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5-NITRO-2'-DEOXYURIDYLATE: A MECHANISM-BASED INHIBITOR OF THYMIDYLATE SYNTHETASE

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SUMMARY

5-Nitro-2'-deoxyuridylate is a potent reversible inhibitor of thymidylate synthetase. Once the reversible binary complex is formed, a nucleophile of the enzyme covalently binds to the 6-position of this inhibitor. The interaction does not require the cofactor of the normal enzymic reaction, and spectrophotometric properties of the complex are ideal for detailed studies of the interaction.

INTRODUCTION

Thymidylate (dTMP)‡ synthetase (E.C.2.1.1.45) catalyzes the conversion of 2'-deoxyuridylate (dUMP) and 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to dTMP and 7,8-dihydrofolate. Fundamental aspects of the mechanism and inhibition of this enzyme have been the subject of numerous studies and have recently been reviewed (1,2). An early event in catalysis involves attack of a nucleophilic catalyst of the enzyme at the 6-position of dUMP to form 5,6-dihydropyrimidine intermediates which remain covalently bound to the enzyme throughout the catalytic sequence and serve to activate moieties of dUMP which are normally inert. The prototype mechanism-based inhibitor of dTMP synthetase is 5-fluoro-2'-deoxyuridylate (FdUMP); in the presence of CH_2-H_4folate a covalent bond is formed between the 6-position of FdUMP and the nucleophilic catalyst of the enzyme. In this paper, we describe preliminary results on the interaction of dTMP synthetase with 5-nitro-2'-deoxyuridylate (NO_2dUMP), a mechanism-based inhibitor which was designed *ab initio* from knowledge of the binding properties and mechanism of this enzyme.

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‡Abbreviations used are: dTMP, 2'-deoxythymidylate; dUMP, 2'-deoxyuridylate; CH_2-H_4folate, 5,10-methylenetetrahydrofolate; NO_2dUrd, 5-nitro-2'-deoxyuridine; NO_2dUMP, 5-nitro-2'-deoxyuridylate.

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MATERIALS AND METHODS

Thymidylate synthetase was obtained from a methotrexate resistant strain of *L. casei* (3) and purified by a modification (4) of a reported procedure (5). NO_2dUMP and 5-nitro-2'-deoxyuridine (NO_2dUrd) were prepared and purified by a reported procedure (6). Their purity was verified by High Performance Liquid Chromatography using a LiChrosorb C-18 column (4.6 x 250 mm); 5 m*M* $(n\text{-Bu})_4N^+HSO_4^-$ and 5 m*M* K phosphate (pH 7.1) in 15% MeOH/H_2O was used as eluant for NO_2dUMP and 3% MeOH/H_2O was used for NO_2dUrd. The standard mixture (NMM buffer) used for enzyme assays contained 60 m*M* N-methylmorpholine-HCl (pH 7.4), 26 m*M* $MgCl_2$, 90 m*M* 2-mercaptoethanol and 1.2 m*M* EDTA. For inactivation experiments, mixtures containing 40 to 55 n*M* dTMP synthetase, specified concentrations of NO_2dUMP and dUMP in a 0.83 dilution of the standard buffer were incubated at 25°; at intervals, 0.10 ml aliquots were removed and diluted into cuvettes containing 1.1 ml of 0.28 m*M* CH_2-H_4folate, 2.5 m*M* H_2CO, 1.85 m*M* dUMP and a 0.9 dilution of the standard buffer; initial velocities were determined spectrophotometrically (7) to monitor the active enzyme remaining. A Cary 118 recording spectrophotometer was utilized to monitor initial velocities and difference spectra.

RESULTS AND DISCUSSION

Recently, we reported a quantitative structure-activity relationship (QSAR) which correlated apparent K_i values for the inhibition of dTMP synthetase by 5-substituted-2'-deoxyuridylates with physical properties of the 5-substituents (8). From this correlation, we calculated that the K_i for NO_2dUMP should be 0.54×10^{-8} *M*. Using a method for rapid determination of initial velocities (8), we have found that NO_2dUMP has an apparent K_i of 2.3×10^{-8} *M*, within the acceptable range of uncertainty of the QSAR relationship.

The strongly electron withdrawing nitro-group also polarizes the 6-position of the heterocycle, making it susceptible to nucleophilic attack. Indeed, 1,3-dimethyl-5-nitrouracil reacts at the 6-position with a variety of nucleophiles to form corresponding 5,6-dihydropyrimidine adducts with favorable equilibrium constants (9). Since the mechanism of dTMP synthetase involves attack of a nucleophile of the enzyme at the 6-position of the substrate, dUMP, it was reasonable to suspect that once reversibly bound, NO_2dUMP might form a covalent bond with the nucleophilic catalyst of the enzyme. When NO_2dUMP and dTMP synthetase were incubated at 25° in the presence or absence of CH_2-H_4folate, we observed a rapid loss of enzyme activity. The fact that inactivation occurs in the absence of CH_2-H_4folate is in striking contrast to the inactivation of dTMP synthetase by FdUMP which *requires* the cofactor. All of the experiments described subsequently were performed in the absence of CH_2-H_4folate.

When 55 n*M* dTMP synthetase was incubated with 1.1 μM NO_2dUMP, we observed 76% inactivation within 1 min. At similar concentrations of components at 0°, the inactivation was clearly first order showing a half-life of 13 min. However, even this concentration of NO_2dUMP was saturating and it was not experimentally feasible to obtain complete inactivation kinetics at low temperatures. Figure 1 shows the inactivation of dTMP synthetase by varying amounts of NO_2dUMP when 1.0 m*M* dUMP was included in the pre-incubation mixture. Two relevant points emerged from this experiment. First, the fact that dUMP protects the enzyme against inactivation suggests that the inhibitor binds at the same site of the enzyme as does the substrate. Second, it is clear that the inactivation follows first order kinetics, indicative of reversible binding followed by irreversible inhibition. The nucleoside 5-NO_2dUrd does *not* inactivate the enzyme, which further supports the notion that NO_2dUMP acts upon the active site of the enzyme since it has been well established that the 5'-phosphate moiety of nucleotides is required for binding to this enzyme. Further kinetic analysis of data of the type presented in Figure 1 is complicated by the fact that plots of $1/k_{obsd}$ *versus* $1/[NO_2dUMP]$ are non-linear and the vertical intercept increases with the amount of dUMP present in the reaction mixture. Because of the dimeric structure of the enzyme (10) numerous kinetically equivalent mechanisms may be conjured and a thorough kinetic analysis of the interaction is a topic of current investigation in this laboratory.

Further evidence that structural changes occur in the heterocycle of NO_2dUMP was obtained by uv difference spectra. The difference spectrum of enzyme (14 μ*M*) and NO_2dUMP (11 μ*M*) *vs.* enzyme (14 μ*M*) shows that the λ_{max} of bound NO_2dUMP is 11 nm higher than NO_2dUMP in the reaction buffer containing 6 m*M* dithiothreitol (λ_{max} 326 *vs.* 337 nm). When the samples were subsequently treated with sodium dodecyl sulfate there was a rapid, *albeit* observeable reversion of the difference spectrum to one identical to that of NO_2dUMP. From this we conclude that the covalently bound NO_2dUMP is not irreversibly modified and the covalent bond can readily be disrupted when the enzyme is denatured. The uv difference

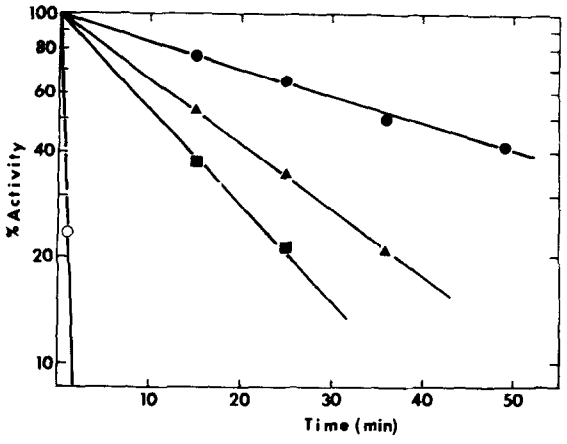

Figure 1. Inactivation of dTMP synthetase by NO_2dUMP: 1.1 μM NO_2dUMP (○); 1 mM dUMP plus NO_2dUMP at concentrations of 0.6 μM (●), 1.1 μM (▲) and 1.7 μM (■). Assay conditions are provided in Materials and Methods.

spectra of enzyme plus NO_2dUMP *vs.* NO_2dUMP shows λ_{max} at 345 nm with a well-defined isosbestic point at 321 nm, further indicating that bound NO_2dUMP is structurally different from the free nucleotide. Using ΔO.D. at 345 nm, difference spectra-titration of dTMP synthetase with NO_2dUMP demonstrates that 2 mol of inhibitor are bound per mol enzyme with $\Delta\varepsilon$ = 4810 per mol of NO_2dUMP. This is consistent with the well-known fact that dTMP synthetase is composed of two identical subunits (10) which can both be covalently bound to FdUMP in the presence of CH_2-H_4folate (11).

Based on the results described here, the reactivity of the 6-position of 5-NO_2-uracil derivatives towards nucleophiles (9) and what is known of dTMP synthetase, a logical mechanism for the inhibition by NO_2dUMP can be deduced. As depicted in Figure 2, we propose that once a reversible complex has been formed, the active site nucleophile adds to the 6-position of NO_2dUMP to form a reversible covalent bond.

While details of the interaction of NO_2dUMP remain to be elucidated, it is clear that it is a potent mechanism-based inhibitor of dTMP synthetase. The covalent complex formed differs in two significant aspects from that formed with the well studied FdUMP. First, unlike FdUMP, covalent bond formation does *not*

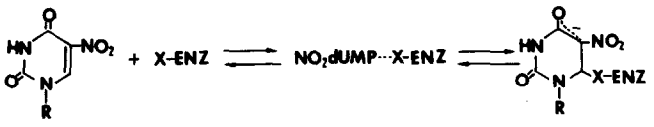

Figure 2. Proposed mechanism of interaction of NO_2dUMP with dTMP synthetase; R = 5-phospho-2-deoxyribosyl.

require the cofactor, CH_2-H_4folate. Second, whereas the FdUMP-CH_2-H_4folate-enzyme complex is stabilized by protein denaturants, denaturation of the binary NO_2dUMP-dTMP synthetase complex results in dissociation of the inhibitor; the latter is expected based on the chemistry of adducts of 1,3-dimethyl-5-NO_2uracil (9) and suggests that reversible binding of NO_2dUMP provides a significant portion of the free-energy of interaction which ultimately results in the covalent complex.

For future investigation of this enzyme, NO_2dUMP possesses a number of desireable features. First, the unstable cofactor CH_2-H_4folate is not required, which will greatly simplify many studies. Second, since the absorbance maxima of NO_2dUMP and the NO_2dUMP-enzyme complex are far from interfering absorbance of protein and other components, detailed studies of the interaction should be amenable to a number of spectrophotometric techniques. Lastly, the potency and nature of inhibition of dTMP synthetase by NO_2dUMP suggests that the corresponding nucleoside might be of significant *in vivo* utility. Indeed, it has been reported that NO_2dUrd has anti-viral activity which was attributed to *in vivo* inhibition of dTMP synthetase (12). Further studies of NO_2dUrd and NO_2dUMP are in progress in this laboratory and will be the topic of a forthcoming report.

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