

Binding of Radiolabeled Folate and 5-Methyltetrahydrofolate to Cow's Milk Folate Binding Protein at pH 7.4 and 5.0. Relationship to Concentration and Polymerization Equilibrium of the Purified Protein

Jan Holm^{1,3} and Steen Ingemann Hansen²

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KEY WORDS: 5-methyltetrahydrofolate; folate; cow's milk folate binding protein; ligand-induced polymerization

INTRODUCTION

The high-affinity folate binding protein (FBP) exists as a membrane-bound folate receptor (FR) as well as a soluble FBP in milk and other body fluids of mammalian species [1]. The presence of a high-affinity FBP was first demonstrated in cow's milk [2]. A large scale purification of FBP from cow's whey powder by means of a combination of cation exchange and methotrexate affinity chromatography made it feasible to characterize this prototype folate receptor protein with regard to physico-chemical nature and ligand binding characteristics [3–7].

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Ultracentrifugation and gel filtration studies performed over a wide range (five decades) of FBP concentrations showed that the protein possessed a remarkable pH- and concentration-dependent aggregation tendency. Thus, while the monomeric form prevailed at 1 nM FBP a polymer composed of more than 32 monomers was formed at 130 μ M FBP at pH 7.4 [4, 5]. At pH 5.0 FBP was virtually unable to polymerize even at high concentrations [4, 5]. The ligand (folate)-induced enhancement of polymerization observed even at pH 5.0 suggested an interrelationship between ligand binding and polymerization equilibria [4, 5]. This hypothesis was compatible with two peculiar characteristics of folate binding, apparent positive cooperativity (convex upward Scatchard plots) and a ligand binding affinity that was inversely proportional to the FBP concentration over four decades suggesting a lower binding affinity of folate for polymeric forms of FBP prevailing at high concentrations of the protein [5].

For the purpose of throwing further light on these phenomena it would be of interest to investigate whether changes in the hydrogen ion concentration exert parallel effects on ligand binding and aggregation. The present study describes the ligand binding characteristics of purified FBP at pH 7.4 and 5.0, and the aggregation behavior of FBP at pH 7.4 and 5.0 in parallel gel filtration experiments with ligand-bound FBP. Due to the instability of 5-methyltetrahydrofolate (5-MTHF), the major form of endogenous folate, radiolabeled folate has typically been preferred as prototype ligand. Another major issue in this study has been to compare the binding behavior of these two folates under conditions suitable to avoid degradation of radiolabeled 5-MTHF.

MATERIALS AND METHODS

The radiochemical [14 C] folate (pteroylglutamate) with a specific activity of 52.4 Ci/mol, [14 C] 5-methyltetrahydrofolate with a specific activity of 58.0 Ci/mol and [3 H] folate with a specific activity of 29–43 Ci/mmol were obtained from Amersham International Ltd., Amersham, UK. The following unlabeled analogs were used: folate (pteroylglutamate) and DL-N-5-methyltetrahydrofolate, 5-MTHF, both supplied by Sigma. FBP was purified from cow's whey powder according to a previously published method which involved a combination of cation exchange chromatography and affinity chromatography [3]. All FBP solutions were dialyzed against 0.2 M acetate buffer, pH 3.5 at 4°C to remove endogenous folate.

Unless otherwise stated, equilibrium dialysis experiments were performed as described previously [5] for periods of 20 hr in 0.17 M Tris-HCl buffer (7°C, pH 7.4 or 5.0) with FBP in the internal (1000 μ l) and radioligand in the external solution (200 ml). Due to a large volume of external solution, the concentration of radioligand was kept constant during the entire dialysis experiment. Triton X-100 at a concentration of 10 g/l was added to both sides of the dialysis membrane [5]. Radioactivity was measured as previously described [5].

In some experiments ascorbic acid (Sigma) was added to the external dialysis medium. Studies on the dissociation of bound radioligand from FBP were performed as previously described in detail [8]. Briefly, aliquots (600 μ l) of a solution of FBP (2 μ M) were dialysed to equilibrium (pH 7.4, 7°C) against the [14 C] labeled ligand.

Some samples were taken for analysis, while the remainder were immersed in a volume of 1000 ml ligand-free buffer containing Triton X-100, 10 g/l. At 24 hours intervals one of the dialysis bags was removed and the external solution replaced by fresh buffer. The amount of radioligand bound at the end of each 24 hours period was calculated from the difference between the counting rates in the internal and external solutions. Radiolabels released from FBP during wash-out analysis were highly diluted owing to a large volume of external buffer (counting rates in the external solutions were very low). Hence, reassociation between radioligand and FBP during wash-out dialysis could be disregarded.

Gel Filtration

Polymerization as a function of the concentration of ligand-bound FPM was studied within a range of 0.1–0.10 μM by use of a gel filtration technique [5]. FBP dissolved in 0.17 M Tris-HCl buffer, pH 7.4 or pH 5.0 was exposed to an excess of [^{14}C] labeled 5-MTHF or [^3H] labeled folate for 3 hours at 7°C, and then applied to a Bio-Gel P-300 (Biorad Laboratories, Richmond, California, U.S.A.) column (2.6 \times 37 cm) equilibrated and eluted with the same buffer at pH 7.4 or 5.0 at 5°C (flow rate 5 ml hour $^{-1}$). The column was calibrated as described [5]. The FBP in the effluent was monitored by radioactivity measurements [5].

RESULTS

Stability of [^{14}C] Labeled Folate and 5-methyltetrahydrofolate

Experiments were performed to analyze stability of [^{14}C] radioligands under different storage conditions. The displacement of [^{14}C] labeled folate and 5-MTHF by unlabeled analogs was studied in equilibrium dialysis experiments (pH 7.4, 7°C) where FBP was exposed to either [^{14}C] folate or [^{14}C] 5-MTHF at a concentration of 100 nM, together with increasing concentrations of the unlabeled analog (Fig. 1). A 50% inhibition of radioligand binding was observed in the presence of equimolar (100 nM) concentrations of unlabeled folate or 5-MTHF. Repetition of the experiment after storage of radiolabels for 20 hours (pH 7.4) at 7°C or 37°C had no effect on the subsequent displacement of [^{14}C] folate, whereas the displacement of [^{14}C] 5-MTHF increased to 67% (7°C) and 95% (37°C). Hence, unless otherwise stated all binding experiments were performed at 7°C. Binding parameters calculated from saturation curves for [^{14}C] folate and [^{14}C] 5-MTHF at 7°C were compared to binding parameters obtained in parallel experiments where both radioligands were used after storage at pH 5.0 in Tris-HCl buffer (20 hr) at 7°C. No changes in maximum radioligand binding or $S_{0.5}$, the concentration of radioligand required for half saturation of binding, were seen (*data not shown*). In a number of experiments we analyzed the effect of ascorbic acid on binding of [^{14}C] 5-MTHF to FBP at 7°C and 37°C. As can be seen the addition of 100 mM ascorbic acid (Fig. 2 and Table 1) which had no effect on binding of [^{14}C] 5-MTHF to FBP at 7°C, increased the binding affinity at 37°C (reduced as compared to that at 7°C) to a value slightly above that at 7°C.

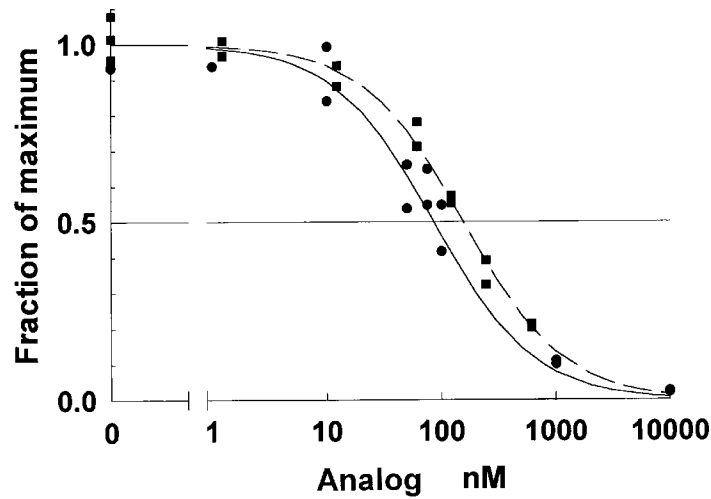


Fig. 1. Competition between labeled and unlabeled folate analogs for binding to FBP. Equilibrium dialysis experiments at 7°C and pH 7.4 with 100 nM [¹⁴C] labeled folate, ● and 5-MTHF, ■ in the external solution together with the corresponding unlabeled analog.

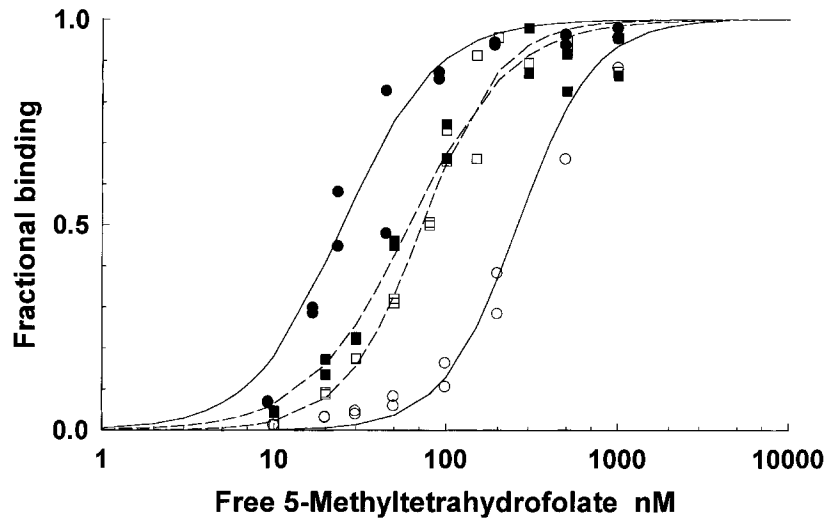


Fig. 2. Effect of ascorbic acid (100 mM) on binding of [¹⁴C] 5-MTHF to FBP (2 μM) at 7°C (squares) or 37°C (circles). Equilibrium dialysis experiments at pH 7.4 with (closed symbols) or without (open symbols) ascorbic acid.

Table 1. Effect of Ascorbic Acid (100 μ M) on Binding of [14 C] 5-MTHF to FBP at 7°C and 37°C.

| | <i>N</i> (nM) | <i>S</i> _{0.5} (nM) | <i>K</i> (l/mol) | <i>h</i> (SD) |
|-----------------------------|---------------|------------------------------|-------------------|---------------|
| Without ascorbic acid, 7°C | 2100 | 80 | 1.3×10^7 | 2.17 (0.17)** |
| With ascorbic acid, 7°C | 1900 | 54 | 1.9×10^7 | 1.71 (0.12)** |
| Without ascorbic acid, 37°C | 1800 | 260 | 3.8×10^6 | 1.60 (0.21)* |
| With ascorbic acid, 37°C | 1900 | 25 | 4.0×10^7 | 1.69 (0.15)** |

Data from equilibrium dialysis experiments at pH 7.4 (Fig. 2) were analyzed in Scatchard and Hill plots.

N, maximum bound [14 C] 5-MTHF to FBP; *S*_{0.5}, the free ligand concentration at half saturation of binding; *K*, overall affinity constant expressed as the reciprocal value of *S*_{0.5}; *h*, the Hill coefficient \pm (SD) calculated from Hill plots ($n = 11-14$) is significantly greater than 1.0: * $p < 0.01$. ** $p < 0.001$.

Binding of Folate and 5-methyltetrahydrofolate to FBP at pH 7.4 and 5.0. Positive Cooperativity and Dependence of Ligand Affinity on Concentrations of FBP

Binding of [14 C] labeled folate and 5-MTHF to FBP was studied at pH 7.4 and 5.0 (7°C). Saturation curves for ligand binding shown in Fig. 3 were obtained at varying concentrations of FBP (10^{-5} – 10^{-7} M). the binding parameters were calculated after analysis of the data in Scatchard and Hill plots (Table 2 and 3). The following conclusions could be drawn:

1. Maximum radioligand binding is proportional to the concentration of FBP, both at pH 7.4 and 5.0 (Table 2 and 3).
2. The binding displays apparent positive cooperativity at both pH values as indicated by Hill coefficients significantly higher than 1.0 and convex upward Scatchard plots (Table 2 and 3).
3. At pH 7.4, the concentration of free folate or 5-MTHF (folates) required for half-saturation of ligand binding (*S*_{0.5}) is almost proportional to the FBP concentration (Table 2 and Fig. 3A), e.g. a 10-fold rise of the FBP concentration is accompanied by a similar increase in *S*_{0.5} and decrease in binding affinity, which equals the reciprocal value of *S*_{0.5}. Furthermore, the affinity of folate for binding to FBP is slightly higher than that of 5-MTHF (Table 2). The binding affinity of folate at pH 5.0 was virtually unaltered as compared to pH 7.4, and so was the dependence on the concentration of FBP. (Fig. 3B and Table 3). The binding affinity of 5-MTHF at pH 5.0 was only two-three fold lower than that of folate at 10^5 M (Fig. 3B and Table 3). The increase in binding affinity observed with decreasing concentrations of FBP was, however, far less pronounced in case of 5-MTHF (Fig. 3B and Table 3), resulting in a binding affinity of 5-MTHF 20-fold lower than that of folate at 100-fold lower concentrations of FBP (10^{-7} M). This is further illustrated in Fig. 6 (upper figure).

Dissociation of Folate and 5-methyltetrahydrofolate from FBP

Aliquots of FBP solutions to which [14 C] labeled folate or 5-MTHF had been bound were dialyzed against ligand-free buffer solutions of varying composition

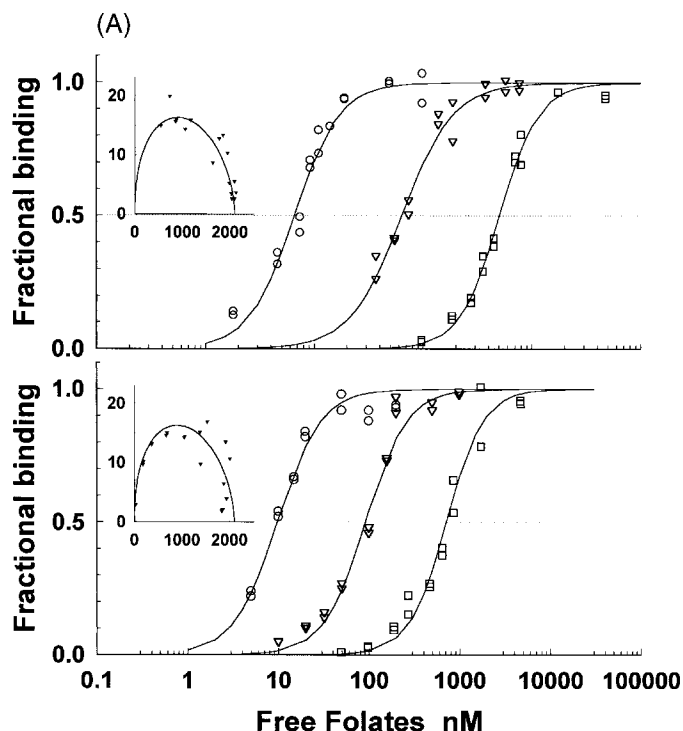


Fig. 3. Saturation curves for high-affinity binding of [^{14}C] labeled folate and 5-MTHF to solutions of FBP, undiluted (\square), 10-times diluted (∇) and 100-times diluted (\circ), cf. Tables 2 and 3. Equilibrium dialysis experiments in 0.17 M Tris-HCl buffer at 7°C (A, pH 7.4; B, pH 5.0). Scatchard plots (ordinate, bound/free radioligand; abscissa, bound radioligand) inserted (10-times diluted FBP solutions). Lower figures, 5-MTHF and upper figures, folate (pteroylglutamate). (Figure 3(B) shown overleaf.)

(Fig. 4). The control experiments in Tris-HCl buffer of pH 7.4 at 7°C showed that dissociation of folate and 5-MTHF was a rather slow process since 80% was still bound after 10 days dialysis (Fig. 4). Further, a rise in the incubation temperature to 37°C did not increase dissociation of 5-MTHF (Fig. 4B). Contrastly, at pH 3.5 only 10% of 5-MTHF was still bound to FBP after 24 hours dialysis (Fig. 4B). A less pronounced rise in the dissociation rate of folate was observed at pH 3.5 (Fig. 4A), e.g. 50% and 10% folate still bound after 24 hours and 3 days dialysis, respectively.

Polymerization at pH 7.4 and 5.0 as a Function of the FBP Concentration

Polymerization of FBP saturated with [^{14}C] 5-MTHF and [^3H] folate was studied in gel filtration experiments. At pH 7.4 the appearance of the elution profile of 5-MTHF depended on the concentration of binding protein in as much as the polymerization degree decreased with declining concentrations of FBP (Fig. 5). This

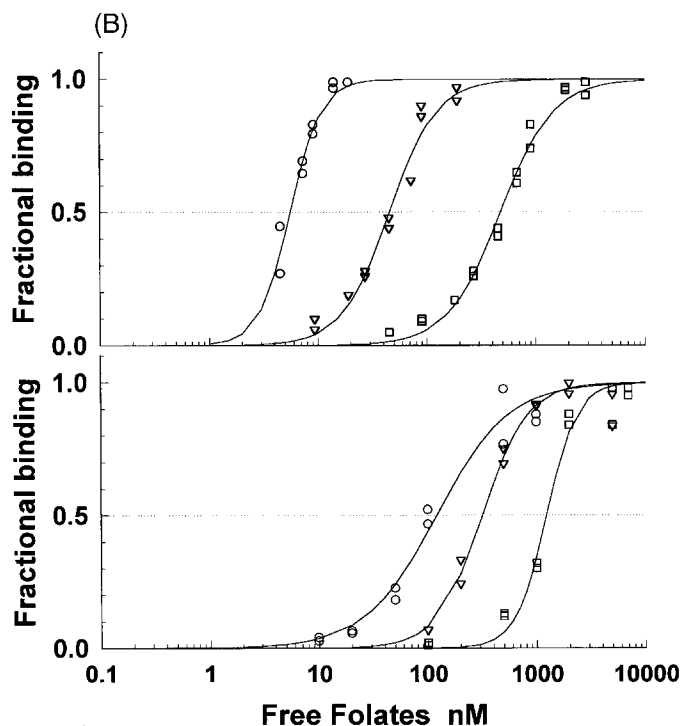


Fig. 3. Continued.

Table 2. Effect of FBP Concentration on Folate and 5-MTHF Binding Characteristics at 7°C and pH 7.4^a.

| Stock solution of FBP | N^b , M | | $S_{0.5}^c$, M | | h^d | |
|--------------------------|----------------------|----------------------|--|--|----------------------|----------------------|
| | Folate | 5-MTHF | Folate | 5-MTHF | Folate | 5-MTHF |
| Undiluted | 2.2×10^{-5} | 2.2×10^{-5} | 48×10^{-8} (2.1×10^6) | 77×10^{-8} (1.3×10^6) | $2.32 \pm 0.07^{**}$ | $1.70 \pm 0.07^{**}$ |
| Diluted 10 times | 2.1×10^{-6} | 2.0×10^{-6} | 6.4×10^{-8} (1.6×10^7) | 9.5×10^{-8} (1.1×10^7) | $1.80 \pm 0.21^*$ | $1.39 \pm 0.07^{**}$ |
| Diluted 100 times | 2.2×10^{-7} | 1.7×10^{-7} | 0.64×10^{-8} (1.6×10^8) | 0.97×10^{-8} (1.0×10^8) | $1.64 \pm 0.08^{**}$ | $1.91 \pm 0.16^{**}$ |

^aData from equilibrium dialysis experiments (7°C) at pH 7.4 with varying concentrations of FBP were analyzed in Scatchard and Hill plots ($n = 10-16$).

^b N , maximum bound ligand.

^c $S_{0.5}$, the free ligand concentration at half saturation; K (l/mol) in parenthesis.

^d h , the Hill coefficient \pm S.D. calculated from observations within a fractional ligand binding range of 0.1–0.9 is significantly higher than 1.0.

^{**} $p < 0.001$.

^{*} $p < 0.01$.

Table 3. Effect of FBP Concentration on Folate and 5-MTHF Binding Characteristics at 7°C and pH 5.0^a

| Stock solution of FBP | <i>N</i> ^b , M | | <i>S</i> _{0.5c} , M | | <i>h</i> ^d | |
|-----------------------|---------------------------|------------------------|---|---|-----------------------|---------------|
| | Folate | 5-MTHF | Folate | 5-MTHF | Folate | 5-MTHF |
| Undiluted | 2.0 × 10 ⁻⁵ | 1.9 × 10 ⁻⁵ | 41 × 10 ⁻⁸ (2.4 × 10 ⁶) | 113 × 10 ⁻⁸ (0.88 × 10 ⁶) | 1.81 ± 0.12** | 1.84 ± 0.12** |
| Diluted 10 times | 2.0 × 10 ⁻⁶ | 2.1 × 10 ⁻⁶ | 4.3 × 10 ⁻⁸ (2.3 × 10 ⁷) | 32 × 10 ⁻⁸ (3.1 × 10 ⁶) | 2.16 ± 0.26* | 2.14 ± 0.07** |
| Diluted 100 times | 1.8 × 10 ⁻⁷ | 1.7 × 10 ⁻⁷ | 0.55 × 10 ⁻⁸ (1.8 × 10 ⁸) | 11 × 10 ⁻⁸ (9.3 × 10 ⁶) | 3.77 ± 0.30** | 1.53 ± 0.15* |

^aData from equilibrium dialysis experiments (7°C) at pH 5.0 with varying concentration of FBP were analyzed in Scatchard and Hill plots (*n* = 8–14).

^b*N*, maximum bound ligand.

^c*S*_{0.5}, the free ligand concentration at half saturation; K (l/mol) in parenthesis.

^d*h*, the Hill coefficient ± S.D. calculated from observations within a fractional ligand binding range of 0.1–0.9 is significantly higher than 1.0.

***p* < 0.001.

**p* < 0.01.

is also illustrated in Fig. 6 where the polymerization tendency of folate-bound FBP in general seems greater than that of 5-MTHF-bound FBP. At pH 5.0, FBP saturated with folate showed a noticeable polymerization tendency over a wide range of FBP concentrations, 0.01–10 μM (Fig. 5 and Fig. 6).

DISCUSSION

One of the issues addressed in the present study was to compare the binding characteristics of 5-MTHF, the major natural form of folate, and pteroylglutamate (folate) to bovine milk FBP. Due to the poor stability of 5-MTHF at 37°C comparative binding experiments were conducted at 7°C. Addition of ascorbate, a substance often used to increase stability of reduced folates, drastically increased the binding affinity of 5 MTHF to FBP at 37°C, but had no effect at 7°C. This finding could suggest an important role for ascorbic acid in 5-MTHF homeostasis.

Apart from a slightly lower “overall” affinity of 5-MTHF for binding to FBP, consistent with a generally weaker affinity of reduced folates for binding to FBP, the data demonstrate resemblance between the two radiolabeled folates with regard to a number of peculiar characteristics (Fig. 3, 4, Tables 2 and 3). Those were (1) positive cooperativity, (2) a ligand binding affinity that increased with decreasing concentrations of the binding protein, (3) a dissociation of ligand from FBP which was extremely slow at pH 7.4, but rapid at pH 3.5. Dissociation of 5-MTHF from FBP (pH 7.4) did not occur at a higher rate when the temperature was raised from 7°C to 37°C. This observation which is surprising in view of the poor stability of 5-MTHF at 37°C suggests a higher stability or protection against degradation of the 5-MTHF molecule after binding to FBP. Furthermore, the extremely slow dissociation of the radioligands at pH 7.4 is consistent with the altered conformational structure of the protein after ligand binding as also demonstrated by previously observed ligand-induced changes in CD-spectrum, tryptophan fluorescence, polymerization pattern

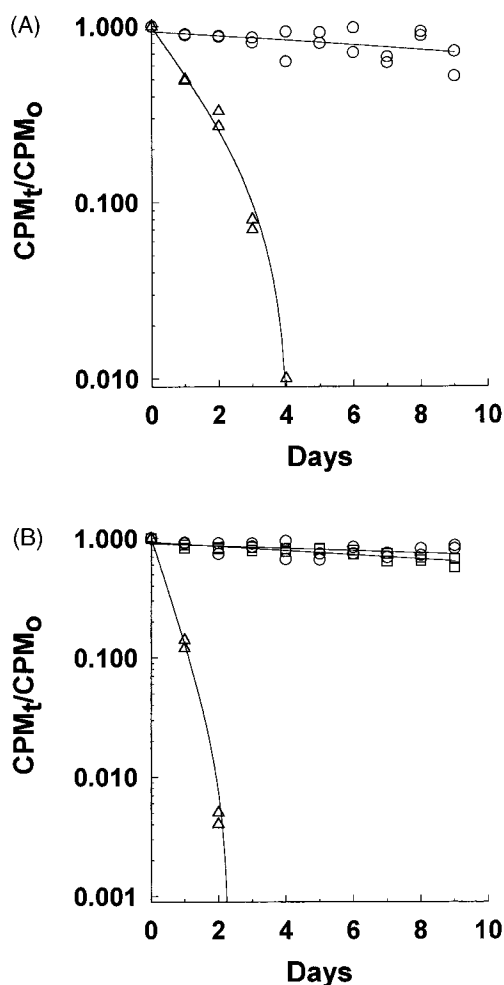


Fig. 4. Dissociation of $[^{14}C]$ labeled folate (A) and 5-MTHF (B) from FBP. Aliquots of FBP solutions were dialysed to equilibrium (pH 7.4, 7°C) against the radioligand (100 nM). Some samples were taken for analysis, (bound ligand₀), while the remainder were dialyzed against large volumes (1000 ml) of ligand-free buffer containing Triton X-100, 10 g/l; Tris-HCL of pH 7.4 at 7°C (O) and 37°C (□, only 5-MTHF), acetate buffer of pH 3.5 at 7°C (Δ). Labeled ligand bound at the end of each wash-out dialysis period = (bound folate)_t. Ratio (bound folate)_t/bound folate₀ as a function of the duration of dialysis (log scale).

and folding stability of bovine milk FBP [4, 7]. No qualitative differences between the characteristics of radioligand binding to FBP at pH 7.4 and 5.0 were observed. The affinity constant for binding of 5-MTHF to FBP at pH 5.0 was lower and showed a drastic decrease with declining concentrations of FBP as compared to pH 7.4. Our

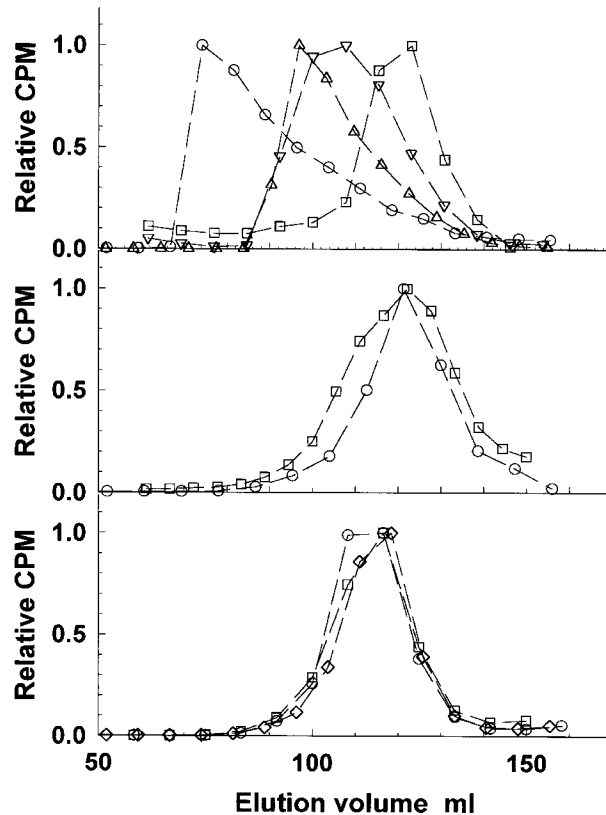


Fig. 5. Degree of polymerization, P (n -mer) as a function of the concentration of FBP: $12.8 \mu\text{M}$ (\circ), $6.4 \mu\text{M}$ (\triangle), $1.2 \mu\text{M}$ (∇), $0.128 \mu\text{M}$ (\square) and $0.012 \mu\text{M}$ (\diamond). The apparent molecular size of the FBP peak, decreasing with increasing elution volume, was estimated from gel filtration experiments in which FBP solution saturated with radiolabeled 5-MTHF at pH 7.4 (top figure) and pH 5.0 (intermediate figure) as well with folate at pH 5.0 (lower figure). Samples were applied to a Bio-Gel P-300 column calibrated as described and equilibrated with Tris-HCl buffer of pH 7.4 and pH 5.0 (5).

ultracentrifugation studies showed a ligand (folate)-induced increase of the polymerization degree of FBP [4]. The phenomenon was most pronounced at pH 6–8, but did also occur to some extent at pH 5.0 where the monomeric form of ligand-free FBP prevails [4]. The present gel-filtration data demonstrate enhancement of the polymerization tendency with increasing concentrations of radioligand-bound FBP at pH 7.4, and to some degree at pH 5.0. In general, 5-MTHF which had a weaker affinity for binding to FBP than folate especially at pH 5.0 seemed less capable of stimulating aggregation of FBP than folate, in particular at pH 5.0. This could indicate a close association between processes involved in ligand binding and ligand-induced polymerization. Changes in the conformational structure of FBP reported to occur subsequent to ligand binding (7) could thus favor aggregation.

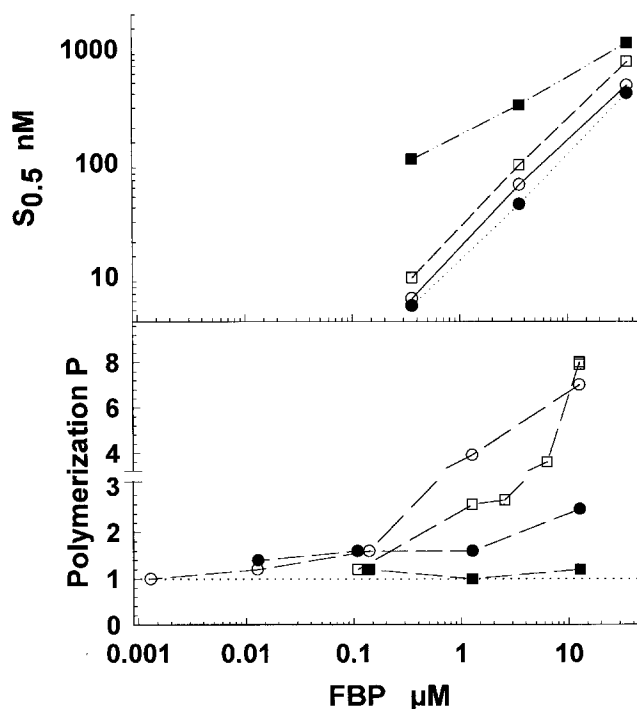


Fig. 6. Free ligand concentration at half saturation of binding ($S_{0.5}$) at 7°C and polymerization as functions of the FBP concentration at pH 7.4 and 5.0. 5-MTHF (squares); folate (circles). Open symbols at pH 7.4 and closed symbols at pH 5.0. Data on polymerization of folate-bound FBP at pH 7.4 taken from reference 5.

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