The Subunit Structure of Horse Spleen Apoferritin: the Molecular Weight of the Oligomer and its Stability to Dissociation by Dilution

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The oligomer molecular weight of horse spleen apoferritin was determined by sedimentation-equilibrium techniques and a value of 443 000 found. It is concluded that the apoferritin molecule consists of 24 subunits. At concentrations as low as 0.01 μM there is no evidence of subunit dissociation.

In previous papers we have shown that the polypeptide chain of horse spleen apoferritin has a molecular weight of 18 500 (Crichton & Bryce, 1970; Bryce & Crichton, 1971). The molecular weight of the oligomer has previously been reported to lie in the range 430 000–480 000 (Rothen, 1944; Harrison, 1959; Richter & Walker, 1967; Björk & Fish, 1971), as determined by a number of physical techniques. These observations, together with the results of other chemical investigations in our laboratory, suggest that the horse spleen apoferritin molecule probably consists of 23–25 chemically identical subunits (Crichton, 1971, 1972). Taking advantage of the u.v.-scanner of the Spinco model E analytical ultracentrifuge, we have redetermined the molecular weight of apoferritin by sedimentation equilibrium under conditions where no aggregation or other non-ideal behaviour was detectable and find a value of 443 000. The oligomer is not dissociated at concentrations from 1 mM to 0.01 μM .

Experimental

Ferritin was prepared from horse spleen by the method of Granick (1942) as modified by Crichton *et al.* (1973), or was purchased from Schwarz/Mann, Orangeburg, N.Y., U.S.A. Apoferritin was prepared by the method described by Crichton (1973), with thioglycolic acid.

Protein samples for ultracentrifugation or gel chromatography were prepared by using either freeze-dried or soluble apoferritin at the appropriate concentration in 1 ml of buffer of the required ionic strength and pH. The protein sample was dialysed against 250 ml of buffer for 24 h at room temperature before analysis. The pH of buffer solutions was determined with a Radiometer (Copenhagen, Denmark) model 25 pH-meter with a scale-expander.

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Sedimentation-velocity and -equilibrium experiments were carried out with a Spinco model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, Calif., U.S.A.) equipped with an electronic speed-control unit, regulated-temperature unit and split-beam-photoelectric-scanner multiplex assembly. Sedimentation patterns were monitored at 280 nm or, in a few cases, at 230 nm. Molecular-weight determinations were performed in dilute solutions with the AnG-T₁ rotor at 1162g and 20°C. The time of centrifugation was 4 days. Sedimentation-velocity experiments were performed in the same rotor with a centrifugal field of 116 200g at 20°C.

Gel chromatography of apoferritin solutions that had been prepared as described above was carried out on columns (1.5 cm × 100 cm) of Sephadex G-75, G-100 and G-150 (Pharmacia, London W.5, U.K.) equilibrated with 0.01 M-sodium phosphate buffer, pH 7.4. The columns were eluted at a flow rate of 20 ml/h and 2 ml fractions were collected. The extinction of the effluent was measured at 280 nm or at 230 nm. Dextran Blue (Pharmacia), myoglobin (C. F. Boehringer und Soehne, Mannheim, Germany) and tryptophan were used as reference materials.

Apoferritin was *N*-methylated by using the procedure of Rice & Means (1971) with [¹⁴C]formaldehyde of specific radioactivity 15 mCi/mmol (The Radiochemical Centre, Amersham, Bucks., U.K.) in 0.1 M-sodium borate buffer, pH 9.0. Excess of reagent was removed by dialysis against the same buffer. Typical results of *N*-methylation gave a value of 97 $\mu\text{Ci}/\text{mmol}$ of protein, corresponding to 5.3 methyl groups/subunit. Radioactivity was determined by mixing 5 μl –1 ml of protein solution in 10 ml of Instagel (Packard Instrument Co., Zurich, Switzerland) and counting in a liquid-scintillation counter (Packard Instrument Co.).

Results and discussion

To establish conditions under which aggregation of apoferritin to higher oligomers did not occur, a series of sedimentation-velocity experiments were carried

out in phosphate buffers at various ionic strengths in the pH range 6–7, and in distilled water. Both in phosphate buffers of molarities 0.01–0.2M and in distilled water no evidence of aggregation was observed. The effect of protein concentration was examined in 0.01M-phosphate buffer, pH 6.4, and on extrapolation to zero concentration a sedimentation coefficient of 17.12S was found. Molecular-weight determinations were carried out over a range of protein concentrations from 0.2 to 1.0mg/ml in 0.01M-phosphate buffer, pH 6.4, by sedimentation-equilibrium techniques, as described above under 'Experimental'. There was no evidence of inhomogeneity (Fig. 1). A value of 0.731 was used for the partial specific volume $\bar{v}$, derived from amino acid analysis of the protein (Crichton, 1969; Bryce & Crichton, 1971). The molecular weight was found to be 443 000.

Previously published values for the molecular weight of horse spleen apoferritin range from 430 000 to 465 000 (Rothen, 1944; Harrison, 1959; Richter & Walker, 1967; Björk & Fish, 1971). However, the values of 465 000 reported by Rothen (1944) from sedimentation-diffusion experiments and of 462 000 by Harrison (1959) from X-ray-diffraction data were both derived by using a value of 0.747 for $\bar{v}$, from pycnometric measurements (Rothen, 1944). The presence of variable amounts of higher oligomers (dimers and trimers), and of a yellow contaminant that is always observed when apoferritin is prepared by the reduction of ferritin with dithionite in the presence of bipyridyl (Granick & Michaelis, 1943), would certainly affect the pycnometric determination of $\bar{v}$. The substitution of 0.731 for $\bar{v}$ in these two cases would result in values for the molecular weight of 440 000 and 437 000 respectively. The other published values of 430 000 by light-scattering techniques (Richter & Walker, 1967) and 440 000–465 000 by

Fig. 1. Sedimentation-equilibrium analysis of horse spleen apoferritin (0.55 mg/ml) in 0.01M-phosphate buffer, pH 6.4

The plot of $\ln C$ against r^2 was constructed from the scanner data at equilibrium (after 4 days at 1162g).

sedimentation-equilibrium analysis (Björk & Fish, 1971), with the meniscus-depletion method of Yphantis (1964), are also in good agreement with the result reported in the present paper.

Since our previous studies showed that the molecular weight of the horse spleen apoferritin subunit is 18 500 (Crichton & Bryce, 1970; Bryce & Crichton, 1971), a value that has been subsequently confirmed independently (Björk & Fish, 1971), we may conclude that the apoferritin molecule most probably consists of 24 polypeptide chains. This number was first suggested by Harrison (1959) from X-ray-diffraction data and subsequently rejected by her in favour of a 20-subunit model (Harrison, 1963). As has been pointed out (Crichton, 1972), the evidence for eicosameric structures in general is rather poor. The subunit molecular weights of apoferritins from human liver and spleen and from horse liver are all about 18 500, and the sedimentation coefficients of the oligomers are all closely similar to that reported here for the horse spleen protein (Crichton *et al.*, 1973). The same is true of the apoferritin from the fungus *Phycomyces* (R. R. Crichton, unpublished work). It therefore seems likely that the subunit structures of all of these proteins are identical.

The stability of the oligomer as a function of dilution was examined by gel filtration with apoferritin at concentrations ranging from 50 mg/ml to 0.5 mg/

Fig. 2. Gel filtration of a sample of N -[^{14}C]methylated horse spleen apoferritin (5 $\mu\text{g}/\text{ml}$) on a column of Sephadex G-150

Conditions were as given in the text. The protein (determined by counting the radioactivity in each fraction) is eluted with the void volume. The apoferritin subunit would be expected to be eluted in the position marked S.

ml, and with *N*-methylated apoferritin at concentrations of 0.5 mg/ml to 5 µg/ml. In all cases the protein was eluted with the void volume on columns of Sephadex G-75, G-100 and G-150 (Fig. 2). It thus appears that, at least at concentrations of the oligomer as low as 0.01 µM, no dissociation can be demonstrated.

The mechanisms by which iron is incorporated into apoferritin and by which it is mobilized from ferritin are poorly understood at present. However, it would seem that models requiring *a priori* the dissociation of apoferritin into subunits (Pape *et al.*, 1967) are unlikely to operate within the cell, where the concentration of ferritin is unlikely to be lower than in the present experiments. However, it must be emphasized that these results do not exclude the possibility that an equilibrium exists between apoferritin oligomers and subunits that lies well in the direction of the oligomer.

We have been informed of a recent study with [¹⁴C]acetylated apoferritin (Jaenicke & Bartmann, 1972) showing that no dissociation of oligomer occurs at protein concentrations of 0.1 µg/ml.

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