# Folate Binding Protein in Bovine Milk

## Occurrence and Properties Studied with Surface Plasmon Resonance

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# Abstract

Surface plasmon resonance (SPR) based biosensors generate real-time binding data and is therefore well suited to analyse binding kinetics of two interacting molecules as well as for concentration measurements. Folate-binding protein (FBP) is present in, among others, milk and may affect intestinal absorption of folate. The objectives of this work were to investigate both the occurrence of bovine FBP in milk and FBP ligand binding properties using the SPR technique.

In order to understand and predict the impact of FBP on folate stability and absorption of native or added folates (fortification) an SPR assay that detects both the free (unliganded) and total FBP content in milk was developed. The assay showed good compliance with an existing ELISA method that measures the total FBP content. It was shown that approx. 50% of the total FBP content in milk is not bound to folate. The SPR assay was further used for screening of the occurrence of free and total FBP in milk from Swedish diary cattle. A large variation in FBP levels among cows was observed.

The binding kinetics between FBP and pure stereoisormeric forms of various folate derivatives were investigated. The results demonstrated that the interactions between FBP and the major forms of folate derivatives differ considerably. Each of the binding responses shown was well described by a 1:1 interaction model. The two folate forms that do not occur in nature, that is, folic acid and [6R]-5-$CH_3H_4$folate, had the highest affinity for FBP, 20 and 160 pmol/L, respectively.

The influence of different folate derivatives on the denaturation temperature of FBP and effect of pasteurization and UHT treatment on FBP binding to folate were studied. The binding of folate increased the denaturation temperature of FBP. The ability of FPB to bind folate was eliminated by UHT treatment but not by pasteurization, regardless of folate form bound.

*Keywords:* folate, folate-binding protein, folate receptor, SPR, bovine milk, kinetic, affinity, aggregation

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# Contents

# Appendix

## Paper I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals I-V.

I. Nygren, L., Sternesjö, Å., and Björck, L. 2003. Determination of folate-binding proteins from milk by optical biosensor analysis. *International Dairy Journal*, 13, (4), 283-290.

II. Nygren-Babol, L., Sternesjö, Å., and Björck, L. 2004. Factors influencing levels of folate-binding protein in bovine milk. *International Dairy Journal*, 14, (9), 761-765.

III. Nygren-Babol, L., Sternesjö, Å., Jägerstad, M., and Björck, L. 2005. Affinity and rate constants for interactions of bovine folate-binding protein and folate derivatives determined by optical biosensor technology. Effect of stereoselectivity. *J Agric Food Chem*, 53, (13), 5473-8.

IV. Nygren-Babol, L. 2007. "Letter to the editor". *Accepted for publication in Bioscience Reports*

V. Nygren-Babol, L., and Landström Karonen, K. The effect of different folate forms on denaturation of bovine folate-binding protein. *Manuscript.*

# Background

## Folate

*Nomenclature and chemical structure*

Folate is the generic term for all B-vitamins that exhibit vitamin activity similar to that of folic acid i.e. pteroyl-L-glutamic acid (PteGlu). The various compounds of this class are designated as "folates". Folic acid is the most oxidised and most stable form of folate, and consists of an aromatic pteridine ring attached to *para*-aminobenzoic acid, which in turn, is linked to one L-glutamic acid residue (**Figure 1**). Folic acid occurs rarely in nature, but it is the synthetic form used for vitamin supplementation and food fortification because of its stability and low cost.

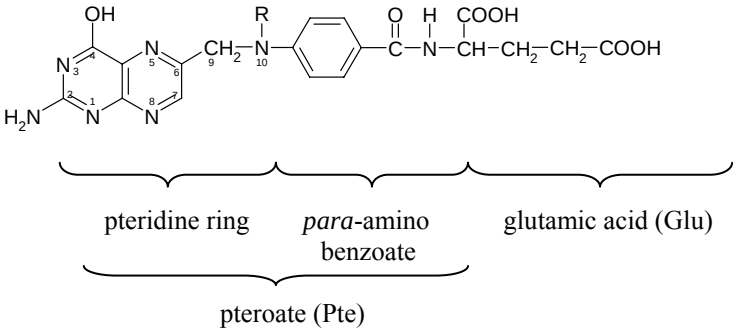



*Figure 1.* Chemical structure of PteGlu.

Folate in body tissues and foods are mainly 5,6,7,8-tetrahydro-pteroylpolyglutamates, which contain a fully-reduced pteridine ring together with additional glutamic acid molecules linked by γ-peptide bounds. Reduced folate can exist with or without one-carbon substituent at the $N^5$, e.g. 5-methyl, 5-formyl, 5-formimino or $N^{10}$, e.g. 10-formyl, positions, or have a single C bridge spanning these positions, e.g. 5,10-methylene, 5,10-methenyl (**Figure 2c**). The pteridine ring can also be partially reduced and exists with or without a one-carbon substituent at the $N^5$ or $N^{10}$ as shown in **Figure 2a** and **2b**. Thus, there are many different derivatives of folic acid exhibiting the common vitamin activity as folate. The vitamin activity of these folates is expressed as long as the essential subunit structure of folic acid remains largely intact. In fact, the number of derivatives is further increased, as all fully reduced folate has two chiral centres: the α-C atom in the glutamic acid moiety and the C-atom in position 6 of the pteroyl moiety (**Figure 2c**). Consequently four possible diastereoisomers exist: [6S, αS], [6S, αR], [6R, αS] and [6R, αR]. The naturally occurring diastereoisomer of tetrahydrofolate ($H_4$folate), 5-methyltetrahydrofolate (5-$CH_3$-$H_4$folate) and 5-

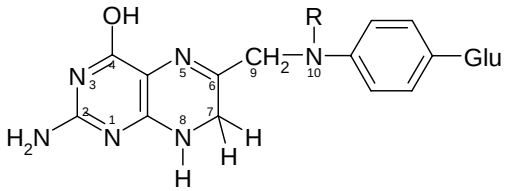
**a &b) Partially oxidized pteridine ring**

| **a)** Substituent (R) | Folate derivative |
|---|---|
| -H | Dihydrofolate ($H_2$folate) |
| -HCO | 10-formyldihydrofolate (10-HCO-$H_2$folate) |
| **b)** Substituent (R) | |
| $-CH_3$ | 5-methyldihydrofolate (5-$CH_3$-$H_2$folate) |
| -HCO | 5-formyldihydrofolate (5-HCO-$H_2$folate) |

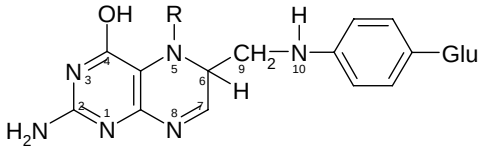
**c) Fully reduced pteridine ring**

| Substituent (R1) | Substituent (R2) | Folate derivative |
|---|---|---|
| -H | -H | Tetrahydrofolate ($H_4$folate) |
| $-CH_3$ | -H | 5-methyltetrahydrofolate (5-$CH_3$-$H_4$folate) |
| -CH=NH | -H | 5-formiminotetrahydrofolate (10-NHCH-$H_4$folate) |
| -HCO | -H | 5-formyltetrahydrofolate (5-HCO-$H_4$folate) |
| -H | -HCO | 10-formyltetrahydrofolate (10-HCO-$H_4$folate) |
| Bridge R1-R2 | | |
| $-CH_2-$ | | 5,10-methylenetetrahydrofolate (5,10-$CH_2$-$H_4$folate) |
| $-CH^+=$ | | 5,10-methenyltetrahydrofolate (5,10-$CH^+$=$H_4$folate) |

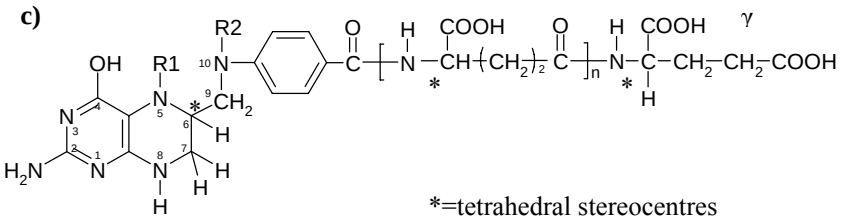
**a)**




**b)**




**c)**




*=tetrahedral stereocentres

*Figure 2 a-c.* Structures and nomenclature of some folate derivatives with partially oxidised and fully reduced pteridine ring. The number of glutamate units (n) linked by a γ-peptide bound varies from 0 to 7.

formyltetrahydrofolate (5-HCO-$H_4$folate) are the [6S, αS]-diastereoisomers, whereas the natural diastereoisomers of 10-formyltetrahydrofolate (10-HCO-$H_4$folate), 5,10-methylenetetrahydrofolate (5,10-$CH_2$-$H_4$folate) and 5,10-methenyltetrahydrofolate (5,10-$CH^+$=$H_4$folate) are the [6R, αS]-diastereoisomers. It should be emphasized that [6R] configurations of the natural 10-substituted and 5,10-bridged compounds are not inverse compared to tetrahydofolate, but according to Cahn-Ingold-Prelog (CIP) nomenclature rules the configuration is [6R]. Therefore, to avoid confusion about the stereochemistry, it has been agreed that all natural diastereoisomers of reduced folate should be defined as L-diastereoisomers and all unnatural ones as D-diastereoisomers (Groehn & Moser, 1999).

### *Biological function*

Folate is essential for a wide range of biochemical pathways involving one-carbon transfer. In particular, folate plays an essential role in cell replication because they are required for synthesis of purines and pyrimindines, the nucleotides base pairs of DNA and RNA. The remethylation of homocysteine to methionine closely involves the metabolism of folate and other B-vitamins. Growth and pregnancies show increased demands for folate, deficiency can lead to impaired cell replication and accumulation of harmful metabolites, such as homocysteine. Impaired cell replication is first observed in fast replicating tissues such as the erythrocytes, causing megaloblastic anaemia (Wickramasinghe, 2006). An elevated plasma homocysteine level caused by a marginal folate intake is a risk factor for vascular disease (Boushey et al, 1995). Furthermore, a marketed protection against neural-tube defects in foetus has been shown in women supplemented with folic acid before conception (MRC Vitamin Study Research Group, 1991; Czeizel & Dudas, 1992).

### *Bioavailability and absorption*

Folate bioavailability refers to the portion of ingested folate that is absorbed and becomes available for metabolic processes or storage. The folate bioavailability is dependent on a number of factors, including intestinal deconjugation of polyglutamyl folate, the food matrix, the instability of certain labile folate during digestion (or before ingestion), and the presence of certain dietary constituents that may enhance folate stability during digestion e.g. folate-binding protein and ascorbate (reviewed by McNulty & Pentieva, 2004).

The absorption of folates occurs mainly in the proximal small intestine (jejunum). Before absorption across the mucosa can take place, dietary folates, which exist predominantly as polyglutamates, need to be hydrolysed to folate monoglutamate by the brush-border γ-glutamyl hydrolase, also called folylpolyglutamate carboxypeptidase or folate conjugase (Halsted, 1980). Many foods contain inhibitors of the intestinal brush-border folate conjugase enzyme and/or folate transport system (Tamura & Stokstad, 1973; Babu & Srikantia, 1976; Tamura et al., 1976). After cleavage to folate monoglutamates, the folate is absorbed by a carrier-mediated process at physiological concentrations, and by passive diffusion at higher concentrations (Selhub et al., 1984). Absorbed folate monglutamtes are

converted to 5-$CH_3$-$H_4$folate during the transit through the intestinal mucosa before being released into the portal vein. Synthetic folic acid is reduced and methylated partly in the intestinal mucosa and partly in the liver (reviewed by Steinberg, 1984). As a consequence of chemical instability of the naturally occurring folate and food mediated inhibition of the enzymes and proteins involved in absorption the bioavailability of folate varies considerably (Gregory, 1997).

### *Intake and dietary sources*

In Sweden, the recommended daily intake (RDI) of folate is 300µg for adults, 400 µg for fertile women, and 500µg for pregnant and lactating women (Becker et al., 2004). Most people in Sweden except for vegetarians and vegans, do not reach the RDI of folate (Livsmedelsverket, 2002). An inadequate folate intake occurs not only in Sweden but worldwide. Therefore, several countries have introduced folic acid fortification, e.g. USA (1998), Canada (1998), Chile (2000). Sweden and many other EU countries are currently discussing whether a mandatory folic acid fortification should be introduced but have not taken any decision so far except for Ireland.

The main dietary sources of folate are foods of plant origin, i.e. vegetables, fruits, berries, fruit juices, cereals and potatoes, contributing 60% of the total intake. Meat, fish and egg products contribute 16% and dairy products contribute an additional 16% of the total intake of folate (Livsmedelsverket, 2002).

## Folate binding protein

### *The family of folate binding proteins*

Absorption and cellular uptake of folate is mediated by two different classes of membrane proteins. One class consists of the reduced folate carrier (RFCs), which are transmembrane proteins that bind reduced folate with a micromolar affinity. The other class is the family of folate-binding protein (FBP or Folbp), also known as the folate receptor (FR). Human FR is encoded by a family of genes whose homologous products are termed FR –α, –β and –γ (Lacey et al., 1989; Sadasivan & Rothenberg, 1989; Elwood, 1989; Ratnam et al., 1989; Shen et al., 1994; Shen et al., 1995). FR–α and –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. However, other folate uptake pathways are generally used by adult tissues (Matherly & Goldman, 2003) and the functions of these membrane-bound FRs are not clear in most tissues. The most commonly expressed receptor isoform, i.e. FR-α, in normal tissues 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 or urine-facing surface of the tubule cells (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).

The origin of soluble folate-binding protein (sFBP) is either from FR–γ, because it is constantly secreted, or from the membrane bound forms. sFBP from FR-α in human malignant tissue culture (KB) and placental cells has been demonstrated to result from both phosholipase cleavage of the GPI anchor and from proteolysis by an $Mg^{2+}$-dependent protease (Luhrs & Slomiany, 1989; Antony et al., 1989; Elwood, Deutsch & Kolhouse et al., 1991; Yang et al., 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 bovine folate binding protein in milk

In 1967 Ghitis reported the presence in bovine milk of a heat labile macromolecular factor that protected milk folate against adsorption to activated charcoal. This observation led to the conclusion that bovine milk contains high affinity folate binding protein (FBP) (Ghitis, 1967). Since then the protein has been extensively studied and well characterized. FBP isolated from bovine milk contains 222 amino acids. The polypeptide contains 8 disulphide bridges and the molecular weight of the protein part is 25,700 Da (Svendsen et.al., 1984). The protein is highly glycosylated with 2 potential sites for N-linked glycosylation. The final molecular weight is about 30,000-35,000 Da (Salter et.al., 1981; Svendsen et.al., 1984). Recently, a 19 amino acid long signal sequence of bovine milk FBP was 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/mol of protein at pH 7.2 (Salter et al, 1981). The folate-binding capacity of FBP varies with pH. 5-methyltetrahydrofolate is totally dissociated at pH below 5 and folic acid at pH slightly above 3 (Salter et al, 1981).

FBP have been isolated by different affinity chromatographic methods (Salter et al., 1972; Treloar, Grieve & Nixon, 2000) sometimes in combination with ion-exchange chromatography (Svenden et al., 1979). The occurrence of FBP in bovine milk is in the range of 5-8mg/L (170-250 nmol/L) (Salter et al., 1972; Wigertz, Svensson & Jägerstad, 1997). There are conflicting results in the literature on how pasteurization affects the FBP content in milk. Wigertz et al (1996) found 20% reduction as measured by ELISA, Ford (1974) found 10% reduction of the folate-binding capacity and Kohashi et al (1985) observed an almost complete reduction of the folate-binding capacity after pasteurization. Further, Wigertz et al (1996) found more than 95% reduction of the FBP content after UHT treatment. Conflicting results from these studies could be caused by the fact that common pasteurization temperatures are very close to those at which denaturation of FBP takes place. Gregory (1982), using gel filtration analysis, found an increased molecular mass after heat treatment and suggested an heat-

induced alternation of FBP as an explanation for the changed structural and functional characteristics of FBP in processed milk products (Gregory, 1982).
The proposed function of FBP in milk is to sequester folate from blood to secure an adequate supply to the neonate (reviewed by Parodi, 1997). Studies have also shown that FBP enhances folate stability under various conditions (Jones & Nixon, 2002). Earlier, FBP was regarded as a stimulant of folate absorption in human (Colman et al., 1981; Salter et al., 1988) but more recent studies have indicated that FBP forms complex with folate, especially with folic acid, resulting in lower folate bioavailability (Arkbåge et al, 2003; Verwei et al, 2004; Witthöft et al, 2006).

### *Sequence homology*

**Figure 3** compares the amino acid sequence of bovine milk FBP (Smith et al., 2005), human FR-α (Elwood, 1989), and chicken riboflavin-binding protein (RfBP) (Zheng et al., 1988a). Amino acids indicated with a star are identical in all sequences in the alignment. The sequence alignment shows that all tryptophan residues in RfBP are conserved in FBP and all 16 cystine residues present in FBP are conserved in RfBP. In the region from residue 36 to 211, where the sequences clearly correspond, the sequence identity is 30%. This indicates a structural and functional similarity of RfBP and FBP (Zhen et al., 1988b; Monaco, 1997). The sequence identity between FBP and human FR-α is 77%. The crystal structure of RfBP has been determined (Monaco, 1997) and shows that all the conserved cystine residues are present in the ligand-binding domain. The structure also shows that five out of six tryptophans present in the protein molecule are clustered in the neighbourhood of the ligand-binding site. The plane formed by the tryptophans is in direct contact with the plane of the ligand. Folate and flavins are functionally distinct but they do share structural resemblance because both are hydrophobic aromatic ring systems. It is therefore plausible that an ancestral protein had the capacity to bind both the vitamins and that a few amino acids replacements could modify the binding specificities (Zhen et al., 1988b).

### *Affinity and aggregation*

Attempts to describe the affinity of the interaction between folate and FBP have been inconclusive since previously reported equilibrium dissociation constants ($K_D$) at neutral pH have exhibited a dependence on the concentration of FBP used for their determination (Hansen, Holm & Lyngbye et al., 1978; Salter et al., 1981; Hansen et al., 1983). In 2004, Nixon, Jones & Winzor interpreted their own and published data and estimated $K_D$ theoretically for the interaction between folic acid and FBP to 20 pM (or even lower). However, no one has confirmed it experimentally so far. The strength and binding kinetics of receptor-folate interactions are essential to elucidate for understanding of folate bioavailability and folate metabolism. Further, this knowledge is important because some methods used for folate analysis, e.g. radio protein binding assay (RPBA), and affinity columns used for folate extraction or enrichment are based on FBP-folate interactions. Variations in both response and recovery have been observed

(Strålsjö et al, 2002; Konings, 1999) and those variations are most likely caused by differences in binding kinetics between the different folate derivatives.

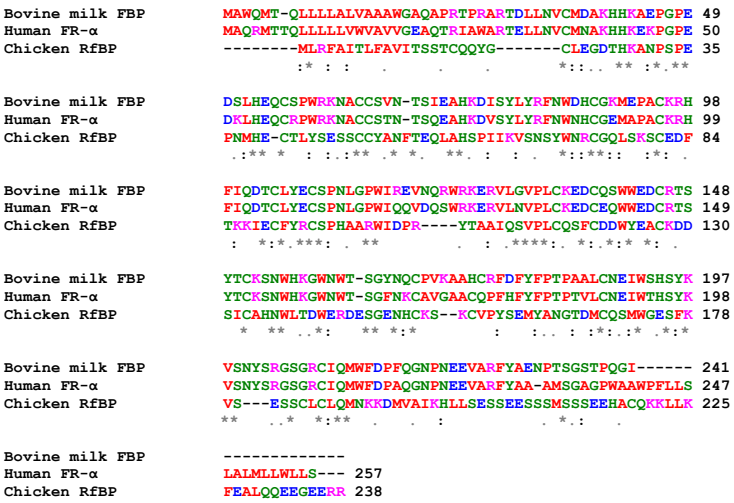



*Figure 2.* Alignment of amino acid sequences. From top; bovine milk FBP (Smith et al., 2005), human FR-α (Elwood, 1989), and chicken RfBP (Zheng et al., 1988a). Positions that have identical amino acids are marked with a star; two dots means that conserved substitution have been observed; one dot means that semi-conserved substitutions are observed. Sequence alignment was preformed in clustralw 1.83 (http://www.ebi.ac.uk/clustalw/).

Aggregation of proteins into insoluble complexes is a common phenomenon in biology. Protein aggregation is widely viewed as a non-specific coagulation of incompletely folded or partially denatured polypeptides, driven by the interaction among inappropriately exposed hydrophobic surfaces. However, refolding studies of chemically denatured polypeptides suggest that protein aggregation *in vitro* is due to specific intermolecular interactions among defined domains within structured folding intermediates (Wetzel, 1996). Evidence of this specificity is found in seeding behaviour of amyloidogenic proteins (Jarrett & Lansbury, 1992) and in the selectivity of aggregate formation by model proteins (Speed, Wang & King, 1996). Even though the exact nature of the FBP aggregates is not known, the aggregation shows a concentration-dependent aggregation enhanced upon addition of folate (Pedersen et al., 1980; Hansen et al., 1983). It has further been observed that liganded (holo) FBP forms hydrophilic water-soluble polymers, whereas unliganded (apo) FBP aggregates have a hydrophobic character (Holm, Hansen & Hoier-Madsen, 2001). Hansen and Holm have suggested that ligand binding is associated with a conformational change that increases the affinity to the ligand and increases FBP aggregation (Holm, Hansen & Hoier-Madsen, 2001; Holm &_Hansen, 2001; Christensen, Holm & Hansen, 2006). This hypothesis is strongly questioned in this thesis (**Paper IV**).

*Measurements*

Traditionally the only direct technique used for determination of FBP has been enzyme-linked immunosorbent assay (ELISA) (Hoier-Madsen, Hansen & Holm, 1986). Before the development of the ELISA, FBP was quantified indirectly by measuring the folate content after absorption to charcoal of excessive unbound folate. The ELISA is based on the recognition of FBP by a polyclonal antibody raised against bovine milk FBP. The method does not distinguish between whether FBP are complexed with folate or not. In recent years there has been an increased demand for an assay that can distinguish the free apo-form from the complexed holo-form of the protein. The surface plasmon resonance (SPR) assay described in this thesis (**Paper I**) fulfils those demands. In 2004 another SPR method was developed by Indyk & Filonzi (2004) this method has an easier analytical scheme and measures only the apo-form.

| Manufacturer | Platforms | Number of publications in 2005 |
|---|---|---|
| Biacore AB | Biacore, Bialite, J, Q, X, 1000, 2000. 3000, Flexchip, S51 | 882 |
| Affinity Sensor | IAsys, IAsys+, IAsys Auto | 40 |
| Texsas Instrument | Spreeta | 20 |
| EcoChemie | Autilab Espirit | 10 |
| Optrel | Multiskop | 10 |
| GWC Technologies | SPRimager II | 8 |
| Nippon Laser & Electronics | SPR-670 | 8 |
| Windsor Scientific | IBIS, IBIS-II | 8 |
| Resonant Probes | SPRTM | 6 |
| Corning | Epic | 4 |
| Toyobo | MultiSPRinter | 4 |
| Analytical μ-system | BIOSUPLAR, BIOSUPLAR-2 | 3 |
| Reichert Analytical Instruments | SR7000 | 3 |
| DKK Corp. | SPR-20 | 2 |
| Genoptics | SPRi-Array | 2 |
| Artificial Sensing | OWLS | 1 |
| Johnson & Johnson Clinical Diagnostics | Unnamed | 1 |
| Vir Biosensor | VirChip | 1 |

*Table 1*. Commercial optical biosensor technologies

## Biosensors

A biosensor is a device that uses label-free techniques to detect biologically active molecules. Typically, the detector molecule must be connected to a sensor that can provide real-time monitoring. Biosensors based on biological materials are now used in a wide variety of disciplines, including medicine, food industry and environmental science. After the development more than a decade ago (Jönsson et al., 1991) of the first biosensor based on the surface plasmon resonance (SPR), the use of this technique has increased steadily. Although there are several SPR-based systems, the most widely used one is the BIAcore (GE Healthcare, Uppsala, Sweden). All commercially available SPR systems found on the market in 2005 (reviewed by Rich & Myszka, 2006) are shown in **Table 1,** including the numbers of publications for each system. As seen in **Table 1**, Biacore totally dominates the use with more than 800 published articles in 2005.

## Analysis with Biacore equipment

The main components of the Biacore system are the interchangeable sensor chip, the microfluidic sample handling system and the SPR detector. Aqueous samples are injected over the sensor chip surface immobilised with one of the interacting partners. Binding of the interactant is detected and quantified in real-time by the surface sensitive detector. The interactant free in solution is referred as the analyte.

### *Optical system*

The underlying physical principles of SPR are complex and a detailed theoretical understanding of the event is not necessary for an adequate working knowledge of the technique. In brief, SPR-based instruments use an optical method to measure the refractive index near (within approx. 300 nm) a sensor surface. In order to detect an interaction, one molecule is immobilised onto the sensor surface. The binding partner (the analyte) is injected in aqueous solution (sample buffer) through the flow cell under continuous flow. When the analyte binds to the immobilised molecule on the sensor surface, the mass at the surface increase, and thereby the refractive index (**Figure 4**). This change in refractive index is measured in real time, and plotted as response or resonance units (RU) versus time (a sensorgram). One RU represents the binding of approximately 1 pg protein/$mm^2$.

The sensorgram provides essentially two kinds of information: (1) the rate of interaction (association, dissociation or both), which provides information on kinetic rate constants and analyte concentration; and (2) the binding level which can provide information on affinity constants and analyte concentration.

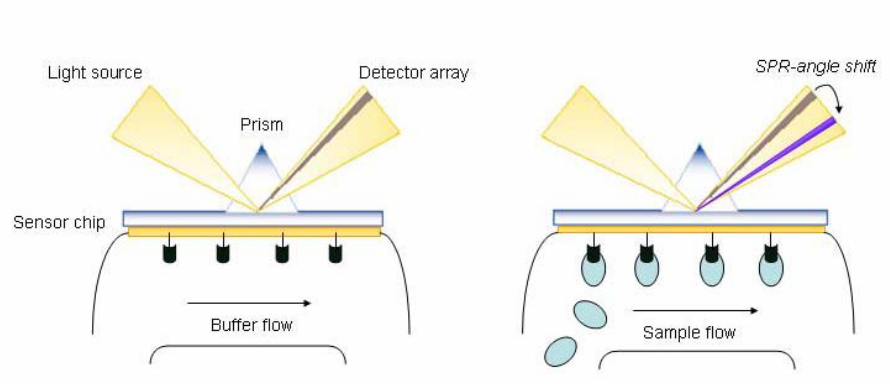



*Figure 4*. Surface plasmon resonance (SPR) biosensor principle. The binding of biomolecules to the sensor surface increase the refractive index, which induces shift of the SPR angle. The shift is directly proportional to the mass increase.

### *Sensor surface*

The sensor chip consists of a gold-coated glass slide embedded in a plastic support platform. The gold film is covered with a coupling matrix e.g. carboxymethylated dextran via a linker layer. Different types of sensor surfaces are available, but the most frequently used surface is carboxymethylated dextran, CM5 (Baird & Myszka, 2001). The coupling matrix is important for the characteristics of the surface. By using a coupling matrix the immobilisation capacity of molecules are enhanced. Furthermore, the ligand will be easier accessed by the interacting molecule, and the hydrophilic structure of the matrix minimises non-specific adsorption of proteins (Johnson, Löfås & Lindquist, 1991; Jönsson et al., 1991).

### *Flow system*

When a sensor chip is docked in the instrument, the integrated micro-fluidic cartridge (IFC) is pressed against the chip surface. Four flow cells are thereby formed; the volume of the flow cells varies between instrument models. The opposite side of the sensor chip, i.e. the glass side, is pressed against a glass prism in the optical unit. The IFC, together with an autosampler, controls the continuous flow of buffer or sample over the sensor surface via a number of loops. For Biacore 1000 and Biacore Q only one flow cell can be monitored at the time. In newer instrument used for kinetic interaction analysis (i.e. from Biacore 2000) a sample can pass over four flow cells in sequence and the response for all the flow cells can be monitored in parallel.

## Concentration analysis

The Biacore instrument can be used for concentration determination of both large and small molecules. Since the assays are based on specific binding between two interaction partners, the values obtained reflect the concentration of analyte which

is active with respect to this binding. There are different approaches to measuring concentration. One is to measure the amount of analyte bound at a fixed time after sample injection has finished. It is also possible to measure the rate of analyte binding at the beginning of sample injection. If the association is limited by mass transport, the binding rate is directly proportional to the analyte concentration and independent of association kinetics. Low molecular weight analytes, which do not give a measurable direct response, can be assayed by using competitive techniques i.e. when a competitive component is measured directly instead of the small analyte. Competitive methods in solution can also be used for very large molecules and particles such as viruses (BIAtechnology Handbook, 1994).

## Kinetic analysis

The advantage of measuring kinetics compared to affinity is that kinetics determines whether a particular complex forms or dissociates within a given time span. Affinity determines how much of the complex is formed at equilibrium, and the same affinity value can result from different combinations of association and dissociation rates.

The fact that the Biacore® generates real-time binding data makes it well suited for analysis of binding kinetics. The monitored data can be analysed to give values for association and dissociation rate constants, and equilibrium constants. **Figure 5** shows schematically the interactions that occur at the sensor surface. The analyte (A) and the ligand (B) interact to form a complex (AB) and a response is generated. Typical ranges for rate constants measurable with Biacore® technology are: association constant ($k_a$), $10^3$-$10^7$ $M^{-1}s^{-1}$; and dissociation constant ($k_d$), $10^{-6}$-$10^{-1}$ $s^{-1}$ (Kinetic and affinity analysis with Biacore, 2003).

For collection of detailed kinetic data it is important to have a good experimental design, good data collection and data processing to avoid artifacts (Myszka, 1997; Morton & Myszka, 1998; Myszka, 1999; Myszka, 2000).

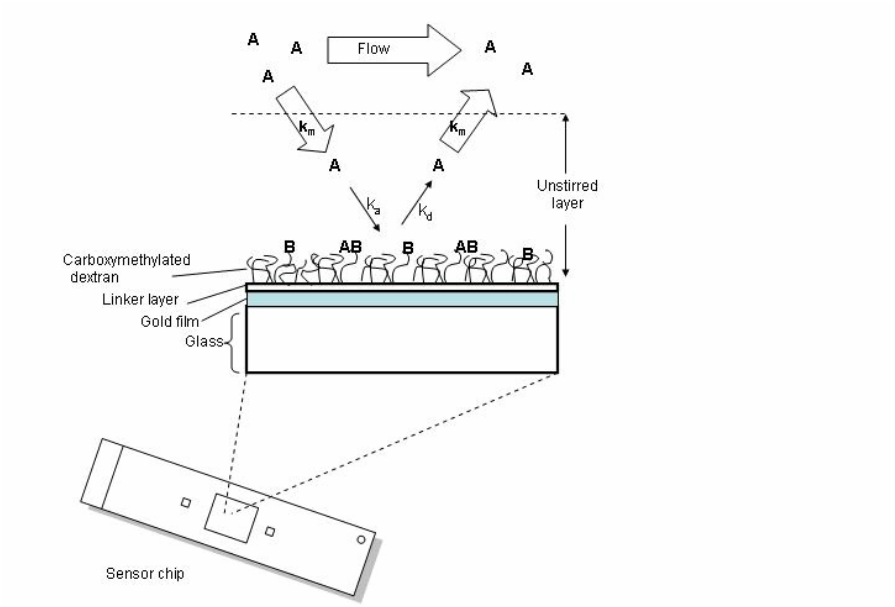



*Figure 5*. Overview of the basic interactions at the biosensor surface. The ligand (B) is attached to the surface via the flexible carboxymethylated dextran. During the association phase, analyte (A) is flowed past the surface. $k_m$ is the mass transport coefficient used to describe the diffusion of analyte through the unstirred solvent layer. $k_a$ and $k_d$ are the rate constants, which describe the formation of the complex (AB).

# Objectives

The limited data up till now published in the literature on concentrations of FBP in raw and heat-processed cow milk are based on an enzyme-linked immunosorbent assay (ELISA) measures total FBP but does not discriminate between free FBP (apo) and folate bound (holo) FBP. In order to understand and predict the impact of FBP on folate stability and absorption of native or added folates (fortification), free and folate-bound FBP in milk should be determined. Moreover there is a lack of data on the interactions between different forms of folate and FBP in foods. This information is needed for the understanding of folate absorption and could also be useful in relation to other folate-binding proteins present in various mammal tissues. Furthermore, FBP is an important constituent in protein-binding methods used to quantify folate in clinical samples and foods. Since the principles behind these methods rely on the interaction between folate and FBP more data on binding kinetics are desirable to fill the gap of limited and inconsistent knowledge in this field. Almost all milk products on the market are pasteurized or UHT-treated, but the effects of heat treatment on FBP concentration and FBP binding capacity are not clearly understood. An interesting question is whether the folate form affects the heat stability of FBP and whether it is possible to more exactly establish FBP's heat denaturation temperature using differential scanning colorimetry (DSC). The SPR technique has a great potential to study protein-vitamin interactions more specific than up till now used techniques. In the present thesis, an SPR assay is developed and validated for quantification of FBP in bovine milk. Moreover, the SPR-based assay is investigated as a suitable method for studying binding kinetics in model systems at ambient temperature and binding capacities of FBP after heat treatment of bovine milk.

The specific objectives in the present work were:

- To develop and validate an SPR-based assay that could differentiate between liganded (holo) and unliganded (apo) form of FBP in bovine milk (Paper I).

- To screen for the amount of total FBP and apo-FBP in individual cow milk samples in relation to breed, lactation stage and lactation number. To investigate the relation between levels of FBP and total protein, fat content and cell count using SPR-based assay (Paper II).

- To investigate the binding kinetics of FBP and different folate derivates, and in addition, the effect of stereoselectivity on the FBP binding properties using SPR-based assay (Paper III).

- To investigate how different folate derivatives influence the denaturation temperature of FBP, and how the heat treatment, i.e. pasteurization and UHT treatment of FBP, affects FBP binding to folate using DSC and SPR-based assay, respectively. (Paper V).

# Methods

Methods used in the present thesis are summarised below, for details see Paper I-III and V.

## FBP Purification

FBP used as standard and for Differential Scanning Calorimetry (DSC) was isolated from whey protein concentrate by affinity chromatography using EAH-Sepharose 4B (GE Healthcare, Uppsala, Sweden). Folic acid was coupled via the free carboxyl group to the matrix. Then 50 g whey protein concentrate was dissolved in 5 L deionised water and the pH was lowered to 3 by addition of 5M HCl under agitation to dissociate bound folate from FBP. The sepharose gel was added to the whey protein concentrate solution and pH was increased to 7 by a slow addition of 5M NaOH. After 3 h agitation the gel was washed on a glass filter with deionized water until a clear eluate was obtained. The gel was then packed into a column and connected to Äkta® FPLC system. The gel was further washed with 0.1 M phosphate buffer, pH 7.2, containing 0.5 M NaCl, until the UV absorbance ($A_{280nm}$) of the eluate was negligible. The bound FBP was dissociated and eluated by 0.1 M acetate buffer, pH 3.5. The FBP containing fraction was collected, dialysed against milliQwater (Millipore, Bedford, MA, USA), freeze-dried and stored at -70°C until further use. To determine the concentration of FBP, the $A_{280nm}$ of a 1mg/mL solution of FBP was used as published by Svendsen et.al. (1979). Purity of the isolated FBP was checked by SDS-PAGE using silver staining technique. Western blotting was further used to characterise the multiple bands that FBP gives due to different levels of glycosylation.

## Biacore assays

Direct binding of FBP to either folate or FBP immobilized on the sensor surface was used for all assays.

### *Immobilisation*

The pteridine ring on folate molecules exhibiting vitamin activity contains an amine group. This ring system is essential for the binding to FBP; therefore direct amine coupling to the activated dextran was not possible. Instead, folic acid and the other derivatives used were converted to their hydroxysuccinimidyl derivatives and coupled to amino groups on the sensor surface. FBP was immobilized directly to the sensor surface through amine coupling.

### *Concentration analysis*

To facilitate measurements of the total FBP content in milk, endogenous folate was removed by a combination of low pH to dissociate the FBP-folate complex followed by a membrane molecular weight cut-off filtration (10kDa) (Millipore, Bedford, MA, USA). To make it possible to perform a cut-off filtration of milk,

whey was prepared; the caseins in the milk would surely clog the filter when lowering pH. Whey, both for total and free FBP measurements, was diluted to a final dilution of 1:10 before injection. FBP used for calibration was diluted in running buffer (HBS-EP) pH 7.4 (10 mmol/L 4-[2-hydroxyethyl] piperazine-1-ethane-sulfonic acid (HEPES), 3.0 mmol/L EDTA, 150 mmol/L NaCl, 0.005% (v/v) surfactant P-20) because of the presence of endogenous folate in milk. The range of the standard curve was set to a level covered the range of the diluted (1:10) normal milk samples. In order to minimize unspecific binding to the sensor surface, 0.1 M phosphate buffer, pH 7.4, containing 0.5 M NaCl was injected before change in response due to bound FBP was determined. Regeneration of the surface was performed by injection of 10 mM glycine buffer, pH 2.0. Calibration was performed both before and after measurements of a series of unknown samples to check the efficiency of the sensor chip regeneration between measurements. As a reference method an ELISA was used (Hoier-Madsen, Hansen & Holm, 1986).

### *Kinetic measurements*

Concentration series of FBP were injected for collection of detailed kinetic data over different folate surfaces. The concentration of the concentration series and the length of time needed for dissociation was monitored and depended on the folate derivative. All binding experiments were carried out with HBS-EP running buffer, pH 7.4, at 25°C, and each concentration was injected in triplicate using random order. Data were processed and analysed by using double referencing (Myszka,1999). To obtain kinetic rate constants, adjusted response data were fitted with nonlinear least-squares regression analysis. Constants reported represent the average of three independent analyses of each FBP-folate interaction.

### *FBP-FBP binding*

To investigate the influence of ligand on FBP aggregation apo-FBP was manually injected over an immobilized FBP surface that was either saturated with folic acid or not. The experiments were carried out with HBS-EP running buffer (pH 7.4) at 25°C.

## Differential Scanning Calorimetry

The thermal properties of FBP-ligand interaction were studied by DSC. The measurements were carried out on a DSC 6200 from Seiko Instruments Inc. (Shizuoka, Japan) over the temperature range of 6–140°C with a scanning rate of 10°C $min^{-1}$. Folate-free freeze-dried FBP was diluted in 0.1 M phosphate buffer, pH 6.7, with and without the investigated folate derivative, to a final concentration of 0 or 1.5mg/mL folate and 10% (w/v) FBP. Transition enthalpy ($\Delta H$ expressed as J/g dry matter) and denaturation temperature ($T_d$) were determined by EXSTAR6000 Thermal Analysis System on a Hewlett Packard computer.

# Results and discussion

## I. Determination of folate-binding proteins from milk by optical biosensor analysis (Paper I)

This paper describes an SPR-based method for detection of FBP in cow milk. The detection is based on the specific interaction between folic acid and FBP. The method measures both the total FBP content and the unliganded (apo) form of the protein. Consequently, the method was able to indirectly differentiate holo-FBP from apo-FBP which was not possible before with such high precision. The assay showed good compliance with an existing ELISA method. The repeatability (CV) of the biosensor assay was high, i.e. at an FBP concentration of 0.38µg/mL it was 1.2-3.6% within-assay on 3 different days and 6.4% between days. Since the biosensor method is based on the specific interaction between FBP and PGA, endogenous folate must be removed before analysis of the total FBP content. This was achieved by a combination of low pH and molecular weight cut-off filtration. Further, it was demonstrated that the pteridine moiety of folate was essential for the interaction. In 2004 Indyk and Filonzi described another SPR-based assay for detection of FBP in cow milk. The analytical scheme of the described methods differed in several aspects. Most importantly, in Indyk and Filonzi's method no sample pre-treatment stage was included since that method measures apo-FBP directly without removal of fat or caseins. The absence of pre-treatment is beneficial because this provides operational simplicity and avoidance of FBP losses. A weakness of such approach is that it is not possible to detect the total FBP concentration because of FBP binding to endogen folate in milk. The method published by Indyk and Filonzi showed approx. 50% higher levels of apo-FBP compared to those obtained by our method. However, apart from a divergent analytical scheme, different FBP standards were used because of absence of an accepted FBP reference material. Furthermore, different milk was used and as shown in **Paper II**, the FBP content in milk varies considerably among cows. The differences in FBP levels between those two methods might also be due to sample storage, which may affect the aggregation of protein as discussed in the methodological consideration below. Additionally, consumer milk was tested by Indyk and Filonzi (2004) and, as established in **Paper V,** heat-treatment, e.g. pasteurization, affects FBP through an increase in aggregation.

## II. Factors influencing levels of folate-binding protein in bovine milk (Paper II)

In this work, the developed SPR assay (**Paper I**) was used for screening of the occurrence of FBP in milk. FBP levels of total and apo-form were determined in individual milk samples from 41 Swedish Red and White dairy cattle and from 25 Swedish Friesian dairy cattle. To evaluate the influence of lactation stage on FBP levels, milk samples were also taken from 5 cows at 1, 3, 10, 30, 60 and 90 days *post partum*. The samples were stored at -80ºC and analysed within a few weeks. The results showed that there was a considerably variation between cows in levels of both total FBP content and apo-FBP, whereas the ratio between total FBP and

its apo-form was relatively constant. The range for the total FBP content was 2.9-11.1 µg/mL (91-347 nmol/L) and this agrees with the ELISA results of 170-210 nmol/L reported by Wigertz et al (1997) measured in raw and pasteurized milk. The range for apo-FBP was 1.1-5.6 µg/mL (34-175 nmol/L). The results show that approx. 50% of the total FBP content is not bound to folate. The total protein content was positively to the amount of apo-FBP (r=0.30), and negatively correlated to the holo-form (r=-0.29). The level of FBP was increased at the beginning of lactation, which is in agreement with the results of Indyk and Filonzi (2004). No differences were found between the two investigated Swedish dairy breeds.

## III. Affinity and rate constants for interactions of bovine folate-binding protein and folate derivatives determined by optical biosensor technology. Effect of stereoselectivity (Paper III)

Paper III is the first study that has investigated binding kinetics between FBP and pure stereoisormeric forms of various folate based on real-time monitoring of association and dissociation rates quantified by SPR biosensor technology. The results demonstrate that there are marked differences in the interactions between FBP and the major forms of folate derivatives. Each of the binding responses was well described by a 1:1 interaction model. Considerable differences were found for both association rate constants ($k_a$) and dissociation rate constants ($k_d$) between the different folate forms. Folic acid exhibited the most rapid association rate constant and the slowest dissociation rate constant and thus had the highest affinity because the affinity are calculated by the quotient of $k_d/k_a$. [6S]-5-HCO $H_4$folate had the most rapid dissociation rate constant and additionally the slowest association rate and thus the lowest affinity. The calculations of equilibrium dissociation constant ($K_D$), showed that the two folate forms that do not occur in nature, i.e. folic acid and [6R]-5-$CH_3H_4$folate, had the highest affinity for FBP, 20 and 160 pmol/L, respectively. Among the naturally occurring folates, [6S]-5-$H_4$folate had the highest affinity (250 pmol/L), [6S]-5-$CH_3H_4$folate almost 10-fold lower (2000 pmol/L) and [6S]-5-$HCOH_4$folate the lowest affinity of all (12000 pmol/L).

*In vitro* and *in vivo* studies indicate that FBP in complex with folic acid results in lower folate bioavailability (Arkbåge et al, 2003; Verwei et al, 2004; Witthöft et al, 2006). Recent published data also show that 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 the recent years, it has become increasingly clear that binding affinity alone may not always be a suitable description of biological activity. Since the equilibrium dissociation constant ($K_D$) are calculated by the quotient of $k_d/k_a$ 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 (reviewed by Andersson & Hämäläinen, 2006). Rich et al (2002) found that estrogen receptor agonists had 100-fold higher

association rates than antagonists, irrespective of dissociation rate. The results in this study show that folic acid has almost 10-fold higher association rate than [6S]-5-$CH_3H_4$folate and almost 50-fold slower dissociation rate. The physiological importance of the differences in rate constants for folic acid compared to those of [6S]-5-$CH_3H_4$folate is not known. The fact that molecules that act on the same receptor but with opposite function, i.e. agonist and antagonists, have been discriminated by kinetics and not by affinity supports the importance of binding kinetics.

The achieved response in competitive binding methods, e.g. RPBA, depends on folate derivative used as a standard and on the folate derivatives in the sample. Simplified, the differences in response depend on difference in affinity between FBP and the different folate derivatives. Yet, strictly speaking, the differences depend on differences in binding kinetics because the assay does not reach equilibrium during the incubation. To reach equilibrium for interactions with such slow dissociation rate as folic acid and [6R]-5-$CH_3H_4$folate will take days at the low concentrations used.

## IV. Letter to the editor (Paper IV)

Christensen et al. (2006) published an article on the interaction between folic acid and bovine FBP using the stopped-flow technique. They interpreted their binding data and concluded the interaction to consist of at least three steps at pH 7.4 and two steps at pH 5.0 and rejected a simple one-step binding model (A + B = AB). These results are contradictory to results from **Paper III** because we were able to fit our kinetic data for the interaction between different folate derivatives and FBP to a simple one-step binding model and no improvement in curve fit was seen when using more complex models. It was, therefore, suggested that the complex binding models described by Christensen et al. were due to rate limiting parallel reactions resulting from the dissociation of pre-formed FBP aggregates caused by the high protein concentration used in the experiment. The binding between folate and monomeric FBP is most likely a simple 1:1 interaction mechanism.

## V. The effect of different folate forms on denaturation of bovine folate-binding protein (Paper V)

The influence of different folate derivatives on the denaturation temperature of FBP was investigated using DSC. The effect of heat treatment, i.e. pasteurization and UHT treatment, on FBP binding to folate was studied using the surface plasmon resonance technique. The folate derivatives studied were (6S)5-HCO-$H_4$folate, (6S)5-$CH_3$-$H_4$folate and folic acid.

The results show that different folate forms affect heat denaturation of FBP differently and this effect is probably related to the affinity of FBP to the different folate forms. Apo-FBP undergoes an endothermic transition with a maximum at 60.5ºC. After ligand binding, the maximum of the denaturation shifts with a transition maximum at 72.4ºC for (6S)5-HCO-$H_4$folate and 78.7ºC for (6S)5-$CH_3$-

$H_4$folate. The highest temperature shift was observed for folic acid with maximum transition temperature at 83.7ºC. These results relate well to the affinity data published in **Paper III**; the order of affinity for FBP was folic acid > (6R)5-$CH_3$-$H_4$folate > (6S)-$H_4$folate > (6S)5-$CH_3$-$H_4$folate > (6S)5-HCO-$H_4$folate. Wasylewski (2000) found that the apo-form of the structurally related Riboflavin-binding protein (RfBP) undergoes endothermic transition with a maximum at 60.8ºC. The endothermic transition maximum increased after riboflavin binding to 72.8°C. These data correspond well with our data. Slightly different results were obtained by Sigurskjold *et al* (1997) for FBP. They found that thermal unfolding of FBP occurs at 48.4ºC, and they measured a denaturation temperature of 72ºC for holo-FBP. However, the publication does not mention which folate derivative was studied.

The results obtained by SPR assay showed that heat treatment close to pasteurization temperature did not eliminate the binding capacity of FBP regardless of folate form bound, but higher temperature of UHT treatment did. Based on these results, UHT or other high temperature treatments of foods containing FBP will most likely increase bioavailability of folic acid and mildly heat treatment, e.g. pasteurization, will reduce bioavailability of folic acid. These effects should be taken into account when considering the folate intake by humans because the bioavailability of folate strongly depends on its interaction with the food matrix. Today most infant formulas are gently heat processed and fortified with folic acid; the bioavailability will probably be reduced because of active FBP and should be considered.

During SPR analysis of FBP an increase in mass on the sensor surface after heat treatment close to pasteurization temperature was observed. The increase was probably due to heat induced aggregation of FBP. This finding is in accordance with the observation of Gregory (1982). He described a heat induced increase in molecular mass of FBP using gel filtration analysis.

# Methodological considerations

The main focus of this thesis has been the use of an SPR-based biosensor to detect and characterise FBP. The SPR-based biosensors have enormous, almost unlimited possibilities, because they measure the interaction between nearly any molecules in real time, without any labelling. However, there are some concerns that the surface of the biosensor might affect binding interaction. Techniques such as ELISA, filter-binding assays and some RIA methods also rely on a surface and have been used for years without being questioned. Many interaction of importance in biology occur on the interface of a surface, e.g. cell surface, and from that point of view it is an advantage to measure interactions at a surface. The main problem is probably the user and not the instrument itself. Pitfalls, when using the instrument both for concentration and kinetic analysis include sample heterogeneity, aggregation and non-specific binding. Being aware of these issues is important especially because the instruments are so easily operated. When reading the annual survey of the optical biosensor literature by Rich & Myszka (2000-2006) it becomes clear that much of the published biosensor data is of low quality and this is because of the lack of sufficient knowledge of the instrument by the users. These instruments require dedicated, well-trained and knowledgeable operators to get most out of them. At the beginning of my PhD work I did not have this knowledge. However, during the following years I have become aware of at least some of the possible problems that might occur when working with SPR technology. In this case the most important complication was protein aggregation.

### *Aggregation*

When working with FBP experiencing aggregation is unavoidable. Much of what have been published about FBP is directly or indirectly related to this phenomenon. Contradictory results both for equilibrium constants and deviations from 1:1 interaction model are in my opinion caused by aggregation. As mentioned earlier, it is important that the biosensor user is aware of possible pitfalls. However, evidently, as discussed in **Paper IV**, it is also possible to fall into the same pitfalls using other techniques and draw conclusions that a complex binding profile is a result of some biologically interesting phenomena.

There are no data in this thesis that show the exact nature of FBP aggregates. However, when working with the SPR instrument differences in the behavior of the aggregates, depending on whether the ligand was present or not, were observed. Moreover, there were differences between the “native-” and heat-induced aggregates, because the heat-induced aggregates appeared to be more stable and did not dissociate when diluting the samples. It is therefore likely that the divergent behavior of the heat-induced aggregates seen during analysis by SPR-biosensor (**Paper V**) is a non-specific coagulation of partially denatured polypeptides. The nature of the aggregation of the native protein is probably due to more specific intermolecular interactions. It is known that presence of ligand increases the aggregation tendency and that holo-FBP forms hydrophilic water-soluble polymers, whereas apo-FBP aggregates have a hydrophobic character (Holm, Hansen & Hoier-Madsen, 2001). The work in this thesis has further shown

that unliganded FBP and liganded FBP aggregates with each other (**Paper V**). Unpublished data indicate that there is a conformational change as suggested by Hansen & Holm (2001) but not during the folate-FBP interaction; instead, the conformational change happens when holo-FBP aggregates. This is in agreement with circular dichroism results of Kaarsholm et al (1993). I therefore suggest that the apo-apo interaction are simple 1:1 interactions (A + B = AB), whereas the holo-holo interaction is followed by a second conformational changing step.

During the work described in **Paper III** I realized the importance of FBP aggregation. Consequently, no consideration for this phenomenon was taken in the two first studies. In **Paper I** an SPR biosensor assay that detected FBP and differentiated between the apo and holo-form of the protein was developed. The SPR biosensor assay showed good compliance with an existing ELISA method and a high repeatability. Later, when working with heat-treated FBP in milk (**Paper V**), it was seen that changes due to heat treatment influenced the response at the sensor surface. It was observed (unpublished results) that freeze storage of the milk samples for longer periods, i.e. 6 months or more, also increased the response especially for the measured apo-FBP. The fact that the response increased more for apo-FBP is probably due to the lack of pre-treatments that dissociate the aggregates. To facilitate measurements of the total FBP content in milk, endogenous folate is removed by a combination of low pH to dissociate the FBP-folate complex followed by a membrane molecular weight cut-off filtration. It is possible that the aggregates formed during freeze storage dissociate due to the low pH. The samples used in the study described in **Paper II** were stored for only a few weeks at -80ºC before analysis, which most likely did not cause aggregation. In addition, differences in FBP level between milk samples and therefore statistical conclusions are valid because all the samples were stored and treated in a similar way.

### *Heterogeneity*

A common FBP source used in purification of bovine FBP is whey powder concentrate (Svendsen et al., 1979; Treloar, Grieve & Nixon 2000). This is also the source used in the present work. Because whey protein concentrate have been exposed for different heat treatments, the conformational purity of FBP cannot be guaranteed. An important issue when working with SPR biosensors is to secure high quality samples to avoid problems with heterogeneity and aggregation. As I realized later, raw milk as a FBP source would have been a better choice. However I need to emphasize that there were no signs of heterogeneity seen in the curve fit for the interaction between folate and FBP, as shown in **Paper V**. Consequently, FBP that is not irreversible destroyed during heat treatment does not change its binding properties to the ligand. Heterogeneity was, on the other hand, observed in the dissociation curves for the FBP-FBP interaction (not shown), probably caused by terminally exposed amino acids.

### *Non-specific binding*

Non-specific binding could either be caused by binding of analyte, in this case FBP to non-specific sites on the surface, or by binding of non-analyte molecules, in this case whey. FBP itself showed no non-specific binding to the sensor surface when diluted in HBS-EP buffer, however, whey did. To counteract non-specific binding from whey, a phosphate buffer with a high salt concentration (0.5 M) was introduced before the change in response due to bound FBP was measured. This successfully reduced the effect of non-specific binding from the whey.

# Conclusions and future research

This thesis describes an SPR-based method for detection of FBP in cow milk. The assay differentiates between the unliganded (apo) and the total FBP content and provides a useful tool for analysis of FBP concentration as well as kinetics of FBP interactions. The method is reliable to use for fresh milk and samples that have been freeze stored for a relatively short period; however, it needs to be further evaluated with respect to FBP aggregation, particularly aggregation induced by long term freeze storage. It cannot be used for milk samples exposed to heat at close denaturation temperatures because of the disproportional increase in response, which is also most likely due to aggregation.

Screening of Swedish dairy cows for FBP concentration in milk shows considerable variation between individuals. The most influential factor affecting FBP levels is the stage of lactation; the levels are high at the beginning of lactation and decrease considerably as lactation progresses. The ratio between FBP and its apo-form is, however, fairly constant and approx. 50% of the FBP remains unliganded. The total protein content is positively related to the amount of apo-FBP and negatively related to the holo-form. Breed does not seem to affect FBP levels.

The analysis of the binding kinetics of interactions between FBP and different folate derivatives demonstrates that the interaction is a simple 1:1 binding (A + B = AB). The rate constants differ between the derivatives and the association rate is almost 10-fold faster for folic acid than any of the other derivative tested. Folic acid and (6R)5-$CH_3$-$H_4$folate, the two folate derivatives that do not exist in nature, have the highest affinity for FBP. The order of affinity for FBP is folic acid > (6R)5-$CH_3$-$H_4$folate > (6S)-$H_4$folate > (6S)5-$CH_3$-$H_4$folate > (6S)5-HCO-$H_4$folate. The impact of the differences in rate constants for the different folate derivatives needs to be further investigated with nutritional studies.

The shift in denaturation temperature due to ligand binding corresponded well with the affinity data; the higher affinity of the ligand to FBP, the higher denaturation temperature of the FBP-ligand complex. Further, all reduced folate derivative tested increases the denaturation temperature of FBP to a temperature close to pasteurization temperature. Pasteurization temperatures do not eliminate the binding capacity of FBP, whereas UHT treatment does. Due to the high proportion of unliganded FBP in fresh and pasteurized milk and the fact that folic acid forms strong complex with FBP, mildly heat-treated cow milk is not a good vehicle for folic acid fortification.

This thesis have shown that SPR-technology provide new possibilities to study binding characteristics of bioactive substances in foods, which, in combination with nutrition studies, would give new knowledge in the field of food science and nutrition. Further, SPR technology could be useful for studying protein aggregation and could provide new useful information about interactions in food systems. Particularly, because proteins are widely used in food industry as texturisers and this is based on the ability of proteins to aggregate.

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