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Purification, properties, and immunological characterization of folate-binding proteins from human leukemia cells

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A new matrix for affinity chromatography using pteroylglutamic acid coupled to an epoxy-activated matrix via hexanediamine resulted in negligible ligand leakage and permitted the purification of soluble and membrane-associated folate-binding proteins from human leukemia cells contained in a human spleen. Two species of membrane-associated folate-binding proteins were purified from the solubilized membrane fraction of the tissue using 2 M guanidine-HCl to elute the proteins from the affinity matrix. The higher molecular weight binding protein had an M_r of approximately 310 000 and the smaller species had an M_r of approximately 28 000 by gel filtration. By SDS-polyacrylamide gel electrophoresis the smaller species of membrane-associated protein had a molecular weight of 35 500, but the molecular weight of the larger membrane-associated species could not be determined by this method because of the high concentration of residual Triton X-100 in the sample which interfered with the silver staining of the gel. Two folate-binding proteins, which by SDS-polyacrylamide gel electrophoresis had molecular weights of 34 500 and 32 000, were purified from the 44 000 × *g* supernatant fraction of the tissue homogenate by acid elution from the affinity matrix. Despite the different cell components from which the soluble and membrane-associated folate-binding proteins were purified, the amino acid compositions were similar, especially with respect to the apolar amino acids. All these forms of folate-binding proteins had higher affinity for oxidized than for reduced folates, and very low affinity for 5-formyltetrahydrofolate and methotrexate. Although these proteins cross-react with one antiserum raised previously to a folate-binding protein from other human leukemia cells, they do not cross-react with the folate-binding proteins purified from two other sources of human leukemia cells, from human placenta, or from the human KB cell line.

Introduction

Folate-binding proteins have been identified in both the soluble cytoplasmic and membrane components of mammalian cells and tissues and from biological fluids (for a recent review, see Ref. 1). The soluble forms of folate-binding proteins have been purified from leukemic leucocytes [2], milk [3,4], human spleen [5,6], and from biological fluids [7,8], while the folate-binding proteins which have been isolated from the choroid plexus [9,10], intestinal mucosa [11], brush borders of renal tubular epithelium [12], human placenta [13], human leukemia cells [6], and the cultured human KB cell line [14] are hydrophobic integral membrane proteins which require detergents for solubilization. Although the soluble and membrane-associated

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folate-binding proteins differ in hydrophobicity, they have similar ligand-binding properties, with an affinity for oxidized folates that is several-fold greater than their affinity for reduced folates. Soluble and membrane-associated folate-binding proteins which have been isolated from the same cells, are antigenically homologous [15,16], but there is also evidence that functionally and structurallly similar folate-binding proteins which are isolated from different human cells and which are functionally and structurally similar differ antigenically [5].

We recently described the purification and properties of a soluble and one form of membrane-associated folate-binding protein from the spleen of a patient with a blast crisis of chronic myelogenous leukemia [6]. In that study, the partially purified preparation contained two membrane-associated folate-binding proteins which could be separated by gel filtration, one having an M_r of approximately 310 000 and the other an M_r of approximately 35 000. During purification by affinity chromatography, the higher molecular weight species was lost and could not be further characterized. We have subsequently obtained the spleen from another patient with a similar hematologic disease, and by modifying the method of coupling the ligand, pteroylglutamic acid, to the affinity matrix, we have improved the yield of the purified folate-binding proteins, and by changing the method of elution of the membrane-associated folate-binding protein from the affinity column, we have been able to purify a high and low molecular weight species. In addition, because of the apparent immunologic heterogeneity of the human folate-binding proteins, we have analyzed the cross-reactivity of a number of folate-binding proteins which have been purified in our laboratory using antisera raised to four different human folate-binding proteins.

Materials and Methods

Pteroylglutamic acid (PteGlu), dihydrofolate (H_2PteGlu), N^5-methyltetrahydrofolate, (methyl-H_4-PteGlu), bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), and 2-aminoethanol were purchased from Sigma Chemical Company (St. Louis, MO). Triton X-100 was obtained from International Biotechnologies Inc. (New Haven, CT). Sepharose 6B and dextran T70 were obtained from Pharmacia (Piscataway, NJ) 1,4-Butane dioldiglycidyl ether, hexanediamine, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were obtained from Aldrich Chemical Company (Milwaukee, WI). Trasylol was obtained from Mobay Chemicals (New York, NY). [^{3}H]PteGlu with a specific activity of 34 Ci/mmol was purchased from Amersham Corporation (Arlington Heights, IL). The [^{3}H]PteGlu was greater than 95% pure as determined by $ZnSO_4$ precipitation [16] and it was stored at −80 °C. Methotrexate and dl-N^5-formyl-H_4PteGlu were purchased from Lederle Laboratories (Pearl River, NY). Methotrexate was purified by the method of Gallelii and Yokoyama [17]. Folylpolyglutamate$_7$ (PteGlu$_7$) was purchased from Dr. Charles Baugh (University of South Alabama, Mobile, AL). Norit-A charcoal was purchased from Amend Drug and Chemical Company (Irvington, NJ). The scintillation cocktail, 3a70, was purchased from Research Products International, (Elkgrove, IL). Glass fiber filter (GF/C) was purchased from Whatman (Clifton, NJ). Marker proteins for SDS-polyacrylamide gel electrophoresis, the protein silver staining kit, and protein-assay kit were purchased from Bio-Rad Laboratories (Richmond, CA).

The concentration of the folate compounds was determined by their absorbance using the published values for their absorption coefficients [18]. The protein concentration in crude preparations was measured by the modified biuret method [19].

Preparation of PteGlu affinity matrix

Epoxy activation of Sepharose 6B. Sepharose 6B (7 ml) was transferred to Whatman No. 1 filter paper on a Buchner funnel, washed with 2 liters of deionized water, then suspended in 5 ml of 1 M NaOH containing sodium borohydride (2 mg/ml) and 1 ml of 1,4-butanedioldiglycidyl ether, and the suspension was stirred gently for 5 h at 25 °C.

Substitution with hexanediamine. The epoxy-activated Sepharose 6B was filtered through a fritted glass funnel under vacuum and washed with 1 liter of deionized water, follwed by 1 liter of 0.1 M sodium bicarbonate buffer, pH 8.0. The moist cake of activated Sepharose was suspended

in 7 ml of 2 M hexanediamine, the pH adjusted to 10.0 by the drop-wise addition of concentrated NaOH, and the suspension rotated slowly for 48 h at 25 °C. Under this alkaline condition, the amino group of hexanediamine couples covalently to the epoxy adduct on the Sepharose 6B. The substituted Sepharose 6B was washed sequentially with 1 liter each of deionized water, 0.1 M sodium bicarbonate, pH 8.0, and 0.1 M acetate, pH 4.0. The uncoupled epoxy groups in the activated Sepharose 6B were then blocked by treating the gel with 7 ml of 1 M 2-aminoethanol, pH 10.0, for 1 h at 25 °C.

Coupling of PteGlu to the substituted Sepharose 6B. The substituted Sepharose was washed with 1 liter of deionized water and stirred gently in 8.5 ml of water containing 122 mg of PteGlu. The pH of the solution was raised to approx. 7.0 by the drop-wise addition of 1 M NaOH in order to dissolve the PteGlu. 2 ml of a freshly prepared solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (100 mg/ml) were added over a period of 5 min and the mixture was then gently rotated in the dark for 60 h at 25 °C. Under these conditions, either the alpha or the gamma carboxyl groups of PteGlu couple covalently to the free terminal amino groups of the hexanediamine.

The Sepharose 6B-PteGlu gel was washed with 2 liters of 0.1 M glycine-NaOH, pH 10.0, containing 1 M NaCl, followed by 2 liters of 0.2 M acetic acid. At the end of the acetic acid wash the absorbancy at 282 nm was zero, indicating that the concentration of PteGlu eluting in the wash was less than 1 μg/l. The gel was then washed with 200 ml of 8 M guanidine-HCl followed by continuous washing with 10 litres of 0.1 M potassium phosphate buffer, pH 7.5, for 16 h at 4 °C. The concentration of PteGlu in this final wash buffer was less than 10 pg/ml as measured by a competitive ligand binding-radioassay [20]. The amount of PteGlu which coupled was approximately 20 μmol/ml of Sepharose.

Purification of soluble folate-binding protein

The source of the folate-binding protein was the spleen from a patient with chronic myelogenous leukemia who developed a blastic crisis. The purification of the folate-binding proteins was carried out at 4 °C and all the buffers contained PMSF (1 mM) and Trasylol (10 U/ml).

The spleen (2100 g) was homogenized in a Waring blender with 3 vol. of 0.14 M potassium phosphate buffer, pH 7.5, and centrifuged at $44000 \times g$ for 30 min to separate the soluble folate-binding protein in the supernate from the membrane-associated folate-binding protein in the pelleted particulate fraction.

The soluble folate-binding protein was purified from the supernate as follows: The endogenous folates were dissociated from the folate-binding protein by lowering the pH to 3.0 by the drop-wise addition of 1 M HCl. After 1 h at this pH the dissociated folates were removed by adding Norit-A charcoal (10 mg/ml) to the mixture, stirring the suspension for 1 h. The charcoal was then pelleted by centrifugation at $13000 \times g$ for 40 min. The pH of the supernatant solution was adjusted to 7.4 with 1 M NaOH. The precipitate which formed during this step was removed by centrifugation at $13000 \times g$ for 40 min. Crystalline $(NH_4)_2SO_4$ was added to the supernatant solution, first to a concentration of 45% (the precipitate that formed was discarded after centrifugation), and then to 85% of saturation. The precipitate which formed was dissolved in 500 ml of 0.01 M potassium phosphate buffer, pH 7.5, containing 0.15 M NaCl and the solution was passed slowly (16 h) through 7 ml of the epoxy-Sepharose 6B-PteGlu gel which was packed into a fritted glass funnel (pore size, 40–60 μ). The effluent was then passed again through the affinity gel over a 3 h period. More than 90% of the folate-binding protein was retained by the affinity gel. Non-specifically bound proteins were removed by washing the affinity gel with 0.01 M potassium phosphate, pH 7.5, containing 1 M NaCl, until the absorbance of the effluent at 280 nm was zero, followed by 0.01 M potassium phosphate, pH 7.5, until the absorbance was less than 0.01. The gel was finally washed with 500 ml of 0.001 M potassium phosphate buffer, pH 7.5.

The folate-binding protein eluted from the affinity resin with 0.01 M acetic acid, pH 3.0, containing 0.1 M NaCl. 4-ml fractions were collected in polypropylene test tubes containing 1.4 ml of 1 M potassium phosphate to raise the pH of the effluent to 7.4. The folate-binding protein in each fraction was measured by the binding of [^{3}H]PteGlu as previously described [6]. The frac-

tions containing the folate-binding protein were pooled, concentrated by ultrafiltration and dialyzed against deionized water.

Purification of the folate-binding protein from the cell membranes

The membrane-associated folate-binding protein was purified as follows. The 44000 × *g* pellet was solubilized with 1% Triton X-100 as described previously [6] and centrifuged at 44000 × *g* for 30 min at 4° C. The pH of the supernatant fraction (2885 ml) containing the solubilized folate-binding activity was lowered to 4.0 with 1 M HCl and stirred in an ice bath for 1 h. The precipitate was pelleted by centrifugation at 10000 × *g* for 15 min. This precipitate contained most of the folate-binding activity and it was dissolved in 0.01 M potassium phosphate, pH 7.5, containing 1% Triton X-100, 1 mM PMSF, and Trasylol (10 U/ml) by stirring for 16 h. Following centrifugation at 16000 × *g* for 15 min, the supernatant fraction was separated and saved, and the pellet was redissolved in 1% Triton X-100 buffer and the centrifugation repeated. Both supernates were combined (total volume, 2850 ml) and its folate-binding capacity was determined as previously described [6].

The solubilized membrane-associated folate-binding protein was also purified by affinity chromatography through epoxy-Sepharose 6B-PteGlu, but the procedure had to be modified, because, unlike the soluble folate-binding protein, the membrane-associated folate-binding protein was denatured at the acid pH required for dissociation of the purified protein from the affinity ligand. Accordingly, 5 ml of the affinity gel was placed in a 15-ml sintered glass funnel (pore size, 40–60 μ) and the solubilized membrane folate-binding protein was slowly passed through it over a 24-h period at 4° C. The gel, which retained 90% of the folate-binding capacity of the preparation, was then washed slowly with 2 liters of 0.01 M potassium phosphate, pH 7.5, containing 1% Triton X-100 (v/v) and 1 M NaCl, followed by 2 liters of 0.01 M potassium phosphate, pH 7.5, containing 1% Triton X-100 alone. The gel was then transferred to a 50-ml sterile polypropylene tube and gently rotated at 25° C in 25 ml of 2 M guanidine-HCl (ultra-pure) in 0.01 M potassium phosphate, pH 7.5, containing 1% Triton X-100 to dissociate the folate-binding protein. After 30 min, the gel was pelleted by centrifugation and the supernatant fraction was separated. This step was repeated 13 times, after which there was no dissociation of any additional folate-binding protein. The guanidine-HCl was removed by dialyzing these supernates for 5 h against 12 liters of 0.01 M potassium phosphate, pH 7.5, containing 1% Triton X-100. The buffer was then changed and the dialysis continued for an additional 19 h. The pooled samples were concentrated on an Amicon filter (PM-10) and the high and low molecular weight folate-binding proteins were separated by filtration through Sephadex G-100 with 0.01 M potassium phosphate buffer containing Triton X-100. The fractions containing the high molecular weight (815 ml) and low molecular weight (954 ml) folate-binding proteins were separately pooled, each pool was passed through the epoxy-Sepharose 6B-PteGlu affinity resin again and eluted into 21 ml and 77 ml, respectively, with 2 M guanidine-HCl. Triton X-100 was removed from the high molecular weight folate-binding protein by butanol extraction [21]. The detergent was removed from the low molecular weight folate-binding proteins using Extracti-gel (Pierce Chemicals, IL).

Measurement of PteGlu binding

The ligand-binding properties of the soluble and the membrane-associated folate-binding proteins, their relative affinities for the folate analogues, and their apparent association constants (K_a) for PteGlu were determined as previously described [6].

Gel filtration chromatography

The purified soluble and membrane-associated folate-binding proteins complexed with [^{3}H] PteGlu were chromatographed through Bio-Gel P-100 (1.6 × 98 cm) and Sephadex G-200 (2.6 × 86 cm), respectively, as previously described [6]. To chromatograph the membrane-associated folate-binding proteins, the column was equilibrated and eluted with 0.01 M potassium phosphate buffer containing 1% Triton X-100.

SDS-polyacrylamide electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [22] using 10% SDS-polyacrylamide and a 1.5-mm-thick slab gel. The purified folate-binding proteins were boiled for 2 min in 2% SDS solubilization buffer before application to the gel.

Amino acid and carbohydrate analysis

The purified folate-binding proteins were hydrolyzed with 6 N HCl containing 4% thioglycollic acid for 24 h at 110 °C. Amino acid analysis was performed using the fluorescamine amino acid analyzer [23]. The content and composition of the carbohydrate in the purified soluble folate-binding protein was determined using gas liquid chromatography following methanalysis, re-*N*-acetylation and derivatization with silyating reagent [24] by Dr. B. Slomiany, New York Medical College, Valhalla, NY.

Analysis of the immunoreactivity of folate-binding proteins

The comparative immunoreactivity of these purified folate-binding proteins as well as a number of other purified folate-binding proteins was determined using monospecific antiserums to these proteins. Antiserums were raised in rabbits to the folate-binding protein purified from CML cells (antiserum L) [2], to the folate-binding protein purified from the spleen of a patient with myelofibrosis (antiserum S) [5], and to the soluble folate-binding protein purified from the culture medium of KB cells (antiserum KB) [15]. Antiserum to the folate-binding protein purified from human placenta (antiserum PL) [13] was raised in a chicken and was kindly provided by Dr. Samuel Waxman, Mt. Sinai Medical Center, New York.

The soluble and the two membrane-derived folate-binding proteins purified in this study were immunoprecipitated with the antiserums L and S by the method previously described [6]. In addition, the cross-reactivity of four other purified folate-binding proteins was determined by the immunoprecipitation of these proteins complexed with [^{3}H]PteGlu by each of the antiserums. The assay was carried out as follows. 200 pg of [^{3}H]PteGlu were incubated for 30 min at room temperature with sufficient folate-binding protein to bind approximately 100 pg of the tracer. A 1 : 50 dilution of the antiserum (and control normal rabbit or normal chicken serum) was then added and the incubation continued overnight at 4 °C. The immune complex was precipitated by the addition of 0.02 ml of goat anti-rabbit (or goat anti-chicken) antiserum followed in 60 min by the addition of 0.3 ml of 14% poly(ethylene glycol). The precipitate was pelleted by centrifugation, washed once with buffer containing 0.1% Triton X-100, and then dissolved in NCS solubilizer (Amersham) and the radioactivity determined.

A second method of studying the immunoreactivity of the folate-binding proteins was to determine the blocking by the antiserum of the binding of [^{3}H]PteGlu. For this assay the same dilution of the folate-binding protein which was used for the immunoprecipitation assay was incubated overnight at 4 °C with a 1 : 50 dilution of the antiserum. Control reactions contained normal rabbit serum or normal chicken serum. Following this incubation, 200 pg of [^{3}H]PteGlu was added to the reaction and after an additional incubation for 30 min at 4 °C, the free [^{3}H]PteGlu was separated by adsorption to hemoglobin-coated charcoal as previously described [6]. The antisera and control sera were dialyzed against 0.01 M potassium phosphate buffer, pH 7.2, before they were used in order to remove endogenous free folates.

Results

Table I summarizes the results of the purification of the soluble and membrane-associated folate-binding proteins. The soluble folate-binding protein was purified over 25 000-fold, and 68% of the folate-binding capacity applied to the affinity gel was recovered. The molar ratio of bound PteGlu to protein was 1.36 : 1. That this ratio (calculated from an M_r of 33 500, which is the mean molecular weight of the two components on SDS-polyacrylamide gel electrophoresis) is greater than unity is most probably due to the experimental error incurred by measuring the folate-binding capacity of a greatly diluted preparation of the folate-binding protein. However, since this ratio is more rather than less than 1, there must be virtually no contaminating protein.

Of the total membrane-associated folate-bind-

TABLE I

PURIFICATION OF SOLUBLE AND MEMBRANE-ASSOCIATED FOLATE-BINDING PROTEINS

Step	Total protein (mg)		Total PteGlu bound (pmol)		Specific activity (pmol/mg protein)		Purification	
	soluble	membrane	soluble	membrane	soluble	membrane	soluble	membrane
Total homogenate	398068		66285		0.17		–	
44000 × *g* supernate	107625	–	6668	–	0.06	–	0.37	–
Triton-solubilized pellet	–	42121	–	9048	–	0.2148	–	1.3
Acidification (+charcoal)	58273	22820	92151 [a]	27170 [a]	1.58	1.19	9.5	7.15
$(NH_4)_2SO_4$ precipitate (45–85%)	14224	–	65408	–	4.6	–	28.0	–
Affinity chromatography	1.54	– [d]	62787 [b]	13022	40771	– [d]	25316 [c]	– [d]

[a] The increase in folate-binding capacity was due to the removal of endogenous folate by adsorption to charcoal following acidification.

[b] This represents a 68% recovery of the protein from the acid-treated 44000 × *g* supernate.

[c] This value was calculated from the specific activity of the acid-treated 44000 × *g* supernate.

[d] These values could not be determined because the protein was eluted from the affinity matrix in 325 ml of 2 M guanidine-HCl containing 1% Triton X-100 and the preparation could not be concentrated because of this detergent.

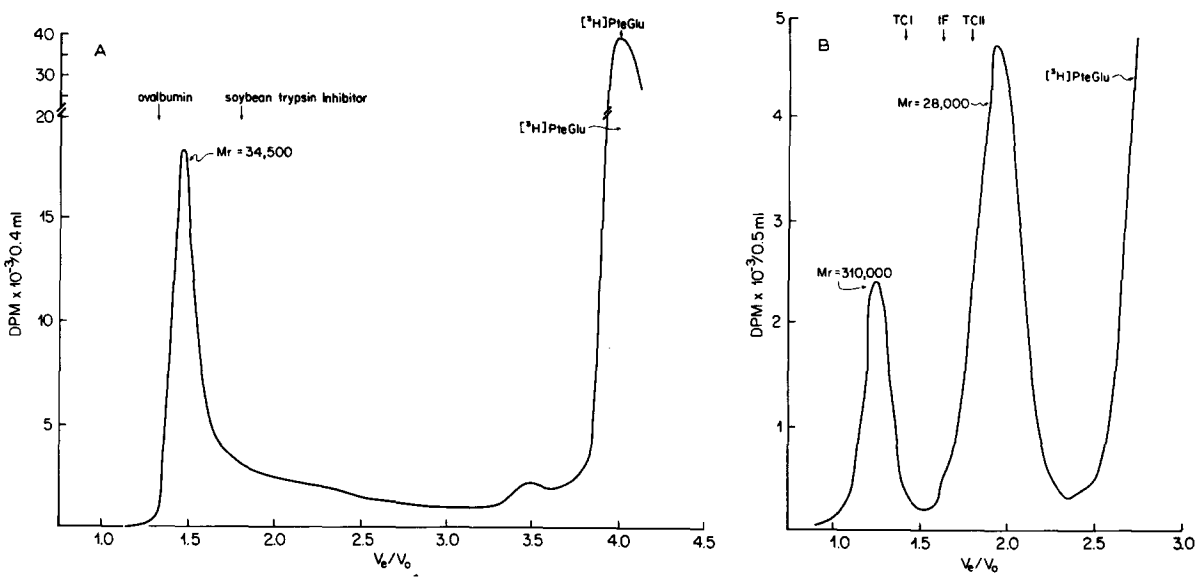

Fig. 1. Gel filtration through Bio-Gel P-100 and Sephadex G-200 of the purified folate-binding proteins complexed with [^{3}H]PteGlu. (A) The soluble folate-binding protein purified from the 44000 × *g* supernate (Bio-Gel P-100 column). (B) The purified folate-binding protein from the Triton X-100-solubilized 44000 × *g* pellet. The column was equilibrated and eluted with buffer containing 1% Triton X-100 (Sephadex G-200 column). The marker proteins were transcobalamin I (TCI, M_r 120 000), intrinsic factor (IF, M_r 60000) and transcobalamin II (TCII, M_r 44 000).

ing capacity (27.2 nmol) applied to the affinity gel, 48% was recovered by elution with the 2 M guanidine-HCl. However, because the folate-binding protein had to be eluted from the affinity matrix in a large volume (325 ml), and the elution buffer also contained Triton X-100, an accurate protein concentration could not be determined. This purified preparation of membrane-associated folate-binding protein contained both the high and low molecular weight forms (approx. 20% and approx. 80%, respectively), (see Fig. 1B below).

The gel filtration profiles of the soluble and membrane-associated folate-binding proteins complexed with [^{3}H]PteGlu are shown in Fig. 1A and B, respectively. The soluble protein eluted from Bio-Gel P-100 with a single peak at a $V_e/V_0 = 1.5$ (apparent $M_r \approx 34\,500$). The purified membrane-associated folate-binding proteins eluted from Sephadex G-100 (the eluting buffer contained Triton X-100) as two peaks within the included volume of the column; the high molecular weight species had an apparent M_r of approx. 310 000 and the smaller species had an apparent $M_r \approx$ 28 000.

Fig. 2 shows the SDS-polyacrylamide gel electrophoresis of the purified folate-binding proteins. The purified preparation from the soluble fraction of the cells resolved into two bands with an $M_r \approx$ 35 000 and $\approx$ 32 000 (Fig. 2A). The very sensitive silver stain shows three additional very lightly stained bands, one above and two below the purified protein bands. These minor bands were not visible when 4 μg of purified folate-binding protein were similarly analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue (not shown), and therefore these contaminants must represent a very small fraction of the purified folate-binding protein. The same two folate-binding protein bands were obtained by SDS-polyacrylamide gel electrophoresis under non-reducing conditions (data not shown).

The SDS-polyacrylamide gel electrophoresis of the purified smaller molecular weight species of the membrane-associated folate-binding protein shows a homogeneous band of $M_r \approx 35\,000$ (Fig. 2B). A band of a contaminating protein was also present but this represented less than 10% of the total stained protein, indicating that the folate-binding protein was greater than 90% pure. The

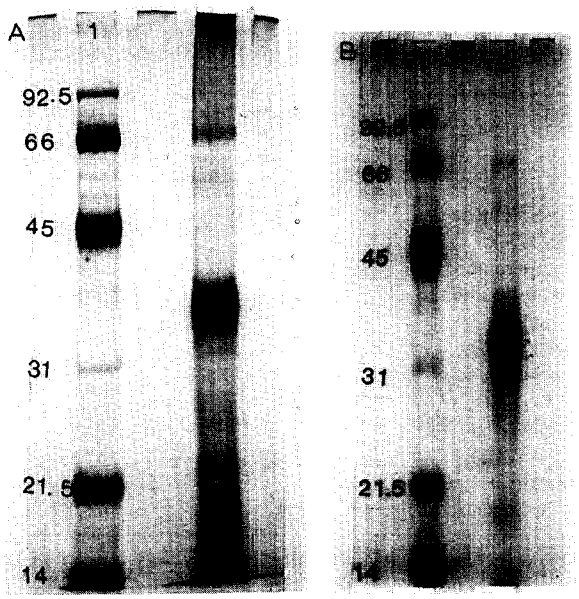

Fig. 2. SDS-polyacrylamide gel electrophoresis of the purified preparations of the folate-binding protein. (A) Lane 1, marker proteins ($M_r \times 10^3$); lane 2, the purified folate-binding protein from the 44 000 × g supernate. (B) Lane 1, marker proteins ($M_r \times 10^3$); lane 2, the purified folate-binding protein from the solubilized 44 000 × g pellet which eluted through Sephadex G-200 with an M_r of 28 000.

M_r of the higher molecular weight membrane-associated folate-binding protein could not be determined by SDS-polyacrylamide gel electrophoresis because the residual Triton X-100 in the sample interfered with the silver stain and we did not have sufficient material to repeat the SDS-polyacrylamide gel electrophoresis for staining with Coomassie blue. This folate-binding protein should, however, have the same degree of purity as the smaller molecular weight membrane-associated species since they were eluted from the affinity matrix together.

Table II lists the amino acid composition of the purified folate-binding proteins. Although the membrane-associated folate-binding proteins and the soluble form of folate-binding protein differ with respect to a number of polar amino acids, they contained a surprisingly similar fraction of apolar amino acids, the soluble containing 28%, and low and high molecular weight membrane-as-

TABLE II

AMINO ACID COMPOSITION OF THE PURIFIED FOLATE-BINDING PROTEINS [a]

Amino acid [b]	Soluble folate-binding proteins	Membrane-associated folate-binding proteins	
		low molecular weight	high molecular weight
Asp	12.9	4.2	8.4
Thr	6.4	5.7	5.4
Ser	8.9	5.0	5.4
Glu	12	11.7	8.5
Gly	6.8	11.7	18
Ala [c]	7.8	11.3	12.2
Val [c]	4.4	6.0	5.5
Met [c]	2.3	9.9	0.33
Ile [c]	2.0	3.6	3.0
Leu [c]	7.5	7.8	5.1
Tyr	3.5	2.8	2.3
Phe [c]	4.2	3.3	2.2
His	6.0	2.5	3.11
Lys	7.8	6.6	4.3
Arg	6.7	5.9	3.3
Carbohydrate composition [d,e]			
fucose	7		
mannose	35		
galactose	33		
N-acetyl-glucosamine	13		
sialic acid	10		

[a] Values are the percent of total residues and are the means of two determinations.

[b] Amino acids Pro, Cys and Trp were not determined. Residues Asn and Gln are identified as Asp and Glu, respectively.

[c] Indicates the hydrophobic amino acids.

[d] Values listed are percent of total carbohydrates.

[e] There was insufficient purified membrane-associated folate-binding protein for this determination.

sociated proteins containing 34 and 28%, respectively.

Mannose and galactose were the predominant sugars comprising the carbohydrate moiety of the soluble folate-binding protein (Table II), with substantially lower amounts of *N*-acetylglucosamine, sialic acid, and fucose. There was an insufficient amount of the purified membrane-associated folate-binding proteins to determine their carbohydrate composition.

The K_a for the binding of [^{3}H]PteGlu by the purified soluble and purified high and low molecular weight species of membrane-associated folate-binding proteins, computed using the double-reciprocal plot [25], was 9.7 nM^{-1}, 96 nM^{-1} and 40 nM^{-1}, respectively.

The relative affinity of the folate-binding proteins for oxidized and reduced folates, and methotrexate, are listed in Table III. Each of the three purified folate-binding proteins has the highest affinity for PteGlu and $PteGlu_7$. The soluble folate-binding protein appeared to have a higher affinity for methyl-H_4PteGlu than for H_2PteGlu, distinguishing it from the two membrane-associated folate-binding proteins which had decreasing affinity for H_2PteGlu, and methyl-H_4PteGlu. All three folate-binding proteins had substantially lower affinity for 5-formyl-H_4PteGlu and methotrexate. Of the two membrane-associated folate-binding proteins, the higher molecular weight species appeared to have a higher affinity for methyl-H_4PteGlu, 5-formyl-H_4PteGlu, and methotrexate.

The soluble and two membrane-associated folate-binding proteins in this study were each quantitatively immunoprecipitated by antiserum raised to the purified folate-binding protein from the spleen of a patient with myelofibrosis [5] but not by an antiserum raised to the purified folate-binding from chronic myelogenous leukemia [2] cells (data not shown).

Table IV shows the cross-reactivity of different folate-binding proteins purified from human cells. Each of the antisera reacted with its homologous antigen in both the binding and blocking assays. The soluble folate-binding protein in this study cross-reacted with a purified folate-binding protein from the spleen of a patient with myelofibrosis [5] but not with the folate-binding proteins purified from chronic myelogenous leukemia cells [2], from the spleen of a patient in the blast crisis of chronic myelogenous leukemia [6], or from KB cells [15]. On the other hand, the folate-binding protein from chronic myelogenous cells cross-reacted with the folate-binding protein from the spleen of a blast crisis patient and had slight cross-reactivity with folate-binding proteins from the spleen of a patient with myelofibrosis and from KB cells. The folate-binding protein from KB cells showed substantial cross-reactivity only

TABLE III

RELATIVE AFFINITY OF FOLATE ANALOGUES FOR THE PURIFIED SOLUBLE AND MEMBRANE-ASSOCIATED FOLATE-BINDING PROTEIN

Folate analogue	Concentration of analogue for 50% inhibition of the binding of [^{3}H]PteGlu (nM)		
	soluble form	membrane-associated forms	
		high molecular weight	low molecular weight
PteGlu	0.58	1.2	0.56
$PteGlu_7$	0.82 (1.4) [a]	1.8 (1.5)	1.65 (2.9)
$H_2PteGlu$	10.0 (17.2)	8.8 (7.3)	7.0 (12.5)
5-Methyl-H_4PteGlu	4.0 (6.9)	11.0 (9.2)	18.0 (32)
5-Formyl-H_4PteGlu	21.0 (36)	25.0 (20.8)	32.0 (57)
Methotrexate	120 (206)	270 (225)	880 (1571)

[a] The number in parenthesis is the ratio of the concentration of the unlabeled folate analogue to the concentration of PteGlu required to inhibit by 50% the binding of [^{3}H]PteGlu by the folate-binding protein.

with the folate-binding protein from the placenta. The antisera also blocked 32–53% of the binding of [^{3}H]PteGlu by the folate-binding proteins that they immunoprecipitated. Where there was little or no cross-reactivity by immunoprecipitation, there was also no blocking of [^{3}H]PteGlu binding.

Discussion

Two classes of folate-binding protein, soluble and membrane-associated, are widely distributed in human and animal tissues, and in biological fluids. Cytosolic and mitochondrial folate-binding proteins purified from rat liver have been associated with some enzyme activity [26–31], but the function of both soluble and membrane-associated

TABLE IV

IMMUNOLOGIC CROSS-REACTIVITY OF HUMAN FOLATE-BINDING PROTEINS [a]

Antiserum [b]	Immunoprecipitation [c]					Immunoinhibition [d]				
	1	L	S	2	KB	1	L	S	2	KB
S	3275	172	3809	0	0	32	0	34	0	0
L	35	4709	946	4102	395	0	38	0	41	0
KB	32	0	0	0	3741	0	0	0	0	51
PL	79	404	138	215	3698	0	0	0	0	53

[a] The folate-binding proteins in this experiment were as follows: 1, the purified soluble form in this study; L, the purified folate-binding protein from chronic myelogenous leukemia cells (2); S, the folate-binding protein from the spleen of a patient with myelofibrosis (5); 2, the folate-binding protein purified from the spleen from a patient with a blast crises of chronic myelogenous leukemia; KB, purified from medium of cultured human KB cells.

[b] The antisera were raised against folate-binding proteins from the spleen of a patient with myelofibrosis (S), the leukemic cell lysates of a patient with chronic myelogenous leukemia (L), the spent culture medium in which KB cells were grown (KB), and the human placenta (PL).

[c] This assay was based on the immunoprecipitation of the folate-binding protein–[^{3}H]PteGlu complex. The values represent the pg of protein precipitated, which was computed from the molar equivalent of the [^{3}H]PteGlu in the immunoprecipitate.

[d] This assay was based on the inhibition by the antiserum of the binding of [^{3}H]PteGlu. The values are expressed as a percent of the control reaction in which there was no antiserum.

folate-binding proteins which have been isolated from other mammalian cells and tissues and which bind the oxidized folates with greater affinity than the reduced folates has not been established.

In this study we have characterized three folate-binding proteins purified from the same tissue. This was facilitated by developing a new ligand matrix for the affinity chromatography whereby PteGlu was coupled to epoxy-activated Sepharose 6B via a hexanediamine intermediate linker. Because the leakage of PteGlu from this matrix was insignificant, the loss of the folate-binding protein during the application of the sample and the washing of the column was reduced. In addition, the use of a guanidine-HCl rather than the customary acidic conditions to dissociate the folate-binding protein from the affinity matrix permitted, for the first time, recovery of a sufficient amount of the high and low molecular weight membrane-associated proteins in pure form to study some of their properties.

Although we were not able to determine the true M_r of the high molecular weight species of the membrane-associated folate-binding protein by SDS-polyacrylamide gel electrophoresis, it is likely that it is a transmembrane integral protein with a substantial hydrophobic domain since it required Triton X-100 for solubilization. The smaller membrane-associated folate-binding protein, however, is likely to be an amphipathic protein with a very small hydrophobic domain because it did not bind sufficient Triton X-100 to increase its apparent molecular weight significantly by gel filtration. The difference observed in its apparent M_r by gel filtration (28 000) and by SDS-polyacrylamide gel electrophoresis (35 000) is likely a consequence of its carbohydrate content which prevents an accurate determination of molecular weight by gel electrophoresis [32]. The membrane-associated folate-binding proteins from human placenta [13], leukemia cells [6] and KB cells [33] have also been separated into a larger molecular weight species which is hydrophobic, and a smaller molecular weight species which requires a detergent for solubilization. The two membrane-associated folate-binding proteins in KB cells were also antigenically and functionally similar to each other and to the soluble forms of folate-binding protein in the cytosol and culture medium [15]. A later study of the folate-binding proteins in KB cells has shown that the molecular weight by SDS-polyacrylamide gel electrophoresis and the number of apolar amino acids in the soluble and membrane-associated species were similar [33]. Similar findings were reported for the soluble and membrane-associated folate-binding protein in human milk [4], so that it has been postulated that the soluble folate-binding protein may be derived from the larger molecular weight membrane-associated species by proteolytic cleavage [13]. This study also shows that the purified membrane-associated folate-binding proteins and the soluble folate-binding protein are similar functionally, antigenically, and by amino acid composition, providing support for the notion that both the smaller species of folate-binding proteins are derived from the larger molecular weight membrane-associated folate-binding protein.

It is noteworthy that there is very little difference in the fraction of the apolar amino acids in the larger hydrophobic molecular weight membrane-associated and the soluble folate-binding protein. This was also observed for the folate-binding proteins in KB cells [33] and human milk [4] and suggests that the hydrophobic properties of the membrane-associated folate-binding proteins may be due to non-polypeptide domains such as glycophospholipids or fatty acids. Such components have recently been identified in integral membrane proteins. For example, a glycophospholipid membrane anchor has been identified for the Thy^{-1} receptor protein on lymphocytes [34], for membrane-associated alkaline phosphatase [35], and for some membrane glycoproteins of trypanosomes [36]. Fatty acids have also been identified as a component of some integral membrane proteins [37,38].

The soluble folate-binding protein in the cytosol is also a glycoprotein, and its resolution into proteins having an M_r of 32 000 and 35 000 by SDS-polyacrylamide gel electrophoresis may be due to some difference in the carbohydrate component of each molecule. In previous studies we have shown that the soluble folate-binding proteins from human leukemic cells [2] could be separated by DEAE chromatography into two components based on the sialic acid content of each molecule.

It is of interest that the soluble species of folate-binding protein has substantially lower affinity for PteGlu ($K_a = 9.7\ nM^{-1}$) than the membrane-associated folate-binding proteins ($K_a = 96\ nM^{-1}$ and $40\ nM^{-1}$ for the high and low molecular weight species, respectively) and also that the low molecular weight membrane-associated folate-binding protein has a lower affinity for the folate analogues than the higher molecular weight membrane-associated folate-binding protein. These findings suggest that these proteins subserve different functions in folate metabolism and that the molecular structure that confers hydrophobicity on the membrane-associated proteins also affects the interaction of folate with the binding site on the protein.

Another interesting finding in this study is that the folate-binding proteins isolated from different human cells appear to be antigenically distinct. While it is true that proteins which cross-react with a polyclonal antiserum may have either similar or homologous antigenic sites, it is also axiometic that two proteins which do not cross-react in the native state with an antiserum raised to one of the proteins lack any homology with respect to the reactive epitopes. The structural configuration of an antigenic site of a protein may extend over a span of polypeptides of approximately 6000 Da [39], so that the folate-binding proteins which have a molecular weight ranging from 30000 to 42000 should have five to seven epitopes, some of which should be on the surface of the native protein. Therefore, the failure to immunoprecipitate the folate-binding protein purified from one source of human leukemia cells with an antiserum raised to a folate-binding protein from another source of human leukemia cells indicates that not a single surface epitope was common to these proteins. Though the ligand binding and molecular size of the folate-binding proteins from the different human cells are similar, there must be some other structural difference to account for this lack of cross-reactivity. A difference in the site of attachment of carbohydrate or of phosphate groups to the protein molecule may alter some of the antigenic properties. It is also possible that these structural differences may reflect evolutionary point mutations which result in single amino acid substitutions and changes in the tertiary structure of the protein. To investigate this phenomenon further, our laboratory is now cloning the folate-binding protein gene to study its structure and polymorphism.

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