared in unbuffered aqueous solution unless otherwise indicated. The pH of the aqueous as well as the guanidinium chloride solutions was adjusted by addition of NaOH or HCl.

Sedimentation velocity experiments were carried out in a Beckman-Spinco Model E analytical ultracentrifuge using 12-mm standard cells with Kel-F centerpieces and schlieren optics. The solutions in 0.1 M NaCl were centrifuged at 50,740 rpm and the guanidinium chloride solutions at 59,780 rpm. Protein concentrations were 0.5–1.0% in all experiments. Measurements were usually started within 30 min after preparation of the solution. Apparent sedimentation constants were obtained at specific temperatures and guanidinium chloride concentrations, and were referred to 20°. In some cases, formal corrections leading to apparent $s_{20,w}$ values were made by use of the equations of Svedberg and Pedersen (1940). The values for viscosity and density of the guanidinium chloride solutions were obtained from the literature (Kawahara and Tanford, 1966).

Electrophoresis. Polyacrylamide gel electrophoresis according to the procedure of Ornstein (1964) was carried out in Tris-glycine buffer (pH 8.3) using 5% acrylamide and 2.5% spacer gels. Preparations were stained with Amido-Schwarz. Densitometer tracings of the gels were made using a Model 2410 linear transport attachment to a Gilford spectrophotometer.

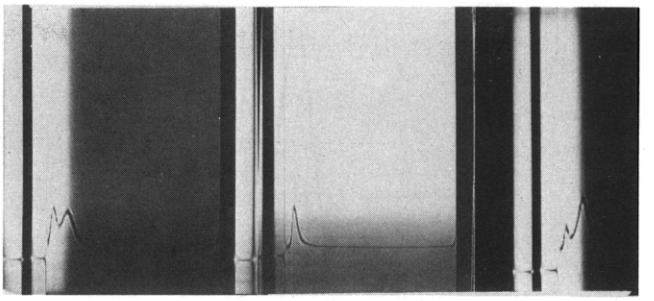

FIGURE 1: Ultracentrifugation patterns of (A) native ferritin preparations, (B) apoferritin (fraction I), (C) the high-density iron-containing ferritin (fraction VIII). Preparations B and C were obtained by means of sucrose density gradient centrifugation of native ferritin. Protein concentrations were 0.75% in 0.1 M NaCl (pH 7.0). Exposures taken 4 min (A and C) and 8 min (B), after a speed of 50,740 rpm was attained. A bar angle of 70° was used.

Circular dichroism spectra were obtained employing a Cary Model 60 spectropolarimeter with a Model 6002 CD accessory. Measurements were made at 27° in cylindrical cells with 1.0-, 0.1-, or 0.005-cm path lengths and duplicate experiments at two or more protein concentrations in the range between 0.02 and 0.1% were performed with no concentration effects observed. The slit width was programmed for a spectral band width of 15 Å at all wavelengths and an absorbance of 2.0 was not exceeded. The mean residue ellipticities $[\theta]$ in (deg cm^2)/dmole were calculated from the relationship: $[\theta] = \theta_{obsd}(MRW)/10lc$. A mean residue weight (MRW) of 113 was used; l = path length in centimeters; c = grams per milliliter.

Electron Microscopy. For negative staining, one drop of a 0.1% protein solution was placed on a Formvar-coated grid. The excess protein was removed by washing with a few drops of distilled water, and the grid was drained with filter paper. A drop of saturated aqueous uranyl acetate solution was added and then drained. The preparation was air-dried and micrographs then obtained using a Siemens Elmiskop I electron microscope with 80-kV accelerating voltage, and at a magnification of 40,000×.

Results

Native horse spleen ferritin consists of molecular species with different iron contents and appreciable amounts of apoferritin. In neutral aqueous solution the apoferritin component exhibited a 18.0S peak in the analytical ultracentrifuge, and the iron-containing molecules appeared as a broad diffuse faster moving band, with an average sedimentation coefficient of about 60 S (Rothen, 1944) (*cf.* Figure 1). We have observed that in 7.0 M guanidinium chloride at pH 7.5, the entire sedimentation pattern was altered and an exceedingly fast moving, colored component appeared. This material, isolated by preparative ultracentrifugation in 7.0 M guanidinium chloride, was essentially free of protein; and probably consisted of aggregates of the mineral core of ferritin. In addition, in 7.0 M guanidinium chloride, two colorless protein peaks of 4.2 S and approximately 0.5 S were obtained. Corrected formally for the effect of the guanidinium chloride, these yielded apparent $s_{20,w}$ values of 16 S and of about 2 S, respectively. The slower moving component probably represented subunits of ferritin. The falue of $s_{20,w}$ = 16 S for the faster component is approximately that of the sedimentation

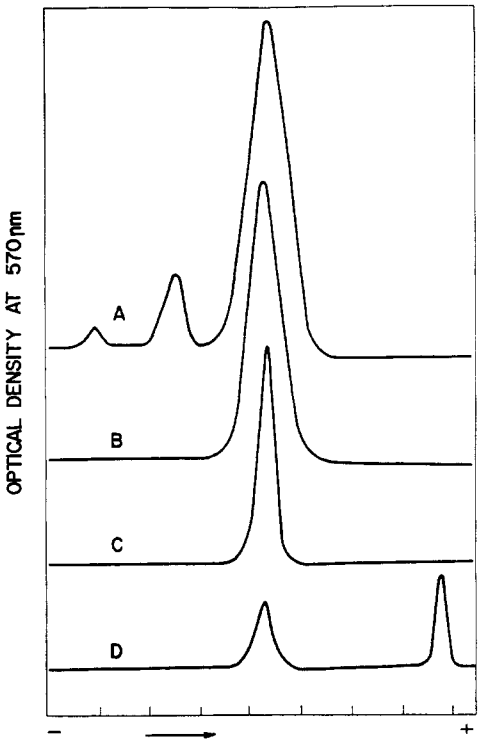

FIGURE 2: Densitometer tracings of polyacrylamide gels. Curve A, native ferritin; curve B, apoferritin; curve C, apoferritin after 4-hr treatment with 7.0 M guanidinium chloride at pH 7.5 and subsequent removal of the guanidine; curve D, apoferritin after 4-hr treatment with 7.0 M guanidinium chloride at pH 4.5 and subsequent removal of the guanidine. The amounts of protein added to each gel were 90, 70, 50, and 50 μg, respectively. The gels were stained with Amido Schwarz and excess dye removed by washing with 7% acetic acid for 24 hr.

constant of the protein moiety of ferritin, suggesting that part of the protein had not been dissociated into subunits by the guanidinium chloride. The sedimentation velocity pattern was not affected by preincubating the ferritin in 7.0 M guanidinium chloride for 48 hr at room temperature or by 10^{-3} M mercaptoethanol. The 4.2S peak disappeared however in 7.0 M guanidinium chloride solution of pH 4.5. Furthermore, lyophilized ferritin, after re-solution in 7.0 M guanidinium chloride of pH 7.5, also showed only the 0.5S component.

To gain further insight into the sedimentation behavior of ferritin in solutions of guanidinium chloride, the native material was fractionated by sucrose density gradient centrifugation into several components of varying iron content as described in the Experimental Section. The apoferritin preparation, was obtained from the gradient as a low-density, colorless fraction. This substance was homogeneous, exhibiting a sharp symmetrical peak ($s_{20,w}$ = 17.9 S) in the analytical ultracentrifuge (Figure 1B).

Native ferritin exhibited three electrophoretic bands on polyacrylamide gels corresponding to approximately 78% monomer, 18% dimer, and 4% trimer, with trace amounts of larger oligomers (Figure 2, curve A). Apoferritin isolated from the sucrose gradient had a single band with a mobility identical with that of the monomer of ferritin (Figure 2, curve B). Ferritin monomers isolated by elution from the polyacrylamide gels, showed no ordinary tendency to aggregate to dimers and trimers even over a period of several weeks. On the other hand, trimer, isolated in the same manner, readily dissociated and gave an electrophoretic pattern that reflected distribution of components similar to that of native ferritin (73:22:5 ratio of monomer:dimer:trimer).

Apoferritin, incubated as long as 48 hr in 7.0 M guanidinium chloride solution of pH 7.5, yielded a peak with an apparent sedimentation constant of 4.2 S. Dilution of the guanidinium chloride to 4 M resulted in the appearance of a 9.8S component similar to that obtained by direct solution of apoferritin in 4 M guanidinium chloride. Removal of the guanidinium chloride by ultrafiltration after dilution with 0.01 M phosphate buffer containing 10^{-3} M dithiothreitol, yielded a product with a 16.7S component. The polyacrylamide gel electrophoretic pattern of this material (Figure 2, curve C), consisted of a single component with a mobility identical with that of the monomer band of ferritin. In 7.0 M guanidinium chloride of pH 4.5, the 4.2S peak did not appear; instead, a very slowly sedimenting component of approximately 0.5 S was observed. These results indicate that no appreciable dissociation or subunit formation occurred at pH 7.5, while complete dissociation appeared to have occurred at pH 4.5. Also, the 4.2S peak obtained for native ferritin in 7.0 M guanidinium chloride of pH 7.5 perhaps was due to the apoferritin component of the ferritin preparation. Removal of the acidic guanidinium chloride also resulted in restoration of the monomer as observed by electrophoresis (Figure 2, curve D), and the appearance of a broad, diffuse 17S component in the analytical ultracentrifuge. In addition, the possibility of nonspecific association of subunits to form lower molecular weight aggregates was indicated by appearance of a more rapidly migrating band in the polyacrylamide gels (Figure 2, curve D) and by occurrence of some precipitation. The protein solutions obtained after removal of the guanidinium chloride were prepared for examination by electron microscopy. The negatively stained apoferritin preparations shown in Figure 3 all have similar overall molecular dimensions, with diameters of about 120 Å. The reassembled subunits appeared to have aggregated specifically to form structures morphologically indistinguishable from native apoferritin.

The sedimentation velocity patterns of the "high-density" fraction of ferritin obtained from the sucrose gradient, with an Fe:N ratio of 2.3 is shown in Figure 1C. The main component appeared to be the colored dense material with a sedimentation coefficient of 60 S. Small amounts of 17S apoferritin were also found in this fraction and in all of the iron-containing fractions isolated from the sucrose gradient. The source of the iron-free molecules found in the high-density fractions was not established. In 7.0 M guanidinium chloride at pH 7.5, this "high-density" ferritin fraction (Fraction VIII) exhibited the 0.5S component accompanied by only very small amounts of the 4.2S material. Under these conditions, apparently only the iron-containing ferritin molecules dissociated into subunits.

Circular dichroism spectra were used to study the conformational characteristics of ferritin and apoferritin and to monitor the changes occurring upon treatment with guanidinium chloride. The CD spectrum of apoferritin (Figure 4) showed negative ellipticity bands at 221 and 209 nm and a positive band at 192 nm. The relatively high ellipticity to absorption ratios exhibited by apoferritin in the vicinity of the 192-nm band, allow one to obtain accurate CD data for the protein in this spectral region. The overall magnitude and profile of the three amide ellipticity bands, suggest that the protein has a substantial helical content, and support our earlier conclusions based on optical rotatory dispersion (ORD) measurements (Listowsky *et al.*, 1967). It should be

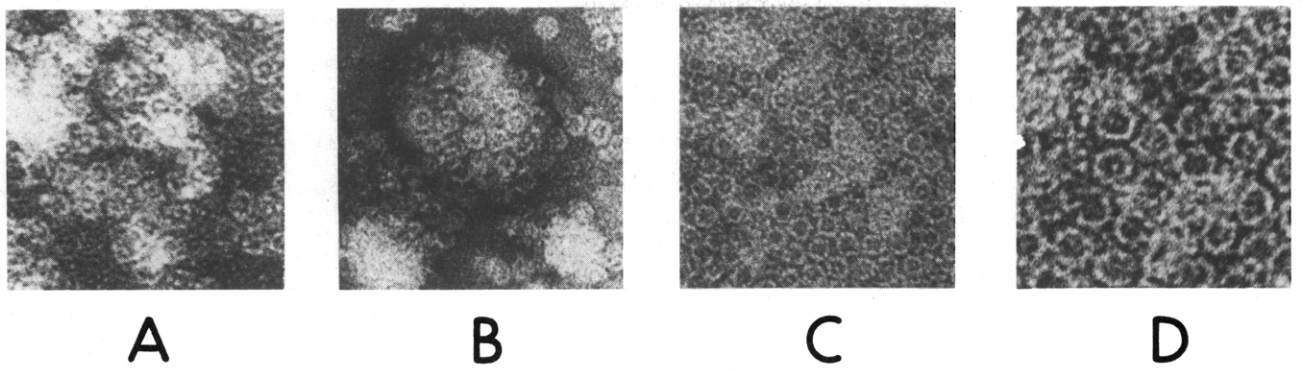

FIGURE 3: Electron micrographs of apoferritin. Magnification for A, B, and C, ×250,000, and ×450,000 for D. A is native apoferritin prepared as described in text. B is apoferritin after treatment with 7.0 M guanidinium chloride at pH 4.5 and subsequent removal of the guanidinium chloride. C is apoferritin after treatment with 7.0 M guanidinium chloride at pH 7.5 and subsequent removal of the guanidinium chloride. D is a further magnification of C.

noted that the peptide ellipticity bands of our native ferritin preparations are somewhat greater in magnitude than those reported by Wood and Crichton (1970). A complex CD pattern was also observed in the 300- to 260-nm spectral region, with multiple ellipticity bands and maxima at 291, 284, 278, and 270 nm. These bands may reflect the spatial orientation of the aromatic amino acid residues (Beychok, 1967). The broad negative ellipticity band above 300 nm described by Wood and Crichton was not observed by us.

The CD spectrum of apoferritin was not altered in guanidinium chloride solutions of concentrations as high as 5.0 M at pH 7.5. However, a gradual decrease in magnitude of the negative ellipticity bands was observed during a 4-hr period of incubation at room temperature in 7.0 M guanidinium chloride (pH 7.5). After 4 hr and until 24 hr under these conditions, the magnitude of the ellipticity at 221 nm reached a value approximately 70% of that obtained with untreated apoferritin (Figure 4). No substantial changes were observed after longer incubation periods, but further denaturation could be achieved by elevation of temperature. Absorbance of the guanidine precluded measurements in the region of the 192-nm band. In 7.0 M guanidinium chloride at pH 4.5, the negative ellipticity band at 221 nm as well as the entire CD spectrum from 300 to 260 nm were not apparent. The magnitude of the molar ellipticity at 221 nm was only about −400 (Figure 4, curve D). Thus the disruption of helical and other ordered structures was almost complete under these conditions. Readjustment of the pH to 7.5 did not restore any ellipticity at 221 nm within 24 hr. Partial reappearance of the 221-nm ellipticity band was achieved, however, by gradual removal of the guanidinium chloride by dialysis against 10^{-3} M dithiothreitol in 0.01 M phosphate buffer of pH 7.4 (Table I). Apoferritin subunits, prepared by treatment with detergent or acetic acid, previously had been shown to have a strong tendency to reassociate (Harrison and Gregory, 1968; Smith-Johannsen and Drysdale, 1969); the electron micrographs reproduced in Figure 3 also support this view.

The CD spectra of the iron-containing ferritin fractions (II–VIII) were identical with those obtained with apoferritin (Table I). Because of the high absorption of the iron core, CD measurements below 205 nm were not as precise as those obtained with apoferritin. Figure 4 shows that the negative peptide ellipticity bands of "high-density" ferritin fraction VIII were almost entirely absent in 7.0 M guanidinium chloride solution of pH 7.5; this contrasts with the case of apoferritin.

The CD spectra of apoferritin and ferritin fraction VIII were virtually unaffected by 10 M urea above pH 5.0. Both ferritin and apoferritin, however, showed a sharp decrease in magnitude of the ellipticity bands when the pH was changed from 5.0 to 4.0. The apparent unfolding in urea could not be reversed by readjustment of pH to 7.5.

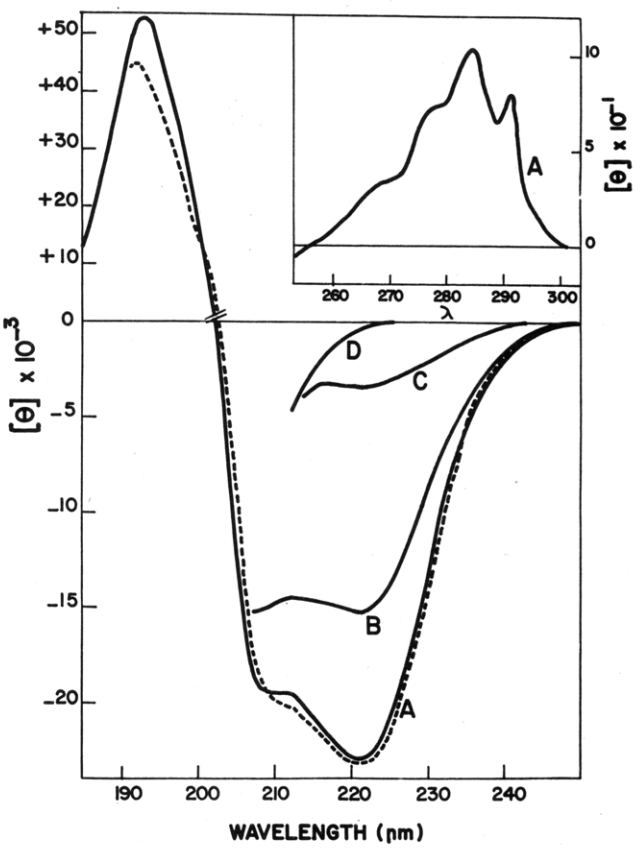

FIGURE 4: Circular dichroism spectra of ferritin and apoferritin: curve A, apoferritin (or a similar curve for native ferritin) in 0.1 M NaCl (pH 7.0); curve B, apoferritin in 7.0 M guanidinium chloride (pH 7.5); curve C, high-density ferritin (fraction VIII) in 7.0 M guanidinium chloride (pH 7.5); curve D, apoferritin in 7.0 M guanidinium chloride (pH 4.5). Dotted line represents the curve computed on the basis of the data shown in Table II. The temperature of all measurements was 27°. Measurements were generally started about 2 hr after preparation of final solution; the values remained constant for at least 8 hr thereafter.

TABLE I: Circular Dichroism Data at the Negative Ellipticity Extrema of Ferritin in Aqueous Medium at 27°.

Experimental Conditions[a]	Native Ferritin		Apoferritin (Fraction I)		"High-Density" Ferritin (Fraction VIII)	
	$-[\theta]_{221} \times 10^{-3}$	$-[\theta]_{209} \times 10^{-3}$	$-[\theta]_{221} \times 10^{-3}$	$-[\theta]_{209} \times 10^{-3}$	$-[\theta]_{221} \times 10^{-3}$	$-[\theta]_{209} \times 10^{-3}$
1. 0.1 M NaCl (pH 7.5)	23.2	20.0	23.2	20.0	23.2	20.0
2. 7.0 M guanidinium chloride (pH 7.5)	8.1	8.3	15.4	15.2	3.6	
3. 7.0 M guanidinium chloride (pH 4.5)	~0.4	5.0	~0.4	6.3	~0.4	
4. Reversal of 3, guanidinium chloride removed by dialysis against 0.01 M phosphate (pH 7.4)			16.8	17.6		
5. 6.0 M guanidinium chloride (pH 7.5)			19.6	16.9		
6. 8.0 M guanidinium chloride (pH 7.5)	4.7	5.2	10.1	10.3	0.5	
7. 10.0 M urea (pH 7.5)	23.4	19.7	23.8	20.4	22.9	20.0
8. 10 M urea (pH 4.0)			6.7	8.1	5.5	7.2

[a] All measurements were made using solutions of at least two different protein concentrations as described in the text. Most of the measurements in guanidinium chloride or urea were made about 4 hr after preparation of final solutions. The reported values remained constant for 24 hr. The experimental errors were less than ±5% in the 0.1 M NaCl solution, and generally less than ±10% at 221 nm and ±15% at 209 nm for the measurements in the guanidinium chloride solutions.

Discussion

The CD spectra reported here suggest that varying iron content does not influence the secondary structure of the protein moiety of ferritin. The ORD differences between native ferritin and chemically prepared apoferritin that were reported previously from this laboratory (Listowsky *et al.*, 1967) have recently been confirmed by CD studies (Wood and Crichton, 1971) and peptide-mapping experiments (Crichton, 1969). The present authors have suggested that structural modifications probably occur in the course of the reductive treatment of ferritin, and these account for the observed differences in optical activity. Apoferritin prepared in this manner is therefore not comparable to the naturally occurring apoferritin such as that isolated for use in the studies reported here. On the other hand, the present study shows that naturally occurring apoferritin, which appears to be structurally identical to the protein moiety of ferritin, may readily be distinguished from iron-containing ferritin by its behavior in solutions of guanidinium chloride. Ferritin is much more susceptible to denaturation by guanidinium chloride at pH 7.5 than is apoferritin.

Dissociation of ferritin or apoferritin into subunits, as observed in sedimentation velocity studies, apparently occurs with attendant conformational changes, as indicated by CD measurements. In neutral guanidinium chloride solutions, the iron-containing protein molecules are preferentially affected and the removal of the iron from the ferritin core is facilitated. This is accompanied by formation of subunits and disruption of ordered structures. The nature of the interaction of guanidinium chloride with the iron core is presently obscure. Electron micrographs of the fast-moving inorganic component obtained by treatment with guanidinium chloride indicate that this material may be composed of clusters of electron-dense, spherical micelles with dimensions identical to those of the iron cores of native ferritin.

In contrast to ferritin, apoferritin preparations remain largely in an aggregated form in 7.0 M guanidinium chloride at pH 7.5. The exceptional stability of apoferritin in the presence of high concentrations of guanidinium chloride or urea is uncommon for globular proteins. In this context, however, one may note that helical polyleucine and polyphenylalanine segments of some synthetic polypeptides also are not completely disrupted by these reagents (Auer and Doty, 1966).

Transitions between folded and unfolded proteins species in guanidinium chloride and urea are most pronounced at a pH of about 4.5, which is close to the isoelectric point of ferritin. Noteworthy are the findings that the ORD and CD spectra of ferritin and apoferritin in buffered or unbuffered aqueous solutions are not influenced by pH in the range from 2.5 to 10. Even at pH 1.5, the changes observed are relatively small compared to those observed in guanidinium chloride. The effect of pH in the presence of urea or guanidinium chloride may therefore be due to cooperative effects of the denaturing agent and the protein in a particular charged state. The main transitions between native and denatured states occurring near pH 4.5 suggests that the native protein structure is stabilized by the presence of carboxylate or other charged groups despite the large concentration of the strong electrolyte, guanidinium chloride. Changes in charge with pH would not cause the same structural changes in the protein in the absence of the denaturing agent.

CD spectra obtained in this laboratory for the helical and the random coil forms of polylysine and polyglutamic acid, and the ellipticity values for the β structure reported in the literature (Greenfield and Fasman, 1969) were employed to interpret the CD data of ferritin and apoferritin in terms of conventional aspects of secondary structure. The relative amounts of the three structural forms, shown in Table II, have been computed from best-fit analysis of the experimental curves (see dotted line in Figure 4). The inherent inaccuracies in such an assessment have been well documented (Saxena and Wetlaufer, 1971; Fasman *et al.*, 1970), but the CD data of some proteins analyzed by this method gave results consistent with those obtained by X-ray diffraction (Greenfield

TABLE II: Relative Amounts of α Helix, β Structure, and Random Coil of Ferritin and Apoferritin.[a]

	f_α	f_β	f_c
1. Ferritin and apoferritin in aqueous solution (pH 2.5–10)	0.52	0.37	0.11
2. Apoferritin in 6.0 M guanidinium chloride (pH 7.5)	0.54	0.18	0.28
3. Apoferritin in 7.0 M guanidinium chloride (pH 7.5)	0.45	0.07	0.48
4. Ferritin fraction VIII in 7.0 M guanidinium chloride (pH 7.5)	0.11	~0	0.89
5. Apoferritin in 7.0 M guanidinium chloride (pH 4.5)	0	~0	1.0
6. Reversal of no. 5 guanidinium chloride removed	0.53	~0	0.47

[a] To solve for f_α, f_β, and f_c, simultaneous equations of the type

$$f_\alpha X_{\alpha,\lambda_n} + f_\beta X_{\beta,\lambda_n} + f_c X_{c,\lambda_n} = Y_{\text{expt},\lambda_n}$$

were generated. The X_{λ_n} values are ellipticities at wavelength λ_n of the indicated structural forms, and are based on values obtained with the reference polypeptides (poly-L-lysine and/or poly-L-glutamic acid). Using up to 21 values of λ_n in the spectral range 240–190 nm, and imposing the added restriction that $f_\alpha + f_\beta + f_c = 1$, all combinations of these simultaneous equations were solved by computer. The values for f_α, f_β, f_c were obtained for each set of equations and averaged for all sets. The standard deviations for α, β, c were calculated and the average recomputed ignoring all points which were not within three standard deviations (3δ) above or below the original average. This process was repeated ten times (which is usually enough to allow the standard deviations to converge) and the final averages were used to generate a new set of Y values, to be compared to the experimental (Y_{expt}) values to obtain a best-fit analysis of the data.

and Fasman, 1969). The results presented in Table II are not intended to indicate absolute amounts of the structural forms, but to approximate relative differences observed under various experimental conditions. On the basis of this analysis, it appears that as much as 90% of the native ferritin and apoferritin exist in ordered forms. (Calculations based on proteins rather than on polypeptides as reference compounds (Saxena and Wetlaufer, 1971) also suggested substantial amounts of ordered structures with decreased β structure (about 15%) and increased α helix (about 60%).)

This highly ordered secondary structure of ferritin may account for its exceptional stability at elevated temperatures or in the presence of denaturing agents. The ratio of the 221 nm:209 nm ellipticities of apoferritin decreases as the guanidinium chloride concentration is increased from 6 to 8 M at pH 7.5 (Table I). If the data in Table II are used as a basis for estimating structure, this change appears to reflect the larger relative decrease in the amounts of β structure as compared to the decrease in α-helix, with the concomitant increase in the random coil form. In the acidic guanidinium chloride solution, the final ellipticity values approximate those expected for the random coil form of the protein.

The reaggregated product obtained after removal of the acidic guanidinium chloride from apoferritin also shows a CD pattern that is almost identical to that of the protein in 7.0 M guanidinium chloride (pH 7.5). One may therefore conclude that neutral guanidinium chloride induces an irreversible denaturation step (which may involve the disruption of β structures) but has very little effect on the state of aggregation or morphologic appearance of the protein. The second major denaturation stage observed in acidic guanidinium chloride or urea solutions is reversible. The reversal of this unfolding is accompanied by specific reaggregation of the protein subunits.

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Properties of the Apoprotein and Role of Copper and Zinc in Protein Conformation and Enzyme Activity of Bovine Superoxide Dismutase†

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ABSTRACT: Different conditions of metal removal from hemocuprein (bovine superoxide dismutase) have been tested to obtain a selective detachment of copper and zinc without inducing irreversible changes in the protein. Electron paramagentic resonance (epr), circular dichroism (CD), and optical spectra, electrophoretic behavior, and superoxide dismutase activity have been used to evaluate the reconstitution capacity of the different types of apoprotein. It has been observed that complete removal of zinc prevents copper from recombination at the native site and leads to the irreversible loss of the properties of the holoprotein. Zinc appears to contribute neither to the ultraviolet CD and absorption spectra of the bovine enzyme, nor to the enzymatic activity. Addition of copper to apoprotein restores the spectral, electrophoretic, and catalytic properties of the holoprotein to an extent proportional to the residual zinc left in the protein after metal depletion. The bovine enzyme contains six cysteic acid residues per 33,000 molecular weight. Since 4 SH groups per mole of protein are titrated in the absence of zinc, it appears that one disulfide bridge is present in the bovine enzyme. One tryptophan per mole is found. Only one lysine residue per 33,000 molecular weight is released by carboxypeptidase B after complete removal of zinc. The bovine enzyme copper is reversibly reduced by ferrocyanide. Addition of stoichiometric amounts of H_2O_2 in the absence of oxygen leads to disappearance of the copper epr signal; the original intensity is slowly recovered by exposure to air. It is proposed that copper is directly involved in the catalytic activity of the bovine enzyme, while zinc stabilizes the protein conformation which provides for a native copper site. The two equivalent copper sites could be located on two subunits. These are not identical but probably of equal size and held together by the single disulfide bridge.

McCord and Fridovich (1969) have shown that an enzymatic activity, namely superoxide dismutase activity, is associated with human and bovine erythrocuprein, the copper and zinc containing protein of red blood cells. This activity is lost by removing copper and partially restored by incubating the apoprotein in the presence of excess cupric ion. Most recently the bovine protein, to which we shall refer as the bovine enzyme, has been further characterized (Bannister *et al.*, 1971; Wood *et al.*, 1971; Keele *et al.*, 1971) and the amino acid composition has been reported. On the basis of electron paramagnetic resonance (epr) and circular dichroism (CD) spectra under various conditions evidence was also presented (Rotilio *et al.*, 1971) for the presence of three to four nitrogen ligands around the copper and for the accessibility of the copper site for water. It became apparent from these studies that reversible and selective removal of the metals, so that protein denaturation is avoided, might lead to decisive new information. The subsequent paper reports the properties of the apoprotein prepared by different procedures: the results obtained will be discussed with a view to the possible role of the two types of metal ions in catalyzing superoxide dismutation and maintaining the native conformation of the protein. Data on the primary structure of the protein will also be reported.

Materials and Methods

Protein and Other Chemicals. All chemicals were reagent grade and were used without further purification. Ultra Pure guanidine-HCl was obtained from Mann Research Laboratories. The bovine enzyme was purified from cattle blood according to McCord and Fridovich (1969). The molecular weight was assumed to be 33,000 (McCord and Fridovich, 1969). Protein concentration was determined by measuring absorbance at 258 nm, with an extinction coefficient (ϵ_{1cm}^{mM}) of 10.3 for the holoprotein and 5.8 for the apoprotein. These values were obtained from dry weight determinations and nitrogen analyses carried out with a Coleman nitrogen analyzer. The nitrogen content was found to be 16%.

Amino Acid Analyses. Amino acid analyses after hydrolysis with 6 N HCl were performed with a Bio-Cal BC-200 instrument, using a single column system. Total cystine–cysteine content was determined as cysteic acid after 6 N HCl hydrolysis in the presence of 0.21 M dimethyl sulfoxide (Spencer and Wold, 1969). Sulfhydryl groups were determined

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