JOURNAL OF BACTERIOLOGY, May 1978, p. 506-513
0021-9193/78/0134-0506$02.00/0

Vol. 134, No. 2
Printed in U.S.A.

Carboxypeptidase Displaying Differential Velocity in Hydrolysis of Methotrexate, 5-Methyltetrahydrofolic Acid, and Leucovorin

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Received for publication 22 September 1977

An enzyme that catalyzes the hydrolysis of folic acid and the antifolate methotrexate nearly 20 times more rapidly than the hydrolysis of 5-methyltetrahydrofolate was extracted from a gram-negative bacterium tentatively identified as a *Flavobacterium* sp. The enzyme was purified 500-fold and found to have a molecular weight of about 53,000. Apparently a metallo-enzyme, it is inhibited by citrate and ethylenediaminetetraacetic acid (EDTA). Ca^{2+}, Co^{2+}, Mg^{2+}, and Zn^{2+} reverse inhibition by EDTA, whereas Ca^{2+} and Zn^{2+} are weak activators in the absence of EDTA. The enzymatic reaction releases the carboxy-terminal glutamyl moiety of derivatives of pteroyl-mono-L-glutamic acid. Substituents on N^5 of the pteridine ring decrease the velocity of hydrolysis. Some non-specificity for the terminal amino acid is expressed. The strikingly different rates of hydrolysis of methotrexate and 5-methyltetrahydrofolate have stimulated interest in this enzyme for its potential clinical value in improving the therapeutic index of methotrexate.

The folic acid analog methotrexate (4-amino-N^{10}-methylpteroylglutamic acid [MTX]) is a potent antineoplastic agent (1). Its clinical application has been advanced with the use of leucovorin (N^5-formyltetrahydrofolic acid) rescue (8, 11, 13, 18, 26), which has been relatively successful in relieving the toxicity of antifolate therapy.

The report of a bacterial carboxypeptidase G_1 (CPD G_1) with hydrolytic activity for MTX introduced the possibility of the enzymatic approach to ameliorating drug toxicity (12, 23). In this report, we describe the partial purification and characterization of another enzyme that catalyzes the hydrolysis of MTX, folic acid (FA), and other derivatives of FA by the reaction shown in Fig. 1. This carboxypeptidase is produced by a bacterium tentatively designated *Flavobacterium* sp. and differs from CPD G and CPD G_1 (15, 19, 23).

MATERIALS AND METHODS

Chemicals. FA (pteroyl-L-glutamic acid) was purchased from Grand Island Biological Co. *dl*-N^5-methyltetrahydrofolic acid was obtained from Sigma Chemical Co. as the barium salt. It was converted to the sodium salt by the addition of an equal volume of 0.1 M sodium phosphate, pH 6.5, to a 1% solution of the folate in 0.1 M sodium bicarbonate–0.05% potassium ascorbate. Leucovorin (*dl*-N^5-formyltetrahydrofolic acid, calcium salt) and MTX were provided by Bernard C. Clark of Lederle Laboratories, American Cyanamid Co., and by Harry B. Wood, Jr., Drug Development Branch, Drug Research and Development, Silver Spring, Md. Chromatographically purified MTX (4) was used for kinetic studies. Purified aminopterin was a gift from Francis M. Sirotnak, Sloan-Kettering Institute. Harry B. Wood, Jr., also provided bioautographically pure samples of methasquin (17), the quinazoline antifolate *N*-[*p*-[[(2,4-diamino-5-methyl-6-quinazolinyl)methyl]amino]benzoyl]-L-aspartic acid. Teropterin, pteroic acid, pteroyl-L-aspartic acid, pteroylglycine, pteroyl-D-glutamic acid, the 4-amino analog of pteroylaspartic acid, and other derivatives of FA were also gifts from Lederle Laboratories. Pteroic acid also was isolated from the spent medium of a bacterium grown with FA as the sole nitrogen source (A. M. Albrecht, J. J. Fenton, and D. J. Hutchison, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P60, p. 151). It was the substrate for the enzymatic synthesis of 5-formyltetrahydropteroic acid by a cell extract of *Streptococcus faecium* subsp. *durans*/A_k (3, 5, 10). Bacterial extract was incubated at 37°C with pteroic acid in a reaction mixture also containing reduced nicotinamide adenine dinucleotide phosphate, ascorbic acid, ammonium formate (as formyl source), ATP, and $MgCl_2$. Conversion of pteroic acid to its reduced, formylated derivative was confirmed bioautographically with *S. faecium* subsp. *durans*/0.

Bacterial cultures. Initially, SKI-CF/6, a water isolate, was the source of carboxypeptidase. SKI-CF/6 consisted of four gram-negative, nonfermentative bacteria tentatively identified as species of *Acinetobacter*, *Flavobacterium*, *Pseudomonas*, and *Vibrio*. Taxonomic data obtained with pure cultures of each bacterial strain will be published elsewhere. Later studies

were carried out with the *Flavobacterium* sp., the predominant carboxypeptidase-producing bacterium of the mixed culture.

Media. The basal medium consisted of 50 mM potassium phosphate, pH 7.0, and the salts provided by the addition of 5.0 ml of salts A and 1.5 ml of salts B per liter of medium (19). Salts A was the clear supernatant solution obtained after several days of storage at 4°C of an aqueous mixture of 1% $MgSO_4$, 0.1% $CaCl_2 \cdot 2H_2O$, and 0.1% $FeSO_4 \cdot 7H_2O$. Salts B contained 0.1% $MnSO_4$ and 0.1% $NaMoO_4 \cdot 2H_2O$. Medium supplemented with 0.2% calcium leucovorin and 0.5% glucose, both added aseptically, was used for the isolation of SKI-CF/6, for maintenance of stock cultures in solid medium, and for the first broth subcultures. Clear, sterile solutions of leucovorin were obtained by autoclaving solutions of 2% calcium leucovorin in the basal medium. This treatment precipitated insoluble calcium salts without affecting the leucovorin concentration and provided material for addition (1 part) to the otherwise complete medium (9 parts).

For enzyme production, the growth medium for SKI-CF/6 contained 0.3% sodium ammonium phosphate instead of leucovorin. For the *Flavobacterium* sp., basal medium was supplemented with 0.3% sodium citrate, 1.0% yeast extract, and the following vitamins (in micrograms per milliliter [14]): biotin, 0.01; thiamine, 0.2; calcium pantothenate and nicotinic acid, each at 0.4; *p*-aminobenzoic acid and riboflavin, each at 0.5; and pyridoxine and pyridoxal, each at 1.

Standard buffer. The standard buffer was 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.2.

Bacterial cultivation procedures. Procedure 1 was initiated with 3- to 4-week-old stock cultures of SKI-CF/6 and carried out at 30°C and 240 rpm in a New Brunswick Scientific Co. shaker, model G26. The resultant 4-day-old subcultures were the 0.5% inocula for 1-liter production cultures grown in 2-liter Erlenmeyer flasks. Production cultures were harvested at 90 h. Bacterial cells were washed by suspension in standard buffer and stored at −20°C as cell suspensions. In procedure 2, 48-h subcultures of the *Flavobacterium* sp. in medium supplemented with leucovorin, glucose, 0.1% yeast extract, and the vitamins indicated above were used as the 0.3% inocula for production cultures. The remainder of procedure 2 resembled procedure 1, except that production cultures of the *Flavobacterium* sp. were harvested 24 h after inoculation.

Partial purification of the enzyme. Bacterial cell suspensions were treated conventionally with acetone (4°C) and dried with ether, and the resultant powder was stored at −20°C.

The following steps were carried out at 0 to 4°C with centrifugation at 27,000 × *g* for 15 min. Acetone powder (10 g) was stirred with 100 ml of standard buffer for 30 min. The mixture was centrifuged, and the pellet was reprocessed with 30 ml of buffer. Supernatant solutions obtained from the first and second extractions were combined to yield the cell extract (fraction 1). Fraction 1 was treated with ammonium sulfate in two steps, each consisting of the slow addition of the salt, stirring for 15 min, and centrifugation. First, 28.8 g of the salt was added to 100 ml of fraction 1, and then 23.5 g was added to 109 ml of the supernatant solution from the first step. The resultant pellet was taken up in buffer, and the hazy suspension was clarified by centrifugation to yield fraction 2. Equal portions (4.9 ml) of fraction 2 were filtered through a column (5 by 45 cm) of Sephadex G-200 in three consecutive filtrations with standard buffer as the eluant. Peak fractions (9.5 ml each) were combined to yield fraction 3. A portion (290 ml) of fraction 3 was stirred for 15 min with 1.3 g of diethylaminoethyl-Sephadex A-50, 40 to 120 μm, previously equilibrated with 10 mM Tris, pH 8.0. After centrifugation of the gel mixture, the gel was discarded and 181 g of ammonium sulfate was added to 280 ml of the supernatant solution (fraction 4). The precipitate was taken up in standard buffer, and the slightly turbid suspension was centrifuged to yield a clear supernatant solution (fraction 5, 12.0 ml).

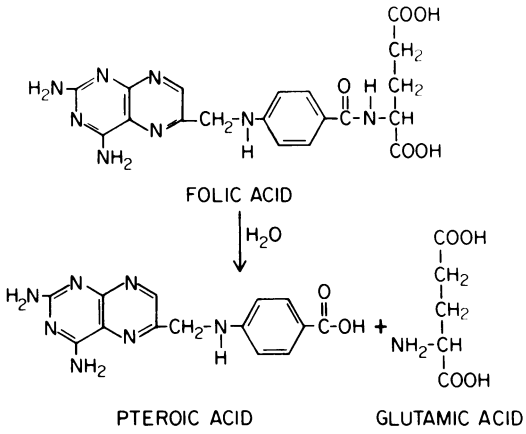

FIG. 1. *Enzymatic hydrolysis of folic acid.*

Enzyme assays. The standard spectrophotometric assay was based upon the decrease in absorbance at 302 nm which occurs when FA is hydrolyzed to pteroic acid (25). Enzyme preparations were routinely preincubated for 5 min at 37°C in 2.9 ml of 50 mM Tris buffer, pH 7.25. After the addition of 0.11 μmol of FA, the reaction was followed for 3 to 5 min. One enzyme unit was that amount of enzyme that catalyzed the hydrolysis of 1 μmol of FA per min under the described conditions. For kinetic studies, enzymatic reactions were incubated for predetermined times, and residual substrates were assayed microbiologically. In kinetic studies, reactions were usually stopped by chilling or by placing tubes in boiling water for 5 min. Potassium ascorbate (0.2% final concentration) was added to reaction mixtures containing 5-methyltetrahydrofolate to stop the reaction and to prevent the oxidation of 5-methyltetrahydrofolate.

Microbiological assays. Disk assays were carried out with *S. faecium* subsp. *durans*/0, *Lactobacillus casei* ATCC 7469, or *Pediococcus cerevisiae* ATCC 8081 and the appropriate medium (9, 14, 27). Samples and reference compounds were applied to paper disks (6.35-mm diameter) that were subsequently aligned on the surface of seeded agar in Pyrex plates (18 by 29 cm). Quantitative determinations were based on the diameter of the zones of growth or inhibition that developed at 37°C. To accentuate the zones of growth

of *L. casei*, a warm solution of 0.1% 2,3,5-triphenyltetrazolium chloride in 1.5% agar was layered over the solid medium. Well-defined, magenta zones developed with further incubation (2 to 3 h, 37°C).

Appropriately seeded agar was also used to identify enzymatic degradation products bioautographically (2, 9). Paper chromatograms of reaction mixtures were placed on seeded agar, and R_f values were determined from the location of zones of growth or inhibition that developed during incubation.

The *L. casei* assay was valuable in determining enzymatic reaction rates with FA and 5-methyltetrahydrofolate as substrates at concentrations below the level of spectral detection. Whereas *L. casei* readily used these folates as growth factors (Fig. 2), it did not respond to pteroic acid and responded weakly to the degradation product of 5-methyltetrahydrofolate (Fig. 3). Similarly, *P. cerevisiae*, unresponsive to 5-formyltetrahydropteroic acid, was used to determine leucovorin (27). *S. faecium* subsp. durans/0, which responded to pteroic and 5-formyltetrahydropteroic acids (Fig. 4), was used to identify the degradation products of FA and leucovorin and to quantify antifolates.

Other procedures. Ascending chromatography was carried out on 1-cm strips of Whatman no. 1 paper with 5% ammonium bicarbonate for 3 to 4 h at 25°C. Ascorbic acid (100 mg/ml), as an antioxidant, was added to the solvent for samples containing 5-methyltetrahydrofolate and/or its degradation product. The filtration behavior of the carboxypeptidase was compared with that of reference proteins on a column of Sephadex G-100 (1.5 by 85 cm) equilibrated with standard buffer containing 0.1 mM $ZnCl_2$ at 4°C. The molecular weight of carboxypeptidase was calculated by the method of Whitaker (32). Release of the glutamyl moiety of MTX was confirmed (6) with an Automatic Amino Acid Analyzer (Beckman Instruments Co., model 119) in the laboratory of Joseph Roberts of Sloan-Kettering Institute. Amino acids released enzymatically from other pteroyl derivatives were quantified by the method of Moore and Stein (24), and protein was determined by the method of Lowry et al. (22).

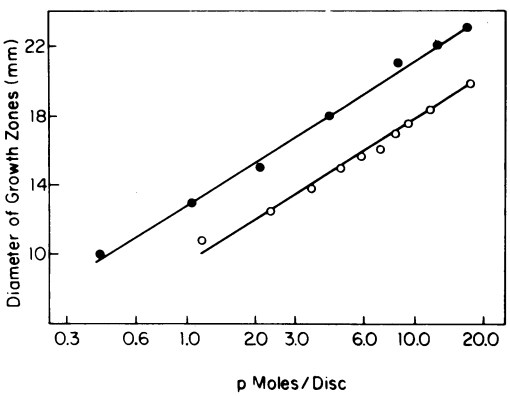

FIG. 2. *Quantitative growth response of L. casei to FA (●) and to dl-5-methyltetrahydrofolate (○).*

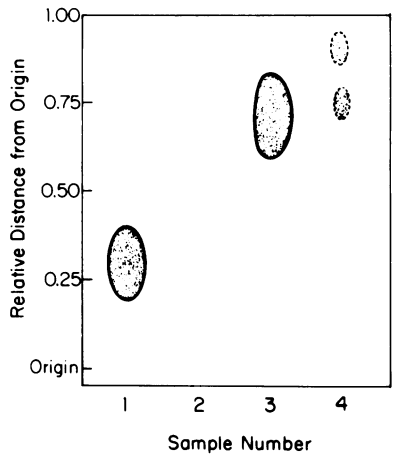

FIG. 3. *Bioautogram, obtained with L. casei, of 5 ng of FA (1), 10 ng of dl-5-methyltetrahydrofolate (3), and the corresponding products obtained after carboxypeptidase-catalyzed hydrolysis (2 and 4, respectively).*

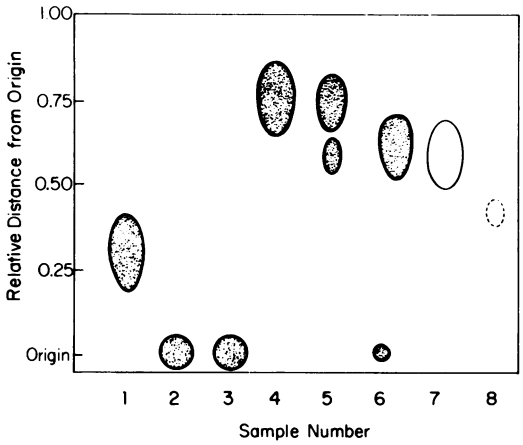

FIG. 4. *Bioautogram, obtained with S. faecium subsp. durans/0, of 5 ng of FA (1), FA degradation product (2), 5 ng of pteroic acid (3), 10 ng of leucovorin (4), the leucovorin-degradation product (5), the enzymatic reaction mixture containing pteroic acid and its reduced, formylated derivative (6), 10 ng of MTX (7), and the MTX degradation product (8). Growth zones are stippled; inhibition zones are not stippled.*

RESULTS

Enzyme purification. The procedure summarized in Table 1 describes the 90-fold purification of the carboxypeptidase extracted from SKI-CF/6. This procedure was initiated with clear extracts of acetone powders that had 5- to 10-fold-higher specific activity than did extracts obtained by ultrasonic disruption of cells. The latter method produced turbid extracts with a

TABLE 1. *Summary of enzyme purification*[a]

Fraction no.	Purification step	Vol (ml)	Total protein (mg)	Total enzyme units	Sp act (U/mg)	Yield (%)
1	Extraction of acetone powder	100	404.0	180	0.44	100
2	Ammonium sulfate fractionation	15	153.8	120	0.78	67
3	Sephadex G-200 filtration	306	18.4	55	3.00	31
4	Diethylaminoethyl-Sephadex A-50, batch treatment	293	2.9	43	12.00	24
5	0.90 Saturation with ammonium sulfate	12	0.8	33	39.43	18

[a] Purification of enzyme produced by approximately 21 liters of culture of SKI-CF/6 (10 g of acetone powder).

specific activity of 0.04 to 0.08 U/mg. Thus, the enrichment achieved by the initial extraction contributed to an overall 500-fold purification. Application of the procedure to the enzyme produced by the pure culture of the *Flavobacterium* sp. gave a sevenfold-greater yield of fraction 5, with a specific activity of 68 U/mg.

Reaction products. The purified enzyme catalyzed the hydrolysis of FA according to the reaction shown in Fig. 1. Pteroic acid (30) and 4-amino-4-deoxy-N^{10}-methylpteroic acid (28) were identified spectrally (Fig. 5) as the products of the degradation of FA and MTX, respectively. Amino acid analysis demonstrated that the reaction mixture containing 4-amino-4-deoxy-N^{10}-methylpteroic acid also contained 1.1 nmol of glutamic acid per nmol of MTX degraded.

As shown in Fig. 5, pronounced spectral change did not accompany the hydrolysis of 5-methyltetrahydrofolate. With leucovorin as the substrate, a slight reaction was observed spectrophotometrically only after prolonged incubation of the reaction mixture. Microbiological methods aided the investigation of the enzyme-catalyzed reaction with these two derivatives of FA. The growth response of *L. casei* to 5-methyltetrahydrofolate provided evidence for the degradation of methylfolate to a product with relatively poor growth factor activity for *L. casei* (Fig. 3). The growth response of *S. faecium* subsp. *durans*/0 demonstrated that the reaction with leucovorin was incomplete, yielding 5-formyltetrahydropteroic acid as the product (Fig. 4). Growth characteristics of *S. faecium* subsp. *durans*/0 also supported the identification of the degradation product of FA as pteroic acid and the weak inhibitory activity of the hydrolytic products of MTX and aminopterin.

Effect of substrate and substrate concentration on reaction velocity. The velocity of MTX degradation, like FA degradation, was greater than the hydrolysis of 5-methyltetrahydrofolate, whereas the reaction with leucovorin was restricted with respect to velocity and extent of cleavage (Fig. 6). Kinetic characteristics calculated from Lineweaver-Burk plots (21) illustrate nearly a 20-fold difference between the rates of degradation of FA or MTX and 5-methyltetrahydrofolate (Table 2). The unusual reaction with leucovorin precluded kinetic analysis of the cleavage of leucovorin.

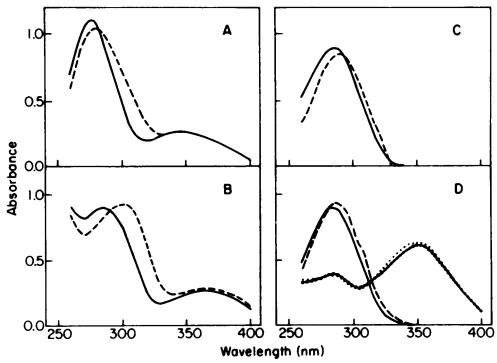

FIG. 5. *Spectral demonstration of carboxypeptidase activity in 0.05 M Tris, pH 7.25. Substrates are represented by broken lines, and enzymatic products are represented by solid lines; leucovorin and the enzymatic reaction mixture containing leucovorin, both after acidification (0.25 N HCl), are represented by the dotted line and solid dotted line, respectively: (A) 40 nmol of FA per ml and (B) 40 nmol of MTX per ml, before and after reaction with 0.04 enzyme unit for 5 min; (C) 30 nmol of dl-barium methyltetrahydrofolate per ml, before and after reaction with 0.2 enzyme unit for 20 min; and (D) 20 nmol of dl-calcium leucovorin per ml, before and after prolonged incubation (90 min) with 0.2 enzyme unit.*

The possibility that leucovorin or its degradation product might be inhibitory was investigated by combining leucovorin and MTX in reaction mixtures. Leucovorin (330 μM) incubated simultaneously with the enzyme and MTX (44 μM) or after preincubation with the enzyme did not inhibit the hydrolysis of MTX.

Substrate specificity. Release of the terminal amino acid provided evidence of hydrolytic activity with other compounds studied as substrates. As shown in Table 3, analogs of pteroyl-L-glutamic acid without N^5 substitution, as well

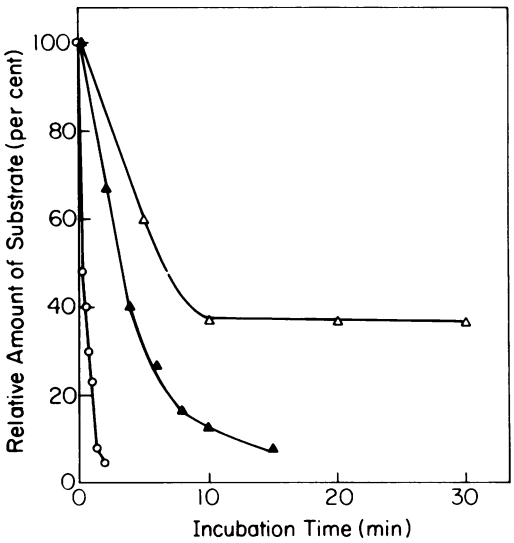

FIG. 6. *Time course of the hydrolysis of 23 nmol of MTX (○), 25 nmol of dl-barium methyltetrahydrofolate (▲), and 30 nmol of dl-calcium leucovorin (△) as catalyzed by 0.02 enzyme unit.*

TABLE 2. *Kinetic values*[a]

Substrate	K_m ($\times 10^{-5}$ M)	V_{max} (μmol/min per mg)
Folic acid, sodium	8.0	200
Methotrexate, potassium	5.0	200
5-Methyltetrahydrofolic acid, barium or sodium	5.7	12

[a] Characteristic values were calculated from double-reciprocal plots of data obtained for reaction mixtures (5 min) of 0.002 enzyme unit and FA ranging from 0.4 to 20 nmol/ml or MTX ranging from 0.2 to 20 nmol/ml and of 0.02 enzyme unit and *dl*-5-methyltetrahydrofolic acid, barium or sodium salt, ranging from 0.8 to 24 nmol/ml.

as *p*-aminobenzoylglutamic acid, were hydrolyzed completely. By contrast, the enzyme was essentially inactive with pteroyl-D-glutamic acid and methasquin (a quinazoline antifolate). The hydrolysis of pteroylglycine, pteroyl-L-aspartic acid, 4-aminopteroylaspartic acid, and teropterin (pteroyl-γ,γ-triglutamic acid) proceeded slowly. Bioautographic data demonstrated that the product of the limited cleavage of teropterin was diopterin (pteroyldiglutamate).

Effect of pH and temperature on the reaction. The degradation of FA or MTX was most rapid in 50 mM Tris buffer near pH 7.2. A similar pH optimum was observed in 50 mM potassium phosphate, although reaction rates in the phosphate system were slightly nonlinear with time. With 5-methyltetrahydrofolate as the substrate, reaction rates were 20% faster in Tris and exhibited broad pH optima ranging between pH 7.0 and 8.0 (Tris) and pH 6.0 and 8.0 (phosphate). The unusual reaction with leucovorin reflected pH optima near pH 7.5 to 8.0 (Tris) and pH 6.5 to 6.8 (phosphate).

With MTX and 5-methyltetrahydrofolate as substrates, the reaction proceeded equally well between 30 and 42°C.

Inhibitors. Degradation products, 100 μM pteroic acid and 100 μM glutamic acid, inhibited the degradation of 40 μM FA by 30 and 10%, respectively. Sulfhydryl agents inhibited more strongly; 2-mercaptoethanol at 10 and 100 μM inhibited by 50 and 95%, respectively, and 3 and 30 μM dithiothreitol inhibited by 20 and 90%, respectively. By contrast, 1 mM potassium ascorbate was harmless, whereas concentrations greater than 3 mM inhibited strongly. Sodium citrate buffers (50 mM) and ethylenediaminetetraacetic acid (EDTA; 300 μM) were also strong inhibitors.

Effect of metals. A pronounced metal requirement was not observed. However, Ca^{2+}, Co^{2+}, Mg^{2+}, and Zn^{2+} reversed the inhibition by 300 μM EDTA (Table 4). K^+, Mn^{2+}, Mo^{2+}, and

TABLE 3. *Substrate specificity*[a]

Compound	Amino acid released (μmol/μmol of compound)	
	Expt 1	Expt 2
Pteroyl-L-glutamic acid (FA)	1.00	1.22
Pteroyl-D-glutamic acid	0.09	
9-Methylpteroyl-L-glutamic acid	1.09	
2,4-Dihydroxypteroyl-L-glutamic acid	1.00	0.85
5-Formyltetrahydrofolic acid, calcium salt (leucovorin)	0.70	0.34
5-Methyltetrahydrofolic acid, barium salt	0.76	0.60
Pteroyltriglutamic acid (teropterin)	0.26	
p-Aminobenzoylglutamic acid	0.98	
Pteroylglycine	0.14	
Pteroyl-L-aspartic acid	0.25	
4-Aminopteroylaspartic acid	0.33	
Methotrexate (MTX)	1.10	1.30
Aminopterin		1.07
Methasquin	0.00	0.10
γ-L-Glutamyl-L-glutamic acid	0.62	

[a] Compounds were incubated (37°C) in 50 mM Tris (pH 7.25) with fraction 3 for periods of time that were longer than that required for the complete hydrolysis of FA and MTX: three times longer (experiment 1) and two times longer (experiment 2). Portions of each reaction mixture were assayed for free amino acid.

TABLE 4. *Effect of EDTA and metal ions*[a]

EDTA (0.3 mM)	Metal (1 mM)	ΔA/min
–	–	0.065
+	–	0.015
+	$MgCl_2$	0.066
+	$ZnCl_2$	0.068
+	$CaCl_2$	0.062
+	$CoCl_2$	0.052

[a] Fraction 5 was preincubated for 5 min at 37°C in 0.05 M Tris buffer (pH 7.2) with the salts indicated before the addition of FA. The reaction was followed spectrophotometrically at 302 nm. ΔA, Change in absorbance.

Na^+ had little, if any, effect. In the absence of EDTA, 10 mM $CoCl_2$ and 10 mM $ZnCl_2$ were inhibitory, and $CaCl_2$ and $ZnCl_2$, between 50 and 300 μM, enhanced activity by 30%. $MgCl_2$ had no effect in the absence of EDTA.

Properties of the enzyme. The filtration behavior of the enzyme through the calibrated column of Sephadex G-100 (Fig. 7) suggests a molecular weight of approximately 53,000 (32).

The enzyme activity of fraction 5 at −20°C was stable for 1 year, whereas the activity of acetone powder was maintained for several months. In dilute solution (4 to 6 μg of protein per ml of standard buffer), fraction 5 had a half-life of 4 h at 4°C. At 25 and 37°C, the half-life values were 90 and 45 min, respectively. The addition of 0.3% bovine albumin to dilute enzyme solutions reduced the decay of activity to an initial 10% decrease within 30 min (4 and 25°C) and less than 10% additional loss within the subsequent 5 h. Although protection by albumin was less effective at 37°C, 0.03% albumin extended the half-life to 6 h.

In dilute solutions protected by 0.03% bovine albumin, the carboxypeptidase was most stable in 10 mM potassium phosphate buffers between pH 7.0 and 8.0. At 4°C, 100% activity was retained for 1 h, with a 10% loss during the subsequent 4 h. At 37°C, the activity decreased by 20% within 30 min without further loss during the next 4 h. Progressively greater lability was noted at pH 6.5 and at pH values of 5.5 and lower.

DISCUSSION

Microbial degradation of FA analogs by *Aerobacter aerogenes* and *Escherichia coli* B was reported more than 20 years ago (31). The mechanism was shown to be oxidative cleavage between C^9 and N^{10}, yielding free pteridines and *p*-aminobenzoylglutamic acid. However, the pertinent enzymes apparently were not isolated. Similar cleavage of FA by an enzyme, tentatively designated pteridine 6-methylaminohydrolase, of a strain of *Pseudomonas acidovorans* was reported recently (7).

To date, only those enzymes that hydrolyze the carboxy-terminal glutamyl moiety of MTX, such as CPD G and CPD G_1, have been extensively purified and characterized (15, 19, 23). In general, the enzyme described in this report resembles those carboxypeptidases in cleaving the glutamyl moiety of specific derivatives of pteroyl-mono-L-glutamate (FA) and in showing some lack of specificity for the carboxy-terminal amino acid. This similarity suggested that the *Flavobacterium* sp. enzyme be designated a carboxypeptidase. Further characterization and specificity studies with homogeneous preparations are necessary to justify the biochemical classification.

Specifically, the *Flavobacterium* sp. carboxypeptidase differs from CPD G and CPD G_1. The *Flavobacterium* sp. enzyme apparently has a 20-fold-higher K_m value for MTX. Perhaps uniquely, however, this enzyme demonstrates maximum velocity in the degradation of the 4-amino analogs (MTX and aminopterin) as well as FA. Because substituents on N^5 of the pteridine ring decrease the velocity of the reaction, the V_{max} value for MTX is about 20 times greater than the V_{max} for 5-methyltetrahydrofolate, and the reaction with 5-formyltetrahydrofolate is restricted, ceasing at 60% of completion.

This *Flavobacterium* sp. enzyme also differs in substrate specificity from the folate amidase produced by a *Flavobacterium* sp. described by Pratt et al. (25) and from the peptidase produced by *Flavobacterium polyglutamicum* (29).

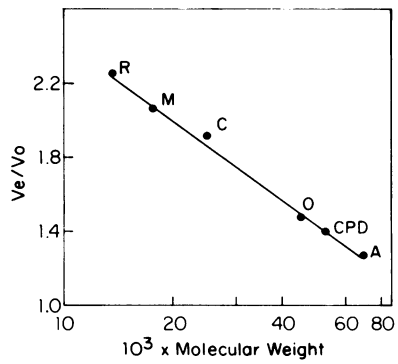

FIG. 7. *Semilogarithmic plot of V_e/V_o versus molecular weight of protein markers and carboxypeptidase (CPD). The void volume (V_o) was determined by elution of blue dextran, and the absorbance of proteins was determined at 280 nm. The volume at peak elution of the proteins (A, bovine albumin; O, ovalbumin; C, chymotrypsinogen A; M, myoglobin; and R, ribonuclease) was regarded as V_e.*

Hydrolysis of teropterin (pteroyl-γ,γ-triglutamate) to diopterin (pteroyldiglutamate) and partial cleavage of γ-L-glutamyl-L-glutamic acid possibly reflect the presence of a contaminating enzyme (29) or, in fact, limited activity of the carboxypeptidase per se with γ-L-glutamyl-L-glutamic acid. Similarly, the presence of a peptidase specific for the aspartyl moiety (20) or non-specificity of the *Flavobacterium* sp. carboxypeptidase toward the carboxy-terminal amino acid could explain the limited cleavage of pteroyl-L-aspartic acid and its 4-amino analog.

An early report of this carboxypeptidase erroneously described its source as an *Acinetobacter* sp. (A. M. Albrecht, E. Boldizsar, and D. J. Hutchison, Abstr. Annu. Meet. Fed. Am. Soc. Exp. Biol. 1976, 3203, p. 787; Fed. Proc. **35**:787, 1976) because this bacterium in the mixed culture grew most rapidly on the characterization media. With the successful isolation of pure cultures of the component strains of SKI-CF/6, the major enzyme-producing bacterium has been identified and tentatively classified as a *Flavobacterium* sp. It requires exogenous nutrients that presumably were synthesized by the other bacterial components of the mixed culture. Although yeast extract and vitamin supplementation effectively promoted growth and enzyme synthesis by the pure culture of the *Flavobacterium* sp., the synergism expressed by SKI-CF/6 requires further studies.

Characteristics of the reaction catalyzed by this carboxypeptidase support its evaluation in experimental therapeutic regimens. The expression of different rates of hydrolysis in vivo of MTX and 5-methyltetrahydrofolate, the predominant plasma folate (16), should result in preferential degradation of the drug when present at toxic concentrations of 5 to 20 nM. Extremely efficient enzymatic degradation of MTX occurs in mice at drug concentrations as low as 10 nM (A. M. Albrecht, unpublished data). However, the efficacy of the enzyme at lower plasma MTX levels and its overall effect in improving the therapeutic index of MTX require further investigation.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA 17839 and CA 08748 from the National Cancer Institute and grant CI-112P from the American Cancer Society.

We gratefully acknowledge the active participation of Robert E. Weaver (Center for Disease Control, Atlanta, Ga.) and Gerald Ghilardi (Hospital for Special Surgery, New York, N.Y.) in the identification of the bacteria. We thank Catherine Staley, who volunteered service throughout the project, and Carol Pratt and John Lewis, who contributed technically to the initial stages of research dealing with microbial degradation of folates.

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