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Occurrence and Significance of Diastereomers of Methotrexate α-Peptides†

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ABSTRACT: The L,L diastereomer of methotrexate-α-alanine (L,L-MTX-Ala) was synthesized by reaction of α-L-glutamyl-L-alanine di-*tert*-butyl ester with 4-amino-4-deoxy-10-methylpteroic acid, followed by removal of the blocking groups. It was identified by HPLC (C_{18} reversed-phase silica gel; acetic acid/CH_3OH) as the slower of two closely spaced components in DL,L-MTX-Ala prepared previously by a different route [Kuefner et al. (1989) *Biochemistry 28*, 2288–2297]. The L,L diastereomer was hydrolyzed by pancreatic carboxypeptidase A (to yield MTX and Ala) twice as rapidly as the DL,L mixture. Analysis of the latter by HPLC established that the slower component was hydrolyzed to MTX and that the unreactive, faster component was D,L-MTX-Ala. DL,L-MTX-Arg was resolved by HPLC (NH_4OAc/CH_3CN) into two closely spaced components, and the diastereomers were partially separated by chromatography on DEAE-Trisacryl ($H_2O \rightarrow 2\%$ NH_4HCO_3). Serum carboxypeptidase N hydrolyzed only the slower HPLC component (to yield MTX and Arg), thereby identifying it as the L,L diastereomer. When tested for cytotoxicity against L1210 cells, L,L-MTX-Arg ($ID_{50} = 1.6 \times 10^{-8}$ M) was more effective than the D,L diastereomer ($ID_{50} = 2.2 \times 10^{-7}$ M). Treatment of MTX with dicyclohexylcarbodiimide and *N*-hydroxysuccinimide (NHS), followed by hydrolysis of the NHS ester, led to racemization in the L-glutamate moiety of MTX as shown by the fact that the product was hydrolyzed by carboxypeptidase G_2 (at the pteroate–Glu bond) only to the extent of ca. 50% compared to the untreated control. These observations have a broad significance, since coupling agents are employed extensively in the derivatization of MTX for attachment to affinity supports and monoclonal antibodies.

Previous studies from this laboratory (Kuefner et al., 1988, 1989) have demonstrated that methotrexate α-peptides (i.e., derivatives in which amino acids are joined via an amide linkage to the α-carboxyl group of MTX[1]) can be hydrolyzed by carboxypeptidases to yield MTX; MTX-α-alanine (MTX-Ala) and MTX-α-arginine (MTX-Arg), for example, were substrates for pancreatic CP-A and serum CP-N, respectively. The MTX peptides were relatively nontoxic, presumably because derivatization of the α-carboxyl suppresses cellular uptake of the drug (Sirotnak et al., 1979). These results suggested that MTX peptide/carboxypeptidase combinations,

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[1] Abbreviations: MTX, methotrexate; MTX-Ala and -Arg, peptides in which the indicated amino acids are linked covalently to the α-COOH of MTX; CP-A, -N, and -G_2, carboxypeptidases A, N, and G_2; DCC, *N*,*N*′-dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; DMF, dimethylformamide; ID_{50}, concentration producing 50% inhibition of cell growth.

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particularly if the enzyme were linked to tumor-specific monoclonal antibodies, might be used to generate high concentrations of free drug in the vicinity of tumors. The feasibility of obtaining this type of *regiospecific* cytotoxicity has been demonstrated with the use of a model system that employed L1210 cells and MTX-Ala distributed uniformly in semisolid agarose and CP-A immobilized on paper disks or beads (Vitols et al., 1989; Huennekens et al., 1990).

Synthesis of MTX peptides utilized a sequence, adapted from the general procedures of Rosowsky et al. (1981) and Piper et al. (1982), that included the isobutyl chloroformate promoted reaction of protected derivatives of the amino acids with MTX γ-*tert*-butyl ester (Kuefner et al., 1989). Although these compounds met various criteria of purity and authenticity (e.g., elemental analysis, absorbance spectra, and NMR spectra), two anomalies were observed: (1) in some HPLC solvent systems, MTX-Ala, MTX-Asp, and MTX-Arg each exhibited approximately equal amounts of two closely spaced peaks, and (2) hydrolysis of MTX-Ala, catalyzed by CP-A, ceased when approximately 50% of the substrate had been consumed. Since Rosowsky and Yu (1978) had noted that treatment of the α-carboxyl of MTX with coupling agents (as a prelude to coupling with amino acids) resulted in racemization of the Glu moiety, it was assumed that the MTX peptides were mixtures of the D,L and L,L diastereomers (with reference to the configurations of Glu and the additional amino acid). The present report provides definitive evidence to support the occurrence of this racemization reaction and describes some chemical and biological properties of the diastereomers of MTX-Ala and MTX-Arg. These observations have broad significance since coupling agents are employed for attaching MTX to solid supports (e.g., for affinity purification of dihydrofolate reductases) or to monoclonal antibodies (for therapeutic purposes), and the racemization resulting from these procedures may reduce the effectiveness of the drug.

Experimental Procedures

Materials

N-Cbz-L-glutamic acid γ-*tert*-butyl ester and L-alanine *tert*-butyl ester hydrochloride were obtained from Sigma. Sources of other purchased materials and the preparation of 4-amino-4-deoxy-10-methylpteroic acid, DL,L-MTX-Ala, and DL,L-MTX-Arg have been given previously (Kuefner et al., 1989). Carboxypeptidase G_2 isolated from a *Pseudomonas* sp. strain RS-16 (Sherwood et al., 1985) and MTX were generous gifts from Drs. R. Sherwood (Public Health Laboratory Service, Salisbury, England) and S. Kerwar (Lederle Laboratories), respectively.

L,L-*MTX-Ala*([*N*-(*4-Amino-4-deoxy-10-methylpteroyl*)-α-L-*glutamyl*]-L-*alanine*). The following procedure, adapted from Rosowsky and Yu (1978), was employed. *N*-Cbz-L-glutamic acid γ-*tert*-butyl ester (2.53 g, 7.5 mmol), triethylamine (1.67 g, 2.3 mL, 16.5 mmol), and isobutyl chloroformate (1.13 g, 1.07 mL, 8.25 mmol) were dissolved in 45 mL of absolute DMF and stirred (with exclusion of moisture) at room temperature. After 15 min, L-alanine *tert*-butyl ester hydrochloride (1.50 g, 8.25 mmol) was added, and the mixture was stirred for 6 h. It was cooled to 4 °C for 15 min, and the precipitate was removed by filtration and washed with absolute DMF. The combined filtrates were evaporated in high vacuum. The oily product was suspended in saturated $NaHCO_3$ and extracted three times with ethyl acetate. The combined organic fractions were dried over Na_2SO_4 and evaporated. The crude product was dissolved in petroleum ether:ether (1:2) and chromatographed on a silica gel column (2 × 25 cm), with the same solvent. The product, *N*-Cbz-L-glutamyl-α-L-alanine di-*tert*-butyl ester, was obtained as a colorless oil: yield 1.26 g (36%); TLC (petroleum ether:ether, 1:2) R_f = 0.67 (even concentrated spots were only faintly visible under UV or visible light after treatment with 15% H_2SO_4 and drying at 120 °C for 15 min); ^{1}H NMR ($CDCl_3$) 7.33 (s, 5 H, phenyl), 6.80 (d, broad, J = 8 Hz, NH), 5.66 (d, broad, J = 8 Hz, NH), 5.12 (s, 2 H, benzyl), 4.6–3.8 (m, 2 H, 2α-CH), 2.5-1.8 (m, 4 H, CH_2CH_2), 1.40 (s, 18 H, 2COOtBu), and 1.24 ppm (d, J = 7 Hz, 3 H, CH_3).

The above product (1.26 g, 2.7 mmol) was dissolved in a mixture of 125 mL of CH_3OH and 7 mL of H_2O, and the solution was saturated with argon. Palladium on charcoal (50 mg) was added under argon, and the mixture was hydrogenated at 10 psi for 12 h. The catalyst was removed by filtration through Celite and washed thoroughly with CH_3OH. The combined filtrates were evaporated in high vacuum (temperature below 35 °C), and the colorless oil was dissolved in 10 mL of anhydrous ether. The solution was filtered and cooled to 4 °C, and 10 mL of a saturated solution of HCl in absolute ether was added. The solution, kept at 0 °C until crystals began forming, was then stored for 12 h at −20 °C. The colorless crystals (α-L-glutamyl-L-alanine di-*tert*-butyl ester hydrochloride) were recovered by filtration, washed with cold ether, and dried in vacuum: mp 142–144 °C; yield 290 mg (29%).

The above dipeptide (250 mg, 0.69 mmol) was linked to 4-amino-4-deoxy-10-methylpteroic acid (231 mg, 0.69 mmol) by using the procedure (triethyl phosphorocyanidate in DMF) described previously for the synthesis of MTX α-methyl γ-*tert*-butyl diester from the pteroic acid and the protected glutamate (Kuefner et al., 1989). After removal of DMF by evaporation in vacuum, the brown residue was taken up in CH_2Cl_2. The solution was extracted twice with 1% NH_4OH, and the aqueous phase was reextracted with CH_2Cl_2; in these procedures, separation of the layers required several hours. The combined organic fractions were dried over Na_2SO_4 and the solvent was removed by evaporation. The product was dissolved in CH_2Cl_2:CH_3OH (10:1) and chromatographed on a silica gel column (3 × 15 cm, 230–400 mesh); the same solvent was used for elution. Fractions containing the product were combined, and the solvent was removed by evaporation. The product, L,L-MTX-α-alanine di-*tert*-butyl ester, was obtained as a yellow amorphous powder: yield 205 mg (47%); TLC (CH_2Cl_2:CH_3OH, 10:1) R_f = 0.51. NMR data were identical with those given previously for the DL,L product (Kuefner et al., 1989). The *tert*-butyl ester groups were removed by acid hydrolysis, as described previously for the preparation of MTX-α-Ala (Kuefner et al., 1989). Characteristics of the L,L product were identical with those reported for DL,L-MTX-Ala.

D,L- *and* L,L-*MTX-Arg*. DL,L-MTX-Arg was synthesized as described previously (Kuefner et al., 1989), except that the D,L and L,L diastereomers were recovered during the final step in the purification procedure (chromatography on DEAE-Trisacryl). Early and late fractions, which contained the D,L form (retention time 27.8 min) and the L,L form (28.2 min), respectively, were pooled, desalted, and taken to dryness; yields, D,L-MTX-Arg, 79 mg (6.2%), and L,L-MTX-Arg, 59 mg (4.6%).

Racemization of MTX by Treatment with DCC/NHS. MTX (122 mg, 0.25 mmol) was dissolved in 10 mL of dry DMF, and the solution was cooled to 4 °C. DCC (52 mg, 0.25 mmol) and NHS (31 mg, 0.28 mmol) were added, and the

mixture was stirred for 1 h at 4 °C, followed by 2 h at room temperature. After addition of water (100 μL), the solution was stirred for 3.5 h at room temperature, passed through a 0.45-μm nylon filter, and evaporated under vacuum (<40 °C). The solid was taken up in H_2O and lyophilized to dryness. As a control, MTX was processed similarly, except that DCC and NHS were omitted.

Methods

Absorbance spectra were measured with a Hewlett-Packard UV/vis spectrophotometer, Model 8450. Concentrations of MTX or MTX peptides were determined spectrophotometrically (ϵ_{mM} = 22.0 at 302 nm; pH 13) (Seeger et al., 1949). HPLC was conducted on a C_{18} reversed-phase silica gel column (Beckman ODS) with solvent systems A (acetic acid/CH_3OH) or B (NH_4OAc/CH_3CN), as described previously (Kuefner et al., 1989). The program for system A, however, was modified: 30% solvent B (2% acetic acid in 50% CH_3OH) for 5 min, followed by a linear gradient from 30 to 70% B over 30 min. This change was necessary because columns currently available from the supplier have been altered to contain an "end-capped" matrix material.

The colorimetric assay for determination of cytotoxicity of MTX peptides against L1210 cells and the coupled spectrophotometric assay for carboxypeptidase-mediated hydrolysis of these compounds have been described previously (Kuefner et al., 1989). L-MTX was measured by its activity as a substrate for CP-G_2 by using the following procedure. MTX (22 μL, 4.5 mM) and Tris buffer (970 μL, 0.1 M, pH 7.3, containing 20 mM $ZnSO_4$) were added to a 1-cm quartz cuvette. CP-G_2 (10 μL, 1.0 unit/mL) was added, and the decrease in A_{320} of the solution was recorded as a function of time with a Gilford spectrophotometer (Model 252). A value of ϵ_{mM} = 8.3 (Levy & Goldman, 1967) was used to calculate the concentration change for the overall reaction (MTX → 4-amino-4-deoxy-10-methylpteroate + Glu). One unit of CP-G_2 will hydrolyze 1 μmol of MTX/min at pH 7.3 and 30 °C (McCullough et al., 1971).

RESULTS

Synthesis of L,L-*MTX-Ala.* This compound was prepared by an unambiguous route, adapted from Rosowsky and Yu (1978), that avoids racemization of the Glu. As described under Experimental Procedures, the dipeptide, L-Glu-α-L-Ala di-*tert*-butyl ester, was linked to 4-amino-4-deoxy-10-methylpteroic acid, and the blocking groups were removed. When L,L-MTX-Ala was subjected to HPLC with acetic acid/CH_3OH (system A, modified as described under Experimental Procedures), a single peak with a retention time of 22.0 min was observed (Figure 1). Conversely, a doublet (21.1 and 22.4 min) was obtained when DL,L-MTX-Ala, synthesized as described previously (Kuefner et al., 1989), was chromatographed under the same conditions. Admixture of the L,L diastereomer and the DL,L mixture produced a selective increase in the 22.0 min peak, from which it was concluded that the faster and slower components are the D,L and L,L diastereomers, respectively.

Hydrolysis of MTX-Ala Diastereomers by CP-A. The L,L and DL,L preparations of MTX-Ala were compared with respect to activity as substrates for pancreatic CP-A. Reactions were followed via the coupled spectrophotometric assay described previously [cf. Figures 4 and 5 in Kuefner et al. (1989)] in which MTX liberated in the CP-A-dependent reaction is detected by its further cleavage, in the presence of excess CP-G_2, at the pteroate–Glu linkage. The rate of hydrolysis of L,L-MTX-Ala was 57.6 μM min^{-1} $unit^{-1}$ of CP-A, which is ca. 2-fold greater than that observed for the DL,L preparation (25.6 μM min^{-1} $unit^{-1}$ of CP-A). This suggested that the D,L diastereomer was not a substrate for CP-A.

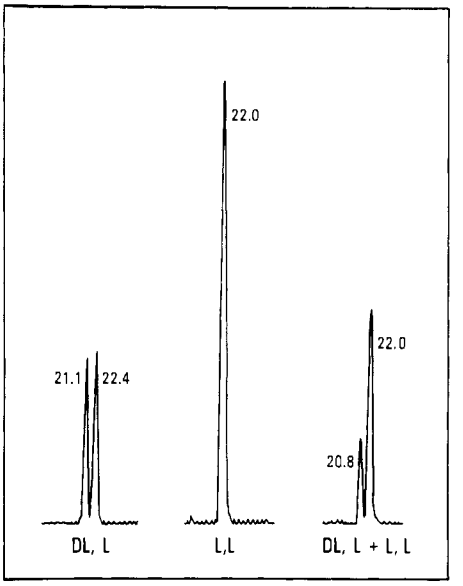

FIGURE 1: HPLC of MTX-Ala preparations. Chromatography was performed with solvent system A (see Experimental Procedures). Left and middle: DL,L-MTX-Ala and L,L-MTX-Ala (each 0.2 mM in 0.1 M Tris buffer, pH 7.3; 20 μL injected). Right: Mixture (1:1) of DL,L-MTX-Ala and L,L-MTX-Ala solutions; 40 μL injected. Full-scale absorbance range: 0.05 (left and middle), 0.1 (right). Numbers indicate retention times (min).

HPLC analysis allowed the CP-A-mediated hydrolysis of DL,L-MTX-Ala to be examined in greater detail, i.e., with respect to the time dependence of substrate disappearance and product appearance and in the absence of CP-G_2. As shown in Figure 2, the progressive increase of MTX was paralleled by the decrease of L,L-MTX-Ala. No change was observed, however, in D,L-MTX-Ala. These results confirmed the conclusion reached in the previous experiment, viz., that only the L,L diastereomer of MTX-Ala is a substrate for CP-A. The HPLC profile also demonstrated that 4-amino-4-deoxy-10-methylpteroate was not formed during the course of the reaction. Thus, unlike CP-G_2, CP-A was incapable of removing Glu from MTX.

Resolution of Diastereomers of MTX-Arg. DL,L-MTX-Arg, prepared as described previously (Kuefner et al., 1989), was partially resolved into two components by chromatography on DEAE-Trisacryl (see Experimental Procedures). When examined by HPLC (system B), early and late fractions each showed single peaks (27.8 and 28.2 min); intermediate fractions contained both peaks. Small amounts (60–80 mg) of each component were recovered from pooled fractions. By analogy with the HPLC data for MTX-Ala (cf. Figure 1), it was assumed that the faster and slower components were the D,L and L,L diastereomers, respectively.

Hydrolysis of MTX-Arg Diastereomers by CP-N. The diastereomers of MTX-Arg and the DL,L mixture were compared with respect to activity as substrates for CP–N in human serum (Schweisfurth et al., 1983). These reactions were followed via the coupled spectrophotometric assay. The L,L diastereomer was about twice as active as the DL,L mixture, and the D,L diastereomer was inert (Figure 3).

Cytotoxicity of MTX-Arg Diastereomers. The MTX-Arg diastereomers were tested for cytotoxicity against L1210 cells. CP-N, a constituent of the fetal bovine serum present in the culture medium, served as the activating agent for the MTX peptide. As shown in Figure 4, the L,L diastereomer (ID_{50} =

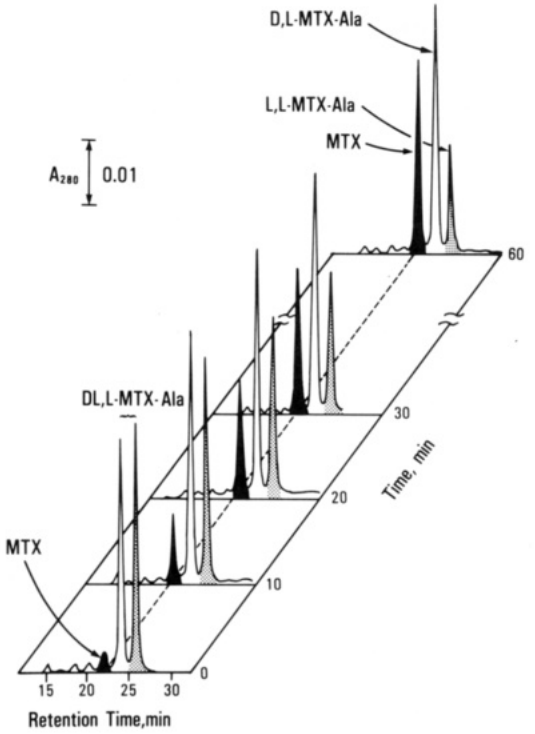

FIGURE 2: HPLC assay of CP-A-mediated hydrolysis of DL,L-MTX-Ala. Replicate assay mixtures (500 μL in microcentrifuge tubes) were prepared as described previously for the spectrophotometric assay (Kuefner et al., 1989), except that CP-A was decreased to 0.04 unit and CP-G_2 was omitted. CP-A was added to initiate the reaction. Temperature, 37 °C. At the indicated times, reactions were stopped by placing the tubes in an ice bath. The tubes were then heated to 90 °C for 1 min, cooled to 4 °C, and centrifuged for 2 min in an Eppendorf microcentrifuge. Supernatants (20 μL) were injected into the HPLC and analyzed with solvent system A (as described previously; Kuefner et al., 1989). Results are displayed as time-dependent changes in the HPLC profile (monitored at 280 nm).

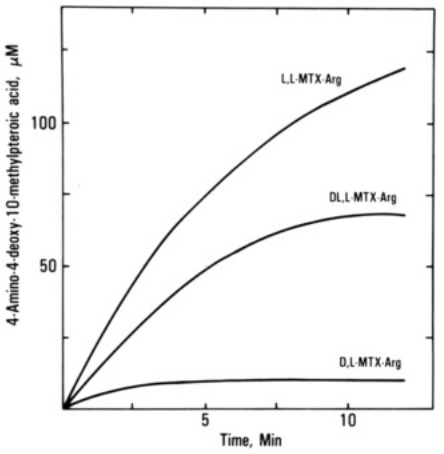

FIGURE 3: Spectrophotometric assay of CP-N-mediated hydrolysis of L,L-, DL,L-, and D,L-MTX-Arg. The assay was performed as described previously (Kuefner et al., 1989), except that 0.016 unit of CP-G_2 was used. Absorbance changes at 320 nm were monitored continuously, and results are expressed as the time-dependent appearance of 4-amino-4-deoxy-10-methylpteroate.

1.8×10^{-8} M) was more toxic than its D,L counterpart (2.0×10^{-7} M). The ID_{50} value for L,L-MTX-Arg was approximately the same as that of MTX (2.2×10^{-8} M), which suggested that hydrolysis of the Glu–Arg linkage was much faster than the other processes (e.g., uptake of MTX and inhibition of dihydrofolate reductase) involved in cell kill.

Racemization of MTX by Coupling Agents. MTX (which contains L-Glu) was treated with DCC plus NHS, and the

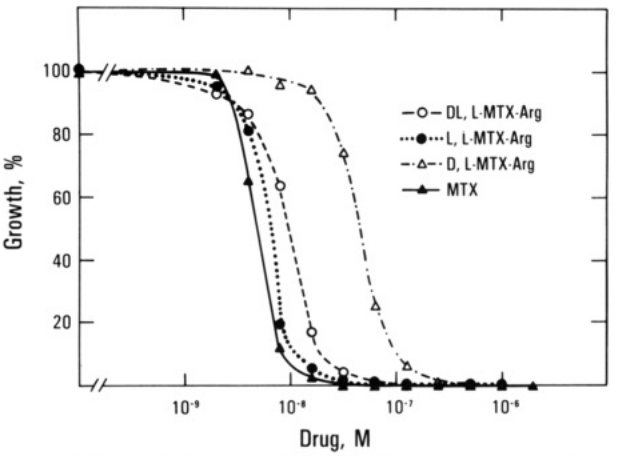

FIGURE 4: Cytotoxicity toward L1210 cells of DL,L-, L,L-, and D,L-MTX-Arg. L1210 cells were grown for 72 h in the presence of the indicated concentrations of the MTX-Arg diastereomers and MTX (as a positive control). For other details, see Kuefner et al. (1989). Each point represents the average of duplicate determinations.

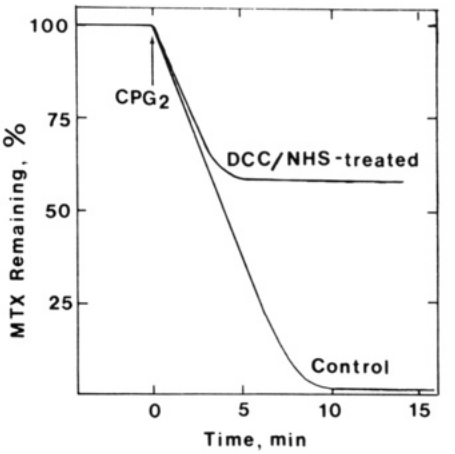

FIGURE 5: Racemization of MTX by treatment with DCC/NHS. MTX treated with those agents and the untreated control (see Experimental Procedures) were dissolved in 0.1 M Tris buffer, pH 7.3, and centrifuged (3000*g*, 30 min). In each sample, total MTX was measured spectrophotometrically and L-MTX was determined by spectrophotometric assay with CP-G_2. Absorbance changes at 320 nm were monitored continuously, and results are expressed as the time-dependent disappearance of MTX.

resulting NHS ester was decomposed with H_2O. Assay of the mixture with CP–G_2 revealed that only ca. 50% of the material was still a substrate for this carboxypeptidase (Figure 5). Since the D enantiomer of MTX is not a substrate for CP-G_2 (Cramer et al., 1984), it was concluded that racemization, catalyzed by the coupling agents, had occurred and that the resulting product was an approximately equimolar mixture of the D and L enantiomers of MTX. HPLC analysis and absorbance spectra (data not shown) verified that no degradation or self-condensation of MTX had occurred.

Discussion

The chemical synthesis of MTX utilizes L-Glu, since naturally occurring folate compounds contain this enantiomer. Although commercial preparations of the drug contain small amounts (0.5–7%) of D-MTX (Cramer et al., 1984), this is due most probably to traces of D-Glu in the starting material. In the present investigation, it was hoped that the conventional procedures used for peptide bond formation would allow L amino acids to be linked to the α-carboxyl of MTX to produce only the L,L diastereomers (with reference to the configurations of Glu and the additional amino acid). However, preparations

of MTX α-peptides, obtained by reacting the γ-*tert*-butyl derivative with protected L amino acids and then removing the blocking groups (Kuefner et al., 1989), appeared to be mixtures of two closely related compounds. Since each preparation could be resolved by HPLC into two components approximately equal in amount, it seemed possible that either the Glu moiety in MTX or the incoming amino acid had racemized during the synthetic procedure. This assumption was strengthened by the fact that only ca. 50% of the MTX-Ala preparation was hydrolyzed (to yield MTX and Ala) by CP-A. Similar results had been obtained previously by Rosowsky and Yu (1978) who found that a preparation of MTX-α,γ-bis(L-Glu) diethyl ester, synthesized by the DCC-promoted reaction of MTX with diethyl L-Glu, could be separated chromatographically into two components that had identical melting points and virtually superimposable IR and NMR spectra. It was proposed that these components were the L,L,L and D,L,L forms and that racemization of the Glu moiety of MTX occurred via a mechanism involving an activating effect of the *p*-aminobenzoyl group on the Glu nitrogen, followed by formation of a resonance-stabilized cyclic intermediate capable of opening to produce Glu in either the L or D configuration. In support of these assumptions, the investigators utilized a synthetic route that would obviate the activating effect of the aroyl group, viz., by first constructing the tripeptide, L-Glu-α,γ-bis(L-Glu) diethyl ester, and then joining it to the pteroic acid. The product, which should have been the L,L,L form, was found to be chromatographically identical with one of the components in the earlier preparation; by exclusion, the other component was assigned the D,L,L structure.

In the present investigation, the L,L diastereoisomer of MTX-Ala was prepared by the unambiguous route of Rosowsky and Yu (1978), i.e., by linking α-L-Glu-L-Ala di-*tert*-butyl ester with 4-amino-4-deoxy-10-methylpteroic acid and removing the blocking groups. The product had an HPLC retention time similar to that of the slower component of the previous MTX-Ala preparation, presumably a DL,L mixture. Admixing equal amounts of the two preparations prior to HPLC resulted in an increased peak height of the slower component (Figure 1). The faster component, therefore, was assumed to be the D,L diastereomer. Subsequently, the faster and slower components in the HPLC doublet of MTX-Arg were also identified as the D,L and L,L diastereomers, respectively. Thus, reversed-phase HPLC provides a rapid and convenient procedure for detecting and quantitating diastereomers of MTX α-peptides. Appropriate solvent systems, however, must be determined empirically: system A (acetic acid/CH_3OH) is suitable for MTX-Ala (and MTX-Asp), while system B (NH_4OAc/CH_3CN) is required for MTX-Arg. Chiral HPLC, yet to be explored, may achieve better separation. It was possible to obtain larger quantities of the diastereomers of MTX-Arg by partial resolution of the DL,L mixture on DEAE-Trisacryl. This procedure was not able to separate the diastereomers of MTX-Ala (or MTX-Asp). Reaction of L-Glu-L-(amino acid) with the pteroic acid, however, provides ready access to the biologically active L,L diastereomers of MTX α-peptides, and the D,L diastereomers (if required) could be obtained by using D-Glu-L-(amino acid).

The L,L and D,L diastereomers of MTX-Ala and MTX-Arg have been examined with respect to biological properties relevant to their potential use as prodrugs in cancer chemotherapy. Since activation of these prodrugs is envisioned to utilize hydrolysis by carboxypeptidases (particularly when the latter are linked to tumor-specific monoclonal antibodies), hydrolysis of MTX-Ala and MTX-Arg by CP-A and CP-N, respectively, were examined in detail. A spectrophotometric assay that coupled the CP-A-mediated hydrolysis of MTX-Ala with further cleavage of the product MTX by CP-G_2 demonstrated that the L,L diastereomer reacted twice as rapidly as the DL,L mixture. HPLC analysis of the latter verified that the L,L diastereomer was the sole source of MTX and that the D,L form was inert (Figure 2). A similar coupled assay in which CP-N was used with CP-G_2 showed that the L,L diastereomer (but not the D,L counterpart) of MTX-Arg could serve as a substrate for the former enzyme (Figure 3). L,L-MTX-Arg was also more toxic to L1210 cells in culture than the D,L form (Figure 4). In the latter experiment, the basis for the toxicity of D,L-MTX-Arg is not clear. Several possibilities exist: (a) although the D,L diastereomer does not appear to be a substrate for CP-N in short-term assays (cf. Figure 3), even very slow hydrolysis over a 72-h period might generate a toxic level of MTX, or (b) since MTX α-peptides are reasonably good inhibitors of dihydrofolate reductases (unpublished results), even very slow uptake might cause sufficient inhibition of the enzyme to block cell replication. To avoid this complication, further experiments to evaluate the chemotherapeutic potential of MTX α-peptides should probably use only the L,L diastereomers.

Further evidence that coupling agents promote racemization of the Glu moiety of MTX was obtained by an experiment in which activation of the α-carboxyl was dissociated from its derivatization. This was accomplished by treatment of the drug with DCC/NHS, followed by hydrolysis of the NHS ester. Assay of the recovered MTX with CP-G_2 revealed that only about 50% of the material had retained its substrate activity; full activity was retained by a control in which the coupling agents were omitted (Figure 5). These results are especially significant, since the conditions employed (DCC/NHS and low temperature) are considered to be optimal for avoiding racemization. Thus, it appears that the synthesis of MTX α-peptides from MTX, regardless of the coupling agent used, will lead to racemization of the glutamate moiety. This can be avoided, however, by first synthesizing the Glu–amino acid peptide and then joining it to the pteroate moiety. Since coupling agents are often used for the synthesis of other MTX (and folate) derivatives, as well as for attaching MTX to affinity matrices or to antibodies, the present investigation may serve to promote further awareness of this racemization reaction and its biological consequences.

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Toward the Solution Structure of Human Insulin: Sequential 2D ^{1}H NMR Assignment of a Des-pentapeptide Analogue and Comparison with Crystal Structure†

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ABSTRACT: 2D ^{1}H NMR studies are presented of des-pentapeptide-insulin, an analogue of human insulin lacking the C-terminal five residues of the B chain. Removal of these residues, which are not required for function, is shown to reduce conformational broadening previously described in the spectrum of intact insulin [Weiss et al. (1989) *Biochemistry 28*, 9855–9873]. This difference presumably reflects more rapid internal motions in the fragment, which lead to more complete averaging of chemical shifts on the NMR time scale. Sequential ^{1}H NMR assignment and preliminary structural analysis demonstrate retention in solution of the three α-helices observed in the crystal state and the relative orientation of the receptor-binding surfaces. These studies provide a foundation for determining the solution structure of insulin.

Insulin, a small polypeptide hormone, provides a model system for biophysical studies of protein folding, dynamics, and recognition. The insulin fold is highly conserved among vertebrate and invertebrate kingdoms, defining an ancestral family of insulin-like regulatory proteins (Blundell & Humbel, 1980). Insulin is composed of two polypeptide chains, the A chain (21 residues) and the B chain (30 residues) linked by two disulfide bonds. As a foundation for comparative studies of insulin-related peptides, we describe complete 2D NMR resonance assignment of an analogue, des-pentapeptide[B26–B30]-insulin (DPI)[1] (Gattner, 1975; Danho et al., 1975; Rieman et al., 1983; Wang & Tsou, 1986). The deleted residues B26–B30 are not generally conserved, and in their absence an amidated analogue exhibits full receptor-binding activity (Fisher et al., 1985; Casaretto et al., 1987; Mirmira & Tager, 1989). An analysis of the aromatic spectrum of DPI at pH 10 has previously been reported (Hua et al., 1989).

The present experiments are based on previous ^{1}H NMR studies of human insulin and proinsulin in 80% H_2O/20% acetic acid–sodium acetate, pH 3 (Cheshnovsky et al., 1983; Weiss et al., 1989, 1990). This solvent system was found to weaken insulin self-association, enabling the monomer to be studied under conditions in which native structure is retained. A remarkable feature of the ^{1}H NMR spectrum of the insulin monomer under these conditions is the extensive variation observed in the linewidths of amide resonances. Such variation is related to exchange among conformational substates. The existence of substates is an intrinsic feature of the insulin fold and is not related to the particular solvent system used in these studies (Weiss et al., 1990). Although of intrinsic interest in relation to insulin dynamics (Chothia et al., 1983; Kruger et al., 1987), exchange-mediated line broadening was found in the earlier studies to limit the application of established sequential ^{1}H NMR assignment methods. This limitation may be overcome by using less aqueous solvent conditions; sequential assignment of insulin has recently been described in 35% acetonitrile (Kline & Justice, 1990). In this paper an alternative strategy is described, based on systematic screening of insulin analogues.

Our results are presented in three parts. In part I the structural effects of successive C-terminal deletions in the B

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[1] Abbreviations: DOI, des-octapeptide-insulin; DPI, des-pentapeptide insulin; DPA, des-pentapeptide-insulin amide; NOE, nuclear Overhauser enhancement; NOESY, 2D NOE spectroscopy; photo-CIDNP, photochemically induced dynamic nuclear polarization; TOCSY, 2D isotropic mixing (Hartmann–Hahn) spectroscopy; DQF-COSY, double-quantum filtered 2D correlation spectroscopy.

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