Carboxypeptidase-Mediated Release of Methotrexate from Methotrexate α-Peptides†

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ABSTRACT: Methotrexate (MTX) α-peptides containing representative neutral (alanine), acidic (aspartic acid), and basic (arginine) amino acids were synthesized by a regiospecific route. Purity and authenticity of MTX-Ala, MTX-Asp, and MTX-Arg were established by TLC, HPLC, elemental analysis, and NMR and absorbance spectra. These peptides were hydrolyzed by carboxypeptidases to yield MTX and the amino acids. Reactions were monitored by using a ninhydrin assay for the amino acids and HPLC and spectrophotometric assays for MTX. Pancreatic carboxypeptidase A (CP-A) hydrolyzed MTX-Ala and, at a much slower rate, MTX-Asp and MTX-Arg. MTX-Ala was also a substrate for pancreatic carboxypeptidase B (CP-B); marginal activity was observed with this enzyme and MTX-Arg. Human serum hydrolyzed only MTX-Arg; biphasic inhibition of this activity by 2-(mercaptomethyl)-3-(guanidinoethyl)thiopropionate was consistent with the known presence of two types of endogenous carboxypeptidase (CP-N). Cytotoxicity of the MTX peptides toward L1210 cells in culture was enhanced considerably in the presence of the appropriate carboxypeptidases. MTX-Ala was much less toxic than MTX (ID_{50} values of 2.0×10^{-6} M and 2.4×10^{-8} M, respectively), but in the presence of CP-A the ID_{50} of the peptide improved to 8.5×10^{-8} M. Similar results were obtained with MTX-Asp/CP-A and MTX-Ala/CP-B combinations. MTX-Arg showed good cytotoxicity (ID_{50} of 5.0×10^{-8} M), due to CP-N activity in the fetal bovine serum of the culture medium; inclusion of CP-B lowered the ID_{50} to that of MTX. Possible clinical uses of MTX peptides are discussed.

Methotrexate (MTX),[1] which is used extensively in cancer chemotherapy, enters eukaryotic cells via an active transport system whose primary substrate is 5-methyltetrahydrofolate [reviewed by Henderson (1986)]. After internalization, MTX is polyglutamylated (which enhances its retention in the cells), and the cytotoxicity of MTX polyglutamates results primarily from their potent inhibition of dihydrofolate reductase [reviewed by Huennekens et al. (1987)]. A membrane-associated folate-binding protein in L1210 mouse leukemia cells, which mediates the transport process, has been solubilized and partially characterized (Henderson & Zevely, 1984; Price & Freisheim, 1987). Substrate specificity of the transport system has been obtained from studies on the uptake or inhibitory activity of various folate compounds. It has been found, for example, that derivatization of the carboxyl groups via amide or ester formation greatly impairs internalization of the substrate; in this respect the α-carboxyl appears to be more critical than the γ-carboxyl (Sirotnak et al., 1979).

The above considerations suggest that MTX α-peptides (i.e., derivatives in which amino acids are joined via an amide linkage to the α-carboxyl of MTX) might have chemotherapeutic potential. These compounds would not be taken up readily by cells, but, if they could be hydrolyzed enzymatically, free MTX would be available for uptake and cell kill. Ideally, it would be desirable for release of the drug to occur only in the vicinity of tumor cells, thereby sparing normal cells from concomitant destruction. This might be accomplished by linking the enzyme to a monoclonal antibody targeted to the tumor.

The alanine, aspartic acid, and arginine derivatives of MTX (MTX-Ala, MTX-Asp, and MTX-Arg), whose structures are shown in Figure 1, were selected as model peptides of MTX containing neutral, acidic, and basic amino acids. The present report describes the synthesis of these compounds via the general procedures developed by Rosowsky et al. (1981) and Piper et al. (1982), their susceptibility to hydrolysis by carboxypeptidases, and the cytotoxicity of the free drug, generated in this manner, for L1210 cells in culture.

Experimental Procedures

Materials

The following were obtained from the indicated commercial sources: 2,4,5,6-tetraaminopyrimidine sulfate hydrate, dihydroxyacetone, *p*-(methylamino)benzoic acid, triphenylphosphine, diethylphosphite, isobutylchloroformate, Pd on charcoal, and Celite (Aldrich); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, *N*-Cbz-L-Glu γ-*tert*-butyl ester, L-Ala *tert*-butyl ester hydrochloride, L-Asp di-*tert*-butyl ester hydrochloride, L-Arg methyl ester dihydrochloride, hippuryl-Phe, hippuryl-Arg, ninhydrin, ninhydrin with aerosol

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[1] Abbreviations: MTX, methotrexate; MTX-Ala, -Asp, and -Arg, peptides in which the indicated amino acids are linked covalently to the α-carboxyl of MTX; CP-A, -B, -N, and G_2, carboxypeptidase A, B, N, and G_2; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; ID_{50}, concentration producing 50% cell kill.

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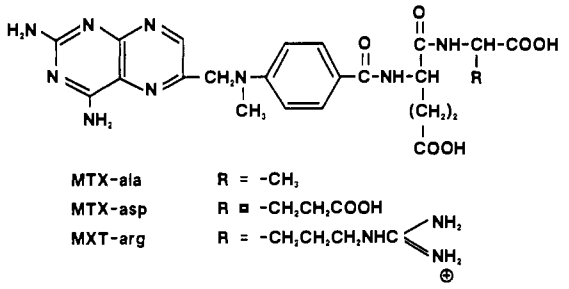

FIGURE 1: MTX α-peptides.

propellant, pancreatic CP-A (bovine; type I; 50 units/mg of protein), pancreatic CP-B (porcine; 200 units/mg of protein), trypsin, chymotrypsin, plasmin, subtilisin, bromelain, leucine aminopeptidase, pronase E from *Streptomyces griseus*, and pancreatic protease type I (Sigma); ficin and proteinase A from *Bacillus subtilis* (Serva); DL-2-(mercaptomethyl)-3-(guanidinoethyl)thiopropionic acid and urokinase (Calbiochem); DEAE-Trisacryl (IBF Biotechnics); C_{18} silica gel (J. T. Baker); and nylon filters (pore size 0.2 μm, Nalge).

Carboxypeptidase G_2 isolated from *Pseudomonas* sp. strain RS-16 (Sherwood et al., 1985) was kindly provided by Dr. J. R. Bertino (Memorial Sloan Kettering Cancer Center). MTX and a cloned human dihydrofolate reductase were gifts from Dr. S. Kerwar (Lederle Laboratories) and Dr. J. Freisheim (Medical College of Ohio), respectively.

Diethyl phosphorocyanidate was prepared by the procedure of Rosowsky et al. (1981), except that the reaction mixture was stirred for 12 h after addition of CNBr. Human serum was obtained by centrifuging whole blood (obtained from the General Clinical Research Center of this institution) at 2500 rpm and 5 °C for 5 min. The supernatant was dialyzed against three changes of 0.85% NaCl and stored frozen. Just prior to use, the serum was diluted 1:4 with saline, made 0.5 mM in $CoCl_2$, and kept at 25 °C for 2 h. The activated serum was then centrifuged (3000 rpm, 10 min, 5 °C) and used immediately.

Synthesis of MTX Peptides

4-Amino-4-deoxy-10-methylpteroic Acid. 2,4,5,6-Tetraaminopyrimidine sulfate hydrate (35.0 g, 0.137 mol) was reacted with dihydroxyacetone (37.0 g, 0.203 mol) to produce 2,4-diamino-6-(hydroxymethyl)pteridine [according to the procedure of Rosowsky et al. (1985)]. Yield: 18.2 g (70%). TLC (system A) R_f = 0.70. Vigorous oxygenation was required during the condensation step to minimize formation of the 6-methylpteridine (R_f = 0.58) as a secondary product. Treatment of the 6-(hydroxymethyl)pteridine (12.25 g, 63.7 mmol) with Br_2 in the presence of triphenylphosphine provided the 6-bromo derivative. The latter, without isolation, was then reacted with *p*-(methylamino)benzoic acid in the presence of diisopropylethylamine to produce 4-amino-4-deoxy-10-methylpteroic acid (Rosowsky et al., 1985). The product, recovered by lyophilization, was obtained as the semihydrate. Yield: 21.8 g (95%). TLC (system B) R_f = 0.57. ^{1}H NMR (DMSO-d_6) 8.73 (s, 1 H, 7-H), 8.20 (broad, 2 H, NH_2), 7.78 (d, J = 9 Hz, 2 H, phenyl), 7.27 (broad, 2 H, NH_2), 6.87 (d, J = 9 Hz, 2 H, phenyl), 4.87 (s, 2 H, CH_2), 3.23 ppm (s, 3 H, NCH_3).

N-Carbobenzoxy-L-glutamic Acid α-Methyl γ-tert-Butyl Diester Hydrochloride. *N*-Cbz-Glu γ-*tert*-butyl ester (16.85 g, 50 mmol) was converted to the α-methyl γ-*tert*-butyl diester by treatment with CH_3I, using the procedure of Rosowsky et al. (1981). Yield 17.2 g (97%). An aliquot (8.68 g, 24.7 mmol) of this intermediate (yellow oil) was used without further purification. Cbz was removed by hydrogenation (10 psi, 12 h, Pd on charcoal), using the method of Klieger and Gibian (1962). After filtration, the solution (at room temperature) was evaporated to dryness under vacuum. The residue was dissolved in 30 mL of CH_3OH/ethyl acetate/ether (1:4:1), and the solution was filtered and cooled to −20 °C. The pH was adjusted to 6.0 with a saturated solution of HCl in absolute ether, and after 1 h, the precipitate was collected by filtration, washed with absolute ether (−20 °C), and dried under vacuum. TLC (system D) R_f = 0.77. The filtrate was cooled again to −20 °C, the pH adjusted to 5.3 with HCl–ether, and additional precipitate was recovered by the above procedure. The combined product (diester hydrochloride), a white, amorphous powder, was obtained in a yield of 6.3 g (80%). [Rosowsky et al. (1981) reported a comparable yield of the free base obtained as an oil.] mp 124–125 °C [Klieger and Gibian (1962), 125–126 °C].

γ-tert-Butyl N-(4-Amino-4-deoxy-10-methylpteroyl)-L-glutamate (MTX γ-tert-Butyl Ester). The procedure of Rosowsky et al. (1981) was modified in the following manner. 4-Amino-4-deoxy-10-methylpteroic acid (4.96 g, 14.8 mmol, containing 0.5 equiv of H_2O) was added to a stirred solution of diethyl phosphorocyanidate (3.62 g, 22.2 mmol) and triethylamine (3.10 ml, 22.2 mmol) in 500 mL of absolute DMF. After the mixture was stirred at room temperature for 3 h, Glu α-methyl γ-*tert*-butyl diester hydrochloride (3.76 g, 14.8 mmol) and triethylamine (4.1 mL, 29.6 mmol) were added and stirring was continued for 72 h. The solvent was removed in vacuum (at 45 °C), and the residue was dissolved in 800 mL of $CHCl_3$. The solution was extracted twice with 500 mL of 1% NH_4OH (allowing time for the slow separation of emulsions), and the organic fraction was dried over Na_2SO_4. Removal of the solvent in vacuum yielded a brown oil (8.0 g). The latter was dissolved in CH_2Cl_2/acetone/CH_3OH (7:7:1) and chromatographed on silica gel. Appropriate fractions were pooled and evaporated to dryness to obtain a yellow, amorphous powder. Yield: 5.66 g (73%). TLC (system E) R_f = 0.46. ^{1}H NMR (DMSO-d_6) 8.60 (s, 1 H, 7-H), 8.28 (m, 1 H, CONH), 7.75 (d, 2 H, J = 9 Hz, phenyl), 7.6–7.4 (m, 2 H, NH_2), 6.83 (d, 2 H, J = 9 Hz, phenyl), 6.59 (broad, 2 H, NH_2), 4.79 (broad, 2 H, $ArCH_2N$), 4.6–4.2 (m, 1 H, α-CH), 3.63 (s, 3 H, $COOCH_3$), 3.20 (s, 3 H, NCH_3), 2.4–1.8 (m, 4 H, CH_2CH_2), 1.33 ppm (s, 9 H, COO-*t*-Bu).

The α-methyl group in the above product was removed by hydrolysis in base with the following modification (which provides a granular, rather than gelatinous, product) of the procedure of Rosowsky et al. (1981). (A) $Ba(OH)_2 \cdot 8H_2O$ (1.57 g, 5 mmol) was added to 40 mL of H_2O, and (B) MTX α-methyl γ-*tert*-butyl ester (2.55 g, 5 mmol) was added to 40 mL of CH_3OH. (A) and (B) were each heated to 50 °C (with stirring) for 15 min and then cooled to room temperature. The two solutions [(A) still slightly turbid] were combined and stirred at room temperature. After 30 min, examination by TLC (system E) indicated that hydrolysis had been completed. Na_2SO_4 (780 mg), dissolved in 5 mL of warm H_2O, was added to the solution, and after 5 min, the fine precipitate of $BaSO_4$ was removed by filtration through Celite. The precipitate was washed with a minimal amount of CH_3OH, and the pH of the filtrate was adjusted to 4.5 with 10% acetic acid. After standing for 12 h at −30 °C, the mixture was filtered through sintered glass to collect the precipitate. The latter was washed with C_2H_5OH and ether, dried in air, and then dried in high vacuum over P_2O_5. The product, MTX γ-*tert*-butyl ester, was obtained as a yellow, amorphous powder. Yield: 2.3 g (90%). TLC (system C) R_f = 0.45. ^{1}H NMR (DMSO-d_6) 8.60 (s,

1 H, 7-H), 8.16–7.93 (m, 1 H, CONH), 7.70 (d, 2 H, J = 9 Hz, phenyl), 7.57–7.40 (m, 2 H, NH_2), 6.80 (d, 2 H, J = 9 Hz, phenyl), 6.59 (broad, 2 H, NH_2), 4.75 (m, 2 H, $ArCH_2N$), 4.6–4.2 (m, 1 H, α-CH), 3.17 (s, 3 H, NCH_3), 2.4–1.7 (m, 4 H, CH_2CH_2), 1.3 ppm (s, 9 H, COO-*t*-Bu).

[*N*-(*4-Amino-4-deoxy-10-methylpteroyl*)-α-*glutamyl*]-L-*alanine Dihydrate* (*MTX-α-alanine*). MTX γ-*tert*-butyl ester (1.28 g, 2.5 mmol) was dissolved in 20 mL of absolute DMF. Triethylamine (528 mg, 736 μL, 5.22 mmol) and isobutylchloroformate (360 mg, 342 μL, 2.64 mmol) were added, and the mixture was stirred (with exclusion of moisture) at room temperature for 15 min; the precipitate of triethylammonium chloride was not removed. Ala *tert*-butyl ester hydrochloride (480 mg, 2.64 mmol) was added, and the mixture was stirred at room temperature for 6 h. Examination by TLC (system C) indicated that all of the MTX *tert*-butyl ester had reacted. After the mixture was cooled to 4 °C, the precipitate was removed by filtration and washed with dry DMF. The combined filtrates were concentrated under high vacuum (temperature below 40 °C), and the yellow oil was suspended in acetone. The mixture was stirred for 6 h and cooled to 4 °C, and the precipitate was recovered by filtration. The material was suspended in 10 mL of CH_3OH, stirred overnight at room temperature, recovered by filtration, washed with CH_3OH, and dried under vacuum. From the filtrate, additional material was obtained by evaporating the solvent, adding a small amount of CH_3OH, and repeating the procedure. The product, the di-*tert*-butyl ester of MTX-α-Ala, was obtained as a bright yellow, amorphous powder. Yield: 829 mg (52%). TLC (system C) R_f = 0.76; (system F) R_f = 0.53. Anal. for $C_{31}H_{43}N_9O_6$ (525.2): calcd C 58.38, H 6.80, N 19.77; found C 57.49, H 6.83, N 19.15. ^{1}H NMR (DMSO-d_6) 8.60 (s, 1 H, 7-H), 8.20 (m, 1 H, NH), 7.97 (m, 1 H, NH), 7.77 (d, 2 H, J = 9 Hz, phenyl), 7.7–7.4 (broad, 2 H, NH_2), 6.82 (d, 2 H, J = 9 Hz, phenyl), 6.57 (broad, 2 H, NH_2), 4.79 (s, broad, 2 H, $ArCH_2N$), 4.5–4.13 (m, 1 H, α-CH), 4.1–3.76 (m, 1 H, α-CH), 3.20 (s, 3 H, NCH_3), 2.5–1.7 (m, 4 H, CH_2CH_2), 1.37 (s, 18 H, *t*-Bu), 1.21 ppm (d, 3 H, J = 7 Hz, CH_3).

The di-*tert*-butyl ester of MTX-α-Ala (959 mg, 1.5 mmol) was suspended in 15 mL of THF, and 20 mL of 2.5 N HCl was added. The solution was heated to 55 °C for 5 h, cooled to room temperature, and neutralized with 5 N NaOH. THF was removed by evaporation, the solution was adjusted to pH 4.5 with HCl, and the yellow precipitate containing a portion of product was recovered by filtration. The precipitate was washed with H_2O and saved. The combined filtrates were desalted by using C_{18} reversed-phase silica gel that had been prepared in the following manner: C_{18} silica gel (15 mL) was placed on a sintered glass funnel (3.5 cm in diameter) and washed with 100 mL of CH_3CN/water (1:1), followed by 200 mL of 10^{-4} N HCl. The combined filtrates were applied to the gel in 20-mL portions. The colorless effluent was discarded, and the gel was washed with 40 mL of 10^{-4} N HCl. If the filtrate was yellow, it was acidified to pH 4, reapplied to the gel, and washed with an additional 40 mL of dilute acid. The product was eluted with 75% CH_3OH until the gel was almost colorless. (Silica gel can be regenerated by washing it alternatively with 25% CH_3OH and CH_3OH and then drying it in air.) The eluate was evaporated at 25 °C under high vacuum (dry ice–acetone trap), and the remaining H_2O was removed by brief lyophilization; extended lyophilization caused loss of product due to sublimation. The dry material was combined with the previous precipitate and suspended in 3 mL of H_2O. NaOH (1 N) was added dropwise (with shaking and gentle heating) until the solid dissolved; excess base impaired the chromatographic step described below. This solution was applied to DEAE-Trisacryl (2 × 15 cm; washed previously with 20 volumes of 5% NH_4HCO_3 and 40 volumes of H_2O). The column was washed with 200 mL of H_2O and eluted with a linear gradient (H_2O → 2% NH_4HCO_3, 1 L each; flow rate 0.5 mL/min). Fractions (15 mL) were examined for product by HPLC (see Experimental Procedures). Appropriate fractions were combined, the pH was adjusted to pH 4.5 (because of the dilution, no precipitate appeared), and the sample was desalted and dried as described above. The product, MTX-α-Ala, was a yellow, amorphous powder. Yield: 581 mg (69%). TLC (system G) R_f = 0.33. Anal. for $C_{23}H_{27}N_9O_8 \cdot 2H_2O$ (561.56): calcd C 49.19, H 5.56, N 22.45; found C 49.61, H 5.78, N 22.20. ^{1}H NMR (DMSO-d_6) 8.31 (s, 1 H, 7-H), 7.92–7.70 (m, 2 H, NH_2), 7.44 (d, 2 H, J = 9 Hz, phenyl), 7.4–7.1 (m, 2 H, 2 × NH), 6.56 (d, 2 H, J = 9 Hz, phenyl), 6.37 (broad, 2 H, NH_2), 4.53 (m, 2 H, $ArCH_2N$), 4.31–3.99 (m, 1 H, α-CH), 3.86 (m, 1 H, α-CH), 2.93 (s, 3 H, NCH_3), 2.15–1.87 (m, 2 H, CH_2), 1.87–1.49 (m, 2 H, CH_2), 0.98 ppm (m, 3 H, J = 7 Hz, CH_3).

[*N*-(*4-Amino-4-deoxy-10-methylpteroyl*)-α-*glutamyl*]-L-*aspartic Acid Dihydrate* (*MTX-α-aspartic Acid*). MTX γ-*tert*-butyl ester (1.28 g, 2.5 mmol) was reacted with Asp di-*tert*-butyl ester hydrochloride (744 mg, 2.64 mmol) via the procedure described above for the synthesis of MTX-α-Ala. The crude product (tri-*tert*-butyl ester of MTX-α-Asp), obtained as an oil, was dissolved in CH_2Cl_2/CH_3OH (10:1) and chromatographed on silica gel (2.5 × 20 cm; 230–400 mesh) using the above solvent for elution. Fractions (10 mL) were examined for product by TLC (see below). Appropriate fractions were evaporated, and the oil was taken up in 15 mL of CH_3OH and stirred for 12 h. The precipitate was recovered by filtration and dried under vacuum. From the filtrate, additional material could be obtained by repeating the precipitation procedure. The product, tri-*tert*-butyl ester of MTX-α-Asp, was obtained as a bright yellow, amorphous powder. Yield: 1.02 g (55.3%). TLC (system C) R_f = 0.9; (system F) R_f = 0.52. Anal. for $C_{36}H_{51}N_9O_8$ (737.86): calcd C 58.60, H 6.97, N 17.09; found C 58.53, H 6.76, N 16.96. ^{1}H NMR ($CDCl_3$) 8.64 (s, 1 H, 7-H), 7.84–7.63 (m, 4 H, NH_2, phenyl), 7.38–7.00 (m, 2 H, 2 × NH), 6.63 (d, 2 H, J = 8 Hz, phenyl), 5.55–5.27 (m, 2 H, NH_2), 4.84–4.50 (m, 4 H, $ArCH_2N$, 2 × α-CH), 3.19 (s, 3 H, NCH_3), 2.90–2.69 (m, 2 H, CH_2), 2.58–2.30 (m, 2 H, CH_2), 2.26–1.99 (m, 2 H, CH_2), 1.36 ppm (s, 27 H, 3 × COO-*t*-Bu).

To remove the blocking groups, the tri-*tert*-butyl ester of MTX-α-Asp (1.02 g, 1.38 mmol) was subjected to acid hydrolysis, as described for MTX-α-Ala. The product, MTX-α-Asp, was obtained as a yellow, amorphous powder. Yield: 164 mg (21%). TLC (system G) R_f = 0.18. Anal. for $C_{24}H_{27}N_9O_8 \cdot 2H_2O$ (605.6): calcd C 47.60, H 5.16, N 20.82; found C 47.16, H 5.41, N 21.11. ^{1}H NMR (DMSO-d_6) 8.35 (s, 1 H, 7-H), 8.0–7.6 (m, 2 H, NH_2), 7.50 (d, 2 H, J = 8 Hz, phenyl), 7.4–7.1 (m, 2 H, 2 × NH), 6.57 (d, 2 H, J = 8 Hz, phenyl), 6.40 (broad, 2 H, NH_2), 4.57 (s, broad, 2 H, $ArCH_2N$), 4.33–3.91 (m, 2 H, 2 × α-CH), 2.98 (s, 3 H, NCH_3), 2.2–1.5 ppm (2 m, 6 H, 3 × CH_2).

[*N*-(*4-Amino-4-deoxy-10-methylpteroyl*)-α-*glutamyl*]-L-*arginine Dihydrate* (*MTX-α-arginine*). MTX γ-*tert*-butyl ester (1.02 g, 2.0 mmol), triethylamine (616 μL, 4.4 mmol), and isobutylchloroformate (304 mg, 288 μL, 2.24 mmol) were dissolved in 12 mL of absolute DMF, and the solution was stirred for 15 min at room temperature. Arg methyl ester hydrochloride (584 mg, 2.24 mmol) was added, and the so-

lution was stirred at room temperature for 4 h, cooled to 4 °C, and filtered. Three volumes of ethyl acetate were added to the filtrate, and after 12 h at −20 °C, the yellow supernatant was discarded, and the oil was suspended in 20 mL of acetone/C_2H_5OH (3:1) and stirred vigorously for 6 h. The precipitate was filtered and dried under vacuum. The product, the γ-*tert*-butyl (Glu) α-methyl (Arg) diester of MTX-α-Arg·HCl, was obtained as an orange-yellow, amorphous powder; it was used, without further purification, as described below. Yield: 1.35 g (94%). TLC (system B) R_f = 0.25. ^{1}H NMR (DMSO-d_6) 8.29 (s, 1 H, 7-H), 8.20–8.00 (m, 1 H, NH), 7.90–7.70 (m, 1 H, NH), 7.59–7.10 (m, 5 H, NH, NH_2, phenyl), 6.96–6.66 (m, 3 H, NH, NH_2), 6.66–6.47 (m, 2 H, phenyl), 6.47–6.29 (m, 2 H, NH_2), 4.54 (broad, 2 H, $ArCH_2N$), 4.30–3.86 (m, 2 H, 2 × α-CH), 3.38 (s, 3 H, $COOCH_3$), 2.97 (s, 3 H, NCH_3), 2.16–1.10 (m, 10 H, 5 × CH_2), 1.10 ppm (s, 9 H, COO-*t*-Bu).

The diester of MTX-α-Arg was first treated to remove the methyl blocking group. The material (1.35 g, 1.88 mmol) was dissolved in 30 mL of CH_3OH by vigorous stirring at room temperature. $Ba(OH)_2·8H_2O$ (2.64 g, 8.36 mmol) was suspended in 120 mL of H_2O, and the mixture was stirred at 50 °C for 10 min. The two solutions were combined and stirred at room temperature for 60 h. An additional amount (593 mg, 1.88mmol) of $Ba(OH)_2$ in 50 mL of warm H_2O (50 °C) was added, and stirring was resumed for another 5 h, or until TLC (system B) indicated complete consumption of the starting material. To hydrolyze the *tert*-butyl group, the solution was then adjusted to pH 1 with 5 N HCl and stirred for 30 min. TLC (system G) verified that both blocking groups had been removed.

Further purification of the product was accomplished via the following procedure. To the above solution, Na_2SO_4 (1.60 g, 11.3 mmol), dissolved in a minimal amount of H_2O, was added, and after 10 min at room temperature, the precipitate was removed by filtration. The filtrate was adjusted to pH 7, and the solution was passed through a nylon filter to remove additional $BaSO_4$. The pH was adjusted to 4.5 with 1 N HCl, which caused some precipitation of product; this was recovered by filtration, washed with H_2O, and saved. The combined filtrates were desalted over C_{18} reversed-phase silica gel, as described above for MTX-Ala. The product was eluted from the gel with 75% CH_3OH and recovered as a solid after removal of solvent. The dry material was combined with the previous precipitate and suspended in 4 mL of H_2O. NaOH (1 N) was added dropwise until the solid dissolved (see precaution regarding this step in synthesis of MTX-Ala). By use of the procedure described previously for the preparation of MTX-Ala, the solution was chromatographed on DEAE-Trisacryl (2.5 × 20 cm), and effluent fractions were monitored by HPLC (system B). In addition to removing impurities, this step also partially separated the D,L- and L,L-diastereomers (see Discussion) of MTX-Arg. Early, intermediate, and late fractions contained the D,L form, the DL,L mixture, and the L,L form, respectively. Appropriate fractions were combined, and the solutions were desalted over silica gel and taken to dryness (CH_3OH removed by high vacuum; H_2O removed by brief lyophilization) as described previously for MTX-Ala. The products were obtained as yellow, amorphous powders. Total yield: 435 mg (34.1%). The more abundant DL,L material was used for characterization of the product. TLC (system G) R_f = 0.19. Anal. for $C_{26}H_{35}ClN_{12}O_6·2H_2O$ (683.1): calcd C 45.71, H 5.76, N 24.60; found C 46.39, H 5.47, N 24.32. ^{1}H NMR (DMSO-d_6) 9.4–9.1 (m, 1 H, NH), 9.1–8.7 (m, 2 H, 2 × NH), 8.6–8.3 (m, 2 H, NH, 7-H), 7.86–7.30 (m, 6 H, 2 × NH, NH_2, phenyl), 6.94–6.33 (m, 4 H, NH_2, phenyl), 4.77 (broad, 2 H, $ArCH_2N$), 4.49–4.18 (m, 1 H, α-CH), 3.98–3.70 (m, 1 H, α-CH), 3.14 (s, 3 H, NCH_3), 2.4–1.1 ppm (m, 10 H, 5 × CH_2).

Methods

Assay Procedures for Enzymatic Hydrolysis of MTX Peptides. (*A*) *TLC.* MTX peptides (ca. 1 mM) were treated with various proteolytic enzymes. In each instance, the pH and buffer composition were optimal for the enzyme used, and the amount of enzyme was ca. 100-fold greater than that required for complete hydrolysis of a standard substrate. Separate controls (enzyme omitted; substrate omitted) were utilized. Mixtures were incubated at 25–40 °C for 24 h. At various time intervals, aliquots were removed and examined by TLC (system G). R_f values: MTX-Ala (0.33); MTX-Asp (0.18); MTX-Arg (0.19); MTX (0.34); Ala (0.26); Asp (0.12); and Arg (0.10).

(*B*) *Ninhydrin.* Hydrolysis of MTX-Ala, catalyzed by CP-A, was measured by the following procedure. CP-A (35 μL, 105 units/mL in 9% LiCl) was added to MTX-Ala (1.2 mL, 1.0 mM in 0.025 M Tris–0.5 M NaCl, pH 7.5), and the mixture was incubated at 25 °C. At various time intervals, 50-μL aliquots were removed and analyzed for Ala according to the ninhydrin method described by Spies (1957). Absorbances at 570 nm were determined by using a Hewlett-Packard spectrophotometer (Model 8450A) and corrected for the zero-time control (enzyme added to substrate, followed immediately by ninhydrin and subsequent processing). Concentrations of the product (Ala) were calculated from a standard curve, and the results are averages of duplicate determinations. One unit of CP-A will hydrolyze 1.0 μmol of hippurylphenylalanine/min at pH 7.5 and 25 °C.

(*C*) *Spectrophotometric.* Hydrolysis of MTX-Ala by CP-A was measured by the following procedure. MTX-Ala (50 μL, 1.0 mM) and Tris buffer (440 μL, 0.1 M, pH 7.3, containing 0.2 mM $ZnSO_4$) were added to a 1-cm quartz cuvette. A_{320} of the solution was determined by using a Gilford spectrophotometer (Model 252), and after 5 min, 5 μL of a solution of CP-G_2 (9 units/mL) was added. At 15 min, 6 μL of a solution of CP-A (31.4 units/mL) was added, and A_{320} was recorded as a function of time. The initial rate was determined from the linear portion (first 5 min) of the curve. A value of ϵ_{mM} = 8.3 (Levy & Goldman, 1967) was used to calculate the concentration change for the overall reaction (MTX peptide → 4-amino-4-deoxy-10-methylpteroate). One unit of CP-G_2 will hydrolyze 1 μmol of MTX/min at pH 7.3 and 30 °C (McCullough et al., 1971).

(*D*) *HPLC.* Hydrolysis of MTX-Ala by CP-A was measured by the following procedure. The mixture for the spectrophotometric assay (see above) was used, except that 0.64 unit of CP-A was present and CP-G_2 was omitted. Reactions in identical samples (at 37 °C) were initiated by addition of CP-A. At various time intervals, one sample was placed in ice to stop the reaction. The samples were heated (90 °C, 1 min) and centrifuged (1 min; Eppendorf microcentrifuge, Model 3414). Aliquots (20 μL) of the supernatants were analyzed by HPLC (system A).

Cytotoxicity of MTX Peptides. These measurements were performed by using a microculture technique and colorimetric assay, adapted from Mosmann (1983) and Finlay et al. (1986). Cytotoxicity of MTX-Ala, activated by CP-A, was measured by the following procedure. Late-log L1210 cells were suspended (10^3 cells/135 μL) in RPMI 1640 medium containing 5% dialyzed fetal bovine serum, 200 units/mL penicillin, and 200 μg/mL streptomycin. Using a multichannel pipettor, 135

μL of the cell suspension was added to all wells (except those in vertical column 1) of a 96-well plate. For wells in column 1, 150 μL of the same medium, but without added cells, served as the spectrophotometric blank. The plate was covered and incubated for 4 h at 37 °C in a CO_2 incubator. Solutions of MTX, MTX-Ala, and CP-A were added (in 15 μL) to columns 3–12 to obtain various final concentrations; water was added to column 2. The plate was covered again (edges sealed with CO_2-permeable tape) and incubated for 72 h in a CO_2 incubator. For colorimetric determination of cell growth, 15 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL in phosphate-buffered saline) was added, and the plate was incubated for 2 h at 37 °C. The reduced dye was dissolved by vigorous pipetting with 165 μL of 2-propanol containing 0.04 N HCl. After 15 min at room temperature, the plate was read in a Titertek Multiskan. Data are plotted as $A_{570-630}$ vs drug concentration.

TLC. Sheets: Baker-flex silica gel IB-F. Solvent systems: (A) ether/CH_3OH (2:1); (B) ethyl acetate/CH_3OH (1:5); (C) $CHCl_3/CH_3OH$/acetic acid (30:5:1); (D) $CH_2Cl_2/CH_3CN/CH_3OH$ (25:25:0.5); (E) CH_2Cl_2/acetone/CH_3OH (7:7:1); (F) CH_2Cl_2/CH_3OH (10:1) (G) 1-butanol/acetic acid/H_2O (4:1:1); (H) petroleum ether/ether (1:2). Visualization: MTX, MTX peptides, and heterocyclic precursors (UV; 254 nm); amino acids (ninhydrin); protected amino acids (sheets dipped in 15% H_2SO_4 and dried at 120 °C for 10 min).

HPLC. A C_{18} reversed-phase silica gel column (Beckman Ultrasphere ODS) with a two-pump Beckman/Altex liquid chromatograph (Model 332) was used; absorbance at 280 nm was determined with a Beckman UV detector, Model 166, interfaced with a Beckman integrator (Model 427). System A: The mobile phase was formed with 2% acetic acid (pump A) and 2% acetic acid in 50% CH_3OH (pump B); flow rate, 1 mL/min; sample size, 20 μL; gradient, 50% A–50% B for 5 min, linear gradient to 100% B for 30 min, and then 100% B for 5 min. System B: The mobile phase was formed with 0.5 M NH_4OAc, pH 5.0 (pump A) and 0.025 M NH_4OAc in 80% CH_3CN, pH 5.5 (pump B); flow rate, 1 mL/min; sample size, 250 μL; gradient, 100% A for 5 min and then linear gradient to 30% B for 35 min.

Other Methods. Absorbance spectra were measured by using a Hewlett-Packard 8450 UV/vis spectrophotometer. Concentrations of MTX and MTX peptides were determined in 0.1 N NaOH by using ϵ_{mM} = 22.0 at 302 nm (Seeger et al., 1949). NMR spectra were measured by using a Varian spectrometer, Model T60-A, and a Bruker spectrometer, Model 100, with tetramethylsilane as the internal standard. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN.

Results

Chemical Synthesis of Methotrexate α-Peptides. The regiospecific route employed for synthesis of these compounds [outlined in Figure 2 in Kuefner et al. (1988)] followed the general procedures developed by Rosowsky et al. (1981) and Piper et al. (1982) for preparation of α- and γ-amides and esters of MTX; modifications introduced in the present study are described under Experimental Procedures. The sequence involved three stages: (a) synthesis of 4-amino-4-deoxy-10-methylpteroic acid; (b) coupling of this compound to a protected glutamic acid (α-methyl, γ-*tert*-butyl), followed by selective removal of the methyl group; and (c) addition of the protected amino acid (Ala, *tert*-butyl; Asp, di-*tert*-butyl; Arg, methyl), followed by removal of the blocking groups. Although the first stage could be omitted by utilizing commercially available 4-amino-4-deoxy-10-methylpteroic acid, the less expensive synthetic procedure was able to produce large quantities (20–30 g) of the compound in good yield (ca. 75%). The second stage, viz., synthesis of MTX γ-*tert*-butyl ester, the common precursor of the MTX peptides, was also conducted on a large scale (10 g) with comparably high yields. Attachment of the amino acid to the open α-carboxyl of MTX and subsequent removal of blocking groups (third stage), however, was limited to smaller preparations, owing to the necessity of using column chromatography for purification of products; the yields were also diminished, particularly in steps involving removal of blocking groups.

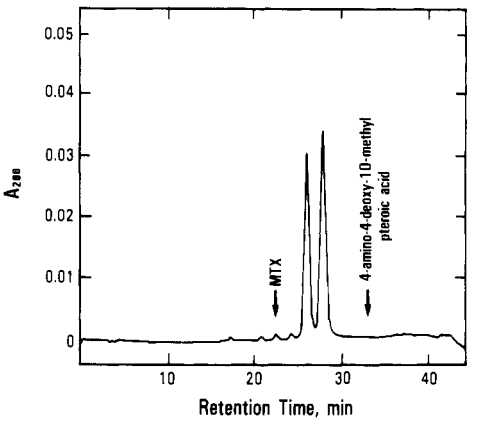

FIGURE 2: HPLC analysis of MTX-Ala. The MTX peptide (20 μL; 0.2 mM in 0.1 M Tris buffer, pH 7.3) was examined by HPLC (system A) as described under Experimental Procedures.

tert-Butyl blocking groups on the γ-carboxyl of the Glu moiety of MTX, the carboxyl of Ala, and both carboxyls of Asp were removed by acid hydrolysis using relatively harsh conditions (concentrated HCl, elevated temperature, and extended time); trifluoroacetic could not be used for this purpose, because of the difficulty in removing it completely from the product. Methyl blocking groups on the α-carboxyl of the Glu moiety of MTX and the carboxyl of Arg were removed by base hydrolysis using $Ba(OH)_2$; the linkage was considerably more labile in the former instance. The final step in purification of the MTX peptides involved chromatography on DEAE-Trisacryl using an NH_4HCO_3 gradient; success in this operation required strict adherence to the protocol described under Experimental Procedures.

Characterization of Products. The MTX peptides were obtained as amorphous powders; attempts to crystallize them have not yet been successful. They were relatively stable to heat and light, soluble (up to ca. 1 mM) in aqueous media (>pH 6), and insoluble in organic solvents except for DMF and DMSO. Purity of intermediates and final products was verified by TLC and HPLC; authenticity of the compounds was established by elemental analyses, absorbance spectra, and NMR spectra (see Experimental Procedures).

No extraneous materials were encountered in the HPLC analyses, but each of the MTX peptides displayed a closely spaced doublet. Representative data for MTX-Ala are presented in Figure 2; peaks of nearly equal height were observed at 25.6 and 27.4 min; MTX and 4-amino-4-deoxy-10-methylpteroate had retention times of 22.5 and 32.7 min. Under the same conditions, the doublet for MTX-Asp had retention times of 19.8 and 20.6 min. MTX-Arg, conversely, appeared as a single peak in system A. It was resolved into a doublet, however, in system B (retention times of 27.8 and 28.2 min). These doublets represent the D,L- and L,L-diastereomers of the MTX peptides (see Discussion). It should be noted, however, that DL,L racemic mixtures were used for all biological experiments described below.

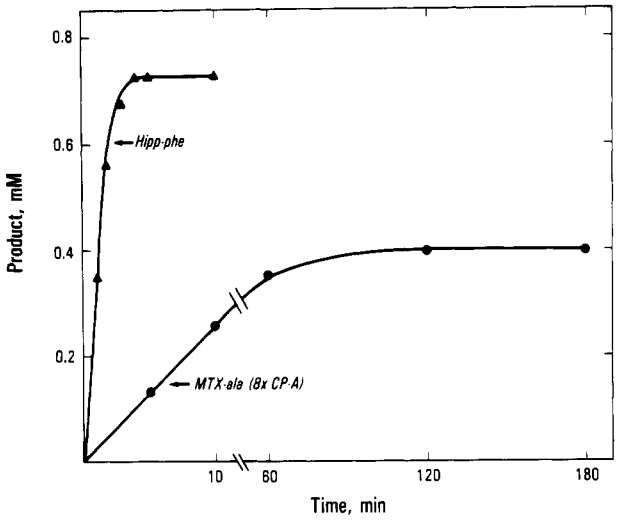

FIGURE 3: Hydrolysis of MTX-Ala and hippurylphenylalanine (Hipp-Phe) by CP-A (ninhydrin assay). For MTX-Ala, see Experimental Procedures. Hydrolysis of Hipp-Phe was measured in the same manner, except that the concentration of CP-A in the stock solution was reduced to 13.1 units/mL, and the standard curve was corrected for the difference in color yield between Ala and Phe.

Qualitative Evaluation of Proteolytic Enzymes for Hydrolysis of MTX Peptides. Representative enzymes were examined for their ability to hydrolyze the MTX peptides. Each enzyme was tested under optimal assay conditions, high concentrations of enzymes and substrates were used, and extremes of time and temperature were investigated. Reaction mixtures were monitored qualitatively by TLC for the appearance of MTX and the amino acids (see Experimental Procedures). No hydrolysis of the MTX peptides was observed with chymotrypsin, trypsin, plasmin, urokinase, pepsin, papain, ficin, bromelain, leucine aminopeptidase, proteinase K, pancreatic protease type I, mouse submaxillary gland protease, or *B. subtilis* protease.

Carboxypeptidases, conversely, were quite effective in hydrolyzing the MTX peptides. MTX-Ala was hydrolyzed by highly purified commercial preparations of pancreatic carboxypeptidases A and B; marginal activity was observed with CP-A/MTX-Asp and CP-B/MTX-Arg combinations. Of the three MTX peptides, only MTX-Arg was a substrate for the endogeneous carboxypeptidase(s) in various sera. In each instance, TLC verified that the only products were MTX and the amino acid; no cleavage of the pteroate–Glu bond was observed.

Characteristics of Carboxypeptidase-Mediated Hydrolysis. Hydrolysis of MTX-Ala by CP-A (the most active substrate/enzyme combination) was examined in detail. Initial measurements were conducted by using a quantitative ninhydrin assay for the liberated amino acid (see Experimental Procedures). Product formation was linear with time, and initial rates were linear with enzyme concentration (data not shown). A comparison of the CP-A-dependent hydrolysis of MTX-Ala and hippurylphenylalanine (a standard substrate for the enzyme) is shown in Figure 3. Both substrates were present at 1 mM, and an 8-fold higher concentration of enzyme was used for MTX-Ala. Under these conditions, the rate of hydrolysis of hippurylphenylalanine was still ca. 10-fold faster than that of the MTX peptide. At the completion of the reaction, only about 40% of the MTX-Ala had been consumed; this was attributed to the use of the DL,L substrate (see Discussion).

The production of MTX via the CP-A-mediated hydrolysis of MTX-Ala was also measured quantitatively by using an

Table I: CP-A-Mediated Production of MTX from MTX-Ala[a]

time (min)	−Δ[MTX-Ala] (mM)	+Δ[MTX] (mM)
10	0.008	0.010
20	0.016	0.017
30	0.024	0.023
60	0.031	0.032

[a] Assay mixture and analysis by HPLC are described under Experimental Procedures. At the indicated times, concentrations of MTX-Ala and MTX were determined by integration of areas under appropriate peaks in the chromatogram. Results [corrected for the amount of MTX present at zero time (0.002 mM)] are expressed as changes in concentration (MTX-Ala decreasing, MTX increasing).

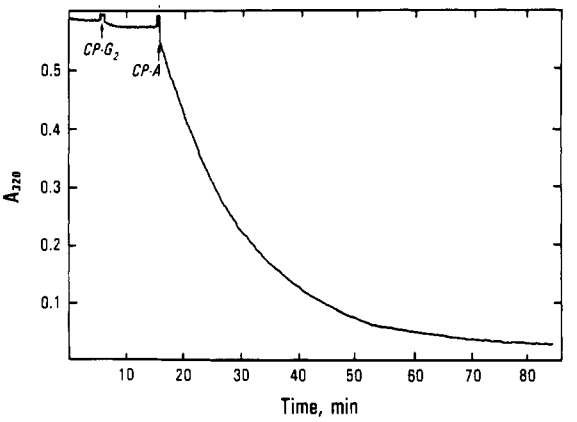

FIGURE 4: Hydrolysis of MTX-Ala by CP-A (spectrophotometric assay). For details, see Experimental Procedures.

HPLC assay (see Experimental Procedures). The solvent system, developed for this assay, provided base-line resolution of MTX, 4-amino-4-deoxy-10-methylpteroate, and the diastereomers of MTX-Ala (cf. Figure 2). Hydrolysis of MTX-Ala by CP-A was examined at the indicated intervals over a 60-min period (Table I), and the time-dependent disappearance of the MTX peptide was paralleled closely by the appearance of MTX. No 4-amino-4-deoxy-10-methylpteroate or other pterin breakdown products were detected.

For subsequent studies, a more convenient spectrophotometric assay was developed (see Experimental Procedures) that allowed the carboxypeptidase-dependent hydrolysis of MTX peptides to be monitored continuously. It was based upon the observation that an unusual group of bacterial carboxypeptidases (CP-G's) (Levy & Goldman, 1967; McCullough et al., 1971) hydrolyzes folate compounds, including MTX, at the pteroate–Glu bond. This reaction is accompanied by a decrease in absorbance at 320 nm. In the presence of CP-G_2, MTX released from MTX-Ala via CP-A (reaction 1) is subjected to additional hydrolysis (reaction 2) with excess CP-G_2,

$$\text{MTX-Ala} \xrightarrow{\text{CP-A}} \text{MTX} + \text{Ala} \quad (1)$$

$$\text{MTX} \xrightarrow{\text{CP-G}_2} \text{4-amino-4-deoxy-10-methylpteroate} + \text{Glu} \quad (2)$$

and the overall rate is determined by the rate of reaction 1. A representative experiment using this assay is shown in Figure 4. No change in A_{320} was observed when CP-G_2 was added to the substrate, which demonstrated that the enzyme was unable to hydrolyze the pteroate–Glu bond in MTX α-peptides. When CP-A was added, however, A_{320} decreased immediately, verifying the sequential operation of reactions 1 and 2. The linear portion of the A_{320} vs time plot (0 → 5 min in this experiment) was suitable for rate measurements.

The spectrophotometric assay was employed to obtain comparative data for the various CP/MTX peptide combi-

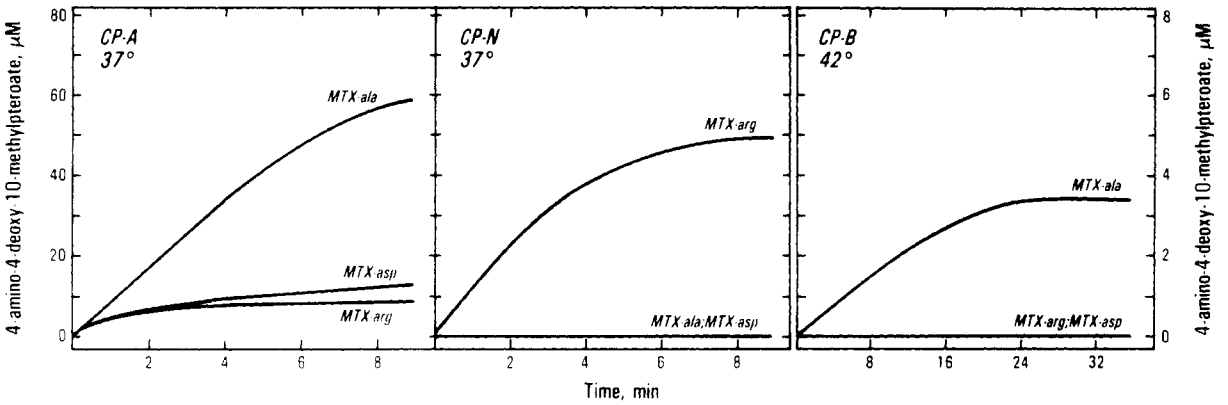

FIGURE 5: Comparison of the hydrolysis of MTX peptides by carboxypeptidases (spectrophotometric assay). The assay system described under Experimental Procedures was used, except that the volume was increased to 0.8 mL. Concentration of MTX peptides, 0.1 mM. Amounts of enzyme: CP-G_2 (0.072 unit); CP-A (0.4 unit); and CP-B (16 units). For CP-N, 710 μL of dialyzed human serum (adjusted to pH 7.3) was used, and buffer was omitted. For CP-B, the temperature was increased to 42 °C (note also change in scale for ordinate values). Results are expressed as the time-dependent appearance of 4-amino-4-deoxy-10-methylpteroate.

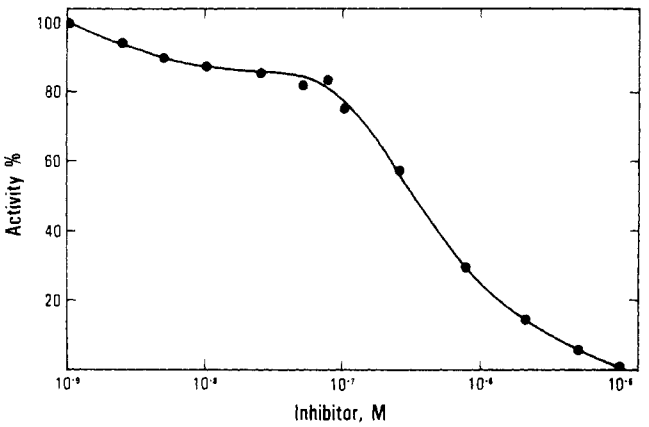

FIGURE 6: Effect of DL-2-(mercaptomethyl)-3-(guanidinoethyl)-thiopropionic acid upon hydrolysis of MTX-Arg by CP-N in human serum (spectrophotometric assay). The assay system described under Experimental Procedures was used, except that MTX-Ala was replaced by MTX-Arg (0.45 mM), buffer was omitted, CP-A was replaced by 440 μL of dialyzed, Co^{2+}-activated human serum (adjusted to pH 7.3), and inhibitor was present at the indicated concentrations. Results are expressed as percent activity (based upon a control with no inhibitor).

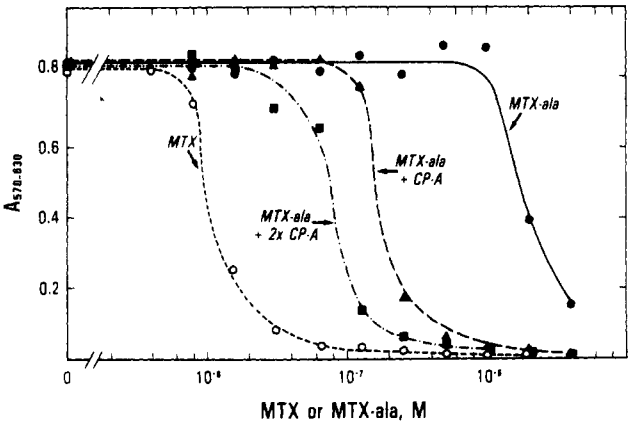

FIGURE 7: Cytotoxicity toward L1210 cells of MTX-Ala activated by CP-A. For details, see Experimental Procedures. Concentrations of MTX and MTX-Ala were present at the indicated concentrations. Amounts of CP-A, 7.85 and 15.7 milliunits/well. Each point represents the average of duplicate determinations.

nations (Figure 5). Substrates were present at 0.1 mM, and sufficient enzyme was used in each instance to provide, within 15 min, a conveniently measured rate for the optimal MTX peptide. As shown in the left panel, CP-A hydrolyzed MTX-Ala readily. Under the same conditions, it also had low activity toward MTX-Asp and MTX-Arg. CP-B was also able to hydrolyze MTX-Ala (right panel), although in this instance it was necessary to use more forcing conditions than in the CP-A/MTX-Ala experiment (16 vs 0.4 unit of enzyme; 42 vs 37 °C). CP-B had no activity toward MTX-Asp or MTX-Arg within the brief time period of the spectrophotometric assay.

Endogenous carboxypeptidase activity in human serum (CP-N) hydrolyzed only MTX-Arg (middle panel). When D,L-2-(mercaptomethyl)-3-(guanidinoethyl)thiopropionic acid, an extremely potent inhibitor (K_i = 2 nM) of highly purified human serum CP-N (Plummer & Ryan, 1981), was tested for its effect upon the serum-dependent hydrolysis of MTX-Arg, a biphasic response curve was obtained (Figure 6). Only about 20% of the activity was affected by nanomolar concentrations of the inhibitor; the remaining activity required micromolar concentrations for complete inhibition. This is consistent with the observation of Schweisfurth et al. (1983), who noted that serum contains two carboxypeptidases, specific for Arg- and Lys-containing peptides, respectively. It would appear that both of these enzymes are capable of hydrolyzing MTX-Arg and that the Arg-specific enzyme (blocked by nanomolar concentrations of the inhibitor) represents ca. 20% of the total CP-N activity. The latter conclusion is consistent with the reported average levels of 36.1 and 144.2 units for the Arg- and Lys-specific activities in human serum (Schweisfurth et al., 1983).

Cytotoxicity of MTX Peptides. Combinations of MTX peptides and carboxypeptidases were tested for cytotoxicity against L1210 mouse leukemia cells in culture. Cells were grown in 96-well plastic plates in the presence of the indicated components, and after 72 h, relative numbers of viable cells were evaluated by the tetrazolium assay described under Experimental Procedures. Results obtained with the CP-A/MTX-Ala combination are shown in Figure 7. MTX-Ala was only weakly cytotoxic (ID_{50} = 2.0 × 10^{-6} M) compared to MTX (ID_{50} = 2.4 × 10^{-8} M). Addition of CP-A at two different concentrations, however, improved the ID_{50}'s to 2.0 × 10^{-7} and 8.5 × 10^{-8} M, respectively. CP-A alone, at the levels employed, had no effect on cell growth. In a similar experiment, the combination of CP-A and MTX-Asp was examined. Unlike MTX-Ala, MTX-Asp (even at 10^{-6} M) showed no toxicity. Inclusion of CP-A at a higher level (39.3 milliunits/well) than that used in the previous experiment produced only a modest degree of cytotoxicity (ID_{50} = 5 × 10^{-6} M). CP-B also enhanced the cytotoxicity of MTX-Ala,

but very high levels of enzyme were required for activation (e.g., CP-B at 1960 milliunits/well gave an ID_{50} of 1.1×10^{-7} M for MTX-Ala). The cytotoxic effect of MTX-Arg is illustrated in Figure 8. MTX-Arg alone had an ID_{50} value of 5×10^{-8} M, due to activation by endogenous CP-N in the fetal bovine serum present in the culture medium. Addition of CP-B lowered this value to the ID_{50} of free MTX.

Discussion

The present investigation was undertaken to determine whether MTX α-peptides (derivatives in which the α-carboxyl of MTX is linked covalently to amino acids) might have potential as cancer chemotherapeutic agents. MTX peptides containing three representative amino acids (Ala, Asp, and Arg) were selected for the initial studies (Figure 1). Synthesis of these compounds in reasonably good yield was achieved via a multistep route. MTX-Asp has been synthesized previously, via a different route, by Piper et al. (1982). MTX-Ala and MTX-Arg, however, are new compounds. Current work in this laboratory is focused upon an alternate route in which the amino acid is coupled directly to MTX. Although this procedure would lead to a mixture of the α-and γ-derivatives, these compounds can be resolved chromatographically (J. Fan, unpublished results) and identified by their biological activity. Thus, α-derivatives of MTX are less inhibitory to dihydrofolate reductase than their γ counterparts (Piper et al., 1982), and as shown in this investigation (Figure 4), they are not susceptible to hydrolysis at the pteroate–Glu bond by CP-G_2; γ-derivatives, however, are hydrolyzed by the enzyme (J. Fan, unpublished results). Of relevance to the feasibility of a direct-coupling route is the report of Rosowsky and Yu (1978), who found that the dicyclohexylcarbodiimide-promoted or diphenyl phosphorazidate promoted interaction of unprotected MTX with the diethyl ester of glutamate produced predominantly the α-derivative.

In the same study, Rosowsky and Yu (1978) obtained evidence that activation of the α-carboxyl of MTX (as a prelude to coupling with amino acids) resulted in racemization of the Glu moiety of the drug to produce D,L- and L,L-diastereomers (with reference to the configurations of Glu and the incoming amino acid). A mechanism involving a cyclic intermediate was proposed to account for this phenomenon. Results from the present investigation support this hypothesis. Thus, each of the MTX α-peptides was resolved by HPLC into a closely spaced doublet (Figure 2), and in the CP-A-mediated hydrolysis of MTX-Ala the extent of reaction approached only 50% (Figure 3). Additional work, in this laboratory, to be described elsewhere, has provided the following information about the diastereomers of MTX α-peptides: (a) L,L-MTX-Ala, prepared by an unambiguous route, was hydrolyzed by CP-A at a faster rate and to a greater extent than the DL,L mixture. (b) The L,L-diastereomer of MTX-Ala was identified as the slower (26.5 min) component in the HPLC profile (Figure 2). (c) The L,L form of MTX-Arg, obtained by partial resolution of the racemic mixture via chromatography on DEAE-Trisacryl (see Experimental Procedures), was also shown to be the slower component of its HPLC doublet. (d) In serum-containing medium, L,L-MTX-Arg exhibited greater cytotoxicity than the D,L form toward L1210 cells.

The MTX α-peptides were not hydrolyzed by a wide variety of proteolytic enzymes. These results were not surprising, since cleavage by a proteolytic enzyme would require specificity for a Glu–Ala, -Asp, or -Arg bond plus tolerance for the bulky pteroate moiety attached to the glutamate. The MTX peptides were hydrolyzed, however, at the Glu–amino acid linkage by carboxypeptidases. Quantitation of the products was accomplished by ninhydrin assay for the amino acid (Figure 3) and by HPLC and reactivity with CP-G_2 for MTX (Table I and Figures 4 and 5).

MTX-Arg was hydrolyzed by serum (Figure 5), and inhibition by 2-(mercaptomethyl)-3-(guanidinoethyl)thiopropionic acid (Plummer & Ryan, 1981) verified that this activity was due to endogenous carboxypeptidase. The biphasic curve obtained when hydrolysis of MTX-Arg was examined as a function of inhibitor concentration (Figure 6) was consistent with the existence of two CP-N activities (specific for -Arg and -Lys) in serum (Schweisfurth et al., 1983). As shown in Figure 5, MTX-Ala was hydrolyzed by highly purified carboxypeptidases A and B. These enzymes also hydrolyzed MTX-Asp and MTX-Arg, but the rates were very much lower than with MTX-Ala. Nevertheless, the CP-A/MTX-Asp and CP-B/MTX-Arg activities were sufficient, in long-term experiments (72 h), to produce cytotoxicity toward L1210 cells (Figure 8).

Detailed kinetic studies have not yet been performed on the CP-mediated hydrolysis of MTX peptides. Data for CP-N must await the availability of the two serum enzymes in purified form. Preliminary experiments on the hydrolysis of MTX-Ala by CP-A at 37 °C have indicated that the K_m is ca. 0.5 mM. This may be compared to the reported value of 1.8 mM (25 °C) for the K_m of hippurylphenylalanine (Folk & Schirmer, 1963). CP-B is not well-suited for kinetic measurements because of its low activity with MTX peptides. Since the rate of hydrolysis of MTX-Ala by CP-B is less than 1% of the rate of the CP-A-mediated reaction (Figure 5), it is possible that the former activity may be due to contamination of CP-B by traces of CP-A.

Combinations of carboxypeptidases and MTX peptides produced cytotoxicities approaching that of the parent drug. CP-A was able to utilize both MTX-Ala (Figure 7) and MTX-Asp for this purpose. The activity of MTX-Ala was also potentiated by CP-B, while MTX-Arg was activated by CP-N and CP-B (Figure 8). In each instance, the mechanism of action appeared to be extracellular release of free MTX, followed by uptake of the drug. Lack of cytotoxicity in the absence of an activating enzyme verified that the MTX peptides did not contain appreciable amounts of free MTX as an impurity, nor was the drug released by chemical or enzymatic hydrolysis during the 72-h incubation.

Previous work by Piper et al. (1982) has shown that α-esters and amides of MTX are not internalized. This has been corroborated indirectly in the present study. The three MTX peptides are reasonably good inhibitors of dihydrofolate reductase (unpublished results). This is not unexpected since, although interaction of the negative α-carboxylate of MTX with a positively charged Arg on the enzyme is a important determinant of drug binding (Freisheim & Matthews, 1984), each of the MTX α-peptides still has a carboxylate in the same general vicinity. Thus, if the peptides were taken up, cytotoxicity would have been expected in the absence of carboxypeptidases; this was not observed (e.g., Figure 7).

MTX peptides are "pro-drugs". The strategy for their activation is similar to that employed by Carl (1983), who prepared several drug–peptides that were candidates for hydrolysis by the serum protease, plasmin. The drugs selected (e.g., adriamycin, phenylenediamine mustard, and Melphalan) had amino groups available for derivatization. Methotrexate (as well as purine and pyrimidine nucleosides) was excluded, since the amino groups on these heterocycles are not readily reactive. In the present study, the α-carboxyl group of MTX has been utilized for linkage to an amino acid, and activation

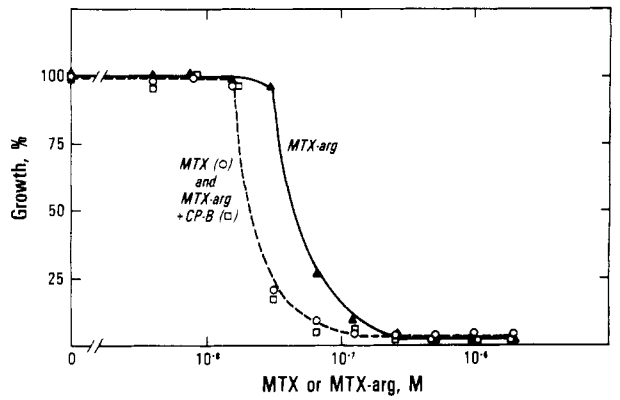

FIGURE 8: Cytotoxicity of MTX-Arg activated by CP-N of fetal bovine serum and CP-B. Experimental details as in Figure 7, except that MTX-Ala was replaced by MTX-Arg (at the indicated concentrations) and CP-A was omitted. Where indicated, CP-B (500 milliunits/well) was present.

of the MTX peptides occurs extracellularly. The latter is in contrast to the approach of Piper et al. (1982), who prepared a series of α- and γ-esters and amides of MTX that were designed for uptake followed by intracellular activation via lysosomal proteases.

There are several possible clinical uses for MTX peptides. MTX-Arg, for example, should have a long half-life in the circulation, thereby providing a reservoir for the slow, continuous generation of the active drug. Encouraging in this respect are reports [e.g., Erdos et al. (1965)] that patients with various malignancies have elevated levels of serum carboxypeptidase. A limitation of this procedure might be lack of selectivity, since both normal and malignant cells would be exposed to the drug. Perhaps the latter problem could be overcome by taking advantage of the fact that certain tumors elaborate proteases similar in specificity to plasminogen activator (Reich, 1975). Thus, selectivity might be achieved by using MTX peptides capable of being activated by these enzymes. Another potential device for producing selectivity would be to immobilize carboxypeptidases in the vicinity of tumors by linking them covalently to tumor-specific monoclonal antibodies. Systemic infusion of MTX peptides might then lead to the generation of high concentrations of MTX in the vicinity of the tumors. Model experiments to test this hypothesis (L1210 cells grown in semisolid agarose containing MTX-Ala, and CP-A immobilized on filter paper disks or agarose beads) have demonstrated *regiospecific* cell kill (Vitols et al., 1989).

Added in Proof

The general strategy of employing an enzyme–antibody conjugate to activate a prodrug in the vicinity of a tumor has been validated by the recent work of Senter et al. (1988). These investigators demonstrated in vitro and in vivo antitumor activity using a phosphatase–monoclonal antibody complex to convert nontoxic etoposide phosphate to the free drug etoposide.

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Registry No. CP-A, 11075-17-5; CP-B, 9025-24-5; MTX γ-*tert*-butyl ester, 79640-76-9; MTX α-methyl γ-*tert*-butyl ester, 79640-68-9; L,L-MTX-ala, 116819-28-4; D,L-MTX-ala, 118375-48-7; L,L-MTX-asp, 71074-48-1; D,L-MTX-asp, 118375-49-8; D,L-MTX-arg·HCl, 118375-46-5; L,L-MTX-arg·HCl, 118375-47-6; MTX-ala di-*tert*-butyl ester, 118355-50-3; MTX-asp tri-*tert*-butyl ester, 118355-51-4; MTX-arg γ-*tert*-butyl (Glu) α-methyl (Arg) diester·HCl, 118355-52-5; *N*-Cbz-Glu γ-*tert*-butyl ester, 3886-08-6; *N*-Cbz-Glu α-methyl γ-*tert*-butyl diester, 56877-41-9; Glu α-methyl γ-*tert*-butyl diester hydrochloride, 6234-01-1; Ala *tert*-butyl ester hydrochloride, 13404-22-3; Asp di-*tert*-butyl ester hydrochloride, 1791-13-5; Arg methyl ester hydrochloride, 18598-71-5; 4-amino-4-deoxy-10-methylpteroic acid, 19741-14-1; 2,4,5,6-tetraaminopyrimidine sulfate, 49647-58-7; dihydroxyacetone, 96-26-4; 2,4-diamino-6-(hydroxymethyl)pteridine, 945-24-4; *p*-(methylamino)benzoic acid, 10541-83-0; *N*-carbobenzoxy-L-glutamic acid α-methyl γ-*tert*-butyl diester hydrochloride, 118355-49-0.

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Dihydrofolate Reductase: Multiple Conformations and Alternative Modes of Substrate Binding†

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ABSTRACT: The complex of *Lactobacillus casei* dihydrofolate reductase with the substrate folate and the coenzyme $NADP^+$ has been shown to exist in solution as a mixture of three slowly interconverting conformations whose proportions are pH-dependent [Birdsall, B., Gronenborn, A. M., Hyde, E. I., Clore, G. M., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1982) *Biochemistry 21*, 5831]. The assignment of the resonances of all the aromatic protons of the ligand molecules in all three conformational states of the complex has now been completed by using a variety of NMR methods, particularly two-dimensional exchange experiments. The resonances of the nicotinamide protons of the coenzyme and the pteridine 7-proton of the folate have different chemical shifts in the three conformations, in some cases differing by more than 1 ppm. Comparison of the COSY spectra of the complex at low pH (conformation I) and high pH (conformations IIa and IIb) with that of the enzyme–methotrexate–$NADP^+$ complex shows only slight differences in the conformation of the protein. The pattern of chemical shift changes in the ligand and the protein indicates that the structural differences are localized within the active site of the enzyme. Nuclear Overhauser effects (NOEs) are observed between the nicotinamide 5- and 6-protons and the methyl resonance of Thr 45 at both low and high pH, indicating that there is no major movement of the nicotinamide ring. By contrast, NOEs are observed between the pteridine 7-proton and the methyl protons of Leu 19 and Leu 27 in conformations I and IIa but *not* in conformation IIb. A model is proposed that can qualitatively account for the observed NOEs and ligand ^1H chemical shifts. In this model, the folate pteridine ring in conformations I and IIa is bound in a way similar to that observed for the pteridine ring of methotrexate in the crystal structure of the enzyme–methotrexate–NADPH complex. In conformation IIb, however, it has a quite different orientation in the binding site, related to that in states I and IIa by a rotation of about 180° about an axis approximately along the C2–NH_2 bond. This latter orientation would account for the observed stereochemistry of reduction of folate by the enzyme. It thus appears that folate is able to bind to the enzyme both in a *productive* and in a quite different *nonproductive* mode, while the inhibitor methotrexate binds only in the nonproductive mode.

Dihydrofolate reductase, which is responsible for maintaining the cellular pools of tetrahydrofolate derivatives, is an NADPH-linked dehydrogenase that catalyzes the reduction of folate and dihydrofolate to tetrahydrofolate. It is inhibited by the important "anti-folate" drugs such as trimethoprim and methotrexate. In recent years, a substantial body of structural information on complexes of the enzyme with inhibitors has become available from X-ray crystallography and NMR spectroscopy [for review, see Roberts (1983), Beddell (1984), Freisheim and Matthews (1984), Blakley (1985), and Feeney (1986)]. Much less information is available concerning substrate binding and the catalytic mechanism. However, studies of the stereochemistry of the reaction have established that in the catalytically functional complex the substrate must bind in an orientation substantially different from that seen for methotrexate in the crystal (Hitchings & Roth, 1980; Charlton et al., 1979, 1985). Thus, in spite of the close structural similarity between folate and methotrexate, the only important difference being the replacement of the 4-oxo substituent of folate by an amino group in methotrexate, there appear to be marked differences in their modes of interaction with the enzyme.

Kinetic and NMR experiments have provided evidence that under a variety of conditions dihydrofolate reductase exists in solution as a mixture of two or more conformational states that interconvert only slowly. This appears to be the case for the enzyme alone (Pattishall et al., 1976; Dunn et al., 1978; London et al., 1979; Cayley et al., 1981) and for the enzyme–trimethoprim–$NADP^+$ (Gronenborn et al., 1981a,b) and

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