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KINETICS OF THYMIDYLATE SYNTHASE INHIBITION BY DISULFIDES

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Summary

The inactivation of thymidylate synthase (5,10-methylene-tetrahydrofolate: dUMP *C*-methyltransferase, EC 2.1.1.45) by a number of disulfides has been examined and found to be a second-order process. The apparent second-order rate constant was strongly influenced by the chemical structure of the disulfide. The data suggest that negatively charged functional groups decrease the reactivity of the disulfides and that positively charged groups enhance the reactivity. GSSG did not react with non-catalytic SH groups, since the number of SH groups of both GSSG-treated and untreated thymidylate synthase was the same. Several sulfhydryl compounds were tested for their ability to reactivate thymidylate synthase that had been inhibited by 2,2′-dithiodipyridine. Complete reactivation was obtained with either dithiothreitol or 2-mercaptoethanol. Reactivation by 2-mercaptoethanol was a second-order process.

Introduction

Thymidylate synthase (5,10-methylene-tetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.145) catalyzes the following reaction:

dUMP + (+)-5,10-CH_2H_4folate → dTMP + H_2folate

where the folate substrate serves as both a one-carbon donor and a reductant. It has been postulated that this reaction is initiated by nucleophilic addition of a cysteinyl sulfur of the enzyme to the 6-position of the uracil ring to form a

Abbreviations: 5,10-CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSS(2-pyridine), the mixed disulfide of GSH and 2-mercaptopyridine; Pipes, 1,4-piperazinediethanesulfonic acid.

carbanion intermediate with high electron density at the C-5 of deoxyuridylate [1,2].

The formation of stable ternary complexes between thymidylate synthase, 5,10-CH_2H_4folate and the potent inhibitor 5-fluoro-2′-deoxyuridylate [3,4] is postulated to involve the same nucleophilic cysteinyl side-chain that participates in the normal catalytic mechanism [5]. Peptide fragments containing cysteine and tightly-bound 5-fluoro-2′-deoxyuridylate have been isolated from pronase digests of thymidylate synthase ternary complexes [6,7] and this cysteinyl residue has been assigned position 198 in the amino acid sequence [8—10].

Numerous reports have appeared concerning the chemical modification and inhibition of thymidylate synthase by various sulfhydryl reagents [5,11—14]. Dunlap and coworkers [15] recently reported that excess *N*-ethylmaleimide, iodoacetamide, methylmethanethiosulfonate and 5,5′-dithiobis(2-nitrobenzoate) [16] reacted with 1.4—1.8 sulfhydryl groups per enzyme dimer, depending on the buffer and reagent used. There was a close correlation between the loss of enzyme activity and the modification of sulfhydryl groups by these reagents, which indicates that the reagents react selectively with cysteinyl side-chains at the active site(s). Not all sulfhydryl reagents react selectively with the essential cysteine since, *p*-chloromercuribenzoate was reported to react with a total of three sulfhydryl groups/enzyme dimer when a 5-fold molar excess of reagent to enzyme was used, although the enzyme was completely inactivated by titrating only 1.4—1.8 sulfhydryl groups with this reagent [15]. Studies on the inhibition of thymidylate synthase by *cis*- and *trans*-$Pt(NH_3)_2Cl_2$, showed that the *cis* isomer reacted with about 1.3 sulfhydryl groups/enzyme dimer with no accompanying loss of activity, whereas the *trans* isomer reacted with about 2.5 sulfhydryl groups with complete loss of enzyme activity [17].

Galivan et al. [18] have previously investigated the reactivity of the essential cysteine residue using several alkylating agents. Lewis et al. [16] and Munroe et al. [19] have shown that the rates of inactivation of thymidylate synthase by methylmethanethiosulfonate and DTNB were decreased by the presence of various polyoxyanions and greatly influenced by pH.

In the present study, the rates of inactivation of thymidylate synthase by a number of disulfides have been examined. Only disulfide inhibitors were used, so that the influence of chemical structure on the reaction rate was principally due to interaction between the inhibitor and groups in the vicinity of the active site cysteine rather than differences in reaction mechanism. We have also determined that GSSG, which is the only disulfide thus far reported not to inhibit thymidylate synthase [11], does not react well with any of the sulfhydryl groups of this enzyme.

Results from this investigation were presented in part at the XIth International Congress of Biochemistry, Toronto, Canada in July, 1979.

Materials and Methods

Materials

Thymidylate synthase from amethopterin-resistant *Lactobacillus casei* was

purified in the presence of 10 mM 2-mercaptoethanol following the procedure of Lyon et al. [20]. Purified enzyme preparations had specific activities of 3.0—3.5 units/mg when assayed in the presence of 25 mM 2-mercaptoethanol by the usual spectrophotometric procedure [11].

Epimeric (±)-H_4folate was prepared by the catalytic hydrogenation of folic acid in acetic acid [21] and was stored at −50 °C as a lyophilized powder under argon in sealed serum bottles [22].

The mixed disulfide GSS(2-pyridine) was prepared by reacting 0.5 mmol GSH with 1 mmol 2,2′-dithiodipyridine dissolved in 10 ml 60% CH_3OH at 37° C. The reaction was monitored by measuring the absorbance at 340 nm of aliquots withdrawn at intervals. After the reaction was complete (approx. 5 min), the mixture was extracted five times with 10-ml portions of $CHCl_3$ to remove unreacted 2,2′-dithiodipyridine and 2-mercaptopyridine. 24 ml acetone were added to the aqueous layer (6 ml) and the resulting precipitate was collected by centrifugation and air dried. The product was dissolved in water and purified by thin layer chromatography on silica gel sheets containing a fluorescent indicator (Eastman Organic Chemicals), using the upper layer of butanol/acetic acid/water (4 : 1 : 5, v/v) as the solvent. The mixed disulfide was located under ultraviolet light and recovered by scraping the band from the chromatogram, eluting with water, and lyophilizing. This procedure yielded 61 mg white solid (29% yield based on GSH). An aqueous solution of this material gave the expected ultraviolet spectra in the presence and absence of excess mercaptoethanol.

Other chemicals were obtained as follows: 4,4′-dithiodibutyric acid, 2,2′-dithiodiacetic acid, 3,3′-dithiodipropionic acid, 2,2′-dithiodipyridine *N*-oxide and 2,2′-dithiodiethanol from Aldrich Chemical Co.; urea (electrophoresis purity grade) from Bio-Rad Laboratories; 2,2′-dithiodipyridine and 2-mercaptopyridine from Calbiochem-Behring Corp.; 2-mercaptoethanol from Matheson, Coleman and Bell; CM-Sephadex and Sephadex G-25 prepacked columns from Pharmacia Fine Chemicals; cystamine dihydrochloride, L-cysteine, L-cystine, L-cystine dimethyl ester, *N*,*N*′-di-(2,4-dinitrophenyl)-L-cystine, DTNB, 6,6′-dithiodinicotinic acid, dithiothreitol, dUMP, GSH, GSSG, and Pipes from Sigma Chemical Co. All other chemicals used were reagent grade.

Methods

Activation of thymidylate synthase and removal of exogenous thiols. The purified enzyme was activated prior to use by dialysis at 5 °C for 12—24 h against 0.05 M potassium phosphate buffer (pH 6.8), 25 mM 2-mercaptoethanol. Exogenous 2-mercaptoethanol was removed from the enzyme by applying the activated enzyme solution to a small CM-Sephadex column (about 1 ml swollen gel per 5 mg protein applied) that had been equilibrated in 0.05 M potassium phosphate buffer (pH 6.8). The column was then washed with 10-times the column volume of cold equilibration buffer, which had been previously deoxygenated by boiling and bubbling with argon. The protein was eluted from the column with cold, deoxygenated 0.1 M potassium phosphate buffer (pH 6.8), 0.5 M KCl. The dethiolated enzyme solutions were stored in stoppered tubes at 5 °C.

Measurement of thymidylate synthase activity. Thymidylate synthase

activity was determined spectrophotometrically by measuring the increase in absorbance at 340 nm due to the formation of H_2folate [23]. The procedure described by Dunlap et al. [11] was used except that Pipes buffer replaced phosphate, thiols were omitted from the cofactor solution and the reactions were initiated with enzyme rather than dUMP. Enzyme concentrations were determined from the absorbance at 278 nm using absorbance coefficients of $1.05 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ or $1.55\ ml \cdot mg^{-1} \cdot cm^{-1}$ [20].

Inhibition of thymidylate synthase by disulfides. Stock solutions of dethiolated enzyme and inhibitors were prepared in 0.1 M potassium phosphate buffer, pH 6.8. Inhibition reactions were carried out at 30°C and begun by mixing measured volumes of inhibitor solutions with measured volumes of enzyme solutions. Enzyme concentrations in the inhibition reaction mixtures were between 6 and 8 μM, depending on the enzyme stock solution used. The inhibitor concentrations in the reaction mixtures are given in Table I. Residual enzyme activities were determined by periodically withdrawing 20-μl aliquots of the inhibition reaction mixtures and adding them to 0.98-ml portions of assay mixture contained in spectrophotometer cuvettes using an Add-A-Mixer apparatus (Precision Cells, Hicksville, NY). Initial velocities (ΔA_{340}/min) were obtained at 30°C from the linear portions of recorder tracings using a Gilford Model 250 spectrophotometer. In the absence of enzyme, the assay mixture lacking mercaptoethanol showed a small but significant increase in absorption, and the initial velocities were corrected for this. In each experiment, it was found that no activity was lost in a control containing the same amount of enzyme but lacking the inhibitor.

Reactivation of 2,2'-dithiodipyridine-inhibited thymidylate synthase by sulfhydryl compounds. Inhibited enzyme was prepared by incubating a solution containing 1.0 ml of dethiolated thymidylate synthase ($2.1 \cdot 10^{-5}$ M) and 1.0 ml of $2 \cdot 10^{-3}$ M 2,2'-dithiodipyridine for 10 min at 30°C. After passing this solution through a 1.5 cm × 5 cm Sephadex G-25 column (equilibrated in 0.1 M potassium phosphate buffer, pH 6.8) 2 ml of $8.6 \cdot 10^{-6}$ M enzyme solution was obtained which was completely inactive when assayed in the absence of thiols.

TABLE I

REACTION MIXTURE INHIBITOR CONCENTRATIONS

Inhibitors	Concentration (mM)
2, 2'-dithiodipyridine	0.01, 0.02, 0.1
2, 2'-dithiodipyridine *N*-oxide	0.01, 0.02, 0.05
6, 6'-dithiodinicotinate	0.05, 0.1, 0.2
GSS (2-pyridine)	0.2, 0.5, 1
cystine dimethyl ester	2, 5
di-(dinitrophenyl)-cystine	2
cystamine	2, 5
2, 2'-dithiodiethanol	5, 10
cystine	1
2, 2'-dithiodiacetate	5, 20, 50
3, 3'-dithiodipropionate	20
4, 4'-dithiodibutyrate	20
GSSG	50

Portions (0.16 ml) of this solution were mixed with 0.04 ml of either dithiothreitol, 2-mercaptoethanol, GSH, cysteine or 2-mercaptopyridine solutions. The resulting mixtures were incubated at 30°C and the rate of reactivation of the enzyme was followed by measuring enzyme activity in 20 μl aliquots withdrawn periodically and assayed as described above.

Sulfhydryl group titration of GSSG-treated thymidylate synthase with DTNB. Dethiolated thymidylate synthase in 0.1 M potassium phosphate buffer, pH 6.8, was divided into three equal portions; one of which was incubated at 30°C for 5 min, another was incubated at 30°C for 30 min with 20 mM GSSG and the third was incubated at 30°C for 1 h with 20 mM GSSG. Each solution was chromatographed on Sephadex G-25 (as above) to remove excess GSSG, a 2.0 ml sample was collected, and the enzyme concentration determined. The three samples were each mixed with 516 mg of solid urea, which gave a final urea concentration of 3.7 M. Aliquiots of each of the three urea-treated samples were titrated with DTNB essentially by the method of Ellman [24]. 20 μl of 0.02 M DTNB solution was added to 0.6 ml of enzyme solution and the absorbance at 412 nm was monitored for about 30 min against a blank containing the same amount of DTNB in 0.6 ml of buffer. The experiments were carried out in 0.1 M potassium phosphate buffer, pH 6.8. A molar absorptivity of 13 600 $M^{-1} \cdot cm^{-1}$ for nitromercaptobenzoate was used to calculate the sulfhydryl group concentrations in the enzyme solutions.

Results

Thymidylate synthase was inhibited to some degree by all of the disulfide compounds tested (Table II) though the relative inhibitory abilities of these compounds differed greatly. The inhibition reactions were conducted in the presence of a molar excess of inhibitor relative to enzyme, so that the inhibitor concentration remained relatively constant throughout the reaction. Under these conditions, semilogarithmic plots of residual activity vs. time were linear

TABLE II

APPARENT SECOND-ORDER RATE CONSTANTS FOR INHIBITION OF THYMIDYLATE SYNTHASE BY DISULFIDES

Numbers in parentheses indicate the number of different inhibitor concentrations used.

Inhibitors	k' ($M^{-1} \cdot min^{-1}$)
2, 2′-dithiodipyridine (3)	$2.5 \cdot 10^4$
2, 2′-dithiodipyridine *N*-oxide (3)	$1.6 \cdot 10^4$
6, 6′-dithiodinicotinate (3)	$2.7 \cdot 10^3$
GSS(2-pyridine) (3)	$1.3 \cdot 10^2$
L-cystine dimethyl ester (2)	46
N, *N*′-di-(dinitrophenyl)-L-cystine (1)	31
cystamine (2)	29
2, 2′-dithiodiethanol (2)	11
L-cystine (1)	5.3
2, 2′-dithiodiacetate (3)	1.5
3, 3′-dithiodipropionate (1)	$6.1 \cdot 10^{-1}$
4, 4′-dithiodibutyrate (1)	$6.0 \cdot 10^{-1}$
GSSG (1)	$9.7 \cdot 10^{-2}$

for the loss of at least the first 70—80% of the enzymatic acitivity for all inhibitors and at all concentrations tested. These linear plots indicate that at a given inhibitor concentration the inhibition of thymidylate synthase by disulfides is a pseudo first-order process with respect to enzyme. Results of a typical experiment with GSS(2-pyridine) as inhibitor are shown in Fig. 1A. The slope of a line corresponds to the pseudo first-order rate constant at that inhibitor concentration. The pseudo first-order rate constant can also be obtained from the time required for the loss of 50% of the activity, $t_{1/2}$, and is numerically equal to $(\ln 2)/t_{1/2}$, where ln 2 is the Naperian logarithm of 2. The value of the pseudo first-order rate constant was dependent on the inhibitor concentration and may be expressed by the equation,

$$k = k'[\text{inhibitor}]^n \quad (1)$$

where k is the pseudo first-order rate constant and n is the average order of the reaction with respect to inhibitor [25]. The value of n can be determined from the slope of plots of log k vs. log [inhibitor] [18,25,26], which is especially suitable for values of n greater than 1, but a value of 1 is readily apparent from a linear plot of k vs. inhibitor concentration. The data in Fig. 1A have been replotted in this manner in Fig. 1B. The linearity of the plot shows that the inhibition is pseudo first-order with respect to inhibitor, i.e. n equals 1. The slope of this plot corresponds to the apparent second-order rate constant, k', for the overall inhibition of thymidylate synthase by GSS(2-pyridine).

Table II shows the apparent second-order rate constants for all of the disulfide inhibitors examined. In some cases only one inhibitor concentration was tested because the rate of inhibition was slow at that concentration and limited solubility or interference with the spectrophotometric assay prevented the use of higher concentrations. In those cases the apparent second-order rate constant was calculated from the equation,

$$k' = k/[\text{inhibitor}] = \frac{\ln 2}{t_{1/2}[\text{inhibitor}]} \quad (2)$$

which can be obtained by rearranging equation (1) assuming $n = 1$.

A number of reports indicate that thymidylate synthase inhibited by sulfhydryl reagents can be reactivated by exposure to thiols [eg. 11,14,16,17] and so several sulfhydryl compounds were tested for their ability to restore activity to thymidylate synthase that had been inhibited by 2,2'-dithiodipyridine.

Dithiothreitol at a concentration of either 1 mM or 20 mM completely reactivated the inhibited enzyme after 1 h at 30°C. Reactivation by 2-mercaptoethanol at 30°C was 7.3% after 1 h, 79.1% after 1 h and 92.6% after 30 min for concentrations of 1 mM, 20 mM, and 50 mM, respectively. The kinetic data are shown in Fig. 2 plotted in the same manner as that for inhibition by disulfides, except that the loss of inhibition is plotted. Linear plots in Fig. 2A indicate that the reactivation process is pseudo first-order with respect to the concentration of inhibited enzyne. Fig. 2B shows that a replot of the pseudo first-order rate constants vs. the corresponding 2-mercaptoethanol concentrations is linear, indicating that the process is pseudo first-order with respect to mercaptoethanol. The apparent second-order rate constant is obtained from the slope of the line.

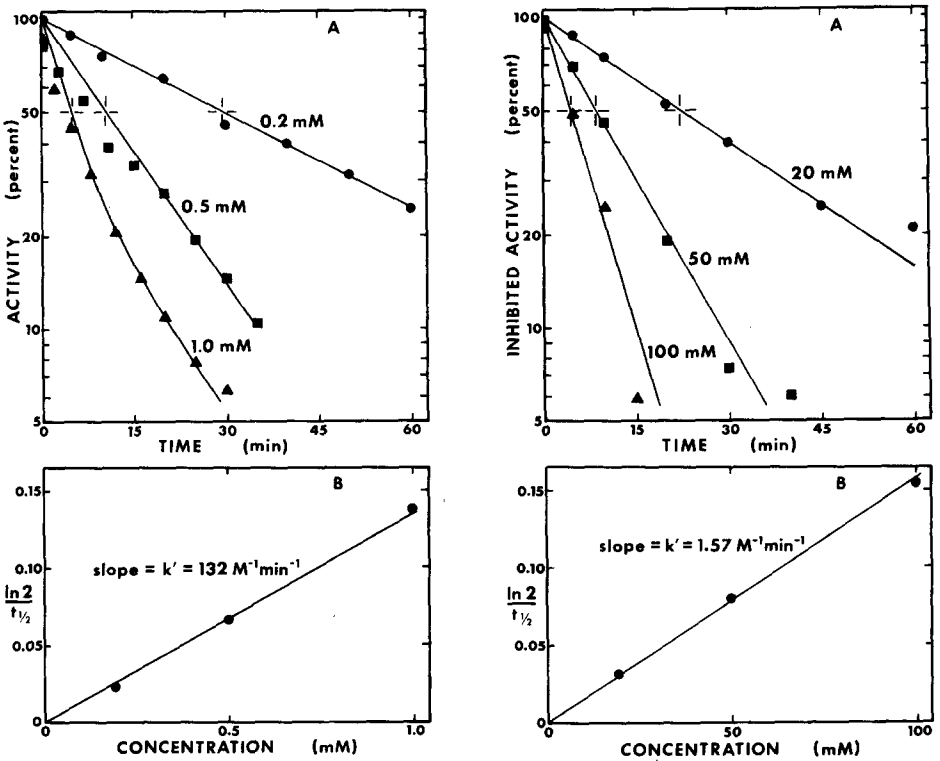

Fig. 1. A, Inhibition of thymidylate synthase ($5.9 \cdot 10^{-6}$ M) by GSS(2-pyridine). Activities are plotted on a log scale and are residual activities remaining after the inhibition times indicated, expressed as percents of the enzymatic activity in reaction mixtures lacking the disulfide. Concentrations of GSS(2-pyridine) in the inhibition reaction mixtures are indicated in the figure. B, Replot of $(\ln 2)/t_{1/2}$ versus the inhibitor concentration. Values for $t_{1/2}$ (time required for the loss of 50% of the activity) were obtained from Fig. 1A.

Fig. 2. A, Reactivation of dithiodipyridine-inhibited thymidylate synthase by 2-mercaptoethanol. Percent inhibited activities are plotted on a log scale and were calculated from the equation:

$$\% \text{ inhibited activity} = \frac{A_\infty - A_t}{A_\infty} \times 100$$

Where A_∞ = activity after 1 h at 30° C with 20 mM dithiothreitol, and A_t = activities after various incubation times (30° C) with 2-mercaptoethanol. The concentrations of mercaptoethanol are indicated in the figure. B, Replot of $(\ln 2)/t_{1/2}$ versus the concentration of 2-mercaptoethanol. Values for $t_{1/2}$ (time required for the recovery of 50% of the activity) were obtained from Fig. 2A.

Cysteine and GSH at 1 mM gave partial reactivation after 1 h (11.5% and 7.2%, respectively), but failed to give significant reactivation at higher concentrations (50 mM cysteine and 100 mM GSH) for reasons that we don't understand. No reactivation was observed when 1 mM or 5 mM 2-mercaptopyridine was used, probably because it exists primarily as the thiopyridone tautomer rather than the thiol.

The interaction of GSSG with thymidylate synthase was investigated further by first treating the native enzyme with GSSG and then titrating the treated enzyme with DTNB in the presence of urea. There were no significant differences between the sulfhydryl contents of thymidylate synthase treated with

GSSG and enzyme that had not been treated, which indicates that GSSG did not react appreciably with either the catalytically important sulfhydryl group or any of the other sulfhydryl groups of thymidylate synthase.

Discussion

From the data presented here as well as that of other researchers [16,19], the inhibition of thymidylate synthase by disulfides appears to be due to a second-order reaction between the enzyme and the inhibitors. Presumably the reaction is between the disulfide and a thiolate anion or activated sulfhydryl group at the active site of thymidylate synthase, resulting in the formation of a mixed disulfide. In most cases semilogarithmic plots of residual activity vs. time were linear throughout the observed inactivation period. However, in a few instances the linearity persisted only throughout the loss of the initial 70—80% of the activity. A possible explanation for such non-linear behavior was suggested by Munroe et al. [19] who reported that the inactivation of thymidylate synthase by DTNB under pseudo first-order conditions was bisphasic at low pH (5.8—6.8) and linear at higher pH (7.5—8.8). They suggested that at low pH the cysteinyl side-chains at the active site of the enzyme can exist either as thiolate anions or as thiols activated by a neighboring base, and that both forms can react with disulfides albeit at different rates.

The apparent second-order constants for the thirteen disulfides shown in Table II differed by as much as 250 000-fold, which clearly demonstrates that the chemical structures of the disulfides are important factors in determining their rates of reaction with this enzyme. The data suggest that negatively charged functional groups decrease the reactivity of the disulfides, and positively charged groups enhance the reactivity. For example, the rate constant for cystine dimethyl ester and cystamine, which lack the negative charge that cystine has, are approx. 6—9 times greater than the rate constant for cystine, whereas the rate constant of dithiodipropionate, which lacks the positive charge present in cystine, is only one ninth that of cystine. The rate constant of dithiodipyridine is approx. 9 times greater than that of the structurally similar but negatively charged dithiodinicotinate. Rate constants for cystamine, dithiodiethanol and dithiodiacetate also show the same relative effects of negative and positive charge. It is of interest to compare the rate constant for the reaction of thymidylate synthase with DTNB (approximately $2 \cdot 10^3$ $M^{-1} \cdot min^{-1}$, 25°C) reported by Munroe et al. [19], with the rate constant for inhibition by dithiodinicotinate ($2.7 \cdot 10^3$ $M^{-1} \cdot min^{-1}$, 30°C), since both of these compounds contain an aromatic ring substituted with a carboxyl group. Galivan et al. [18] noted a similar influence of charge on reactivity from rates of inhibition of thymidylate synthase by iodoacetate and iodoacetamide. Disulfides that contain aromatic groups were, in general, better inhibitors than the aliphatic disulfides, which may explain why *N,N'*-di-(dinitrophenyl)-L-cystine was a better inhibitor than expected on the basis of charge alone. The mixed disulfide GSS(2-pyridine) is more aromatic and has less negative charge than GSSG which may explain the 1300-fold difference in the rate constants of these two inhibitors. Since both dUMP and CH_2H_4folate have aromatic moieties, the substrate binding site(s) of the enzyme may have a stronger

influence on the orientation and reaction rate of aromatic disulfides than on aliphatic disulfides. We do not feel that the earlier report [11] that thymidylate synthase was not inhibited by GSSG is in conflict with the slight inhibition by GSSG that we observed, since the former is based on an inhibitor concentration of 10 mM and an incubation period of 15 min while we used 50 mM GSSG for periods up to 1 h.

A possible explanation for the relationship between charge and reactivity of the disulfide inhibitors is an ionic attraction or repulsion by a negatively charged group at or near the essential cysteinyl residue of thymidylate synthase. This negatively charged group might be the base proposed by Munroe et al. [19] to be involved in the activation of the catalytic cysteinyl residue. It is highly unlikely that it is the C-terminal carboxyl group, however, since Galivan et al. [18] have shown that treatment with carboxypeptidase A had no effect on the rate or extent of the reaction of *N*-ethylmaleimide with thymidylate synthase.

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