On the Mechanism of Horse Spleen Apoferritin Assembly: A Sedimentation Velocity and Circular Dichroism Study

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ABSTRACT: The apoferritin shell is known to assemble spontaneously from its subunits obtained at acid pH upon neutralization. The reassembly of apoferritin from horse spleen has been followed by means of sedimentation velocity and circular dichroism experiments as a function of the pH and the nature of the assembly buffer in order to obtain information on the assembly pathway. In all the buffer systems tested the subunits sediment as a single peak of varying sedimentation and diffusion coefficients, and shell assembly starts at pH values around 3.5. In dilute glycine–acetate buffers the subunits are essentially dimeric up to this pH value. Therefore, the dimeric building blocks of the apoferritin shell that are apparent in the X-ray structure represent the first assembly intermediates. When the pH is increased to 4.0–4.3, the weight-average sedimentation velocity of the subunits increases to 3.6–4.7 S, respectively, and the subunit population becomes heterogeneous. Concomitantly, significant changes in the circular dichroism properties of the aromatic residues take place. On the basis of the X-ray structure, where aromatic residues appear to be located at or near the fourfold symmetry axes, these data suggest that assembly proceeds from dimers through tetramers and octamers. In the pH range 4.5–6.5 the reassembly process cannot be followed due to reversible precipitation of the subunits near their isoelectric point; at neutral pH values essentially quantitative reassembly is obtained. A further series of experiments in other buffer systems has shown that the presence of chloride or phosphate markedly alters the behavior of the subunits: at acid pH values the weight-average sedimentation coefficient is much higher than that in glycine–acetate buffers, but the circular dichroism spectrum in the near-UV region remains featureless. Moreover, at neutral pH values massive precipitation of the subunits takes place. These data point to specific binding of chloride and phosphate to the subunits and suggest that the interaction leads to the formation of assembly misfits.

Apoferritin, the protein moiety of the iron storage molecule ferritin, has a rather unique structure being assembled from 24 structurally equivalent subunits related by 432 symmetry (Rice et al., 1983). The same structural arrangement is shared by all tissue ferritins, despite the fact that they consist of families of closely related hybrid polymers, the isoferritins, assembled from different proportions of two polypeptide chains, the H and L chains (Arosio et al., 1978). The two types of chain differ in molecular weight (about 21 000 for H and 19 000 for L) and have extensive sequence homologies; their ratio is species- and tissue-specific; for example, it is 9 L/1 H in horse spleen and 1 L/8.5 H in horse heart (Heusterspreute & Crichton, 1981; Addison et al., 1983; Boyd et al., 1985). The assembly of apoferritin is well suited to the functional role of the protein since it allows the formation of a central cavity, where iron crystallites can be harbored, and of channels (along the threefold and fourfold axes), which provide a means for iron to enter and leave the protein shell.

The unique structural arrangement of the 24 apoferritin subunits imparts to the protein shell an unusual stability toward dissociation into subunits. Thus, at neutral pH, apoferritin can stand heating to 80 °C for 10 min or incubation in 10 M urea or 1% sodium dodecyl sulfate at room temperature (Listowsky et al., 1972; Smith-Johannsen & Drysdale, 1969). Possibly due to this remarkable stability, most studies on the association–dissociation properties of apoferritin have dealt with the search for experimental conditions that lead to its dissociation. In particular, it has been established that exposure of apoferritin to pH values below 3.0 (Crichton & Bryce, 1973), to concentrated guanidine hydrochloride (Listowsky et al., 1972), or to the combined action of low pH and high concentrations of urea (Otsuka et al., 1981) yields subunits that reassemble into apoferritin-like molecules (as judged by their physicochemical and spectroscopic properties and by their appearance in the electron microscope) after neutralization and/or removal of the denaturant, provided the formation of aspecific aggregates is minimized, e.g., by the use of low protein concentrations and thiol protecting agents. Other aspects of the dissociation and reassembly reactions have received relatively less attention. Crichton and co-workers inferred on the basis of circular dichroism (Wood & Crichton, 1971), UV difference spectroscopy (Crichton & Bryce, 1973), and chemical modification data (Crichton, 1973) that dissociation of horse spleen apoferritin entails a large change in the environment of several aromatic residues (four or five tyrosines and one tryptophan per subunit), which become exposed to solvent, and that a significant hysteresis occurs in the reassociation reaction. Regarding the existence of stable intermediates, Crichton (1972) obtained evidence by gel filtration that in the course of both dissociation and reassociation subunit dimers and tetramers are formed and that these subunits appeared to be in slow equilibrium on the gel filtration time scale. Stefanini et al. (1979) reported on the formation of 7S subunits, tentatively identified as hexamers, when reassembly is induced at acid pH by the presence of 8-anilino-1-naphthalenesulfonic acid. Concerning the recognition mechanisms that account for the assembly of the highly specific quaternary structure of apoferritin, Otsuka et al. (1980) demonstrated that formation of the isoferritin hybrid shells occurs in a random manner also with L and H subunits from different species, since there is no apparent preference for assembly of homologous subunits.

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This paper presents data bearing on the mechanism of reassembly of subunits obtained after acid dissociation of horse spleen apoferritin. Reassembly was followed by sedimentation velocity. The ultracentrifuge patterns show that different low molecular weight subunit types are stabilized under different solvent conditions and that the assembly process is highly cooperative as indicated by the absence of detectable subunit peaks of sedimentation velocity higher than 4.7 S. In order to discriminate between the two assembly pathways that have been proposed on the basis of the X-ray structure, i.e., dimer–tetramer–octamer and dimer–trimer–hexamer (Rice et al., 1983; Ford et al., 1984), the sedimentation velocity data have been supplemented with circular dichroism (CD) ones in the near-UV region. Advantage has been taken of the different location of the aromatic residues with respect to the three distinct intersubunit contacts that are generated by the 432 geometry of the molecule. Thus, the extensive contact along the dimer interface is characterized by the presence of several aromatic residues on the outside surface of the molecule while hydrophilic residues face the internal cavity; the contacts around the fourfold channels likewise have a remarkably hydrophobic character and are rich in aromatic residues, while the contacts around the threefold channels are lined with hydrophilic residues. The significant changes that take place in the CD spectrum of aromatic residues concomitantly with the increase in molecular weight of the subunits indicate that condensation of dimers proceeds via tetramers and octamers.

Materials and Methods

Horse spleen ferritin was prepared as described previously (Stefanini et al., 1982); apoferritin was obtained by reduction of iron with sodium dithionite and chelation with α,α'-bipyridyl (Stefanini et al., 1975). Apoferritin concentration was calculated from the absorption at 280 nm by using the extinction coefficient $E^{1\%}_{1cm} = 9.0$ (Bryce & Crichton, 1973).

Apoferritin dissociation was achieved by dialyzing apoferritin solutions at concentrations around 4–5 mg/mL for 24 h at 4–6 °C against 200 mM glycine–HCl buffer at pH 1.8; this treatment ensured complete dissociation of the protein as indicated by control sedimentation velocity experiments.

Apoferritin reassociation was carried out routinely in the cold (4–6 °C) by dialyzing overnight apoferritin subunits at pH 1.8 against buffers of higher pH. The buffers in the acid range were prepared by adjusting the pH of a 40 mM glycine solution with 1 N HCl (in the range 2.0–3.5) or 1 M CH_3COOH (in the range 3.5–4.3); 0.6% β-mercaptoethanol was also added. The final concentration of HCl was 40 or 4 mM at pH values of 2.0 and 3.5, respectively, and that of CH_3COOH was 65 or 2.0 mM at pH values of 3.5 and 4.3. The buffers around neutrality were 10 mM [bis(2-hydroxyethyl)amino]-tris(hydroxylmethyl)methane (Bis-Tris) or tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) containing 2 mM dithiothreitol. The apoferritin solutions were analyzed in a Spinco Model E ultracentrifuge equipped with an RTIC unit at 44 000 or 52 000 rev/min and at a temperature of 10–12 °C. The sedimentation coefficients were reduced to s_{20} by standard procedures. The amount of reassociated apoferritin was evaluated on enlarged tracings of the ultracentrifuge patterns from the area of the 17S component and of the 23S component, if present. The areas of the peaks were measured by planimetry and were corrected for radial dilution.

Diffusion coefficients were calculated from the areas of the schlieren diagrams and were corrected for radial dilution and for the movement of the boundary in the centrifugal field according to Elias (1961).

Iron incorporation into reassociated apoferritin was followed by measuring the absorption of the ferric oxide micelle at 350 nm; ferrous ammonium sulfate was used as the source of iron (Stefanini et al., 1976).

Iron reduction was followed spectrophotometrically at 400 nm after a solution of reconstituted ferritin in 20 mM imidazole buffer at pH 6.4 was mixed anaerobically with varying amounts of sodium dithionite. The concentration of dithionite was determined by titration of known ferricyanide solutions (Stefanini et al., 1978).

Analytical polyacrylamide gel electrophoresis was performed at acid pH in 7.5% acrylamide gels with a 37:1 ratio of acrylamide to N,N'-methylenebis(acrylamide) according to the method of Davis (1964). All samples were used in the native state; gels were run for 2 h at 300 V and at 4 °C.

Circular dichroism experiments were carried out in the UV region with a Jasco J500 spectropolarimeter equipped with a DP500 data processor. The data obtained in the near-UV region are expressed as $\Delta\epsilon$ on the basis of the relationship $\Delta\epsilon = [\theta]^*/3300$, where $[\theta]^*$ is the molar ellipticity calculated with an average polypeptide chain M_r of 20 000 (Strickland, 1974).

Results

Reassembly Reaction

Time Course and Effect of Protein Concentration. A first series of experiments was carried out in 40 mM glycine–CH_3COOH buffers containing 0.6% β-mercaptoethanol in the pH range 4.0–4.3, where reassembly into whole molecules is only partial according to earlier investigations (Harrison & Gregory, 1968; Crichton & Bryce, 1973). Dissociated apoferritin solutions in 200 mM glycine–HCl buffer at pH 1.8 were equilibrated with the reassembly buffer at 4–6 °C on a Sephadex G-25 column; the final protein concentration was 1.5–3 mg/mL. The solutions were analyzed in the ultracentrifuge within 15 min from the pH change, and the time course of reassembly into the 17S apoferritin molecule was followed for about 24 h thereafter. In many preparations the amount of reassembled protein reaches its maximum value already at the time of the first ultracentrifuge run; in others it continues to increase somewhat for several hours (Figure 1). However, this slow phase in the assembly reaction is contrasted by the slight tendency of the subunits to precipitate, a feature that appears to be related to the "age" of the apoferritin stock solution. The omission of β-mercaptoethanol from the reassembly buffer has no significant effect on the amount of reassociated apoferritin in accordance with the negligible ionization of the –SH groups in this pH range.

A second series of experiments was performed at pH values around neutrality. In these experiments the dissociated apoferritin solutions at pH 1.8 were dialyzed first against 40 mM glycine–CH_3COOH buffers at pH 3.5–4.0, where no or partial reassociation into whole molecules takes place; β-mercaptoethanol was added to give a final concentration of 0.6%, and the solution was brought to the desired pH by addition of small volumes of 1 M Tris. The final protein concentration was 1.5–3 mg/mL. As in the reassembly experiments at acid pH, in most preparations the amount of reassociated molecules reaches its final value already at the time of the first ultracentrifuge run (i.e., about 15 min after the pH change); in others a small part of the material reassociates at a slow rate with a half-time in the order of hours. Representative results are included in Figure 1.

The protein concentration was varied next at pH 4.0–4.3. In agreement with earlier investigations (Crichton & Bryce, 1973; Otsuka et al., 1980) at protein concentrations higher than about 4 mg/mL massive precipitation occurs, due to

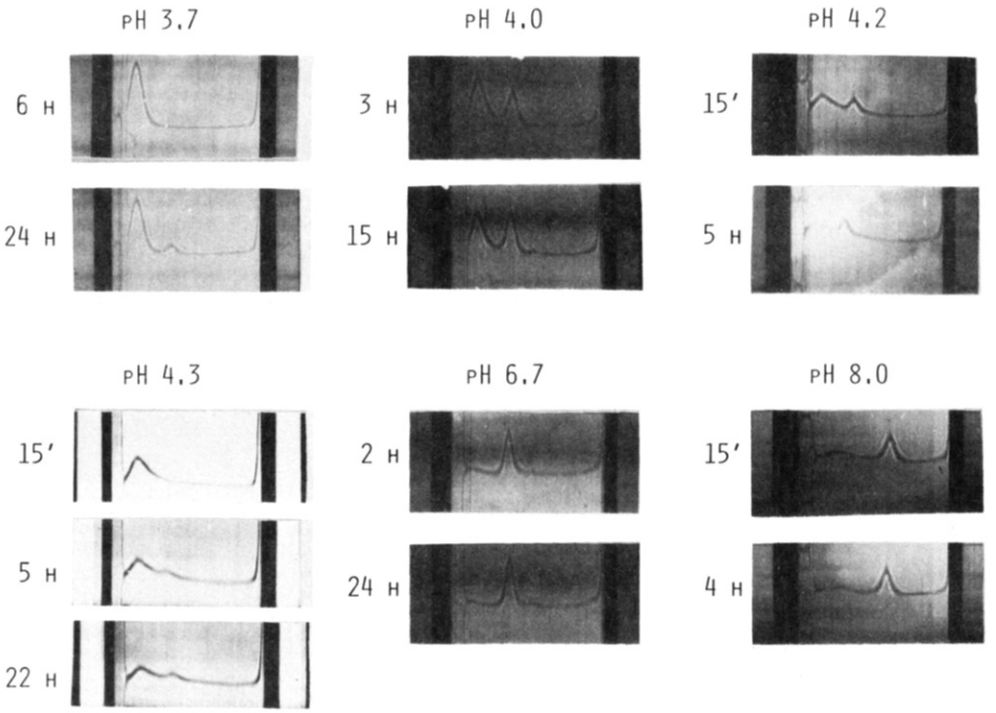

FIGURE 1: Extent of reassociation of apoferritin subunits as a function of time of exposure to reassembly buffer. The conditions for dissociation and reassociation are detailed in the text. The photographs of the samples at pH 3.7, 4.0, 4.3, and 6.7 were taken 45 min after a full speed of 44000 rev/min was reached; those of the samples at pH 4.2 and 8.0 were taken 30 and 45 min, respectively, after 52000 rev/min was reached. The final protein concentration was 1.5–3.0 mg/mL.

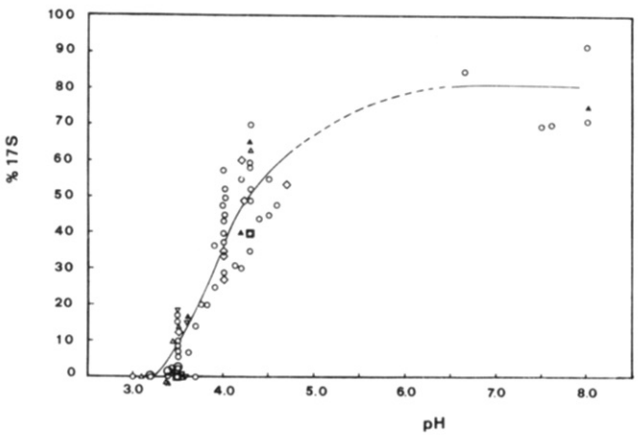

FIGURE 2: Reassociation of apoferritin as a function of pH of reassembly buffer. Dissociation was achieved at pH 1.8; samples were analyzed in the analytical ultracentrifuge after exposure to the reassembly buffer for 15–20 h. The data were obtained on about 30 different apoferritin preparations. Reassembly buffer: glycine–HCl or CH_3COOH at a concentration of (◇) 10, (○) 40, and (□) 200 mM; (△) 40 mM NaCl + CH_3COOH; (▽) 40 mM NaH_2PO_4 + CH_3COOH; (▲) subunits at pH 3.2 brought to the indicated pH with 1 M Tris.

formation of aspecific aggregates. Over the accessible concentration range (0.5–3 mg/mL) the amount of reassembled material does not change significantly in any given subunit preparation but does vary in different preparations (see Figure 2). On the basis of these data the assembly experiments were performed routinely by overnight dialysis (at 4–6 °C) of the dissociated apoferritin solutions at concentrations of 1–3 mg/mL against the desired buffer.

pH Dependence. The pH dependence of the reassembly reaction was studied in 40 mM glycine–HCl or CH_3COOH buffers containing mercaptoethanol and in 10 mM Bis-Tris or Tris-HCl buffers containing dithiothreitol as detailed under Materials and Methods. In accordance with the data obtained by Crichton and Bryce (1973) in other buffer systems, assembly of the subunits into the 17S apoferritin molecule starts at pH values around 3.4 and is 50% complete at pH values of 4.0–4.3 (Figure 2). In the pH range 4.6–6.5, no experiments can be performed due to precipitation of the protein near its isoelectric point. When the pH is increased further, the greatest part of the protein goes back into solution and reassociates substantially (70–90%). Reassembled apoferritin has the same sedimentation velocity (16–17 S) as the native molecule (Figure 3A), while the subunits sediment under all accessible conditions as a single peak with a weight-average velocity that increases from 2.6 ● 0.1 S at pH 2.0–3.0 to 4.7 ± 0.6 S at pH 4.3. The increase in sedimentation velocity is paralleled by an increase in the diffusion coefficient of the subunit peak (Table I and Figure 3B). Moreover, in many experiments at pH values 4.0–4.3, the base line between the subunit peak and the 17S one is elevated significantly, a finding that points to the presence of assembly intermediates.

Table I: s_{20}, D_{20}, and Molecular Weight of Apoferritin Subunits at Acid pH in Dilute Glycine Buffer

pH	s_{20} ($\times 10^{13}$)[a]	D_{20} ($\times 10^{7}$)[a]	M_w[a,b]
2.0–3.0	2.6 ● 0.1 (5)	6.4 (3)	35600 (3)
3.5	3.0 ± 0.3 (9)	7.5 (7)	38500 (7)
4.0	3.6 ± 0.3 (11)	>8[c]	
4.3	4.7 ± 0.6 (13)	≫8[c]	

[a] The number of experiments is given in parentheses. [b] Calculated from s_{obsd} and D_{cor} on the basis of the relationship $M_w = sRT/[D(1 - \bar{V}\rho)]$, using a partial specific volume $\bar{V} = 0.740$. [c] Accurate determination difficult (see text).

Apoferritin dimers (s_{20} = 23 S) are formed in small amounts during reassembly at pH values higher than 4.0, albeit with slower kinetics than the 17S monomers, as brought out by the experiments reported in Figure 1.

Effect of Solvent Composition. Lastly, the effect of solvent composition on the extent of reassembly and on the sedimentation velocity of the subunits was studied at pH 3.5 and 4.3. The results summarized in Table II show that a change in the molarity of the glycine–CH_3COOH buffers between 10

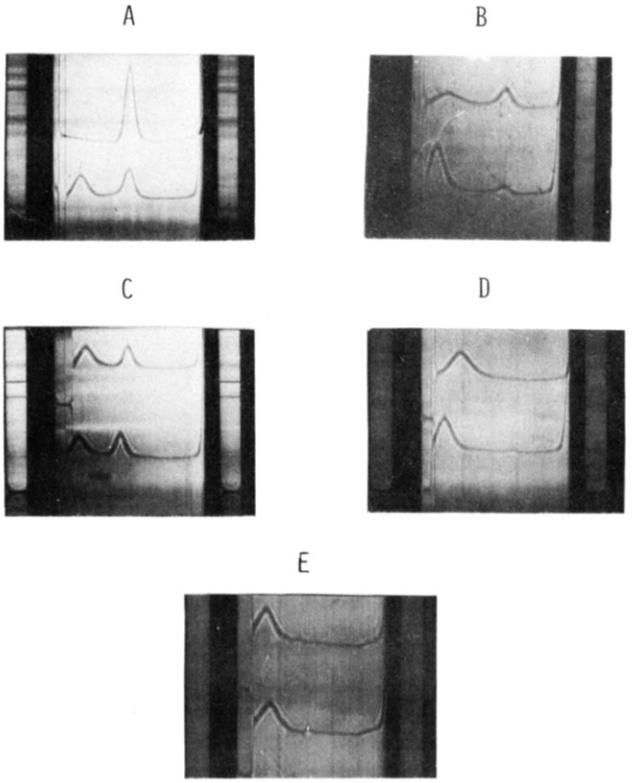

FIGURE 3: Sedimentation velocity patterns of apoferritin subunits as a function of pH and nature of reassembly buffer. (A) Native apoferritin in 100 mM Tris-HCl at pH 7.0 (top). Buffer composition: (A) 40 mM glycine–CH_3COOH at pH 4.3 (bottom); (B) 40 mM glycine–CH_3COOH at pH 4.3 (top) and 3.5 (bottom); (C) 40 mM (top) and 10 mM (bottom) glycine–CH_3COOH at pH 4.3; (D) 40 mM glycine–CH_3COOH at pH 3.5 with 40 mM NaCl (top) and without NaCl (bottom); (E) 200 mM glycine–CH_3COOH at pH 3.5 (top) and 200 mM glycine–HCl at pH 3.5 (bottom). Photographs were taken 55 min (B and D) or 80 min (E) after a full speed of 52 000 rev/min was reached and 55 min after full speed of 44 000 rev/min (A and C) was reached. Protein concentration was 2–3 mg/mL.

Table II: Effect of Solvent Composition on Sedimentation Velocity of Apoferritin Subunits at pH 3.5 and 4.3[a]

solvent composition	pH	subunit peak %	subunit peak s_{20}
10 mM glycine + CH_3COOH (26 mM)	3.5	100	2.7
40 mM glycine + CH_3COOH (64 mM)	3.5	100	3.0
40 mM glycine + CH_3COOH (64 mM)	3.5	100	2.7
40 mM glycine + HCl (4 mM)	3.5	95	2.8
200 mM glycine + CH_3COOH (300 mM)	3.5	100	3.0
200 mM glycine + HCl (16 mM)	3.4	100	3.7
500 mM glycine + HCl (40 mM)	3.5	100	3.9
40 mM glycine + CH_3COOH (64 mM) + NaCl (40 mM)	3.5	100	4.5
40 mM NaCl + CH_3COOH (5 mM)	3.5	100	5.0
40 mM NaH_2PO_4 + CH_3COOH (20 mM)	3.5	100	5.1
40 mM NaOH + HCl (43 mM)	3.4	100	4.1
40 mM NaOH + CH_3COOH (306 mM)	3.5	100	3.9
40 mM NaOH + HCOOH (110 mM)	3.5	100	4.2
40 mM NaOH + lactic acid (135 mM)	3.5	100	3.9
10 mM glycine + CH_3COOH (1 mM)	4.3	50	4.0
40 mM glycine + CH_3COOH (2 mM)	4.3	45	4.1
40 mM glycine + CH_3COOH (2 mM)	4.3	30	4.7
200 mM glycine + CH_3COOH (16 mM)	4.3	60	4.1
40 mM NaCl + CH_3COOH (0.2 mM)	4.3	40	6.4

[a] Protein concentration 1.5–2.5 mg/mL; temperature 10–12 °C.

and 200 mM (which corresponds to an increase in ionic strength from 0.8 to 15 mM) does not produce significant differences in the sedimentation velocity of the subunits (see also Figure 3C). In contrast, the addition of 40 mM NaCl causes an increase both in the sedimentation and in the diffusion coefficient of the subunit peak (Figure 3D). A similar effect (Figure 3E) is obtained when CH_3COOH is substituted with HCl in the glycine buffers of higher molarity (e.g., 200 mM, I = 16 mM). This latter observation suggests that the effect of NaCl can be ascribed to specific binding of the anion to the apoferritin subunits. In order to substantiate this contention, a series of experiments was performed at pH 3.5 with buffer solutions containing different anions and Na^+ as a cation (in these experiments the ionic strength was 40 mM). The sedimentation and diffusion coefficients of the subunits were significantly higher than those in the glycine–CH_3COOH buffers. The nature of the buffer does not seem to influence markedly the amount of reassociated material at acid pH values. However, when subunit solutions at pH 3.5 in 40 mM NaCl or NaH_2PO_4 are dialyzed vs. neutral pH buffers, massive precipitation is always observed. Moreover, sedimentation velocity experiments carried out on the protein remaining in solution indicate that the extent of reassembly never exceeds 40–50%.

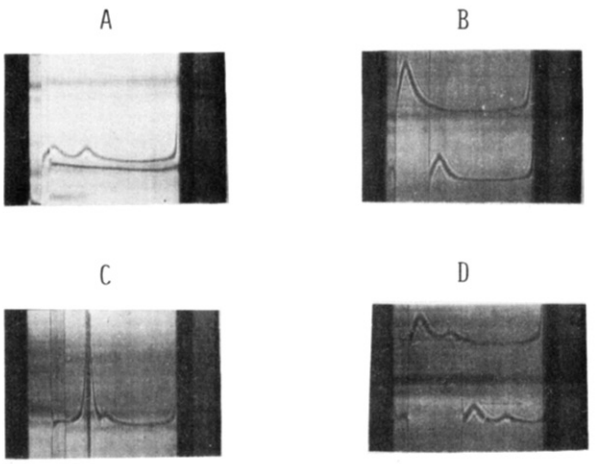

FIGURE 4: Schlieren patterns of fractions obtained from partially reassociated apoferritin by preparative ultracentrifugation: (A) stock solution (2 mg/mL) reassociated in 40 mM glycine–CH_3COOH at pH 4.0; (B and C) two of the upper supernatant fractions and pellet analyzed within 4 h after the preparative ultracentrifugation; (D) same two supernatant fractions after 48 h. Photographs were taken 40 min after a full speed of 44 000 rev/min was reached. For further details, see text.

"Nature" of Reassembly Reaction. The possible existence of a finite equilibrium between apoferritin subunits and whole molecules in partially reassembled solutions was assessed by means of the following experiment. A dissociated apoferritin solution (15 mL at 2 mg/mL) was reassembled partially by extensive dialysis against 40 mM glycine–CH_3COOH buffer containing β-mercaptoethanol at pH 4.3. It contained 3.6S subunits and 17S whole molecules in roughly equal amounts. This solution was subjected to a preparative ultracentrifugation for 4 h at 45 000 rev/min in a Spinco 70 Ti rotor. Four supernatant fractions and a pellet were collected and analyzed within 4 h by sedimentation velocity. The three upper supernatant fractions, which amounted to a total of ~13 mL, contained only 3.5S–3.7S subunits; the fraction nearest to the pellet (~1 mL) contained subunits and some 30% whole molecules and was not analyzed further. The pellet resuspended in the dialysis buffer was practically devoid of subunits but contained 23S dimers. In the three upper supernatant fractions, 48 h after their separation from the pellet, reassembly of the subunits into 17S molecules was substantial although it did not reach the level of the initial solution; during this time no changes in the composition of the pellet occurred.

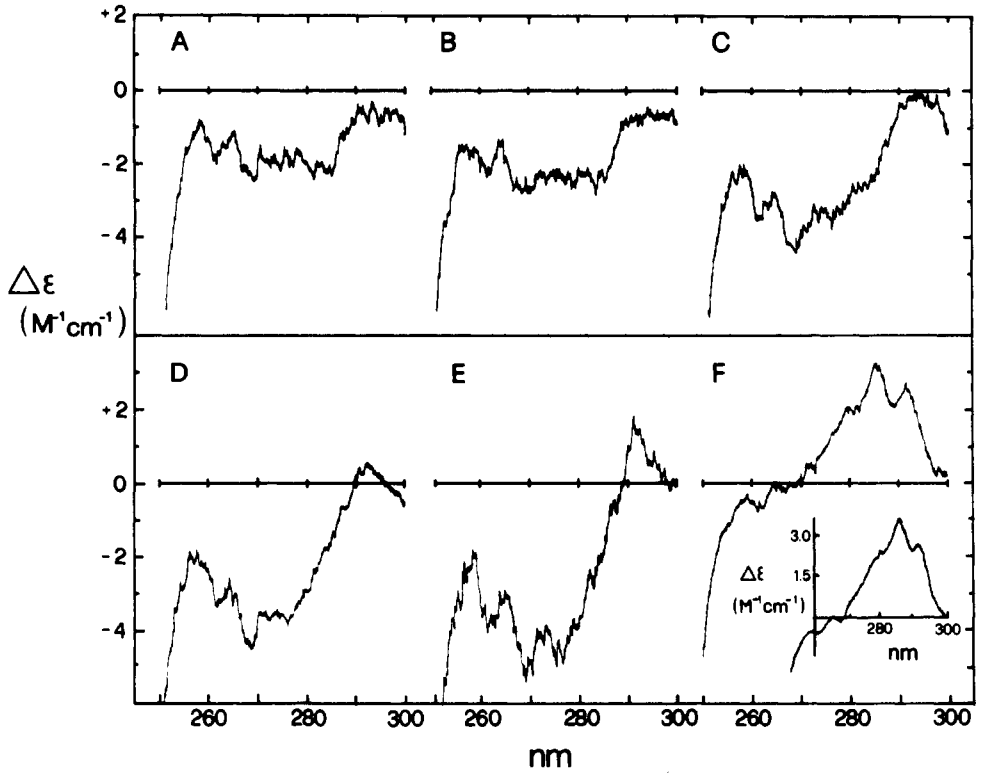

FIGURE 5: Near-UV circular dichroism spectra of apoferritin subunits at acid pH values. Buffer: 10 mM glycine–CH_3COOH at pH 2.0 (A), 2.5 (B), 3.2 (C), 3.5 (D), and 4.0 (E and F). (E) and (F) refer respectively to subunits and reassembled apoferritin separated from a partially reassociated mixture by preparative ultracentrifugation. The inset shows the spectrum of native apoferritin at pH 3.5. Protein concentration was 0.8 (A–D) or 1 (E and F) mg/mL.

Representative sedimentation velocity patterns of this experiment are given in Figure 4.

Characterization of Subunits

Diffusion Coefficients and Molecular Weights. The diffusion coefficients measured in dilute glycine buffers in the pH range 2.0–3.5 have been used in combination with the s values to calculate the molecular weight of the subunits by assuming $\bar{V} = 0.740$ (Table I). At higher pH values diffusion increases significantly (see Figure 3A). However, an accurate evaluation of the diffusion coefficient is difficult due to the presence in many experiments of an elevated base line between the subunit peak and that pertaining to the reassembled material. In other experiments, especially those at pH 4.3, the D values show a marked time dependence indicative of molecular weight heterogeneity in the subunit population.

CD Experiments. As a means to further characterize the various subunit types of different sedimentation velocity that are obtained in the pH range 2.0–4.0 in dilute glycine buffers (see Table I), their near-UV CD spectra were measured. In fact, due to the localization of many aromatic residues in the vicinity or at the intersubunit contacts, the near-UV CD spectral features of the native molecule and of the subunits differ markedly. Thus, native horse spleen apoferritin displays a characteristic spectrum with two positive peaks at 293 and 286 nm and negative ones between 270 and 260 nm due to the immobilization of the tryptophan residue and of at least some of the tyrosines and phenylalanines in a hydrophobic environment. This spectrum is maintained also at pH 3.5 (inset to Figure 5). In contrast, the subunits at pH 3.0 in 10 mM glycine–HCl buffer display only a negative CD spectrum below 290 nm (Stefanini et al., 1982).

Figure 5 shows the near-UV CD spectra of apoferritin subunits as a function of pH in 10 mM glycine buffers and at protein concentrations of 0.8–1 mg/mL. At pH values ≤ 2.5, where the weight-average s_{20} value of the subunits is 2.6 S, the ellipticity is slightly negative over the whole spectral range analyzed (300–250 nm); at pH 3.2–3.5, where dissociation is still complete but the weight-average sedimentation velocity of the subunits increases to 3.0 S, a small positive dichroic band at 293 nm with negative shoulders at 286 and 283 nm and intense negative peaks between 270 and 260 nm become evident. Over this pH range the subunits essentially reacquire the secondary structure of the native molecule as indicated by their far-UV CD spectra (data not shown). At pH 4.0 the 3.6S subunits, isolated by preparative ultracentrifugation from a mixture containing about 50% reassembled apoferritin, exhibit spectral features similar to those of the subunits at pH 3.5 but have a significantly higher rotatory power at 292 nm, while the pellet shows the spectrum of the native molecule (positive peaks at 293 and 286 nm in the tryptophan and tyrosine region and negative ones in the phenylalanine region, between 270 and 260 nm).

In the presence of 40 mM NaCl and at pH 3.5, the subunits despite their high s_{20} value ($\sim$5.0 S) display a negative CD spectrum of smaller rotatory power than that characteristic of the subunits in dilute glycine buffers at the same pH (data not shown).

Properties of Reassembled Apoferritin. Reassembled apoferritin has the same physicochemical properties, e.g., sedimentation velocity, electrophoretic mobility in 7.5% polyacrylamide gels, and CD spectra in the UV region, as the native molecule.

The functional properties of the reassembled molecule were also studied in parallel with those of the native one. The reassembled polymer incorporates ferrous ions and reconstitutes ferric micelles inside the protein shell like native apoferritin, albeit with somewhat slower kinetics. However, the incubation product is indistinguishable from that of the native protein in terms of the size distribution of the ferric micelles,

as indicated by sedimentation velocity analyses, and in terms of the rate of iron release by dithionite at pH 6.4.

Discussion

Apoferritin subunits are known from previous work to have a strong tendency to reassociate into apoferritin-like molecules characterized by the same physicochemical properties as the native protein. Hence, distinct assembly mechanisms and highly specific subunit interactions must underlie the acquirement of the unique arrangement of the 24 subunits in the apoferritin shell. Analysis of subunit packing in the high-resolution X-ray structure of horse spleen apoferritin shows that the subunits are organized in dimeric building blocks, which are ideally suited to the generation of a molecular assembly with 432 symmetry. Higher levels of organization are also apparent: octamers, that create the fourfold channels, and hexamers, that create the threefold channels. Hence, it may be envisaged that either octamers or hexamers represent the route by which the condensation of dimers proceeds during shell assembly (Rice et al., 1983; Ford et al., 1984).

This sedimentation velocity study on the reassociation of subunits obtained at low pH provides information on this point as well as on other features of the assembly reaction. Over the pH range where shell formation occurs, the schlieren patterns always show only two peaks, one pertaining to the reassembled molecule and the other to subunits. The presence of only one subunit peak indicates that if several subunit types are present, they are in rapid equilibrium on the centrifuge time scale (Gilbert, 1959). This result cannot be compared easily with the data reported by Crichton (1972, 1975), who obtained evidence for a slow equilibrium between the subunits in gel filtration but did not provide information on the time scale of the experiments. In contrast to the fast equilibration of subunits, there is no finite equilibrium between subunits and whole molecules since the latter have no tendency to dissociate when separated from the subunits. This finding is not unexpected given the remarkable stability of the apoferritin shell but proves the fidelity of the reassembly reaction just as the similarity of the physicochemical and functional properties of the native and of the reassembled protein. A further observation is that at moderately acid pH part of the subunits assembles rapidly, while the rest goes very slowly. Moreover, the fraction that is assembly-competent varies from preparation to preparation and with the "age" of the stock solution. This conformational heterogeneity reflected in the competence to reassemble could be related to a "conformational drift" of the subunits that leads to their progressive disorganization as described by Weber and co-workers (Weber, 1986; King & Weber, 1986a,b).

The subunit peak has a weight-average sedimentation velocity that depends on pH and buffer composition. In particular, in dilute glycine buffers the sedimentation coefficient is 2.6 S at pH values between 2.0 and 3.0 but increases to 3.0 S at pH 3.5, where assembly of the apoferritin shell starts, and to 4.7 S at pH 4.3, which corresponds to the apparent pK of the assembly reaction. It is interesting that the progressive increase in the subunit sedimentation velocity initiates around pH 3.5, concomitantly with the appearance of assembled shells. At this pH, molecular weight calculations using the s and D values show the subunits to be essentially all dimeric. These data therefore lend experimental support to the proposal based on the apoferritin X-ray structure that dimers represent the basic building block for shell assembly (Rice et al., 1983; Ford et al., 1984). The X-ray data also show that dimer formation entails burying of several aromatic residues (e.g., Tyr-28, Phe-78, Try-89) as anticipated by the difference spectroscopy data of Crichton and Bryce (1973). Indeed, a comparison of the present near-UV CD spectra taken over the pH range 2.0–3.5 indicates that at the higher pH values tyrosine (283 and 286 nm) and tryptophan (286 and 290 nm) peaks become evident and phenylalanines (250–270 nm) increase their rotatory strength as a result of their embedding in a hydrophobic environment. On the other hand, the presence of hydrophilic residues, and in particular of carboxylates, on the internal surface of the dimer contact accounts for the pH dependence of the assembly reaction.

The subsequent steps in the assembly pathway are difficult to define unequivocally on the basis of the sedimentation velocity data alone, since the subunit population becomes heterogeneous as the pH is increased to 4.0 and above. Heterogeneity is clearly indicated by the marked increase in the diffusion coefficient, whose value in some experiments (e.g., at pH 4.3) becomes also strongly time dependent. Therefore at this latter pH one can only estimate that the weight-average molecular weight is at least 65 000 on the basis of the s_{20} value and of the nomogram of Wyman and Ingalls (1943) assuming a partial specific volume, $\bar{V}$, of 0.740 and $f/f_0 = 1.2$. This average molecular weight is compatible with the existence in fast equilibrium of dimers–tetramers–octamers or dimers–trimers–hexamers. In these equilibria, octamers (complete around the fourfold axes) and hexamers (complete around the threefold axes) are likely to be more stable than tetramers and trimers, respectively, and hence should be the most conspicuous high molecular weight components in the distribution. The X-ray data suggest a means to discriminate between these two assembly mechanisms given the different nature of the intersubunit interactions, which are predominantly hydrophobic in the octamer and predominantly hydrophilic in the hexamer. On this basis one would expect a change in the CD spectra solely if the assembly pathway goes through octamers. That this may be indeed the route is indicated by the significant differences between the CD spectra at pH 3.5 and 4.0 (Figure 5D,E). Such differences can be ascribed to the high molecular weight fraction of the subunit population (~20% on the basis of the s_{20} value); in turn this assignment requires the high molecular weight subunits to have the same CD spectrum as the whole molecules.

This present work does not provide information on the assembly pathway beyond octamers. In any case, shell formation appears to proceed in a very cooperative fashion since subunits with higher levels of aggregation are never apparent as distinct schlieren peaks.

A further characteristic of the reassembly reaction brought out clearly by this study regards the influence of buffer composition. Indications that the extent of reassembly varied with the buffer used have been obtained by Harrison and Gregory (1968). Moreover, Crichton and Bryce (1973) reported that the amount of protein remaining in solution during reassembly and the apparent pK of the reaction depend on the ionic strength of the buffer. These data indicate that these effects may be ascribed to the specific binding of buffer components and in particular of anions. Thus, marked differences in the sedimentation and diffusion coefficients of the subunits are observed in the presence of different anions in buffers of the same ionic strength (e.g., in 200 mM glycine–CH_3COOH and glycine–HCl at pH 3.5, where $I = 16$ mM). With respect to acetate and formate, Cl^- and $H_2PO_4^-$ induce the formation of subunits with a different molecular weight distribution. However, several observations suggest that in Cl^- and $H_2PO_4^-$ reassembly misfits are produced. In fact, no changes in the CD spectrum of the subunits takes place in NaCl as a function

of pH in the range 2.5–4.0, and at higher pH values an irreversible, massive precipitation of the protein occurs. One may speculate that Cl^- and $H_2PO_4^-$ bind at sites located at or near one of the intersubunit contacts, thus favoring the formation of "incorrect" intermediates. It is intriguing that a possible anion binding site has been identified tentatively near the threefold channel (Harrison et al., 1986).

The occurrence of anion binding in turn raises the question whether glycine, the major buffer component in most of the experiments reported here, does itself bind to the subunits since it is present mainly in the zwitterion form at the pH values under consideration. The answer to this question is not clear-cut: on one hand, the data in 10 and 40 mM glycine–CH_3COOH do not differ significantly (Figure 3C), suggesting that at these concentrations any interaction between glycine and the subunits is weak; on the other hand, all the s_{20} values in glycine buffers are consistently lower than those measured in the absence of glycine with Na^+ as a cation (Table II).

In conclusion, these data confirm the general features of the apoferritin reassembly reaction after neutralization of the subunits obtained at acid pH and indicate that the differences observed previously in various buffer systems may be ascribed to the specific binding of anions. In addition, they provide evidence for the stability of the basic dimeric building block evident in the X-ray structure and indicate that assembly of the apoferritin shell proceeds from dimers through tetramers and octamers.

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