AGGREGATION OF A FOLATE-BINDING PROTEIN FROM COW'S MILK

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Sedimentation equilibrium experiments of folate-binding protein from cow's milk performed in the analytical ultracentrifuge at pH 5.0 showed a single molecular species with a molecular weight of 30,000. In contrast, at pH 7.4 sedimentation equilibrium indicated molecular weights ranging from about 30,000 to at least 300,000. Sedimentation velocity experiments at pH 5.0 showed a single symmetrical peak with $s_{20,w}^0 = 2.8$ S in agreement with a molecular weight of about 30,000. At pH 6.0 was seen a faster moving boundary ($s_{20,w} \sim 6$ S) in addition to the slow one. At higher pH two peak maxima were seen: a slow one with sedimentation coefficients of about 5S and a fast one increasing from 9S to 11S with increasing pH. Addition of folate further increased the sedimentation rates. By assuming that GILBERT's rapid monomer-n-mer equilibrium theory was valid also for a lower polymer in equilibrium with a higher polymer our data can be interpreted as an equilibrium between a tetramer and a polymer consisting of more than 16 monomers. In the case of folate addition the results may similarly be interpreted as an equilibrium between an octamer and a polymer composed of more than 32 monomers.

1. INTRODUCTION

In a previous report (12) the isolation of a folate-binding protein (FBP) from cow's milk and some of its properties were described. The molecular weight was found to be 30,000 at pH

5.0. However, studies of the binding of folate to FBP at physiological pH (7.4) indicated aggregation of the protein at this pH (6, 7). In order to obtain further evidence for the nature of this aggregation a series of studies were conducted in

the analytical ultracentrifuge in which pH and the concentrations of FBP and folate were varied.

The present report confirms that extensive aggregation of the FBP takes place and that the presence of folate enhances this aggregation.

2. MATERIALS AND METHODS

FBP from cow's milk was prepared according to a previously published method (12) and used in the experiments in concentrations from 0.5 to 10 mg · ml⁻¹. Sedimentation coefficients were determined at 20 °C in a Spinco Model E ultracentrifuge at 59,780 rev · min⁻¹ using schlieren optics. The velocities of the boundaries were determined by measuring the position of the schlieren curve maxima. Molecular weights were determined by sedimentation equilibrium experiments, performed with interference optics at 20,410 rev · min⁻¹ using the long column meniscus depletion method described by CHERVENCA (1). One series of experiments were performed in 0.2 M-Na-acetate buffers at pH from 3.5 to 7.9. Since the capacities of the buffers above pH 6 were very low, pH was measured both before and after the experiments and found to change less than 0.1 units. Some experiments were performed in a NaCl-phosphate buffer pH 7.4, with ionic strength 0.2. In the acetate buffer at pH 3.5 was similarly added NaCl to an ionic strength of 0.2 to prevent a retardation of the protein due to formation of a potential gradient equivalent to an externally applied electric field (10).

3. RESULTS

The molecular weight of FBP at pH 5.0 was determined by a sedimentation equilibrium experiment. The slope of the plot $\ln f$ versus x^2 (see Figure 1) showed a linear dependence indicating the absence of concentration dependent aggregation at pH 5.0. A molecular weight of $30,000 \pm 2,000$ was calculated, using a partial specific volume of $0.71 \text{ ml} \cdot \text{g}^{-1}$ estimated from the relative amino acid composition and a carbohydrate content of 3 wt. % (12).

In contrast, a sedimentation equilibrium experiment performed at pH 7.4, as used in the folate-binding experiments (6, 7), showed aggregation as reflected in an upward concavity of the

Figure 1. Plot of $\ln f$ versus x^2 .

Sedimentation equilibrium ultracentrifugation of FBP at 20 °C and at pH 5.0. The slope, $\frac{d \ln f}{dx^2}$, of the line is proportional to the molecular weight, $M_w = \frac{2RT}{(1-\bar{v}\rho)\omega^2} \frac{d \ln f}{dx^2}$, where R is the gas constant, T the absolute temperature, ω the angular velocity, ρ the density of the solution, and f an expression for the protein concentration at a certain distance x from the center of rotation (10).

Figure 2. Plot of $\ln f$ versus x^2 .

Sedimentation equilibrium ultracentrifugation of FBP at 20 °C and at pH 7.4.

Figure 3. Sedimentation velocity experiments of FBP.

a) pH 5.0, 48 min after start, $10.3 \text{ mg} \cdot \text{ml}^{-1}$. b) pH 5.75, 48 min after start, $8.0 \text{ mg} \cdot \text{ml}^{-1}$. c) pH 6.0, 48 min after start, $10.1 \text{ mg} \cdot \text{ml}^{-1}$. d) pH 6.5, 26 min after start, $6.8 \text{ mg} \cdot \text{ml}^{-1}$. e) pH 6.7 with addition of folate, 19 min after start, $3.6 \text{ mg} \cdot \text{ml}^{-1}$. Sedimentation is to the right in these pictures.

plot $\ln f$ versus x^2 (Figure 2). The slope obtained at the top section and the bottom section of the cell corresponded to apparent molecular weights of about 30,000 and 300,000, respectively.

In sedimentation velocity experiments symmetrical peaks were observed at pH 3.5 and 5.0 (Figure 3a). Between the pH-values 5.0 and 6.0 the sedimentation velocity was slightly increased with a broadening of the peaks indicating the beginning of an aggregation (Figure 3 b). At pH above 6.0 the peaks broadened further and showed two distinct maxima (Figures 3 c and 3 d). Addition of folate increased the sedimentation velocity of both the slow and the fast peaks (Figure 3 e). It was also observed that the concentration of the fastest moving boundary increased both with increasing pH and with folate addition. At low protein concentrations and when the equilibrium was displaced to the extremes it was only possible to determine the velocity of one boundary although a shoulder on the main peak was seen.

Sedimentation coefficients as determined from the individual peaks in a large number of experiments are listed in Table I. At pH 5.0 extrapolation to infinite dilution indicated $s_{20,w}^0 = 2.8 \text{ S}$ (Figure 4). The sedimentation coefficient decreased as a function of increasing protein concentration with a slope of $0.015 \text{ S per mg} \cdot \text{ml}^{-1}$, viz. within the range expected for globular protein molecules. A sedimentation coefficient obtained at pH 3.5 coincided with the pH 5.0 curve indicating that no drastic unfolding or denaturation took place.

In Figure 5 it is attempted to illustrate the influence of pH on the sedimentation velocity. Without folate and at a protein concentration between 7 and $8 \text{ mg} \cdot \text{ml}^{-1}$ the sedimentation velocity increased only slightly between pH 5.0 and 6.0. At pH 6.0 two maxima were present which travelled with velocities of 3.2S and 6.3S,

Tabel I

Sedimentation coefficients, $s_{20,w}$, of FBP in 0.2 M-Na-acetate buffers.

pH	FBP-conc	slow	$s_{20,w}$ fast
(a)			
3.5*)	7.1	2.72	
5.0	3.7	2.77	
—	7.1	2.73	
—	10.3	2.68	
5.5	8.0	2.8	
5.75	7.7	3.4	
—	8.0	3.3	
6.0	3.9	3.4	
—	7.1	3.2	6.3
—	10.1	2.6	6.0
6.5	6.8	5.1	9.0
7.4	1.2		10.0
—	1.9		10.4
—**)	1.1		9.0
—**)	2.3		10.2
—**)	4.5		12.3
7.9	5.7	4.9	10.7
(b)			
5.0	1.5	3.0	
—	3.1	3.55	
6.0	3.1	7.5	10.1
—	3.2	7.2	10.2
—	3.9		10.0
6.7	3.1	6.9	11.9
—	3.6	7.0	12.7
7.4	1.2		11.7
—	1.9		13.8
—	3.7		15.7
—**)	0.6		10.6
—**)	0.9		12.4
—**)	1.9		14.3

*) NaCl added to ionic strength 0.2. **) NaCl-phosphate buffer with ionic strength 0.2. (a) Folate free. (b) With folate in concentrations between 5 and 10 fold the numbers of FBP molecules.

respectively. At the pH-values 6.5 and 7.9 the velocities were further increased, with sedimentation coefficients for the fast boundary increasing from 9.0S to 10.7S, while the slow boundary appeared to reach a constant value of about 5S. In experiments with addition of folate a precipitate was formed at protein concentrations of 7 to 8 mg·ml⁻¹. However, when the concentration was decreased to less than 4 mg·ml⁻¹ FBP remained dissolved and sedimenta-

Figure 4. Sedimentation coefficients, $s_{20,w}$, at pH 5.0 as a function of protein concentration. Figure 5. Sedimentation coefficients, $s_{20,w}$, as a function of pH.

P is the degree of polymerization expected for a pure polymer sedimenting with the $s_{20,w}$ -values of the ordinate. — FBP without folate, the protein concentration was from 7 to 8 mg·ml⁻¹. ---- FBP with folate added, the protein concentrations was from 3 to 4 mg·ml⁻¹. Two boundaries are seen above pH 5.0, with folate, and above pH 6.0, without folate.

tion experiments could be performed. The sedimentation of the complex as a function of pH showed the same pattern with folate as without except that all velocities were increased (Figure 5).

4. DISCUSSION

The understanding of the behaviour of aggregating protein systems during sedimentation experiments originate from two papers by GILBERT (4, 5). He studied in model systems the behavior of rapid monomer-*n*-mer reactions. He had to apply several restrictive assumptions such as diffusion-free sedimentation, absence of concentration dependence of sedimentation rates and a uniform centrifugal field. The essential features he found for the aggregating systems were: (a) Monomer-dimer systems will exhibit concentration gradient curves with only a single maximum, although the curve may be asymmetric. The position of this maximum will correspond, neither to the sedimentation coefficient of the dimer nor to that of the monomer, but will lie between them, approaching one or the other as the equilibrium is shifted to either side. (b) Monomer-*n*-mer systems ($n > 2$) may exhibit two maxima in their concentration gradients. One of these will travel with the velocity of the monomer while the other travels with a velocity intermediate between that of monomer and *n*-mer.

Assuming that FBP undergoes a rapid association-dissociation reaction we have attempted to interpret our data according to GILBERT's monomer-*n*-mer theory. At pH above 6.0 and at a protein concentration from 7 to 8 mg·ml⁻¹ the constant sedimentation velocity of the slow boundary was significantly higher than the sedimentation velocity observed for the monomer at pH values below 5.0. Presuming the slow peak to represent a globular protein complex we have tentatively assumed that the slow form might be a tetramer since the *s*-value of such a polymer would agree with the observed sedimentation coefficient. The size of the high polymer in equilibrium with this tetramer is reflected in the increased sedimentation velocity of the fast boundary but from sedimentation velocity experiments it is not possible to accurately determine the size.

However, a crude estimate may be obtained by using an extension of GILBERT's theory by FUJITA (3) which include the effect of a sector-shaped cell in a centrifugal field. This estimate showed that the sedimentation coefficient for the high polymer form in equilibrium with the tetramer would correspond to a polymer consisting of 20 to 26 monomer units if it was of globular shape. In the case of folate addition, at concentrations from 3 to 4 mg·ml⁻¹ and above pH 5.5, the same kind of consideration would suggest an octameric form to be in equilibrium with a polymer composed of more than 32 monomers.

This crude estimate of the size of the high polymer forms is consistent with the sedimentation equilibrium experiment at pH 7.4 which produced a protein concentration at the bottom section of the cell of 3 to 4 mg·ml⁻¹ and showed an apparent molecular weight of about 300,000 indicating a degree of polymerization of $n \geq 10$. Since the aggregation is a function of the protein concentration (e.g. the $\ln f$ versus x^2 slope in the same experiment at very low concentration correspond to a monomer) it is likely that the polymerization at a concentration from 7 to 8 mg·ml⁻¹ would be as found in the sedimentation velocity experiments, viz. $n > 16$ (Figure 5).

That FBP molecules aggregate has previously been shown by more indirect methods such as gel filtration and studies of the binding of folate. Thus, SALTER et al. (9) showed that the molecular weight of FBP was concentration dependent when determined at pH 7.4 by gel filtration on Sephadex G-100. This finding was interpreted as aggregation taking place with increasing FBP concentration.

In the present paper the value of 30,000 determined in the analytical ultracentrifuge was obtained at pH 5 where the molecule is in a monomeric state while molecular weights of 35,000 (6, 9) for the cow's FBP and 39,000 (8) for FBP from goat were obtained at pH 7.3–7.4 by gel filtration. In these cases probably a monomer (dominating species) is in a rapid equilibrium with polymers resulting in a slight elevation of the molecular weight.

Equilibrium dialysis studies of the binding of folate to FBP at pH 7.4 displayed positive cooperativity which could be explained assuming a concomitant aggregation to polymers (7).

The present study using the analytical ultracentrifuge confirms the aggregation of FBP molecules. This aggregation is enhanced upon binding of folate and is strongly pH-dependent with a great change around pH 6.

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