INHIBITION OF THYMIDYLATE SYNTHETASE BY SHOWDOMYCIN AND ITS 5'-PHOSPHATE

Thomas I. Kalman

Department of Biochemical Pharmacology, School of Pharmacy
State University of New York at Buffalo, Buffalo, New York 14214

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SUMMARY: Thymidylate synthetase of dichloromethotrexate resistant Lactobacillus casei was found to be rapidly and irreversibly inactivated by showdomycin (3-β-D-ribofuranosylmaleimide). Prior treatment of the enzyme with hydroxyethyldisulfide prevented the inhibitory effect of showdomycin. The enzyme could also be protected by its substrate, deoxyuridylate, against inactivation by the antibiotic. Reduction of the 3,4-double bond by catalytic hydrogenation destroyed the enzyme inhibitory activity of showdomycin. Phosphorylation of its 5'-hydroxyl group greatly enhanced the inhibitory potency of the antibiotic, presumably by conferring substrate-like specificity. These results suggest that the maleimide moiety of showdomycin alkylates a reactive sulfhydryl group at the active site of thymidylate synthetase, and are consistent with a sulfhydryl addition-elimination mechanism of the enzyme catalyzed reaction.

Thymidylate synthetase (EC 2.1.1.b) a key enzyme in the de novo pathway of DNA-thymine biosynthesis catalyses the reductive methylation of deoxyuridylate. Santi and Brewer proposed (1) that the mechanism of this reaction may involve the addition-elimination of a nucleophilic group of the enzyme across the 5,6-double bond of the substrate. It has also been suggested (2-4) that the functional nucleophile is an enzymic SH-group and that its participation leads to the formation of a transient thioether linkage (5) between enzyme and substrate at position 6 of the nucleotide next to the glycosidic bond. The antibiotic showdomycin, 3-β-D-ribofuranosylmaleimide (6), has a reactive electrophilic carbon atom next to its glycosidic bond at a position analogous to C-6 of the substrate, therefore, if the above mechanistic hypothesis is correct, showdomycin and its 5'-phosphate should be able to effectively alkylate the functional SH-group at the active site of the enzyme.

This communication describes the effects of showdomycin and its 5'-phosphate on purified thymidylate synthetase of dichloromethotrexate resistant Lactobacillus casei (7), and discusses their relevance to the mechanism of the enzyme catalyzed reaction.

MATERIALS AND METHODS

Showdomycin, 2-mercaptoethanol, dithiothreitol (DTT) and disodium deoxyuridylate (dUMP) were obtained from Calbiochem. Hydroxyethyldisulfide was purchased from Aldrich and purified by distillation. d,l-L-Tetrahydrofolic acid was supplied by Sigma.

Thymidylate synthetase of dichloromethotrexate resistant L. casei was purified according to the method of Leary and Kisliuk (7) and incubated for 15 min. at 30° in the presence of 5 mM DTT at pH 7 prior use, unless otherwise stated. Enzyme activity was assayed spectrophotometrically as described by Wahba and Friedkin (8), using a Gilford 2000 multiple absorbance recorder at 0.1 O.D. full scale. The regular assay mixture contained the following: Tris-acetate, 70 mM, pH 7.4; mercaptoethanol, 80 mM; $MgCl_2$, 20 mM; d,l-L-tetrahydrofolate, 0.3 mM; CH_2O, 12 mM; EDTA, 0.3 mM; dUMP, 1.0 mM and sufficient enzyme to produce an absorbance change of 0.010-0.015 O.D. units/min. at 340 mμ.

Inactivation experiments were conducted by incubating the enzyme(including 0.4 mM DTT) at 30° in the presence or absence of inhibitors and protecting agents in complete assay mixtures containing 5.5 mM ascorbate in place of mercaptoethanol and no dUMP, unless otherwise indicated. At various time intervals aliquots were diluted 25-fold into regular assay mixtures and their enzyme activities were determined spectrophotometrically.

Catalytic hydrogenation of showdomycin (10 mg in 2.0 ml abs. EtOH) was performed as described by Darnall et al. (6).

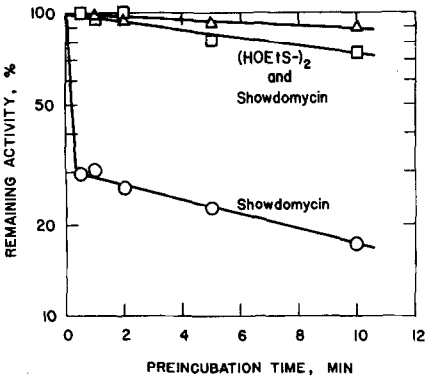

Figure 1. Time Course of the Inactivation of Thymidylate Synthetase by Showdomycin. Control (△); 5.0 mM showdomycin (O); 5.0 mM showdomycin, after incubation of the enzyme in the presence of 0.1 M hydroxethyldisulfide at 30° for 15 min. (□).

Showdomycin-5'-phosphate was prepared according to the method of Yoshikawa et al. (9) and isolated by preparative TLC on cellulose, using 1 M NH_4OAc:EtOH, 2:3 (v/v) as the developing solvent and further purified by repeated chromatography.

RESULTS AND DISCUSSION

Incubation of thymidylate synthetase in the presence of 5.0 mM showdomycin resulted in rapid loss of catalytic activity (Figure 1). Enzyme preparations, which have been exposed to air and not been subjected to dithiothreitol (DTT) treatment were inactivated to a much lesser extent or not at all, suggesting that the susceptibility to inhibition by the antibiotic may depend on the reduced state of a sensitive SH-group of the enzyme at the time of interaction with the inhibitor. Almost complete protection against inactivation by showdomycin was obtained, when prior to its contact with the inhibitor the DTT-treated enzyme was incubated with hydroxyethyldisulfide (Figure 1), which inhibits thymidylate synthetase in the absence of thiols (10), presumably through mixed disulfide formation with the enzyme. In the regular assay

medium, the excess mercaptoethanol destroys the free showdomycin and reactivates the hydroxyethyldisulfide treated enzyme allowing for the measurement of remaining activity. The protecting effect of hydroxyethyldisulfide strongly suggests that the inactivation is a result of the reaction of the antibiotic with enzymic SH-groups.

When the chemically reactive electrophilic double bond of the maleimide ring of the antibiotic was reduced by catalytic hydrogenation, the resultant 3,4-dihydroshowdomycin was found to be devoid of enzyme inhibitory activity, which confirmed the expectation that showdomycin may act as an alkylating agent in this enzyme system.

It was of interest to determine whether or not the binding of the substrate to the enzyme would influence the inhibitory action of showdomycin. Figure 2 shows the results of an experiment, in which increasing concentrations of dUMP were present during preincubation of the enzyme with the antibiotic. It is apparent that complete protection is achieved at a concentration of 5×10^{-4}M, corresponding to 100 times the K_m-value of dUMP. Thus, saturation of the enzyme by its substrate prevents the reaction of the "essential" SH-group with showdomycin, indicating that the reactive cysteinyl residue is most likely part of the active site of thymidylate synthetase.

It was thought that by converting showdomycin to its 5'-phosphate, one may induce specific binding of the inhibitor to the active site, since thymidylate synthetase has no significant affinity to nucleosides, only to nucleotides. This binding was not expected to be strong, however, because the presence of the 2'-hydroxyl greatly decreases the affinity (11). Figure 3 shows the comparative enzyme inhibitory effects of showdomycin and its 5'-phosphate at an equal concentration (10^{-4}M). A fresh enzyme preparation of high SH-content was used in these experiments, without prior treatment with DTT. It is ap-

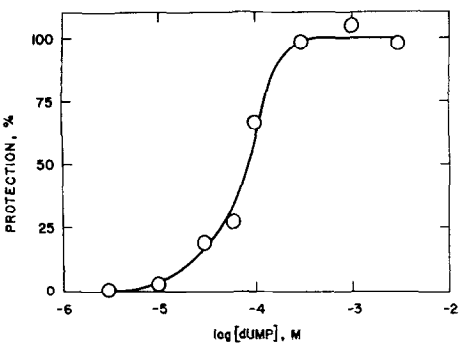

Fig. 2.

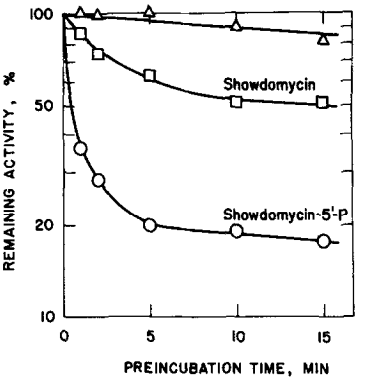

Fig. 3.

Figure 2. Substrate Protection against Inactivation of Thymidylate Synthetase by Showdomycin.

Figure 3. Inactivation of Thymidylate Synthetase by Showdomycin and Its 5'-Phosphate in Thiol Free Medium. Control (△); 0.1 mM showdomycin (□); 0.1 mM showdomycin-5'-phosphate (O).

parent that showdomycin-5'-phosphate is a much more effective inhibitor of the enzyme, its initial rate of inactivation being more than 10 times higher than that of the unphosphorylated antibiotic. It can be predicted that the 2'-deoxy-derivative of showdomycin-5'-phosphate would be a very potent affinity label of thymidylate synthetase.

The observed enhanced rate of inactivation by the phosphorylated antibiotic demonstrates that the approach of the reactive SH-group by the enzyme-bound inhibitor is favorable. If we assume that showdomycin-5'-phosphate binds to the active site in a similar fashion to that of the substrate, then the SH-group, which presumably becomes alkylated by C-4 of the maleimide ring, must be in the vicinity of the analogous C-6 position of the pyrimidine ring of dUMP, when the latter binds. These conclusions are consistent with a mechanism of the alkylation step of the thymidylate synthese catalyzed reaction, which involves a nucleophilic attack of an enzymic SH-group at position 6 of dUMP.

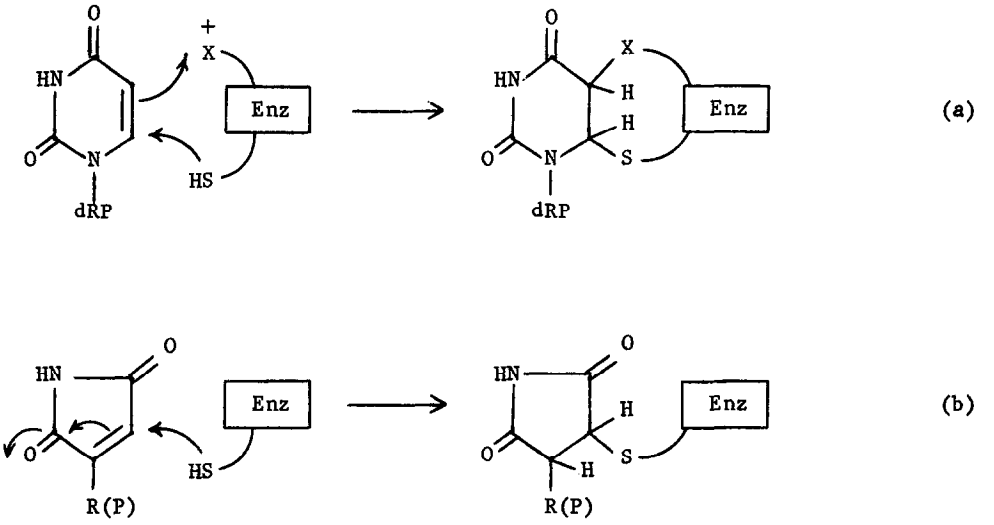

Figure 4. Analogy between the Postulated Catalytic Role of an Active Site SH-Group and Its Interaction with Showdomycin and Showdomycin-5'-phosphate. Abbreviations: X^+, electrophilic precursor of the methyl group of thymidylate; dR, deoxyribosyl; P, 5'-phosphate.

Figure 4 illustrates the analogy between the postulated concerted nucleophilic attack of the functional SH-group and the alkylating electrophile across the 5,6-double bond of dUMP (2,4) during the enzyme catalyzed reaction (scheme a) and the hypothetical mechanism of the enzyme inactivation involving the nucleophilic addition of the "essential" SH-group to the 3,4-double bond of the maleimide ring of showdomycin or its 5'-phosphate (scheme b).

It should be pointed out that its in vitro enzyme inhibitory effects described in this communication may have no relation to the in vivo biological activity of the antibiotic. Several enzymes of pyrimidine metabolism have been reported to be inhibited by showdomycin (12), but a direct relationship between these specific effects and the cytotoxic action of the antibiotic could not be established.

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