Dahlquist, F. W. (1978) *Methods Enzymol. 48*, 270–299.
Erecinska, M., Sierakowska, H., & Shugar, D. (1969) *Eur. J. Biochem. 11*, 465–471.
Feldherr, C. M., Richmond, P. A., & Noonan, K. D. (1977) *Exp. Cell Res. 107*, 439–444.
Feller, M., Richardson, C., Behnke, W. D., & Gruenstein, E. (1977) *Biochem. Biophys. Res. Commun. 76*, 1027–1035.
Folin, O., & Ciocalteu, V. (1927) *J. Biol. Chem. 73*, 627–650.
Goldstein, I. J., & Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem. 35*, 127–340.
Gonatas, N. K., Kim, S. U., Stieber, A., & Avrameas, S. (1977) *J. Cell Biol. 73*, 1–13.
Greene, W. C., Parker, C. M., & Parker, C. W. (1976) *J. Biol. Chem. 251*, 4017–4025.
Grundmann, E. (1966) in *General Cytology: An Introduction to Function and Morphology of the Cell*, p 242, Williams and Wilkins, Baltimore, MD.
Jett, M. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 2313.
Jett, M., Seed, T., & Jamieson, G. A. (1977) *J. Biol. Chem. 252*, 2134–2142.
LeVine, D., Kaplan, M. J., & Greenaway, P. J. (1972) *Biochem. J. 129*, 847–856.
March, S. C., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149–152.
Martin, B. M., Wasiewski, W. W., Fenton, J. W., II, & Detwiler, T. C. (1976) *Biochemistry 15*, 4886–4893.
Nagata, Y., & Burger, M. M. (1974) *J. Biol. Chem. 249*, 3116–3122.
Nicolson, G. L. (1974) *Int. Rev. Cytol. 39*, 89–190.
Nicolson, G., LaCorbiere, M., & Delmonte, P. (1972) *Exp. Cell Res. 71*, 468–472.
Ozanne, B., & Sambrook, J. (1971) *Nature (London) 232*, 156–160.
Peters, B. P., Ebisu, S., Goldstein, I. J., & Flashner, M. (1979) *Biochemistry 18*, 5505–5511.
Reisner, Y., Lis, H., & Sharon, N. (1976) *Exp. Cell Res. 97*, 445–448.
Reunanen, M. A., & Soini, E. J. (1974) *Liq. Scintill. Counting 3*, 86–93.
Stanley, P., & Carver, J. P. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5056–5059.
Stanley, P., Sudo, T., & Carver, J. P. (1980) *J. Cell Biol. 85*, 60–69.
Steck, T. L., Weinstein, R. S., Straus, J. M., & Wallach, D. F. H. (1970) *Science (Washington, D.C.) 168*, 255.
Virtanen, I., & Wartiovaara, J. (1976) *J. Cell Sci. 22*, 355–364.
Wallach, D. F. H., & Winzler, R. J. (1974) in *Evolving Strategies and Tactics in Membrane Research*, Springer-Verlag, New York.
Walsh, F. S., Barber, B. M., & Crumpton, M. J. (1976a) *Biochemistry 15*, 3557–3563.
Walsh, F. S., Barber, B. H., & Crumpton, M. J. (1976b) *Bicohem. Soc. Trans. 4*, 251–252.

Structure, Assembly, Conformation, and Immunological Properties of the Two Subunit Classes of Ferritin†

Shinobu Otsuka,‡ Hiroshi Maruyama, and Irving Listowsky*

ABSTRACT: The two subunit types of human liver ferritin were purified to homogeneity. Both subunits reassembled in a well-defined manner and formed spherical particles that resembled natural apoferritin in electron micrographs. Affinity chromatography methods were employed to obtain preparations of antibodies that interacted exclusively either with the H or with the L polypeptides, demonstrating that distinct immunological properties may be ascribed to each subunit of ferritin. The amino acid compositions of the subunits were similar, but the larger H subunit had fewer leucine, phenylalanine, and arginine residues. It is therefore improbable that H subunits undergo proteolytic processing and are precursors for L subunits. Circular dichroism data indicated that homopolymers assembled from L-type subunits had substantially more ordered secondary structures and greater α-helical contents than their H counterparts. Small differences in the environment of tryptophan residues were evident from fluorescence spectra of each homopolymer. In isoelectric focusing experiments reassembled H or L homopolymers migrated as families of proteins within discrete p*I* ranges which are probably representative of subpopulations of each subunit type. The H homopolymer focused at lower p*I*'s than the L component. These data substantiate the contention that both subunits are authentic polypeptide moieties of ferritin with some common structural features, but the results also underscore prominent dissimilarities in their properties.

Ferritin has 24 subunits arranged in the form of a hollow sphere which envelops an iron micelle in its central cavity (Crichton, 1973; Harrison et al., 1977; Drysdale et al., 1977; Richter, 1978; Munro & Linder, 1978; Aisen & Listowsky, 1980). The protein is found in most mammalian tissues and is synthesized in response to iron (Drysdale & Munro, 1966; Linder-Horwitz et al., 1969; Fagard & Saddi, 1977). It provides a mobilizable reserve of iron in a nontoxic form for the organism's requirements. Ferritins from different organs of a single species may have common peptide sequences but often exhibit small differences in electrophoretic mobility, amino acid composition, and peptide map patterns (Linder & Munro, 1973; Crichton et al., 1973; Bomford et al., 1977; Massover, 1978; Alpert et al., 1979; Kohgo et al., 1980). In addition, a purified ferritin preparation from a single source may consist of a family of closely related proteins ("iso-

† From the Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461. *Received October 22, 1980.* This work was supported by Grants HL 11511 and AM 17702 from the National Institutes of Health.

‡ Present address: Department of Medicine, Cancer Research Institute, Sapporo Medical College, Sapporo, Japan.

0006-2960/81/0420-5226$01.25/0

ferritins"), that are resolved by isoelectric focusing methods (Drysdale, 1970, 1974; Ishitani et al., 1975a,b; Adelman et al., 1975; Russell & Harrison, 1978; Russell et al., 1978). Since it is doubtful that there are one or more different ferritins from every tissue, tissue specificity and microheterogeneity have been attributed to the existence of a family of ferritin molecules with varying proportions of two different subunit types (Drysdale et al., 1977; Arosio et al., 1978; Wagstaff et al., 1978a,b). The subunit with an electrophoretic mobility corresponding to a molecular weight of ~21 000 has been designated as the "H" type (heavy or heart), and the smaller polypeptide with a molecular weight of ~19 000 is the "L"-type subunit (Drysdale et al., 1977). The two subunits have similar amino acid compositions and peptide sequences in common, and both are synthesized in cell-free systems by using unfractionated rat liver mRNA[1] (Arosio et al., 1978). We have recently described methods for fractionation and purification of these two structurally similar subunits (Otsuka & Listowsky, 1980). In this report homogeneous H and L subunits of human liver ferritin are characterized. Both subunit species assemble into spherical "apoferritin-like" molecules and shares some common structural features, but each is structurally distinct with differences in composition, conformation, and immunological properties.

Experimental Procedures

Materials

Ferritin was prepared from human liver by methods described previously (Lavoie et al., 1978). Natural apoferritin was isolated from the ferritin by density gradient centrifugation (Niitsu & Listowsky, 1973). CNBr-activated Sepharose 4B was obtained from Pharmacia Fine Chemicals. Urea was ultrapure grade from Schwarz/Mann, and acrylamide and other electrophoretic reagents were obtained from Bio-Rad Laboratories. Sodium dodecyl sulfate ($NaDodSO_4$) (Pierce Chemicals) was recrystallized from hot ethanol. Ampholines were from LKB, and buffers, thiol reagents, and other solutions were prepared from reagent grade chemicals.

Methods

Dissociation and Reassembly of Apoferritins. The protein was dissociated into subunits by incubation in 9 M urea, pH 2.5, for 10 min, readjustment of the pH to 7.0, and centrifugation at 57000*g* for 2 h to pellet the inorganic iron micelles (Listowsky et al., 1972). Homogeneous H- and L-type subunits were prepared by the electrochromatography procedures described previously (Otsuka & Listowsky, 1980). For reassembly of discrete (H- and L-containing) apoferritin components, subunit solutions (0.5–1.0 mg/mL) in 9 M urea were dialyzed successively into 4 M urea, 2 M urea, and 0.01 M sodium phosphate buffer, pH 7.0, in the presence of 1 mM dithiothreitol. The dithiothreitol was then dialyzed out into large volumes of 0.01 M phosphate buffer, pH 7.0.

Electron Microscopy. A Siemans 1A electron microscope was used for these experiments. Natural apoferritin and reassembled H- and L-type homopolymers in aqueous solutions (~0.2 mg/mL protein) were prepared for electron microscopy by procedures described earlier (Ishitani et al., 1975a). The protein specimens were negatively stained with 1% uranyl acetate solution, and micrographs were obtained at a primary magnification of 94 000×. One drop of protein solution was applied to carbon-coated Formvar grids and dried. Catalase molecules were used to calibrate the micrographs. Histograms of size distribution of apoferritin molecules were obtained by measuring ~225 intact molecules for each preparation.

Electrophoresis. Nondenaturing gels, $NaDodSO_4$–polyacrylamide gels, and protein samples used for electrophoresis were prepared by methods described earlier (Lavoie et al., 1978, 1979). Relative amounts of H or L subunits in reassembled mixtures were estimated by densitometric scans of $NaDodSO_4$ gels. A Gilford spectrophotometer with a Model 2410 linear transport was used to scan cylindrical gels, and Clifford Densicomp Model 445 was used for slab gels. An LKB 2117 multiphore apparatus was used for slab gel isoelectric focusing. Solutions of 2% pH 4–6 ampholine were used for electrofocusing under conditions described elsewhere (Lavoie et al., 1978).

Amino Acid Analysis. Amino acid analyses were performed in triplicate by using a Beckman 119 BL auto analyzer. Samples (0.1–0.2 mg of protein) were hydrolized in 1.0 mL of constant-boiling 6 N HCl in evacuated sealed tubes at 110 °C for 20–24 h. Several different preparations each of H and L subunits were analyzed.

Circular Dichroism and Fluorescence. A Cary Model 60 spectropolarimeter with 6001 CD attachment was used for circular dichroism measurements. The instrument was calibrated with *d*-10-(+)-camphorsulfonic acid. A 1-cm path length cell was used, and the temperature of the cell compartment was 25–27 °C. A mean residue weight of 109 was used to calculate molar ellipticities ($[\theta]$ in deg·cm^2·dmol^{-1}).

Fluorescence spectra were obtained by using a Perkin-Elmer Model 650-10 S fluorescence spectrophotometer. Uncorrected excitation and emission spectra were obtained with slit widths of 5 nm. Protein concentrations were estimated by methods described previously (Listowsky et al., 1972).

Affinity Chromatography. Affinity columns were prepared by coupling H and L subunits to Sepharose supports. CNBr-activated Sepharose 4B (1 g) was swollen and washed on a scintered-glass filter with 200 mL of a 1.0 mM HCl solution. Purified H or L subunit preparations (3 mL of 100 μg/mL each) were dissolved in 0.5 M NaCl in 0.1 M $NaHCO_3$ buffer, pH 8.6, and mixed with the gel suspension. The suspension was agitated for 12 h at 4 °C and washed with the above buffer to remove unbound protein. The unreacted groups were inactivated with 1 M ethanolamine, in the bicarbonate buffer, and three subsequent cycles with 0.5 M NaCl in 0.1 M sodium acetate buffer, pH 4.0, and 1.0 M NaCl in 0.1 M sodium borate buffer, pH 8.0, removed noncovalently bound protein.

The affinity column was washed with phosphate-buffered saline (PBS) (0.02 M sodium phosphate and 0.18 M NaCl, pH 7.3), and antisera were applied (see below). Unbound components were eluted with PBS buffer and bound antibodies eluted with 3 M KSCN solution. Sheep anti-human placental ferritin sera (Marcus et al., 1978), which were enriched in antibodies that interacted with acidic subunits, were used to isolate anti-H antibodies. Rabbit anti-human liver ferritin antisera raised against L-rich ferritins (Marcus et al., 1978) were used to isolate anti-L antibodies.

Immunodiffusion. Ouchterlony double-immunodiffusion studies were performed in 1% agarose gels prepared in a 0.15 M NaCl solution, pH 7.0. Protein samples (0.3–0.7 mg/mL) and antisera were applied to indicated wells, and diffusion was allowed to proceed for 48 h at 20 °C. Gels were stained with Coomassie Brilliant Blue and destained.

Results

Dissociation, Isolation, and Reassembly of Subunits. Human liver ferritin, like most other ferritins, is stable in the

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; mRNA, messenger ribonucleic acid.

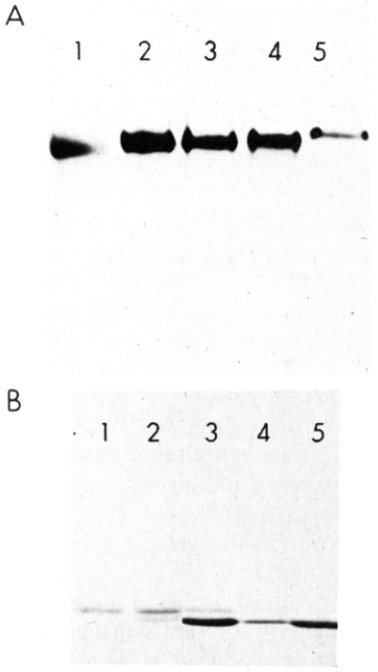

FIGURE 1: Polyacrylamide gel electrophoresis of apoferritin components assembled from subunits. Plate A is an electrophoretic pattern in a nondenaturing slab gel prepared by using a 5–22% acrylamide gradient run from top to bottom as shown. Plate B is an $NaDodSO_4$ gel (11–30% concave acrylamide gradient). Assembled apoferritins (200–400 μg/mL) were dissociated by heating samples at 100 °C for 3 min in 1% $NaDodSO_4$–1% mercaptoethanol solutions. Approximately 100 μL was applied to each channel. Lane 1 is a homopolymer of H-type subunits (20 μg), and lane 5 is a homopolymer of L subunits (25 μg). The components in lanes 2–4 were reassembled from mixtures of the two subunits containing 60%, 30%, and 10% H-type subunits, respectively. Lane 2 had 35 μg of protein, lane 3, 40 μg protein, and lane 4, 32 μg of protein.

presence of high concentrations of urea at neutral pH (Listowsky et al., 1972). The protein was therefore incubated in 9 M urea at pH 2.5 for 10 min and the pH readjusted to 7.0; these conditions promote its dissociation into subunits (Listowsky et al., 1972). Homogeneous H and L subunit types were prepared by electrochromatography procedures (Otsuka & Listowsky, 1980).

After removal of urea by stepwise dialysis procedures (cf. Experimental Procedures), the reassembled apoferritin moieties were examined by polyacrylamide gel electrophoresis and electron microscopy. Figure 1A shows that H or L homopolymers, and reassembled mixtures of the two subunits, migrate in single zones in nondenaturing electorphoretic media. There are insignificant amounts of nonspecific subunit aggregates under these conditions (usually more than 90% of the protein migrated in one zone), but if the reconstitution was carried out in the absence of thiol reagents or if subunit concentrations were high (>1 mg/mL), greater amounts of nonspecific aggregates were detected by gel filtration or in nondenaturing gels. Figure 1B shows $NaDodSO_4$–polyacrylamide gel electrophoresis patterns of the subunits of H and L homopolymers and mixtures of the two components obtained by dissociating the assembled proteins.

Electron micrographs of reassembled H- and L-type homopolymers are shown in Figure 2A,B. Reconstituted molecules that are nearly spherical particles of about 110–130 Å in diameter predominate, and these resemble natural ferritin and apoferrin molecules. The size distribution among the H-type components appears to be slightly greater than among the L homopolymer components (Figure 2C). A small number of imperfect spheres and other fragments are also visible on the

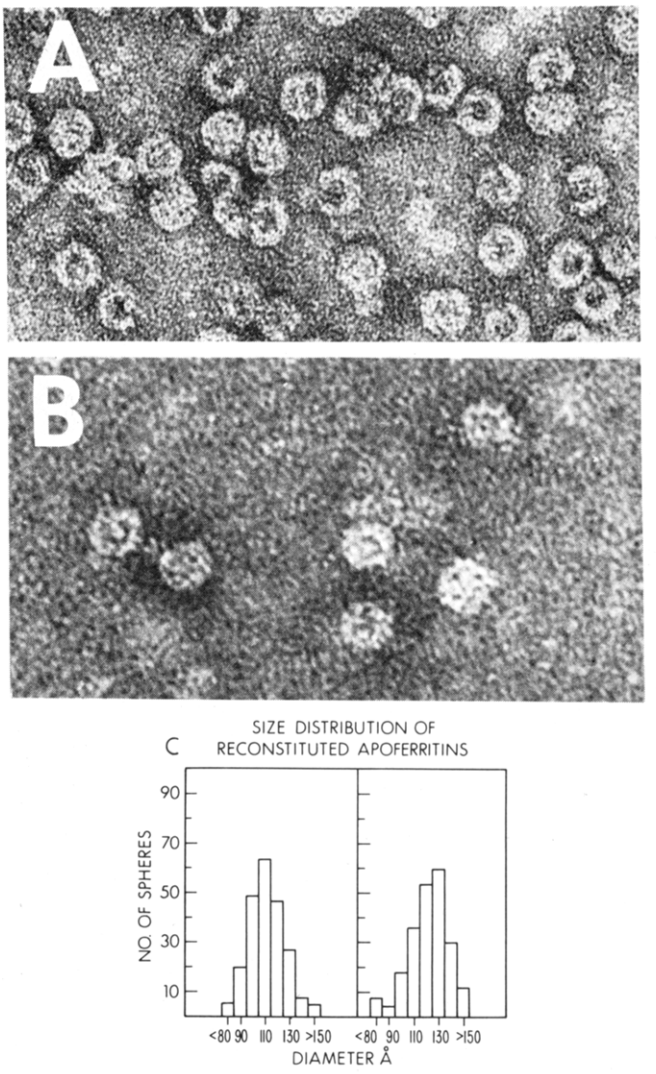

FIGURE 2: Electron micrographs of reassembled apoferritins. Plate A is a component reassembled from L-type subunits. Plate B is a reassembled H-type homopolymer. Primary magnifications were 94 000× and the magnification shown is 470 000×. Plate C shows histograms of the size distribution of reconstituted L (left panel) and H (right panel) apoferritins. Approximately 225 intact spheres in five different fields were chosen randomly for measurement of diameters with the aid of a calibrated magnifier. Final magnification in the micrographs was 470 000×.

sections of the grid shown in Figure 2A.

Solubility properties may also be used to distinguish between the subunit species. Thus the L-type components are readily soluble in water or low ionic strength buffers and crystallize in the presence of $CdSO_4$. H components are not precipitated by $CdSO_4$ and are sparingly soluble in low ionic strength solutions (i.e., <0.01 M sodium phosphate buffer, pH 7.0).

Circular Dichroism and Fluorescence. Circular dichroism spectra of reassembled H and L homopolymers are compared to the spectrum of natural human liver ferritin in Figure 3. Assembly of L subunits restores most of the secondary structure associated with natural ferritin. The spectrum of this preparation is characterized by bands at 221 and 208 nm. H homopolymers, however, have considerably less ordered secondary structure than their L counterparts. For H homopolymers ellipticity magnitudes at the shorter wavelength band (205 nm) are greater than those of the 221-nm band, with a considerable diminution in intensity of both bands as compared to those of the L component. A reassembled hybrid molecule (30% H, 70% L) has an ellipticity magnitude of −14 200 at 221 nm, which indicates an almost additive nature

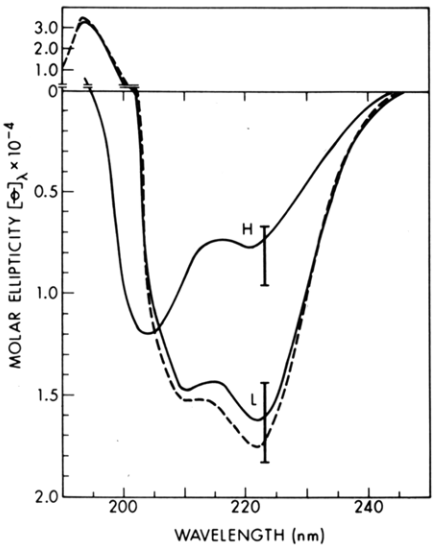

FIGURE 3: Circular dichroism spectra of reassembled H- and L-type polymers. The dotted line represents a spectrum of natural human liver ferritin. H and L are reassembled homopolymers. Protein concentrations were 0.05–0.1 mg/mL (1-cm cell) and 0.4 mg/mL (1-mm path length cell) in 0.01 M sodium phosphate buffer, pH 7.0. Ellipticity magnitudes were independent of protein concentration in these ranges. Other conditions are cited under Experimental Procedures.

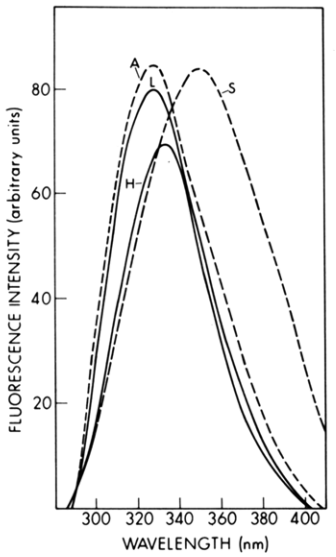

FIGURE 4: Fluorescence emission spectra. All spectra were uncorrected with an excitation wavelength of 280 mm. Protein samples were 0.3–0.6 mg/mL in 1 mM sodium phosphate buffer, pH 7.0, and data were normalized to the same protein concentration. L and H are homogeneous components reassembled from the corresponding subunits of human liver ferritin, A is natural apoferritin, and S is natural apoferritin equilibrated in the presence of 9 M urea, pH 2.5, to generate dissociated and unfolded subunits of ferritin.

of the conformation of the two subunits in their heteropolymeric context.

Fluorescence spectra of H and L homopolymers and natural and denatured apoferritins are shown in Figure 4. Natural apoferritin and reassembled L components have intrinsic emission maxima near 330 nm (excitation 280 or 296 nm). The tryptophan emission for H homopolymers is red shifted to 335 nm. These maxima are at longer wavelengths than those reported previously for horse spleen ferritin (Stefanini et al., 1976). The spectra do not change if these preparations are equilibrated in 9 M urea at neutral pH. Acid–urea denatured samples of all components, however, have emission maxima near 358 nm (Figure 4).

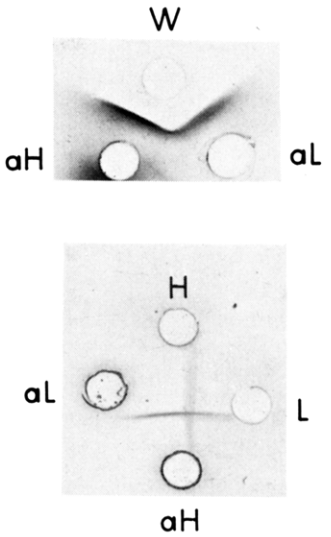

FIGURE 5: Immunological analysis of human ferritin subunits. Ouchterlony double-immunodiffusion reactions between whole human liver ferritin (W), reassembled H-type subunits (H) (1 drop of 120 mg/mL), and reassembled L-type homopolymers (164 μg/mL) (L), with anti-H sera (aH) and anti-L sera (aL). The anti-L serum was prepared by passing rabbit anti-human liver ferritin sera through an H subunit affinity column and adsorbing the eluant on the L column (cf. text). The anti-H component was prepared by passing sheep anti-human placental feritin sera through an L subunit affinity column followed by the H column. The bound antiserum components from the second column in each case were released by 3 M potassium thiocyanate.

Immunological Studies. Affinity columns of H and L subunits were prepared by coupling each purified component to cyanogen bromide–Sepharose 4B. Sheep anti-human placental ferritin IgG was passed through the L subunit affinity column to adsorb the anti-L component, and rabbit anti-human liver ferritin IgG was passed through the H subunit column. The antisera were then passed through the homologous columns to be adsorbed and eluted with 3 M KSCN. Interactions of the purified antibodies with assembled apoferritins were examined by Ouchterlony immunodiffusion methods.

The data in Figure 5 show that the two subunit types are immunologically distinguishable; we were not able to detect cross-reactivity at several concentrations of antigen (unpublished). Both antisera give immunoprecipitin lines of fusion with whole human liver ferritin (Figure 5) or with reconstituted hybrid molecules made up of H and L subunits (not shown).

Isoelectric focusing patterns of H- and L-type homopolymers, and a component reconstituted from a 1:1 mixture of H and L subunits, are shown in Figure 6. These are compared to results obtained with natural human liver ferritin (which contained about 70% L subunits and 30% H). Each preparation focused as a family of isoproteins within discrete p*I* ranges. Thus, the H apoferritin is distinctly more acidic than the L component, and the electrofocusing range of the hybrid mixture spans the P*I*'s of both components.

Discussion

Analyses of homogeneous preparations of the H and L subunit classes of human ferritin provide compelling evidence that the two polypeptides are discrete structural entities and are authentic subunits of ferritin. These data apparently refute previous contentions that the H subunit is an aggregate of proteolytic fragments of the L subunit (Bryce *et al.*, 1978) or that the two bands observed in gels may represent conformational isomers not completely dentured and with different amounts of bound detergent (May & Fish, 1977). The H and

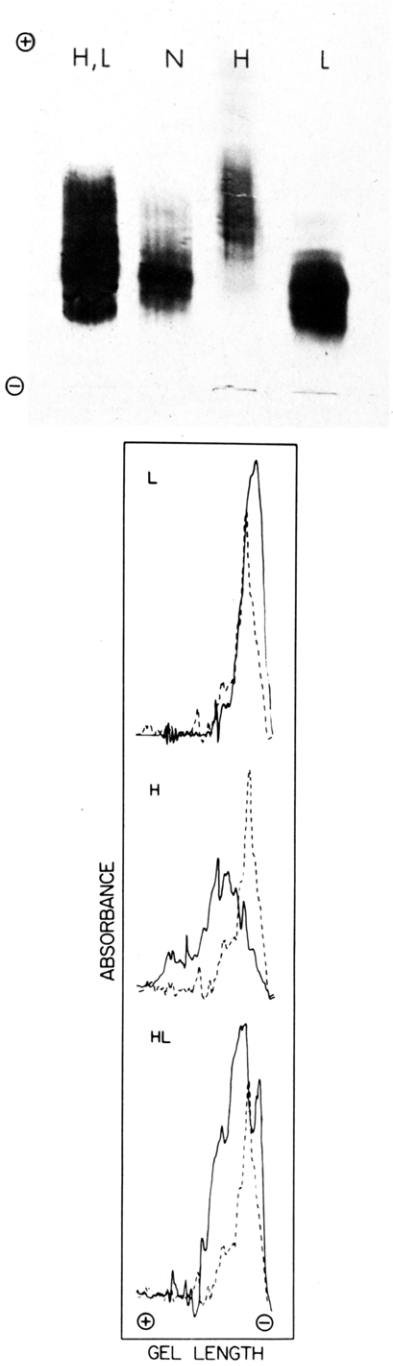

FIGURE 6: Isoelectric focusing of reassembled components. Gel electrofocusing was performed in an acrylamide slab gel containing a 2% pH 4–6 ampholine solution. Other conditions are described under Methods. HL was reassembled from a 1:1 mixture of H and L subunit types. N is a natural liver ferritin preparation (which contained about 70% L-type subunits and 30% H). H is a homopolymer reassembled from H-type subunits, and L is a homopolymer reassembled from L-type subunits. Electrofocusing data of natural human liver ferritin (dotted line) are superimposed upon the scans of the reassembled L, H, and HL moieties.

L subunits have certain structural similarities but also display properties that distinguish them from one another. Both subunits have common elements of primary structure and similar amino acid compositions (Table I), and previous peptide mapping results (Arosio et al., 1978) indicated that there is extensive homology in primary structure of the two subunits.

After removal of denaturant, both subunits tend to reassemble in a defined manner to form apoferritin-like components. These aggregates have electrophoretic mobilities similar to those of natural human liver ferritin (Figure 1A) and assume the characteristic morphological appearance in electron micrographs that resembles authentic apoferritin molecules (Figure 2).

Table I: Amino Acid Composition of the Two Subunits of Human Ferritin[a]

residue	H subunit	H[b]	L subunit	L[b]
Asx	28 (±1.6)	22	22 (±1.3)	16
Thr	8 (±0.8)	7	7 (±0.5)	7
Ser	11 (±0.9)	12	9 (±0.7)	9
Glx	30 (±1.2)	24	25 (±1.0)	21
Pro	4 (±0.7)		4 (±0.5)	
Gly	12 (±1.7)	11	13 (±1.3)	15
Ala	17 (±1.3)	13	16 (±1.4)	13
Cys	2 (±0.4)		1–2 (±0.05)	
Val	7 (±0.6)	7	7 (±0.4)	7
Met	4 (±0.5)	3	4 (±0.6)	2
Ile	6 (±0.7)	6	4 (±0.6)	5
Leu	23 (±1.1)	20	27 (±0.9)	19
Tyr	7 (±0.6)	8	6 (±0.7)	5
Phe	6 (±0.4)	7	8 (±0.4)	6
Lys	13 (±1.0)	11	12 (±0.9)	10
His	7 (±0.9)	7	6 (±0.5)	5
Arg	9 (±0.8)	9	11 (±0.7)	9

[a] The data are expressed as residues per subunit (to the nearest whole number), assuming a molecular weight of 21 000 for the H subunit and 19 500 for the L subunit. The values are approximate means ± standard deviations, based on data obtained for four different preparations of H and five preparations of the L subunits. [b] Data from Arosio et al. (1978).

The data in Figure 3 indicate that homopolymers of reassembled H subunits are conformationally distinct from those assembled from L subunits. The L component has substantially more ordered secondary structure (~50% α helix) than the H apoferritin (~30% α helix) (cf. Listowsky et al. (1972) for data analyses). Fluorescence studies not only show that both H and L peptides contain tryptophan residues but also suggest differences in tryptophan environments of the two subunits (Figure 4). These results are unexpected if considered in terms of similarities in primary structure of the two subunit types. Moreover, reconstituted apoferritins are not as stable as natural apoferritin in the presence of denaturants and are partially denatured in 10 M urea at neutral pH (unpublished observations). The fidelity of the denaturation–dissociation and reassembly procedure described here may thus be questioned. It is possible that the unexpected CD spectra of H homopolymers result from methodological problems during reassembly and that a "quasi"-native secondary structure of the protein is restored by these methods. Further studies are required to resolve this issue. It should be noted, however, that the L subunit was treated in the same way, and sizes, morphological appearances, electrophoretic mobilities, and other properties are restored by the reassembly procedure. In addition, the results are consistent with earlier observations that ferritin from human heart, placenta, and other tissues that have enriched populations of H subunits has a more unfolded secondary structure than liver or spleen ferritins which are enriched in L-type subunits (Lavoie et al., 1979).

Antisera prepared by affinity chromatography methods provide an unequivocal approach to differentiate between H and L components. Thus in spite of extensive sequence homologies, it is possible to obtain specific populations of antibodies that react exclusively with H types and others that react only with L subunits. These data imply that there are structural features unique to *both* H and L subunits and also are consistent with the hypothesis that L is not generated from H.

The data in Table I indicate that the smaller L subunit has more leucine, phenylalanine, and arginine than the H subunit. Arosio et al. (1978) also found more leucine in the L subunit of horse spleen ferritin. These results appear to rule out the possibility that H polypeptides undergo proteolytic processing and are precursors for L subunits. It is also unlikely that other posttranslational modifications of a polypeptide synthesized as a single chain account for the subunit heterogeneity of ferritin.

The antisera may also be used to support the notion that natural ferritin is a true hybrid system with individual molecules containing both subunits (Otsuka et al., 1980). It had been suggested that tissue ferritins are mixtures of two homopolymers and give rise to families of proteins by minor posttranslational modifications (Russell et al., 1978). Indeed, isoelectric focusing studies (Figure 6) show that polymers assembled from homogeneous H or L subunits still generate multiple bands, and these may reflect subpopulations of each subunit type. It is therefore noteworthy that both anti-H and anti-L antibodies give a line of fusion in their reaction with natural human liver ferritin (Figure 5). If a mixture of reassembled H and L polymers is used as the antigen, it does not yield lines of fusion in Ouchterlony experiments.

A need to resolve the controversy regarding the authenticity and interrelationship between the two subunits is underscored by recent metabolic studies which show that the two subunit systems of most ferritins may have important physiological implications. The relative amounts of H and L subunits vary in different tissues of a single species (Arosio et al., 1977, 1978; Drysdale et al., 1977; Drysdale, 1977; Hazard & Drysdale, 1977; Powell et al., 1975). It is likely that hybrid molecules containing different proportions of the two subunits account for the observed tissue specificity of ferritin. More important, however, is the apparent differential regulation of the two subunits in response to iron release to the tissues. In rats the amount of L subunit increases to a much greater extent than does the amount of H subunit after iron administration (Kohgo et al., 1980). Thus, there are much greater proportions of L subunits in tissue replete with iron. Conversely, ferritins from iron-poor sources such as heart, placenta, and certain malignant tissues are usually enriched in H-type subunits. Indeed, direct correlations have been made between nonheme iron content and H and L subunit distribution of ferritin and various human and rat tissue (Wagstaff et al., 1978a,b; Bomford et al., 1978; Kohgo et al., 1980).

The H and L polypeptides may be primary gene products or may originate from a single precursor by posttransciptional processing. It is reasonable to assume that H and L subunits have different mRNAs, especially since both subunits are synthesized in cell-free translation systems (Arosio et al., 1978). At present, however, there are no clear indications about the formation of two ferritin subunits. Nevertheless, distinctions made on the basis of structural studies such as those described here may provide a basis for elucidating their role in mammalian iron metabolism. Furthermore, specific H and L antisera (i.e., Fig. 5) may be essential to provide a more unified approach for analysis of the extensive immunoassays of serum ferritins as indices of reticuloendothelial iron stores, malignancies, and other physiological factors (Alfrey, 1978).

Acknowledgments

We are grateful to Dr. D. M. Marcus for providing antisera and for his helpful discussions regarding the immunological results. We also thank J. Fant for her help with the electron microscopy experiments and I. Moisa for his skilled technical assistance.

References

Adelman, T. G., Arosio, P., & Drysdale, J. W. (1975) *Biochem. Biophys. Res. Commun. 63*, 1056–1062

Aisen, P., & Listowsky, I. (1980) *Annu. Rev. Biochem. 49*, 357–393.

Alfrey, C. P. (1978) *CRC Crit. Rev. Clin. Lab. Sci. 9*, 179–208.

Alpert, E., Quaroni, A., & Goldenberg, D. M. (1979) *Biochim. Biophys. Acta 581*, 193–197.

Arosio, P., Yokoto, M., & Drysdale, J. W. (1977) *Br. J. Haemotol. 36*, 201–209.

Arosio, P., Adelman, T. G., & Drysdale, J. W. (1978) *J. Biol. Chem. 253*, 4451–4458.

Bomford, A., Lis, Y., McFarlane, I. G., & Williams, R. (1977) *Biochem. J. 167*, 309–312.

Bomford, A., Berger, M., Lis, Y., & Williams, R. (1978) *Biochem. Biophys. Res. Commun. 83*, 334–341.

Bryce, C. F. A., Magnusson, C. G. M., & Crichton, R. R. (1978) *FEBS Lett. 96*, 257–262.

Crichton, R. R. (1973) *Struct. Bonding (Berlin) 17*, 67–134.

Crichton, R. R., Millar, J. A., Cumming, R. L. C., & Bryce, C. F. A (1973) *Biochem. J. 131*, 51–59.

Drysdale, J. W. (1970) *Biochim. Biophys. Acta 207*, 256–258.

Drysdale, J. W. (1974) *Biochem. J. 141*, 627–632.

Drysdale, J. W. (1977) *Ciba Symp. Found, 51*, 41–57.

Drysdale, J. W., & Munro, H. N. (1966) *J. Biol. Chem. 241*, 3630–3637.

Drysdale, J. W. Adelman, T. G., Arosio, P., Casareale, D., Fitzpatrick, P., Hazard, J. T., & Yokota, M. (1977) *Semin. Hematol. 14*, 71–88.

Fagard, R., & Saddi, R. (1977) *Biochimie 59*, 765–773.

Harrison, P. M., Banyard, S. H. Hoare, R. J., Russell, S. M., & Treffry, A. (1977) *Ciba Symp. Found. 51*, 19–40.

Hazard, J. T., & Drysdale, J. W. (1977) *Nature (London) 265*, 755–756.

Ishitani, K., Niitsu, Y., & Listowsky, I. (1975a) *J. Biol. Chem. 250*, 3142–3148.

Ishitani, K., Listowsky, I., Hazard, J., & Drysdale, J. W. (1975b) *J. Biol. Chem. 250*, 5446–5449.

Kohgo, Y., Yokota, M., & Drysdale, J. W. (1980) *J. Biol. Chem. 255*, 5195–5200.

Lavoie, D. J., Ishikawa, K., & Listowsky, I. (1978) *Biochemistry 178* 5448–5454.

Lavoie, D. J., Marcus, D. M. Otsuka, S., & Listowsky, I. (1979) *Biochim. Biophys. Acta 579*, 359–366.

Linder, M. C., & Munro, H. N. (1973) *Am. J. Pathol. 72*, 263–282.

Linder-Horowitz, M., Ruettinger, R. T., & Munro, H. N. (1969) *Biochim. Biophys. Acta 200*, 442–448.

Listowsky, I., Blauer, G., Englard, S., & Betheil, J. J. (1972) *Biochemistry 11*, 2176–2182.

Marcus, D. M., Zinberg, N., & Listowsky, I. (1978) *Immunodiagnosis of Cancer* (Herberman, R., & McIntire, R., Eds.) pp 473–499, Marcel Dekker, New York.

Massover, W. H. (1978) *Biochim. Biophys. Acta 532*, 202–206.

May, M. E., & Fish, W. W. (1977) *Arch. Biochem. Biophys. 182*, 396–403.

Munro, H. N., & Linder, M. C. (1978) *Physiol. Rev. 58*, 317–396.

Niitsu, Y., & Listowsky, I. (1973) *Arch. Biochem. Biophys. 158*, 276–281.

Otsuka, S., & Listowsky, I. (1980) *Anal. Biochem. 102*, 419–422.

Otsuka, S., Listowsky, I., Niitsu, Y., & Urushizaki, I. (1980)

J. Biol. Chem. 255, 6234-6237.
Powell, L. W., Alpert, E., Isselbacher, K. J., & Drysdale, J. W. (1975) Br. J. Haematol. 30 47-55.
Richter, G. W (1978) Am. J. Pathol. 91, 363-396.
Russell, S. M., & Harrison, P. M. (1978) Biochem. J. 173, 91-104.
Russell, S. M., Harrison, P. M., & Shinjo, S. (1978) Br. J. Haematol. 38, 296-298.
Stefanini, S., Chiancone, E., & Antonini, E. (1976) FEBS Lett. 69, 90-94.
Wagstaff, M., Worwood, M., & Jacobs, A. (1978a) Br. J. Haematol. 39, 624-625.
Wagstaff, M., Worwood, M., & Jacobs, A. (1978b) Biochem. J. 173, 969-977.

Subunit Structure and Physical Properties of the Hemocyanin of the Giant Isopod *Bathynomus giganteus*†

K. E. Van Holde* and Michael Brenowitz‡

ABSTRACT: The hemocyanin of the deep sea dwelling isopod *Bathynomus giganteus* has been isolated. From the determination of the sedimentation coefficient ($s^0_{20,w}$ = 16.6 S) and the diffusion coefficient ($D_{20,w}$ = 3.1 × 10^{-7} cm^2/s at 1.12 mg/mL), a molecular weight of 4.7 × 10^5 was calculated. The 16S molecule is the largest aggregate observed even under conditions of temperature, pressure, and concentration simulating in vivo conditions. The dissociation of the 16S molecule to 6S monomers is dependent on pH and divalent cation concentration. The sedimentation coefficient and calculated molecular weight are consistent with a hexameric native molecule composed of six monomers of molecular weight about 70 000–80 000, as has been observed for other hemocyanins. The constituent subunits are heterogeneous on both sodium dodecyl sulfate (two bands, 70 200 and 71 800 molecular weight) and nondenaturing alkaline electrophoresis (three bands). In the presence of 10 mM $CaCl_2$, the hexamer is stable to above pH 9.0. Upon dialysis vs. buffer containing 10 mM ethylenediaminetetraacetate, the hemocyanins begin to dissociate into monomers at pH 8.0. The pH-dependent hexamer–monomer dissociation is slowly equilibrating and completely reversible and obeys the law of mass action. The reaction can be described by a cooperative mechanism with one proton binding per monomer. The oxygen binding properties of the hexamer hemocyanin are characterized by a large positive Bohr effect (P_{50}'s at pH 9 and 7 are 5.6 and 57.5, respectively) and moderate cooperativity (n_H = 1.8–3.0). Divalent cations do not appreciably affect either the oxygen affinity or the cooperativity of the hexamer. Complete dissociation to monomers yields noncooperative binding, as expected. The oxygen binding curves of partial dissociation mixtures are well represented by the weighted sum of the curves for "pure monomer" and "pure hexamer" conditions. Comparison of the oxygen binding properties of this deep sea animal with coastal and intertidal species suggests that physiological requirements are more important than phylogenetic relationships in determining the properties of the oxygen-transport protein of a particular species.

The subunit structure, equilibria, and oxygen binding of hemocyanin have been the subject of numerous studies [for reviews, see Van Holde & Van Bruggen (1971), Antonini & Chiancone (1977), and Bonaventura et al. (1977)]. Recent interest in these proteins has centered mainly around two topics: the assembly of the subunits into the native oligomers and the homotropic and heterotropic interactions which influence oxygen binding. Arthropod hemocyanins are assemblies of ca. 70 000-dalton polypeptides organized into hexameric building blocks. In most cases, larger polymers are formed. The aggregation state to which the subunits assemble is species dependent with native molecules ranging in size from a single hexamer (in *Panulirus* and *Penaeus* hemocyanins for example) to an eight-hexamer, 48-subunit molecule found in *Limulus* hemocyanin (Kuiper et al., 1975; Brouwer et al., 1978; Johnson & Yphantis, 1978). The hexamers, dodecamers, etc. isolated from different species have similar morphology when examined in the electron microscope. This observation supports the application of information gained from studies of a single species to the properties of arthropod hemocyanins in general.

The stability of the oligomer is usually dependent on a combination of factors including pH, ionic strength, divalent cation concentration, and oxygen binding. Different conditions are often required to stabilize each level of aggregation [for examples, see Miller & Van Holde (1974), Kuiper et al. (1975), and Brenowitz et al. (1980)]. It is thus necessary to study each association–dissociation reaction separately to understand its importance to the assembly of the whole molecule. An additional complication in studying assembly is heterogeneity of the constituent polypeptides (see reviews). Differences in the roles of a particular subunit in assembly have been demonstrated for hemocyanins from a number of arthropod species (Jeffrey et al., 1978; Lamy et al., 1977; Bijlholt et al., 1979). For example, the apparent role of the dimeric "linker" subunits which have been found in some hemocyanins is to bridge across the two hexamers stabilizing a dodecamer. This explains the observation that hexamers

† From the Marine Biological Laboratory, Woods Hole, Massachusetts 02543. *Received January 16, 1981.* This research was supported in part by Grant GM 00265 to the Marine Biological Laboratory and in part by National Institutes of Health Grant 15460 and Office of Naval Research Grant N 00014-79-C-0178 to Drs. Celia and Joseph Bonaventura, Marine Biomedical Center, Duke University Marine Laboratory.

* Address correspondence to this author at the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331.

‡ Present address: Department of Biochemistry, Duke University Marine Laboratory, Beaufort, NC 28516.

0006-2960/81/0420-5232$01.25/0