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Conformation of Ferritin and Apoferritin in Solution. Optical Rotatory Dispersion Properties*

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ABSTRACT: Ferritin exhibits a plain optical rotatory dispersion (ORD) curve in the spectral region from 600 to 250 mμ. The curve obtained in the far-ultraviolet region with a trough at 233 mμ and peak at 198 mμ is typical of a protein containing an appreciable content of helical segments. The rotatory parameters which were obtained using the Moffitt–Yang and Shechter–Blout treatments of the data in the visible region of the spectrum suggest that nearly half of the native protein exists in the helical form. Ferritin fractions of varying iron content were obtained by ammonium sulfate fractionation or by density gradient centrifugation. The rotatory properties were independent of the iron content. Apoferritin prepared after chemical reduction of ferritin at pH 4.7, however, showed rotational changes consistent with additional folding in the molecule. The optical rotatory properties of ferritin and apoferritin were unaffected by 10 M urea. In concentrations as great as 3 M guanidinium chloride, the rotatory properties of apoferritin were unchanged. However, between 3 and 6 M guanidinium chloride, the rotations changed in a manner compatible with an unfolding of the molecule. The optical rotatory properties remained constant over a wide pH range, but significant changes were observed at pH values of 2.5 and 11. The rotations of ferritin and apoferritin at 233 mμ in several organic solvents were similar to, although somewhat lower than, the rotations in aqueous solutions. Therefore disruption of hydrophobic interactions did not induce additional α-helix formation.

Horse spleen ferritin is a well-characterized protein, uniquely suited for its function of storing iron in an amount equivalent to over 20% of its weight (Granick, 1942). The iron occurs as a hydrated ferric oxide–phosphate micelle core surrounded by a protein shell (Farrant, 1954). The iron micelles were originally thought to be composed of four subunits in a square-planar arrangement or of six subunits situated at the corners of a trilateral prism (Farrant, 1954; van Bruggen *et al.*, 1960). More recently, high-resolution electron micrographs suggest a polyhedral-type structure of variable shape (Haggis, 1965). The quantity of iron in highly purified preparations of native ferritin is variable, and the protein can be fractionated on the basis of its iron content by means of ammonium sulfate precipitation (Mazur *et al.*, 1950) or by high-speed density gradient centrifugation (Fischbach and Anderegg, 1965). The electrophoretic heterogeneity (one major and two or three minor components) has been ascribed to different states of molecular aggregation rather than to different iron contents (Harrison and Gregory, 1965; Suran and Tarver, 1965).

The molecular dimensions of ferritin and apoferritin have been estimated from data obtained by X-ray diffraction and electron microscopy studies. From X-ray diffraction measurements on "wet" ferritin crystals, Harrison (1963) concluded that the protein moiety is a hollow sphere with an external diameter of approximately 122 A and an internal diameter of 76 A. Dry ferritin crystals visualized in electron micrographs have somewhat smaller dimensions (Farrant, 1954; Kuff and Dalton, 1957; Labaw and Wykoff, 1957). Low-angle, X-ray-scattering studies of ferritin in solution (Fischbach and Anderegg, 1965; Bielig *et al.*, 1966) disclose dimensions that correspond to those obtained by X-ray diffraction of the wet crystals. Scattering from iron micelles of ferritin molecules containing a full complement of iron show they are uniform, nearly spherical aggregates having a diameter very close to that of the center cavity of apoferritin. Similar dimensions for the micelles were computed from studies of denatured ferritin by X-ray scattering (Kleinwachter, 1964).

Apoferritin, prepared after chemical reduction of the iron to facilitate its removal from the protein

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(Behrens and Taubert, 1952), consists mainly of a component having a sedimentation constant of 17.6 S and a molecular weight of about 480,000 (Rothen, 1944; Harrison, 1963). A lyophillized sample of apoferritin has been degraded into approximately twenty 2.1S subunits by treatment with sodium dodecyl sulfate (Hofmann and Harrison, 1963). Evidence from peptide fingerprints of tryptic digests indicates that the subunits are composed of identical peptide chains (Harrison and Hofmann, 1962), and the pentapeptide N-terminal sequence has also been determined (Suran, 1966).

Ferritin and apoferritin are indistinguishable on the basis of electrophoretic mobility (Mazur and Shorr, 1950), viscosity, and vasodepressor (Mazur *et al.*, 1950) and immunological properties (Granick, 1943). In addition, electron micrographs of apoferritin show protein shells similar to those of ferritin (van Bruggen *et al.*, 1960), and the dimensions of apoferritin are almost identical with those of the protein moiety of ferritin (Harrison, 1964).

Spectropolarimetric techniques have had wide application for the study of conformational aspects of protein structure (Urnes and Doty, 1961). Small organic molecules or metal ions that are bound to or incorporated into protein structures often can alter protein conformation and have a pronounced influence on optical rotatory properties (Vallee and Ulmer, 1965; Listowsky *et al.*, 1965). The present study demonstrates that the optical rotatory disperson properties of native ferritin are independent of its iron content. Removal of the iron after chemical reduction, however, produces distinct changes in these properties. The effects of pH, denaturing agents, and organic solvents on the optical rotatory properties of ferritin and apoferritin have also been examined.

Experimental Section

Materials and Methods. Ferritin was isolated from horse spleen according to Granick's (1942) modification of Laufberger's method. Additional samples of concentrated solutions of six-times-crystallized ferritin were obtained from Pentex Co., Kankakee, Ill. All ferritin samples studied were free of cadmium and had comparable optical rotatory dispersion (ORD) properties. Apoferritin was routinely prepared from ferritin by reduction of the iron with sodium hydrosulfite in 0.1 M acetate buffer (pH 4.7) followed by removal of the iron as the α,α-bipyridyl complex by dialysis (Granick and Michaelis, 1943; Behrens and Taubert, 1952). In 0.1 M Veronal buffer (pH 8.6), a 1:1 mixture of 0.5% ferritin and 0.5% apoferritin appeared as a single symmetrical component in the moving-boundary electrophoresis apparatus.

Solvents were purified by distillation when necessary. Urea and guanidinium chloride were recrystallized before use. Unless otherwise stated, solutions were made up in distilled water. The pH was adjusted by addition of HCl or NaOH. Protein solutions in the organic solvents generally also contained 1–2% water, since to avoid denaturation the protein had not been dried completely. Solutions were allowed to stand for 1–6 hr and were clarified by centrifugation prior to any spectropolarimetric investigation. Protein concentrations were generally determined by the method of Lowry *et al.* (1951) and checked by a micro-Kjeldahl procedure. Iron contents were determined by the method of Wong (1928). Fractionation of ferritin by ammonium sulfate was carried out according to the procedure of Mazur *et al.* (1950). The protein fractions shown in Table II, having iron:nitrogen (Fe:N) ratios of 2.2, 1.0, and 0.6, respectively, were precipitated from the solutions containing 26, 36, or 50% saturated ammonium sulfate in that order.

For the density gradient centrifugations, a Beckman-Spinco Model L-2 ultracentrifuge equipped with a No. 50 fixed-angle rotor was used. Gradients were established by layering equal volumes of 60, 50, and 40% sucrose and allowing the tubes to stand for 24 hr (Charlwood, 1963). The tubes were centrifuged at 45,000 rpm for 4 hr. Protein fractions were collected in 14 equal aliquots. The ferritin which was fractionated in the gradient exhibited a decreasing iron content from the bottom to the top of the tube. Sucrose was removed from the fractions by dialysis prior to spectropolarimetric measurements.

Spectropolarimetric Measurements. The Cary Model 60 spectropolarimeter was employed for the ORD measurements. The slit widths of the polarimeter were programmed to maintain a wavelength resolution of better than ± 0.75 mμ throughout the spectral range studied. Cells of 1- or 0.1-cm light paths were used for all of the measurements, and the temperature within the cell compartment was 27°. Absorbancies were always kept below a value of 2.0, and protein concentrations were adjusted accordingly. Because of increasing light absorption in the visible region for ferritin samples containing the full complement of iron, these had to be measured using at least five different concentrations or path lengths to obtain rotations through the entire spectral range of 600–195 mμ. In the spectral range from 400 to 300 mμ the observed rotations were only 3–4 mdeg (with a precision of ± 0.5 mdeg), and, therefore, a large number of rotational values were obtained for the analysis of the data. Measurements in the spectral region below 240 mμ were repeated with at least two different concentrations of protein.

Analysis of ORD Data. The reduced mean residue rotation [m'] was calculated from the specific rotation using the equation

$$[m']_\lambda = \frac{3\mathrm{MRW}}{(n^2+2)100}[\alpha]_\lambda$$

A mean residue weight (MRW) of 113 was calculated from the amino acid composition of apoferritin (Harrison and Hofmann, 1962). Refractive index (n) at various wavelengths was calculated by conventional methods using values reported by Fasman (1963)

at specific wavelengths. In the case of 6 M guanidinium chloride solutions, the refractive index was determined experimentally in the visible region and extrapolated to lower wavelengths.

The parameters of the Moffitt–Yang (1956) equation

$$[m']_\lambda = \frac{a_0\lambda_0^2}{(\lambda^2 - \lambda_0^2)} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

were calculated using a λ_0 value of 212 mμ, and the b_0 value was obtained from the slope and a_0 from the intercept of plots of

$$[m']_\lambda \frac{(\lambda^2 - \lambda_0^2)}{\lambda_0^2} \text{ vs. } \frac{\lambda_0^2}{(\lambda^2 - \lambda_0^2)}$$

The modified two-term Drude equation was also employed (Shechter and Blout, 1964a)

$$[m']_\lambda = \frac{A(\alpha,\rho)_{193}\lambda_{193}^2}{(\lambda^2 - \lambda_{193}^2)} + \frac{A(\alpha,\rho)_{225}\lambda_{225}^2}{(\lambda^2 - \lambda_{225}^2)}$$

with $A(\alpha,\rho)_{193}$ and $A(\alpha,\rho)_{225}$ obtained from plots of

$$[m']_\lambda \frac{(\lambda^2 - \lambda_{193}^2)}{\lambda_{193}^2} \text{ vs. } \frac{\lambda_{225}^2}{(\lambda^2 - \lambda_{225}^2)}$$

with the slope giving $[(\lambda_{225}^2 - \lambda_{193}^2)/\lambda_{193}^2]A(\alpha,\rho)_{225}$ and the intercept giving $(\lambda_{225}^2/\lambda_{193}^2)[A(\alpha,\rho)_{225} + A(\alpha,\rho)_{193}]$.

Optical rotation values in the spectral range between 500 and 270 mμ resulted in linear or near-linear plots for these data. The following type of relationship (Urnes and Doty, 1961) was used to calculate the fraction of the protein in the helical form (f_H) given in Table I.

$$P = f_H P^H + (1 - f_H)P^C$$

where P is the experimentally obtained parameter and P^H and P^C are the parameter values for the helical and random-coil forms, respectively. Average values obtained for poly-L-glutamic acid at pH 4 and for paramyosin (Riddiford, 1966; Shechter and Blout, 1964a,b) and the value for poly-L-glutamic acid at pH 7 (Simmons *et al.*, 1961) have been used as values representative of the helical and random-coil forms, respectively. Thus, values of $b_0 = -630$, $A(\alpha,\rho)_{225} = -2050$, $A(\alpha,\rho)_{193} = +2900$, $[m']_{233} = -15{,}000$, and $[m']_{198} = +72{,}000$ represent reference values for 100% helix and $b_0 = 0$, $A(\alpha,\rho)_{225} = -60$, $A(\alpha,\rho)_{193} = -750$, $[m']_{233} = -1800$, and $[m']_{198} = -500$ are values for the random-coil form.

Results

The ORD curves for ferritin and apoferritin in the spectral range of 600–195 mμ are shown in Figure 1. Although ferritin has a very broad absorption band in both the visible and near-ultraviolet regions, a plain dispersion curve is obtained. The curve has a trough at 233 mμ ($[m']_{233} = -7200$), intersects zero at 223 mμ, and has a peak at 198 mμ ($[m']_{198} = +37{,}000$). The shapes of both curves in the far-ultraviolet region are typical for a protein having appreciable helical content (Jirgensons, 1965).

The values for the rotations at the trough and peak and the constants obtained from the various treatments of the rotatory data are shown in Table I. These parameters, as noted previously, may be employed to obtain an approximation of helical content. There is relatively close agreement in the fraction helix calculated from each of the parameters shown in Table I. Apoferritin, prepared after reduction of ferritin at pH 4.7, has lower negative rotations in the visible region than native ferritin, and larger extrema at 233 and 198 mμ (Figure 1). The a_0, $-b_0$, $A(\alpha,\rho)_{193}$, and $-A(\alpha,\rho)_{225}$ values are also greater for apoferritin (Table I). The possibility that the protein undergoes an uncoiling during the reductive process is precluded on the basis of these values. Indeed the trend indicated by these parameters is toward a greater degree of folding in the protein after iron is removed. It should be noted that the two proteins are indistinguishable when studied by several other physical methods (Harrison, 1964).

The iron content affects ease of precipitation of ferritin by ammonium sulfate, and the iron-rich fractions may be precipitated with lower concentrations of ammonium sulfate than is required to precipitate the iron-poor fractions (Mazur *et al.*, 1950). Employing this mild technique to fractionate ferritin, the fraction with the lowest iron content obtained had an Fe:N ratio of 0.6. The optical rotations at the 233-mμ trough obtained for several fractions of different iron content are shown in Table II. For these fractions, it is clear that the rotation is relatively independent of iron content. Ferritin fractionated by high-speed sucrose gradient centrifugation yielded fractions almost free of iron. The $[m']_{233}$ values obtained with these fractions were also independent of the Fe:N ratio (Table II).

One would expect that high concentrations of urea would have a pronounced influence on the optical rotatory properties of helical proteins (Urnes and Doty, 1961). However, the ORD curves of ferritin or apoferritin are unchanged in concentrated urea solution (Figure 2). The $[m']_{233}$ values in 10 M urea shown in Table III are the same as those obtained in water. Ferritin dried by lyophylization becomes almost water-insoluble and its ORD properties in 10 M urea (Figure 2) then are similar to those of other denatured proteins. The large decreases in $-b_0$ value (-70) and in the 233-mμ trough ($[m']_{233} = -3800$) are compatible with a large degree of unfolding in the protein molecule.

The behavior of ferritin and apoferritin in guanidinium chloride was distinctly different. In concentrations as high as 3 M, the optical rotatory properties of apoferritin were unchanged, as in the case with urea. However, between 3 and 6 M guanidinium chloride,

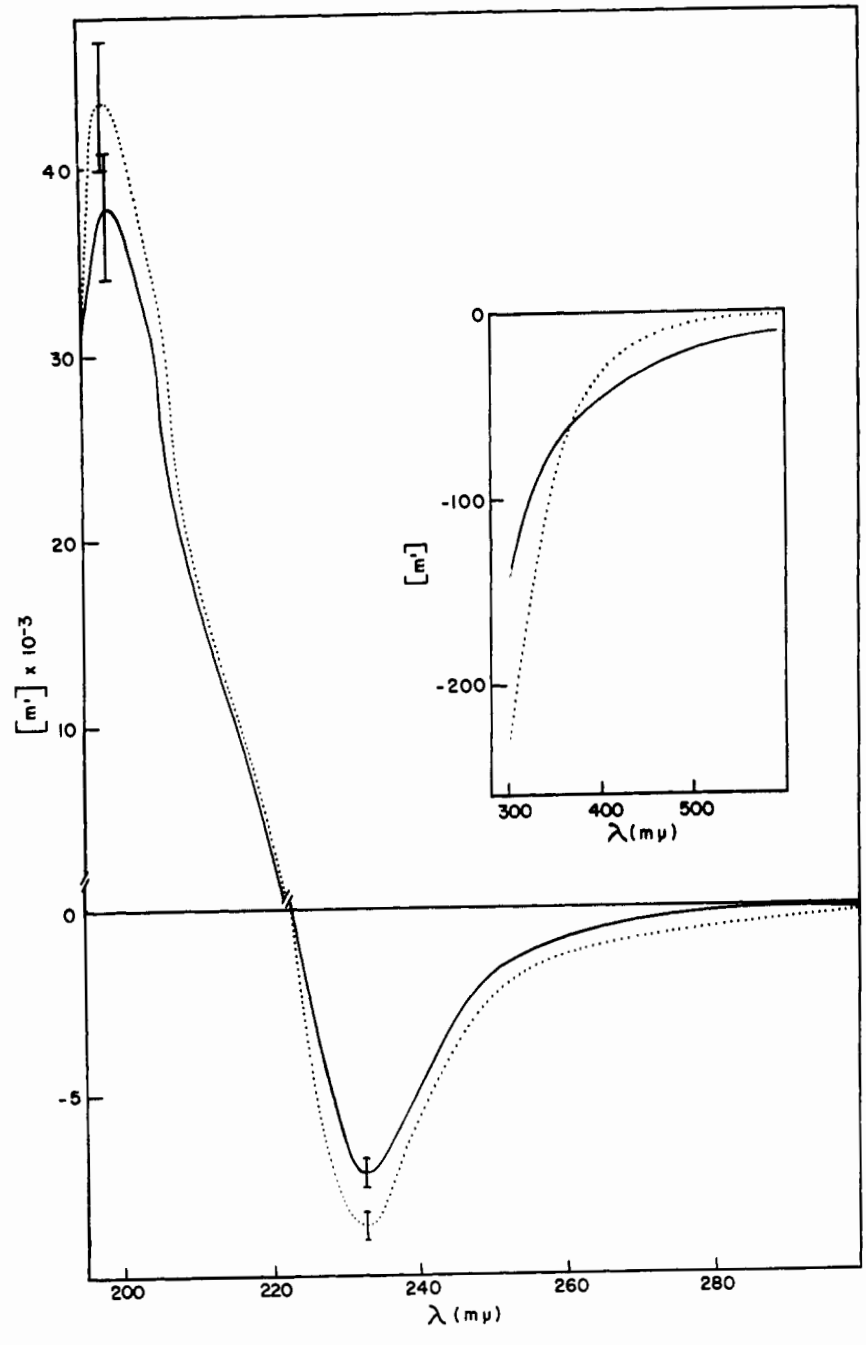

FIGURE 1: Optical rotatory dispersion curves for ferritin (solid line) and apoferritin (dotted line) in aqueous solution.

TABLE I: Optical Rotatory Dispersion Parameters and the Calculated Helical Contents of Ferritin and Apoferritin.

	a_0	b_0	f_H	$A(\alpha,\rho)_{225}$	$A(\alpha,\rho)_{193}$	f_H[a]	$[m']_{233} \times 10^{-3}$	f_H	$[m']_{198} \times 10^{-3}$	f_H
Ferritin	−20	−260	0.41	−840	+1140	0.45	−7.2	0.41	+37 ± 4	0.52
Apoferritin	+40	−340	0.54	−1010	+1400	0.53	−8.6	0.52	+44 ± 4	0.61

[a] The fraction helix is an average value obtained from the $A(\alpha,\rho)_{225}$ and $A(\alpha,\rho)_{193}$ values. In each case the value calculated from $A(\alpha,\rho)_{193}$ was somewhat greater than that calculated from $A(\alpha,\rho)_{225}$.

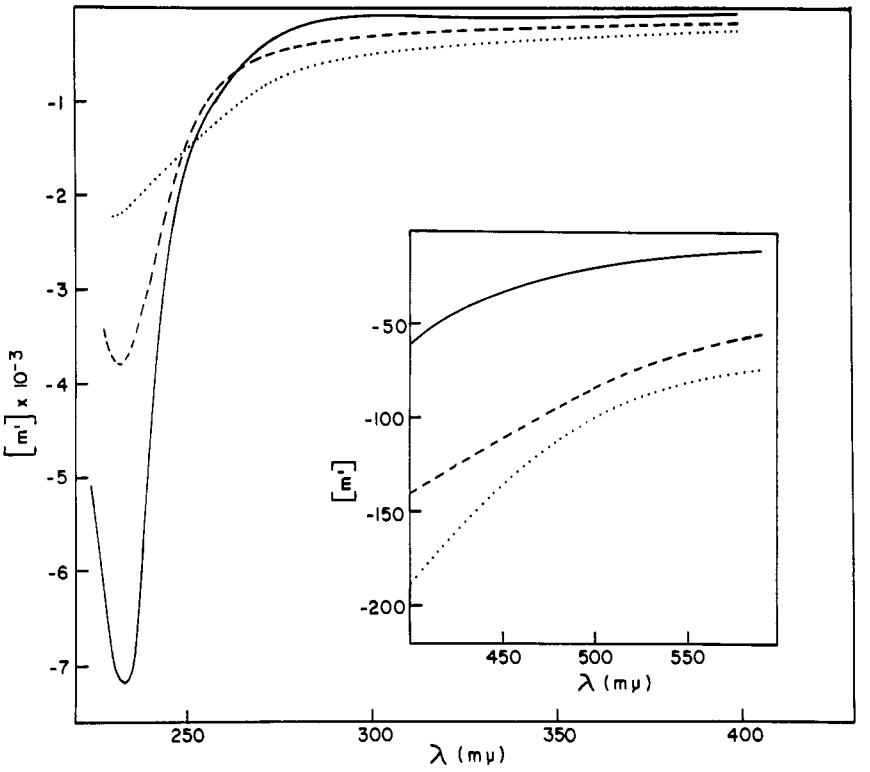

FIGURE 2: Optical rotatory dispersion curves in 10 M urea and 6 M guanidinium chloride. The solid line represents the curve for native ferritin in 10 M urea. The dashed line represents the curve for lyophillized ferritin in 10 M urea. The dotted line represents the curve for ferritin in 6 M guanidine hydrochloride, pH 6.

he optical rotatory properties changed in a manner compatible with disruption of helix. It can be seen in Figure 2 that ferritin and apoferritin in 6 M guanidinium chloride yield a dispersion curve consistent with that of an unfolded protein.

The effects of varying pH on the rotations in the far ultraviolet are shown in Figure 3. The values obtained at pH 8.6 are equivalent to those obtained at pH 7. In addition, the rotations at the peak and trough are not significantly different at pH 4.6, the pH at which the iron is removed after reduction. At pH 2.5 or 11.0, however, the rotations at the Cotton effect extrema are diminished, suggesting that a significant although not complete unfolding of the protein had occurred.

Both ferritin and apoferritin could be dissolved in several organic solvents in concentrations high enough to permit accurate spectropolarimetric measurements. To determine in a general way contributions of hydrophobic interactions in maintaining the native conformation of both proteins, optical rotations at the

TABLE II: Reduced Mean Residue Rotations at the 233-mμ Trough for Ferritin Samples of Varying Iron Content.

Obtained by Ammonium Sulfate Precipitation		Obtained by Sucrose Density Gradient Centrifugation	
mg of Fe/mg of N	$[m']_{233} \times 10^{-3}$	mg of Fe/mg of N	$[m']_{233} \times 10^{-3}$
2.2	−7.0	2.9	7.1
1.0	−7.3	1.7	7.0
0.6	−7.1	0.31	7.0
		0.15	7.3
		0.08	7.2

TABLE III: Rotations at the 233-mμ Trough for Ferritin and Apoferritin in Several Solvents.

Solvent	$[m']_{233} \times 10^{-3}$ for Ferritin	$[m']_{233} \times 10^{-3}$ for Apoferritin
10 M urea in water	−7.1	−8.4
6 M guanidinium chloride	−2.2	−2.4
Ethanol	−6.3	−7.7
Ethylene glycol	−6.1	−7.7
2-Methyoxyethanol	−6.2	−7.5

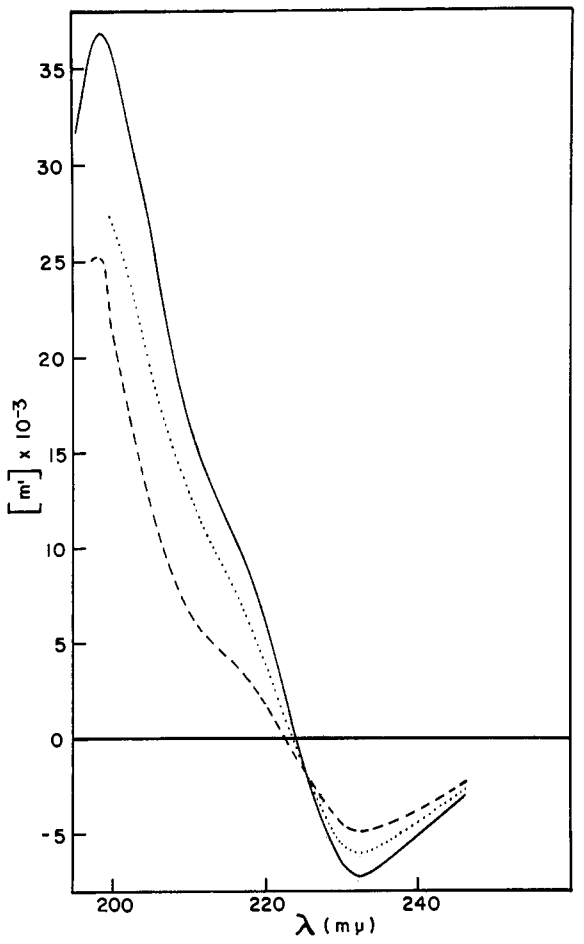

FIGURE 3: Ultraviolet optical rotations for ferritin at various pH values. The solid line is the dispersion curve obtained at pH 8.6 and 4.7. The dashed line is the curve obtained at pH 2.5. The dotted line is the curve obtained at pH 11.0.

233-mμ trough were measured in several solvents (Table III). The rotations of ferritin in all of the solvents shown were similar to although somewhat lower than the rotations in aqueous solution. This was also true for apoferritin.

Discussion

Native ferritin is relatively stable at elevated temperatures and in the presence of 10 M urea solutions. The iron is tenaciously retained within the protein shell although its removal can be facilitated by prior reduction at low pH. These and other characteristic properties of native ferritin reflect the nature of the three-dimensional structure of the protein. The spectropolarimetric studies reported in this paper are an attempt to probe this complex structural network.

The protein exists to a very large extent in the helical conformation. The ORD parameters commonly employed as indices of helix content suggest that nearly half of the native protein molecule is in helical conformation. The values for these parameters are similar to those obtained for myoglobin (Harrison and Blout, 1965; Breslow *et al.*, 1965), and indicate greater helical contents for ferritin and apoferritin than for 27 of the 28 helical globular proteins studied by Jirgensons (1965).

In general, there exists a certain degree of uncertainty when ORD parameters are used to calculate absolute helical content for a protein. Thus, the theoretical treatment of the data does not take into consideration the rotatory contributions of protein side chains, of short helical segments (Goodman *et al.*, 1962), and of ordered structures other than the α helix. In addition, the uncertainties in the reported values for polyglutamic acid (Yang and McCabe, 1965), used as a standard of reference, are not considered in the calculations. However, considering the close agreement in the fraction helix calculated from each of the parameters shown in Table I, satisfactory estimates of the helical contents of ferritin and apoferritin are obtained.

The amino acid composition of ferritin (Harrison *et al.*, 1962) suggests that the protein is potentially capable of maintaining extensive helical conformation. The proline content (1–2%) is low, and the valine, isoleucine, threonine, and serine residues, all of which would tend to disrupt helical regions, occur in relatively small amounts. The compositional predisposition to helix formation is indeed realized in the protein molecule.

The structural modification of the protein molecule attendant to chemical removal of iron after hydrosulfite treatment is presently not known. The changes in the optical rotatory properties could indicate an increased folding of the molecule after complete removal of the iron. This occurs only when the protein undergoes chemical reduction at pH 4.7 since the fractions of native ferritin of low iron content (Table II) have optical rotatory properties similar to those of the high iron content fractions. It therefore seems likely that the protein has undergone a chemical change, possibly of a reductive nature, upon treatment with hydrosulfite. Thus, although the surface properties of ferritin and the chemically produced apoferritin are the same, and the two proteins have been compared interchangeably in the literature, they do have structural differences.

The inability of 10 M urea to promote conformational changes in ferritin and apoferritin accords with the finding that this reagent does not dissociate the protein into subunits (Hofmann and Harrison, 1963), or denature the protein. In contrast, 6 M guanidinium chloride results in optical rotatory changes consistent with helix disruption in the ferritin molecule (Table III, Figure 2). It should be noted that under these conditions the iron is rapidly released from the protein.

The rotatory properties of ferritin and apoferritin remain unchanged in the pH range between 4.5 and 9.0. Some unfolding does occur however at the high and low pH ranges. Profound changes in the physical properties occur when the protein is lyophillized or dried. Apparently the water associated with the mole-

cule is an integral part of the structure, and its removal is accompanied by a collapse of the protein structure as indicated by the observed changes in the optical rotatory properties.

In contrast to the case with a number of other proteins in nonaqueous solution (Herskovits and Mescanti, 1965; Herskovits, 1966; Tanford and De, 1961; Kientz and Bigelow, 1966), the ORD properties of ferritin and apoferritin do not indicate additional helical formation in high concentrations of organic solvents (Table III). A concentration of organic solvent (*e.g.*, ethylene glycol) of at least 50% is required to induce a significant rotational change in ferritin. The rotations in a concentration of organic solvent greater than 98% remain constant for very long periods of time, and do not differ greatly from those obtained in water. Therefore, it is unlikely that ferritin undergoes an unfolding followed by a refolding into a different conformation, as observed for β-lactoglobulin (Tanford and De, 1961). Since the disruption of hydrophobic interactions does not produce substantial changes in the rotatory properties, the tendency for a large degree of conformational flexibility in the protein molecule apparently does not exist. The small changes in optical rotatory properties of ferritin in nonaqeuous solvents appear to be unrelated to the observed liberation of iron under these conditions. This is evident from the fact that changes of the same order of magnitude occur with apoferritin in organic solvents.

In cases where a chromophoric moiety binds or interacts at an asymmetric site, an extrinsic Cotton effect in the region of absorption of the optically inactive chromophore may be induced (Betheil, 1961; Ulmer and Vallee, 1965; Harrison and Blout, 1965). The binding of only one iron molecule to conalbumin and transferrin generates Cotton effects in the region of absorption of the complex (Ulmer and Vallee, 1963). There are also other examples where the incorporation of non-heme iron into protein structures results in ORD changes (Vallee and Ulmer, 1965). Ferritin however is not comparable to these other proteins containing small molecules because of the rather unique manner in which the iron micelle is confined within the protein. Indeed, although the iron represents a large portion of the ferritin molecule, there are no significant changes in the ORD parameters associated with the variable iron content of ferritin.

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Stimulation of Mitochondrial Respiration and Phosphorylation by Transport-Inducing Antibiotics*

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ABSTRACT: The stimulation of the respiration of rat liver mitochondria has been compared under various conditions of energy demand. In the presence of 10 mM P_i and 3 mM succinate or glutamate plus malate, the rates accompanying the active accumulation of K^+, induced by valinomycin or dinactin, can exceed the maximal values attained either with dinitrophenol or during oxidative phosphorylation. Both the latter rates, however, can be increased by raising the substrate to 10 mM. Under certain circumstances oxidative phosphorylation successfully competes for mitochondrial energy with the antibiotic-induced uptake of K^+. Although the absolute rate of phosphorylation can even be enhanced in conditions of stimulated K^+ movement, this is secondary to the increased respiratory rate and the P:O ratio is not increased. Another transport-inducing antibiotic, gramicidin, exhibits an inherent uncoupling activity not shared by valinomycin and dinactin which is substantiated by the inability of gramicidin to stimulate mitochondrial phosphorylation, as well as other tests with submitochondrial particles. It correlates with the inability of gramicidin to support respiratory rates as high as those obtainable with the other transport-inducing antibiotics. The increases in both the phosphorylation rate and maximum uncoupled respiration which accompany antibiotic-induced K^+ transport suggest that the passage of metabolically active anions (P_i, nucleotides, and substrates) across the mitochondrial membrane can be facilitated by the concomitant and energy-dependent uptake of K^+. The possible significance of this suggestion in relation to metabolic regulation in general is discussed.

Conventional uncouplers of oxidative phosphorylation such as DNP are supposed to act by catalyzing the discharge or hydrolysis of an energized intermediate or state which can energize the conversion or convert ADP[1] to ATP. It has been proposed (Mitchell, 1961) that the state discharged is specifically that of a preexisting proton gradient so that the uncoupler short circuits the energy stored across a charged membrane. Another class of agents, found among the toxic antibiotics (Pressman, 1965a), increases mitochondrial permeability to monovalent cations, with various degrees of specificity for K^+. Mitochondria, although washed extensively during preparation, normally retain a considerable amount of K^+; its release from the mitochondria can be facilitated by agents which increase cation permeability. In the presence of an energy source the increased permeability activates a process which not only replenishes the K^+ which leaks out, but even increases the total quantity associated with the particle. Since a large number of such agents are

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[1] Abbreviations used: ADP and ATP, adenosine 5′-di- and -triphosphates; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone; TTBI, tetrachlorotrifluoromethylbenzimidazole.