The preparation and properties of folate-binding protein from cow's milk

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An improved affinity-chromatographic method for the preparation of folate-binding protein from cow's milk is described. Under dissociating conditions the protein appeared homogeneous in the ultracentrifuge, with a molecular weight of $35\,000 \pm 1500$, but it was heterogeneous on electrophoresis and ion-exchange chromatography and evidently consisted of several glycoproteins with similar molecular weights that all bound folic acid. Overall, the protein contained a high proportion of half-cystine (18 residues/molecule) and 10.3% of carbohydrate. At saturation it bound approx. 1 mol of folate/mol of protein at pH 7.2. Equilibrium-dialysis measurements of the binding of folic acid and 5-methyltetrahydrofolate to the purified protein gave non-linear Scatchard plots, the shapes of which depended on pH. The results were interpreted in terms of ligand binding to a polymerizing system in which the affinity of ligand for monomer was greater than its affinity for polymer. When the protein concentration was similar to that in cow's milk, dissociation constants (K_d) for folate and 5-methyltetrahydrofolate were 3 nM and 5 nM respectively at pH 7.2 and 37°C, whereas K_d for the binding of folate to monomer was about 50 pM. The properties of the binding protein are discussed in relation to its possible role in folate absorption in the gut.

The natural folate in cow's milk, 5-CH₃-H₄folate is bound to a minor whey protein that may be isolated by affinity chromatography on a column containing folate coupled to aminohexyl-Sepharose (Salter *et al.*, 1972). Adaptations of this method have been used to prepare folate-binding proteins from whey powder (Svendsen, *et al.*, 1979), goat's milk (Selhub & Grossowicz, 1973; Rubino *et al.*, 1977) and human milk (Waxman & Schreiber, 1975). We found, however, that the use of aminohexyl chains to link folate to Sepharose resulted in hydrophobic interactions with milk proteins that did not bind folate, thereby decreasing the specificity of the affinity column. Folate linked to Sepharose through a hydrophilic chain produced an affinity gel with higher specificity, and in the present paper we describe an improved method of preparation of the cow's milk-binding protein and some of its molecular and ligand-binding properties.

Materials and methods

[G-³H]Folate (potassium salt; 11.3 Ci/g) and 5-¹⁴CH₃-H₄folate (barium salt; 87 mCi/g) were

Abbreviation used: 5-CH₃-H₄folate, 5-methyltetrahydrofolic acid.

obtained from The Radiochemical Centre (Amersham, Bucks., U.K.), folate was from Koch-light Laboratories (Colnbrook, Bucks., U.K.), and 5-CH₃-H₄folate from Sigma (London) Chemical Co. (Poole, Dorset, U.K.).

Sepharose 6B and Sephadex G-25 were obtained from Pharmacia Fine Chemicals (Hounslow, Middx., U.K.), and DEAE-cellulose (DE-52) was from Whatman Biochemicals (Maidstone, Kent, U.K.).

Buffer A contained 0.02 M-Na₂HPO₄, 0.15 M-NaCl and 1 mM-ascorbic acid, and the pH was adjusted to 7.2 with H₃PO₄. Buffer B was the same, but with ascorbate omitted.

Preparation of folate-Sepharose affinity adsorbent

The hydrophilic spacer arm [NH₂-CH₂-CH(OH)-CH₂-NH-CH₂-CO-NH-CH₂-CH(OH)-CH₂-NH-] was synthesized by coupling 1,3-diaminopropan-2-ol to CNBr-activated Sepharose 6B, bromoacetylating the product, and allowing it to react with more 1,3-diaminopropan-2-ol (O'Carra *et al.*, 1974). Folate was coupled to the Sepharose derivative (100 ml settled volume) by means of a carbodi-imide condensation at pH 6.2 (Salter *et al.*, 1972). The resultant yellow gel, which contained about 0.7 mg of folate/ml, was washed on a filter

with 5 litres of 0.1 M-NaHCO₃ and then in a column with 500 ml of 6 M-urea and with buffer B. It was stored in buffer A.

Purification of folate-binding protein

Step 1. Rennet whey was prepared as previously described (Ford *et al.*, 1969) except that skimmed raw cow's milk (10 litres) was used and 3 M-CaCl₂ (0.34 ml/litre) was added before the pH was adjusted to 6.2. Subsequent steps were all performed at 0–5°C.

Step 2. Solid (NH₄)₂SO₄ (250 g/litre) was added slowly to the stirred whey, and after 4 h it was filtered through Whatman no. 42 filter paper. More (NH₄)₂SO₄ (190 g/litre) was added to the filtrate and the precipitate was collected by centrifugation at 2000 g for 45 min, suspended in buffer A and dialysed against the same buffer until free from (NH₄)₂SO₄.

Step 3. After the pH had been adjusted to 5 with H₃PO₄ solution (10%, v/v) to dissociate native 5-CH₃-H₄folate from the binding protein, the dialysed solution was mixed with 10 ml of the folate-Sephadex affinity adsorbent, previously washed with 0.05 M-sodium acetate buffer, pH 5.0, containing 6 M-urea and with buffer A, and the suspension was stirred for 5 min. The pH was then re-adjusted to 7.2 with NaOH and stirring was continued for a further 5 min to allow the gel to adsorb the binding protein. The gel with adsorbed binding protein was recovered by centrifugation, packed into a small column and washed with 200 ml of buffer A. The binding protein was eluted from the gel with 0.05 M-sodium acetate buffer, pH 5.0, containing 6 M-urea, and fractions containing protein were pooled and dialysed against buffer B until the pH was 7.2 and urea had been removed.

Partial separation of multiple forms of folate-binding protein on DEAE-cellulose

A solution of about 5 mg of folate-binding protein (binding capacity about 40 µg of folate) in buffer B was dialysed against 20 mM-Tris/HCl buffer, pH 9.3, containing 4 M-urea, and applied to a column (2.5 cm × 21 cm) of DEAE-cellulose (Whatman DE-52) equilibrated with the same buffer. The column was then eluted with 250 ml of this buffer, followed by further 250 ml portions of buffer, containing successively higher concentrations of NaCl (5 mM, 10 mM, 15 mM, 20 mM, 30 mM and 40 mM). The absorbance of the eluate was measured at 230 nm and fractions corresponding to absorbance peaks were pooled for measurement of folate-binding capacity and, after dialysis against 1 mM-NH₃ and concentration, for analysis by polyacrylamide-gel electrophoresis.

Polyacrylamide-gel electrophoresis

Disc electrophoresis at pH 8.3 was done with the

discontinuous system described by Davis (1964), except that the small-pore gel contained 6% instead of 7% acrylamide, and both small-pore and large-pore gels contained 3 M-urea. Solutions of protein for electrophoresis were dialysed against 1 mM-NH₃ and freeze-dried. The protein was dissolved in electrophoresis buffer containing 3 M-urea to give a concentration of about 1 mg/ml, and up to 50 µl of solution was applied to each gel. A constant current of 4 mA/tube was applied for 1 h. When electrophoresis was complete, gels were fixed in 40% (w/v) trichloroacetic acid and stained either for protein with Coomassie Blue G-250 or for carbohydrate by the periodate/Schiff method, as described by Anderson *et al.* (1974).

Electrophoresis in the presence of sodium dodecyl sulphate was done with gels prepared with 7% acrylamide in 0.05 M-sodium phosphate buffer, pH 8.0, containing 5 mM-EDTA, 5 mM-2-mercaptoethanol and 0.1% (w/v) sodium dodecyl sulphate. The same buffer mixture was used as the electrode-compartment buffer, and with an increased concentration of sodium dodecyl sulphate (3%, w/v) to dissolve freeze-dried binding protein.

Amino acid analysis

For reduction and carboxymethylation, the binding protein (10 mg) was dissolved in 2.5 ml of 0.5 M-Tris/HCl buffer, pH 8.5, containing 6 M-guanidinium chloride and 0.3 M-2-mercaptoethanol, and the solution was incubated at 37°C for 4 h in the absence of O₂. The solution was then diluted with 10 ml of the Tris buffer and the pH was kept at 8.5 with 5 M-KOH while 233 mg of iodoacetic acid was added slowly at room temperature. The pH was maintained at 8.5 for a further 15 min, then 1.1 ml of 2-mercaptoethanol was added. The modified protein (yield 8 mg) was isolated by gel filtration on a column (2.5 cm × 50 cm) of Sephadex G-25 (fine grade) equilibrated with 1 mM-NH₃, and freeze-dried.

The modified protein was hydrolysed *in vacuo* in 3 M-mercaptoethanesulphonic acid (protein concn. 1 mg/ml) at 118°C for 24, 48 and 72 h (Penke *et al.*, 1974). Amino acid analyses were obtained with an LKB automatic analyser (type 4102).

Carbohydrate analysis

The protein was hydrolysed in methanolic HCl, and the resultant glycosides were analysed by g.l.c. as described by Chambers & Clamp (1971).

Ultracentrifugation

Runs were made at 20°C in a Spinco model E ultracentrifuge equipped with Rayleigh interference optics, with a rotor type AN-H and cells with 12 mm six-channel filled-Epon centrepieces and sapphire windows. The high-speed sedimentation-equilibrium method of Yphantis (1964) was used. For sedimen-

tation under non-denaturing conditions, the protein was dissolved in 20 mM-Tris/HCl buffer, pH 7.5, or in 50 mM-sodium acetate buffer, pH 5.5, each containing 0.1 M-KCl, or in either buffer containing KCl and 5 μ M-folate. For sedimentation under denaturing conditions, the protein was dissolved in the Tris buffer containing 6 M-guanidinium chloride.

Equilibrium dialysis

The apparatus for equilibrium dialysis consisted of 12 Teflon cells each of which was divided into two half-cells of 5 ml capacity, separated by Visking dialysis membrane (effective diameter 2.5 cm), and 4 ml of protein solution was placed in one half-cell and 4 ml of radioactive ligand solution in the other. The cells were rotated at 8 rev./min in a water bath at constant temperature in the dark. Equilibrium between protein-bound and free ligand was reached in 24 h at 20°C and above, or in 48 h at lower temperatures. Since very low binding-protein concentrations were used in some experiments, bovine serum albumin (1 g/litre) was added to both half-cells to minimize possible adsorption of binding protein to surfaces. Ligand binding by the albumin alone was not detected, and [3 H]folate was recovered quantitatively from the cells after dialysis.

Measurement of first-stage binding of folate to bacterial cells

Binding of folate and 5-CH₃-H₄folate to *Lactobacillus bifidus* N.C.D.O. 1452 cells was measured as 'instantaneous' or first-stage uptake of graded amounts of folate by a constant weight of cells, as described by Ford (1974).

Results

Purification and multiple forms of folate-binding protein

The yield of folate-binding protein from 10 litres of skimmed raw milk averaged 20 mg, a recovery of about 25% of the total binder (free and complexed with folate) present in the milk initially. In polyacrylamide-gel electrophoresis at pH 8.3 in the presence of urea, different preparations of the protein gave three to five slowly moving bands (Figs. 1a and 1b), which all reacted with both protein and carbohydrate stains. Electrophoresis in the presence of sodium dodecyl sulphate produced mainly a single broad band with diffuse edges (Fig. 1g).

The components of a preparation that showed five bands on electrophoresis were partly separated by chromatography on DEAE-cellulose at pH 9.3 in the presence of urea (Fig. 2). No protein was detected in peaks 1 and 6, but polyacrylamide-gel electrophoresis showed that the five components initially observed in the preparation were unequally distributed amongst peaks 2, 3, 4 and 5 (Figs. 1c-1f),

Fig. 1. Polyacrylamide-gel electrophoresis of cow's-milk folate-binding protein and separated fractions

Gels (a)-(f) contained 3 M-urea and gel (g) contained 0.1% (w/v) sodium dodecyl sulphate; other conditions are given in the text. (a) and (b), different preparations of binding protein; (c)-(f), fractions of the preparation shown in (b) obtained by DEAE-cellulose chromatography (see Fig. 2); (g), electrophoresis of the preparation shown in (b) in the presence of sodium dodecyl sulphate.

Fig. 2. Cow's-milk folate-binding protein fractionated by DEAE-cellulose chromatography in the presence of 4 M-urea

The stepwise increments in NaCl concentration in the eluting buffer are indicated at the top; other details are given in the text. The separate peaks are numbered to assist comparison with the text and with Fig. 1, which shows electrophoretic patterns obtained from the starting material and from four of the peaks.

which accounted for 18, 25, 31 and 23% of the folate-binding capacity respectively.

Stability and storage of purified binding protein

The binding protein remained soluble but lost 7% of its binding capacity when stored at -10°C at low concentration (0.2 mg/ml) in buffer B for 6 months. Addition of glycerol or 5-CH₃-H₄folate before storage had no effect, but the loss of binding capacity was doubled in the presence of Triton X-100 (0.5 μ l/ml). Solutions (0.2 mg of protein/ml)

in buffer B stored at 5°C were stable for several days, but then they gradually became turbid and binding activity was lost. Addition of 2-mercaptoethanol had no effect, and more concentrated solutions gave precipitates more quickly.

Composition, absorbance and partial specific volume

The amino acid and carbohydrate composition is given in Table 1. The carbohydrate content of 10.3%, as part of a glycoprotein of mol.wt. 35 000 (see below), indicates a polypeptide-chain mol.wt. of 31 400.

Absorbance measurements correlated with the recovery of material from hydrolysis, and with dry-weight measurements, gave a value for $A_{1\text{cm}}^{1\%}$ of 18.4 at 280 nm.

A partial specific volume of 0.713 ml/g was calculated from the amino acid and carbohydrate composition, with partial specific volumes for amino acids and monosaccharides taken from Cohn & Edsall (1943) and Gibbons (1966) respectively.

Sedimentation behaviour

Although the binding protein showed heterogeneity during polyacrylamide-gel electrophoresis, this was not apparent at sedimentation

equilibrium in the ultracentrifuge when the protein was dissolved in 6 M-guanidinium chloride. A mol.wt. of $35\,000 \pm 1500$ was obtained with protein concentrations ranging from 0.4 to 0.8 mg/ml. Protein (0.2 mg/ml) dissolved in acetate buffer, pH 5.5, also appeared homogeneous, with a similar molecular weight, but considerable heterogeneity was evident when folate was added, with material sedimenting more rapidly than monomer accumulating towards the base of the solution column. Protein (0.2 mg/ml) dissolved in Tris buffer, pH 7.5, showed no marked heterogeneity with or without folate, but it existed in aggregated form, since the molecular weight was about 120 000 in the absence of folate and about 170 000 in its presence.

Ligand-binding studies

Effect of temperature. Equilibrium dialysis was carried out at temperatures ranging from 0 to 60°C with a binding-protein concentration (0.1 μM) comparable with that in cow's milk. Protein and ligands were dissolved in buffer A (pH 7.2). Scatchard (1949) plots of the results at different temperatures for both folate and 5-CH₃-H₄folate were generally similar to the one for folate at 37°C and pH 7.2 shown in Fig. 3(b). Hill (1913) plots of the results had slopes averaging 1.12. Apparent dissociation constants (K_d) obtained from the binding curves (free ligand concentration at half saturation) are given in Fig. 4(a). At 37°C the K_d values for folate and 5-CH₃-H₄folate were 3 nM and 5 nM respectively.

Effect of pH. Equilibrium dialysis was done at 37°C with protein and ligands dissolved in appropriate buffers containing 1% ascorbic acid and with *I* 0.1. Scatchard plots of the results obtained in acid solution with folate as ligand were concave upward (Fig. 3a), and the slopes of the corresponding Hill plots at pH 5.1 and pH 6.0 were 0.91 and 0.95 respectively. In contrast, Scatchard plots of the results obtained in neutral and alkaline solution were convex upward (Fig. 3b). With folate as ligand, Hill plots of the results had slopes of 1.20, 1.32 and 1.36 at pH 7.2, 8.0 and 8.8 respectively. K_d values obtained from the binding curves showed (Fig. 4b) that folate was strongly bound to the protein over a much wider range of pH than was 5-CH₃-H₄folate.

Effect of binding-protein concentration. The results of equilibrium-dialysis experiments covering a wide range of protein concentrations at pH 7.2 gave Scatchard plots that were all convex upward. The corresponding Hill coefficients ranged from 1.3 (0.08 μM -protein) to 2.8 (0.002 μM -protein), but showed no consistent correlation with protein concentration. On the other hand, K_d values obtained from the binding curves indicated an increase in affinity between binding protein and both folate and 5-CH₃-H₄folate as the protein concentration was decreased (Table 2).

Table 1. *Composition of cow's-milk folate-binding protein*
Values were calculated on the basis of a molecular weight of 35 000, and rounded off to the nearest integer.

Residue	Composition (no. of residues/molecule)
Aspartic acid	31
Threonine	16
Serine	19
Glutamic acid	29
Proline	19
Glycine	16
Alanine	18
Valine	12
Methionine	4
Isoleucine	10
Leucine	14
Tyrosine	11
Phenylalanine	10
Histidine	11
Lysine	16
Arginine	18
Tryptophan	5
Half-cystine	18
Fucose	1
Mannose	7
Galactose	3
N-Acetylglucosamine	5
N-Acetylgalactosamine	2
Sialic acid	<1

Fig. 3. Scatchard (1949) plots of the binding of folate to cow's-milk folate-binding protein at acid pH (a) and neutral and alkaline pH (b)
O, pH 5.1; ●, pH 6.0; △, pH 7.2; ▲, pH 8.0; ■, pH 8.8; r is the degree of saturation.

Fig. 4. Effect of temperature (a) and pH (b) on the affinity of cow's-milk folate-binding protein for folate and 5-CH₃-H₄folate at pH 7.2
O, Folate; ●, 5-CH₃-H₄folate. The protein concentration was 0.1 μM .

Table 2. Effect of binding-protein concentration on its affinity for folate and 5-CH₃-H₄folate at pH 7.2

Binding-protein concentration (μM)	K_d (nM)	
	[³ H]Folate	5- ¹⁴ CH ₃ -H ₄ folate
0.002	0.11	—
0.02	0.67	—
0.08	2.9	—
0.133	3.7	4.5
0.2	—	5.8
0.5	—	12
1.0	41	62

Fig. 5. Effect of pH on stoichiometry of ligand binding to cow's-milk folate-binding protein
O, Folate; ●, 5-CH₃-H₄folate.

Stoichiometry of binding. The folate-binding capacity of the protein was independent of its concentration in the range 0.002–1 μM , and of temperature in the range 0–37 $^{\circ}C$, but varied with

pH (Fig. 5). The maximum capacity for both folate and 5-CH₃-H₄folate ranged from 0.8 to 0.9 mol of ligand bound/mol of protein (mol.wt. 35 000) for

different preparations. 5-¹⁴CH₃-H₄folate was a mixture of the two diastereoisomers about the 6-position, but both isomers were evidently bound to the protein, since when the protein was in excess it bound over 98% of the radioactive ligand.

Affinity of L. bifidus cells for 5-CH₃-H₄folate and folic acid

L. bifidus cells bound 5-CH₃-H₄folate most strongly when the pH was neutral or slightly acidic, *K_d* values for first-stage binding being 4.2 nM, 2.7 nM, 10 nM and 11 nM at pH 6, 7, 8 and 9 respectively. *K_d* values for folate at these pH values were closely similar.

Discussion

The purified binding protein from fresh cow's milk consisted of several glycoproteins, each of which bound folate and had a molecular weight of about 35 000. As with the binding proteins from whey powder (Svendsen *et al.*, 1979), goat's milk (Rubinoff *et al.*, 1977) and human milk (Waxman & Schreiber, 1975), which were also heterogeneous, the cause of the heterogeneity has not yet been determined. The protein in cow's milk is not easily denatured, since it was reported that the protein isolated from whey powder still bound about 0.5 mol of folate/mol, although the preparation of whey powder had involved severe heat treatment of the milk (vacuum evaporation at 68°C and spray-drying at 180°C) (Svendsen *et al.*, 1979). However, such treatment evidently had affected the molecule, since the protein from whey powder contained less carbohydrate than did the protein that we describe, and one preparation contained only glucosamine residues. The proteins from cow's milk (Table 1) and goat's milk (Rubinoff *et al.*, 1977) contained the monosaccharides characteristic of many glycoproteins, indicating that fresh milk is preferable to whey powder as a source of binding protein in native form. Despite the differences in carbohydrate content, the binding proteins from cow's milk and whey powder were similar in amino-acid composition, and both proteins were similar to the goat's-milk protein (Rubinoff *et al.*, 1977), except for glycine and half-cystine content. The differences are probably explained by different hydrolytic techniques, destruction of cysteine occurring when the unmodified goat's-milk protein was hydrolysed with HCl (Rubinoff *et al.*, 1977). The higher half-cystine content is more likely to be correct, and the probability of a high degree of cross-linking in the structure is consistent with the robust nature of the bovine protein.

On the basis of a molecular weight of 35 000, the purified cow's-milk protein formed an equimolar complex with folate and with 5-CH₃-H₄folate at

saturating ligand concentrations, but ligand-binding under other conditions was non-linear. Various reasons for the non-linear binding may be considered. These are (a) heterogeneity of the protein, (b) co-operative interaction between aggregated monomers (ligand-induced isomerization), since its sedimentation behaviour showed that it existed in polymerized form, and (c) association-dissociation equilibria between monomer and polymers affected by ligand binding (Nichol *et al.*, 1967; Nichol & Winzor, 1976; Cann, 1978). Hansen *et al.* (1978), working with partly purified binding protein from cow's milk, observed non-linear folate binding at neutral and alkaline pH values and attributed the behaviour to co-operativity. However, binding curves for both heterogeneous and co-operative systems are independent of protein concentration, whereas those for reversibly polymerizing systems are dependent on concentration. Since the cow's-milk protein showed reversible concentration-dependent aggregation (Ford *et al.*, 1969), and different binding curves (Fig. 6) and apparent *K_d* values for the ligand (Table 2) were obtained at different protein concentrations, the non-linear binding is evidently an example of ligand binding to a reversibly polymerizing system.

Polymerization of the ligand-free protein occurred to a much greater extent at pH 7.5 than it did at pH 5.5, although saturation with folate increased polymerization at both pH values. These results suggest that the different Scatchard plots of folate

Fig. 6. Reciprocal plots of the binding of 5-CH₃-H₄folate to folate-binding protein at different protein concentrations

The protein concentrations were: ○, 0.50 μM; ●, 0.20 μM; △, 0.08 μM; *r* is the degree of saturation. Conditions were pH 7.2 and 37°C.

binding (Fig. 3) were due at least in part to a change from a ligand-mediated mechanism of polymerization in acid solution to a ligand-facilitated one at neutral and alkaline pH (Cann, 1978). In the first case the ligand binds to monomer and induces polymerization, and Scatchard plots are concave upward, whereas in the second case the ligand binds to polymers and stabilizes them, and Scatchard plots are convex upward. Binding curves at pH 7.5 indicated that, even at a concentration as low as 2 nM, the purified ligand-free protein still existed in polymerized form. The decrease in apparent K_d for folate and 5-CH₃-H₄folate as the protein concentration was decreased (Table 2) showed that both ligands bound more tightly to the monomer than to polymeric forms of the protein. In this respect the folate-binding system differs from the model systems described by Cann (1978), but the property is consistent with equilibrium binding represented by Scatchard plots that are either concave or convex upward (Nichol *et al.*, 1967). Values from Table 2 of K_d for folate, when plotted against log (protein concentration) (x), fitted reasonably well to the exponential equation:

$$10^{10} K_d = 0.504 + 363.2 e^{1.03x}$$

giving a K_d of about 50 pM for the binding of folate to monomer at pH 7.2. The greater affinity between ligand and monomer than between ligand and polymer indicates competition between ligand binding and protein polymerization, and this provides an additional explanation for convex-upward Scatchard plots (Nichol *et al.*, 1967) such as those obtained with the cow's-milk protein at neutral and alkaline pH. Concave-upward plots such as those obtained in acid solution represent non-competitive ligand binding, the loss in competition presumably resulting from the greatly decreased tendency of the ligand-free protein to polymerize under these conditions.

Hansen *et al.* (1979) isolated a folate-binding protein from cow's whey that differed from the protein we describe because, on dilution, it changed its mode of ligand binding at pH 7.4 from one represented by convex-upward Scatchard plots to one represented by concave-upward plots. In addition, inclusion of a substance separated from the protein during its purification prevented the change. The change in binding mode was not explained, but its dependence on protein concentration indicates a mechanism based on association-dissociation equilibria, with the protein isolated by Hansen *et al.* (1979) dissociating to monomer more readily on dilution than did the protein that we isolated. On the same basis, the substance that prevented the change in binding mode acted by preventing dissociation. One explanation for the differences between the two proteins is that they result from differences in the purification procedures.

Ford (1974) suggested that the binding protein in milk protected endogenous folate (5-CH₃-H₄folate) from utilization by bacteria in the gut, an analogy with the action of vitamin B₁₂-binding proteins in sow's milk, which completely inhibit the growth of *Lactobacillus leichmannii* unless vitamin B₁₂ is added in excess of the binding capacity of the milk (Gregory & Holdsworth, 1955). The K_d of 5 nM for 5-CH₃-H₄folate, applied to the usual concentrations of binding protein and 5-CH₃-H₄folate in cow's milk (0.2 μ M and 0.1 μ M respectively), indicates that only about 95% of the folate would be protein-bound. However, no free folate was detected in cow's milk (Ford *et al.*, 1969), nor was an appreciable amount of folate in milk used by gut bacteria (Ford, 1974). A possible explanation is that the binding protein in milk exists in the monomeric form, as indicated by gel filtration (Ford *et al.*, 1969), rather than in the polymerized form that it assumed at a similar concentration in isolation. The K_d of 50 pM for folate binding to monomer indicates that only about 0.05% of the folate in milk is not protein-bound, much more in agreement with observation. The lower K_d also explains how the binding protein could compete effectively with bacteria for folate, since the K_d value for 5-CH₃-H₄folate bound to *L. bifidus* cells is greater than 1 nM. The folate-protein complex dissociates below pH 5 *in vitro* and presumably also in the stomach. However, the protein retains its binding activity after acid treatment, as demonstrated by its elution in active form from the affinity gels, so the folate-protein complex should re-form in the duodenum, where the pH becomes neutral again.

The binding protein might facilitate folate absorption, since concentrations of both protein-bound folate and free binder in the plasma of kids rose markedly when they received large amounts of folate-protein complex in colostrum (Ford *et al.*, 1972). However, after the intestine has lost its neonatal permeability to proteins, the action of the binder could be limited to interaction with receptors in the intestinal mucosa. According to Blair (1977), 5-CH₃-H₄folate is transported across the mucosal membrane of rat intestine after conversion into neutral compounds in an acidic micro-environment, pH 3.5, existing at the membrane surface. Dissociation of folate from protein in such an environment would solve the problem of how the high-affinity complex is broken to make the folate available for metabolic processes.

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