Inhibition of Thymidine Kinase by $P^1$-(Adenosine-5')-$P^5$-(thymidine-5')-pentaphosphate*

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Potential bisubstrate analogs, with adenosine and thymidine joined at their 5' positions by polyphosphoryl linkages of varying lengths (Ap,dT, where n = the number of phosphoryl groups), were examined as inhibitors of cytosolic thymidine kinase from blast cells of patients with acute myelocytic leukemia. The number of phosphoryl groups), were examined as inhibitors of cytosolic thymidine kinase from blast cells of acute myelocytic leukemia.

Pathway, catalyzes the transfer of phosphate from binding by the cytosolic enzyme, these compounds were defrayed in part by the payment of page charges. This article of carbamoyl phosphate synthetase comprised two distinct regions capable of binding ATP (16). Apdglucose and Apdglucose have been found to be relatively weak inhibitors of hexokinases (17, 18).

Hampton and associates (18) recently showed that ApdAT inhibited rat spleen mitochondrial thymidine kinases effectively, but was considerably less effective against the cytosolic enzyme. The present study shows that ApdAT, ApdAT, and ApdAT are stronger inhibitors of human cytosolic thymidine kinase. In experiments designed to determine enzyme affinities for their inhibitors in a different way, we were surprised to find that these compounds were bound with considerably higher affinity at another site, resulting in stabilization of the enzyme toward thermal inactivation.

EXPERIMENTAL PROCEDURES

Materials—Nucleosides, nucleotides, phosphocreatine, dithiothreitol, alkaline phosphatase (type III), agarose-bound alkaline phosphatase, venom phosphodiesterase (type VII), bovine serum albumin, and creatine kinase (type I) were obtained from Sigma. [Mg-32P] Thymidine (60–90 Ci/mmol) was purchased from ICN Radiochemicals. Whatman DE81 filter paper discs were obtained from Fisher.

Analytical Methods—Proton magnetic resonance spectra of ApdAT and ApdAT were recorded using Varian EM 390 and Bruker WM 250 spectrometers, operating at 90 MHz and 250 MHz, respectively. 31P NMR spectra were obtained using a Bruker WM 250 spectrometer operating at 101.3 MHz, employing acquisition and delay times of 2.2 and 2.0 s, respectively. Inverse gated proton decoupling was used to eliminate nuclear Overhauser effects. Mass spectra of inhibitors were obtained on a VG micromass mass spectrometer (model ZAB 2F) using fast atom bombardment with either positive or negative argon ions. Mass spectra of the tetracationic salt of $P^1P^5$-di(adenosine-5')-tetraphosphate established that the mass/charge for the molecular ion corresponded to that expected for the singly ionized free acid.

In addition to the spectroscopic methods described above, inhibitors were analyzed for purity in TLC systems A (cellulose developed in 0.25 M NH4HCO3-2-propanol (35:65)) and B (polyethyleneimine cellulose developed in 0.9 M LiCl). After digestion for 1 h using phosphodiesterase (0.5 mg/ml), alkaline phosphatase (0.5 mg/ml), and MgCl2 (1.0 mM), product mixtures were again analyzed in TLC System A using authentic adenosine and thymidine standards (Table I). Extinction coefficients of ApdAT and ApdAT were determined by measuring the absorbance at 282 nm before and after enzymatic digestion, and comparison with the absorbance of equimolar mixtures of adenosine and thymidine of known concentration (Table I).

Synthesis of Inhibitors—Tributylammonium salts of nucleotides (20) were prepared by first converting their barium or calcium salts (0.1 mmol) to pyridinium salts by treatment with Dowex 50W-X8 (pyridinium form, 30 g) in water. Mixtures were evaporated under reduced pressure to a volume of 10–20 ml, and 1 molar equivalent of tributylamine/mol of phosphorus was added, along with sufficient ethanol to produce a homogeneous solution. The tributylammonium salt was then evaporated to dryness several times, from ethanol and then from dry pyridine.

ApdAT (20), ApdAT, ApdAT, and ApdAT were synthesized by the

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1. The abbreviations used are: ApdA, $P^1P^5$-(adenosine-5')-pentaphosphate; ApdA, $P^1P^5$-(adenosine-5')-pentaphosphate; ApdAT, $P^1$-(adenosine-5')-$P^5$-(thymidine-5')-hexaphosphate; ApdAT, $P^1$-(adenosine-5')-$P^5$-(thymidine-5')-pentaphosphate; ApdAT, $P^1$-(adenosine-5')-$P^5$-(thymidine-5')-tetraphosphate; ApdAT, $P^1$-(adenosine-5')-$P^5$-(thymidine-5')-triphosphate.
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TABLE I

| Compound | Product yield | TLC mobility | Phospho-imidazole base ratio | Mass/charge, molecular ion | Calculated | Observed |
|----------|---------------|-------------|-----------------------------|--------------------------|------------|----------|
| $\text{Apd}^T$ | 4.1 | 0.31-0.69 | 21.30% | 1.55 | 790 | 790 |
| $\text{Apd}^T$ | 20 | 0.26-0.41 | 24.40% | 2.05 | 812 | 812 |
| $\text{Apd}^T$ | 7.5 | 0.21-0.23 | 22.10% | 2.65 | 890 | 890 |
| $\text{Apd}^T$ | 3.0 | 0.17-0.11 | 23.70% | 3.0 | 972 | 972 |

$^a$ Determined chemically (for phosphorimidazole analyses by NMR), see integrated intensities in Footnotes e, h, and k.
$^b$ Ref. 20.

$\text{Apd}^T$ was eluted last, at salt concentrations between 1.35 and 1.55 M, and these functions were repeatedly evaporated (60°C) under reduced pressure until less than 5 g of ammonium acetate remained. The residue was dissolved in water (400 ml) and subjected to chromatography on DEAE-Sephadex (2.5 x 25 cm) eluting with a gradient of NH$_4$HCO$_3$ (2000 ml from 0.1-0.7 M). ApdT emerged in the second of two broad peaks that partially overlapped. Fractions of the front-running material were desalted by repeated evaporation under reduced pressure. ApdT now appeared to be greater than 95% pure by TLC and NMR criteria (Table III).

Enzyme Assays—Thymidine kinase was prepared as previously described (3, 4) and assayed at 37°C in a final volume of 100 μl that contained Tris-HCl (50 mM, pH 7.5), dithiothreitol (2 mM), NaF (7.5 mM), bovine serum albumin (1.2 mg/ml), creatine phosphate (13 mM), and creatine kinase (22.8 units/ml) in a 2.5 x 25 cm column, eluting with a linear gradient of aqueous NH$_4$HCO$_3$ (2000 ml, 0-8 M).

Several peaks of UV-absorbing material resulted. In each case, the peak containing the desired compound, emerging at a salt concentration of 0.45-0.65 M, was identified by its absorption maximum (262 nm), resistance to alkaline phosphatase digestion, and decreased UV absorbance in the same wavelength region.

For the synthesis of $\text{Apd}^T$, $\text{Apd}^T$, and $\text{Apd}^T$, tributylammonium adenosine nucleotide (ADP, ATP, or adenosine 5'-triphosphate, 0.1 mmol) was dissolved in a minimal volume of dry dimethylformamide and stirred for 2 h with 1,1'-carbonyldiimidazole (0.5 mmol). Methanol (0.6 mmol) was then added, the solution was stirred for 30 min, and tributylammonium dTMP (0.25 mmol) was added in a minimal volume of dry dimethylformamide. For the synthesis of ApdT, this procedure was reversed and tributylammonium dTTP (0.1 mmol) was first converted to the imidazolide, and then allowed to react with tributylammonium ATP (0.55 mmol). After standing for 48 h at room temperature, reaction mixtures were evaporated to dryness under reduced pressure, dissolved in methanol-water (1:1), adjusted to pH 11.5 with triethylamine, and stirred for 2 h. After evaporation to dryness under reduced pressure, the residue was redissolved in water and subjected to chromatography on DEAE-Sephadex (2.5 x 25 cm), eluting with a linear gradient of aqueous NH$_4$HCO$_3$ (2000 ml, 0-8 M).

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inhibition, ApdT and ApdT were nearly as effective, and ApdT and ApA were much less effective.

In view of the resemblance of these compounds to possible reaction intermediates, it was of interest to examine the stability of the strongest inhibitor, ApdT, in the presence of the enzyme. Rates of reactions catalyzed by both cytosolic and mitochondrial enzymes were found to be constant for at least 1 h either in the absence or presence of sufficient ApdT to inhibit the reactions by 90%, suggesting that no significant breakdown of ApdT had occurred during that period. To test the stability of ApdT in the presence of the enzymes more directly, ApdT (20 μM) was incubated with MgCl₂ (100 μM) and thymidine kinase (6.7 × 10⁻³ units) in standard assay buffer without substrates or ATP-regenerating components. After 1 h at 37 °C, the reaction was quenched by immersion in boiling water for 2 min, and the denatured protein was removed by centrifugation. Aliquots (20 μl) of the supernatant fluid were analyzed by high performance liquid chromatography using a Zorbax-SAX column (4.6 mm × 25 cm), eluting with NH₄H₂PO₄ (1.1 M, pH 3.5) and monitoring the eluate absorbance at 254 nm. Negligible hydrolysis of the inhibitor was found to have occurred; experiments with control samples, incubated without enzyme or ApdT, showed that 5% hydrolysis would have been detected.

Fig. 1 shows the dependence of initial reaction rates on thymidine concentration (0.5–20 μM) in the presence of ApdT (0, 15, and 30 μM) and ATP (2.0 mM). Fig. 2 shows double reciprocal plots of initial reaction velocity as a function of changing ATP concentration in the presence of saturating concentrations of thymidine (50 mM) in the absence (circles) and presence of ApdT (0.475 μM, triangles; 1.9 μM, squares). Nonlinear double reciprocal plots were observed, suggesting that ATP was bound cooperatively by cytosolic thymidine kinase (5). The substrate concentration required for half-maximal velocity was estimated from linear plots of 1/v versus 1/(ATP)² (Fig. 3). Table III shows Kᵢ values observed for ApdT, determined from double reciprocal plots of initial reaction rates as a function of changing concentrations of each of the two substrates, in the presence of saturating concentrations of the co-substrate.

To avoid complications arising from the cooperative binding of ATP by cytosolic thymidine kinase, the affinities of ApdT, ApαdT, and ApβdT were estimated by monitoring activity at substrate concentrations (ATP = 7.5 μM, thymidine = 100 nM, MgCl₂ = 100 μM) far below their Kᵢ values, in the absence and presence of increasing concentrations of inhibitors. Kᵢ values were estimated from the reciprocal of the slope of a plot of uninhibited initial velocity divided by inhibited initial velocity as a function of inhibitor concentration (Fig. 4). Kᵢ values determined in this way were 1.2 μM for ApdT, 310 nM for ApαdT, 120 nM for ApβdT, and 190 nM for ApγdT.

After monitoring activity as a function of time, it became apparent that the enzyme lost activity rapidly at 37 °C in the
from the half-time for inactivation (Fig. 5). The solid line is a theoretical curve calculated for a dissociation constant of 2.5 nM assuming that no depletion of ApdT had occurred. The inset shows experimental data obtained at six concentrations of ApdT (nM).

ApdT yielded an apparent dissociation constant of 2.5 nM for ApdT (Fig. 5). This dissociation constant was considered an upper limit, since binding was so tight that some depletion of ApdT could not be avoided under these conditions. Protection from inactivation was unaffected by the addition of MgCl₂ (100 μM). Under the same experimental conditions, the rate of inactivation was reduced 47% by 1.0 μM ApdT, 55% by 30 nM ApdT and 88% by 20 nM ApdT. Dissociation constants were estimated from these results to be 1.1 μM for ApdT, 29 nM for ApdT, and 2.7 nM for ApdT.

**DISCUSSION**

ApdT was earlier shown to inhibit rat cytosolic thymidine kinase with an affinity comparable with the apparent affinity of the substrate ATP (18). The rat cytosolic enzyme apparently differs from the human enzyme in binding ATP non-cooperatively, and in being somewhat more sensitive to inhibition by ApdT (18). The present inhibitors were bound by the human enzyme substantially more tightly than ApdT, consistent with the possibility that these compounds bear a somewhat closer resemblance to the phosphoryl donor and acceptor, in a spatial relationship similar to that which they may adopt during direct phosphorly transfer. The ordered sequential kinetics of substrate binding by human liver cytosolic (5) and mitochondrial (14) thymidine kinases accord with a mechanism involving direct phosphate transfer. In keeping with the established specificity of thymidine kinase for thymidine as an acceptor (5), ApA was found in the present study to be relatively ineffective as an inhibitor (Table II).

Somewhat surprisingly, the affinities of the most effective (ApdT; Kᵢ = 0.12 μM) and the least effective (ApdT; Kᵢ = 1.2 μM) inhibitors examined in the present study differed by only one order of magnitude. This lack of discrimination by thymidine kinase stands in marked contrast to the behavior of adenylate kinase, which showed 10⁷-fold higher affinity for ApA (Kᵢ = 2.5 nM) than for ApA (Kᵢ = 24 μM) (15). Since ApA was very weakly bound by thymidine kinase (Table II), it seems likely that both ends of these two-headed inhibitors are involved in enzyme-inhibitor interaction. The insensitivity of binding to chain length, in inhibitors of the type ApdT, suggests that the inhibitors may adapt in different ways to fit...
the stereochemical requirements of the active site, or that recognition sites on the enzyme may be somewhat flexible in their relative orientation. In this latter connection, it appears possible that the binding sites are aligned in such a way as to encourage nucleophilic displacement on phosphorus, but are sufficiently mobile to allow some latitude in the distance separating the phosphoryl donor and acceptor; thus, inhibitors with different numbers of phosphoryl groups might be accommodated with similar affinities by the active site.

The unexpected ability of these inhibitors to protect thymidine kinase from thermal inactivation, at concentrations substantially below those at which half-maximal inhibition was achieved, indicates the presence on the enzyme of an additional binding site or sites. In view of the considerations that went into their design, it is surprising that these two-headed analogs should adhere with such high affinity to noncatalytic sites on the enzyme. These sites differ from the inhibitory site not only in their absolute affinity, but also in their relative affinity for ligands. Thus, Ap,dT was bound at least as tightly as Ap,dT, Ap,dT was bound an order of magnitude less tightly, and Ap,dT protected the enzyme only at the high concentrations that also produced inhibition.

Detailed information is not yet available concerning the overall structure of this enzyme, but it is worth noting that inhibition of cytosolic thymidine kinase by dTTP has been found to result in an increase in cooperativity, so that more than two molecules of ATP appear to be bound (5). Thus, each subunit of this dimeric enzyme probably contains at least one allosteric binding site for ATP and one for dTTP, in addition to binding sites for the substrates ATP and thymidine. Inhibitor binding at any two of these sites, on the same or different subunits, could presumably result in thermal stabilization of the enzyme.

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