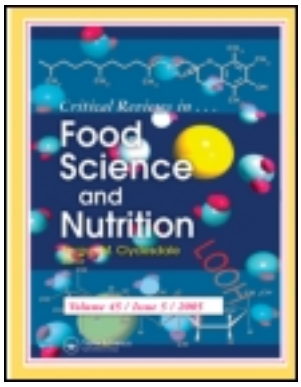

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Folate-Binding Protein in Milk: A Review of Biochemistry, Physiology, and Analytical Methods

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Folate-Binding Protein in Milk: A Review of Biochemistry, Physiology, and Analytical Methods

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Folate-binding protein (FBP) was discovered in cow's milk around 40 years ago. Bovine FBP belongs to a family of several folate-binding proteins. In milk, it is a soluble whey protein with the ability to sequester folate from blood plasma. Bovine FBP is a well-characterized protein in terms of amino acid sequence and binding characteristics. Affinity and binding kinetics towards various folate forms have been intensively studied because they are crucial in using bovine FBP as an analytical tool. Shortly after the identification of bovine FBP, a competitive protein-binding assay for measuring serum and blood folate concentrations was introduced. Another analytical application of bovine FBP is in affinity chromatography, as a clean-up/concentration step for analysis of folates in foods and biological samples by liquid chromatographic methods. Concentrations of FBP in milk and dairy products have been determined by ELISA and Surface Plasmon Resonance-biosensor techniques. Since the initial reports of FBP in cow's milk, its physiological role has been discussed, especially regarding its effects on folate absorption from milk and dairy products. This review summarizes recent biochemical, analytical, food science, and nutritional advances regarding folate-binding protein in milk.

Keywords Bovine FBP, binding kinetics, folate absorption

LIST OF ABBREVIATIONS

5-CH_3-H_4folate	= 5-methyl-tetrahydrofolate
5-HCO-H_4folate	= 5-formyl-tetrahydrofolate
10-HCO-folic acid	= 10-formyl-folic acid
Da	= dalton
DSC	= differential scanning calorimetri
ELISA	= enzyme-linked immunosorbent assay
FBP	= folate-binding protein
Folbp	= folate-binding protein
FR	= folate receptor
GPI	= glycosylphosphatidylinositol
H_4folate	= tetrahydrofolate
HPLC	= high performance liquid chromatography
k_a	= association rate constant
k_d	= dissociation rate constant
K_D	= dissociation constant in equilibrium
LC	= liquid chromatography
LOD	= limit of determination
LOQ	= limit of quantification
MS	= mass spectrometry
NMR	= nuclear magnetic resonance
RfBP	= riboflavin-binding protein
RFC	= reduced folate carrier
RPBA	= radio protein-binding assay
sFBP	= soluble folate-binding protein
SFF	= stop-flow fluorescence
SPR	= surface plasmon resonance
SAX	= strong anion exchange
SD	= standard deviation
SPE	= solid phase extraction
TIM	= dynamic in vitro gastrointestinal model
UHT	= ultra high treatment

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INTRODUCTION

The naturally occurring folate in milk, 5-methyltetrahydro folate, is almost completely bound to folate-binding protein (FBP). In 1967, the presence in cow's milk of a heat-labile

macromolecular factor that protects milk folate against adsorption to activated charcoal was reported (Ghitis, 1967). These folate binders were subsequently separated and identified as proteins in both bovine and human milk (Ford et al., 1969). Since then, FBP has been extensively studied and is well-characterized. The protein has a significant sequence similarity with chicken riboflavin-binding protein (RfBP), the crystal structure of which has been determined (Monaco, 1997). Attempts to report equilibrium constants for the interaction between folate and FBP proved inconclusive for many years. The recent development of the Surface Plasmon Resonance (SPR)-based biosensor technique has made it possible to study the binding kinetics of various folate forms (Nygren-Babol et al., 2005) and has confirmed the theoretical equilibrium dissociation constant K_D for the interaction between folic acid and FBP to be in the pM range at physiological pH (Nixon et al., 2004).

Although knowledge about the affinity of the interaction between different folate derivatives and FBP was initially limited, the protein was soon being used as a tool for analyzing folate status in serum, plasma, and blood. A radioprotein-binding assay was developed and replaced the microbiological assay for folate determinations in clinical samples from the mid-1970s (Waxman et al., 1971). Another analytical application appeared during the 1980s, when FBP was introduced in affinity chromatography for concentration and purification of folates from biological samples (Selhub et al., 1980).

The development of more specific analytical methods, for example, ELISA and SPR, allowed free, bound, and total FBP to be quantified in milk and body fluids (Hoier-Madsen et al., 1986; Nygren et al., 2003; Indyk and Filzoni, 2004). Both techniques have generated further data concerning concentrations of FBP in cow's milk and other dairy products (Forssén et al., 2000; Nygren-Babol et al., 2004; Indyk et al., 2006).

Since the discovery of FBP in cow's milk, the physiological role of the protein has been discussed. It has been suggested that milk FBP sequesters folate from blood to secure an adequate supply to the neonate, as well as preventing reduction of milk folate by hindering oxidation during ingestion (Ford et al., 1969; 1972; Ford, 1974). It has also been suggested that FBP assists in the absorption of folate by preventing its uptake by intestinal bacteria, and that it directly promotes the transport of folate across the intestinal mucosa (Ford et al., 1972; Ford, 1974). Studies examining the effects of FBP on folate absorption and bioavailability have produced inconsistent results, most probably because many of these studies lacked specific, validated analytical methods. In addition, most of the studies were performed using animal models.

Recently, more advanced absorption and bioavailability models such as the dynamic in vitro gastrointestinal model (TIM), ileostomy studies and intervention studies, all human-based studies, have produced new knowledge concerning the role of FBP in the absorption of dietary folate (Verwei et al., 2005; Witthöft et al., 2006; De Jong et al., 2005).

This review summarizes and discusses recent knowledge on FBP in milk against the background of older findings, including previous reviews concerning bovine FBP produced during the 1980s and 1990s (Anon., 1982; Wagner, 1982; Editoreal, 1985; Kane and Waxman, 1989; Henderson, 1990; Parodi, 1997). The current review is divided into three parts: (i) biochemistry, (ii) FBP analysis, and (iii) food science/nutrition.

BIOCHEMISTRY

The Family of Folate-Binding Proteins

Absorption and cellular uptake of folate is mediated by two different classes of membrane proteins. One class is the family of folate-binding proteins (FBPs or Folbps), also known as folate receptors (FR). The other class, which is not further discussed in this review, consists of the reduced folate carrier (RFCs), which are transmembrane proteins that bind reduced folate with micromolar affinity. A variety of FBP analogs have been isolated from different species, in both soluble and particulate state. In humans, FBP is encoded by a family of genes, the homologous products of which are termed FR-α, FR-β, and FR-γ (Lacey et al., 1989; Sadasivan and Rothenberg, 1989; Elwood, 1989; Ratnam et al., 1989; Shen et al., 1994; 1995). FR-α and FR-β are attached to the cell membrane by a glycosyl-phosphatidylinositol(GPI) anchor. FR-γ is constitutively secreted because it lacks a signal for GPI modification (Shen et al., 1995). The membrane-associated forms of FBP are capable of transporting folate into the cell. In normal tissues, the most commonly expressed receptor isoform, FR-α, occurs largely at the apical/luminal surface of epithelial cells, where it is not supplied with folate from blood circulation. In proximal kidney tubules, FR mediates reabsorption of folate from urine due to the expression of FR on the apical (luminal) side of the tubule cells (Birn et al., 2005; Weitman et al., 1992). Gene knockout studies have shown that FR-α is essential for nerve tube development in the embryo, but folate supplementation allows normal development (Piedrahita et al., 1999). Knockout of the FR-β gene in mice is associated with an apparently normal phenotype, but may increase the risk of arsenic-induced neural tube defects, especially when dietary folate is restricted (Wlodarczyk et al., 2001). Soluble folate-binding protein (sFBP) originates either from FR-γ, because it is constantly secreted, or from the membrane-bound forms. The sFBP from FR-α in human malignant tissue culture (KB) and placental cells has been demonstrated to originate both from phospholipase cleavage of the GPI anchor and from proteolysis by an Mg^{2+}-dependent protease (Luhrs and Slomiany, 1989; Antony et al., 1989; Elwood et al., 1991; Yang eta l., 1996). FR-β is processed intracellularly by two independent pathways, one resulting in GPI anchor addition and another resulting in its secretion (Wang et al., 1997).

Soluble Folate-Binding Protein in Cow's Milk

Soluble bovine FBP (Salter et al., 1972; Treloar et al., 2000; Nygren et al., 2003) has been isolated from cow's milk by different affinity chromatographic methods, sometimes in combination with ion-exchange chromatography (Svendsen et al., 1979). FBP isolated from cow's milk contains 222 amino acids, the partial sequence was determined in 1979 (Svendsen et al., 1979) the complete sequence five years later (Svendsen et al., 1984). The polypeptide contains eight disulphide bridges and the molecular weight of the protein part is 25,700 Da (Svendsen et al., 1984). The protein is highly glycosylated, with two potential sites for *N*-linked glycosylation. The final molecular weight is about 30,000–35,000 Da of which the glycosylated part constitutes between 7–10% (Svendsen et al., 1979; 1984; Salter et al., 1981). A 19 amino acid signal sequence of bovine milk FBP has recently been sequenced (Smith et al., 2005). The signal sequence is cleaved off before secretion into the milk. At saturation, FBP binds approx. 1 mol of folate per mol of protein (Salter et al., 1981). The folate-binding capacity of FBP varies with pH, as 5-methyltetrahydrofolate (5-CH_3-H_4folate) is totally dissociated at pH values below 5 and folic acid at values slightly above 3 (Salter et al., 1981). In contrast to bovine milk, human mik contains two forms of FBP; one particulate and one soluble (Antony et al., 1982; Svendsen et al., 1982).

Sequence Homology

There is a significant sequence similarity between FBP and chicken riboflavin-binding protein (RfBP) (Zheng et al., 1988). In the region where the sequences clearly correspond, the sequence identity is more than 30%. This indicates a structural and functional similarity between RfBP and FBP (Monaco, 1997; Zheng et al., 1988). The crystal structure of RfBP has been determined and shows that the 16 cysteines that form the ligand-binding domain in RfBP are conserved in the proteins (Monaco, 1997). The structure also shows that five out of six tryptophans present in RfBP are clustered in the neighborhood of the ligand-binding site. However, FBP consists of a higher number of tryptophans, namely 13. The plane formed by the tryptophans in the structure of RfBP is in direct contact with the plane of the ligand. Folate and flavins are functionally distinct, but share a structural resemblance because both are hydrophobic aromatic ring systems. It is therefore plausible that an ancestral protein had the capacity to bind both vitamins and that a few amino acid replacements could modify the binding specificity (Zheng et al., 1988).

Affinity and Binding Kinetics

Attempts to describe the affinity of the interaction between folate and FBP have proven inconclusive, since the previously reported equilibrium dissociation constants (K_D) at neutral pH exhibited a dependence on the concentration of FBP used for their determination (Salter et al., 1981; Hansen et al., 1978; 1983). A study in 2004 theoretically interpreted original and existing data and estimated K_D for the interaction between folic acid and FBP to be 20 pM, or even lower (Nixon et al., 2004). This constant was confirmed experimentally in 2005, when K_D for the interaction between folic acid and FBP was determined to be 20 ± 9 pM using SPR technology (Nygren-Babol et al., 2005). That study also revealed marked differences in binding kinetics between FBP and the major forms of folate derivatives. Considerable differences were found between the different folate forms for both association rate constants (k_a) and dissociation rate constants (k_d) (Table 1). Folic acid exhibited the most rapid association rate constant and the slowest dissociation rate constant. Among the naturally occurring folates investigated, (6S)-5-H_4folate had the highest affinity (250 pmol/L), (6S)-5-CH_3–H_4folate almost 10-fold lower (2000 pmol/L) and (6S)-5-HCO–H_4folate the lowest affinity of all (12000 pmol/L). The results also showed a large difference in affinity for the two stereoisomeric forms of 5-CH_3H_4folate, as the affinity for the (6R)-5-CH_3H_4folate was 160 pmol/L, more than 10-fold higher than that for the naturally occurring 6S-isomer.

Kinetic behavior and distribution differ between folic acid and reduced folate in humans (Wright et al., 2005). It is likely that these differences are caused by different binding kinetics to folate receptors in the body. During recent years, it has become increasingly clear that binding affinity alone may not always be a suitable descriptor of biological activity. Since the equilibrium dissociation constant (K_D) is calculated by the ratio k_d/k_a the same affinity value can result from different combinations of association and dissociation rates. In cases where the association or the dissociation rate dictates biological activity, affinity alone may be a directly misleading measure of activity (Andersson and Hämälainen 2006). Estrogen receptor agonists have been found to have 100-fold higher association

Table 1 Affinity and Rate Constants of FBP/Folate Interatction[a]

Folate	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_D (pM)
PteGlu	$(1.0 \pm 0.4) \times 10^6$	$(1.3 \pm 0.4) \times 10^{-5}$	20 ± 9
(6*R*)-5-CH_3-H_4folate	$(3.0 \pm 0.4) \times 10^5$	$(4.7 \pm 1.7) \times 10^{-5}$	160 ± 50
(6*S*)-H_4folate	$(3.8 \pm 0.3) \times 10^5$	$(9.7 \pm 3.0) \times 10^{-5}$	250 ± 60
(6*S*)-5-CH_3-H_4folate	$(2.8 \pm 0.3) \times 10^5$	$(5.7 \pm 1.0) \times 10^{-4}$	2000 ± 200
(6*S*)-5-HCO-H_4folate	$(1.2 \pm 0.2) \times 10^5$	$(3.2 \pm 1.0) \times 10^{-3}$	12000 ± 3000

[a]Mean values ± standard error obtained from three or more independent measurements on different surfaces.
From Nygren-Babol et al., 2005. Reprinted with permission of the American Chemical Society.

rates than antagonists, irrespective of dissociation rate (Rich et al., 2002) while folic acid has almost 10-fold higher association rate than (6S)-5-CH_3–H_4folate and almost 50-fold slower dissociation rate (Nygren-Babol et al., 2005). The physiological importance of the differences in rate constants for folic acid compared with those of (6S)-5-CH_3–H_4folate is not known. The fact that molecules that act on the same receptor but with an opposing function, that is, agonists, have been discriminated by kinetics and not by affinity stresses the importance of binding kinetics.

Binding Mechanism and Aggregation

It is well documented that FBP has a remarkably concentration-dependent aggregation tendency for the native protein (Salter et al., 1981; Hansen et al., 1983; Pedersen et al., 1980). Even though the exact nature of the FBP aggregates is not known, the aggregation is concentration-dependent and enhanced upon addition of folate (Hansen et al., 1983; Pedersen et al., 1980). It has also been observed that liganded (holo) FBP forms hydrophilic water-soluble polymers, whereas unliganded (apo) FBP aggregates have a hydrophobic character (Holm et al., 2001).

Contradictory results have been reported on the binding mechanism of the FBP-folate interaction. Results from a stop-flow fluorescence (SFF) study led the authors to propose a two-step binding mechanism at pH 5.0 and a three-step binding mechanism at pH 7.4 (Christensen et al., 2006). The first step was envisioned as being the ligand-entering step and the second as a conformational change. The third step that only occurs at pH 7.4 was not explained in that study, but has later been suggested to be a consequence of ligand-induced polymerization (Nygren-Babol, 2007). By contrast, a one-step binding model was proposed based on SPR binding data at pH 7.4 (Nygren-Babol et al., 2005), as no improvement in curve fit was seen when more complex models were used. The discrepancy between these two studies could be explained by the existence of rate-limiting, parallel reactions resulting from dissociation of pre-formed FBP aggregates due to the high protein concentration used in the SFF experiment (Nygren-Babol, 2007). However, it is well-established that affinities obtained using SPR-biosensor analyses, for example, Biacore, agree well with those determined by solution-based methods (Rich and Myszka, 2000; Day et al., 2002; Navratilova et al., 2007). It is important that careful experimental design is used, regardless of analytical method, in order to minimize potential artefacts that could easily be interpreted as complex-binding mechanisms. When a binding model that includes structural changes is suggested, it needs to be verified with techniques detecting structural changes, such as NMR or circular dichroism. A circular dichroism study has observed a conformational change in FBP, but could not disclose whether this change was a consequence of initial aggregation or ligand binding (Kaarsholm et al., 1993). On the other hand, no change was observed in conformation during ligand binding for the structurally related RfBP (Wasylewski, 2000). Therefore, it seems reasonable that the structural change in FBP observed by circular dichroism was a consequence of initial aggregation and not of ligand binding.

Heat Stability of FBP (Denaturation)

In Milk

Most of the studies that have analyzed FBP in milk report that heating of cow's milk, for example, pasteurization or UHT-treatment, affects the folate-binding capacity of FBP (Areekul et al., 1978; Gregory, 1982; Kohashi and Iwai, 1985; Ford et al., 1977). Whereas UHT treatment undoubtedly seems to inactivate FBP, the results are not consistent for pasteurization. For example, a >90% reduction in folate-binding capacity was observed in pasteurized milk (75°C for 15 sec), when folate binding was monitored using ^{3}H-labelled folic acid (Areekul et al., 1978). Similar results were reported for a comparison of unprocessed milk with milk pasteurized at 63°C for 30 min, 75°C for 15 sec, and 120°C for 1 sec, where an almost complete reduction in folate-binding capacity after pasteurization was observed (Kohashi and Iwai, 1985). In contrast, it has been reported that around 90% of the folate-binding capacity remains in pasteurized human milk (Ford et al., 1977). A later study (Wigertz et al., 1996) found only a 20% reduction in folate-binding proteins in pasteurized milk compared with unheated milk, while a recent study (Achanta et al., 2007) found no significant decrease in FBP content in folic acid-fortified milk after pasteurization. Both these latter studies quantified FBP by ELISA. Previous conflicting results, in contrast to these latter studies, could have been caused either by the use of different methodologies to establish changes in folate-binding capacity or by the fact that common pasteurization temperatures are very close to those at which denaturation of FBP takes place.

A study using gel filtration analysis found increased molecular mass after heat treatment (Gregory, 1982) indicating heat-induced alternation of FBP as an explanation for the changed structural and functional characteristics of FBP in processed milk products. Heat-induced alternation of FBP was also suggested to be the reason for the increase in molecular mass detected on the sensor surface when measuring the active FBP content in milk after pasteurization using SPR-biosensor technology (Nygren-Babol and Karonen, 2009).

In Buffer

The thermal unfolding of FBP has been studied by Differential Scanning Calorimetry (DSC) (Nygren-Babol and Karonen, 2009; Sigurskjöld et al., 1997). It was found that ligand binding increased the thermal stability of FBP from 60.5°C for apo-FBP to 72.4°C for (6S)-5-HCO–H_4folate, 78.7°C for (6S)-5CH_3–H_4folate and 83.7°C for folic acid (0.1 M phosphate buffer, pH 6.7; protein conc. 10% w/v) (Nygren-Babol and Karonen, 2009). The shifts in denaturation temperature after ligand

binding correspond well with previously published affinity data (Table 1) (Nygren-Babol et al., 2005). Somewhat different results for the thermal stability of apo-FBP were obtained in another study (Sigurskjöld et al., 1997) which found that thermal unfolding of apo-FBP occurred at 48.4°C and that ligand binding increased the thermal stability to 72°C for FBP in complexes with folate. However, this publication (Sigurskjöld et al., 1997) does not mention the buffer, protein concentration, or folate derivative used, and it is therefore reasonable to assume that the different results are due to different experimental conditions.

FBP ANALYSIS

Radioprotein-Binding Assays (RPBA) of Clinical Samples

Shortly after the identification of folate-binding proteins in cow's milk, the competitive protein-binding assay was introduced for the measurement of serum folate concentrations (Waxman et al., 1971). The principle of the competitive binding assay is based on competition between folates in the sample or standard and a known amount of labeled folate for the limiting binding sites on a folate-binding protein from cow's milk. Charcoal is used to adsorb the unbound folate. The charcoal-bound folate is removed by centrifugation, while the unbound folates remain in the supernatant. The folate concentration of the sample is calculated from the amount of radiolabel remaining in the supernatant. The original study (Waxman et al., 1971) used a non-radioactive mixture of (6R, S)-5-CH_3–H_4folate as standard and the (6S)-stereoisomer of radioactive (^{3}H) 5-CH_3–H_4folate as competitive radioactive folate. It was concluded from the results that only the active (6S)-5-CH_3–H_4folate bound to FBP. Moreover, it was clearly shown that neither 5-HCO–H_4folate nor methotrexate (antifolate) bound to FBP.

Ten years later, the relative activities of purified folate standards were determined using in-house-synthesized racemic mixtures and pure stereoisomers (Shane et al., 1980). These folate derivatives were tested in four different commercial radioassay kits, of which two were based on bovine FBP. For one kit (Bio-Rad), only small differences (<5%) in relative activity were found between folic acid and the two stereoisomers of 5-CH_3–H_4folate, (6R, S) and (6S), respectively (Shane et al., 1980). However, the Schwartz-Mann kit showed the following relative activities: folic acid 0.93, (6S)-5-CH_3–H_4folate 1.00 and (6,R,S)-5-CH_3–H_4folate 1.27, indicating a ~30% difference between certain folate derivatives. One explanation for these differences was that commercial assay kits differ in the type of diluting system, and either buffer, serum, or human serum is used, which might influence the results (Shane et al., 1980).

Today, competitive binding methods are routinely used for folate analysis in clinical samples. The most common standard in clinical RPBA kits of today is folic acid, because of its greater stability compared with native folates. At pH 9.3 the relative binding activities for folic acid and the diastereoisomer mixture (6R,S)-5-CH_3–H_4folate to FBP have been shown to be equal (Salter et al., 1981; Givas and Gutcho, 1975) and in serum (6S)-5-CH_3–H_4folate is the major folate form.

A stable-isotope-dilution tandem mass spectrometry method (LC-MS/MS) was recently developed and validated for analysis of human serum samples (Pfeiffer et al., 2004). Serum folate in the normal range (<50 nmol/L) was found to be dominated by 5-CH_3–H_4folate (93.3%). These authors also compared the folate concentrations from 32 serum samples analyzed by LC-MS/MS, microbiological assay and RPBA. By carefully selecting the standards, quite satisfactory agreement in mean total folate concentrations could be achieved for all three methods (Pfeiffer et al., 2004). However, in a more extensive study conducted by the same group based on 327 serum samples, analyzed by LC-MS/MS, microbiological assay and RPBA (Bio-Rad QuantaPhase II radioassay), good agreement was only found between data obtained by LC-MS/MS and the microbiological assay, while the RPBA method produced much lower results (Fazili et al., 2007).

RPBA for Analysis of Folate in Foods

In spite of the convenience of the RPBA method, its application to food analysis has failed because of problems with variations in ligand-binding affinity for the multiple folate forms present in many foodstuffs (Wigertz and Jägerstad, 1995; Strålsjö et al., 2002). Particularly low affinity to folate-binding proteins (compared with folic acid) has been observed for 5-HCO-H_4folate (Wigertz and Jägerstad, 1995; Strålsjö et al., 2002; Gregory et al., 1982) whereas H_4folate has a much higher affinity (Wigertz and Jägerstad, 1995; Strålsjö et al., 2002) and the affinity for 5-CH_3-H_4folate is in the same range as for folic acid when a pH of 9.3 is used in the assay (Strålsjö et al., 2002). This stresses the necessity of having only one dominant form of folate in the food sample, preferably 5-CH_3-H_4folate, which rules out the use of RPBA as a method of choice for analyzing the cocktail of native folate forms present in many food items. For milk, fruits, and berries, where 5-CH_3-H_4folate is the major folate form, acceptable agreement in folate content has been reported for RPBA compared with HPLC or microbiological assays (Wigertz and Jägerstad, 1995; Strålsjö et al., 2002). In general, however, the food matrix is usually a mixture of many folate forms, resulting in low precision and poor agreement with the microbiological assay when RPBA is applied to food sample (De Souza, and Eitenmiller, 1990; Finglas et al., 1993).

Affinity Chromatography with FBP as a Clean-Up and Concentration Step in Folate Analysis by LC

The failure to obtain similar protein binding with all folate derivatives when using RPBA in food analysis created a need to develop reliable and efficient LC methods. Because of the varying numbers of native folate vitamers present in foods, and because many folate vitamers are labile, efficient methods for clean-up and concentration of the food folates prior to separation

by liquid chromatography were necessary. The use of affinity chromatography with FBP from cow's milk was therefore introduced (Selhub et al., 1980). This research group developed columns consisting of folate-binding protein covalently bound to agarose (Sepharose) beads, which have been successfully used to extract a variety of folate forms from biological samples (tissue, whole blood, and plasma/serum) (Selhub et al., 1988; Selhub, 1989; Varela-Morerras et al., 1991; Bagley and Selhub, 2000). Although the initial study (Selhub et al., 1980) claimed that all folate derivatives were quantitatively bound to FBP, regardless of affinity, later studies on food samples showed that recovery of 5-HCO-H_4folate was a major obstacle due to its low affinity. For example, a 10% and 20% loss of 5-HCO-H_4folate was reported when the column was loaded to 25% and 50% of column capacity respectively (Pfeiffer et al., 1997), which was in agreement with other investigations (Selhub, 1989; Kariluoto et al., 2001). Therefore, it is of critical importance not to overload the column, with less than 25–30% of the total loading capacity being recommended for biological samples containing a mixture of various folate forms (Selhub, 1989; Pfeiffer et al., 1997; Kariluoto et al., 2001). It is also necessary to check the folate-binding capacity of the columns regularly because the binding capacity decreases after several extractions. For instance, it decreased by 58% after 18 extractions and 26% and 47% after 12 and 22 extractions, respectively (Konings, 1999; Freisleben et al., 2003). The situation becomes even more complicated if internal standards are used, because the useful capacity of folate-binding sites is reduced (Freisleben et al., 2003). Moreover, many commercial folates used as standards are racemic. Racemic folate solutions do not bind uniformly to the columns. For instance, the non-natural and biologically inactive (6R)-isomer has a 10-fold higher affinity for bovine FBP than the natural (6S)-folate isomers (Nygren-Babol et al., 2005). Therefore, the recovery rates of racemic folate standards need to be determined before they are added to biological samples as internal standards.

Further limitations are that FBP columns are not commercially available, which complicates their routine use. Instead, they have to be prepared in-house from partly purified bovine FBP and affi-gel, commercially available (Kariluoto et al., 2001; Konings, 1999), or other types of stationary phase for immobilization of FBP (Nelson et al., 2003). As a consequence, column binding characteristics may vary among research groups and among batches. In comparison with SPE columns, the FBP-based affinity chromatography columns are much more expensive.

The main advantage of affinity chromatography is that folate-binding protein is highly specific towards folate (Pfeiffer et al., 1997) and therefore gives much cleaner chromatograms, resulting in 10-fold lower LOD and LOQ compared with SAX (Freisleben et al., 2003); however, some researchers report unsatisfied specificity when using affinity chromatography (Kariluoto et al., 2001; Ruggeri et al., 1999).

Due to these obstacles with FBP-based affinity chromatography, it is most probable that sample purification with optimized and validated solid phase extraction (SPE) methods will be the choice in LC methods of the future.

Concentration Analyses of FBP

The first direct technique used for determination of FBP concentration in cow's milk and dairy products was an enzyme-linked immunosorbent assay (ELISA) (Hoier-Madsen et al., 1986). Before the development of the ELISA, FBP was quantified indirectly by measuring the folate content after adsorption to charcoal of excessive unbound folate (Ghitis, 1967). Later, FBP was quantified in terms of folate-binding capacity following addition of graded amounts of folic acid or by incubation with radio labeled folic acid. Bound folic acid was then analyzed after dialysis (Ford et al., 1972; Salter et al., 1972), or after being selectively trapped by filtering on cellulose-nitrate (Selhub et al., 1984).

The ELISA developed by Hoier-Madsen et al. (1986) is of a two-site "sandwich" type and is based on the recognition of FBP by a polyclonal antibody raised against bovine milk FBP. This method has demonstrated high specificity due to the absence of cross-reactivity, and high reproducibility and sensitivity. When whey protein concentrate (65%) is used as a reference sample, the CV% is generally below 15%. Analyses of FBP-spiked milk samples have resulted in recoveries ranging from 80–110% (Arkbåge, 2003a). The method does not distinguish between whether FBP is complexed with folate or not. It has been observed that the FBP content detected decreases as the free folic acid fraction increases (Verwei et al., 2003; Achanta et al., 2007). The reason for this is unknown but it might be caused by the increased FBP aggregation after ligand binding, which results in a reduced number of antigen epitopes being exposed.

In addition to ELISA, two different Surface Plasmon Resonance-based assays (SPR-biosensor technology) have been developed for direct measurements of FBP in milk (Nygren et al., 2003; Indyk and Filonzi, 2004). The underlying physical principles of SPR are complex, but since a detailed theoretical understanding of the physics is not necessary for an adequate working knowledge of the technique, they are not discussed here. However, SPR-biosensor technology enables the interaction between biomolecules to be monitored in real time using label-free assay formats. One of the interacting molecules is immobilized on the sensor surface. The other molecule (the analyte) is then injected in solution over the sensor surface. As analytes from the injected sample bind to the interacting partner immobilized on the sensor surface, a change in SPR response is detected. The response level is proportional to the change in mass at the surface. The changes in amount of bound mass can be detected down to a few picograms or less per square millimeter of surface, corresponding to concentrations in the picomolar to nanomolar range in the bulk sample solution. SPR-biosensors provide information on affinity and binding kinetics (association, dissociation rates) of an interaction and the concentration of specific molecules present in the samples. The

analytical procedure for the two SPR-based FBP assays developed to date, differ in several aspects. The method developed by Nygren et al. (2003) can distinguish the free apo-form from the complexed holo-form of the protein, but includes a pre-treatment stage in order to remove endogenous folate from the samples. The Indyk and Filonzi (2004) method does not include any pre-treatment and therefore detects only the apo-form of the protein. The Nygren et al. (2003) method has been reported to give mean values of 5.8 μg/mL (range, 2.9–11.1 μg/mL; n = 66) for the total FBP content and 2.9 μg/mL (range, 1.1–5.6 μg/mL; n = 66) for the apo-FBP in milk samples from individual dairy cows. The Indyk and Filonzi (2004) method gives somewhat higher levels of apo-FBP, 4.6–8.0 μg/mL (mean 6,7 μg/mL; n = 29. Apart from a divergent analytical scheme, different FBP standards are used because of the absence of an accepted FBP reference material. In addition, pasteurized consumer milk was tested by Indyk and Filonzi (2004) and it is possible that heat-induced aggregation of FBP caused by pasteurization had increased the molecular mass of FBP. Since SPR-biosensors detect changes in mass on a sensor surface, this could have influenced the values reported in that study.

FOOD SCIENCE AND NUTRITION

FBP Occurrence in Milk and Dairy Products

The first quantitative estimation of FBP presented for cow's milk was about 8 mg/L (~250 nmol/L), based on folic acid-binding capacity measured by addition of graded amounts of folic acid followed by dialysis (Salter et al., 1972). Similar figures for FBP content expressed as folic acid-binding capacity were reported in a subsequent study for human milk samples (180–270 nmol/L) (Selhub et al., 1984). More recent quantitative data on the FBP concentrations in cow's milk before and after gentle processing, that is, pasteurization and drying, are summarized in Table 2. Analyses of total FBP in milk from five different local farms (n = 10) using ELISA (Hoier-Madsen et al., 1986) showed an average of 211 ± 7 nmol FBP/L in unheated milk (Wigertz et al., 1996). A later study reported data on FBP occurrence in an extensive survey of milk samples (n = 66) collected from two Swedish dairy breeds, at different stages and numbers of lactations (Nygren-Babol et al., 2004). The analyses were performed on defatted and non-heated milk using SPR-biosensor technology (Nygren et al., 2003). The range for total FBP in all samples was 91–347 nmol/L, which was of similar magnitude to that reported previously (Wigertz et al., 1996). The SPR method is also able to discriminate between total FBP and unbound FBP (apoprotein). The range reported for apo-FBP (non-liganded FBP) was 34–175 nmol/L (Nygren-Babol et al., 2004). These results indicate that approximately 50% of the total FBP content was unliganded. This is in agreement with data reported previously of 90.1± 4.2 nmol/L 5-CH_3-H_4folate in unheated milk samples analyzed by HPLC, whereas the same samples contained twice as much total FBP (211 ± 7 nmol/L) (Wigertz et al., 1996).

Whereas no significant differences were observed between breeds or number of lactations (1st and 2nd) as regards FBP, the stage of lactation seemed to have a great impact (Nygren-Babol et al., 2004). Milk was sampled from five cows at 1, 3, 30, 60, and 90 days post-partum. Total FBP and unliganded apo-FBP varied widely between the cows at the beginning of lactation, for example, on day 1 the levels of total FBP ranged between 625–1250 nmol/L (20–40 mg/mL). This concentration decreased later in the lactation (after 30 days) to 160–200 nmol/L (5–6 mg FBP/mL). The ratio of total FBP to apo-FBP, on the other hand, remained fairly constant throughout the study period, suggesting that around 50% of FBP was unbound (Fig. 1). A similar pattern for FBP concentrations related to the stage of lactation in cow's milk was also reported for one animal in a recent study (Indyk et al., 2006). In fact, this phenomenon was first reported in a study by Ford (1974). Milk samples were collected from one goat at parturition and again on day 7 and day

Table 2 FBP concentrations (nmol/L or mg/kg) reported for unheated milk, pasteurized milk and milk powder. Mean ± SD or range

Dairy product	n	Treatment	Total FBP nmol/L; mg/kg	Method of analysis	Reference
Cow's milk	1	Raw, unheated	8	Binding capacity	Salter et al., 1972
Human milk	14	Freshly pumped	180–250	Binding capacity	Selhub et al., 1984
Cow's milk	10	Raw, unheated	211±7	ELISA	Wigertz et al., 1996
Cow's milk	66	Raw, unheated defatted	90–350; 3–11	SPR-Bia	LNB et al., 2004
Cow's milk[a]	10	Pasteurized	168 ± 20	ELISA	Wigertz, 1996
Cow milk[a]	128	Pasteurized	199–211	ELISA	Wigertz,1997
Cow milk[a]	29	Pasteurized	210* 6.7* 140–310 4.5–10	SPR-BIA (Apo-FBP)	Indyk, 2004; 2006
Cow's milk[a]	48	Skim milk powder	1960 ± 460	ELISA	Wigertz, et al.,1997
Cow's milk	?	Commercial milk powder	310–1875; 10–60	SPR-BIA (Apo-FBP)	Indyk, 2006
Cow's milk	?	Colostrum powder	2500–3125; 80–100	SPR-BIA (Apo-FBP)	Indyk, 2006
Cow's milk	5	Solid/powder	2280 ± 1350	Binding capacity	Swiatlo, et al.,1990
Goat's milk	7	Solid/powder	4480 ± 1980	Binding capacity	Swiatlo, et al., 1990
Human milk	19	Solid/powder	1850 ± 160	Binding capacity	Swiatlo, et al.,1990

*Mean and range

[a]Total FBP nmol/L (mg/kg); cow's milk 250 (8); 90–350 (3–11); 210 (6.7); 140–310 (4.5–10).

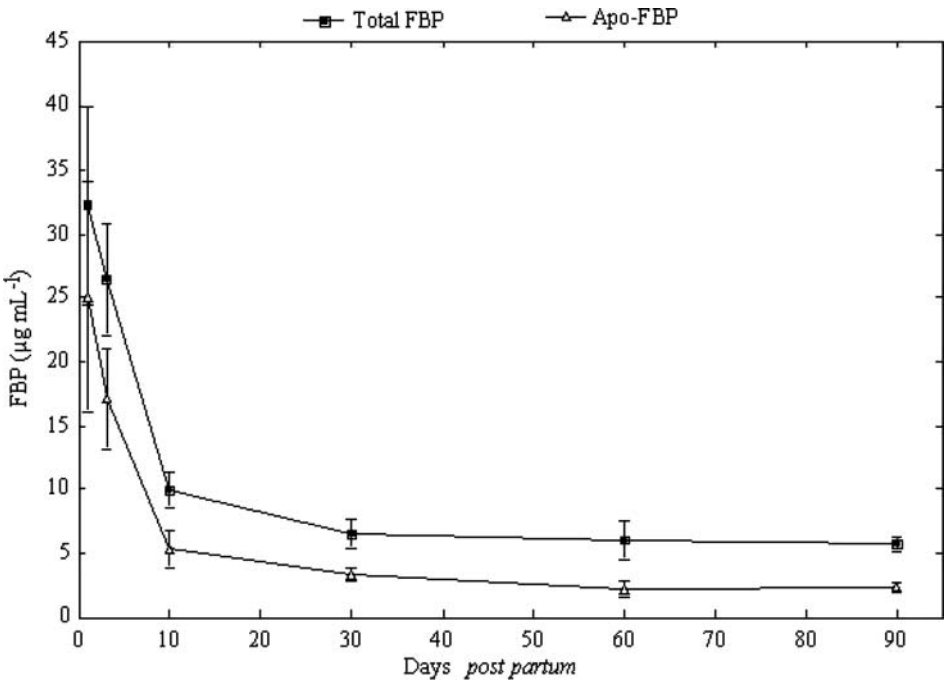

Figure 1 Levels of total FBP and apo-FBP (mean values ± SD) in milk during the first 90 days of lactation for five dairy cows at the Jälla experimental dairy farm (SLU). From: Nygren-Babol et al. (2004). Reprinted with permission from Elsevier.

30 of lactation. Folic acid-binding capacity was monitored as retained folic acid after dialysis. The folate-binding capacity in the colostrum (day 1) amounted to 600 μg/L (~1,333 nmol/L) which gradually decreased to 550 μg/L (~1,200 nmol/L, day 2), 250 μg/L(~600 nmol/L, day 7), and 130 μg/L (~300 nmol/L) after 30 days of lactation (Ford, 1974). Corresponding figures for folate concentration were 210 μg/L (colostrum) (~470 nmol/L) to 5 μg/L (~10 nmol/L) (day 30), thus indicating a pronounced excess of FBP compared with folate in goat's milk (Ford, 1974).

Like all proteins, FBP is heat-sensitive (see FBP denaturation above). Following pasteurization at 74°C/15 s, the total FBP in cow's milk from five different local farms decreased by 20% ($P < 0.05$; $n = 10$) to 168 nmol/L, from 211 nmol/L in unheated milk from the same batches (Table 2) (Wigertz et al., 1996). In contrast, UHT milk (heated for 140°C/5 sec) and yogurt (heated for 90°C/10 min prior to inoculation) contained only traces of FBP (< 10 nmol/L) (Wigertz et al., 1996; 1997). These results are in line with reports of FBP denaturation temperatures in the range 70–80°C, depending on type of ligand binding (Nygren-Babol and Karonen, 2009).

Freeze-drying or spray-drying for the manufacture of milk powder seems to retain most of the FBP in an active state. A 1990 study by Swiatlo et al. determined folate-binding capacity, analyzed according to Selhub et al. (1984) in human, in cow's, and in goat's milk. Morning and evening milk samples from five cows and seven goats, respectively, were pooled and freeze-dried. Human milk was collected from 19 apparently healthy women and pooled prior to freeze-drying. Solids of human milk and cow's milk contained close to 2000 nmol/kg (1850 ± 160 and 2280 ± 1350, respectively) whereas goat's milk contained twice that figure (4480 ± 1980 nmol/kg). Skim milk powder, spray-dried by Arla Foods (Kågeröd, Sweden) in a current of hot air at 140–150°C retained most of its FBP (~2000 nmol/kg; n = 48) (Table 2) (Wigertz et al., 1997). Similar figures (320–1920 nmol/kg) have been reported for FBP in commercial milk powder samples (unknown number of samples) (Indyk et al., 2006).

Analyses of other dairy samples for total FBP showed cottage cheese (n = 16) to be a rich source of FBP (540 ± 3 nmol/kg), whereas whey and whey cheeses contained only 30–97 nmol/FBP per kg; (n = 8–12) (Wigertz et al., 1997). Hard cheeses manufactured from whey-separated milk contained less than 25 nmol FBP/kg (n = 36) (Arkbåge, 2003; Wigertz et al., 1997).

Milk FBP and Folate during Suckling

Milk has evolved for the nutritional support of the infant mammal. It has been suggested that FBP in milk may act initially in the mammary gland as a trapping agent to accumulate folate from blood plasma into milk (Ford et al., 1972; Ford, 1974). This suggestion was substantiated by monitoring folate concentrations in milk and plasma from two goats following intramuscular injection of folic acid. The difference in milk folate and plasma folate during the first two days following parturition was more than ten-fold. Furthermore, there was marked increase in FBP concentration in goat's milk close to parturition (colostrum), which was accompanied by increasing folate concentrations

in the goat's milk for around one week (Ford et al., 1972). The folate status of the kids was elevated over the same period. The authors reported that during a period lasting only a few hours from birth, the gut of the newborn ruminant transmits intact proteins freely and non-selectively, and it is believed that the young animal receives into its circulation all the proteins that occur in the colostrum (Ford et al., 1972; Ford, 1974).

A folate-transport mechanism mediated by FBP from goat's milk in the small intestinal brush-border membrane vesicles of a 6-day-old goat was found using a rapid filtration assay (Salter and Blakeborough, 1988). In the presence of FBP prepared from goat's milk and bound to ^{14}C- 5-CH_3–H_4folate, 50–100 times higher amounts of ^{14}C- 5-CH_3–H_4folate were taken up by the vesicles compared with free ^{14}C- 5-CH_3–H_4folate. The uptake occurred in the pH range 4.5–6.5, with an optimum pH of 5–5.5. The authors concluded that the study produced evidence showing the existence of a folate transport mechanism mediated by the milk FBP at the intestinal brush-border of neonatal goat kids. They also suggested that the uptake appeared to involve binding of FBP to a receptor in the cell membrane, but concluded that further studies were needed to confirm the existence of these special receptors.

Another species-specific study was performed on suckling rats (Mason and Selhub, 1988). Pups aged 12–14 days were given FBP isolated from rat milk and bound to (^{3}H)-folic acid and the absorption of unbound folic acid and folic acid bound to rat milk FBP was compared in the small intestine of the suckling rats using an in vivo intestinal loop technique. In contrast to free folic acid, folic acid-bound FBP was absorbed more rapidly in the ileum than in the jejunum. In parallel, the mucosa of the distal (ileum) small intestine showed four-fold higher folate-binding capacity than that of the proximal intestine (duodenum and jejunum combined). The authors concluded that the folic acid-FBP complex is absorbed by a different mechanism than that responsible for the absorption of free folic acid, and that absorption does not require prior dissociation of the vitamin-binding protein complex. Instead, the authors discussed the resemblance of the absorption pattern of FBP-bound folic acid to the characteristics of endocytotic absorption of macromolecules, a well-documented feature of the suckling mammal's intestinal physiology. An early study (Clarke and Hardy, 1969) observed absorption of macromolecular substances in the intestine of young rats to be greater in the ileum than in the jejunum, which was the same pattern observed in suckling rats (Mason and Selhub, 1988). A recent in vitro study by Birn et al. (2005b) on rats have demonstrated megalin, expressed in several epithelia, including intestine and kidney proximal tubule as receptor to be able to bind and mediate cellular uptake of complexed folate-FBP. Megalin is a large multiligand endocytic receptor and member of the low-density lipoprotein receptor family which could be a mechanism for intestinal end renal endocytic uptake of FBP as suggested by these authors (Birn et al., 2005).

A recent paper by Rosenberg and Selhub (2006) reports that human milk folate is similarly absorbed bound to protein, largely intact, through the permeable infant intestine after birth. The infant intestine remains permeable to proteins, including immunoglobulins, for weeks or months post-natally, after which the intestine matures and develops less permeable junctions between cells. The milk folate-FBP complex is not preceded by prior digestion in the stomach or intestinal lumen. The acid pH in the stomach is not well-developed in the infant stomach, making intact passage of the FBP-folate complex possible (Nagrita et al., 1996). It has also been suggested that the glycosylation of FBP in human milk, amounting to ∼22%, may help it survive proteolytic digestion (Lönnerdal, 2003). Human milk folate concentration ranges between 109–204 nmol/L, which is 5–10 fold higher than that of maternal plasma (Tamura and Picciano, 2006). A positive relationship exists between human milk folate and folate-binding protein concentrations, while human milk folate-binding capacity exceeds that for folate by ∼68 nmol/L (Tamura and Picciano, 2006). The folate-binding capacity of human milk is reported to range between 180 and 270 nmol/L (Selhub et al., 1984). The excess folate-binding capacity may act to concentrate human milk folate for secretion against a concentration gradient. Plasma folate concentrations in breast-fed infants are generally high (45–68 nmol/L) in the first six months of life and decline to 23–45 nmol/L at 12 months, when foods other than human milk contribute a sizeable proportion of total intake (Tamura and Picciano, 2006). This pattern indicates good bioavailability of folate from human milk with the assistance of FBP.

Can Bovine FBP Affect Folate Absorption and Bioavailability in Adults of other Species?

Animal Experiments

Many scientists have investigated a possible role of FBP from cow's milk in the absorption and bioavailability of folate in adults of other species, particularly rats. Studies on adult rats have indicated that although free folic acid is rapidly absorbed from the jejunum, absorption of folic acid, either bound to purified bovine FBP or in the presence of crude cow's milk, occurs primarily in the ileum (Mason and Selhub, 1988; Tani et al., 1983; Izak et al., 1972; Colman et al., 1981), Whether overall absorption is affected by FBP is still unclear; some investigators have observed an increase (Salter and Blakeborough, 1988; Colman et al., 1981) some have observed no difference (Tani et al., 1983), and some have observed lower overall absorption of FBP-bound folate (Izak et al., 1972; Said et al., 1986). Lower excretion of folate into the urine of rats was observed when folic acid was administered with bovine FBP by stomach tube, and this result was attributed to a more gradual absorption of folic acid, thereby decreasing the blood folate peak and reducing urinary folate loss (Tani and Iwai, 1984). Since the total absorption of free and FBP-bound folic acid was the same, the authors concluded that FBP improved folate bioavailability.

Another study on rats used a depletion-repletion model (Swiatlo et al., 1990). Male Sprague-Dawley rats were depleted for 12 weeks, followed by a repletion period of 4 weeks. The test-feed contained 20% solid milk powder from either human, cow's, or goat's milk, which was given together with graded amounts of added folic acid (0, 200, 400, and 600 μg/kg feed). The total binding capacity of raw lyophilized milk solids was then analyzed by existing methods (Selhub et al., 1984). Mean values for binding capacity were 1.85 $\pm$ 0.16, 2.28 $\pm$ 1.35, and 4.48 $\pm$1.98 μmol folic acid/kg milk powder for human, cow, and goat milk, respectively. The feed contained 20% milk solids, thus resulting in 370, 450, and 900 nmol folic acid binding capacity per kg. Also added to the feed during the repletion period of 4 weeks were 0, 556, 1113, and 1669 nmol folic acid per kg feed. This means that the intakes of FBP and folic acid were equimolar only at the lowest test dose of folic acid (200 μg/kg experimental diet). Thus, for the higher doses of added folic acid, the FBP present amounted to up to 50% of the folic acid on a molar basis. The enhancing effect of milk on folic acid bioavailability concluded for this feeding study did not consider the variation in molarity between FBP and folic acid with increasing folic acid dose, and thus no effect of FBP on folate bioavailability can be drawn. Interestingly, goat's milk solids with the highest folate-binding capacity showed the lowest relative bioavailability, yet this was not significantly different from the experimental diet without milk or FBP.

Another experimental study performed on adult rats examined the impact of FBP on the bioavailability of reduced folate forms. Both single doses of ^{3}H-reduced folates and 4-week feeding experiments were performed (Jones et al., 2003). Equimolar amounts of one reduced folate form at a time was given with and without FBP isolated from a whey protein concentrate and mixed with different milk proteins. The FBP activity after purification was determined by quenching tryptophan fluorescence upon binding of folate (Kaarsholm et al., 1993). The results suggested that the addition of FBP-rich foods to folate-rich food could enhance the bioavailability of natural folates, but that the outcome of such combination would depend on interactions with other components of the diet.

Human Studies In Vitro

TIM is a dynamic, in vitro gastrointestinal model developed at the TNO Nutrition and Food Research in the Netherlands, simulating the gastrointestinal conditions of a healthy human adult (Minekus et al., 1995). Bioaccessible folate is defined as the fraction of folate available for absorption, that is, the amount of folate released from the food matrix and thereby capable of passing over the membranes (molecular cut-off of 5 kDa) in the jejunum and ileum during passage through the gastrointestinal tract. Results from TIM studies on folate bioaccessibility in yogurt and pasteurized milk fortified with either the natural diastereoisomer (6S)-5-CH_3H_4folate or folic acid, with and without addition of FBP in equimolar amounts (250–350 vs $\sim$350 nmol), are shown in Fig. 2. Between 70 and 80% of the folate from both fortified yogurt and pasteurized milk was found to be bioaccessible. After addition of FBP (335–354 nmol) to yogurt, folate bioaccessibility decreased, with a more pronounced decrease in folic acid-fortified yogurt (340 nmol) compared with yogurt fortified with (6S)-5-CH_3H_4folate (275 nmol), to 34 and 57% bioaccessibility, respectively (Verwei et al., 2003; 2005; Arkbage et al., 2003). In contrast, FBP in pasteurized milk did

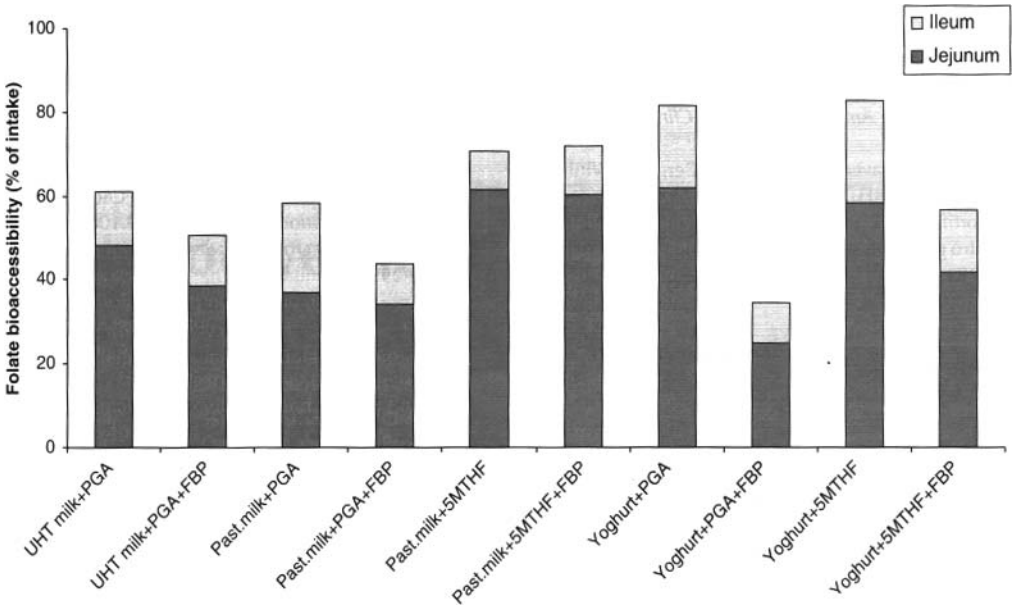

Figure 2 Folate bioaccessibility from the jejunum (dark grey) and ileum (light gray) compartment of the TIM system (as% of intake) from several folic acid (PGA) or 5-CH_3-H_4folate (5MTHF)-fortified milk products tested in the absence or presence of additional FBP. Quoted with kind permission from Verwei et al. (2005).

not affect the bioaccessibility of (6S)-5-CH_3–H_4folate, whereas the bioaccessible fraction of folic acid added to pasteurized milk was reduced by 11–14% by FBP (Fig. 2) (Verwei et al., 2003; 2005; Arkbage et al., 2003).

Caco-cells, the human colon carcinoma cell line, have been used with test solutions of 1μM of either (^{3}H) folic acid or (^{14}C- 6 R,S)-5-CH_3–H_4folate to which 0. 25 μM bovine FBP was added to obtain various molar ratios of FBP:folate (0.25:1;0.5:1,0.75:1,1:1, 1:5:1, and 2:1) (Verwei et al., 2005b). Compared with the reference compounds, folic acid and 5-CH_3–H_4folate showed a moderate permeability across Caco-2-cells, which indicated that folate absorption from the intestinal lumen, was not likely to be complete. The transport from the apical to the basolateral side was higher for folic acid than for 5-CH_3–H_4folate. With increasing amounts of FBP, decreased transport and intracellular accumulation occurred for both folate forms up to equimolar amounts of folate and FBP. FBP in excess of 1:1 molar ratio had no further effect. Whether there are specific receptors for FBP from human or cow's milk in neonates or infants is still unknown. A receptor mechanism for milk FBP in the small intestine, if it exists, is most probably species-specific.

Human Studies In Vivo

A human ileostomy model was used to study relative folate bioavailability and the fate of FBP on folate retention after gastrointestinal passage of UHT-milk, pasteurized milk, and fermented milk fortified with physiological amounts (400–500 nmol) of the major folate form in milk, (6S)-5-CH_3–H_4folate (as calcium salt) (Witthöft et al., 2006). Two of the milk samples (pasteurized milk and fermented milk) were also enriched with a whey protein concentrate containing FBP (Table 3). The model determined folate absorption from single test food doses compared with pharmaceutical folate doses. Nine healthy ileostomy volunteers with presaturated folate body stores underwent strictly standardized independent study days (two or four weeks apart) in random order. After an overnight fast, they received a single portion of each dairy product, or an oral dose of pharmaceutical preparation of (6S)-5-CH_3–H_4folate (Ca-salt). Apparent and relative absorption were calculated from the area under the plasma concentration curve (AUC) and kinetic modeling. In addition, collection of ileostomal effluents in individual fractions over 10 h post-dose not only reflected non-absorbed folate but also showed the time pattern of the gastrointestinal passage of folate and "survival" of intact FBP (Witthöft et al., 2006).

The ileostomy study investigated intake of a physiological dose (200–250 μg) of 5(6S)-Ca-5-CH_3–H_4folate. The ingestion of FBP amounted to around half the folate intake on a molar basis, which corresponded to an intake of 1–2 L milk. This amount of FBP intake in combination with up to 43% survival throughout gastrointestinal transit explained the lowering effect on apparent folate absorption, to 55–60% compared with 86% in UHT milk without FBP (Table 3) (Witthöft et al., 2006). The decreasing effect on absorption of folate in the presence of FBP could have been more pronounced if the milk diet had been enriched with folic acid, since the affinity of FBP is much higher for folic acid than for (6S)-Ca-5-CH_3–H_4folate (Nygren-Babol et al., 2005).

In order to investigate whether milk fortified with folic acid enhanced the folate status of humans and whether the presence of endogenous FBP in pasteurized milk affected the bioavailability of folic acid in fortified milk, a 4-week double-blind intervention study based on 69 healthy, free-living subjects (mostly women) aged 18–49 was performed (De Jong et al., 2005). In addition to a fully controlled diet containing 140 $\pm$ 25 μg total folate/day (326 nmol/day), the groups consumed either 500 mL pasteurized milk or UHT milk with and without fortification with approximately 200 μg folic acid. The pasteurized milk, but not the UHT milk, contained native FBP. Table 4 shows the intake of folate-binding proteins and dietary folate, including milk folate, in the four groups studied. Four weeks' consumption of fortified milk increased folate concentration in serum and red blood cells by 6.6–7.0 nmol/L ($p < 0.001$) and 32–36 nmol/L ($p < 0.01$), respectively. Similarly, plasma homocysteine concentrations were lowered by almost 1 μmol/L ($P = 0.01$) in subjects consuming folic acid-fortified milk. The bioavailability of folic acid from pasteurized milk containing active FBP relative to that of folic acid from UHT milk without FBP was 74–94%, depending on the parameter used. This effect of endogenous FBP was not significant, most probably since the FBP concentration

Table 3 Intake (nmol/portion) of (6S)-Ca-5-CH_3-H_4folate and FBP added to different milks, relative excretion (% of dose) with stomal effluent over 10 h post-dose and apparent absorption (% of dose), respectively. Median values and ranges for nine subjects

	Intake of 5-CH_3–H_4folate nmol	Intake of FBP nmol	Relative excretion of 5-CH_3–H_4folate % of dose	Relative excretion of FBP % of dose	Apparent absorption % of dose
UHT milk	438	None	7[b]	None	86[a]
	406–509		4–25		
Pasteurized milk	542	263	20[c]	24[a]	55[b]
	542–542	263–263	13–51	3–43	
Fermented milk	426	336	16[bc]	4[b]	62[ab]
	241–431	156–442	5–29	0–32	

[a,b,c]Median values within columns with different superscript letters are significantly different ($P < 0.05$)
For details, see Witthöft et al., 2006.

Table 4 Intakes of dietary folate and FBP from four milk products[a]

	UHT milk	UHT milk + folic acid	Pasteurized milk	Pasteurized milk + folic acid
Folate (μg/500 mL)[b]	20 ± 2	201 ± 8	23 ± 2	233 ± 7
(nmol/500mL)	45 ± 5	455 ± 18	52 ± 5	528 ± 16
FBP(nmol/500 mL)[c]	<1	<1	80 ± 11	$70 + 8$
Additional dietary folate intake (nmol)	315 ± 55	$315 + 55$	$315 + 55$	315 ± 55

[a]Values are means $\pm$ SD

[b]Folate contents of all four milk products significantly different, $P < 0.01$.

[c]FBP content of both pasteurized milks significantly different from that of both UHT milks, $P < 0.001$; pasteurized milk + folic acid significantly different from pasteurized milk, $P = 0.05$.

Modified from De Jong et al. (2005).

versus folate intake on a molar basis was low, only amounting to 1:8–1:10 (De Jong et al., 2005).

The Stability of Bovine FBP during Gastrointestinal Transit

The fate and stability of free or folate-bound FBP from cow's milk during transit through the gastrointestinal tract of adults is dependent on the pH and the action of proteolytic enzymes. The pH-dependency of FBP affinity for different folate compounds has already been mentioned. This phenomenon must be considered regarding the mechanism of FBP on folate absorption, since the pH varies from acidic conditions in the stomach to neutral pH (6–7) in the ileum. At acidic pH, the FBP-folate complex is dissociated. Studies in rats fed either free folic acid or folic acid bound to bovine milk FBP showed that under acidic gastric conditions (pH < 4.5), folic acid was released from FBP and recombined in the small intestine (jejunum, pH 6–7) (Tani et al., 1983). This result was confirmed in a recent study of protein binding from a whey concentrate to folic acid and (6R, S)-5-CH_3–H_4folate using gel-exclusion chromatography of samples passing through the dynamic TIM system (Verwei et al., 2004). Before gastric passage, folic acid and (6R, S)-5-CH_3–H_4folate were bound mainly to whey concentrate FBP (76–79%), whereas 25% was free. Folic acid seemed to quickly revert to FBP in the duodenum. In contrast, the FBP-bound fraction of (6R, S)-5-CH_3–H_4 folate gradually decreased to 5% after gastric transit. Hence, although folic acid entered the proximal part of the intestine bound to FBP, 5-CH_3–H_4folate appeared to be present mainly as free folate in the duodenal lumen (Verwei et al., 2004).

The evidence that the capacity of FBP to bind folate is reversible and is recovered after passage through the acidic stomach raises another issue, namely the extent to which FBP is resistant to digestion by pepsin and intestinal proteases. Early studies reported that in vitro digestion with pepsinogen, and especially trypsin, inactivated the folate-binding capacity of FBP (Ford, 1974). Another study on 6-day old goat kids bottle-fed FBP-bound folic acid in goat's milk showed that gastric acidity and gastrointestinal digestive enzymes had little effects on the binding characteristics of FBP (Salter and Mowlem, 1983).

Studies on the stability of FBP after passage through the stomach and the entire small intestine using the dynamic TIM model showed that 34% and 17% of the FBP added to yogurt fortified with folic acid or (6S)-5-CH_3-H_4folate was found in the ileal delivery, that is, resisted digestion (Ford, 1974; Arkbåge <Year of publication for Arkbage not provided in reference list>; Verwei et al., 2003). The corresponding FBP stability values after gastrointestinal transit of pasteurized milk were 13% and 0–1% for milk fortified with folic acid and (6S)-5-CH_3-H_4folate, respectively.

In the human ileostomy model study, between 0–43% of the ingested FBP dose was excreted in the stomal effluent collected over 10 h post-dose. The median excretion of FBP for the nine ileostomy volunteers was 4% and 24% for fermented milk and pasteurized milk, respectively. The individual variation was pronounced (Witthöft et al., 2006).

Moreover, stabilization of milk folates may be a role of FBP and would improve the bioavailability of milk folate to newborns and other consumers. FBP was shown to stabilize two of the most labile folate derivatives normally occurring in food, that is, 5-CH_3–H_4folate and H_4folate (Jones and Nixon, 2002). The degradation rates of the very labile H_4folate and moderately labile 5-CH_3–H_4folate were measured with the compounds free or bound to either soluble or immobilized bovine milk FBP. FBP binding increased the stability from 2- to >1000 fold, depending on buffer and temperature conditions. H_4folate was stable for >100 days when bound to soluble FBP in 0.1 M phosphate buffer pH 6.7 at 4°C, but had a half-life of <1 h in the same buffer condition when it was free.

CONCLUDING REMARKS

Binding affinity alone is not always a suitable description of biological activity, and therefore studies of binding kinetics are important. The implications of differences in binding kinetics of different folate derivatives on folate metabolism are not known. To our knowledge, no other member of the FBP family except for bovine FBP has been studied in respect of the rates of the binding events, that is, association and dissociation rates. Knowledge of the binding kinetics for the human folate receptors might have great significance in understanding the role of folate metabolism

in health and disease, and also in evaluating metabolic effects of various folate fortification forms.

Since the end of 1970s, FBP has been used routinely for quantification of total folate in clinical samples. Due to the varying binding properties of bovine FBP towards other different folate forms, for example, H_4-folate, formylated folates, and stereoisomers of reduced folates, caution in interpretation of quantitative results from folate analysis of biological samples is strongly recommended. The same recommendation applies when using FBP affinity chromatography in purification/concentration of various folate forms in biological samples. However, it seems that the hitherto dominant role of FBP as an analytical tool for sample purification and folate analysis in biological samples is being supplanted by stable isotope dilution LC-MS/MS techniques combined with solid phase extraction (SPE) for sample cleaning/concentration. The latter method provides both total and all individual folate species (as monoglutamates) in the same run, whereas RPBA gives total folate, that is, the sum of various folate forms, especially folic acid and/or 5-CH_3–H_4folate, in clinical samples and foods.

More recent and specific methods based on ELISA and SPR-Bia for direct quantification of FBP give similar concentrations to the few previously reported results based on folic acid-binding capacity. Screening of active FBP in milk and dairy products shows raw milk and pasteurized milk to retain their native FBP content. Also, there is evidence that FBP occurs in excess compared with the folate content, whereby as much as half the total FBP might be unbound to folate, that is, free. Milk powder manufactured by freeze-drying or spray-drying also retains most of its FBP. Moreover, cottage cheese and whey products contain FBP, whereas UHT milk and yogurt lose their FBP by denaturation due to high processing temperatures. Hard cheese contains negligible amounts of FBP, probably due to separation of the whey proteins during manufacturing.

The suggested role of FBP as a folate-trapping protein in milk has been well documented in a number of mammal species over the years.

Stabilization of milk folates may be one role of FBP and would improve the bioavailability of milk folate to newborns and other consumers. FBP has also been shown to increase the stability of two of the most labile folate derivatives normally occurring in food, that is, 5-CH_3–H_4folate and H_4folate, by up to 1000-fold, depending on buffer and temperature conditions.

There is convincing evidence from studies on goat kids and rat pups that FBP-bound folate plays a physiological role during suckling by being absorbed per se, especially early in the lactation. This is most likely also an important mechanism for newborn infants. No species-specific receptors for a FBP present in the intestinal mucosa have yet been confirmed for mammals.

Although FBP without doubt plays an essential role for folate bioavailability in neonates, earlier suggestions that bovine FBP could have a general enhancing effect on the absorption and/or bioavailability of dietary folate in adult consumers have not been confirmed. Intake of cow's milk containing up to equimolar amounts of folate and FBP actually decreases folate uptake significantly in human gastrointestinal-simulating models such as adult ileostomists and TIM. The decreasing effect of FBP is dose-dependent and also dependent on folate form. Folic acid is more affected by FBP than 5-CH_3–H_4folate which is related to the various affinity characteristics of FBP towards different folate forms.

The infant situation is probably the most important one to consider in view of these recent results. For infants, a much higher survival of FBP could be expected due to a less mature gastrointestinal system, for example, the gastric pH is higher than for adults and the intestinal mucosa more permeable. Milk formula prepared from spray-dried milk powder could contain active FBP. As milk formula in general is fortified with folic acid, the effect of FBP on folic acid bioavailability could be substantial, especially during the lactation period when milk formula is the sole dietary source of folate. Gruels used during the infant period are another source that might provide FBP-folic acid complexes owing to frequent fortification with folic acid. Responsible producers of milk formula should consider either denaturing the FBP or replacing folic acid with (6S)-5-CH_3–H_4folate as fortificant. It is important to pay attention to the fact that the denaturation temperature of FBP depends on the folate derivative with which FBP is complexed.

The role of bovine FBP on folate absorption for adults should be negligible, since the daily intake of FBP originating from dairy products in a mixed diet is low, probably less than 10% of the total folate intake. Exceptions to this could be consumers with high intakes of cottage cheese and whey products, because these food items seem to be quite rich in active FBP.

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