Mode of Action of Site-Directed Irreversible Folate Analogue Inhibitors of Thymidylate Synthase†

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Received July 15, 1997; Revised Manuscript Received January 20, 1998

ABSTRACT: 5,8-Dideazafolate analogues are tight binding but not irreversible inhibitors of thymidylate synthase (TS). However, when a chloroacetyl (ClAc) group is substituted at the N^{10}-position of 2-desamino-2-methyl-5,8-dideazafolate (DMDDF), the resulting compound, ClAc-DMDDF, although still a reversible inhibitor ($K_I = 3.4 \times 10^{-3}$ M), gradually inactivates thyA-TS irreversibly at a rate of 0.37 min^{-1}. The corresponding iodoacetyl derivative alkylated the enzyme somewhat slower ($k_3 = 0.15$ min^{-1}) than ClAc-DMDDF but was bound more tightly ($K_I = 1.4 \times 10^{-5}$ M), resulting in a second-order rate constant (k_3/K_I) of inactivation that was 100-fold greater than that of ClAc-DMDDF. A tryptic digest of the ClAc-DMDDF-inactivated enzyme yielded a peptide on HPLC, which revealed that cysteine-146, the residue at the active site that is intimately involved in the catalytic process, had reacted with ClAc-DMDDF to form a covalent bond. This derivative was confirmed indirectly by Edman analysis and more directly by mass spectrometry. Deoxyuridine 5′-monophosphate, a substrate in the catalytic reaction, protected against inactivation. Similar to previously described *Lactobacillus casei* TS inhibition studies with sulfhydryl reagents [Galivan, J., Noonan, J., and Maley, F. (1977) *Arch. Biochem. Biophys*. *184*, 336−345], the kinetics of inhibition suggested that complete inhibition occurs on reaction of only one of the two active site cysteines, although sequence and amino acid analysis revealed that iodoacetate and ClAc-DMDDF had reacted with both active site cysteines. These studies demonstrate that a sulfhydryl reactive compound that is directed to the folate binding site of TS may diffuse to the active site cysteine, and form a covalent bond with this residue. How this inhibition comes about is suggested in a stereoscopic view of the ligand when modeled to the known crystal structure of *Escherichia coli* TS.

Thymidylate synthase (TS)[1] (EC 2.1.1.45) has been a chemotherapeutic target since it was discovered to be a major provider of a nucleotide (dTMP) that is essential for DNA synthesis (*1*). One of the first nucleotide derivatives to be developed as an anti-cancer agent is FU (*2*) which is converted in situ to FdUMP, the actual inhibitor of TS (*3*) due to its ability to form a tight ternary complex with TS and CH_2H_4PteGlu, the second substrate of TS (*4*, *5*). Because of the many shortcomings of FU in the clinic, more recent emphasis has been placed on developing folate analogue inhibitors of TS, based on earlier studies which demonstrated that 5,8-dideaza analogues of folate inhibit this enzyme (*6*). One such inhibitor, 10-propargyl-5,8-dideazafolate (CB3717) (*7*), was found to inhibit TS in the nanomolar range, which on polyglutamylation in situ (*8*) was even more effective, particularly in the presence of its nucleotide substrate (*9*, *10*). A definitive location of this inhibitor in the TS dimer was established by X-ray crystallography, both in its monoglutamate (*11*, *12*) and in its polyglutamate forms (*13*). With this information in mind, the propargyl group was replaced with a functionally reactive chloroacetyl group to determine if the resulting analogue of CB3717 could become an irreversible inhibitor of TS. This paper describes what occurs when 2-desamino-2-methyl-N^{10}-chloroacetyl-5,8-diazafolate is incubated with *Escherichia coli* TS.

MATERIALS AND METHODS

Synthesis of N^{10}-Chloroacetyl-2-desamino-2-methyl-5,8-dideazafolic Acid. Di-*tert*-butyl-L-glutamate was coupled to 4-nitrobenzoic acid by the isobutyl chloroformate method,

† This work was supported in part by grants from the National Cancer Institute, CA44355 (F.M.) and CA27101 (M.G.N.) USPHS/HHS, and also by grants from the American Cancer Society, DHP-45 (W.R.M.) and DHP-178 (M.G.N.).

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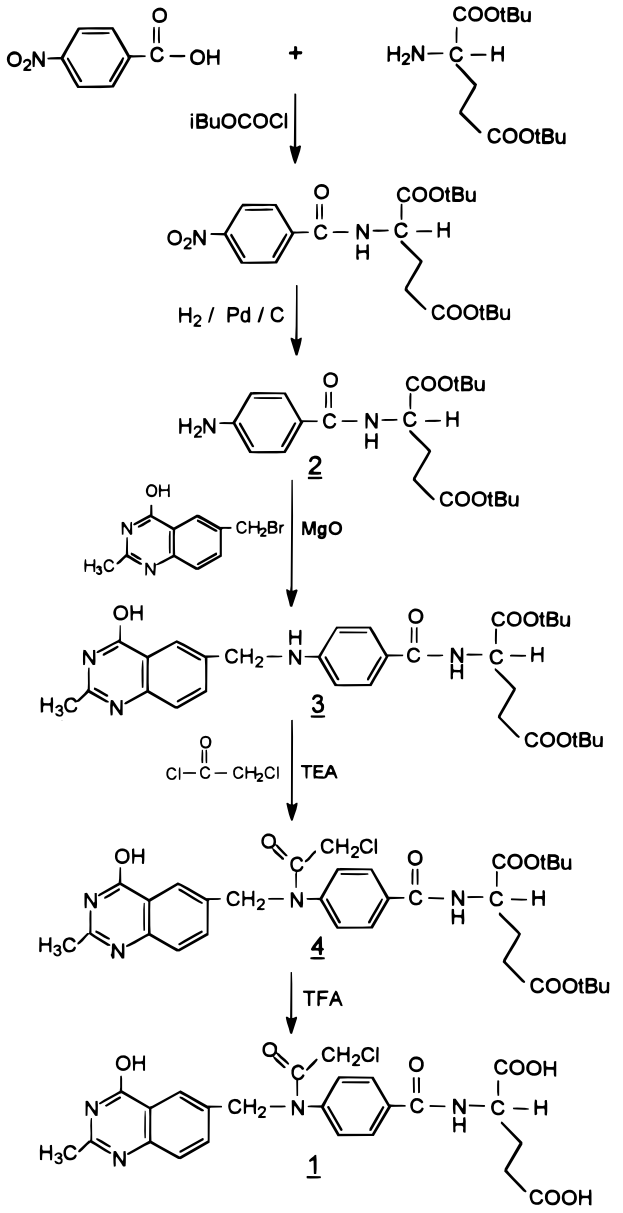
[1] Abbreviations: TS, thymidylate synthase; CB3717, 10-propargyl-5,8-dideazafolate; ClAc-DMDDF, N^{10}-chloroacetyl-2-desamino-2-methyl-5,8-dideazafolate; IAc-DMDDF, N^{10}-iodoacetyl-2-desamino-2-methyl-5,8-dideazafolate; IAc, iodoacetate; $IACNH_2$, iodoacetamide; CM, carboxymethyl; FU, 5-fluorouracil; FdUMP, 5-fluoro-2-deoxyuridine 5′-monophosphate; dTMP, thymidine 5′-monophosphate; CH_2H_4PteGlu, 5,10-methylenetetrahydrofolate; TFA, trifluoroacetic acid; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone.

S0006-2960(97)01728-5 CCC: $15.00
Published on Web 03/11/1998

Scheme 1: Method Employed To Synthesize ClAc-DMDDF



and the resultant di-*tert*-butyl-4-nitrobenzoylglutamate was hydrogenated with Pd/C to di-*tert*-butyl-4-aminobenzoyl-L-glutamate (**2**) (*7*). Reaction of **2** with 6-bromomethyl-4-hydroxy-2-methylquinazoline gave di-*tert*-butyl-2-desamino-2-methyl-5,8-dideazafolate (**3**) (*14*), which was converted then to **4** by reaction with chloroacetyl chloride (Aldrich). Compound **4**, after chromatographic purification, was deprotected to the target compound **1** by treatment with TFA. The crude product thus obtained was purified to >98% purity by reverse phase chromatography on a C-18 silica gel column using 12% acetonitrile in water as the eluting solvent. Compound **1** was characterized by UV (λ_{262} = 11 649 nm; λ_{305} = 4846 nm) and mass spectrometry (MH^+ = 515). The reaction sequence leading to compound **1** is summarized in Scheme 1. More extensive details on the synthetic procedures will be published elsewhere (Nair, M. G., in preparation). Compound **1** was dissolved in 5% $NaHCO_3$ and stored in this solution, since it appeared to be more stable than in 0.01 M NaOH, which led to new peaks at 278 and 293 nm and a change in retention time on HPLC.

Synthesis of N^{10}-Iodoacetyl-2-desamino-2-methyl-5,8-dideazafolic Acid. This compound was synthesized by a modification of the procedure of Kampf et al. (*15*). ClAc-DMDDF was dissolved in acetonitrile−water (80:20) to which was added a 5-fold excess of NaI. This solution was stirred at room temperature for 3−5 days, during which time the exchange reaction was followed by HPLC using a 25 × 0.46 cm μBondapak C_{18} column (Waters Associates, Milford, MA). The column was eluted isocratically with a solution of 1.26 mM NH_4OAc, 0.018 N acetic acid, and 13.8% acetonitrile. IAc-DMDDF eluted at 26 min compared to 18 min for ClAc-DMDDF. Purification of the iodo compound was effected in the same way as compound **1**. The iodo derivative on mass spectroscopy showed a M+1 peak at 607.

Assay for TS Activity in the Presence and Absence of Inhibitors. Homogeneous thyA-TS was prepared essentially as described previously (*9*). Assays were performed at 30 °C in 1 cm quartz cuvettes containing 0.65 mL of water, 0.25 mL of a folate cocktail, 0.05 mL of 1 M $MgCl_2$, and 0.05 mL of 10 mM dUMP. The folate cocktail contained 20 mM ascorbic acid, 0.2 M β-mercaptoethanol, 0.2 M Tris-HCl (pH 7.5), 0.012 M formaldehyde, and 1.6 mM (RS) tetrahydrofolate. To determine the kinetics of inhibition, various concentrations of each inhibitor were added to TS in 25 mM potassium phosphate (pH 7.5) at 30 °C. At various times, aliquots were removed and assayed for TS activity (*16*). The increase in absorbancy at 340 nm, due to dihydrofolate formation, was followed for 2.5 min with data points taken every 10 s using the kinetic program of a Beckman 7400 spectrophotometer equipped with a Pelltier temperature regulator. Control experiments without inhibitor showed the enzyme did not decrease more than 3−5% in 60 min. For more exact details, see the legend to Figure 1.

Tryptic Peptide Mapping of ClAc-DMDDF-Inhibited TS. To uninhibited *E. coli* TS or ClAc-DMDDF-inhibited enzyme (26 nmol) in 40 μL was added 150 μL of H_2O and 96 mg of urea, followed by 200 μL of a solution containing 0.4 M NH_4HCO_3. After addition of 5 μL of 0.5 M DTT, the solution was incubated at 55 °C for 15 min, and then cooled to room temperature at which point 70 μL of 0.2 M iodoacetate in 8 M urea was added. After 1 h, 0.1 mL of 0.5 M DTT was added and the incubation continued for 1 h followed by exhaustive dialysis against 30% acetic acid at 4 °C. The dialysate was lyophilized in a 12 mL conical centrifuge tube and the resulting protein dissolved in 0.1 mL of a solution containing 0.4 M NH_4HCO_3 and 8 M urea. This solution was diluted to 0.5 mL with water to which was added 15 μL of a 2% solution of TPCK-trypsin. After 18−24 h at 37 °C, the digest was diluted 15-fold with water and lyophilized. The lyophilized peptides were dissolved in 0.5 mL of 0.1% TFA and separated by reverse-phase HPLC using an Ultrasphere C-18 column (25 × 0.46 cm, Altex). The solvent system consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in 90% acetonitrile (solvent B). After injection of the sample onto the reverse-phase column that had been equilibrated with solvent A, the column was washed with solvent A for 10 min. A nonlinear gradient (0−8% B, 5 min; 8−50% B, 185 min; 50−70% B, 30 min; 70−100% B, 30 min) was used to effect the separation of tryptic peptides.

Chymotryptic Digestion of ClAc-DMDDF-Modified Peptides. The fractions containing the modified tryptic active

site peptide and small amounts of a contaminating peptide were pooled and lyophilized. The lyophilized sample (about 4 μg) was taken up in 0.2 mL of 20 mM NH_4HCO_3−1 mM $CaCl_2$. The sample was then digested with *N*-α-*p*-tosyl-L-lysine chloromethyl ketone−chymotrypsin (2%) overnight at 37 °C and lyophilized. The digested sample was dissolved in 0.1% TFA, and the peptides were separated by HPLC using the following nonlinear gradient. For the chymotryptic peptides, the following nonlinear gradient was used: 0−3% B, 5 min; 3−50% B, 215 min; 50−70% B, 15 min; 70−100% B, 15 min. The flow rate was 0.5 mL/min, and 1 mL fractions were collected. The eluate was monitored by measuring the absorbancy at 230, 262, or 309 nm. Monitoring the eluate at 230 nm was conducted mainly to obtain a tryptic map of the *E. coli* TS. When larger amounts of enzyme were reacted with ClAc-DMDDF, the peptide elution profile was monitored at 309 nm to locate those peptides containing the inhibitor.

Tryptic Peptide Mapping of Iodoacetic Acid and Iodoacetamide Inhibited TS. TS (38 nmol in 30 μL) was added to 0.6 mL of a solution containing 50 mM NH_4HCO_3 and 5 mM iodoacetic acid or 5 mM iodoacetamide at room temperature. In the case of the former iodo derivative, about 75% of the TS activity was impaired in 12 min, while in the latter case 90% of the TS activity was lost in about 1.5 min. At these respective times, 10 μL of 1 M DTT was added to each reaction to stop further inactivation of the enzyme by the iodo compounds. After 1 h at room temperature, 480 mg of urea was added to each tube, and the volume was brought to 1 mL with water to bring the urea concentration to 8 M. 4-Vinylpyridine (5 μL) was added to each and the solutions were incubated at 37 °C for about 4 h, after which 20 μL of β-mercaptoethanol was added, and the incubation continued for another 2 h. The resulting solutions were dialyzed overnight at 4 °C against two 1 L volumes of 10 mM NH_4HCO_3. Following lyophilization of each sample, the protein was taken up in 0.2 mL of 20 mM NH_4HCO_3−8 M urea, and then diluted with 0.6 mL of 20 mM NH_4HCO_3. Each protein was then digested with 10 μg of TPCK-trypsin overnight at 37 °C and lyophilized. The trypsinized samples were dissolved in 0.1% TFA, and the peptides were separated by HPLC as above.

Peptide Sequence Analysis. Peptide sequencing was carried out on the isolated peptide fractions by automated Edman degradation with a Model 477A Applied Biosystems pulsed liquid sequencer equipped with an on-line Model 120A phenylthiohydantoin-amino acid analyzer. When more than one peptide was present, the previously determined amino acid sequence of *E. coli* TS (*17*) as well as the positions of Arg and Lys in the sequence was used to identify the peptides present. In this way, almost all of the 21 potential tryptic peptides in each monomer of the dimer TS were identified. In general, the longer the peptide, the longer was the retention time on HPLC.

In a few cases, the peptide sequences containing the inhibitor were obtained on a Finnigan-MAT TSQ-700 triple quadrupole mass spectrometer equipped with a Finnigan electrospray ionization source. This method was used also when the modified PTH-amino acid under study was isolated by split-stream separation prior to Edman sequence analysis.

Amino Acid Analysis. The HPLC-purified active site containing tryptic peptides (about 0.6 nmol of each) from ClAc-DMDDF-treated and untreated TS were hydrolyzed in 0.5 mL of constant-boiling HCl for 24 h at 110 °C. Norleucine was included as an internal control. The amino acid analyses were carried out using a Beckman System Gold amino acid analyzer with ninhydrin as the detecting agent.

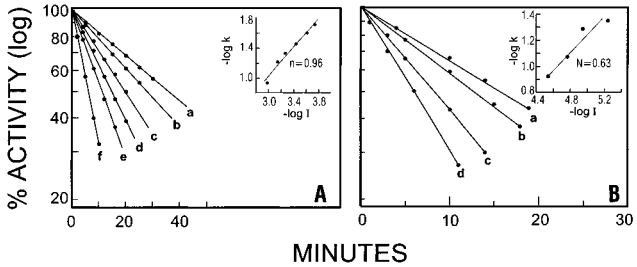


FIGURE 1: Kinetics of irreversible inactivation of TS by ClAc-DMDDF and IAc-DMDDF. The reactions were initiated by the addition of TS to solutions containing varying concentrations of inhibitor in 25 mM potassium phosphate, pH 7.5, at 30 °C. Aliquots of 10 μL were removed at the indicated times and assayed for residual TS activity as described under Materials and Methods. In panel A, the millimolar concentrations of ClAc-DMDDF present were (a) 0.183, (b) 0.245, (c) 0.371, (d) 0.550, (e) 0.726, and (f) 1.06 in the presence of 2.4 μM TS. In panel B, the respective micromolar concentrations of IAc-DMDDF were (a) 5.76, (b) 11.4, (c) 17.2, and (d) 29.1. The TS concentration was 7.2 μM. The order of the reaction, *n* (inset), was obtained from a plot of −log *I* vs −log *k*, according to eq 1.

RESULTS

Based on the findings of Jones et al. (*18*), that N^{10}-substituted 5,8-dideazafolates are effective inhibitors of L1210 TS, particularly if the substituents are three or less carbon atoms in length, it was not unreasonable to assume that N^{10}-ClAc-DMDDF and IAc-DMDDF would also be inhibitory. The presence of a halogen on the N^{10}-alkyl substituent provided another dimension to this normally reversible inhibitor in that it could become irreversible with time by reacting covalently with a sulfhydryl, amino, or hydroxyl group within the enzyme. The fact that the precise location of the dideazafolate with *E. coli* TS was known from X-ray crystallographic analyses (*11*) revealed which amino acids might be involved, the most likely being the active site Cys-146. Earlier studies measuring the inactivation of *Lactobacillus casei* TS by the alkylating agents iodoacetamide and iodoacetic acid (*19*, *20*) indicated that reaction with one of the two active site cysteines of this enzyme was sufficient to completely inactivate this TS. A similar analysis was undertaken using *E. coli* TS, in which the rates and extent of inactivation of this enzyme by ClAc-DMDDF and IAc-DMDDF were compared with such alkylating agents as chloroacetate, chloroacetamide, and their corresponding iodo derivatives.

Inhibition Studies. The kinetics of inactivation of TS by ClAc-DMDDF and IAc-DMDDF were examined by plotting the reciprocal of the half-time ($t_{0.5}$) of inactivation against inhibitor (I) concentration. In each case, a straight line was obtained reflecting the pseudo-first-order nature of the inactivation (Figure 1). To obtain the average order (*n*) for each of the reactions, which generally indicates the number of reactive sites in the enzyme protein, the equation of Edwards and Keech (*21*) was employed:

$$\log k = \log k' + n \log I \qquad (1)$$

Table 1: Kinetic Parameters Associated with the Irreversible Inhibition of TS by ClAc- and IAc-DMMDF[a]

inhibitor	k_3 (min^{-1})	K_I (M)	k_3/K_I (M^{-1} min^{-1})
ClAc-DMDDF	0.368	3.43×10^{-3}	107
IAc-DMDDF	0.146	1.42×10^{-5}	10300

[a] Determined by the use of eq 4 originally developed by Kitz and Wilson (*22*).

where n is obtained by plotting $-\log k$ vs $-\log I$ (see insets of Figure 1), and k is the apparent first-order rate constant, while k' is a function of the inhibitor. Since the rate of enzyme loss is linear for each of the inhibitors (Figure 1), k can be substituted for by $0.693/t_{0.5}$ from their relationship in eq 2:

$$k = \frac{0.693}{t_{0.5}} \tag{2}$$

and as shown in the insets to Figure 1, the n value for each inhibitor approaches unity, suggesting that reaction at only one of the two identical subunits of *E. coli* TS is sufficient to inhibit the enzyme. Similar results were obtained with chloracetate, chloroacetamide, and iodoacetamide (data not shown) as presented earlier with *L. casei* TS (*20*). From the ordinate intercepts of these plots, it is also possible to obtain an apparent rate constant (k') for each inhibitor that is a function of the concentration of the inhibitor (*21*). In comparing the relative k' values of chloroacetamide to chloroacetate, the former was found to be about 25-fold more active than the latter in inactivating TS, while iodoacetamide was about 400 times more active than chloroacetamide (data not shown).

Because of the complex manner in which active site affinity reagents such as ClAc- and IAc-DMDDF react with TS, the equation of Kitz and Wilson (*22*) was employed. This equation is based on the premise that when an enzyme (E) binds with a slow reacting irreversible inhibitor (I) a reversible complex, [E·I], is formed initially, which becomes irreversible with time at a rate equal to k_3 (eq 3).

$$E + I \overset{K_I}{\rightleftharpoons} [E\cdot I] \xrightarrow{k_3} E{-}I \tag{3}$$

The irreversible part of the reaction will be shown in the present study to be due to the acylation of Cys-146 by the chloracetyl or iodoacetyl moieties of DMDDF. By plotting the reciprocal of the observed pseudo-first-order rate constant $1/k_{app}$ for each inhibitor concentration (I), k_3 and K_I

$$1/k_{app} = \frac{K_I}{k_3(I)} + 1/k_3 \tag{4}$$

can be calculated from eq 4, where $1/k_3$ is the intercept and K_I/k_3 is the slope of the line. As indicated in Table 1, the rate of enzyme acylation (k_3) does not differ greatly between ClAc- and IAc-DMDDF, but the specificity of inhibition as defined by the second-order rate constant k_3/K_I is about 100-fold greater for the latter compound than the former. The fact that an n value of about 1.0 was obtained for each reactant (Figure 1) suggests, but does not prove, that alkylation at one site is sufficient to cause complete inactivation of the enzyme. It is of interest to note that Brouillette et al. (*23*) using a BrAc derivative of dUMP (5-α-bromoacetyl-2′-deoxyuridine 5′-phosphate) found that this compound irreversibly inactivated *L. casei* TS with a similar k_3 value as that reported for IAc-DMDDF in Table 1.

Identification of the Reactive Site. Tryptic peptide mapping on the control (untreated) *E. coli* TS showed the HPLC eluate at 69 min (monitored at 230 nm) to contain a peptide with the amino acid sequence MALAPCHAFFQFYVADGK corresponding to residues 141−158 of the active site region (*17*). The cysteine residue at cycle 6 on sequence analysis of this peptide did not show up because of its instability when left unblocked, but was inferred from the Pro at cycle 5 and His at cycle 7. However, when the TS was treated with ClAc-DMDDF or other alkylating agents, such as iodoacetate, iodoacetamide, or 4-vinylpyridine, the respective active site tryptic peptides eluted with retention times of 136−137 min. In the case of ClAc-DMDDF, which was monitored by its absorbance at 309 nm (Figure 2A), the mass of the active site peptide was increased by 479 mass units, which corresponds to ClAc-DMDDF minus its Cl group. Although sequence analysis of the Ac-DMDDF-containing peptide did not reveal an identifiable PTH residue at the sixth position of the peptide, unlike the corresponding iodoacetate-, iodoacetamide-, or vinylpyridine-containing peptides, amino acid analysis of the Ac-DMDDF-containing peptide on hydrolysis in 6 N HCl for 24 h yielded *S*-(carboxymethyl)-cysteine.

When *E. coli* TS was treated with iodoacetamide until about 10% of the enzyme activity remained, followed by ClAc-DMDDF, the major modified active site tryptic peptide eluted at about 138 min (monitored at 230 nm). The amino acid sequence of this peptide was consistent with residues 141−158, but with the cysteine alkylated to a carboxamidomethyl thioether residue [*S*-(acetamidomethyl)cysteine]. The latter was confirmed by amino acid analysis and mass spectrometry since acid hydrolysis of the peptide yielded *S*-(carboxymethyl)cysteine, and the mass of the peptide containing amino acid residues 141−158 was 59 mass units greater than the unalkylated peptide. When the HPLC elution of an aliquot of the above tryptic digest was monitored at 309 nm (Figure 2B), a peak at 137.0 min corresponding to residues 141−158 modified by an acetyl-DMDDF moiety was obtained, which as expected represented about 10% of the enzyme originally present. If iodoacetamide had only reacted with one of the two cysteines in the active site of each subunit, this value would have been much higher. The 44.9, 66.2, and 82.4 min are background peaks that were obtained when *E. coli* TS was treated with ClAc-DMDDF alone (Figure 2A).

It is of interest to note that treatment of TS with 4-vinylpyridine, followed by digestion with trypsin, also yielded an HPLC peak at about 136 min (monitored at 230 nm), which was shown to correspond to the modified active site amino acid sequence involving residues 141−158. It appears, therefore, that modification of Cys-146 with iodoacetate, ClAc-DMDDF, or vinylpyridine yields a tryptic peptide in each case that migrates to the same region, although as indicated above if this cysteine is not modified, the peptide elutes much earlier (69 min).

Quantitation of Cysteine Reactive Sites. As was shown earlier, *L. casei* TS is very sensitive to inactivation by sulfhydryl reagents (*5*, *19*, *20*, *24*−*26*). Kinetic studies on the rate of *L. casei* TS inactivation suggested that the alkylation of a single subunit of the two identical subunits

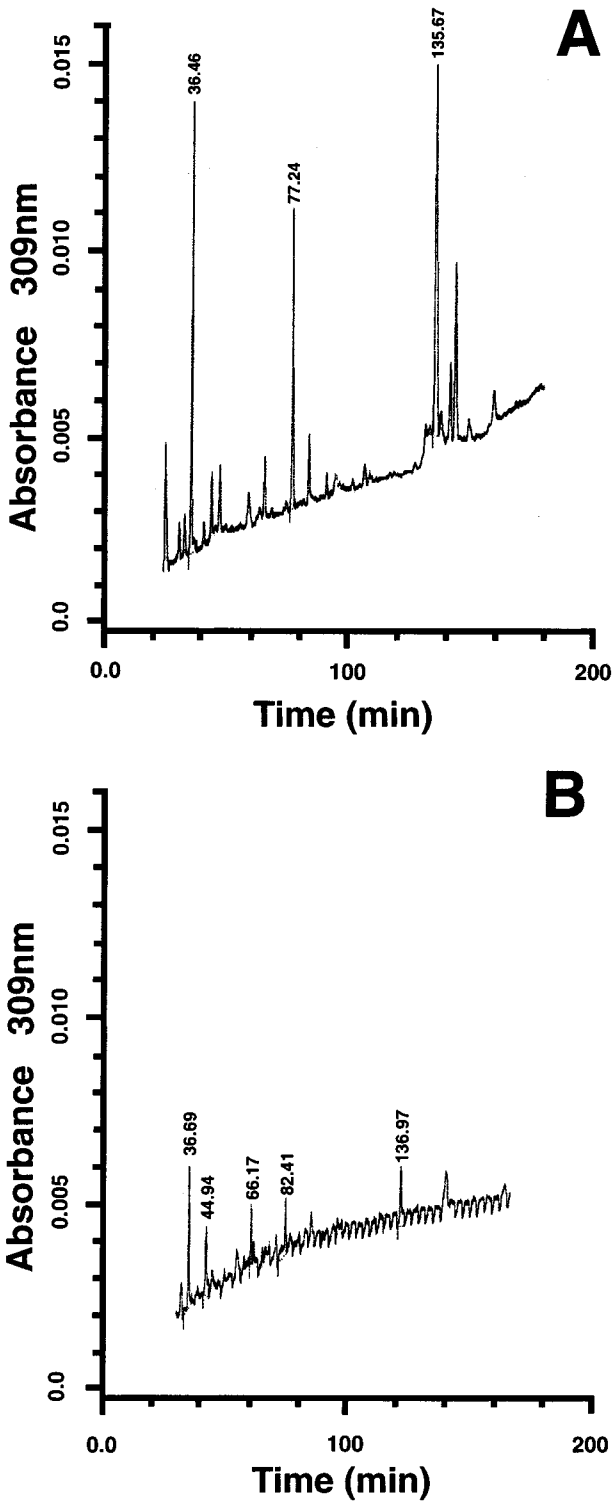


FIGURE 2: HPLC pattern of tryptic peptides resulting from the treatment of TS with ClAc-DMDDF until enzyme activity was completely lost (panel A). The tryptic digest (7 nmol) was chromatographed using a C18-RP Altex column and monitored at 309 nm. A large peak (not drawn) at 7.6 min was present due to urea eluting in the void volume. Fractions were collected and lyophilized. The composition of peptides at the indicated times was ascertained by Edman sequence analysis and by mass spectrometry. A similar fractionation of tryptic peptides derived from the treatment of TS with iodoacetamide until about 10% of the enzyme activity remained, at which point the enzyme was reacted with ClAc-DMDDF (panel B).

in the holoenzyme results in its complete inactivation (*19*, *20*, *24*, *26*), which was consistent with the data from peptide analysis (*24*). Whether similar results would be obtained following inactivation of *E. coli* TS by ClAc-DMDDF was not known, although earlier studies (*9*) had shown 1 mol of *E. coli* TS could bind 2 mol of 10-propargyl-5,8-dideazafolate.

To determine whether one or both subunits of *E. coli* TS are modified by ClAc-DMDDF, the enzyme was allowed to react with this compound until about 90% of the activity had been eliminated, at which point an excess of dithiothreitol was added to destroy the inhibitor. The enzyme protein was then denatured with 8 M urea, and its residual cysteines were reacted with iodoacetate or vinylpyridine. Following dialysis and lyophilization, the protein was taken up in a solution of 20 mM NH_4HCO_3−2 M urea and trypsinized with TPCK-trypsin. The resulting peptides were separated by HPLC to isolate the *S*-acetyl-DMDDF-modified active site peptide (indicated as C_{146}DDF in Figure 3B). Although slightly contaminated with another peptide, treatment of the isolated peptide (underlined in panel B) with chymotrypsin yielded a pure C_{146}DDF peptide on sequencing (Figure 3C). When this peptide was hydrolyzed in 6 N HCl at 110 °C for 18 h and subjected to amino acid analysis, 1 mol of CM-C_{146}/mol of active site peptide was obtained, which results from the cleavage of DMDDF from the DMDDF-carboxymethylated peptide. The underlined peptides in Figure 3C represent small amounts of CM-C_{146} peptides, one slightly larger than the other. This study has been repeated by replacing the iodoacetate treatment of TS, following inactivation of TS by ClAc-DMDDF with 4-vinylpyridine, which yields *S*-(pyridylethyl)cysteine on hydrolysis in 6 N HCl. In each case, only minor quantities of CM-Cys_{146} or pyridylethyl-C_{146} were obtained on acid hydrolysis, indicating that each of the two subunits of the *E. coli* TS had been alkylated (Table 2). Similar results were obtained on treatment of the enzyme with iodoacetate until about 80% of the enzyme had been inactivated, or with iodoacetamide until the enzyme was completely inactive. The residual cysteines were then reacted with vinylpyridine. Isolation of the respective active site peptides by HPLC, followed by amino acid analysis, revealed that the alkylated peptides were predominantly S-carboxymethylated, emphasizing the fact that both subunits had been S-carboxymethylated. In support of this finding, the same result was obtained when the intact enzyme, which contains five cysteine residues per subunit, was treated with iodoacetate until inactive, followed by denaturation with urea and vinylpyridine treatment. As shown in Table 2, only one of the five cysteines per subunit was completely S-carboxymethylated, that at the active site (Cys-146). Peptide sequence analysis on the isolated peptides was in basic agreement with the amino acid analysis data, although not as quantitative. By contrast, when *L. casei* TS, which contains only two cysteines per subunit, was treated with iodoacetate until inactive followed by vinylpyridine and active site isolation, only half of the active site peptide was S-carboxymethylated with the other half being pyridylethylated (data not shown). This finding is in good agreement with the earlier studies on *L. casei* TS (*19*, *20*, *24*), which indicated that reaction of one of the two active site cysteines with iodoacetate was sufficient to give complete inactivation of the enzyme.

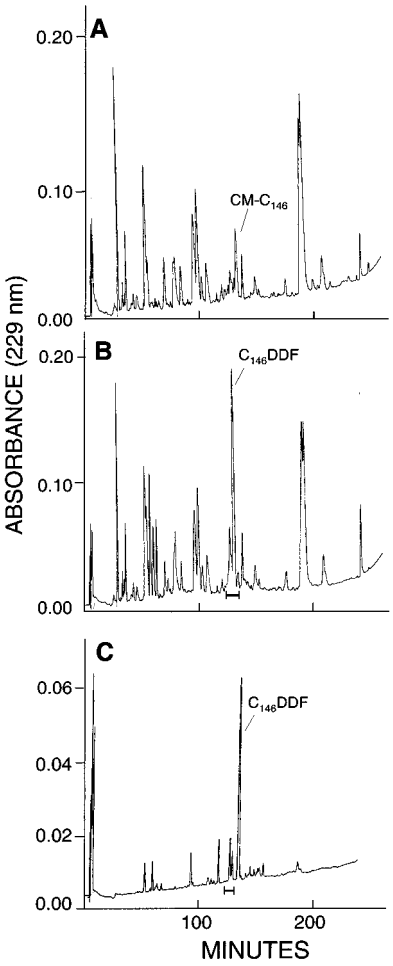


FIGURE 3: HPLC elution profile of peptides from TS following treatment with sulfhydryl reagents and then trypsin (A and B) or chymotrypsin (C). In panel A, the enzyme was denatured and treated with iodoacetic acid, and then trypsin; in panel B, the enzyme was treated with ClAc-DMDDF until inactive, followed by denaturation and exposure to iodoacetic acid, and then trypsin. The active site peptide pool modified by CIAc-DMDDF and possibly by iodoacetate (underlined) was treated with chymotrypsin and rechromatographed. The carboxymethylated active site peptides are underlined in panel C, and it is clear that these are more definitively separated from C_{146}Ac-DMDDF (designated as C_{146}DDF in panels B and C) than in panel B. For further details, see Materials and Methods.

Modeling of ClAc-DMDDF to the Active Site of TS. Using the known three-dimensional structure of *E. coli* TS ligated to dUMP and PDDF (*11*), ClAc-DMDDF was modeled into the structure to replace PDDF, in an orientation too distant from the sulfhydryl of Cys-146 to support a chemical reaction, and sterically blocked from reacting with the pyrimidine ring of dUMP (Figure 4). Including dUMP with the inhibitor leads to reduced inhibition of TS (data not shown), suggesting the model studies reflect the correct binding arrangement. In contrast, the inhibitor is likely to adopt multiple conformations in the TS active site on binding in the absence of nucleotide. A structure of the CB3717−TS binary complex has been reported in which binding induced the TS closed conformation, trapping the inhibitor in the active site (*27*). In this structure, the inhibitor displayed multiple conformations. If a similar arrangement occurred with ClAc-DMDDF, which is likely since the compounds are similar, then the ClAc-DMDDF reactive group would easily encounter the Cys-146 sulfhydryl.

Table 2: Extent of Alkylation of *E. coli* TS and Active Site Peptide by Iodoacetate, Iodoacetamide, and ClAc-DMDDF

sample hydrolyzed	CM-cysteine (nmol)	(pyridylethyl)cysteine (nmol)
IAc-treated TS[a]	0.14	0.65
	(1.08)[e]	(3.92)
active-site peptide from	2.17	0.47
IAc-treated TS[b]	(0.82)	(0.18)
active site peptide from	2.33	0.06
$IAcNH_2$-treated TS[c]	(0.98)	(0.02)
active-site peptide from	0.68	0.04
ClAc-DMDDF-treated TS[d]	(0.94)	(0.06)

[a] TS holoenzyme was treated with iodoacetate until completely inactive followed by denaturation with 8 M urea, and the addition of 4-vinylpyridine. Following extensive dialysis against 10% acetic acid, the sample was lyophilized. [b] TS was treated with iodoacetate until it was about 80% inactivated, followed by vinylpyridine. The active-site peptide was isolated as described under Materials and Methods. [c] Same as in *b*, but TS was treated with iodoacetamide until the TS was basically inactive. [d] Same as in *c*, but TS was treated with ClAc-DMDDF until the enzyme was inactive. The protein and HPLC-isolated peptide samples were hydrolyzed in constant-boiling HCl for 24 h and subjected to amino acid analysis. See Materials and Methods for additional details. [e] The numbers in parentheses have been normalized to represent the number of cysteines in a subunit of the protein or peptide, respectively. As shown earlier (*17*), the number of cysteines per subunit of *E. coli* TS is 5.0, with the number of subunits per holoenzyme being 2.0.

The greater reactivity of ClAc-DMDDF relative to chloroacetate and chloroacetamide is no doubt due to the "caged" nature of its binding, which results in a correspondingly greater local concentration of the inhibitor in the proximity of Cys-146. This is in contrast to the halogenated acetic acid and acetamide derivatives, which can only gain access to Cys-146 by diffusing into the active site from the surrounding solution. To achieve the same relative degree of inactivation of TS as ClAc-DMDDF, higher concentrations of chloroacetate and chloroacetamide would be required, which in the case of chloroacetate is complicated by its limited access to the active site due to its negative charge. This effect was clearly seen in the case of *L. casei* TS inactivation by iodoacetamide and iodoacetate, where the former compound was 2 orders of magnitude more reactive than the latter (*20*), and also in the present studies, where iodoacetamide was found to inactivate *E. coli* TS 400 times more effectively than chloroacetamide. The presence of dUMP sterically protects TS against the inactivation by alkylating agents (*5*, *19*, *20*) due to its proximity to the active site cysteine (*11*, *12*). However, a 10-fold higher concentration of dUMP was required to protect TS against inactivation by ClAc-DMDDF than that required to provide the same degree of protection against chloroacetamide (data not shown). This effect was somewhat surprising as it might be expected, based on equilibrium dialysis studies, where it was shown that folate analogues (*28*, *29*) enhance the binding of dUMP, that ClAc-DMDDF might also improve the binding of dUMP. Since this was not observed, other factors must be considered such as the impairment of the alkylating groups access to Cys-146 by dUMP. In this vein, it would be of interest to determine whether CB3717 or a comparable folate analogue might increase the effectiveness of the site-directed irreversible nucleotide inhibitor, 5-bromoacetyl-dUMP (*23*), by enhancing its binding.

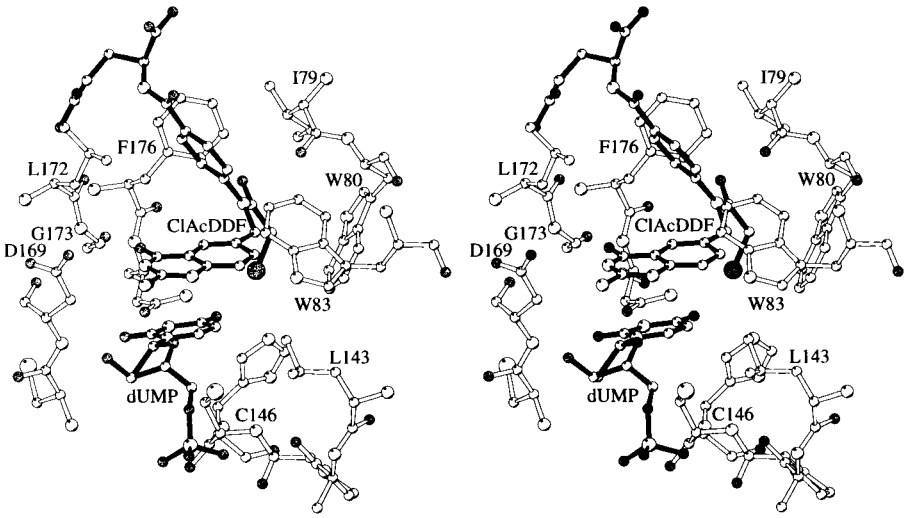


FIGURE 4: Stereoview of ClAc-DMDDF modeled into the TS active site, showing the proximity of the chlorine to dUMP and Cys-146. ClAc-DMDDF and dUMP are indicated with filled bonds, protein with open bonds. Chlorine, sulfur, and phosphate atoms are shown with large spheres, nitrogen atoms with intermediate spheres, and oxygens and carbons with small spheres. Oxygens and chlorine are half-filled. This figure was produced with the program MOLSCRIPT (*39*).

DISCUSSION

The kinetic results in Figure 1 suggest that ClAc-DMDDF and IAc-DMDDF inactivate *E. coli* TS by reacting with a single functional group in the dimer as was suggested in our earlier studies on the effect of alkylating agents on the inactivation of *L. casei* TS (*20*). This reactive group, based on previous studies from our laboratory (*24*) and others (*30*), corresponds to the active site of TS, which in the case of *L. casei* TS is Cys-198 (*31*), and in *E. coli* TS is Cys-146 (*17*). However, it is obvious from the peptide isolation and sequencing data in this paper that each of the two active site cysteines of *E. coli* TS had been alkylated following inactivation of *E. coli* TS by ClAc-DMDDF, although as shown in our earlier studies with *L. casei* TS, reaction of only one of the two active site cysteines with a sulfhydryl reagent such as iodoacetate or 5-HgdUMP was sufficient to completely inactivate this enzyme (*20*, *24*). The significance of these apparent conflicting findings, where alkylation of one of the two active site cysteines in *L. casei* TS results in complete inactivation of this enzyme, while the inactivation of *E. coli* TS is associated with the alkylation of the active site on each of the enzyme's subunits, is not entirely clear at this time. One possibility suggested by the kinetic order of inactivation, but which is by no means proven, is that although both subunits are alkylated in the case of E. coli TS only one of the active sites is involved in the catalytic process. This thesis is supported in part by the asymmetry of ligand binding reported earlier (*32*, *33*) and the asymmetry of inhibition of the enzyme by sulfhydryl reagents (*20*), and also by the asymmetry of enzyme inhibition by phenylglyoxal (*34*). In addition, our recent demonstration that certain inactive *E. coli* TS mutants complement each other in such a manner as to restore one intact active site per dimer with the same specific activity as that of the wild-type enzyme (*35*) is consistent with the kinetic order of inactivation by alkylating agents where reaction at both active sites may occur, but the reaction at one is sufficient to inactivate the enzyme. That this finding is not a peculiarity of *E. coli* TS was obtained in comparable studies with *Bacillus subtilis* TS (F. Maley, in preparation). Additional supportive evidence has been obtained recently from thermodynamic stabilization (*36*) and X-ray crystallographic studies *(37*) showing that distinct conformational differences can exist between the two subunits of *E. coli* TS. Although other explanations are possible, the most reasonable one presently is that although ligand binding to both subunits can be observed in equilibrium dialysis and X-ray diffraction studies, this results in a conformational state wherein only one of the two subunits is functionally active, or as suggested recently (*38*) that "catalysis is probably confined to only one subunit at a time". However, the latter suggestion would appear to be ruled out by the finding that an active site heterodimer consisting of $C_{146}S$ on one peptide and $C_{146}W$ on the other possesses the same k_{cat} as WT-TS (*35*).

While IAc-DMDDF was not modeled into the *E. coli* TS structure, it is of interest to note that its K_I is about 240-fold lower than ClAc-DMDDF (Table 1), although its conversion to the irreversible complex is about 3-fold slower. This effect might be explained by the greater van der Waals interaction of the iodine atom within the active site pocket due to its larger size, which could cause IAc-DMDDF to diffuse to the active site cysteine more slowly than ClAc-DMDDF.

One problem with the DMDDF derivatives employed in this study that remains unexplained is their relatively poor K_I values, compared to CB3717. As indicated earlier (*14*), the corresponding propargyl derivative of DMDDF is not as inhibitory as CB3717, which is due possibly to the loss in hydrogen bonds to the carbonyl of Ala-263 and through water to Asp-169 (*11*) as a consequence of replacing the 2-amino group in the latter compound with a methyl group. However, this would not explain the fact that the loss involved is several orders of magnitude. At present, the most likely explanation appears to be associated with the 10-acetamido substituent, which could impose a steric barrier to binding at the folate site. The critical nature of substituents at this position has been discussed by Jones et al. (*18*). It would be of interest therefore to prepare N^{10}-ClAc- and N^{10}-IAc-5,8-dideazafolate and compare them as inhibitors of TS with the corresponding DMDDF derivatives used in this study.

ACKNOWLEDGMENT

We express our appreciation to Judith Valentino for her excellent assistance in preparing the manuscript and to Dr. Charles Hauer III and Robert Stack of the Wadsworth Center's Mass Spectrometry Unit for their aid in identifying the Ac-DMDDF-peptides.

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BI9717284