Functional specificity of jejunal brush-border pteroylpolyglutamate hydrolase in pig

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Chandler, Carol J., David A. Harrison, C. Anthony Buffington, Nilda A. Santiago, and Charles H. Halsted. Functional specificity of jejunal brush-border pteroylpolyglutamate hydrolase in pig. Am. J. Physiol. 260 (Gastrointest. Liver Physiol. 23): G865–G872, 1991.—To determine the functional specificity of intestinal brush-border pteroylpolyglutamate hydrolase (PPH), we compared the regional location of in vivo hydrolysis of pteroylglutamate (PteGlu) with the location of activity and immunoreactivity of the enzyme in the pig. After in vivo incubations, PteGlu, hydrolytic products were recovered from intestinal segments in the jejunum but not in the ileum. Brush-border PPH activity in fractionated mucosa was 10-fold greater in the jejunum than in the ileum, whereas the activity of intracellular PPH was increased in the distal ileum. Antibodies to purified brush-border PPH identified a major protein band at 120 kDa and a minor protein band at 195 kDa in solubilized jejunal brush border. Immunohistochemistry identified the enzyme only on the brush-border surface of the jejunum, whereas an immunoblot of solubilized brush-border membranes identified brush-border PPH in the jejunum but not in the ileum. The parallel of the regional location of in vivo hydrolysis of PteGlu, with the location of brush-border PPH activity and immunoreactivity demonstrates the functional specificity of this enzyme in folate digestion.

Folic acid; intestinal absorption; folate hydrolase

FOLATES are a family of vitamins required for the transfer of one-carbon units during amino acid and nucleic acid synthesis. The principal clinical effect of folate deficiency is megaloblastic anemia, where a defect in deoxyribonucleic acid synthesis may result in alterations of cell proliferation and function in the small intestine (13). Folates are nearly ubiquitous in the diet, occurring as pteroylpolyglutamates (PteGlu) in liver and other animal tissue, in leafy vegetables, in legumes, and in many fruits (30). Dietary deficiency is rare in developed countries, where folate deficiency more commonly results from abnormalities of intestinal absorption or metabolism of the vitamin. Diseases associated with folate malabsorption include celiac and tropical sprue. Folate absorption is also impaired by the chronic use of alcohol, diphenhydantoin, and the anti-inflammatory drug sulfasalazine (14).

The intestinal absorption of dietary folates involves at least two stages: hydrolysis of PteGlu, and transport of the pteroylglutamate (PteGlu) derivative across the intestinal epithelium (14). In 1969, Rosenberg et al. (26) showed that folate hydrolysis required a reaction in the intestinal mucosa because in vitro incubation of PteGlu, in everted rat gut sacs or with homogenates of human jejunal biopsies resulted in its conversion to PteGlu. Others (18) demonstrated that the activity of the enzyme pteroylpolyglutamate hydrolase (PPH) (EC 3.4.12.10) in human intestinal mucosa is several hundredfold greater than its activity in bile, pancreatic, or intestinal secretions. Subsequently, an intracellular soluble PPH was isolated and purified from rat intestinal mucosa (9). However, human jejunal perfusion studies in which [14C]-PteGlu was hydrolyzed to intermediate chain-length folates and PteGlu suggested a hydrolytic process that was active on the mucosal surface (15, 17).

Subsequently, two groups independently described two separate PPH enzymes in the jejunal mucosa of the human and the pig, one in the brush-border membrane and the other a soluble intracellular enzyme (6, 7, 12, 25, 32). A species comparison study identified intestinal brush-border PPII in both the human and the pig but not in the rat or monkey (32). A recent study demonstrated a pig pancreatic PPH, which is an endopeptidase with an acid pH optimum that may provide initial cleavage of folate before complete hydrolysis by brush-border PPH (2). Purified human intestinal brush-border PPH is a heat-stable, zinc-dependent exopeptidase with optimum activity at pH 6.5 (6), whereas human intestinal intracellular PPH is a heat-labile endopeptidase that is found in the lysosomal fraction with a pH optimum at 4.5 (31). Human and pig intestinal brush-border PPH are both exopeptidases with similar substrate affinities, pH optima, and responsiveness to zinc (6, 7, 12), making the pig a suitable model of folate digestion.

The physiological role of each intestinal PPH in the intestinal absorption of dietary folates is controversial. On one hand, the endopeptidase mechanism of soluble rat intestinal PPH (9) would require an absorption process in which PteGlu, crosses the brush-border membrane before intracellular hydrolysis. On the other hand, the pattern of appearance in the lumen of hydrolytic products of PteGlu, during jejunal perfusion in both the human (13, 17) and the pig (24) is consistent with the exopeptidase mode of action of brush-border PPII in each species (6, 7, 12) and suggests that PteGlu, are hydrolyzed before transport of PteGlu into the enterocyte. Clinical studies of the activities of both PPH en-
zymes in human jejunal biopsies showed that brush-border PPH is affected by mucosal disease, whereas soluble intracellular PPH is not (16). An observation on the varied availability of folate in different foods (30) may be explained by the recent report that the activity of brush border PPH is selectively inhibited by different dietary constituents (3). Zinc dependency of brush-border PPH (6, 7, 12) may account for selective inhibition of PteGlu₂₉ absorption in human subjects fed zinc-deficient diets (29).

The present study using the pig as an experimental animal model was designed to demonstrate the specificity of brush-border PPH for folate digestion. The regional location of hydrolysis of PteGlu was defined by in vivo incubations in jejunal and ileal segments, followed by chromatographic analysis of reaction products. These data were compared with the regional locations of activities of both brush-border and intracellular PPH in segments of intestine between the pylorus and colon. The jejunal location of brush-border PPH was confirmed by immunohistochemistry and immunoblotting. The parallel of the in vivo location of hydrolysis of PteGlu, with the activity and immunological reactivity of brush-border PPH demonstrates that brush-border PPH functions specifically in the digestion of dietary folate.

METHODS

Materials. PteGlu, [¹⁴C]PteGlu₂₉, and [¹⁴C]PteGlu₃ were synthesized and generously provided by Dr. Carlos Krumdieck, University of Alabama at Birmingham. [¹⁴C]PteGlu was obtained from Amersham (Arlington Heights, IL). Chromatography materials used during the purification of the enzyme were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ) (Sephacryl S-200), Sigma Chemical (St. Louis, MO) (DEAE-cellulose), and Bio-Rad Laboratories (Richmond, CA) (Bio-gel A1.3). All other chemicals used were the purest available from commercial sources. The sources of apparatus and commercial kits used in immunological studies are indicated in the text. Locally bred chow-fed 1- to 2-year-old miniature Hanford pigs were used for in vivo studies and as a source of intestinal tissues. Surgery and tissue harvest were performed at the Animal Resources Service of the University of California, Davis. All procedures were reviewed and approved by the Animal Welfare Committee of the University of California, Davis.

In vivo hydrolysis of PteGlu. The activities of intestinal brush-border and intracellular PPH were measured in mucosal scrapings from the duodenum, the jejunum at the ligament of Treitz, the small intestine in equidistant segments between the ligament of Treitz and the ileocecal valve, the distal ileum, and the colon. The tissue was excised, rinsed in cold saline, scraped free of underlying tissue, wrapped in Parafilm (American Can, Milford, MA), and centrifuged and placed in a vial containing 0.1 ml of 10% trichloroacetic acid. Each segment was then removed, opened, and flushed with 10 ml of ice-cold saline, which was added to each original sample. Samples were centrifuged to remove protein, and supernatants were filtered before analysis. The samples were analyzed for PteGlu, by ion-pair high-performance liquid chromatography (HPLC) using a modification of the method of Allen and Newman (1) with a Beckman HPLC (Beckman Instruments, Fullerton, CA) and a μBondapak C₁₈ column (Waters, Millipore, Milford, MA). [¹⁴C]PteGlu, [¹⁴C]PteGlu₂₉, and [¹⁴C]PteGlu₃ were used as standards.

Enzyme assays. PPH activity was measured by a modification (6) of the charcoal absorption method of Krumdieck and Baugh (19), with 12 μM [¹⁴C]PteGlu as substrate. Brush-border PPH was measured in 33 mM 3,3-dimethylglutarate buffer at pH 6.5 with 0.1 mM zinc acetate, and intracellular PPH was measured in 33 mM sodium acetate buffer at pH 4.5. Interfering activity from each enzyme was controlled by measuring activities with and without the addition of 0.17 mM p-hydroxymercuribenzoate, an inhibitor of the intracellular enzyme (25). Enzyme activities used for controls in different experiments included the brush-border enzymes sucrase (5) and aminopeptidase (34) and the lysosomal enzyme N-acetylgalactosaminidase (21).

In vitro hydrolysis of PteGlu. The activities of intestinal brush-border and intracellular PPH were measured in mucosal scrapings from the duodenum, the jejunum at the ligament of Treitz, the small intestine in equidistant segments between the ligament of Treitz and the ileocecal valve, the distal ileum, and the colon. The tissue was excised, rinsed in cold saline, scraped free of underlying tissue, wrapped in Parafilm (American Can, Greenwich, CT), and stored frozen at -80°C. Mucosal samples were homogenized in 10 mM phosphate buffer, pH 6.5, using a Polytron PT10∕8 generator (Brinkmann, Westbury, NY) and centrifuged at 40,000 g for 30 min. The supernatant was decanted, and the membrane fraction was suspended in homogenization buffer. We avoided different recoveries for the brush-border enzymes when preparing brush-border vesicles (12) by measuring PPH activities in the total membrane and supernatant fractions. For comparisons, activities of the brush-border enzyme sucrase (5) and the lysosomal enzyme N-acetylgalactosaminidase (21) were measured in each membrane and supernatant fraction.

PPH purification. Brush border PPH was purified from the proximal 60 cm of jejunum obtained from pigs at the campus slaughterhouse (Table 1). Within 5 min of killing, a jejunal segment was removed and flushed with ice-cold saline; the mucosa was then scraped free of underlying tissue, wrapped in Parafilm, and stored at -80°C. As previously described (7), a brush-border membrane fraction was prepared from -10 g mucosal samples following the method of Selhub and Rosenberg (27), with a 20-fold purification and 39% recovery of brush-border PPH activity, and stored at -80°C. Vesicles from four
to five small preparations were diluted to 5.5 mg protein with 25 mM potassium phosphate buffer, pH 6.3. Papain, previously activated with 10 mM cysteine, was added to a final concentration of 12 U/ml, and the sample was incubated for 15 min at 37°C. Papain was used to release the protein from the brush border in a hydrophilic form to maximize antigenicity during immunization with the final purified enzyme. After the addition of zinc acetate to 0.1 mM, the sample was chilled on ice, centrifuged at 20,000 g, and incubated for 20 min. The supernatant was decanted and immediately applied to a 4 x 15 cm Sephacryl S-200 column (Pharmacia), equilibrated with buffer [10 mM tris(hydroxymethyl)aminomethane (Tris), 0.1 mM zinc acetate, and 2 mM mercaptoethanol at pH 8.5] at a flow rate of 2 ml/min. The fractions containing enzyme activity were pooled and applied to a 1.5 x 30 cm DEAE cellulose column equilibrated with the same buffer at a flow rate of 1 ml/min. The column was washed with 100 ml of buffer, and the enzyme was eluted with a 0–0.2 M NaCl gradient in buffer (400 ml total volume). Two other brush-border enzyme activities, sucrase and aminopeptidase, were eliminated at this step. Fractions containing PPH activity were pooled and concentrated to 2 ml with an Amicon 10 Stir Cell (YM 30 membranes) (Amicon Division, W. R. Grace, Danvers, MA). The sample was loaded at a flow rate of 10 ml/h onto a Biogel A1.5 column (1.5 x 90 cm) that was equilibrated in buffer containing 0.1 M NaCl. The column was eluted with the same buffer, collecting 2-ml fractions. A single sharp peak with uniform specific activity was recovered at a position corresponding to ~400 kDa. Fractions containing enzyme activity were pooled and concentrated with a stir cell, resulting in a 9,700-fold purification with 17% yield and specific act of 780 nmol . min⁻¹ . mg protein⁻¹.

**Antibody production.** BALB/c mice were injected intraperitoneally with 5 μg papain-purified brush-border PPH in Ribi adjuvant (Ribi ImmunoChem Research, Hamilton, MT) and boosted with 2.5- and 7.5-μg injections at 1 and 3 mo. Mouse polyclonal antibody was identified in tail vein sera by an enzyme-linked immunosorbent assay (ELISA) against the purified enzyme. Two days after the last antigen booster injection, 50 μg of *Salmonella typhimurium* Mitogen (STM, Ribi) was injected intraperitoneally. Splenic cells were harvested the next day and fused with SP2/O.Ag14 myeloma cells following the protocol of Oi and Herzenberg (23). After fusion, the cells were plated in 12–96 wells (Falcon, Becton-Dickinson, Oxnard, CA) with the addition of either 10⁶ spleen or 10⁷ peritoneal macrophage feeder cells per well. On the first day postfusion, hypoxanthine-aminopterin-thymidine and STM were added to the incubation media, each in final concentration of 2.5 μg/ml. Through the use of the ELISA, six clones tested positive for brush-border PPH antibodies. The hybridomas from each were subcloned by limiting dilution, and the subclones with the highest supernatant titer by ELISA were used for large-scale antibody production in pristane-primed mouse ascites (23). Through the use of specific antibodies to each subclass (Zymed Laboratories, San Francisco, CA), MAb-3 was typed as immunoglobulin G (IgG) and was purified by affinity chromatography with Bio-Rad Affi-Gel Protein A (10) and used for future studies.¹

**Immunospecificity.** To determine the specificity of the polyclonal antibody response, varied amounts of sera from immunized or control mice were incubated overnight in phosphate-buffered saline (pH 7.8) with 0.6 U of intracellular PPH or papain-solubilized and partially purified brush-border PPH. Washed Pansorbin cells (Calbiochem, La Jolla, CA) were added and incubated for 1 h at 4°C. The samples were centrifuged, and PPH activity was measured in each supernatant. A separate experiment was designed to exclude the possibility of antibody cross-reactivity toward different brush-border proteins with similar size. In this and all subsequent experiments, membranes were solubilized with the detergent Triton X-100 in the presence of the protease inhibitors leupeptin, antipain, pepstatin, soybean trypsin inhibitor, and phenylmethylsulfonyl fluoride to preserve the authenticity of membrane proteins. Jejunal brush borders were prepared as described above with the addition of 0.05 mg/ml of the protease inhibitors in all buffers. The brush borders were suspended in 25 mM Tris, pH 7.5, 20 mM NaCl, and 0.5% Triton X-100, rotated for at least 4 h at 4°C, and centrifuged at 40,000 g for 1 h. All brush border PPH activity was recovered in the jejunal supernatant. The supernatants were pre-treated twice for 1 h each with Pansorbin cells at 4°C, centrifuged, and incubated overnight at 4°C with mouse control serum or antisera to brush-border PPH, followed by Pansorbin treatment and centrifugation. The initial solubilized brush border and final supernatants were assayed for PPH as well as for sucrase (5) and aminopeptidase (34), two brush-border enzymes with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) migration rates similar to PPH. The precipitated pellet was solubilized in Laemmli buffer and analyzed by SDS-PAGE in a 5 x 7 cm 6% polyacrylamide gel (20) and a Bio-Rad Mini-Protein II system.

Further characterization of brush-border PPH was achieved by immunoblot using polyclonal antisera. Triton-solubilized jejunal brush-border membranes prepared in the presence of protease inhibitors were separated by SDS-PAGE in a 6% polyacrylamide gel at 100 V. The separated proteins were immediately transferred to a Problot membrane (Applied Biosystems, Foster City, CA) using Tris-glycine buffer (pH 8.3) at 15 V overnight at 4°C in a Bio-Rad Mini-transblot cell. After blocking for 2 h with 1% dry nonfat milk in Tris buffered saline, the membrane was incubated overnight at room temperature.

¹ Antibody production was performed by David A. Harrison and was presented at the second annual meeting of the Pew National Nutrition Foundation, 1989.
ature with polyclonal antiserum or control mouse serum diluted in Tris-buffered saline with 0.1% milk. The membrane was washed three times with the same buffer and incubated for 2 h at room temperature with alkaline phosphatase-linked goat antiamino IgG (Bio-Rad) in the same buffer. After additional washing, immunoreactive protein was identified by alkaline phosphatase stain using the Bio-Rad kit.

The specificity of purified MAb-3 was demonstrated by immunoprecipitation with Sepharose-linked antibody. Purified MAb-3 was covalently coupled to CNBr-activated Sepharose beads (Pharmacia) according to the manufacturer's instructions. Control CNBr beads were treated to identical conditions without the addition of antibody. For immunoprecipitation, 0.5 ml of Triton-solubilized jejunal membrane were incubated with 0.35 ml of 50 mM Tris in 150 mM NaCl, pH 7.8, and 0.150 ml control Sepharose bead suspension in the same buffer for 2 h at 4°C. After centrifugation, the supernatant was decanted, and 0.125 ml of MAb-3-linked Sepharose beads in the same buffer were added and incubated at 4°C for an additional 2 h. After centrifugation, the postincubation supernatants were assayed for brush-border PPH activity, and the pellets were washed and solubilized in Laemmli buffer for SDS-PAGE.

Immunological localization of brush-border PPH. Brush-border PPH was localized by immunohistochemistry in the different intestinal sections using an avidin-biotin-conjugated peroxidase method (4). Samples of pig jejunum and ileum were rapidly removed at killing, rinsed in cold saline, blotted, and frozen in O.C.T. Compound (Miles, Elkhart, IN). Frozen sections (5 µm) were dried on glass slides for 20 min at room temperature, fixed in acetone for 30 min, and washed for 5 min in phosphate-buffered saline, pH 7.4. Endogenous peroxidase activity was blocked with a 10-min bath of 1% H$_2$O$_2$ in methanol. MAb-3 was diluted in buffer and applied to each section for 60 min. After rinsing, biotinylated goat antimouse IgG/IgM was applied for 30 min. The slides were rinsed again, and an avidin-biotin-horseradish peroxidase complex was applied for 30 min. Peroxidase activity was measured using diaminobenzidine tetrahydrochloride as substrate. Background affinity and activity were controlled using nonrelated mouse ascites fluid, a gift of Dr. Harry Greenberg, Stanford University.

Immunoblotting with MAb-3 was used to confirm the regional location of brush-border PPH. As described above, the brush-border membranes were solubilized in buffer containing 0.5% Triton X 100 with protease inhibitors and centrifuged at 40,000 g for 20 min, resulting in complete recovery of PPH activity in the jejunal supernatant. Jejunal and ileal samples, each containing 0.5 mg protein, were electrophoresed on a 5 × 7 cm 6% SDS-polyacrylamide gel at 100 V using the method of Laemmli (20) with a Bio-Rad Mini-Protean II System and immediately transferred to an Immobilon-PVDF membrane (Waters, Millipore) using Tris-glycine buffer (pH 8.3) at 15 V overnight in a Bio-Rad Mini-transblot cell. Antibody staining was detected as described above, using MAb-3 and alkaline phosphatase-linked goat antimouse IgG.

RESULTS

In vivo hydrolysis of PteGlu$_3$. After a 5-min incubation with PteGlu$_3$ in the jejunal segments, HPLC of the luminal aspirate products demonstrated that 70% of the substrate was hydrolyzed to PteGlu$_2$ and PteGlu. By contrast, PteGlu$_3$ remained unhydrolyzed after 5-min incubation in the ileal segment (Fig. 1).

Distribution of brush-border and intracellular PPH. Intestinal distribution of activities of brush-border and intracellular PPH is shown in Fig. 2. Brush-border PPH activity was greatest in the duodenum and proximal jejunum and was decreased by an order of magnitude in the ileum and colon. The activity of the brush border enzyme sucrase was greater in the jejunum and ileum than in the duodenum and colon. In contrast, intracel-
FIG. 2. Intestinal distribution of brush-border and intracellular pteroylpolyglutamate hydrolase (PPH). Enzyme activities were measured in membrane fractions (A) or in supernatants (B) from homogenates of mucosa removed from duodenum (DUOD), from jejunum at the ligament of Treitz (JEJ), from 5 equidistant sites along small intestine (1–5), from distal ileum at ileocecal junction (ILE), and from colon (COL). Error bars represent SE derived from average activities in mucosa from 4 pigs. Statistical analysis was carried out by analysis of variance. * P < 0.001. Brush-border PPH activity predominated in proximal jejunum, whereas intracellular PPH activity was greatest in terminal ileum and colon.

lular PPH activity was low in the proximal segments of the intestine and was elevated at least twofold in the distal ileum and colon. The activity of N-acetylglucosaminidase, lysosomal enzyme, was evenly distributed throughout the digestive tract.

Immunological specificity of brush-border PPH. Polyclonal antiserum was tested for specificity toward brush-border PPH and intracellular PPH and for determination of the molecular weight of jejunal brush-border PPH. As shown in Fig. 3, incubation of partially purified papain-treated brush border or intracellular supernatant with varied amounts of polyclonal antiserum, followed by addition of Pansorbin cells, precipitated all brush-border PPH activity but no intracellular PPH activity. In separate experiments, jejunal brush borders solubilized with Triton X-100 in presence of protease inhibitors with varied amounts of polyclonal antiserum, followed by addition of Pansorbin cells, precipitated all brush-border PPH activity but no intracellular PPH activity. In separate experiments, jejunal brush borders solubilized with Triton X-100 in presence of protease inhibitors were treated with control sera or polyclonal antiserum followed by addition of Pansorbin cells. Brush-border PPH activity was completely precipitated from Triton-solubilized jejunal brush borders by polyclonal antiserum, whereas sucrase and aminopeptidase activity remained in solution. SDS-PAGE identified a single immunoprecipitated protein band at 120 kDa (Fig. 4A). Half of the brush-border PPH activity was also precipitated with MAb-3-linked Sepharose beads, and SDS-PAGE again showed a protein band at 120 kDa (Fig. 5). An immunoblot of Triton-solubilized jejunal membranes with polyclonal antiserum revealed a major band at 120 kDa and a minor band at 195 kDa (Fig. 4B).

Immunological localization of brush-border PPH. For immunohistochemistry, MAb-3 was incubated with fresh frozen tissue sections from pig jejunum and ileum and detected with an avidin-biotin-conjugated peroxidase method. As shown in Fig. 6, immunohistochemistry of the two regions clearly showed the presence of the enzyme in the brush-border surface of jejunal enterocytes...
and its absence from the ileum. Immunoblotting of the Triton-solubilized jejunal brush-border membranes with MAb-3 revealed a prominent band at 120 kDa and a minor band at 195 kDa. No bands were detected by immunoblots prepared from solubilized ileal brush-border membranes (Fig. 7).

DISCUSSION

Proof of the hypothesis that brush-border PPH functions specifically in the hydrolysis of PteGlu, requires demonstration that the site of in vivo hydrolysis is identical to the site of PPH activity and to the immunological location of the enzyme. In the present studies, we used the pig as an experimental animal model because, like the human, it expresses both intracellular and brush-border PPH (12, 32). We combined three approaches: in vivo incubation of PteGlus in jejunal and ileal intestinal segments, measurement of activities of brush-border and intracellular PPH in different segments along the intestine, and immunological localization of brush-border PPH by immunohistochemistry and immunoblot techniques. The results are summarized as follows.

First, in vivo intestinal incubations in the fasted anesthetized pig demonstrated that the intestinal hydrolysis of PteGlu occurs in the jejunum and is absent in the ileum. Because these studies used isolated and washed intestinal loops, the potential effect of pancreatic PPH on substrate hydrolysis was excluded. The in vivo study confirms our previous finding using jejunal and ileal intestinal perfusion of PteGlu through surgically implanted tubes in an awake animal (24).

Second, the activity of brush-border PPH paralleled the location of in vivo hydrolysis of PteGlu, maximal in the jejunum and negligible in the ileum. By contrast, the activity of intracellular PPH was greater in the terminal ileum than in the jejunum. These data, which showed different distributions of enzyme activities in the pig intestine, complement studies of human jejunal biopsies, which showed specific clinical effects on brush-border PPH that were not shared by intracellular PPH (16) and underscore the biochemical distinctions between the two enzymes (6, 7, 12, 32).

Third, we used purified pig jejunal brush-border PPH to prepare polyclonal and monoclonal antibodies for characterization and immunological confirmation of the regional location of the enzyme. MAb-3 identified the enzyme by immunohistochemistry on the brush-border...
surface of jejunal villus enterocytes. Further confirmation of the regional location of the enzyme was achieved using immunoblots with MAb-3, in which brush-border PPH was identified by SDS-PAGE of jejunal brush-border as two protein bands that were absent in solubilized ileal brush border.

Jejunal brush-border PPH was characterized by several methods in the present studies. The molecular mass of 400 kDa obtained by gel filtration of the purified enzyme should be interpreted with caution, since the protease papain was used in the initial solubilization of brush borders for the purification process. Nevertheless, purity of the enzyme used for immunization was assured by the exhaustive purification process resulting in a sharp peak with uniform specific activity and by the identical protein bands obtained in experiments using both polyclonal antibody and MAb-3. The subunit molecular weights obtained by immunological methods can be considered reliable, since authenticity of brush-border proteins was assured by the use of protease inhibitors while preparing Triton-solubilized jejunal membranes. The specificity of the immune response was shown by immunoprecipitation experiments in which polyclonal antisera precipitated all of the activity of brush-border PPH but none of the activity of either intracellular PPH (Fig. 3) or other jejunal brush-border hydrolases. Immunoprecipitation experiments using Triton-solubilized membranes and either the polyclonal or monoclonal antibodies resulted in a single protein band at 120 kDa (Figs. 4A and 5). Incomplete precipitation by MAb-3 may be explained by incomplete affinity for the protein associated with the activity of brush border PPH. A minor band detected at 195 kDa using the more sensitive immunoblot method with both the polyclonal (Fig. 4B) and monoclonal antibody (Fig. 7) may represent a precursor of the 120-kDa active protein. Similar size precursors have been identified for other brush-border hydrolases (6, 33). Consistent with the present results, Gregory et al. (12) used nondenaturing PAGE of Triton-solubilized brush-border membranes to identify a native molecular mass for pig brush-border PPH of 237 kDa, which may represent a dimer of the 120-kDa protein found by SDS-PAGE in the present study. Further definition of the relationship of the molecular weights will require additional studies of enzyme synthesis and amino acid and nucleotide sequencing.

Others (2) have recently described a pig pancreatic PPH that is an endopeptidase with an acid pH optimum and somewhat lower affinity for PteGlu3 than intestinal brush-border PPH. Comparison of results from the pancreatic study and the present study indicate a substantially greater capacity of the brush-border enzyme for folate hydrolysis. According to the prior report, fasting pig pancreatic secretions have the capacity to hydrolyze 3.7 nmol PteGlu3 per hour, with a maximal sixfold stimulation after meals (2). The present data show that the specific activity of brush-border PPH in pig jejunal mucosa is 0.08 mU/mg protein, with the capacity to hydrolyze 0.08 nmol PteGlu3·min⁻¹·mg protein⁻¹ (Table 1) or, by calculation, 4.8 amol PteGlu3·h⁻¹·g protein⁻¹. Thus only 1 g of jejunal mucosal protein has 1,000 times greater capacity for PteGlu3 hydrolysis than 1 h of fasting pancreatic secretion and >100 times the capacity for PteGlu3 hydrolysis than maximally stimulated pancreatic secretion. As proposed (2), the initial hydrolysis of long-chain PteGlu by stimulated pancreatic PPH may provide shorter chain-length PteGlu3 for completion of hydrolysis by brush-border PPH.

By showing the parallel of regional location of in vivo hydrolysis of PteGlu3 with the enzymatic and immunological location of brush-border PPH, these data confirm the functional specificity of this enzyme in folate digestion. The function of intracellular PPH in humans and in pigs is uncertain and could relate to enteroocyte folate metabolism rather than to digestion of dietary folates. Because brush-border PPH is essential for the absorption of dietary folates, further study of the regulation of synthesis and activity of brush-border PPH assumes paramount importance in understanding the process of assimilation of this vitamin.

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