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HUMAN JEJUNAL BRUSH BORDER FOLATE CONJUGASE

CHARACTERISTICS AND INHIBITION BY SALICYLAZOSULFAPYRIDINE

ANN M REISENAUER and CHARLES H HALSTED *

Department of Internal Medicine, TB 192, School of Medicine, University of California, Davis, CA 95616 (U S.A)

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Summary

Human jejunal brush border folate conjugase (EC 3.4.22.—) was partially purified and characterized. Three drugs known to be associated with clinical folate deficiency were tested for inhibition of the partially purified enzyme. Using jejunal mucosa from obese patients undergoing intestinal bypass surgery, brush border folate conjugase was purified 50—80-fold by centrifugation, Triton X-100 solubilization and DEAE-Sephadex and Sephacryl S-200 chromatography. Using synthetic pteroyldiglutamyl[^{14}C]glutamate as substrate, the enzyme was found to have a pH optimum of 6.5 and an apparent K_m of 1.6 μM. Incubation of the enzyme with synthetic pteroyl[^{14}C]glutamylhexaglutamate resulted in a spectrum of shorter-chain ^{14}C-labeled pteroylglutamates at 60 min. Pteroyl[^{14}C]glutamate was the major product at 120 min, with quantitative recovery of free glutamate in the incubation medium. Salicylazosulfapyridine was a competitive inhibitor of the enzyme (K_i = 0.13 mM), while ethanol, diphenylhydantoin and salicylazosulfapyridine metabolites had no effect. These data suggest that brush border folate conjugase is an exopeptidase which progressively hydrolyzes glutamyl units from pteroylpolyglutamate, leaving pteroylmonoglutamate as the folate form available for intestinal transport. Inhibition of brush border folate conjugase by salicylazosulfapyridine provides a mechanism for folate malabsorption and deficiency in chronic users of this drug

* To whom reprint requests should be addressed

Introduction

During the process of intestinal folate absorption, dietary polyglutamyl folates ($PteGlu_n$) are hydrolyzed to pteroylmonoglutamate (PteGlu) by a mucosal enzyme known as folate conjugase (EC 3.4.22.—), or pteroylpolyglutamate hydrolase [1]. As compared to its activity in human biliary, pancreatic and intestinal secretions, folate conjugase is concentrated several hundred-fold in the jejunal mucosa [2]. Cellular fractionation studies of rat and guinea pig mucosa described intestinal folate conjugase as an intracellular and probably lysosomal enzyme [3,4]. However, our studies in human volunteers suggested that intestinal folate conjugase acts on the mucosal surface, since the jejunal perfusion of pteroyl[^{14}C]glutamylhexaglutamate (^{14}C-labeled $PteGlu_7$) was followed by the stepwise appearance of all possible hydrolytic products in distal aspirates of the luminal contents [5]. Subsequently, we identified two separate folate conjugase activities in the normal human jejunum, one intracellular and the other located in the brush border fraction, with different molecular weights, pH optima and inhibition characteristics [6]. *p*-Hydroxymercuribenzoate, a known inhibitor of cytosolic peptide hydrolases [7], completely inhibited the intracellular enzyme but had no effect on folate conjugase activity in the brush border fraction. The objectives of the present study were to partially purify and characterize human jejunal brush border folate conjugase, and to test the effects of three drugs associated with low serum folate levels on its activity. Results of these experiments have previously appeared in abstract form [8,9].

Methods

Substrates Pteroyldiglutamyl[U-^{14}C]glutamate (^{14}C-labeled $PteGlu_3$) and pteroyl[U-^{14}C]glutamyl-hexaglutamate (^{14}C-labeled $PteGlu_7$) were synthesized by the solid phase method [10] and were gifts of Dr. Carlos Krumdieck, University of Alabama.

Enzyme assay. Based on preliminary kinetic studies, folate conjugase activity was measured at each purification step by the charcoal adsorption method [11], using 13 μM ^{14}C-labeled $PteGlu_3$ (1.08 Ci/mol) as substrate, 33 mM Tris-HCl buffer, pH 7.5/66 μM glutamic acid/enzyme to a volume of 1.5 ml. 0.5 mM *p*-hydroxymercuribenzoate was added to each assay to inhibit possible contaminating intracellular folate conjugase [6]. Enzyme activity is expressed as cpm ^{14}C-labeled glutamate liberated/ml or as nmol substrate hydrolyzed/mg protein per 30 min. Protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA).

Purification procedure Human jejunal mucosa was obtained from patients undergoing elective jejunoileal bypass surgery and stored at —70°C until use. In five separate experiments, 15—45 g mucosa were weighed, minced and homogenized in 10 mM Tris-HCl, pH 7.0/5 mM 2-mercaptoethanol/1 mM zinc acetate using a Waring Blendor (30 s with the Powerstat variable transformer at a setting of 60) to give a 5% (w/v) solution. Following filtration through coarse nylon, a crude membrane fraction was prepared by 20 min centrifugation at 25 000 × *g*. After two washings, the pellet was resuspended in buffer and frozen

overnight at −70°C. In another experiment, a brush border fraction was prepared by the method of Schmitz et al. [12], starting with a 2% homogenate (w/v). Purity of the resultant fraction was indicated by 9-fold concentration of sucrase [13], a brush border marker enzyme. After thawing and re-centrifugation, the crude membrane or the brush border fraction was homogenized in buffer containing 1% (v/v) Triton X-100 to give a 20% (w/v) solution. After stirring for 30 min at 5°C and centrifugation at 25 000 × *g* for 20 min, enzyme activity was completely recovered in the supernatant. The Triton X-100 concentration, monitored by the method of Garewal [14], was reduced to 0.1% by the addition of Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) with stirring at 5°C for 60—90 min [15]. After removal of the Bio-Beads, the solution was concentrated by ultrafiltration through an Amicon PM-10 Diaflo membrane (Amicon Corporation, Lexington, MA) under nitrogen at 60 lbs/inch^2. The Amicon PM-10 retentates from each fraction were chromatographed on DEAE-Sephadex A-50 columns (Sigma Chemical Co., St. Louis, MO). (Fig. 1). In each chromatogram, peak 1 eluted in the void volume and was assumed to represent enzyme sequestered in Triton X-100 micelles. Rechromatography of peak 1 resulted in its partial (30%) redistribution to peak 2. Peak 2, which appeared in an identical position in each chromatogram and contained the most concentrated enzyme activity, represented brush border folate conjugase. Subsequent purification and characterization were based on the crude membrane fraction since it yielded a 10-fold greater enzyme recovery after DEAE-Sephadex chromatography. Peak 2 enzyme activity was concentrated to 2 ml by Amicon ultrafiltration and applied to a Sephacryl S-200 column (1 × 88 cm) (Pharmacia Fine Chemicals, Uppsala, Sweden). The majority of the enzyme was eluted with buffer containing 0.1% Triton X-100/0 1 M NaCl in a single peak near the void volume.

Enzyme characterization. Using ^{14}C-labeled $PteGlu_3$ as substrate, the pH optimum of the partially purified enzyme was determined over a range from pH 3.5 to 8.5. The kinetics of the reaction were studied at pH 6.5 using substrate concentrations ranging from 1.2 to 26 μM ^{14}C-labeled $PteGlu_3$ in 33 mM sodium phosphate buffer. To determine whether brush border folate conjugase acts as an endopeptidase or exopeptidase, the partially purified enzyme was incubated at timed intervals with 21.5 μM 1.0 Ci/mol ^{14}C-labeled $PteGlu_7$, in 50 mM sodium phosphate buffer at pH 6.5 in the presence of *p*-hydroxymercuribenzoate. Since the ^{14}C label was positioned in synthesis of ^{14}C-labeled $PteGlu_7$ on the glutamate next to the pteridine ring structure, the hydrolytic products could be identified by chromatography on DEAE-cellulose. The ninhydrin assay [16] was used to quantitate free glutamate in the reaction mixture before and after the 120 min incubation, which contained 85.8 nmol ^{14}C-labeled $PteGlu_7$ in 4 ml.

Drug studies Inhibition of brush border folate conjugase activity was tested using ethanol, diphenylhydantoin, salicylazosulfapyridine and its metabolites, sulfapyridine and 5-aminosalicyclic acid. Ethanol (95%) was diluted to final concentrations of 0.5, 1.0, 3.0 and 5.0% (v/v). Diphenylhydantoin (Phenytoin Sodium injection, Parke-Davis Co., Detroit, MI) 50 mg/ml, was diluted to 25, 100, 200 and 300 μg/ml. Salicylazosulfapyridine (Pharmacia Fine Chemicals, Uppsala, Sweden) was dissolved by heating one 500-mg tablet in a boiling water

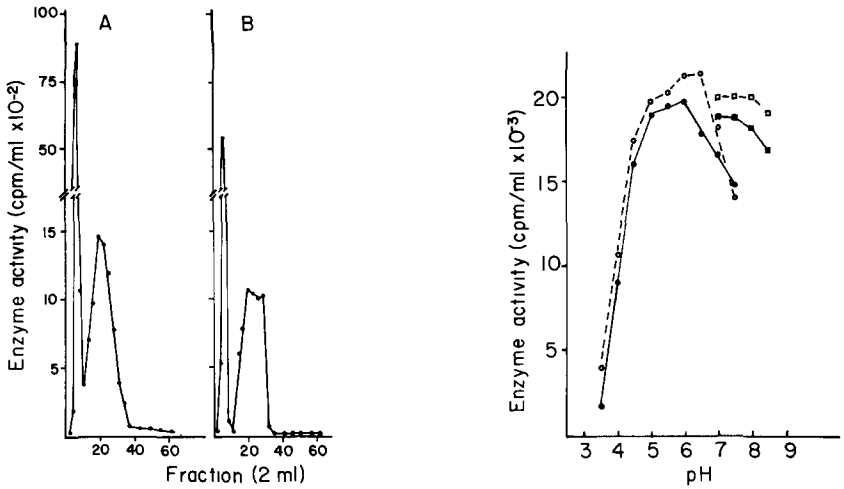

Fig 1 Comparison of DEAE-Sephadex elution patterns of folate conjugase activity in Triton-solubilized (A) brush border or (B) crude membrane fractions The Amicon PM-10 retentate of the Triton X-100 solubilized brush border fraction (12 mg protein) or crude membrane fraction (20 mg protein) was applied to the DEAE-Sephadex A-50 column (1 X 30 cm) and eluted by a linear gradient of 0—0 2 M NaCl in buffer (10 mM Tris-HCl/5 mM 2-mercaptoethanol/1 mM zinc acetate, pH 7 0/0 1% Triton X-100), 200 ml each Fractions (2-ml) were collected and analyzed for enzyme activity at pH 7 5 with *p*-hydroxymercuribenzoate Enzyme activity is expressed as cpm ^{14}C-labeled glutamate liberated/ml fraction per 15 min

Fig 2 Dependence of brush border folate conjugase on pH The enzyme was assayed from pH 3 5 to 8 5 with (- - - - - -) and without (———) 0 5 mM *p*-hydroxymercuribenzoate, using the charcoal adsorption method [11]. 17 mM 3 3-dimethylglutarate-NaOH buffer (○, ●) was used to determine enzyme activity from pH 3 5 to 7 5, and 33 mM Tris-HCl buffer (□, ■) was used to determine activity from pH 7 0 to 8 5 Enzyme activity is expressed as cpm ^{14}C-labeled glutamate liberated/ml enzyme per 30 min

bath in 5 mM potassium phosphate buffer, pH 7.0, and then diluted to 0.3, 0 6, 0.9 and 1.5 mM. Sulfapyridine and 5-aminosalicyclic acid (Pharmacia Fine Chemicals, Uppsala, Sweden) were dissolved in 0.1 M NaOH and 0.1 N HCl, respectively, and diluted similarly. The pH of the incubation solutions was not changed by the addition of the various drugs.

Results

Enzyme purification. Table I summarizes the purification process and shows the results of a representative experiment. Final purification ranged from 48 to 80-fold. The partially purified enzyme was stable up to 3 months at —70°C.

pH optimum. Brush border folate conjugase was optimally active at pH 6.0—6.5 (Fig. 2). Enzyme activity was slightly enhanced by the presence of *p*-hydroxymercuribenzoate, and was most active in 33 mM sodium phosphate buffer, pH 6.5 (data not shown).

Hydrolysis of ^{14}C-labeled PteGlu$_7$. Different chromatographic spectra of ^{14}C-labeled pteroylpolyglutamates were observed following timed incubations of ^{14}C-labeled PteGlu$_7$ with brush border folate conjugase (Fig. 3). Longer chain length ^{14}C-labeled PteGlu$_n$ compounds predominated after 15 min

TABLE I

PURIFICATION PROCEDURE FOR HUMAN INTESTINAL BRUSH BORDER FOLATE CONJUGASE

Enzyme activity measured by the charcoal adsorption method [11] and expressed as cpm ^{14}C-labeled glutamate liberated/ml per 30 min

	Purification step	Total protein (mg)	Total enzyme activity 1 10^{-5}	Specific activity (nmol/mg)	Purification (-fold)	Recovery (%)
1	Homogenate	2228	47 7	3 76	1 0	100
2	25 000 X *g* pellet	980	29 3	5 26	1 4	61 5
3	Pellet + 1% Triton X-100	—	31 2	—	—	65 4
4	Bio-Bead-treated pellet	218	26 3	21 2	5 6	55 2
5	Amicon PM-10 retentate	195	12 6	11 4	3 0	26 5
6	DEAE-Sephadex					
	Peak 1	12 7	2 72	37 6	10	5 7
	Peak 2	13,7	9,75	125 0	33	20 4
7	Retentate of DEAE Peak 2	13 9	4 54	57 8	15	9 5
8	Sephacryl S-200					
	Peak 1	5 6	5 80	182 0	48	12 2
	Peak 2	3,7	0 39	18 6	5	0 8

incubation, whereas ^{14}C-labeled PteGlu and ^{14}C-labeled $PteGlu_2$ were the major peaks after 60 min. In a separate experiment, an identical 60 min chromatogram was obtained when *p*-hydroxymercuribenzoate was excluded from the incubation medium. The major product after 120 min incubation was ^{14}C-labeled PteGlu. The calculated complete hydrolysis of 85.8 nmol ^{14}C-labeled $PteGlu_7$ to ^{14}C-labeled PteGlu yields 515 nmol free glutamate. By the ninhydrin

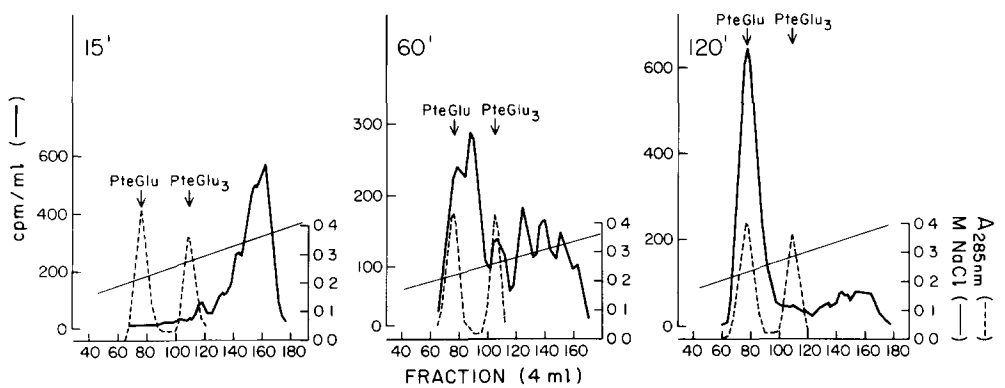

Fig 3 Chromatograms of the products of incubation of brush border folate conjugase with ^{14}C-labeled $PteGlu_7$ for 15, 60 and 120 min The partially purified enzyme (0 5 mg protein) was incubated with 21 5 μM substrate in 50 mM sodium phosphate buffer at pH 6 5 with 0 5 mM *p*-hydroxymercuribenzoate in a volume of 2 ml After stopping the reaction with 0 5 ml 10% trichloroacetic acid the incubation products were neutralized, applied to DEAE-cellulose column (0 7 X 28 cm) and eluted by linear gradients of 0 1—0 5 M NaCl in 5 mM sodium phosphate buffer, pH 7 0 (500 ml each) Prior to chromatography, unlabeled PteGlu and $PteGlu_3$ were added as markers and detected by absorbance at 285 nm (- - - - - -) Fractions (4-ml) were collected and screened for radioactivity (cpm/ml) (———) and conductivity (M NaCl) (———) 1 ml of each fraction was counted in 10 ml scintillation fluid (4% Cab-O-Sil (Eastman Kodak, Rochester, NY) in Multisol (Isolab Inc , Akron, OH)) See text for interpretation of results

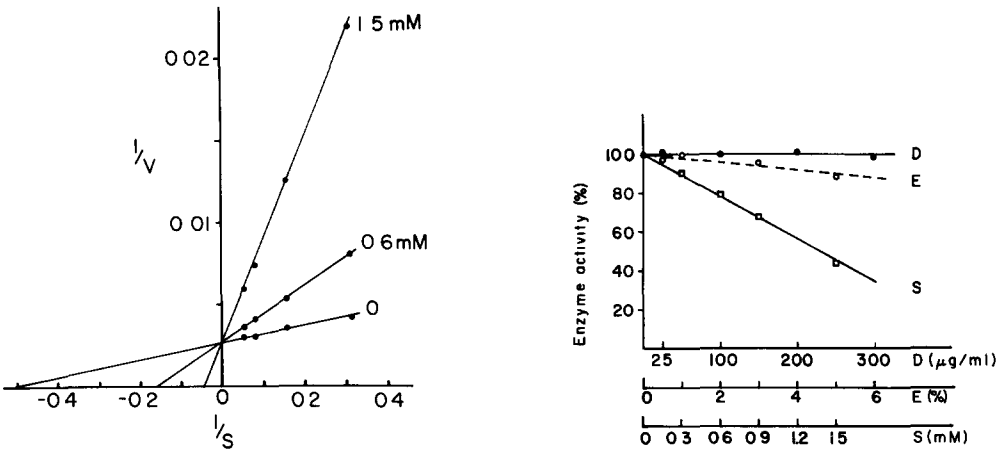

Fig 4. Lineweaver-Burk plot of the inhibition of brush border folate conjugase by salicylazosulfapyridine Enzyme activity was assayed at four substrate concentrations without (0 mM) and with two concentrations of the drug (0 6 and 1 5 mM) The concentration of ^{14}C-labeled PteGlu$_3$ ranged from 3 2 to 19 μM Using the 0 line, the apparent K_m of brush border folate conjugase was calculated as 1.6 μM ^{14}C-labeled PteGlu$_3$, with V of 368 nmol/mg protein per 30 min. Salicylazosulfapyridine was a competitive inhibitor with an apparent K_i of 0 13 mM

Fig 5 Effect of diphenylhydantoin (D), ethanol (E) and salicylazosulfapyridine (S) on brush border folate conjugase activity. Enzyme activity was assayed at pH 6 5 using various concentrations of these drugs, and is expressed as percent of the control activity Of these drugs only salicylazosulfapyridine was found to inhibit the enzyme

assay, the measured difference in free amino groups between the 0 and 120 min incubation was 484 nmol, yielding a free glutamate recovery of 94%

Enzyme kinetics. Zero-order kinetics were obtained at ^{14}C-labeled PteGlu$_3$ concentrations greater than 10 μM. A Lineweaver-Burk plot yielded an apparent K_m of 1.6 μM ^{14}C-labeled PteGlu$_3$ and V of 368 nmol substrate hydrolyzed/mg protein per 30 min (Fig. 4). Similar K_m and V values were calculated using the Eadie method, 1.9 μM and 375 nmol/mg protein per 30 min, respectively.

Drug studies Brush border folate conjugase activity was not inhibited by ethanol or diphenylhydantoin at any concentration tested, but showed linear inhibition with increasing concentrations of salicylazosulfapyridine (Fig. 5). On the other hand, the metabolites of salicylazosulfapyridine did not inhibit the enzyme (data not shown). Fig. 4 shows that salicylazosulfapyridine is a competitive inhibitor of the enzyme, with an apparent K_i of 0.13 mM.

Discussion

The present study extends our previous observations of two separate folate conjugase activities in human jejunal mucosa [6] by characterization of the surface-active brush border enzyme. Partially purified human jejunal brush border folate conjugase has maximal activity at pH 6.5 and an apparent K_m of 1.6 μM ^{14}C-labeled PteGlu$_3$. The data suggest that this enzyme is an exopeptidase which is capable of progressive hydrolysis of ^{14}C-labeled PteGlu$_7$ to ^{14}C-

labeled PteGlu with quantitative liberation of free glutamate. Enzyme activity was not affected by physiological levels of ethanol, diphenylhydantoin or salicylazosulfapyridine metabolites, whereas salicylazosulfapyridine itself was a competitive inhibitor.

Previous evidence that folate conjugase is an exopeptidase includes the demonstration of stepwise hydrolysis of $PteGlu_6$ during its incubation with human liver conjugase [17], and the progressive appearance within the luminal contents of all possible hydrolytic products of ^{14}C-labeled $PteGlu_7$ during its jejunal perfusion in human volunteers [5]. The similarity of the sequence of hydrolysis of ^{14}C-labeled $PteGlu_7$ by brush border folate conjugase (Fig. 3) and that observed during the human jejunal perfusion of ^{14}C-labeled $PteGlu_7$ [5] provides evidence that this enzyme is essential for the digestion of dietary polyglutamyl folates Species differences may account for a previous demonstration that folate conjugase purified from chick intestine behaved as an endopeptidase in liberating $PteGlu_2$ and $PteGlu_5$ as end products of hydrolysis with $PteGlu_7$ [18].

We are confident that the partially purified enzyme represents the brush border enzyme and not an intracellular contaminant since enzyme activity was assayed at pH 7.5 in the presence of *p*-hydroxymercuribenzoate, conditions which completely inactivate the intracellular enzyme [6]. Furthermore, identical DEAE-Sephadex elution profiles were obtained after chromatography of either the Triton-solubilized crude membrane or brush border fractions (Fig. 1). The purification process was complicated by the requirement for Triton X-100, a non-ionic detergent which solubilizes membrane proteins and forms micelles. However, the complete recovery of enzyme activity after Triton X-100 solubilization suggests that the detergent had no deleterious effects on this enzyme.

Ethanol, diphenylhydantoin and salicylazosulfapyridine were chosen for enzyme inhibition studies since the chronic use of each drug is associated with low serum folate levels in humans [19,20,21]. The concentrations used in our studies were based on previous direct intraluminal measurements [22,23] or an estimate of intraluminal levels [21]. Our data suggest that folate malabsorption in chronic alcoholism or during diphenylhydantoin therapy is not due to impaired hydrolysis of $PteGlu_n$, but may be ascribed to the previously observed effect of use of these drugs on the transport of PteGlu [23,24] The present study extends the list of folate-dependent enzymes competitively inhibited by salicylazosulfapyridine, all with similar apparent inhibition constants (K_i) [25]. The failure of 5-aminosalicyclic acid and sulfapyridine to inhibit brush border folate conjugase is consistent with a previous observation that salicylazosulfapyridine, but not its metabolites, inhibited the uptake of PteGlu by rings of intestinal tissue [21] Salicylazosulfapyridine, a drug which is poorly absorbed and not metabolized in the small intestine [26], appears to have a dual effect on folate absorption inhibition of the intestinal hydrolysis of $PteGlu_n$ as demonstrated in this study, and, as shown previously, inhibition of the intestinal transport of PteGlu [21]

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