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Folate Conjugase: Two Separate Activities in Human Jejunum

Abstract. *Previous perfusion studies of the human jejunum suggested that conjugated folate is hydrolyzed on the mucosal surface. The techniques of cell fractionation and DEAE and gel chromatography led to the identification of two separate folate conjugase activities in human jejunal mucosa: one membrane-bound and concentrated in the brush border, the other soluble and intracellular. These enzyme activities exhibit different* pH *optima, molecular weights, and inhibition characteristics. Folate conjugase in the brush border may accomplish the initial digestion of dietary pteroylpolyglutamates.*

The intestinal absorption of dietary folate requires hydrolysis of the predominant pteroylpolyglutamate forms to pteroylmonoglutamate (*1*). The hydrolytic enzyme, a γ-glutamyl hydrolase referred to as folate conjugase, is concentrated in the mucosa of the small intestine (*2*). The cellular location of this enzyme, and hence the sequence of folate digestion and membrane transport, is poorly understood. If mucosal folate conjugase is primarily lysosomal as reported (*3*), then pteroylpolyglutamates should enter the epithelial cell prior to hydrolysis. In contrast, the observation that perfusion of the human jejunum with ^{14}C-labeled pteroylheptaglutamate results in the appearance of its hydrolytic products in the intestinal lumen suggests that surface hydrolysis of pteroylpolyglutamates precedes cellular entry (*4*). We report evidence for two separate folate conjugase activities in human jejunal mucosa: one soluble and intracellular, and the other membrane-bound and concentrated in the brush border.

Segments of human jejunum were obtained at surgery from obese patients undergoing elective jejuno-ileal bypass. The mucosa was dissected from the remainder of the jejunal tissue, frozen immediately, and stored at −70°C until use. We fractionated the tissue by a modification of the method of Schmitz *et al.* (*5*). In a Waring Blendor, a 1 percent homogenate of mucosa was prepared in 50 m*M* mannitol, 2 m*M* tris (*p*H 7.1) at 5°C and filtered, yielding a whole homogenate fraction (WH). Calcium chloride was added to the homogenate to a final molarity of 10 m*M*. The mixture was stirred for 10 minutes and centrifuged at 2000*g* for 10 minutes, producing a "cell debris" precipitate (fraction P_1). Centrifugation of the supernatant at 20,000*g* for 15 minutes resulted in a second pellet which was washed twice to give a brush border fraction (BB) in which membrane particles could be identified by electron microscopy. The supernatant was further centrifuged at 50,000*g* for 2 hours, resulting in a final pellet (fraction P_3) and a final supernatant (fraction S). Each pellet was resuspended in the mannitol-tris buffer before assay. All fractions were assayed for protein (*6*), sucrase (*7*), and folate conjugase.

Folate conjugase activity was assayed with the use of a synthetic pteroyltriglutamate, pteroyldi-γ-[^{14}C]glutamate as the substrate, and the charcoal precipitation method (*8*). On the basis of a preliminary kinetic study with whole homogenates showing linear enzyme activity with respect to incubation time and enzyme concentration and zero order kinetics at concentrations greater than 6.5 μ*M*, we chose a substrate concentration of 13 μ*M* and a reaction time of 15 minutes. An initial experiment indicated that folate conjugase activity in fraction S was maximal at *p*H 4.5 while the enzyme in fraction BB was active over a broad *p*H range, with major activity at *p*H 7.5.

The results of six fractionation experiments are shown in Table 1. Fractions P_1 (cell debris) and S (final intracellular supernatant) accounted for 96 percent of the total protein. Sucrase, a brush border marker enzyme, was distributed nearly equally in the P_1 and BB fractions, while its specific activity was concentrated 12-fold in BB. At *p*H 4.5, 73 percent of folate conjugase was found in S while, at *p*H 7.5, the enzyme was distributed among P_1, BB, and S, with 12 percent recovery in BB. The specific activity of folate conjugase in S was eight times greater at *p*H 4.5 than at *p*H 7.5, but similar at

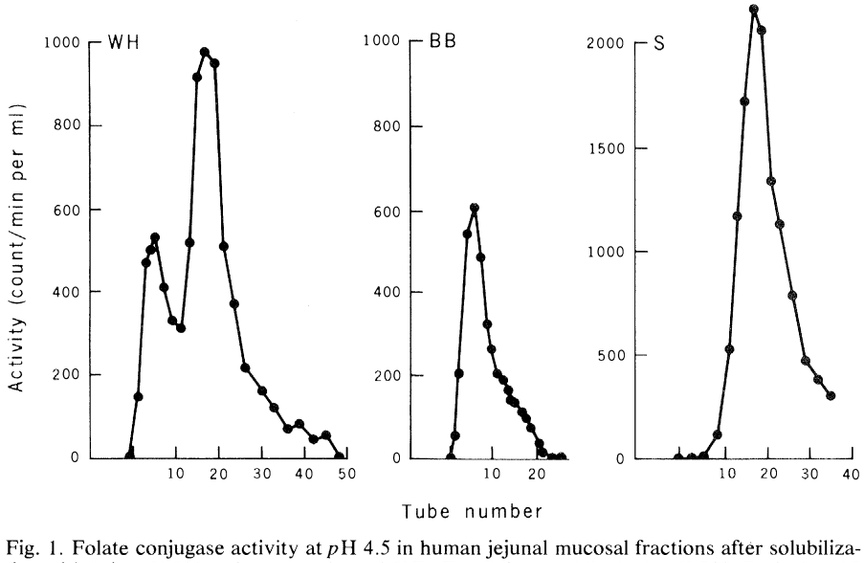

Fig. 1. Folate conjugase activity at *p*H 4.5 in human jejunal mucosal fractions after solubilization with Triton X-100 and passage through DEAE-cellulose and Sephadex G-200. Peaks 1 and 2 of the whole homogenate (WH, left panel) correspond in elution volume to peaks obtained after separate treatment of brush border (BB, center panel) and intracellular supernatant (S, right panel) fractions. Approximate molecular weights are: peak 1 (BB) 91,000, and peak 2 (S) 45,000. Subsequently, peak 1 (BB) was found optimally active at *p*H 7.5 and not inhibited by PHMB, while peak 2 (S) was optimally active at *p*H 4.5 and completely inhibited by PHMB.

Table 1. Fractionation of human jejunal mucosa. Results expressed as means ± standard error of the mean (N = 6).

Fraction*	Protein (percent)	Sucrase		Folate conjugase (pH 4.5)		Folate conjugase (pH 7.5)	
		Percent	Specific activity†	Percent	Specific activity‡	Percent	Specific activity
WH	100	100	415 ± 27	100	2.96 ± 0.22	100	0.483 ± 0.070
P_1	39 ± 2	47 ± 3	499 ± 29	14 ± 1	1.04 ± 0.03	36 ± 3	0.476 ± 0.035
BB	2.6 ± 0.3	32 ± 4	5170 ± 520	2.3 ± 0.3	2.67 ± 0.20	12 ± 2	2.36 ± 0.17
S	57 ± 2	1.7 ± 0.4	12.1 ± 2.4	73 ± 4	3.82 ± 0.41	39 ± 9	0.499 ± 0.067
P_3	1.1 ± 0.2	5.6 ± 1.2	2150 ± 190	0.7 ± 0.2	1.99 ± 0.24	2.9 ± 0.5	1.31 ± 0.13
Recovery	100 ± 3	86 ± 5		90 ± 4		90 ± 6	

*WH, whole homogenate; P_1, cell debris (2000g for 10 minutes precipitate); BB, brush border (20,000g for 15 minutes precipitate); S, soluble intracellular supernatant; P_3, final 50,000g for 2 hours pellet. †Specific activity in units per gram of protein (sucrase). ‡Nanomoles of substrate hydrolyzed per milligram of protein for 15 minutes (folate conjugate).

each pH in BB. However, relative to its whole homogenate activity, folate conjugase was concentrated fivefold in BB at pH 7.5; but there was no concentration of activity in BB at pH 4.5 or in S at either pH. The addition of 0.5 mM p-hydroxymercuribenzoate (PHMB), an inhibitor that binds to sulfhydryl groups and that has been shown to discriminate between cytoplasmic and brush border peptide hydrolases (*9*), decreased folate conjugase activity in S at pH 4.5 by 91.7 ± 1.4 percent (S.E.M.); at the same time inhibitions in BB at pH 4.5 and at pH 7.5 were 26.4 ± 2.0 percent and 7.6 ± 2.7 percent, respectively. These data suggest two separate mucosal folate conjugase activities: one membrane-bound, concentrated in the brush border, active at pH 7.5 and not inhibited by PHMB, and the other a soluble intracellular enzyme optimally active at pH 4.5 and inhibited by PHMB.

We further characterized the two mucosal folate conjugase activities as follows. Using a Potter-Elvejem glass-Teflon homogenizer, we prepared an 8 percent homogenate of jejunal mucosa in 5 mM β-mercaptoethanol, 10 mM tris (pH 7.0), followed by solubilization with 1 percent Triton X-100 at 5°C for 30 minutes. After centrifugation at 50,000g for 2 hours, initial purification of the resulting supernatant was accomplished by application to a DEAE-cellulose column (10 by 1.0 cm) and elution with the mercaptoethanol-tris buffer. This purification step yielded a single broad peak of enzyme activity. Fractions containing folate conjugase activity were pooled and concentrated by ultrafiltration with an Amicon PM 30 membrane. The concentrate, containing 70 to 80 percent of the initial folate conjugase activity, was then applied to a Sephadex G-200 column (40 by 2.0 cm) and eluted with mercaptoethanol-tris buffer containing 0.15M NaCl. Fractions (2 ml) were collected and assayed for folate conjugase activity at pH 4.5. Gel filtration of the solubilized whole homogenate resulted in two distinct peaks of activity (Fig. 1). With aldolase, bovine serum albumin, and ovalbumin as marker proteins, the molecular weights of the peaks were estimated as 91,000 (peak 1) and 45,000 (peak 2). In order to identify the two peaks, we first prepared BB and S fractions. The fractions were homogenized with β-mercaptoethanol-tris and treated with 1 percent Triton as described, and then each was separately chromatographed on DEAE-cellulose and Sephadex G-200. The elution volume of folate conjugase activity from solubilized BB corresponded to peak 1 of the whole homogenate, while S activity corresponded to peak 2. The fractions in each peak were pooled and assayed for folate conjugase activity over a pH range from 3.5 to 8.5, with the use of a series of 0.1M acetate, Mes, Tes, and tris-HCl buffers. Folate conjugase in the intracellular supernatant (S) peak was optimally active at pH 4.5, while the optimum pH in the BB peak was 7.5. At each pH, the addition of 0.5 mM PHMB completely inhibited supernatant folate conjugase activity but had no effect on brush border activity.

Our data indicate the existence of two separate jejunal mucosal folate conjugase activities, based on criteria of pH optima, inhibition by PHMB, and molecular weight. One enzyme activity, membrane-bound and concentrated in the BB fraction, has an approximate molecular weight of 91,000, a neutral pH optimum, and is not inhibited by PHMB. The second enzyme activity is soluble and intracellular, has an approximate molecular weight of 45,000, an acid pH optimum, and an apparent requirement for sulfhydryl groups in the active site since it is completely inhibited by PHMB. While certain characteristics of the brush border and supernatant folate conjugase activities may have been altered by treatment with detergent, nevertheless, prior to Triton solubilization, the brush border and supernatant fractions obtained by centrifugation exhibited distinct differences with regard to pH optima and PHMB inhibition. Although precise localization was not attempted, it is probable that the intracellular activity represents previously described lysosomal folate conjugase (*3*). Further characterization of these two mucosal folate conjugase activities will enhance understanding of pteroylpolyglutamate hydrolysis and absorption. Conceivably, pteroylpolyglutamates are initially hydrolyzed by brush border folate conjugase, thus permitting entry of folates of lower molecular weight into the cell.

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