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Affinity labelling of the folate-binding protein in pig intestine

Ann M. REISENAUER
Department of Internal Medicine, University of California Davis, Davis, CA 95616, U.S.A.

A specific transport system for folate and a high-affinity folate-binding protein have been identified in pig intestinal brush-border membranes. To determine if the binding protein plays a role in folic acid (PteGlu) uptake in to the cell, the inactivation of folate binding and transport by *N*-hydroxysuccinimide esters of folic acid (NHS-PteGlu) was compared. In addition, the number of brush-border proteins modified by the affinity reagent was assessed. Brush-border vesicles were incubated with various concentrations of NHS-PteGlu or NHS-methotrexate. Transport and binding of [^{3}H]PteGlu by the vesicles were measured at 37 and 4 °C respectively by using the vacuum-filtration technique. NHS-methotrexate and NHS-PteGlu specifically inhibited PteGlu transport. Incubating the vesicles with 1 μM-NHS-PteGlu inactivated [^{3}H]PteGlu transport by 60% and binding by 80%. Half-maximal inhibition of both transport and binding was observed at similar concentrations of the affinity reagent (0.05 and 0.07 μM-NHS-PteGlu respectively). Treating the vesicles with radiolabelled NHS-PteGlu followed by gel electrophoresis and autoradiography revealed a specifically labelled protein with an M_r of 56000. These results indicate that the intestinal folate-binding and transport proteins are identical and that the function of the folate-binding protein is to transport folate into the cell.

INTRODUCTION

Folate-binding proteins are found on the plasma membrane of a variety of mammalian cells, including the intestinal mucosa. Cell-surface folate binders in mouse L1210 cells, human KB cells and rat renal-tubular cells appear to play a role in folate transport [1–6]. In contrast, the function of the intestinal folate-binding protein has not been established. In both the rat and pig, the intestinal folate-binding protein has a high affinity for PteGlu and other folate derivatives [7,8]. PteGlu binding to pig intestinal brush-border membranes is saturable, pH-dependent, and competitively inhibited by methyl- and polyglutamyl-folates [8]. The binding component has a high affinity for PteGlu ($K_d = 0.08$ μM) and 5-methyl-H_4(tetrahydro)PteGlu ($K_d = 0.8$ μM). Pteroylpolyglutamate hydrolase (PPH), the enzyme that digests dietary folate, is also found on the brush-border membrane in the pig intestine, but has a much lower affinity for PteGlu ($K_m = 45$ μM) and distinct metal-ion requirements and inhibition characteristics [8].

In the intact intestine, folate absorption is a saturable and pH-dependent process that involves a specific, carrier-mediated transport system with broad specificity for various folate compounds [9]. A specific transport system for folate as well as a high-affinity folate-binding protein has been identified in pig intestinal brush-border membranes [8,10]. The transport process is pH-dependent, with maximal uptake at pH 5.2, shows high affinity for PteGlu ($K_m = 0.7$ μM), and is inhibited by various folate analogues [10]. Folate binding and transport are similar in the pH optima, order of affinities for different folate compounds, requirements for the complete folate structure, and susceptibilities to thiol-group-reactive inhibitors [8,10].

The present study used the technique of affinity labelling to determine whether the intestinal folate-binding protein functions in folate transport across the brush-border membrane. Affinity-labelling reagents introduce a covalent bond between the ligand (in this case PteGlu) and an amino acid residue in the binding site of the protein. *N*-Hydroxysuccinimide (NHS) esters of PteGlu (NHS-PteGlu) were used to link PteGlu covalently to the intestinal folate-binding protein. Carbodi-imide-activated folates and *N*-hydroxysuccinimide esters of methotrexate have previously been used to specifically and covalently modify membrane transport proteins in *Lactobacillus casei* and L1210 cells and are potent inhibitors of folate transport in these cells [11,12]. The inhibition of PteGlu binding and transport in brush-border vesicles treated with NHS-PteGlu was compared. In addition, the number of brush-border proteins modified by the affinity reagent was assessed.

MATERIALS AND METHODS

Materials

[3′,5′,7,9-^{3}H]PteGlu (20–70 Ci/mmol) was purchased from American Radiolabeled Chemicals and was purified before use by DEAE-cellulose chromatography [13]. The DEAE-purified [^{3}H]PteGlu was approx. 90% pure as assessed by ion-pair h.p.l.c. [14]. PteGlu, methotrexate, NHS, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) and SDS were purchased from Sigma Chemical Co. Dimethyl sulphoxide (Me_2SO) was obtained from Fisher Scientific and stored in the presence of a molecular sieve [0.3 nm (3 Å), particle size 8–12 mesh; Sigma] to remove residual water. Low-molecular-mass standards for SDS/PAGE and the Bio-Rad protein assay were obtained from Bio-Rad Laboratories. EN^{3}HANCE was acquired from NEN Research Products. Scintisol was purchased from Isolab.

Preparation of brush-border vesicles

The proximal jejunum was removed from 50–100 kg pigs and washed in cold saline (0.9% NaCl). The mucosa was collected, frozen with solid CO_2 and stored at −70 °C. Brush-border membrane vesicles were prepared by calcium precipitation and differential centrifugation [15] and resuspended in 140 mM-mannitol/10 mM-Hepes, pH 6.8 (Buffer A) before use. The purity of the vesicles was monitored by using the marker enzyme sucrase [16]. In these experiments, mean sucrase purification was 19-fold.

Abbreviations used: (NHS-)PteGlu, (*N*-hydroxysuccinimide esters of) pteroylglutamate (folic acid); EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; Me_2SO, dimethyl sulphoxide; PPH, pteroylpolyglutamate hydrolase; the term 'folate' is used generically to described various folate compounds.

* Present address and address for correspondence and reprint requests: Department of Pediatrics, S324, Stanford University Medical Center, Stanford, CA 94305-5119, U.S.A.

Preparation of NHS-activated folate

NHS esters of methotrexate or PteGlu were prepared by dissolving 1 μmol of methotrexate or PteGlu in 1 ml of anhydrous Me_2SO containing 20 mM-NHS and 20 mM-EDC and incubating the mixture for 1 h at 22 °C as previously described [12].

Treating the vesicles with NHS-activated PteGlu or methotrexate

Aliquots (0.75 ml) of NHS-PteGlu or NHS-methotrexate were diluted to the desired concentration with 2 mM-NHS and 2 mM-EDC in anhydrous Me_2SO (Solvent A), added to the brush-border vesicles (0.25 ml; 10–20 mg of protein/ml) and incubated for 10 min at 22 °C. Control or untreated vesicles were incubated with Solvent A alone. These conditions were shown to maximize subsequent inhibition of PteGlu transport in the preliminary experiments described below. The vesicles were collected by centrifugation at 30000 *g* (15 min at 4 °C), washed twice with Buffer A, pH 7.4, to remove unchanged affinity reagent and resuspended in this buffer before use in the PteGlu transport and binding assays.

Preliminary studies showed similar inhibition of PteGlu uptake when the vesicles were preincubated with 0.1 μM-NHS-PteGlu for 5, 10 or 30 min at 22 °C. Inhibition of PteGlu uptake increased linearly when the vesicles were incubated with 1 μM-NHS-PteGlu or -NHS-methotrexate and increasing amounts of Solvent A. The integrity of the solvent-treated vesicles was demonstrated by the characteristic 'overshoot' in D-glucose transport in the presence of a sodium gradient as described by Hopfer *et al.* [17]. Electron microscopy revealed a relatively pure preparation of sealed membrane vesicles.

Transport of PteGlu

The initial velocity of [^{3}H]PteGlu uptake was measured at 37 °C in the presence of a pH gradient using the vacuum-filtration technique as previously described [10]. At this temperature PteGlu uptake is inversely proportional to the osmolarity of the external buffer, a result consistent with intravesicular transport [10]. In the present study, brush-border vesicles (0.8–1.2 mg of protein) were incubated with 0.5 μM-[^{3}H]PteGlu, 50 μM-NaCl, 0.1 mM-$MgSO_4$ and 50 mM-Mes, pH 5.5 (Buffer B). The reaction was stopped by adding 2 ml of ice-cold 70 mM-NaCl/10 mM-Hepes, pH 7.4 (Buffer C), and the mixture was immediately applied to a Whatman GF/C glass-fibre filter. The filters were washed twice with 2 ml of ice-cold Buffer C, dissolved in 10 ml of Scintisol and assayed for radioactivity by liquid-scintillation counting. The results, corrected by control incubations in which the vesicles were preincubated with excess PteGlu (1 mM) before adding the labelled substrate, are expressed as pmol of [^{3}H]PteGlu transported/min per mg of protein. The time course of [^{3}H]PteGlu uptake by the solvent-treated vesicles was linear to 45 s of incubation (results not shown).

Binding of PteGlu

Binding of [^{3}H]PteGlu to the brush-order membranes was determined at 4 °C by using the vacuum-filtration technique [8]. Under these conditions, increasing the osmolarity of the external buffer does not affect the amount of PteGlu associated with the vesicles, a result consistent with membrane binding [8]. Briefly, the vesicles were incubated with 0.25 μM-[^{3}H]PteGlu for 5 min in Buffer B. The reaction was stopped by vacuum filtration and the filters were washed with 6 ml of ice-cold Buffer C. The results, corrected for non-specific binding as described above, are expressed as pmol of PteGlu bound per mg of protein.

Labelling with NHS-[^{3}H]PteGlu

Radiolabelled NHS-PteGlu was prepared by a modification of the procedure of Henderson & Huennekens [18]. [^{3}H]PteGlu (2 nmol; 26 μCi/nmol) was acidified by adding HCl (5 nmol), and the mixture was evaporated to dryness with a Speed Vac concentrator (Savant). The isotope was dissolved in 0.2 ml of anhydrous Me_2SO containing 1 mM each of NHS and EDC, and the mixture was incubated for 1 h at 22 °C. The reagent was added to brush-border vesicles (7.6 mg of protein) and resuspended in 225 mM-sucrose/20 mM-Hepes, pH 6.8 (Buffer D) to give a final concentration of 1 μM-NHS-[^{3}H]PteGlu. The vesicles were incubated with the affinity reagent for 10 min at 22 °C, washed twice, then resuspended in Buffer D. Control vesicles were preincubated with excess PteGlu (2 mM) before adding the affinity reagent.

Gel electrophoresis

Electrophoresis was performed in 10% (w/v) polyacrylamide slab gels containing SDS by the method of Laemmli [19]. Brush-border membranes treated with NHS-[^{3}H]PteGlu were solubilized in62.5 mM-Tris(pH 6.8)/2.3% SDS/5%(v/v)2-mercaptoethanol by boiling for 3 min. The samples were applied to the gel, and electrophoresis was performed with 12 mA of constant current. The gels were stained with 0.2% Coomassie Blue R-250. After destaining, the gels were immersed in EN^{3}HANCE for 1 h, soaked in distilled water for 30 min and dried on filter paper for 3 h at 60 °C. For autoradiography, the dried gel was exposed to X-ray film (Kodak XAR-5) for 14 days at −70 °C.

RESULTS

The effect of treating the vesicles with Solvent A alone on subsequent PteGlu transport was assessed. The affinity of the transport protein for PteGlu was compared in control and solvent-treated brush-border vesicles. The control and solvent-treated vesicles were resuspended in Buffer A, pH 7.4, and Solvent A respectively, incubated for 10 min at 22 °C, and washed twice with buffer as described above. As shown by the Lineweaver–Burk plot in Fig. 1, the affinity of the transport protein for PteGlu was identical in the control and solvent-

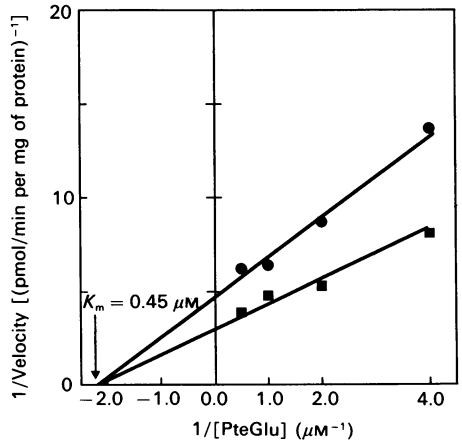

Fig. 1. Lineweaver-Burk plot of PteGlu uptake in control and solvent-treated brush-border vesicles

Vesicles were incubated with Buffer A (●) or Solvent A (■) for 10 min at 22 °C as described in the Materials and methods section. [^{3}]PteGlu uptake was measured at 30 s. Each point is the mean result from three experiments. The K_m of 0.45 μM was determined from the intercept on the abscissa.

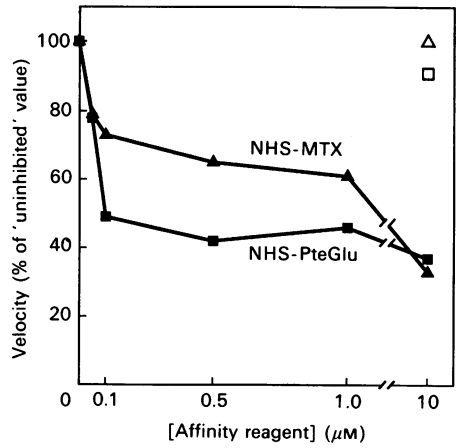

Fig. 2. Inactivation of PteGlu transport by NHS-methotrexate and NHS-PteGlu

Brush-border vesicles were incubated with various concentrations of NHS-methotrexate (NHS-MTX) (▲) or NHS-PteGlu (■) and washed to remove unchanged affinity reagent. [^{3}H]PteGlu transport was measured after 1 min incubation. 'Uninhibited' velocity was determined by using vesicles treated with Solvent A alone. In certain experiments, the vesicles were preincubated with excess (1 μM) methotrexate or PteGlu before adding 10 μM-NHS-methotrexate (△) or -NHS-PteGlu (□). Results are the means for three experiments.

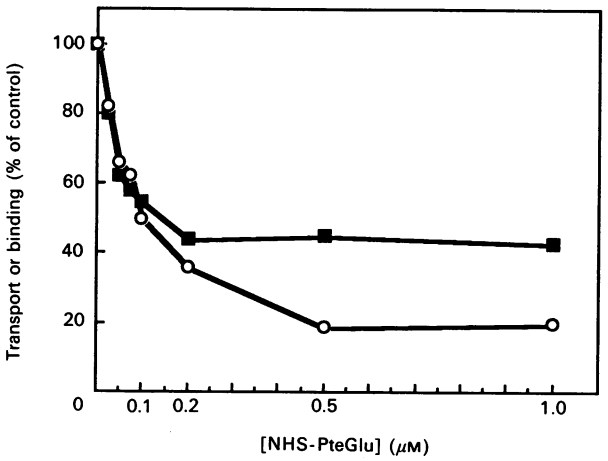

Fig. 3. Comparison of the inactivation of PteGlu binding and transport by NHS-PteGlu

Brush-border vesicles were incubated with various concentrations of NHS-PteGlu and washed twice before measuring [^{3}H]PteGlu transport (30 s at 37 °C) (■) or binding (5 min at 4 °C) (○). Results are means for three or four experiments.

treated vesicles (K_m = 0.45 μM-PteGlu). However, the apparent $V_{max.}$ was increased in the solvent-treated vesicles.

To compare the inhibition of PteGlu uptake by NHS-methotrexate and NHS-PteGlu, brush-border vesicles were incubated with various concentrations of each affinity reagent. At 10 μM, both reagents inhibited PteGlu transport by 60–70% (Fig. 2). However, NHS-PteGlu was the more effective inhibitor at lower concentrations and was used in subsequent experiments. Excess methotrexate or PteGlu added before treatment with 10 μM-NHS-methotrexate or -NHS-PteGlu prevented subsequent inactivation by the affinity reagent (Fig. 2, open symbols). These data indicate specific covalent attachment of the affinity reagents to the transport protein.

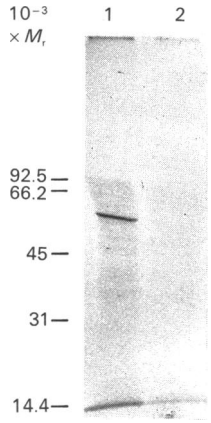

Fig. 4. Affinity labelling of brush-border membranes with radiolabelled NHS-PteGlu

Brush-border membranes were incubated with 1 μM-NHS-[^{3}H]PteGlu and analysed by SDS/10%-(w/v)-PAGE and autoradiography as described in the Materials and methods section. The autoradiogram obtained from the dried gel shows: lane 1, brush-border membranes treated with NHS-[^{3}H]PteGlu; lane 2, membranes that had been preincubated with 2 μM-PteGlu before adding the radiolabelled affinity reagent. The same amount of protein (190 μg) was applied to each lane. The positions of M_r standards are marked on the left. They are phosphorylase *b* (92500), BSA (66200), ovalbumin (45000), carbonic anhydrase (31000) and lysozyme (14400).

Fig. 3 compares the inactivation of PteGlu binding and transport with increasing concentrations of NHS-PteGlu. Half-maximal inhibition of both transport and binding was observed at similar concentrations of the affinity reagent (0.05 and 0.07 μM-NHS-PteGlu respectively). These values correlate closely with the previously determined affinity of the folate-binding protein for PteGlu (K_d = 0.08 μM) [8]. Treating the vesicles with 1 μM-NHS-PteGlu inhibited [^{3}H]PteGlu transport by 60% and binding by 80%.

To assess the number of proteins recognized by the affinity reagent, brush-border vesicles were treated with NHS-activated [^{3}H]PteGlu, solubilized in Laemmli sample buffer containing 2-mercaptoethanol, and analysed by gel electrophoresis and autoradiography. A representative autoradiogram is shown in Fig. 4. The same amount of membrane vesicles treated with the radiolabelled affinity reagent was applied to both lanes. However, the vesicles in lane 2 had been preincubated with excess PteGlu before adding the affinity reagent. Autoradiography revealed the presence of a principal radioactive protein with an M_r of 56000 (lane 1). When the vesicles were preincubated with excess PteGlu, labelling of this protein was abolished (lane 2). Specific labelling of a 56000-M_r brush-border membrane protein was observed in three experiments. Autoradiograms of gels run in the presence or absence of 2-mercaptoethanol were identical (results not shown). Specific covalent labelling with NHS-[^{3}H]PteGlu (0.19 pmol/mg of protein) was comparable with PteGlu binding to control vesicles measured in a previous study [8].

DISCUSSION

The biological function of a limited number of cell-surface folate-binding protein of the mouse L1210 leukaemia cell plays a role in folate uptake. In this cell line, the transport of folate compounds is specifically inhibited by both NHS esters and photoaffinity analogues of methotrexate [1,2,12]. These reagents covalently modify a single plasma-membrane protein [1,2]. In addition, the ability of the L1210 cell to transport folate correlates with the amount of the folate-binding protein on the cell surface

[2,3]. Immunochemical studies suggest that the folate-binding protein in human KB cells plays a role in folate uptake. An antiserum to the human placental folate-binding protein not only recognizes the KB-cell folate binder, but also inhibits folate uptake by intact cells [4]. In the rat kidney, the order of substrate affinities of renal-tubular folate absorption correlates closely with the affinities of the brush-border membrane folate-binding protein for various forms of folate [5,6].

The present study provides evidence that the intestinal folate-binding and -transport proteins are identical and that the function of the folate-binding protein is to transport folate into the enterocyte. The role of this protein in folate uptake is supported by several observations. Methyl-, formyl- and polyglutamyl-folates inhibit PteGlu binding and transport in the intestine [7,8,10,14]. Thus the binding protein and transporter recognize physiological folate compounds as well as PteGlu. We have previously demonstrated that folate binding and transport by pig intestinal brush-border vesicles have similar properties, including similar pH optima and orders of affinities for different folate compounds [8,10]. In the present study, NHS-activated PteGlu specifically inhibited folate transport in pig jejunal brush-border vesicles. Similar concentrations of NHS-PteGlu produced half-maximal inactivation of both PteGlu binding and transport. These data suggest that the same protein mediates both processes. In addition, the affinity reagent specifically labelled a 56000-M_r brush-border protein. The amount of covalent labelling with NHS-PteGlu was similar to the amount of PteGlu-binding activity reported previously [8]. The results of these studies indicate that the intestinal folate-binding protein functions in folate transport across the brush-border membrane.

Although NHS-PteGlu specifically inhibited PteGlu transport, saturating concentrations of the affinity reagent did not inactivate PteGlu uptake completely. A 100-fold increase in the concentration of NHS-PteGlu only slightly enhanced the inhibition of PteGlu transport (Fig. 2). The residual uptake observed may be related to minor contaminants in the [^{3}H]PteGlu substrate which either diffuse across the membrane or are otherwise transported in a manner not inactivated by the affinity reagent. Uptake of radiolabelled contaminants could account for the approx. 2-fold difference between uninhibited transport and binding at 0.5 and 1 μM-NHS-PteGlu. An alternative possibility is that the affinity reagent may not have quantitatively reacted with all of the transport proteins. This possibility is supported by the incomplete inhibition of PteGlu binding. It is unlikely that diffusion of PteGlu into the vesicle contributes to the residual uptake observed. A significant diffusional component would lead to a deviation from linearity on the Lineweaver–Burk plot, with points at high concentrations diverging towards the origin. Fig. 1 does not show this.

The pig intestinal folate-binding protein identified by affinity labelling with NHS-[^{3}H]PteGlu in the present study and the folate-binding protein purified from pig choroid plexus have similar M_r values (56000 and 51000 respectively) [20]. In other species and in carcinoma cell lines, particulate folate-binding proteins are somewhat smaller molecules. As estimated by SDS/PAGE, the M_r values of particulate folate-binding proteins purified from rat kidney [21], human placenta [22], human KB cells [23] and mouse L1210 cells [1,2] range from 30000 to 50000.

Both a folate-binding protein and a folate hydrolase (PPH) are found on the brush-border membrane in the pig intestine [8,24]. These proteins have distinct affinities for PteGlu and different M_r values. The affinity of the binding protein for PteGlu (K_m = 0.08 μM) is approx. 500-fold greater than that of the hydrolytic enzyme (K_i = 45 μM-PteGlu for PteGlu$_3$ hydrolysis) [8]. PPH is a large protein with native M_r of approx. 400000 and 700000 in pig and human intestine respectively, and subunits of M_r 115000 and 145000 in humans [25,26]. In contrast, the folate-binding protein is a much smaller protein (subunit M_r 56000) (Fig. 4). These data indicate that two distinct brush-border proteins are involved in folate absorption: a folate-transport and a hydrolytic enzyme.

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