Structural Heterogeneity and Subunit Composition of Horse Ferritins†

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ABSTRACT: Structural, spectroscopic, and immunological properties of horse ferritins extracted from spleen, liver, and heart were studied to test the hypothesis that the different tissue ferritins are hybrids composed of variable proportions of two subunit types. The weight-average molecular weights determined by sedimentation velocity and gel filtration increase from 460 000 for spleen to 480 000 for liver and to 515 000 for heart apoferritin; moreover, the diffusion coefficients prove that each tissue-specific ferritin consists of a population of hybrid molecules. The intrinsic fluorescence and the near-UV circular dichroism (CD) spectra change as a function of the subunit composition of the three ferritins. The fluorescence emission maximum, which occurs at a very low wavelength (315 nm) in horse spleen apoferritin, is shifted to increasingly higher wavelengths in the liver and heart proteins (320 and 325 nm, respectively), indicating that the tryptophan and tyrosine residues become less rigidly immobilized with an increase in the H-subunit content. The tryptophan residues behave as fully solvated in the monomeric subunits at acidic pH values. In accordance with the fluorescence data, the near-UV CD spectra show that the tryptophan environment is highly asymmetric in spleen apoferritin, progressively less so in liver and heart apoferritins, and completely relaxed in the dissociated subunits. Moreover, they show that the environment of tyrosines and phenylalanines differs markedly in the spleen and heart apoproteins. The ferritins studied appear to have immunogenic sites which are specific for the H and L subunits on the basis of enzyme-linked immunoassay and double-diffusion experiments.

Ferritin is an iron-containing protein widely distributed in nature (Granick, 1946; Crichton, 1973; Harrison et al., 1974) but present in highest concentration in iron-storage tissues like liver and spleen. It is also found in other tissues, like heart and kidney, which typically do not have a major iron-storage function (Munro & Linder, 1978). Ferritins from different mammalian tissues can be distinguished electrophoretically (Alfrey et al., 1967; Gabuzda & Pearson, 1968); in turn, ferritins from a single tissue have themselves been shown to consist of a family of closely related proteins or isoferritins. Isoferritins differ in surface charge and thus can be separated by electrophoresis (Bomford et al., 1977; Gianazza & Arosio, 1980), isoelectric focusing (Drysdale, 1970), and ion-exchange chromatography (Urushizaki et al., 1971). Isoferritins also have different immunological properties (Niitsu et al., 1980; Hazard et al., 1977; Jones & Worwood, 1978). The isoferritin profiles in the different tissue ferritins vary (Drysdale, 1977; Halliday et al., 1976) and can change in several physiological and pathological conditions (Powell et al., 1974; Richter, 1965) and during development (Linder et al., 1975).

Ferritin heterogeneity has been suggested to originate at a molecular level from the presence of two subunit types, called H and L, which assemble in different proportions in the multimeric ferritin molecule (Drysdale, 1977; Arosio et al., 1978). As an alternative hypothesis, posttranslational modifications have been proposed to occur (Harrison et al., 1977). The H and L subunits differ in several structural properties, such as molecular weight, H having an M_r of about 21 000 and L of about 18 500 (Arosio et al., 1978), and amino acid and peptide composition (Alpert et al., 1979; Linder et al., 1975) and seem to be different gene products (Kohgo et al., 1980; Watanabe & Drysdale, 1981). The structural differences of the subunit types are not reflected in the gross morphology of the assembled shell, since ferritins from different organisms and tissues all form isomorphous crystals (Clegg et al., 1980). Therefore, it can be safely assumed that all ferritin molecules consist of 24 subunits arranged in the form of a hollow sphere which encloses an iron core of variable size (Harrison, 1963; Banyard et al., 1978). The isoferritins contained in iron-storage tissues are mainly composed of L subunits and can be crystallized easily from solutions of $CdSO_4$ (Granick, 1946), while the isoferritins with a higher content of H subunits are usually present in very low amounts and cannot be crystallized from solutions of the same salt (Hazard et al., 1977; Arosio et al., 1978). From a functional viewpoint, L-rich isoferritins extracted from human tissues appear to accumulate iron faster than the H-rich ones (Russel & Harrison, 1978).

In the present paper, structural and immunological properties of horse ferritins extracted from spleen, liver, and heart have been examined to test the hybrid isoferritin model. Horse isoferritins were chosen as they are available in big quantities, differ significantly in subunit composition, and cover a wide range in the isoferritin isoelectric focusing spectrum. This system may serve as a reference model for human isoferritins.

Molecular weight, intrinsic fluorescence, UV circular dichroism, and immunoreactivity vary as a function of the relative proportions of H and L subunits. Direct experimental evidence for the validity of the hybrid model is provided by the comparison of the diffusion coefficient of liver ferritin (40% H) and that of a mixture of spleen (90% L) and heart (90% H) apoferritins containing the same proportion of subunits as the liver protein.

Materials and Methods

Ferritins were prepared from horse tissue obtained from freshly killed animals and processed within the day. Liver and heart ferritins were purified essentially as described by Arosio et al. (1978). The purification steps consisted of homogenization of the tissue, heat extraction at 75 °C, ammonium sulfate precipitation at 60% saturation, sedimentation by ultracentrifugation at 300000*g* for 2 h, preparative gel filtration

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on Sepharose 6B (the whole ferritin monomer peak was collected), and preparative gel electrophoresis on a 6–22% gradient of polyacrylamide. The proteins were extracted from the gel as follows: the brown ferritin band was cut from the gel and ground by passing it through a syringe; the protein was eluted electrophoretically into a dialysis bag in 25 mM Tris–glycine, pH 8.3, at 120 V for 3–4 h. Subsequently, ferritin was dialyzed against 20 mM sodium phosphate, pH 7.4. Spleen ferritin was purified after the ammonium sulfate precipitation by two crystallizations from 5% cadmium sulfate solutions (Granick, 1946). The purity of the preparations was assessed by polyacrylamide gel electrophoresis (Arosio et al., 1976). The percentage content of the two subunit types was determined by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis and corresponded to 90% L in spleen, 40–50% H in liver, and 85–90% H in heart ferritin.

Apoferritins were prepared upon reduction of iron with sodium dithionite and chelation with α,α'-bipyridyl; subunits were obtained by exposure to 67% acetic acid followed by a prolonged dialysis against 0.01 M glycine hydrochloride buffer at pH 3.0 (Harrison & Gregory, 1968). Ferritin and apoferritin concentrations were determined by the method of Lowry et al. (1951) or Richterich (1969). Alternatively, apoferritin concentration was calculated from the absorption at 280 nm by using the extinction coefficient $E^{1\%,1cm}_{280nm} = 9.0$ (Bryce & Crichton, 1973). The protein concentration is expressed in terms of the molecular weight of the polypeptide chains (M_r 18 500).

Sedimentation velocity experiments were performed at 10–12 °C in a Spinco Model E ultracentrifuge at 42 000 and 56 000 rpm. Sedimentation coefficients were measured from the movement of the maximum ordinate of the schlieren peak and corrected to s_{20} by standard procedures. Molecular weights were estimated from the value of the sedimentation coefficient by assuming $\bar{V} = 0.732$ and $f/f_0 = 1.2$ (Wyman & Ingalls, 1941). Diffusion coefficients were calculated from the areas of the schlieren diagrams in the sedimentation velocity experiments and were corrected for radial dilution and for the movement of the boundary in the centrifugal field (Elias, 1961).

Gel filtration experiments were carried out on a Sepharose 4B column (72 × 1.6 cm) equilibrated with 20 mM phosphate buffer, pH 7.4, at 4 °C. The flow rate of 8.5 mL/h was maintained by gravity (80-cm hydrostatic pressure); 1.1-mL fractions were collected. For each fraction, the optical density at 420 nm due to the iron micelle was recorded and the protein content determined according to Lowry et al. (1951). The peaks determined in both ways were coincident. The column was calibrated with β-galactosidase, horse spleen ferritin, and its dimer. The ferritin dimer was prepared by incubation of a solution at 5 mg/mL with 2% glutaraldehyde for 6 h at pH 7.4; it was separated from the monomer and oligomers by preparative polyacrylamide gel electrophoresis. K_d was calculated as $(V_e - V_0)/V_i$ (Axelsson, 1978); the void volume (V_0) and the inner volume (V_i) were determined from the elution peaks of Blue Dextran 2000 and 2-mercaptoethanol, respectively.

Isoelectric focusing experiments were performed on 0.3-mm-thick polyacrylamide gel slabs [T = 4.5% acrylamide, C = 4% *N,N'*-methylenebis(acrylamide)] with 2% (w/v) ampholines covering the pH range 5–7 essentially as described by Gianazza & Arosio (1980). The samples were run for 6 h at 800 V and 3 W.

Enzyme-Linked Immunoassay (ELISA). The method of Zuyderhoudt et al. (1978) was followed. Rabbit γ-globulins were precipitated from the immunosera by addition of ammonium sulfate at 40% saturation. The solid-phase antibodies were prepared in 10 × 75 mm polystyrene test tubes by incubating 0.5 mL of the γ-globulin fractions diluted 1000 times in 20 mM phosphate buffer at pH 7.4. Undiluted aliquots of the same γ-globulin fractions were labeled with horseradish peroxidase in 0.08 M sodium periodate, as described by Miedema et al. (1972). Standard ferritins and labeled antibodies were diluted before use in 20 mM phosphate buffer, pH 7.4, containing 1% bovine serum albumin. In order to obtain similar enzymatic activities, the labeled anti-spleen and anti-liver antibodies were diluted 800-fold, while the anti-heart antibody was diluted 250-fold.

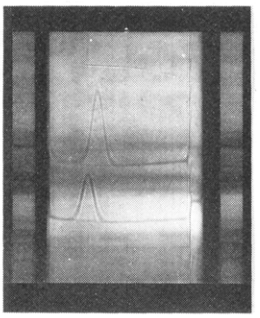

FIGURE 1: Sedimentation velocity patterns of apoferritins from horse heart (top) and spleen (bottom). Protein concentration was 2–3 mg/mL in 10 mM Tris-HCl buffer at pH 7.0.

Ouchterlony double-diffusion experiments were performed in 1% agarose gels, 0.14 M NaCl, and 20 mM Tris-HCl at pH 7.4. Wells were filled with ferritin samples (0.3 mg/mL) and absorbed antisera. The antisera were absorbed until disappearance of the precipitin line between the antibody and the absorbing antigen in double-diffusion experiments. Plates were allowed to diffuse for at least 18 h at 4 °C and were then stained for iron with Prussian Blue.

Fluorescence experiments were performed with a FICA 55L corrected spectrofluorometer at 20 °C. The relative quantum yield, φ, was determined by using *N*-acetyltryptophanamide as a standard compound. The value of φ was calculated from the optical densities of the sample and of the standard at the excitation wavelength and from the areas, A, enclosed by their respective emission spectra according to the equation $\varphi = (A_{sample}/OD_{sample})(OD_{standard}/A_{standard})$ (Demas & Crosby, 1971).

CD experiments were carried out by using a Cary 60 spectropolarimeter with a 6002 CD attachment. Cylindrical cells of 0.1 or 1.0 cm were employed. The data obtained in the far-UV region are expressed in terms of molar ellipticities, $[\theta]$, in deg cm^2 dmol^{-1}, calculated by using a mean residue weight of 113. The data obtained in the near-UV region are expressed as $\Delta\epsilon$ by using the expression $\Delta\epsilon = [\theta]^*/3300$, where $[\theta]^*$ is the molar ellipticity calculated on the basis of an average subunit molecular weight of 18 500, 18 750, and 19 000 for the spleen, liver, and heart proteins, respectively (Strickland, 1974).

Results

Sedimentation Velocity Experiments. The sedimentation velocity patterns of ferritins from horse liver and heart show a broad iron distribution which resembles that of spleen ferritin. In the different preparations analyzed, the weight-average sedimentation coefficients of spleen ferritin ranged from 59 to 69 S as reported previously (Stefanini et al., 1976a), while those of liver and heart ferritins ranged between 50 and 58 S and between 33 and 55 S, respectively. Polydispersity

Table I: Sedimentation Coefficients, Molecular Weights, and Subunit Composition of Horse Ferritins

	% content in H subunit	s_{20} (S)	mol wt: calcd from s_{20}	mol wt: calcd from subunit composition[a]	mol wt: determined by gel filtration
spleen	10	16.5	460 000	440 000	454 000
liver	40	17.0	480 000	470 000	474 000
heart	85	18.0	515 000	515 000	500 000

[a] Based on the following molecular weight values for the subunits: H = 21 200; L = 18 800 (Arosio et al., 1978).

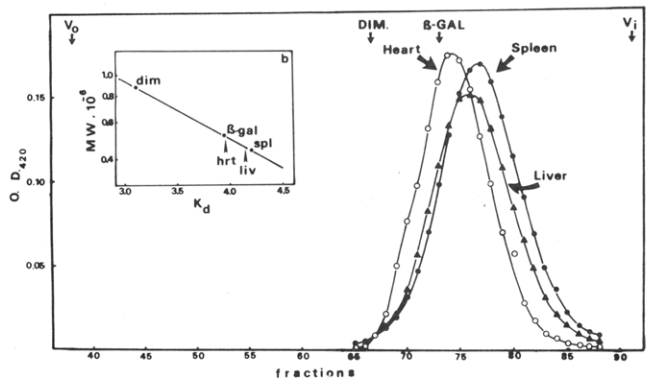

FIGURE 2: Elution profiles of horse heart, spleen, and liver ferritins from a Sepharose 4B column. The inset shows the calibration plot for the molecular weight determination.

is lost after removal of the iron; thus, all apoferritins sediment as single, symmetrical peaks (Figure 1). The values of the sedimentation coefficients of the apoferritins in 200 mM imidazole buffer at pH 6.4, summarized in Table I, increase as a function of the increase in H-subunit content, determined by $NaDodSO_4$ gel electrophoresis (Arosio et al., 1978). Table I also shows for the sake of comparison the molecular weights calculated on the basis of the subunit composition and the apparent molecular weights obtained from the gel filtration experiments to be described below.

The diffusion coefficients of all apoferritins are time independent and correspond to (2.5–3.0) × 10^{-7} cm^2/s. In contrast, a mixture of spleen and heart apoferritins containing the same proportion of H and L subunits as liver apoferritin yields a significantly higher diffusion coefficient [D_{20} = (5.0–5.5) × 10^{-7} cm^2/s].

Gel Filtration Experiments. The elution volumes of equine ferritins were measured in gel filtration experiments performed with a Sepharose 4B column at pH 7.4 in 20 mM phosphate buffer. Under these experimental conditions, horse ferritins consistently eluted in the order heart, liver, and spleen. Figure 2 shows typical elution profiles and the apparent molecular weights obtained from the calibration plot. The molecular weight values are included in Table I.

In another experiment, the relationship between the elution volume and the isoelectric focusing profile of ferritins was examined. A mixture of heart and liver ferritins, containing all the components of the isoelectric focusing spectrum in appreciable amounts, was run on a Sepharose 4B column. The eluate was collected in five fractions (1–5) of equal volume which were likewise subjected to isoelectric focusing. Figure 3 shows that no redistribution occurs during the gel filtration experiment and that only the central fraction (3) has a focusing pattern which resembles that of the unfractionated material. The fraction eluting first contains mainly basic heart-type components, while the fraction eluting last contains only acidic spleen-type components.

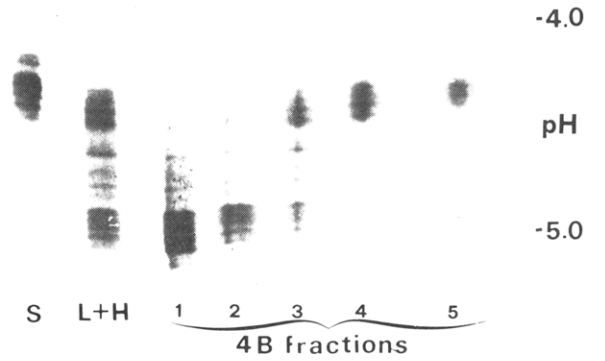

FIGURE 3: Isoelectric focusing patterns of fractions taken during the elution of a mixture of horse liver and heart ferritins from a Sepharose 4B column. Fractions of 1–5 are in increasing order of elution. The patterns of the unfractionated mixture (L + H) and of spleen ferritin (S) are included for comparison.

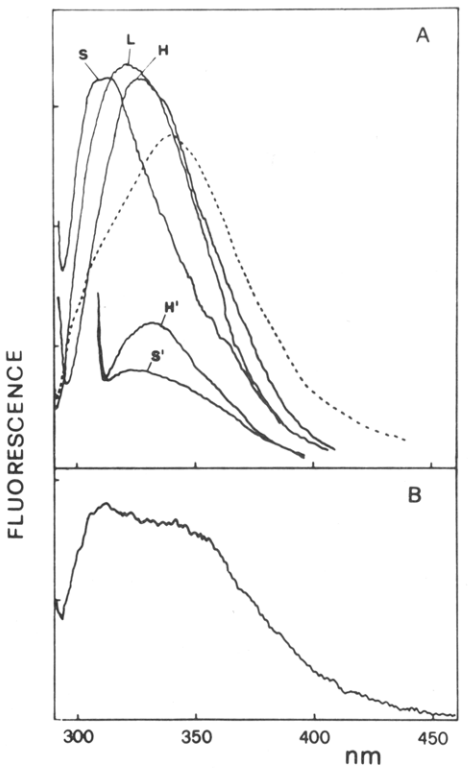

FIGURE 4: Fluorescence emission spectra of horse spleen, liver, and heart apoferritins and their subunits. (A) Apoferritins from spleen (S), liver (L), and heart (H) in 10 mM Tris-HCl buffer at pH 7.0. Monomeric subunits ($s_{20} \simeq 2.0$ S) in 0.01 M glycine hydrochloride buffer at pH 3.0 (broken line). Excitation was at 280 nm. Apoferritins from spleen (S′) and heart (H′) in 10 mM Tris-HCl buffer at pH 7.0. Excitation was at 295 nm. Protein concentration was 0.15 mg/mL. (B) Heart and spleen apoferritin subunits in 6 M guanidine hydrochloride at pH ≤2.0. Excitation was at 280 nm. Protein concentration was 0.25 mg/mL.

Fluorescence Experiments. The intrinsic fluorescence of horse spleen apoferritin is characterized by an emission maximum at an unusually low wavelength (315 nm) for a tryptophan-containing protein (Longworth, 1971); a large contribution from tyrosines was also reported (Stefanini et al., 1976b). As shown in Figure 4A, liver and heart apoferritins have emission maxima at 320 and 325 nm; these do not change as much as in the case of apoferritin upon excitation at 295 nm. Figure 4A also shows that the differences among the tissue-specific apoferritins disappear upon dissociation into

Table II: Fluorescence Emission Maxima (λ_{max}) and Relative Quantum Yields (φ) of Horse Apoferritins

	heart		liver		spleen	
	λ_{max}	φ	λ_{max}	φ	λ_{max}	φ
apoferritin, 0.01 M phosphate buffer, pH 7.0	325		320		315	
subunits, 0.01 M glycine hydrochloride buffer, pH 3.0	340	0.50			340	0.90
unfolded subunits, 6 M guanidine hydrochloride, pH ≤2.0		0.44				0.44

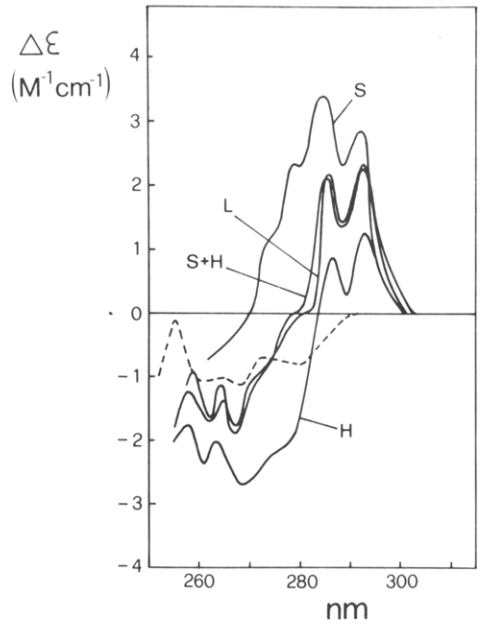

FIGURE 5: Near-UV circular dichroism spectra of horse apoferritins and their subunits. Spleen (S), heart (H), liver (L), and an equimolar mixture of spleen and heart (S + H) apoferritins in 10 mM Tris-HCl buffer at pH 7.0; subunits (broken line) in 0.01 M glycine buffer at pH 3.0. Protein concentration was 1 mg/mL.

monomers; i.e., all preparations exhibit the same emission maximum at 340 nm. These subunit preparations all had a weight-average sedimentation coefficient, s_{20}, between 2.0 and 2.2 S, corresponding to M_r values between 20 000 and 22 000. Previous experiments carried out with spleen subunits having an s_{20} of 4 S (M_r 40 000) exhibited a fluorescence maximum at 328–330 nm. Thus, it appears that the position of the fluorescence maximum depends on the polymerization state of the protein. Unfolding of the subunits, which is obtained in 6 M guanidine hydrochloride at pH ≤2.0, further shifts the tryptophan emission to lower energy, while a peak at 310 nm, related to the fluorescence of tyrosyl residues, becomes apparent (Figure 4B). In the unfolded subunits, the shape of the emission spectrum and the quantum yield are identical for the different tissue-specific apoferritins; in contrast, when the subunits retain their tridimensional structure (at pH 3.0), the quantum yields differ (Table II). The fluorescence yields of apoferritin polymers have not been reported since the presence of even minute amounts of incorporated iron strongly quenches the protein fluorescence (Stefanini et al., 1976b).

CD Experiments. The near-UV circular dichroism spectra of the apoferritins in 10 mM Tris-HCl buffer at pH 7.0 are shown in Figure 5. Spleen apoferritin exhibits two positive peaks with different intensities at 292 and 285 nm and a shoulder at 278 nm in accordance with literature data (Wood & Chrichton, 1971; Listowsky et al., 1972; Silk & Breslow,

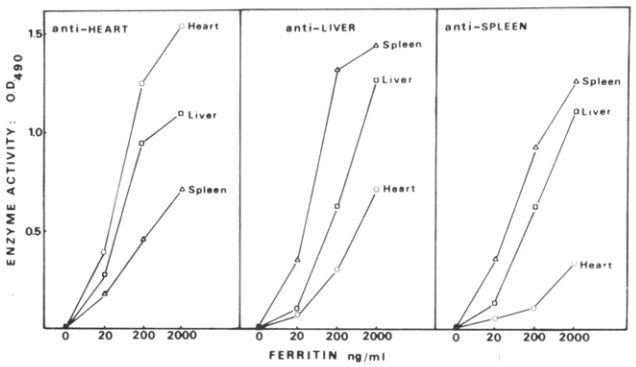

FIGURE 6: Enzyme immunoassays of horse heart, liver, and spleen ferritins with anti-heart, -liver, and -spleen antibodies. For details, see Materials and Methods.

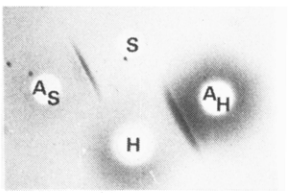

FIGURE 7: Double-diffusion plates of absorbed anti-heart (A_H) and anti-spleen (A_S) antibodies against heart (H) and spleen (S) ferritins. The gels were stained for iron with Prussian Blue.

1976; Leach et al., 1976). In contrast, heart apoferritin shows two positive bands at 293 and 286 nm and a negative absorption between 283 and 255 nm with peaks at 268 and 262 nm. The positive rotatory power is markedly decreased with respect to spleen apoferritin. The spectrum of liver apoferritin is intermediate and does not differ significantly from that of a mixture of heart and spleen apoferritins containing the same proportion of H and L subunits as the liver protein.

Dissociation of all three apoferritins into subunits leads to the disappearance of the positive bands, while the negative peaks broaden (Figure 5).

The CD spectra of spleen and heart apoferritins in 10 mM Tris-HCl buffer at pH 7.0 were also measured in the far-UV region. The molar ellipticity at 222 nm is somewhat higher for the protein extracted from spleen, in agreement with literature data (Lavoie et al., 1979).

The subunits in 10 mM glycine hydrochloride buffer at pH 3.0 show a significant decrease in intensity in the far-UV region; concomitantly, the ellipticity value at 221 nm becomes smaller than that at 208 nm, indicating a decrease in the α-helix content.

Immunological Experiments. An immunoenzymatic system (ELISA) was developed by employing antibodies elicited by heart, spleen, and liver ferritins in order to obtain information on their immunogenic sites. Figure 6 shows the binding assays of the three proteins with the different antibodies. The anti-heart antibody has the highest affinity for heart, lower affinity for liver, and even lower for spleen ferritins. The order of affinity is reversed in the system involving the anti-spleen antibody. These findings are in agreement with data obtained on human ferritins (Arosio et al., 1976; Jones & Worwood, 1978; Niitsu et al., 1980). Interestingly, the anti-liver antibody shows the same order of affinity observed with the anti-spleen antibody.

It is possible to increase the specificity of the antigen–antibody reaction by previous cross-absorption of the anti-spleen antibody with heart ferritin and of the anti-heart antibody with spleen ferritin (Figure 7). However, since neither antigen contains a single type of subunit, when an excess of antigen

is added, both antibodies loose their immunoreactivity (data not shown).

Discussion

The data presented in this paper point out several structural characteristics of horse ferritin in the framework of the hybrid model. First, the molecular weights and diffusion coefficients of the proteins extracted from spleen, liver, and heart have been determined. To date, no direct and systematic measurements of these parameters had been reported. The molecular weights of the various tissue ferritins differ and change as expected on the basis of the relative proportion of H and L subunits. Thus, the values calculated from the sedimentation velocity and gel filtration experiments closely correspond to the values calculated by using the respective subunit composition. Moreover, the combined gel filtration and isoelectric focusing experiments confirm the correlation between size and surface charge previously observed in human isoferritins (Drysdale et al., 1977; Arosio & Albertini, 1980) and show that no redistribution due to subunit exchange occurs. This finding is in keeping with the existence of hybrid molecules which cover the whole isoelectric focusing pattern.

The diffusion coefficients provide direct experimental proof of the hybrid model, since diffusion is very sensitive to molecular heterogeneity. Thus, in a solution containing two macromolecular species of slightly different molecular weight, the diffusion coefficient increases markedly due to additional boundary spreading through heterogeneity (Gilbert & Gilbert, 1980). The various tissue-specific apoferritins all appear to be monodispersed and have similar diffusion coefficients. In contrast, the mixture of spleen and heart apoferritins, containing H and L subunits in the same proportion as the liver protein, has a markedly higher diffusion coefficient. These data can be explained solely if in all tissue-specific ferritins both subunit types coexist in hybrid molecules.

Information on tertiary and quaternary structure in the various apoferritin molecules is provided by the fluorescence and the near-UV circular dichroism experiments which probe the environment of the aromatic residues. As reported previously, horse spleen apoferritin shows an intrinsic fluorescence spectrum with a maximum at 315 nm, resulting from contributions of tyrosyl and tryptophanyl side chains (Stefanini et al., 1976b). Liver and heart apoferritins have fluorescence maxima at higher wavelengths and exhibit a lower contribution from the tyrosine residues to the overall fluorescence (see Figure 4A). These data point to a more rigid structure in spleen apoferritin with respect to the other two proteins, since immobilized tryptophanyl side chains emit at higher energy than freely rotating ones (Burstein et al., 1973). The large contribution of tyrosines can likewise be related to their rigidity. In fact, globular proteins which contain both tyrosines and tryptophans usually show only the emission from the latter residues. This has been attributed to the lower quantum yield of tyrosines and to the possibility of an efficient nonradiative energy transfer. This type of energy transfer (Förster type) is governed by several parameters and in particular depends on the distance between the acceptor and donor dipoles and on their mutual orientation during the excited state (Förster, 1948). Obviously, when both partners are prevented from rotation, a decreased efficiency of transfer can be expected. The constraints around the single tryptophanyl (Heusterspreute & Crichton, 1981) and at least some of the six tyrosyl residues present per subunit, which account for the emission spectrum of spleen apoferritin, become less severe with the increase in H-subunit content. The constraints are completely relaxed in the monomeric subunits of all apoferritins at acidic pH values. Thus, upon dissociation, there is a dramatic change in the emission properties of the tryptophan residue which appears to be almost fully solvated (emission maximum at $\simeq$340 nm). It is of interest that although the fluorescence emission of the monomers is qualitatively similar in all apoferritins, the relative quantum yield differs. Thus, spleen subunits have a value which is roughly twice as large as that of the heart ones. The relative quantum yield becomes the same only when the subunits are completely unfolded in 6 M guanidine at low pH ($\leq$2), and therefore this property appears to be related to a different tertiary structure of the subunits. In the unfolded subunits, the energy transfer between tyrosine and tryptophanyl residues is again less efficient for the statistical increase of distances, and the tyrosine fluorescence becomes apparent as a separate peak.

Complementary evidence on the environment of the tryptophan and of tyrosine residues in the different tissue-specific ferritins comes from the near-UV CD spectra. In the region above 280 nm, all apoferritins show two positive bands, albeit of different intensity, at 292–293 and 285–286 nm. This fine structure can be attributed to tryptophanyls, which typically exhibit the 0–0 ^1L_b band at a wavelength that coincides with the absorption band ($\simeq$292 nm) and the 0 + 850-cm^{-1} ^1L_b band, of the same sign and nearly as much intensity, about 7 nm toward shorter wavelengths (Strickland, 1974). Since only one tryptophan residue is present per subunit, the progressive decrease in intensity, which parallels the increase in H-subunit content, may be accounted for in terms of an increase in the motility of the tryptophan side chains (Strickland, 1974), in agreement with the fluorescence data discussed above. In spleen apoferritin (90% L), the rotatory strength at 292 nm approaches the maximum theoretical value per mole of tryptophan ($\Delta\epsilon$ = 2.5), indicating that the tryptophan residue is immobilized in a highly asymmetric, hydrophobic environment. However, the higher intensity of the 286-nm band, with respect to the 292-nm one, points to an overlap with other CD bands. These can be attributed to tyrosyls since their CD maximum (0 + 800-cm^{-1} band) is known to occur around 277 nm and their 0–0 band about 6 nm toward the red (Strickland, 1974). In heart apoferritin (90% H), the 277-nm band is negative, suggesting that the tyrosyl residues are in a different conformation. Moreover, at variance with spleen apoferritin, the protein from heart exhibits a fine structure also in the region below 270 nm, where two sharp negative bands are present at 268 and 262 nm. These transitions can be ascribed to phenylalanines whose environment therefore must differ in the two types of polymer. Liver apoferritin (the sample used contained 50% H subunit) has a CD spectrum intermediate between those of spleen and heart. The spectrum over the whole region closely corresponds to that of a mixture of spleen and heart apoferritin with the same proportion of H and L subunits as the liver protein. The dissociated subunits of all apoferritins exhibit a relatively weak negative CD spectrum in the tyrosyl and phenylalanyl region, but no tryptophanyl peaks. All these and the previously available spectroscopic data (Stefanini et al., 1976b; Leach et al., 1976; Crichton & Bryce, 1973) can be interpreted by assuming that both subunit types can exist in two conformations. The transition between the "unlocked" conformation characteristic of the monomer and the "locked" conformation characteristic of the polymer occurs in the assembly process. Moreover, the conformation of each type of subunit in the polymer is not affected by the nature of the partner subunit. The transition into the "locked" conformation involves a tightening in the tryptophan environment, which is more marked in the case

of the L subunits, and changes in the interaction of tyrosines and phenylalanines with their surroundings.

The preliminary immunological data lend further support to the hybrid nature of isoferritins and provide information on the specificity of the immunoresponse elicited by the polymers of different tissues. The double-diffusion experiments indicate that antibodies raised against spleen and heart ferritins can be made specific for their own antigen after absorption and suggest that there are some of the immunogenic sites characteristic of each of the two assembled structures. Since horse spleen and heart ferritins contain 90% of L and H subunits, respectively, it is likely that the two subunit types are responsible for the specificity in the immunoresponse. However, the presence of the other subunit type, albeit in very small amounts, prevents a complete lack of cross-reaction. The ELISA method shows that horse liver ferritin, composed of similar amounts of H and L subunits (40% H and 60% L), has a behavior which is intermediate between those of spleen (90% L and 10% H) and heart (15% L and 85% H) ferritins with all the antibodies used. Moreover, the antibodies raised against liver ferritin have characteristics similar to those elicited by spleen ferritin, suggesting that no new immunogenic determinants, neither sequential nor conformational, appear in the middle of the isoferritin spectrum. This finding is in accordance with the observation that specific antibodies are elicited by L-rich ferritins (such as human liver) and by H-rich ferritins (such as HeLa) (Hazard et al., 1977). In addition, the similarity of the immunoresponse of horse liver (60% L) and spleen (90% L) ferritins and the weakness of the immunoresponse of heart ferritin (85% H) suggest that the L subunit is much more immunogenic than the H subunit. The relative ease of raising H-specific antibodies by using HeLa ferritin (80% H) as immunogen, but not human heart ferritin (60% H), is in keeping with this interpretation and suggests that production of anti-H-specific antibodies may require a critical ratio between the two subunit types (Arosio et al., 1976).

In conclusion, all the properties of horse ferritins investigated in the present work can be interpreted in the framework of the hybrid model of ferritin, which involves molecular species made up by different proportions of two types of subunits.

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Reactions of α-Chymotrypsin with 4-(Trifluoromethyl)-α-bromoacetanilide†

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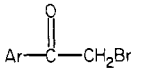
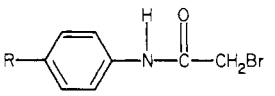
ABSTRACT: 4-(Trifluoromethyl)-α-bromoacetanilide is structurally similar to a large number of compounds that inactivate α-chymotrypsin by alkylating the methionine-192 residue or occasionally serine-195. Fluorine nuclear magnetic resonance (NMR) experiments suggest that this material reacts with the enzyme at two distinct loci. One of these involves alkylation of methionine while reaction at a second site, which does not appear to be near the active site, diminishes the proclivity for reaction at methionine. Solvent effects (H_2O/D_2O) and fluorine–proton Overhauser experiments indicate that the reporter group attached to methionine closely contacts the protein surface and is thereby shielded from solvent while the CF_3 group at the second site is more accessible to solvent.

Selective chemical modification of amino acid residues remains an important tool for structural studies of proteins, especially when the reagent used for the modification has a feature that is easily detected spectroscopically. In an early study Schramm & Lawson (1963) demonstrated that molecules having the general structure I are effective affinity labels



I

for α-chymotrypsin, and more recent work with a greatly expanded series of inactivators has illuminated those features of I that lead to rapid and selective reactions with this enzyme (Lawson & Rao, 1980; Lawson, 1980). In most cases alkylation at a methionine residue, presumed to be Met-192, of the protein was observed. However, a few structures were found to react at a serine residue, which likely was Ser-195. The Ar group of structure I is usually derived from a substituted aniline. Although substituents on the aromatic ring of Ar have some influence on the rate of the protein modification reaction, a diverse collection of para substituents on α-bromacetanilide (II) was found to have only relatively minor



II, R = H, CH_3, F, CH_3O, NO_2

effects on the reaction rate; in all cases these compounds were found to rapidly alkylate methionine (Lawson & Rao, 1980).

With the goal of introducing a fluorine NMR[1] reporter group into chymotrypsin, we earlier prepared a set of trifluoromethyl-substituted analogues of II and examined the inactivation of chymotrypsin by these structures (Bittner & Gerig, 1970). At the ortho and meta positions the CF_3 derivatives reacted 8–9 times more slowly than the corresponding CH_3-substituted α-bromoacetanilides. However, at the para position the CF_3 substituent slowed the reaction nearly 2 orders of magnitude relative to the rate when the para substituent was CH_3. Landis & Berliner (1980a,b) have reported additional studies of these fluorine-labeled derivatives of chymotrypsin and showed via fluorine chemical shifts that indole, a good competitive inhibitor of the enzyme known to bind in the "tosyl pocket" (Steitz et al., 1969), is able to displace the trifluoromethylaryl group from its normal location in or on the enzyme structure. Alkylation of the enzyme by these reagents was also demonstrated to have substantial effects on the kinetics of the hydrolysis of specific and nonspecific substrates (Landis & Berliner, 1980b).

Upon starting high-resolution fluorine NMR studies of the trifluoromethyl-labeled enzyme derivatives, we were perplexed by the appearance of two major signals in the spectrum. Landis & Berliner (1980a) have reported similar observations and have assigned one of the resonances to partially autolyzed or denatured protein. In the case of the ortho and meta CF_3-substituted derivatives, this explanation may well be correct, for chromatographic procedures substantially reduce the contribution of one signal to the spectrum. However, the same methods do not appreciably remove the second component from the fluorine spectrum of the 4-(trifluoromethyl)-

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[1] Abbreviations: NMR, nuclear magnetic resonance; GPNA, *N*-glutarylphenylalanine *p*-nitroanilide; fid, free induction decay; ^{19}F NMR, fluorine-19 nuclear magnetic resonance; NOE, nuclear Overhauser effect; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

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