Binding Characteristics of Folate to High Affinity Folate Binding Protein Purified from Porcine Serum

Masahiro NATSUHORI, Maki OKADA¹, Ryo IDA¹, Kazuaki SASAKI¹, Minoru SHIMODA¹ and Ei-ichi KOKUE¹

Laboratory of Veterinary Radiology and Radiation Biology, Kitasato University School of Veterinary Medicine and Animal Sciences, Towada, Aomori 034–8628, ¹Laboratory of Veterinary Pharmacology, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fachu, Tokyo 183–8509, and ²Central Research Laboratory, Hitachi, Ltd., Kokubunji, Tokyo 185–8601, Japan

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ABSTRACT. High affinity folate binding protein (HFBP) in porcine serum was purified 2,000-fold to a specific activity of 1.4 nmol of tetrahydrofolic acid (THF) bound per mg of protein, using folic acid (FA) coupled EAH-Sepharose gel affinity chromatography. Binding activity of purified HFBP to folate was examined by ultrafiltration method linked to high-performance liquid chromatography with electrochemical detection or to liquid scintillation counting. Binding affinity of HFBP to folate was characterized by dissociation constants (Kd): 13, 17, and 31 pM for tritiated FA (³HFA), THF, and N⁵-methyltetrahydrofolic acid (5MF), respectively. FA, THF, and 5MF significantly inhibited binding of HFBP to ³HFA, and according to the magnitude of intensity of the binding inhibition, the order of affinity of each folate was confirmed to be FA > THF > 5MF. Binding activity was rather high and stable for THF and 5MF at pH ranging from 6.0 to 10.0. The binding activity, however, rapidly decreased at pH below 6.0 and over 10.0. No binding activity was observed pH below 3.0 and over 12. Gel filtration analysis showed that the prepared HFBP solution had specific binding activity at around 77 kDa of apparent molecular weight, which was 82 kDa by SDS-PAGE. It is considered that this specific and stable binding enables THF to distribute in porcine plasma.—KEY WORDS: binding protein, folate, serum, swine.

Different from other mammalian species, pigs have a unique composition of plasma folate, namely tetrahydrofolic acid (THF) and N⁵-methyltetrahydrofolic acid (5MF) [1, 12, 13]. In a previous study, Mantzos et al. [11] have demonstrated that porcine serum has a much higher capacity of folate binding than other animal species. O'Connor and Picciano [14] and Natsuhori et al. [13] also reported that most of plasma folate appeared to be bound to high affinity folate binding protein (HFBP) in pigs. Recently Sasaki et al. [17] clearly demonstrated that most of porcine plasma folate existed as a bound form with HFBP and the binding capacity of HFBP was more than 3 times of the levels of plasma folate. Furthermore, they demonstrated that plasma HFBP protects THF from degradation. The authors therefore suggested that HFBP may have a role in governing plasma folate distribution in pigs.

HFBP has been known to be distributed in various biological fluids and tissues including milk, plasma or serum, cerebrospinal fluid, saliva, urine, liver, kidney, small intestine, spleen, placenta, choroid plexus, neutrophils, granulocytes, and various cancer cells in several mammalian species [2, 3, 5, 9, 19]. Although some of these HFBPs were purified and their biochemical properties were characterized, their physiological functions in folate distribution, transport, and intracellular uptake are still unclear [2, 3, 5, 9, 19]. Due to its uniquely high capacity of specific folate binding, further research is required concerning porcine plasma HFBP. This study was therefore undertaken to identify some binding properties of purified porcine serum HFBP to folic acid, THF, and 5MF.

MATERIALS AND METHODS

Blood: Porcine blood was obtained from slaughterhouse (Tachikawa Meat Inspection Center, Tokyo, Japan). Porcine serum was separated by centrifugation at 2,000 × g for 5 min. The pooled serum was adjusted pH to 3.0 with 1.0 N HCl and kept at 4°C for 2 hr, in order to degrade endogenous folates, as described by Sasaki et al. [17]. After re-adjusting pH to 7.4 with 1.0 N NaOH, the serum was stored at -80°C until used. After this treatment, THF and 5MF were not detected in the treated serum using high-performance liquid chromatography with an electrochemical detector (HPLC-ECD).

Chemicals and reagents: Folic acid (FA), N-ethyl-N'-[(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), sodium carbonate anhydrous, sodium acetate trihydrate, sodium dihydrogen-phosphate dihydrate, and disodium hydrogenophosphate were from Wako Pure Chemical (Osaka, Japan). (6R,S)-5,6,7,8-tetrahydrofolic acid trihydrochrolide and (6R,S)-N⁵-methyl-5,6,7,8-tetrahydrofolic acid calcium salt were from Dr. Schircks Laboratory (Switzerland). These folates were further purified by anion exchange chromatography using QAE-Sephadex A-25 (Pharmacia Biotech, Tokyo, Japan) as described by Reed and Scott [15]. Tritium labeled folic acid ([3',5',7,9-³H] folic acid potassium salt, 962 GBq/mmol (26 Ci/mmol) of specific activity, and 97% of radiochemical purity) was from Amersham (Buckinghamshire, UK). All other reagents used in this experiment were of analytical grade.

Preparation of HFBP: Fifty ml of EAH-Sepharose 4B gel (Pharmacia LKB, Uppsala, Sweden) was coupled with...
FA by EDC condensation with slight modification of the description by Salter et al. [16]. The Sepharose gel was suspended in an equal volume of water, FA (30 mg) dissolved in 30 ml of 50 mM NaHCO₃ was added, and the pH was adjusted to 6 with 1 N HCl. Then EDC (0.5 g) was added in the suspension and stirred for 2 hr. The pH of the suspension was kept 6 during this coupling process by adding 1 N HCl as necessary. After this reaction, the gel became pale yellow and the mixture was washed with 500 ml of the following each solution through glass filter, i.e., 0.1 N NaOH containing 0.5 M NaCl, 0.1 N HCl containing 0.5 M NaCl, and 50 mM phosphate buffer (pH 7.4). In order to diminish the remaining unsubstituted amino group, the Sepharose-FA gel was mixed with 50 mM NaHCO₃ (30 ml) containing sodium acetate (30 mg) and EDC (0.5 g) for further coupling or “blocking” reaction at pH 6 for 2 hr. Again, after this reaction the Sepharose-FA gel was washed by the similar way, and packed in a column (3.0 × 12 cm). Phosphate buffer (50 mM, pH 7.4) was used for thorough washing of the gel at a flow rate of 2 ml/min for 2 days.

For the preparation of HFBP, 3 litters of the folate-free porcine serum was mixed with 50 ml of FA coupled EAH-Sepharose 4B gel and kept for 2 hr at 4°C. The mixture was then packed into a column and washed by phosphate buffer (pH 7.2–5.5 of gradient), phosphate buffer containing 0.5 M NaCl (pH 7.2), and acetate buffer (pH 4.5), and then HFBPs were eluted with 0.2 N acetic acid (pH 2.7) at a flow rate of 2 ml/min. The elution profiles were monitored by UV detector (Model 112, Gilson Medical Electronics Inc., Middleton, WI) at 280 nm and pH monitor (Pharmacia, Uppsala, Sweden), and each fraction of the absorption peak was collected by fraction collector (model FC 203, Gilson). Obtained fractions were dialyzed against 20 mM phosphate buffer (pH 7.4) for overnight at 4°C. The dialyze was kept in a deep freezer at -80°C until the binding kinetic analysis was performed. Total protein concentration of the dialyze of each fraction taken through affinity chromatography was determined spectrophotometrically by using commercially available kit (Bio-Rad Protein Assay Kit, Dye reagent, Bio-Rad Lab. Inc., CA).

**HPLC system:** THF and 5MF concentrations were analyzed by using the following HPLC system: a pump (LC-9A, Shimadzu, Kyoto, Japan), a fixed-loop injector (model 7125, Rheodine, Cotani, CA), an analytic column (Radial-Pak, type 5NVP4µ, Waters, Milford, MA), an electrochemical detector (model E-502, Irica, Kyoto, Japan), and a data processor (Chromatopak CR4A, Shimadzu, Kyoto, Japan), as described previously [12]. The mobile phase was a mixture of 20 mM acetate buffer (pH 3.6) containing 0.1 mM EDTA and acetonitrile (98:2, v/v). The flow rate was 0.8 ml/min. The applied potential of the ECD was +300 mV. The HPLC procedure has a good reproducibility with good recovery (97 ± 1% for THF and 96 ± 3% for 5MF, means ± sd, n=5), as described by Natsuhori et al. [13].

**Binding activity of HFBP to folate:** Protein binding activity of folate (THF, 5MF, and 3HFA) to each fraction, or to prepared HFBP was examined by using a ultrafiltration kit (Amicon® centrifree micropartition system, Grace Japan K.K., Tokyo, Japan) as described previously [17]. In this analysis, recovery of folate by ultrafiltration was 87%, which was independent of the folate analogues. Sodium ascorbate (Wako Pure Chemical, Osaka, Japan) was added to HFBP solution as antioxidant (0.5%, W/V). To the HFBP sample (1 ml), 50 µl of folate solution was added and then mixed, and kept for 5 min in order that the binding equilibrium was completed. After ultrafiltration of the sample by centrifugation at 2,000 × g for 5 min, the ultrafiltrate (100 µl) was injected into HPLC-ECD system to determine free concentrations of THF or 5MF. Free concentration of 3HFA was determined by liquid scintillation counting (Aloka Liquid Scintillation System, model SC-700, Tokyo, Japan) using scintillation solvent (Biofluor, Biotechnology systems, NEN® Research Products, MA). Total folate levels were also determined by using HPLC-ECD system for THF and 5MF, and liquid scintillation counting for 3HFA as described previously [12, 13].

Effect of pH on HFBP binding to folate (THF and 5MF) was examined in solutions adjusted at various pH. The various pH buffers were prepared with solution containing sodium borate, sodium acetate, and sodium phosphate buffer (40 mM each), and sodium ascorbate (0.5%) at various pH adjusted by NaOH or phosphoric acid. Then an equal volume of the prepared HFBP solution and the buffer were mixed. The pH of the final mixture at 37°C was again measured just before the ultrafiltration. Relative binding activity was then calculated based on the maximum binding activity.

**Binding kinetic analysis:** In this analysis, to obtain dissociation constants of HFBP to folates, the maximum binding capacity of the prepared HFBP solution was set to be about 160 nM for THF and 5MF, and about 18 nM for 3HFA binding. The concentrations of free (Cf) and bound (Cb) folate were depicted in a Scatchard plot. Since the Scatchard plot showed property of one binding site, the following equation was applied and regressed based on the obtained Cf and Cb data.

\[
Cb = \frac{B_{\text{max}} \times Cf}{K_d + Cf} \quad \ldots \text{Eq. 1}
\]

In Eq. 1, \(B_{\text{max}}\) and \(K_d\) are maximum binding capacity and dissociation constant of the folate-protein binding, respectively. Analysis was performed by using a non-linear least squares method with computer curve fitting program “MULTI” [21].

Inhibition of 3HFA binding to HFBP by each folate (FA, THF, 5MF) was also examined in phosphate buffer (20 mM) containing sodium ascorbate (0.5%) at optimum pH (pH 7.4). The maximum binding capacity of the prepared HFBP solution was adjusted to be approximately 18 nM. Binding activities of 3HFA were measured in aliquots of the solution to which 3HFA (final conc. 18 nM) and folate (final conc. ranging from 0 to 144 nM) were simultaneously mixed.
this case, there was a certain amount of competing inhibitor of the binding in the protein solution. Therefore the following equation was applied;

\[
Cb = \frac{B_{\text{max}} \times S_{0}}{S_{0} + (1 + I / K_i) \times K_d} \quad \text{...Eq. 2}
\]

where \( C_b \), \( S_0 \), \([I]\), \( K_d \) and \( K_i \) are bound concentration of \( ^{3}\text{HFA} \), concentration of substrate (\(^{3}\text{HFA}, 18 \text{nM}\)), concentration of competing agent (\( \text{FA, THF, and 5MF, 0 to 144 nM} \)), dissociation constant of folic acid, and dissociation constant of competing agent, respectively. Then Eq. 2 was modified to the following formula (Eq. 3.),

\[
\frac{1}{C_b} = \frac{K_d}{B_{\text{max}} \times S_0} + \frac{I}{B_{\text{max}} \times S_0} \times \frac{K_i}{K_d} \quad \text{...Eq. 3}
\]

According to Eq. 3, Dixon plot analysis was performed to see differences of the slope of regression line. Since the slope is expressed by the ratio of \( K_d / K_i \) which is divided by constants (\( B_{\text{max}} \) and \( S_0 \)), the higher value of the slope represents higher affinity (lower values of dissociation constants) of the competing agent for binding proteins, which directly indicates the order of the affinity of folate to HFBP.

**Gel filtration and electrophoresis:** Gel filtration analysis was carried out to determine the apparent molecular weight of HFBP that bound with \( ^{3}\text{HFA} \), and to isolate the fraction of the solution containing the target protein. Guard column (TSK-guardcolumn SWXL, 6.0 × 40 mm, Tosoh, Tokyo, Japan) and analytical column (TSK-Gel G3000SWXL, Tosoh) were used to the HPLC system. Mobile phase was 0.1 M phosphate buffer containing 0.1 M sodium sulphate (pH 7.4) and flow rate was 0.8 ml/min. Monitored wavelength of the UV detector was 280 nm. Appropriate amount of calibration proteins (HMW and LMW Gel Filtration Calibration Kit, Pharmacia) were dissolved by 100 \( \mu \)l of the mobile phase and 10 \( \mu \)l of the solutions were injected to the HPLC for the estimation of apparent molecular weight of HFBP. The prepared HFBP solution (about 200 nM of adjusted binding capacity) containing 0.5% ascorbate was mixed with \( ^{3}\text{HFA} \) (final conc. 200 nM), and 100 \( \mu \)l of the aliquot was injected to the HPLC system. Fractions (0.4 ml/fraction) were collected by using a fraction collector (Model FC-203, Gilson) linked to HPLC system, and radioactivity (DPM) of each fraction was measured by liquid scintillation counting. The fraction that had the most binding activity was then applied to SDS-PAGE to determine the molecular weight of porcine serum HFBP. An appropriate amount of marker proteins and Coomassie Brilliant Blue R-250 (Fluka, Switzerland) were used.

**RESULTS**

**Preparation of porcine serum HFBP by using affinity chromatography:** Figure 1 shows elution profile of porcine serum affinity chromatography. Only final peak (f4) eluted by 0.2N acetic acid had specific binding activity for folate after dialysis, as summarized in Table 1. Compared to serum, the prepared HFBP solution was 2,100-fold purified with yield of 41% and the specific binding activity was 1.4 nmol of THF per mg of protein at pH 7.4.

**Binding activity of folate to HFBPs:** Figure 2 shows effect of pH on binding of HFBP to THF and 5MF. Binding activity was rather high and stable for both folate at pH ranging from 6.0 to 10.0. The binding activity, however, rapidly decreased at pH below 6.0 and above 10.0. No binding activity was observed pH below 3.0 and pH at 12. Scatchard plot showed high affinity with a single binding site for each folate examined (Fig. 3). Dissociation constant (Kd) of \( ^{3}\text{HFA} \), THF, and 5MF for the purified HFBPs was calculated to be 13, 17, and 31 pM, respectively. Biphasic (high and low affinity) binding was not observed in the prepared HFBP. The ratio of Kd for FA to Kd for FA, THF, and 5MF were 1, 0.76, and 0.42, respectively. Dixon plot demonstrated that binding of prepared HFBP to \(^{3}\text{HFA} \) was inhibited by FA, THF, and 5MF, respectively (Fig. 4). The slope of each regression line in figure 4 was calculated to be 2.8 (± 0.3) × 10⁻⁵, 2.0 (± 0.1) × 10⁻⁵, and 1.1 (± 0.1) × 10⁻⁵.

![Fig. 1. Elution profile of porcine serum high affinity folate binding protein by using affinity chromatography](image)

**Table 1. Purification of porcine serum high affinity folate binding protein**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Bmax (nM THF)</th>
<th>TP (µg/ml)</th>
<th>Specific activity*</th>
<th>Yield (%)</th>
<th>Purification</th>
</tr>
</thead>
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<td>0.69</td>
<td>100.0</td>
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</tr>
<tr>
<td>Elution peak fraction</td>
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<tr>
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<td>42400</td>
<td>0.11</td>
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</tr>
<tr>
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<td>990</td>
<td>0.00</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
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<td>920</td>
<td>78.3</td>
<td>1.4</td>
<td></td>
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<tr>
<td>f4</td>
<td>1329</td>
<td>920</td>
<td>1445</td>
<td>41.2</td>
<td>2093</td>
</tr>
</tbody>
</table>

Bmax, maximal binding capacity for tetrahydrofolate (THF); TP, total protein; *Specific activity, pmol THF bound per mg of protein. Elution peak fractions correspond to peaks in Fig. 1.
The ratio of the slope for competing agents (FA, THF, and 5MF) to the slope for FA, which represents the ratio of the relative intensity of binding inhibition for FA to the competing agents (FA, THF, and 5MF), were 1, 0.71, and 0.39, respectively. Accordingly, the ratio of Kd for \(^3\)HFA to Kd for \(^3\)HFA, THF, and 5MF (from Scatchard plot) and the relative intensity of binding inhibition (from Dixon plot) showed similar values, indicating that the order of affinity of folate to HFBBP is FA > THF > 5MF.

Gel filtration and electrophoresis: Gel filtration analysis of prepared HFBBP solution mixed with \(^3\)HFA demonstrated that the prepared HFBBP solution (final eluate of the affinity chromatography) contained several proteins of which some were potent binder for folate (Fig. 5). In Fig. 5, while there were 6 UV absorption peaks detected (p1–p6), only 3 major peaks of \(^3\)HFA radioactivity were observed. One initial radioactive peak was observed in fraction 14–18, and the apparent molecular weight of the corresponding UV peak (p1) was more than 700 kDa. However, the most specific binding activity was observed in fraction 22–25 with apparent molecular weights of the corresponding UV peak of 77 kDa (p4). The small radioactive peak in the fraction 29–32 was unbound \(^3\)HFA, which coeluted with ascorbate, since the last, huge UV absorption peak (p6) did not contain protein. This UV peak (p6) was appeared only after the addition of \(^3\)HFA into the HFBBP solution. Therefore the p6 was ascorbate dissolved in \(^3\)HFA solution. No free \(^3\)HFA was detected in the fraction from 1 to 27, but fraction 30–35 contained free \(^3\)HFA. SDS-PAGE of the fraction containing the most specific activity (p4) showed a band with a molecular weight of 82 kDa.

DISCUSSION

Characteristics of HFBBP from biological fluids (plasma, milk, cerebrospinal fluid) and tissues (kidney, liver, placenta) in several animal species have been summarized and reviewed by several authors [2, 3, 5, 9, 19]. Although porcine HFBBPs derived from choroid plexus [18] and kidney [7, 8], were identified, purified and characterized, porcine serum HFBBP has not been purified and characterized, yet. In this study, porcine serum HFBBPs were purified about 2,100 fold to a specific binding activity of 1.4 nM of THF per 1 mg of protein.

Binding activity of HFBBP to folates (THF, 5MF) was rather constant in buffered solution at variable pH ranging from 6 to 10, although the activity dramatically dropped at the pH below 6.0 and over 10.0. Kamen and Caston [8] have observed similar binding activity of porcine HFBBP purified from kidney. They reported that at pH values greater than 9.5 or less than 5.5, the binding activity was dramatically decreased. Suleiman and Spector [18] have reported similar observation of pH dependent binding activity of HFBBP purified from porcine choroid plexus, where the activity decreased below pH 6.0 and above pH 8.0. Iwai et al. [6] however, reported rather pH sensitive
respectively. Binding activity of 3 HFA was inhibited by FA to Kd for FA, THF, and 5MF were 1, 0.76, and 0.42, respectively (Fig. 3). The ratio of Kd for (Kd) of purified HFBP were 13, 17, and 31 pM for FA, binding of folate in porcine serum. Dissociation constants Sasaki extremely high affinity for folate. Hansen HFBP. binding activity against variation of pH than bovine milk pH 8 and 9. Porcine serum HFBP may have more stable binding activity of purified bovine HFBP to [14C]FA from porcine plasma. The ratio of the slope for competing agents (FA, THF, and 5MF) to the slope for FA, which represents the relative intensity of binding inhibition for FA to the competing agents (FA, THF, and 5MF), were 1, 0.71, and 0.39, respectively. Accordingly, the similar values between the ratio of Kd in Scatchard plot and the ratio of the slope observed in Dixon plot confirmed that the order of affinity of folate to HFBP is FA > THF > 5MF. Thus, as described elsewhere [2, 9, 19], serum HFBP has higher affinity for the oxidized form of folate than for the reduced form, which was also demonstrated for porcine serum HFBP.

Molecular weight of porcine serum HFBP was considered to be 82 kDa. However, Kamen and Caston [8] reported that molecular weight of porcine kidney HFBP was 38.5 kDa. Suleiman and Spector [18] reported 51 kDa of HFBP from porcine choroid plexus. Moreover, HFBPs isolated from several biological fluids have been reported to be 30–40 kDa [10]. These differences of molecular weight of HFBPs suggest that porcine serum HFBP is essentially different protein from those in kidney and choroid plexus, and those in tissues and biological fluids in other species.

Recently, stereo selective binding of tetrahydrofolates to human serum albumin has been reported [10]. In spite of the usage of racemic tetrahydrofolate in this study, most of folate bound almost completely and no apparent biphasic binding was observed. Therefore it may be concluded that porcine serum HFBP does not significantly distinguish racemic tetrahydrofolates, in spite that purified HFBP had significant differences in the order of affinity to folate analogues. Most likely, the oxidation/reduction status of a pterin structure of folate is a determinant for dissociation constant of HFBP binding.

Several roles of serum HFBPs were proposed as follows: like other binding proteins for the transport of small molecules such as vitamin B12, iron, and thyroid hormone, HFBPs transport the absorbed or released folates to target cells, scavenge inactive or oxidized folates and carry them to liver for metabolic conversion, trap folates in fetal circulation for fetal growth, store and protect physiologic form of folates (5MF) from degradation [9]. In this respect, Sasaki et al. [17] recently demonstrated that porcine plasma HFBPs have much higher capacity than the plasma level of folate and the HFBPs tightly bind with folate and protect significant amount of THF from degradation. Previous results [13] demonstrated that intravenously injected FA is rapidly metabolized and yielded significant levels of both THF and 5MF in plasma, where no unbound forms of THF and 5MF were detected.

It is of interest that THF had higher affinity for HFBP than 5MF, and had relatively similar affinity (Kd value) to FA, since THF is the principal folate together with 5MF in porcine plasma. These results indicate that THF have priority over 5MF in HFBP binding in plasma. However, the uniquely high capacity of HFBP which is higher than the folate levels in plasma diminishes this difference of affinity, since almost all plasma folates bind with HFBP. Nevertheless, the unique folate metabolism and rather high binding capacity of this HFBP may be at least determinants of the unique distribution of plasma folate in pigs. Further comparative approach on research of HFBP in body fluids and tissues in relation to folate receptor-mediated intracellular uptake and release of folate and/or HFBP from tissues will give important information concerning the mechanism of folate homeostasis and its regulation system in the body, and may elucidate this unique folate distribution in pigs.

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