# DISSOCIATION-ASSOCIATION PROPERTIES OF APOFERRITIN IN THE MILLIGRAM AND MICROGRAM RANGE

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Summary. Concentration dependent association of the 17 S unit of horse spleen apoferritin leads to dimer formation at $c \sim 0.1$ mg/ml; at $c > 0.4$ mg/ml trimers and higher aggregates are formed. Dissociation into subunits is not detectable at concentrations as low as $10^{-2}$ or $10^{-4}$ mg/ml using sedimentation velocity or gel chromatography, respectively. In accordance with iron incorporation into the preformed protein shell as the basic mechanism of ferritin formation, the results suggest the quaternary structure of apoferritin to be stable under quasi physiological conditions.

The mechanism of ferritin formation is not fully understood as far as the sequence of subunit association and $Fe^{++}$ incorporation is concerned.

According to Harrison (1) and Crichton (2) iron ions are incorporated into the pre-formed shell of the monomeric apoferritin. On the other hand, Pape et al. (3) considered formation of the iron micelle to be the precursor reaction which then is followed by the assembly of apoferritin subunits around the $Fe^{++}$-core.

In case the second mechanism applies, partial disintegration into subunits is to be expected under conditions favouring dissociation, e.g. low apoferritin concentrations. Contrary to this assumption, Björk and Fish (4) were unable to detect any change of the native quaternary structure at $0.1 < c_p < 1$ mg/ml, while Richter and Walker (5) and Harrison (6) reported aggregation to oligomers in the same range of concentration. The present experiments show that no apoferri-

tin subunits are detectable at concentrations as low as $10^{-4}$ mg/ml. At $c_p > 0.1$ mg/ml dimers and higher oligomers are observed.

## MATERIALS AND METHODS.

Apoferritin from horse spleen, A grade, and dithiothreitol were purchased from Calbiochem; $^3H$ acetic anhydride (specific activity 500 mCi/mmole) from Radiochemicals, Amersham. All other substances were of A grade purity (Merck, Darmstadt). Bidistilled water was used throughout.

Sedimentation velocity experiments were performed in an analytical ultracentrifuge, Beckman, model Spinco E, with scanning equipment; sedimentation coefficients were corrected for temperature and viscosity. For partial acetylation apoferritin was reacted with $^3H$ acetic anhydride according to (7); only ~6 residues per 444000 dalton were reacted. A Tricarb, Model 3380 (Packard) was used for scintillation counting. Ascending gel chromatography with Sephadex G 100 was performed in Pharmacia columns using Perpex pumps (LKB, Uppsala) and a Gilson fraction collector. Circulardichroism spectra were recorded with a Dichrographe II from Jouan-Roussel, Paris.

## RESULTS

### Ultracentrifugation

Sedimentation velocity experiments at apoferritin concentrations $0.02 < c_p < 2.0$ mg/ml in 0.05 M sodium acetate pH 4.6, sodium phosphate pH 7.0, and Tris/HCl pH 9.0 lead to identical results. At protein concentrations above 0.1 mg/ml dimers (24 S) and trimers (33 S) were observed (Fig. 1).

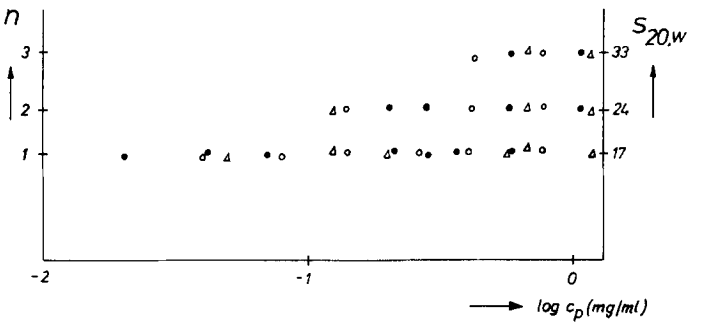


Fig. 1. Sedimentation coefficient $S_{20,w}$ and degree of association n of apoferritin as function of protein concentration, 20° C.
o 0.05 M sodium acetate pH 4.6; 36000 - 44000 rpm
● 0.05 M sodium phosphate pH 7.0; 36000 rpm
▲ 0.05 M Tris/HCl pH 9.0; 36000 - 44000 rpm
12 mm double sector cells, scanner at $\lambda$ = 235 and 280 nm; $S_{20,w}$ obtained from linear log r vs t plots.
Values at $c_p < 0.1$ mg/ml belong to a monodisperse system; the multicomponent systems at $c_p > 0.1$ mg/ml consist of monomers and dimers or monomers, dimers and trimers.

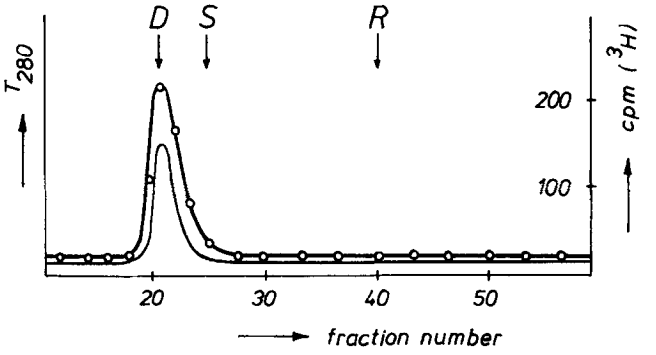


Fig. 2. Gel chromatography of native and $^3$H-acetylated apoferritin on Sephadex G 100, citrate pH 6.5, I = 0.03, 4° and 20° C.
—— native apoferritin, $c_p$ = 0.4 - 4 mg/ml, transmittance at 280 nm
o—o $^3$H-acetylated apoferritin, $c_p = 10^{-4}$ mg/ml, cpm with PPO/POPOP cocktail.
Columns were calibrated with dextrane blue (D), bovine serumalbumin (S), and ribonuclease (R).

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With decreasing concentration the 33 S component disappears at $c_p \leq 0.4$ mg/ml; at 0.1 mg/ml only monomeric apoferritin (17 S) is present. No evidence for higher aggregates could

be detected at $c_p < 0.1$ mg/ml. Under conditions of high dilution (below 10 µg/ml) neither association to oligomers nor dissociation into subunits takes place. Within the limits of error (± 1%) apoferritin represents a monodisperse system consisting of 17 S monomers only.

## Gel chromatography

In order to check the sedimentation data by an independent method gel chromatography was applied using unlabelled and labelled apoferritin, down to protein concentrations as low as $10^{-4}$ mg/ml. Parallel CD measurements were performed as additional means of characterizing the state of association and conformation. Using Sephadex G 100 gel chromatography causes monomeric apoferritin to be eluated with the void volume while subunits will penetrate into the gel matrix. If there exists an equilibrium between the apoferritin monomer and its subunits, these will form either a trailing edge of the monomer peak or a separate fraction, depending on the rate of dissociation. As shown in fig. 2 only one symmetrical peak was observed at concentrations $0.4 < c_p < 4$ mg/ml recording the transmittance at 280 nm. According to the position of blue dextrane ($M > 2 \cdot 10^6$ dalton) this peak corresponds to the void volume of G 100.

For measurements in the concentration range $10^{-4} < c_p < 10^{-1}$ mg/ml $^3$H-acetylated apoferritin was applied. Again no anomalous asymmetry or splitting of the peak could be detected (fig. 2). To ensure that the labelling procedure did not change the native conformation CD spectra of native and acetylated apoferritin were compared; no differences between the spectra could be observed (fig. 3a).

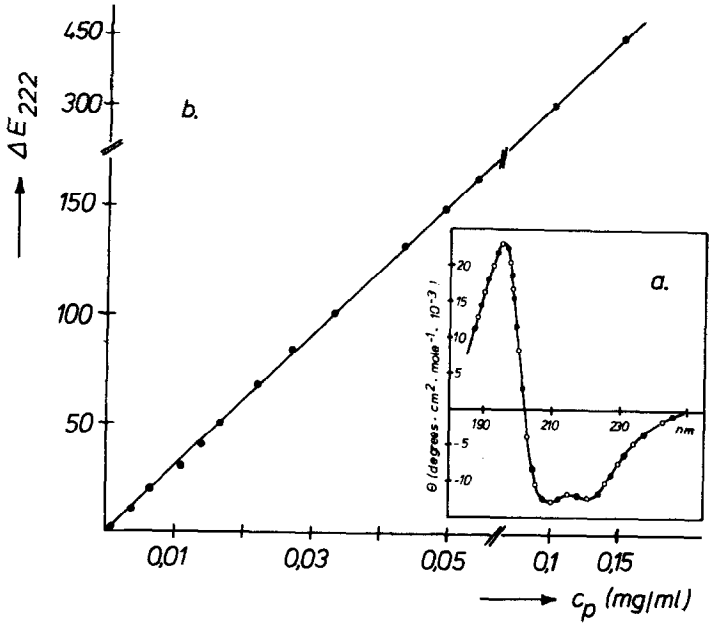


Fig. 3. Circular dichroism of apoferritin, 0.05 M sodium phosphate pH 7.0, 20° C

a. CD spectrum of native (o) and $^3$H-acetylated (●) apoferritin;
$c_p$ = 1.5 mg/ml, path length d = 0.1 mm.

b. Extinction difference ( $\Delta E = E_l - E_r$) at 222 nm as a function of protein concentration, path length d = 10 mm.

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Since previous experiments (8-10) proved dissociation into subunits to be accompanied by conformational changes, concentration dependent ellipticity measurements were performed. Adding µl-portions of a concentrated stock solution of apoferritin to a constant buffer volume (3ml) led to a strictly linear relationship in the $\Delta E$ vs $c_p$ plot (fig. 3b). This result again suggests the native quaternary structure of apoferritin to be stable under the given conditions. Neither dissociation nor drastic conformational changes were observed.

## DISCUSSION

The results at apoferritin concentrations of 0.1-1.0 mg/ml

confirm the data of Richter and Walker (5) who reported a concentration dependent equilibrium between the apoferritin monomer and higher oligomeric forms; on the other hand they contradict the assumption that the degree of association does not change with increasing apoferritin concentration (4). The fact that $c_p$ alone is the effective parameter in the dissociation-association reaction proves the oligomers to be associated merely by weak intermolecular forces. Below a limiting concentration the equilibrium is shifted to the monomeric state.

As shown by sedimentation velocity ($c_p \geq 0.02$ mg/ml) as well as gel chromatography and circulardichroism measurements ($10^{-4} < c_p < 4$ mg/ml) no significant amount of apoferritin subunits could be detected within the limits of error. Therefore, the conclusion may be drawn that the potential monomer $\rightleftharpoons$ subunit equilibrium under the given conditions must be shifted completely to the formation of the stable 17 S monomer. From these results association of protein subunits around the preformed iron micelle does not seem to represent a probable model for the mechanism of ferritin formation.

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