# BINDING OF HYDROPHOBIC COMPOUNDS TO APOFERRITIN SUBUNITS

## Effects on the polymerization state

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## 1. Introduction

Apoferritin is a very stable polymer made up by 24 identical subunits forming a nearly spherical, hollow shell in which a ferric hydroxide core is enclosed. Dissociation of apoferritin into subunits occurs only at extremes of pH; thus, in the acid range, dissociation begins below pH 2.8 and the subunits reassociation begins above pH 3.5 [1]. The importance of hydrophobic interactions at the inter-subunit contact regions has been clearly established [1,2] and binding of hydrophobic molecules by the subunits could be anticipated.

This work reports experiments on the binding of two hydrophobic compounds to apoferritin subunits. ANS was chosen because of its widespread use as a hydrophobic fluorescent probe; BZ, which is an anti-inflammatory drug, since it has been shown to bind to hydrophobic sites in serum albumin [3]. Both compounds bind to apoferritin subunits, but not to the native protein, and induce their polymerization.

## 2. Materials and methods

The sodium salt of ANS was purchased from Eastman Kodak; its concentration was determined using the extinction coefficient $\epsilon^{M}_{1\,cm} = 5000$ at 350 nm. BZ was obtained from Angelini Labs; its extinction coefficient is $\epsilon^{1\%}_{1\,cm} = 195$ at 310 nm [3].

*Abbreviations:* ANS, 8-anilino-1-naphtalensulfonic acid; BZ, 1-benzyl-3-indazoleoxyacetate

Horse spleen ferritin was obtained from Oti (Parma) and was used after purification on a Sephadex G-200 column. Apoferritin was prepared by reduction of ferritin with sodium dithionite as in [4]. Apoferritin subunits were obtained at 4°C by exposure of the polymer to 67% acetic acid for 1 h followed by dialysis in 0.01 M glycine–HCl buffer at pH 3.0–3.2 [5]. The concentration of apoferritin and its subunits was calculated from the $A_{280}$ using the extinction coefficient $E^{1\%}_{1\,cm} = 9.0$; concentrations are expressed in terms of the molecular weight of the polypeptide chain (18 500).

A Cary 14 or a Beckman DB-GT recording spectrophotometer was used for the absorbance measurements.

Sedimentation velocity experiments were performed at 10°C in a Spinco Model E ultracentrifuge at 42 000 rev./min in the case of apoferritin and at 56 000 rev./min in the case of the subunits. Sedimentation coefficients were measured from the movement of the maximum ordinate of the Schlieren peak and corrected to $s_{20,w}$ by standard procedures. Molecular weights were estimated from the value of the sedimentation coefficients assuming $\bar{V} = 0.740$ and $f/f_o = 1.2$ [6].

Spectrofluorimetric experiments were carried out at 20°C in a Fica 55L spectrofluorimeter. Inner filter effects were corrected for when required.

## 3. Results

### 3.1. *Fluorescence experiments*

Binding of ANS to apoferritin subunits was



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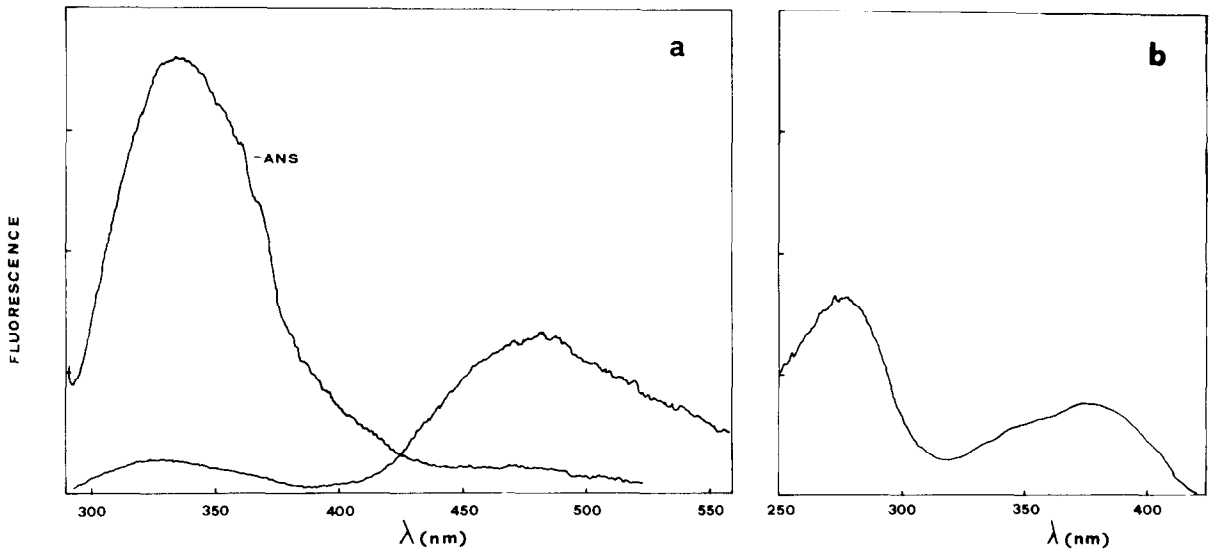


Fig.1. Effect of ANS on the fluorescence emission (a) and fluorescence excitation (b) spectra of apoferritin subunits. Protein, $1.1 \times 10^{-5}$ M; ANS, $3 \times 10^{-5}$ M in 0.04 M glycine–acetate buffer at pH 4.2. (a) Excitation wavelength 280 nm; (b) emission wavelength 480 nm.

detected by means of fluorescence experiments performed at pH 3.2 in 0.01 M glycine–HCl. Addition of ANS quenches the fluorescence of the subunits, while the fluorescence of ANS becomes apparent (fig.1a). The total quenching of protein fluorescence is an expression of the highly efficient energy transfer from the aromatic residues to the bound ANS. Another manifestation of energy transfer is given by the excitation spectrum which shows a maximum at 280 nm in addition to that at 375 nm when ANS is present (fig.1b).

Titrations of the subunits with ANS were carried out under similar experimental conditions in order to measure the affinity and stoichiometry of binding; however, saturation could not be achieved even at high ANS/protein ratios. Similar titration experiments with apoferritin at pH 7 and 3.2 indicated that ANS binding to the polymer is negligible also at low pH, where some unfolding and/or denaturation of the protein may be expected (fig.2).

BZ differs from ANS in that its fluorescence (emission maximum at 357 nm) does not change upon binding [3]. Its reaction with apoferritin subunits could be followed only indirectly by observing the fluorescence of ANS in competition experiments between the two fluorophores. Addition of BZ to a solution of apoferritin subunits containing ANS brings about an increase in the fluorescence of ANS. Conversely, the fluorescence of ANS is enhanced in titration experiments carried out in the presence of BZ

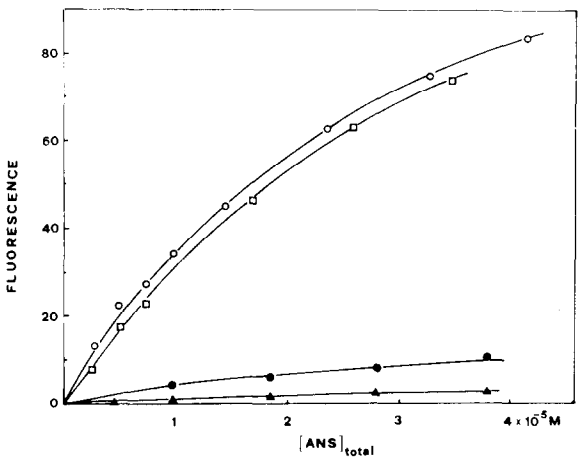


Fig.2. Fluorimetric titrations of apoferritin and its subunits with ANS. Apoferritin subunits at $1.1 \times 10^{-5}$ M in: (○) 0.01 M glycine–HCl buffer at pH 3.2; (□) 0.04 M glycine–acetate buffer at pH 4.2. Apoferritin at $1.1 \times 10^{-5}$ M in: (▲) 0.1 M phosphate buffer at pH 7.0; (●) 0.01 M glycine–HCl buffer at pH 3.2.

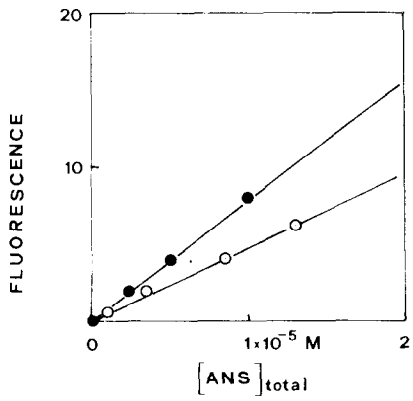


Fig.3. Fluorimetric titration of apoferritin subunits with ANS in the presence and absence of BZ. Protein, $1 \times 10^{-5}$ M in 0.01 M glycine–HCl buffer at pH 3.0. (○) no BZ added; (●) $5 \times 10^{-4}$ M BZ added. Excitation wavelength 370 nm, emission wavelength 480 nm.

(fig.3). Equilibrium dialysis experiments with apoferritin at pH 7 in 0.1 M phosphate buffer give no evidence of binding of BZ to the polymer.

### 3.2. *Sedimentation velocity experiments*

Sedimentation velocity experiments were carried out at pH 3.0–3.2 in 0.01 M glycine–HCl buffer. Under these conditions apoferritin subunits sediment as a single, fairly symmetrical peak; the weight average sedimentation coefficient, $s_{20,w}$, varied in different preparations from 2.0–2.3 S, which correspond to mol. wt $2.0–2.3 \times 10^4$. Table 1 summarizes the $s_{20,w}$ values obtained upon addition of ANS and/or BZ; it clearly shows that binding of both compounds brings about a polymerization of the apoferritin subunits. Thus, when ANS or BZ are in slight excess over the protein, the weight average sedimentation coefficient of the subunits increases; when the reagents are in 5–10-fold excess, a distinct, high molecular weight component appears with $s_{20,w}$ 5–7 S, corresponding to a mol. wt $\sim 10^4$. When both ANS and BZ are added to the same protein solution, the formation of the high molecular weight component appears to be enhanced independently of the order of addition of the reagents. Representative Schlieren patterns are shown in fig.4. When the amount of ANS or BZ is in very high excess the material precipitates. Thus, in order to check whether further association into apoferritin shells may occur in the presence of ANS or BZ, in another series of experiments assembly was promoted by increasing to pH 8 in 0.01 M Tris–HCl buffer containing 0.2 mM dithiothreitol [5]. Complete reassociation of the subunits was obtained both in the absence and in the presence of ANS or BZ; however the apoferritin shells obtained in the presence of ANS did not show the characteristic fluores-

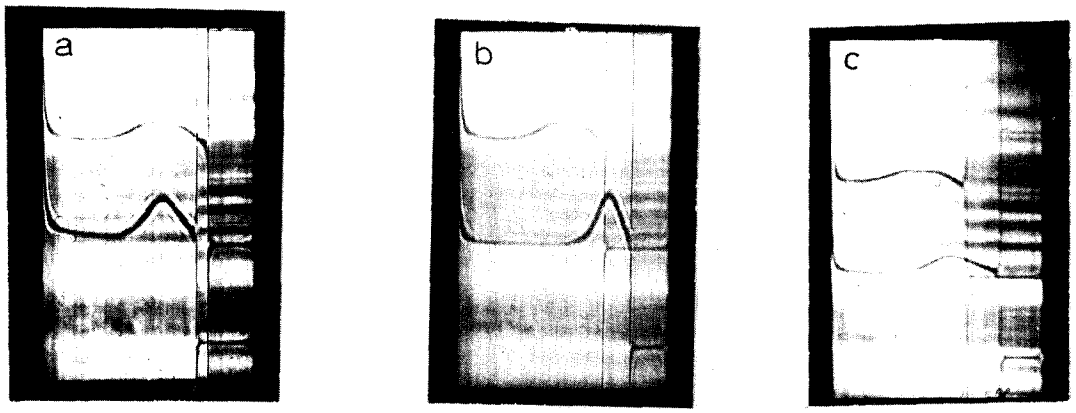


Fig.4. Sedimentation velocity patterns of apoferritin subunits in the presence of ANS and/or BZ. Protein, $0.9 \times 10^{-4}$ M in 0.01 M glycine–HCl buffer at pH 3.2. (a) Bottom, ANS, $4.4 \times 10^{-4}$ M; top, ANS, $4.4 \times 10^{-4}$ M, BZ, $4.4 \times 10^{-4}$ M. (b) Bottom, BZ, $4.4 \times 10^{-4}$ M; top, BZ, $4.4 \times 10^{-4}$ M, ANS, $4.4 \times 10^{-4}$ M (added in the opposite order compared to (a)). (c) Bottom, ANS, $1 \times 10^{-3}$ M; top, as in (a) after 6 days. Photographs taken 75 min after reaching full speed of 52 000 rev./min. Sedimentation is from right to left.

Table 1
Effect of ANS and BZ on the sedimentation velocity of apoferritin subunits

| Preparation no. | Protein (mol/l × $10^4$) | ANS (mol/l × $10^4$) | BZ (mol/l × $10^4$) | $s_{20,w}$ |
|---|---|---|---|---|
| 1 | 2 | – | – | 2.3 |
| 1 | 2 | 2.4 | – | 2.9 |
| 1 | 2 | 9.0 | – | 6.5 |
| 2 | 0.9 | – | – | 2.1 |
| 2 | 0.9 | 4.4 | – | 3.0 |
| 2 | 1 | 10 | – | 2.3–7.0 |
| 3 | 2 | – | – | 1.9 |
| 3 | 2 | – | 20 | 2.3 |
| 3 | 2 | – | 80 | 1.9–5.3 |
| 2 | 0.9 | – | – | 2.1 |
| 2 | 0.9 | – | 4.4 | 2.5 |
| 2 | 0.9 | 4.4 | 4.4 | 1.8–5.3 |
| 2 | 0.9 | 4.4 | 4.4 | 2.6–4.9 |

Buffer: 0.01 M glycine–HCl at pH 3.2; temp. 9°C

cence of the bound fluorophore.

Sedimentation velocity experiments with apoferritin and ANS in 5-fold excess showed no changes in the sedimentation coefficient of the protein.

## 4. Conclusions

Apoferritin subunits bind hydrophobic molecules like ANS and BZ while the assembled molecule does not. A quantitative evaluation of the affinity and stoichiometry of binding has not been possible in view of the polymerization induced by the binding itself. The ANS binding sites are likely to involve tryptophan residues buried at the intersubunit contact regions, since both tryptophans [1] and the ANS binding sites become accessible upon dissociation of apoferritin into subunits. The efficiency of energy transfer between tryptophans and ANS suggests their close proximity, since the efficiency is known to be inversely related to the sixth power of the distance between the donor and acceptor group [7].

The binding sites for BZ may well differ from the ANS binding sites since no evidence of ANS displacement by BZ was obtained by fluorescence spectroscopy. The binding of BZ actually increases the fluorescence of bound ANS.

ANS and BZ promote partial reassociation of the subunits under conditions where they have practically no tendency to reassociate; the oligomers formed have mol. wt $\leqslant 10^4$, which correspond to hexamers of the polypeptide chains. When the pH is raised and assembly of the subunits into apoferritin shells is promoted, the presence of ANS or BZ does not hinder assembly; however, the polymer does not contain the hydrophobic probes, in accord with the lack of binding to the polymer. This finding indicates that the oligomers, formed at low pH and which contain ANS (or BZ) do not assemble further into the polymer; upon formation of the apoferritin shell the bound ANS is displaced.

The polymerization of apoferritin subunits induced by ANS or BZ may be of general relevance in studies with other systems; it suggests that these probes should be used with caution in the case of multimeric proteins or more complex structures like cell membranes [8,9], since their binding may perturb significantly the physical state of the system.

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