IRREVERSIBLE INHIBITION OF THYMIDINE KINASE FROM *ESCHERICHIA COLI*

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1. Introduction

The concept of nonclassical antimetabolites [1] allows for major structural changes of enzyme inhibitors at that part of the molecule which does not directly interfere with its binding to a specific site of the protein. This idea has led to the evaluation of active-site-directed irreversible inhibitors bearing functional groups that might form covalent bonds to reactive residues within the enzyme [2]. Specificity of this irreversible desactivation requires the formation of a reversible enzyme–inhibitor complex prior to the chemical reaction.

It has become clear from previous studies [3,4] that thymidine kinase (ATP-thymidine 5′-phosphotransferase; EC 2.7.1.21) from *E. coli* exhibits bulk tolerance towards substitution at the 5′-position of its substrate thymidine. We have extended these investigations, and wish to report on the irreversible interaction between thymidine kinase and several 5′-derivatives of thymidine and its analogs.

2. Materials and methods

2.1. *Preparation of thymidine kinase*

The enzyme was purified from crude extracts of *E. coli* by salt precipitation (0–45% ammonium sulfate saturation) and affinity chromatography on Sepharose to which 6-(*p*-amino) benzyluracil was attached covalently [5]. From analysis on denaturing polyacrylamide gels, enzyme purity was estimated at 18%; the specific activity was determined to be 1103 units/mg protein. Enzyme preparations obtained by this procedure were essentially free of esterase activity as followed by the hydrolysis of *p*-nitrophenylacetate.

2.2. *Synthesis of thymidine derivatives*

Synthesis has been described in detail for 4-thiothymidine [6], 5′-amino-5′-deoxythymidine [7], and its *N*-bromoacetyl derivative [8]. Sulfonylfluoride-substituted derivatives were obtained by reacting the nucleosides with *O*-, *m*- and *p*-fluorosulfonyl-substituted benzenesulfonyl chlorides in anhydrous pyridine at −20°C [9]. Compounds were characterized by elemental analysis and spectroscopic data. The 5′-chloro-,-bromo- and -iodoacetic acid esters of thymidine were kindly provided by Dr J. P. Neenan.

3. Results

Following previous observations that preincubation of thymidine kinase from *E. coli* with 5- and 6-substituted uracils carrying sulfonylfluoride and bromoacetyl groups leads to a time- and concentration-dependent decrease of catalytic activity (W. Rohde, unpublished results), we have extended similar studies towards 5′-substituted thymidine and thymidine analogs. Figure 1 depicts a typical desactivation pattern when the enzyme is incubated in the presence of the *m*- and *p*-sulfonylfluorides III and IV, respectively. No loss of enzymatic activity is observed with the *o*-sulfonylfluoride II under these conditions. The *N*-bromoacetyl derivative I of 5′-amino-5′-deoxythymidine does not desactivate the enzyme upon preincubation, nor do the 5′-halogenacetyl esters of thymidine (data not shown).

Desactivation rates are influenced by the presence of ligands interacting with the allosterically regulated

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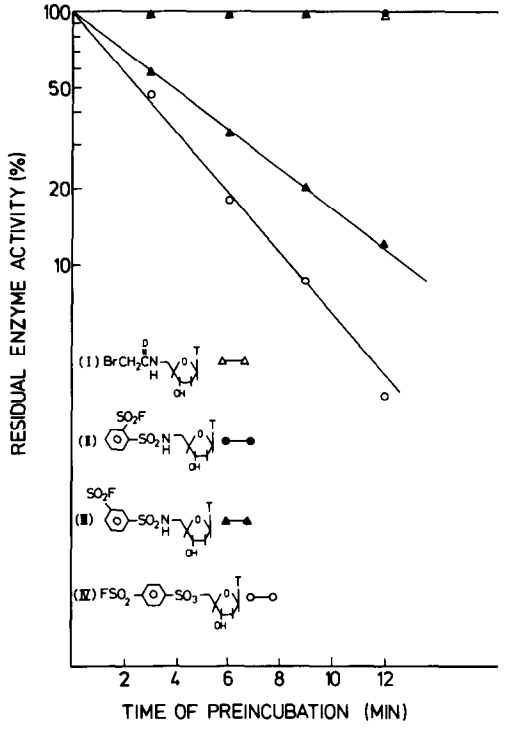

Fig.1. Time-dependent inactivation of thymidine kinase; 20 enzyme units were incubated at 37°C in total vol. 0.1 ml, containing 50 mM Tris · HCl, pH 8.0, 10% DMSO and 0.5 mM of the corresponding thymidine derivatives I–IV. At the indicated time intervals, 10 μl samples were withdrawn and assayed for residual enzyme activity [5].

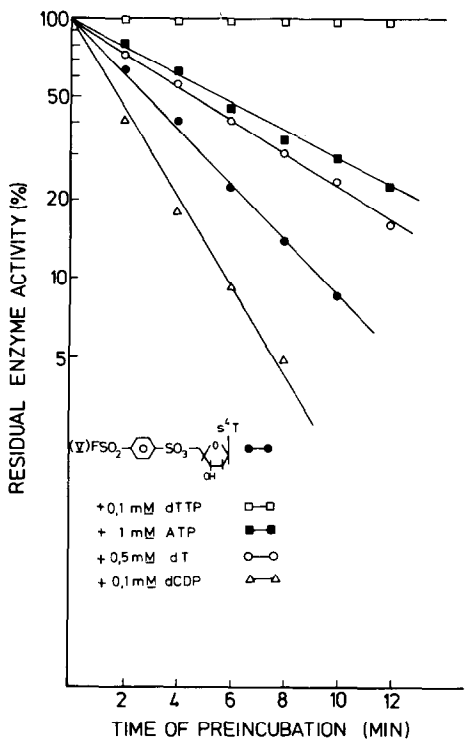

Fig.2. Effect of allosteric regulators on the inactivation of thymidine kinase by the 4-thiothymidine derivative V. Experimental conditions were the same as described for fig.1, except for the addition of the indicated ligands.

enzyme [10]. Figure 2 shows that dCDP, an activator of enzyme activity that binds to a site distinct from the ATP- and thymidine-binding sites, brings about an increase of the apparent first-order rate constant k_{app} of desactivation. On the other side, the substrate thymidine as well as ATP, the phosphate-donating cofactor of the enzymatic reaction, exhibit measurable protective effects. This protection against desactivation becomes even more drastic with dTTP, the in vivo feedback inhibitor. In the presence of this allosteric and competitive inhibitor with regard to thymidine, irreversible inhibition is not observed during the time course followed. Similar effects on desactivation rates were obtained for the sulfonylfluorides III and IV. Thymidine kinase, preincubated with the 4-thiothymidine derivative V until total loss of enzymatic activity and reisolated by gel filtration on G-25 Sephadex, shows a decrease of the A_{280}/A_{330} ratio. This reflects the covalent attachment of this inhibitor with the characteristic 4-thiothymidine chromophor (λ_{max} 330 nm, ϵ 20 000).

With enzyme preparations obtained by different purification procedures [3], desactivation of thymidine kinase levels off rapidly. This was shown to be due to the presence of esterases which compete for sulfonylfluorides by covalent bond formation via the SO_2F moiety. When, e.g., α-chymotrypsin (9×10^{-6} M) is preincubated with inhibitor III at concentrations of 1, 2.5 and 5×10^{-4} M, enzyme activity decreases with $t_{1/2}$ of 6.6, 2.4 and 0.83 min, respectively. This desactivation of the protease resembles its interaction with phenylmethyl sulfonylfluoride which forms a covalent bond to the active site-specific serine 195 [11]. In contrast, thymidine kinase is not inactivated by phenylmethyl sulfonylfluoride.

4. Discussion

Several thymidine derivatives have been evaluated as irreversible inhibitors of thymidine kinase from *E. coli*. The *m*- and *p*-fluorosulfonylsubstituted thymidine derivatives III, IV, and V irreversibly interact with the enzyme, presumably because of juxtapositioning of the SO_2F moiety and a reactive amino acid residue close to the active site. The *ortho* derivative II does not decrease catalytic activity, nor does the structurally unrelated phenylmethyl sulfonylfluoride act as an irreversible inhibitor. Together with the finding that allosteric regulators of enzyme activity influence the desactivation pattern, it seems reasonable to assume that interaction of these thymidine derivatives with the enzyme is a specific process. Clearly, detailed kinetic data are necessary to decide unequivocally whether these compounds might serve as affinity labeling reagents.

The use of 4-thiothymidine derivatives provides an opportunity for spectroscopic studies on enzyme–inhibitor interaction. Furthermore, 4-thiothymidine readily exchanges with elemental ^{35}S [12]; the ^{35}S-labeled compound V might thus serve to identify the amino acid residue involved in covalent bond formation. The role which thymidine kinase might play in DNA regulation [13] stresses the importance of specific and possibly irreversible inhibitors of this enzyme. It is clear, however, from our data that sulfonylfluorides because of their interaction with the ubiquitous esterases do not serve this purpose.

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