The Purification and Characterization of the Low Molecular Weight Human Folate Binding Protein Using Affinity Chromatography†

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ABSTRACT: The low molecular weight folate binding protein (FABP) has been purified 10000-fold to a specific activity of 7.2 μg of pteroylglutamic acid (PGA) bound per mg of protein. This purified FABP represents two protein bands that bind PGA on polyacrylamide disc gel electrophoresis, elutes from DEAE-cellulose in 0.001 *M* phosphate buffer, stains positive with PAS, elutes from concanavalin A Sepharose affinity columns with methyl α-mannoside, and shows three major peaks (p*I* = 6.8, 7.5, 8.2) by isoelectric focusing. The binding of PGA to purified FABP is dependent on pH and is inhibited by urea. PGA bound to purified FABP is not available for uptake by HeLa cells. These data characterize purified FABP to represent two basic glycoproteins which bind folates by noncovalent bonds.

Specific folate binding proteins (FABP)[1] with similar characteristics have been found in normal sera (Waxman and Schreiber, 1973a) and in serum from folate deficients (Waxman and Schreiber, 1973a), some uremics (Hines et al., 1973), some pregnant women (daCosta and Rothenberg, 1973), as well as in milk (Waxman and Schreiber, 1973a, Metz et al., 1968), and in leukocyte lysates from some patients with chronic myelogenous leukemia (Rothenberg, 1970). FABP has also been found in cow's milk (Ghitis, 1967), hog kidney (Kamen and Caston, 1974), and the brush border membranes of rat small intestinal epithelial cells (Leslie and Rowe, 1972). The characteristics of FABP include a rapid association and slow dissociation rate for the binding of tritiated pteroylglutamic acid ([^{3}H]PGA) and a preference for the binding of oxidized folyl mono- and polyglutamates as compared to the reduced folates (Waxman and Schreiber, 1973a). FABP appears to represent two groups of proteins, one eluting as a β globulin with a molecular weight of approximately 40000 (low molecular weight FABP) and another larger protein excluded by G-200 Sephadex (high molecular weight FABP) (Waxman and Schreiber, 1973a; Markkanen and Peltola, 1971). Human milk, an apocrine secretion, is much higher in FABP content than serum (Waxman, 1975). For these reasons we have previously suggested that FABP represents a cellular, perhaps membrane derived protein, important in regulating cellular uptake, distribution, and storage of various folate coenzymes (Waxman and Schreiber, 1973a; Waxman, 1975).

Studies of structure and function of these proteins have been handicapped by the difficulty encountered in their purification. Purification and isolation of FABP is limited by the small amounts present. The problem is apparent when one considers that 1 l. of human milk contains only 0.7 mg of unsaturated total (low plus high molecular weight) FABP.

To achieve homogeneous protein preparations, milk must be purified almost 10000-fold. Purification of this degree with reasonable yields is beyond the scope of conventional techniques. Affinity chromatography is an attractive method for consideration and has been used successfully in partially purifying folate binding protein from cow's milk (Salter et al., 1972). We now report the purification of low molecular weight FABP by combining affinity chromatography with DEAE-cellulose chromatography. The purification and properties of human milk low molecular weight FABP form the basis of this report.

Materials and Methods

[^{3}H]PGA (40 Ci/mmol) was purchased from Amersham/Searle Corp., Arlington Heights, Ill. The purity of this compound varied from 90 to 95% as determined by descending paper chromatography in 0.1 *M* phosphate buffer (pH 7.4). Human milk was a pool from Mother's Milk Bank, Wilmington, Del. Norit A (neutral) charcoal is a product of Amend Chemical Co. (Irvington, N.J.). The DEAE-cellulose was purchased from Schleicher and Schuell Inc., Keene, N.H. The HeLa cell line was provided by the laboratory of Dr. I. A. Jaffe.

Preparation of the Affinity Gel. CNBr-activated Sepharose 4B (Pharmacia, Batch 7877) (7.5 g) was swollen in 0.001 *M* HCl and then washed with 2 l. of 0.001 *M* HCl to remove the stabilizing dextran and lactose; 50 ml of 12% 1,6-diaminohexane, adjusted to pH 10 with concentrated HCl, was added to the washed gel and stirred for 16 hr at 4°C. The gel suspension was then filtered and washed with 1 l. of distilled water. The gel was resuspended in 40 ml of distilled water containing 6 mg of folic acid (Folvite, Lederle Labs) and stirred at room temperature protected from light. The pH was lowered to 6 with 1 *N* HCl and 100 mg

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[1] Abbreviations used are: FABP, folate binding protein; PGA, pteroylglutamic acid; PAS, periodic acid-Schiff; HBSS, Hank's balanced salt solution.

of *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (K&K Lab., Plainview, N.Y.) was added in 10-mg aliquots and stirred for 60 min while keeping the pH constant at 6. The gel was filtered, washed with 1 l. of 0.05 *M* sodium bicarbonate, and resuspended in 40 ml of 0.125% sodium acetate. The pH was readjusted to 6 and 100 mg of carbodiimide was added as above. The gel was filtered, washed with 100 ml of distilled water, and packed into a 2.2 × 40 cm glass chromatography column. The column was kept at 4°C and the PGA bound affinity gel was washed with 0.02 *M* Na-KPO_4 buffer (pH 7.2) containing 0.1 *M* NaCl until the washing contained less than 1 ng of PGA/ml as determined by radioassay (Waxman and Schreiber, 1973b).

Preliminary Preparation of Milk Proteins. Untreated (pasteurization temperatures destroy FABP) pooled human milk was shipped frozen and stored at −10°C. The samples were thawed and centrifuged at 19500 rpm for 30 min at 4°C. The fat was removed from the supernatant by passing through glass wool. The fat-free supernate was pooled, 2-mercaptoethanol added to a final concentration of 0.005 *M*, and the pH brought down to 3.5 with concentrated HCl. Norit A neutral charcoal was added in a concentration of 50 g/l. and the pH readjusted to 3.5. After stirring overnight at 4°C the slurry was centrifuged at 19500 rpm for 30 min and clarified by passing through glass wool and the resultant solution neutralized with 1 N NaOH.

Purification of the Folate Binding Protein. The acid-charcoal treated milk (5 l.) was passed through the affinity gel by gravity flow and the eluate (high molecular weight FABP) was collected and stored. The column was washed successively with 1 l. of 0.02 *M* phosphate buffer (pH 7.2) containing 0.1 *M* NaCl, 500 ml of 1 *M* NaCl, and 1 l. of 0.02 *M* phosphate buffer (pH 7.2) containing 0.1 *M* NaCl. The FABP (low molecular weight) was then released from the gel by stripping with two 25-ml aliquots of 0.2 *M* acetic acid (Selhub and Grossowicz, 1973). These were dialyzed overnight against distilled water, assayed for protein and folate binding capacity, and stored at −10°C after lyophilization.

DEAE-Cellulose Chromatography. DEAE-cellulose with an exchange capacity of 0.87 mequiv/g was suspended in 1 *N* NaOH for 1 hr. The slurry was poured onto a Buchner funnel and washed with water until the pH was 7.0. The cellulose was equilibrated in 0.001 *M* phosphate buffer (pH 6.3) and packed into a 2.2 × 32 cm glass column. FABP (unlabeled or labeled with [^{3}H]PGA) was passed through and eluted with stepwise additions of phosphate buffers of pH 6.3 with increasing salt concentration.

Protein Assay. Protein was determined by the method of Lowry et al. using bovine serum albumin as the standard (Lowry et al., 1951). Dihydrofolate reductase activity was determined by the method of Rothenberg (1966).

Determination of Folate Binding Protein. FABP was measured by the method of Waxman and Schreiber (1973b). Samples were assayed in siliconized glass tubes and adjusted to a volume of 1.2 ml with 0.1 *M* phosphate buffer (pH 7.4). [^{3}H]PGA (0.5 ng) was added and the reactions were incubated for 30 min at 23°C. Hemoglobin-coated charcoal was used to adsorb free [^{3}H]PGA and after centrifugation aliquots of the supernate were counted in 15 ml of scintillation cocktail (1 l. of toluene containing 0.7% 2,5-diphenyloxazole and 10% Bio-Solv BBS3) in a Beckman LS 250 liquid scintillation system to a counting error of 1% or less. Appropriate corrections were made for protein content and the results reported in nanograms bound per milligram of protein.

Polyacrylamide Disc Gel Electrophoresis. Protein solutions were subjected to analytical disc gel electrophoresis in Tris-glycine buffer (pH 8.3) using a modification of the method of Davis (1964). Gels were stained for protein with Amido Schwarz and Coomassi Brilliant Blue and for sugar using the periodic acid-Schiff (PAS) reaction (Zacharius et al., 1969). An unstained gel was fractionated with a Savant Autogel divider into 12-drop fractions and measured for radioactivity.

Sodium Dodecyl Sulfate Gel Electrophoresis. Protein samples were incubated for 3 hr at room temperature with an equal volume of 0.01 *M* phosphate buffer (pH 7.1) containing 20% glycerine, 2% dodecyl sulfate, and 2% 2-mercaptoethanol. Electrophoresis, staining, and destaining procedures and molecular weight calculations were carried out with known marker proteins (cytochrome *c*, ovalbumin, bovine serum albumin) as described by Weber and Osborn (1969) using a 10% gel.

Sephadex Gel Filtration. The molecular weights of the folate binding proteins were estimated using a 2.2 × 85 cm column of Sephadex G-200 equilibrated with 0.1 *M* phosphate buffer containing 0.5 *M* NaCl and 0.02% sodium azide at pH 7.2. [^{3}H]PGA-tagged samples in a total volume of 2.0 ml of buffer were applied directly to the top of the column; 5-ml fractions were collected in an LKB 7000 fraction collector and protein was monitored at 280 nm using a Uvicord II. The gel filtration data are expressed in terms of K_{av} as defined by Laurent and Killander (1964). Blue dextran 2000 was used to determine the value of V_0. Phenol red was used to determine the value of V_t and the column was calibrated with the following proteins of known molecular weight: ribonuclease (13700), chymotrypsin A (25000), ovalbumin (45000), bovine serum albumin (67000), and aldolase (158000).

Sucrose Density Gradient Analysis. Protein samples dissolved in 0.1 *M* phosphate buffer (pH 7.4) and tagged with [^{3}H]PGA were dialyzed overnight against 0.9% NaCl at 4°C. The dialysand was placed on 5 ml of a 10–40% linear sucrose gradient in 0.01 *M* Tris buffer containing 0.1 *M* NaCl and 0.001 *M* Na_2EDTA at pH 7.0. Following centrifugation at 65000 rpm for 6 hr in an SW 65 rotor, 0.3-ml fractions were collected and analyzed for protein, radioactivity, and sucrose concentration.

Isoelectric Focusing. Protein samples were submitted to isoelectric focusing in an LKB 8100 ampholine column with a capacity of 110 ml. Ampholytes (1%) having a pH range of 3–10 or 5–8 were stabilized with sucrose in a linear gradient of 5–50%. Electrofocusing was started with an output voltage of 400 V and an initial power output of 3 W. Focusing was complete within 3 days at which time the column was emptied and 2-ml fractions were collected. Folate binding activity of each fraction was determined by the ability to bind [^{3}H]PGA as described above. Protein was determined by optical density at 280 mn in a Gilford spectrophotometer.

Concanavalin A Sepharose Affinity Chromatography. Concanavalin A Sepharose (Pharmacia) was packed into a 1.0-cm column to a height of 6.0 cm and washed through with 200 ml of buffer (0.1 *M* Tris (pH 7.2) containing 1 *M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, and 1 m*M* $CaCl_2$). Purified FABP was incubated with an excess of [^{3}H]PGA and dialyzed overnight against 0.9% saline to remove unbound [^{3}H]PGA. The [^{3}H]PGA–FABP was placed on the column

Table I: Purification of Folate Binding Protein from Human Milk.

Item	Volume (ml)	Total Protein (mg)	Total FABP (μg of Folic Acid Bound)	Recovery of Total FABP (%)	Specific Activity (μg of Folic Acid Bound/mg of Protein)	Purification
Clarified milk	6000	56415	40.5	100	0.0007	1
Acid charcoal treated milk	5000	7350	38.7	95	0.005	7
Affinity column eluates						
(1) Unretained milk	5000	7262	17.1	42	0.0025	
(2) 0.02 *M* phosphate buffer (0.1 *M* NaCl) pH 7.2	1000		0.16	0.3		
(3) 1 *M* NaCl	500		0.18	0.4		
(4) 0.2 *M* acetic acid	50	22.5	15.2	37	0.67	1000
DEAE-cellulose (0.001 *M* phosphate buffer pH 6.3 fraction)		1.8	13.1	32	7.2	10000

and washed with buffer until the eluate was free of radioactivity. The bound FABP was stripped from the column by the addition of 5% methyl α-mannoside; 4.0-ml fractions were collected and aliquots counted for [^{3}H]PGA activity.

Uptake Studies in HeLa Cell Cultures. The uptake of [^{3}H]PGA and the effect of purified FABP on this process were studied as previously described (Waxman and Schreiber, 1974c). HeLa cells were grown in monolayer culture using Minimal Essential Medium-Earle's salts containing 10% fetal calf serum and 4 m*M* glutamine. For the [^{3}H]PGA uptake experiments, the monolayer was washed with Hank's balanced salt solution (HBSS) and the [^{3}H]PGA, alone or with various pure FABP, was incubated in the monolayer culture for 3 hr at 37°C with HBSS as the culture medium. The FABP obtained from the isoelectric focusing column was separated from the sucrose and ampholytes by G-25 Sephadex column chromatography before use in the uptake experiments. The monolayer was then washed two times with HBSS and the cells were harvested and dissolved in 1.0 ml of NCS reagent (Amersham/Searle) prior to counting. Radioactivity recovered was calculated as percent uptake per 1×10^7 cells.

Results

Purification of the Low Molecular Weight Folate Binding Protein. The preliminary preparation of pooled human milk (unpasteurized) was purified sevenfold by acid charcoal adsorption (Table I). Approximately 42% of the milk FABP when passaged through the PGA-Sepharose affinity column was not retained. This FABP was predominantly the high molecular weight peak (>200000 mol wt) as determined by Sephadex G-200 gel filtration and was not further studied (Figure 1). The retained FABP (low molecular weight) was eluted in 0.2 *M* acetic acid from the PGA-Sepharose column with a recovery of 37% of the total (whole milk) FABP with a specific activity of 0.67 μg of PGA bound per mg of protein. This represents nearly a 150-fold purification. The FABP was rapidly stripped from the PGA-Sepharose column. Prolonged exposure to 0.2 *M* acetic acid did not remove the remainder (21%) of the FABP from the column. There was no increase in the recovery of FABP removed from the affinity column when dialyzed in the presence of excess PGA. The FABP was purified to homogeneity by passage through DEAE-cellulose. The FABP was recovered in a single peak using 0.001 *M* phosphate buffer (pH 6.3) and had a specific activity of 7.0 μg of PGA bound per mg of protein. This represents a 10000-fold purification and was used for the remainder of the studies. Stepwise elution in buffers of increasing ionic strength did not recover additional peaks of FABP.

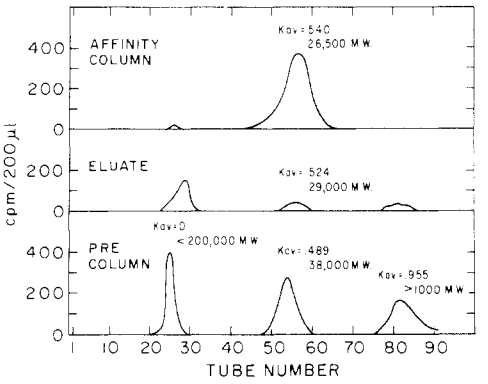

FIGURE 1: Determination of the apparent molecular weight of FABP by gel filtration. [^{3}H]PGA-labeled FABP was passed through a 2.2 × 85 cm column of G-200 Sephadex equilibrated with a 0.1 *M* phosphate buffer containing 0.5 *M* NaCl and 0.02% sodium azide. PRE-COLUMN, clarified milk; ELUATE, milk FABP that did not adhere to affinity column; AFFINITY COLUMN, milk FABP that adhered to affinity column and eluted in 0.2 *M* acetic acid.

Characterization of Purified FABP. FABP was more stable at −10°C than at −70°C, 4°C, or room temperature as determined by its ability to bind [^{3}H]PGA. The addition of saturating amounts of PGA appeared to stabilize FABP binding capacity. FABP incubated with 8 *M* urea did not bind [^{3}H]PGA. The binding of PGA to FABP is pH dependent with a maximum from pH 6.8 to 10.0 in 0.1 *M* Na-KPO_4 buffer. FABP did not exhibit dihydrofolate reductase activity.

Sucrose Density Gradient Centrifugation. The FABP was recovered in a single peak with a buoyant density of 23.4% sucrose. This FABP had a specific activity of 7.2 μg bound per mg of protein (Figure 2).

Polyacrylamide Disc Gel Electrophoresis. When 30–60 μg of the folate binding protein-[^{3}H]PGA complex was subjected to polyacrylamide disc electrophoresis and stained for protein, two bands were observed (Figure 3) Unstained gels were fractionated into 12-drop fractions and the distribution of [^{3}H]PGA was determined by measuring the radioactivity of the individual gel fractions. A double peak of radioactivity was observed which coincided with the

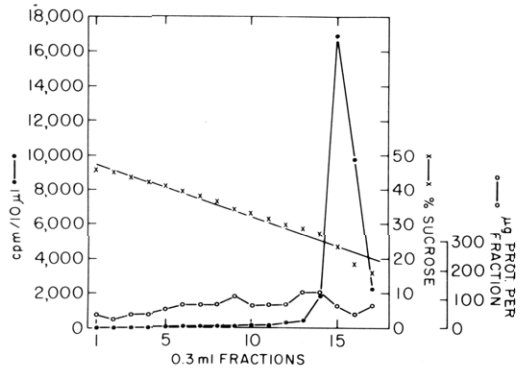

FIGURE 2: Sucrose density gradient of [^{3}H]PGA-labeled FABP recovered from affinity column.

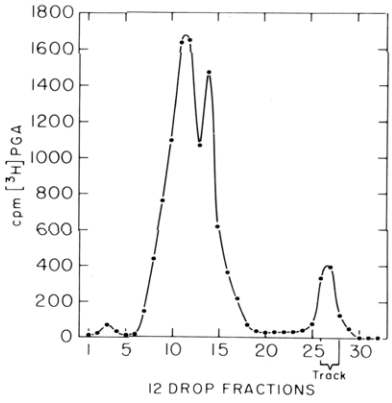

FIGURE 4: [^{3}H]PGA–FABP peaks in fractions of polyacrylamide disc gel electrophoresis of 30 µg of FABP containing 220 ng of bound PGA.

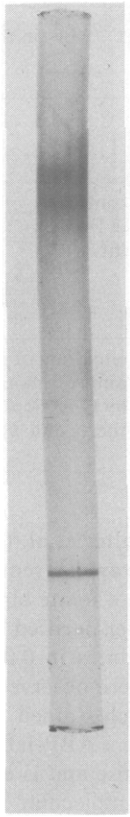

FIGURE 3: Polyacrylamide disc gel electrophoresis of 30 µg of FABP containing 220 ng of bound PGA. The band in the lower 1/3 of the gel is the tracker dye.

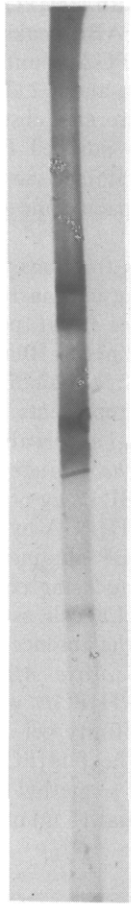

FIGURE 5: Sodium dodecyl sulfate polyacrylamide electrophoresis of 30 µg of FABP.

gel region that stained for protein (Figure 4). A minor peak of radioactivity was recovered in the region of the tracking dye which probably represents either free [^{3}H]PGA or breakdown products of the [^{3}H]PGA. A similar gel, following exhaustive washing with 10% Cl_3CCOOH stained faintly pink with PAS in the region of the two protein bands of FABP.

Molecular Weight Determination by Gel Filtration. When 50 µg of the FABP–[^{3}H]PGA complex was applied to the calibrated column of Sephadex G-200, a major symmetrical peak of radioactivity was observed with an apparent molecular weight of 26500 (Figure 1). A minor peak of radioactivity was observed in the void volume with an apparent molecular weight of >200000. The major peak of radioactivity recovered in the pure milk FABP was approximately 30% smaller in weight than the native milk FABP.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. When 30 µg of FABP was subjected to dodecyl sulfate polyacrylamide gel electrophoresis and stained for protein, three double bands were observed (Figure 5). The molecular weight estimation based on the position of the bands was 87000:80000, 36500:30000, 19000:11500. The larger molecular weight bands are difficult to interpret and may represent denatured FABP.

Isoelectric Focusing Experiments. Partially purified FABP (1 mg with a specific activity of 1 µg of PGA bound per mg of protein) eluted from the PGA-Sepharose affinity column was further purified and analyzed using isoelectric focusing in ampholytes with a pH range of 3–10 (Figure 6).

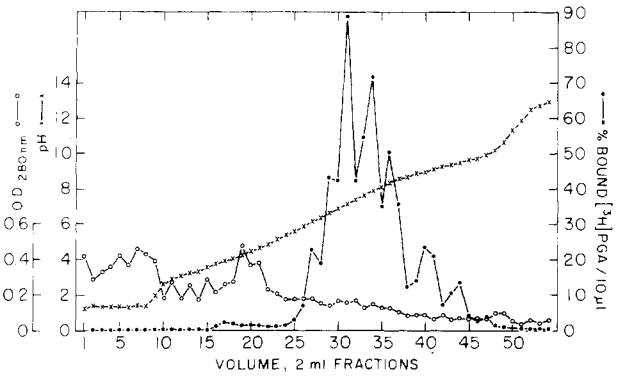

FIGURE 6: Isoelectric focusing of partially purified FABP* in pH range 3–10.

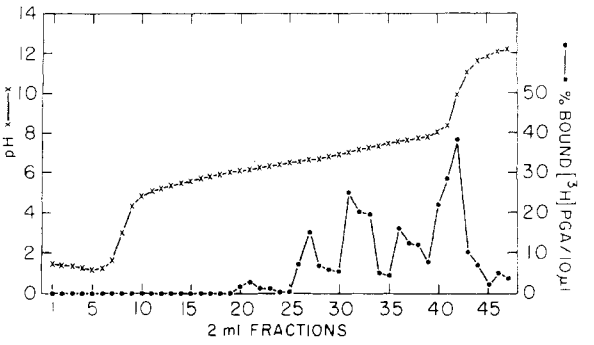

FIGURE 7: Narrow range isoelectric focusing (pH 5–8) of pooled pure FABP peaks obtained from fractions 25 to 38 of the pH 3–10 isoelectric focusing column.

Similar patterns were obtained whether the FABP was unsaturated or saturated with [^{3}H]PGA prior to isoelectric focusing. Three distinct FABP peaks with a specific activity of greater than 7 μg of PGA bound per mg of protein were recovered at pH 6.8, 7.5, and 8.2. Three minor peaks of folate binding activity were also observed. Most of the total protein in the partially purified FABP was separated at lower pH levels and contained no FABP activity, but did contain iron binding capacity and probably represents lactoferrin (fractions 5–22).

The three FABP peak fractions were pooled and submitted to isoelectric focusing in a narrow pH (5–8) ampholyte column (Figure 7). Three FABP peaks in similar locations (pH 6.7, 7.2, 7.8) to the pH 3–10 ampholyte gradient were recovered. The peak of folate binding activity at the end of the gradient (pH >10) represents free [^{3}H]PGA since the radioactivity was charcoal adsorbable.

Effect of FABP in the Uptake of [^{3}H]PGA to HeLa Cells. The effect of FABP (7 μg of PGA bound/mg of protein) on the uptake of [^{3}H]PGA by HeLa cells was studied. [^{3}H]PGA bound to FABP obtained from the three FABP peaks of the isoelectric focusing column was less available for uptake into the HeLa cell as compared to a similar amount of [^{3}H]PGA in the absence of FABP (Table II).

Concanavalin A Sepharose Affinity Chromatography. FABP saturated with [^{3}H]PGA was retained (87%) onto the Con A Sepharose affinity gel after ten column washes with the Tris buffer. The [^{3}H]PGA–FABP complex was rapidly eluted by the 5% methyl α-mannoside with over 60% recovered in the initial 15 ml of eluate.

Table II: The Effect of Pure FABP on the Uptake of [^{3}H]PGA into HeLa Cells.

Materials	% Uptake of 0.5 ng of [^{3}H]PGA/ 10^7 Cells
[^{3}H]PGA alone (control)	7.2
[^{3}H]PGA and pure FABP[a] isoelectric point	
pH 6.8	1.4
pH 7.5	1.1
pH 8.2	1.2

[a] FABP (7.4 μg of PGA bound/mg of protein) recovered in the three isoelectric focusing points of human milk FABP. Ampholytes and sucrose were removed by G-25 Sephadex chromatography. Experiments were done in triplicate and were reproducible within 10% error.

Discussion

Low molecular weight folate binding protein has been purified from human milk using affinity chromatography as the major purification technique. An initial preparation using acid charcoal improved specific activity by probably releasing and removing bound endogenous folate, as well as denaturing or precipitating some of the milk protein. The high molecular weight FABP (>200000) was separated from the low molecular weight FABP since it was excluded by the PGA-Sepharose affinity column and recovered in the milk eluate.

The remainder of the milk FABP representing low molecular weight FABP binds tightly to the PGA-Sepharose and remains bound while the PGA-Sepharose is washed with large volumes of a number of solutions of varying salt concentrations. We found it more effective to use 0.2 *M* acetic acid to desorb FABP from the PGA-Sepharose (Selhub and Grossowicz, 1973) rather than the 8 *M* urea at pH 5 which was used by Salter et al. (1972) for the cow's milk FABP. The FABP is rapidly removed by the acetic acid without significant loss of folate binding activity.

The FABP was further purified by DEAE-cellulose chromatography where it elutes in 0.001 *M* phosphate buffer. Two bands of FABP were observed on polyacrylamide disc gel electrophoresis which stained positive for both protein and sugar. Most of the FABP labeled with [^{3}H]PGA adsorbs to Con A Sepharose and is eluted with 5% methyl α-mannoside. The lower molecular weight determination by Sephadex gel filtration of the purified FABP as compared to the native FABP may be due to interaction of the sugar residues on the protein with the Sephadex beads. Therefore, the FABP which adheres to the PGA-Sepharose column represents at least two basic proteins, one or both being glycoprotein. In support of this, we have observed that FABP isolated from goat milk contains sialic acid in its structure (Rubinoff et al., 1975).

Dodecyl sulfate polyacrylamide gel electrophoresis of pure FABP revealed three double bands of protein. Two bands were similar in size to the FABP activity recovered by Sephadex gel filtration. The smaller bands suggest that the two FABP may break down into two subunits or monomers which are held together by hydrogen bonds. The larger bands are difficult to explain but may represent concentration dependent tetramers similar to that found with purified cow's milk FABP (Salter et al., 1972). It is possible that the conditions of low pH in eluting the protein from the affinity column may have been harsh enough to cause some deamidation and/or irreversible conformational changes to account for the larger number of bands observed.

Isoelectric focusing of post PGA-Sepharose column FABP revealed three major peaks and three minor peaks of FABP at near neutral or alkaline pH. Three peaks of FABP with similar isoelectric points have been found in cow's milk (Waxman and Schreiber, 1974a) and goat's milk even when the latter was immunologically homogeneous and demonstrated a single N-terminal amino acid (Rubinoff et al., 1975). The multiple peaks of FABP may represent isoproteins and may be related to a varying amount of sugar moieties in the glycoprotein, similar to that described for the "R" type vitamin B_{12} binding proteins (Stenman et al., 1968). The majority of the contaminating protein which was recovered from the PGA-Sepharose column with the FABP separated at a lower pH and contained iron-binding capacity and was, presumably, lactoferrin. No iron binding activity was recovered in the three peaks of FABP. The isoelectric points of the FABP are most consistent with β or γ globulins. These findings are similar to other studies on unpurified human (Markkanen and Peltola, 1971) and pig (Mantzos et al., 1974) serum FABP which suggest that FABP may represent a group of proteins which include a macroglobulin, β globulin and γ globulin.

There does not appear to be a functional difference between the various pure FABP separated by isoelectric focusing since all three peaks retarded uptake of [^{3}H]PGA into HeLa cells. The inhibition of uptake was previously reported for crude human milk and serum FABP and indicates that the final preparation of pure FABP retains its native characteristics. There is preliminary evidence that FABP preincubated with the cell may enhance attachment of the PGA to the membrane of the HeLa cell within the first 5 min of exposure (Waxman and Schreiber, 1974c) and this is being further studied in our laboratory at the present time. It has been previously shown that there is an initial, rapid uptake of PGA and methotrexate which may be moderated by membrane attachment sites (Lichtenstein et al., 1969). The glycoprotein nature of FABP and the finding of similar FABP in the lymphocyte membrane (Waxman, 1975) and also in the brush border membranes of the rat small intestinal mucosa cell (Leslie and Rowe, 1972) lend further evidence to its role as a component of a membrane carrier system for folate transport. The release of FABP bound methyl tetrahydrofolate may result by exchange with a more oxidized folate or a folyl polyglutamate.

PGA can be separated from FABP by urea, guanidine, low pH, and negative ions. This may be due to disruption of hydrogen bonds, alteration of protein conformation, or indirect alteration of hydrophobic bonding. PGA has a greater affinity for pure FABP and can dissociate or exchange with methyl tetrahydrofolate previously bound to FABP (Waxman and Schreiber, 1975). This could explain why there is unsaturated FABP in serum in the presence of considerably more unbound folate since serum folate is mainly methyl tetrahydrofolate. Moreover, recent evidence obtained by radioassay and immunologic methods shows that a small portion of serum folate is in the oxidized form and is bound to FABP (Rothenberg et al., 1973; Waxman et al., 1974).

The importance of FABP in milk remains to be determined. It is clear that the FABP bound oxidized folate is unavailable for cellular uptake and could significantly affect a folate dependent cell or organism such as inhibiting the growth of folate-dependent bacteria (Ford, 1964). Therefore, FABP in milk could affect the flora of the gastrointestinal tract, or remove folate from mother to child during lactation. FABP in the cell may be important in the regulation of folate coenzyme function since it avidly binds dihydrofolate and can significantly affect DNA synthesis (Rothenberg, 1970; Waxman, 1975). Finally, FABP-bound folyl polyglutamate is not converted to monoglutamate by conjugase (Waxman et al., 1974). Thus, the distribution of folyl polyglutamates may be regulated by FABP since FABP binds oxidized folyl polyglutamates as well as monoglutamates.

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Subunit Interactions in Yeast Glyceraldehyde-3-Phosphate Dehydrogenase†

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ABSTRACT: The spontaneous inactivation of yeast glyceraldehyde-3-phosphate dehydrogenase was found to fit a simple two-state model at pH 8.5 and 25°. The first step is a relatively rapid dissociation of the tetramer to dimers with the equilibrium largely in favor of the tetramer. In the absence of NAD^+ the dimer inactivates irreversibly. The apoenzyme is quite stable with a half-life for complete activity loss proportional to the square root of the enzyme concentration. Perturbations of the protein structure (by pH, ionic strength, and specific salts), which have no effect on the tetrameric state of the molecule, result in an alteration of the cooperativity of NAD^+ binding, the reactivity of the active-site sulfhydryl group, and the catalytic activity of the enzyme. Covalent modification of two of the four active-site sulfhydryl groups has profound effects on the enzymic activity which are mediated by changes in the subunit interactions. Sedimentation analysis and hybridization studies indicate that the interaction between subunits remains strong after covalent modification. Under normal physiological and equilibrium dialysis conditions the protein is a tetramer. Equilibrium dialysis studies of NAD^+ binding to the enzyme at pH 8.5 and 25° reveal a mixed cooperativity pattern. A model consistent with these observations and the observed half-of-the-sites reactivity is that of ligand induced sequential conformational changes which are transferred across strongly interacting subunit domains. Methods for distinguishing negatively cooperative binding patterns from mixtures of denatured enzyme and multiple species are discussed.

Analysis of subunit interactions is an important tool for delineating the nature of protein conformational changes (Koshland, 1970). Although the nature of the communication between subunits is fundamentally similar to the communication between sites within the same peptide chain, there are some advantages to the study of intersubunit effects. It is difficult to directly relate conformational changes within a subunit to energy changes, but cooperativity can be expressed in terms of ligand binding constants and these in turn to the free energy of subunit interactions. Moreover, association–dissociation experiments can give further insight into the energetics of the subunit contacts.

A particularly attractive protein for these studies is glyceraldehyde-3-phosphate dehydrogenase since the crystallography of the enzyme (Watson et al., 1972; Buehner et al., 1973, 1974), its primary structure (Jones and Harris, 1972), its mechanism (Krimsky and Racker, 1963; Furfine and Velick, 1965; Trentham, 1971; Orsi and Cleland, 1972; Harrigan and Trentham, 1973), and its cooperativity patterns (Conway and Koshland, 1968; DeVijlder and Slater, 1968; Velick, 1970; Smith and Velick, 1972) have been investigated extensively. Studies on the acylation and alkylation of the yeast enzyme clearly reveal a half-of-the-sites reactivity pattern which has been described in terms of negatively cooperative interactions (Stallcup and Koshland, 1973a–c). The NAD^+ binding pattern is more ambiguous with evidence of both concerted and sequential binding patterns (Kirschner et al., 1966; Kirschner, 1971; Cook and Koshland, 1970; Sloan and Velick, 1973). Moreover the intersubunit communication in association–dissociation is not clear. Accordingly studies were initiated to focus on the subunit interactions with the view of clarifying their properties and of relating them to dehydrogenases from other sources.

Materials and Methods

GPD[1] from *Saccharomyces cerevisiae* was prepared as described previously (Stallcup et al., 1972) except that the cells were broken with a Manton Gaulin-APV homogenizer (Hetherington et al., 1971) instead of by grinding with alumina. This more rapid process reduced the time required for obtaining the crude extract (from 1 hr to 5 min) and resulted in a twofold increase in final yield of enzyme (~400

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[1] Abbreviations used are: GPD, glyceraldehyde-3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12]; G3P, glyceraldehyde 3-phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxylmethyl)-aminomethane; N_S, number of substrate molecules bound per mole of enzyme, e.g., $[NAD^+]_b/[E_t]$.