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Inhibition of terminal deoxynucleotidyl transferase by adenine dinucleotides

Unique inhibitory action of Ap5A

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Terminal deoxynucleotidyltransferase (TdT) exhibits strong sensitivity to ATP and its dinucleotide analogues, Ap2A, Ap3A, Ap4A, Ap5A and Ap6A. Similar to ATP, all of the dinucleotides appear to be competitive inhibitors of TdT catalysis with respect to substrate deoxynucleoside triphosphates and effectively block the UV-mediated substrate cross-linking to TdT. Among the various dinucleotides, Ap5A and Ap6A (diadenosine 5′-5′ penta- and hexaphosphate, respectively) are significantly more effective than dinucleotides containing 2, 3 or 4 phosphate backbones. Furthermore, Ap5A is found to be the only dinucleotide which has reactivity at both substrate- and primer-binding domains in TdT.

Terminal deoxynucleotidyltransferase; Adenine dinucleotide inhibitor

1. INTRODUCTION

TdT is a DNA polymerase class of enzyme which is exclusively found in immunocytes and their precursors [1]. While the biological function of TdT has not been clarified, its biochemical properties have been extensively investigated [2–5]. During our investigations on the substrate-binding site directed reagents of DNA polymerase [6], we noted a unique inhibitory effect of ATP on TdT catalysis [7]. ATP was found to be a classical competitive inhibitor of TdT with respect to substrate dNTPs [8]. Subsequently, using 8-azido ATP as an affinity labeling reagent, we demonstrated that the substrate-binding site resides in the 26 kDa subunit of calf TdT [9]. Based on the unique sensitivity of TdT catalysis to ATP and Mn-dATP [8,10], we proposed a regulatory role for adenine nucleotides in the expression of TdT-containing cells. Indeed, TdT positive cells appear to respond to external ATP stimulus in a mitogenic manner (i.e. stimulation of DNA synthesis [11]). To gain further insight into the biological regulation/function of TdT, we also investigated the effect of ATP and other ribonucleotides on the overall composition of TdT-catalysed DNA products. While there was no significant difference in the overall composition of DNA product, which remained rich in deoxyguanosine residues [12], the mean length of DNA product was severely restricted (Basu and Modak, unpublished). Thus, ATP appears to be quite a useful biochemical and biological effector molecule in the study of TdT. In our continued efforts to understand the possible regulatory connec-

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Abbreviations: Ap2A, Ap3A, Ap4A, Ap5A and Ap6A, diadenosine (5′-5′)di-, tri-, tetra-, penta- and hexaphosphate, respectively; TdT, terminal deoxynucleotidyltransferase; dNTP, deoxynucleoside triphosphate

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tion between adenine nucleotides and TdT, we examined the sensitivity of TdT catalysis to a number of diadenosine phosphates; the phosphate moiety in these compounds, however, was unusual in that all the phosphates were sequestered in a 5′-5′ anhydride linkage. The results indicate that all of the diadenosine phosphates are efficient inhibitors of TdT catalysis. Thus, diadenosine phosphates, containing varying numbers of phosphates, constitute a new class of inhibitor which appears to inhibit TdT catalysis by competing with substrate dNTP. One of the diadenosine phosphates, namely Ap5A, was unusual in that it exhibited reactivity at both substrate- and primer-binding domains in TdT.

2. MATERIALS AND METHODS

dGTP, ATP, Ap2A, Ap3A, Ap4A, Ap5A, Ap6A, NAD and NADP were obtained from Sigma, [α-^{32}P]dTTP was purchased from Amersham while [^{3}H]dGTP was the product of ICN. 45 kDa TdT was isolated from a chromatin extract of calf thymus to homogeneity using a simplified purification procedure (Pandey and Modak, to be published elsewhere).

2.1. *Enzyme assay*

The standard assay conditions for TdT assay have been described [10]. Activated calf thymus DNA and [^{3}H]dGTP were used as the primer and substrate respectively. Mn^{2+} was used as a divalent cation.

2.2. *UV-mediated cross-linking of [α-^{32}P]dTTP to TdT*

Cross-linking of labeled dTTP to TdT was carried out to determine the extent of enzyme-substrate (ES) complex formation, essentially as in [13]. Briefly, 5 μg homogeneous TdT (45 kDa) was cross-linked with 5 μCi [α-^{32}P]dTTP in a buffered solution containing Mn^{2+} as a divalent cation, by exposure to UV irradiation. The extent of cross-linking of TdT in the presence or absence of various inhibitors was determined by separation of labeled enzyme on 12% SDS-polyacrylamide gels followed by autoradiography [13].

3. RESULTS

3.1. *Effect of various diadenosine phosphates on TdT catalysis*

A typical dose-response pattern of diadenosine (5′-5′)di-, tri-, tetra-, penta- and hexaphosphate as well as ATP on the DNA synthesis catalysed by TdT is shown in fig.1. It is clear from the results that Ap2A, Ap3A and Ap4A exert an inhibitory effect which is comparable to that of ATP, while Ap5A and Ap6A are significantly more effective inhibitors of TdT catalysis. When different molecular mass species of calf thymus TdT (i.e. 58, 45 and 32 kDa) were used as test enzymes, nearly identical results were obtained (not shown).

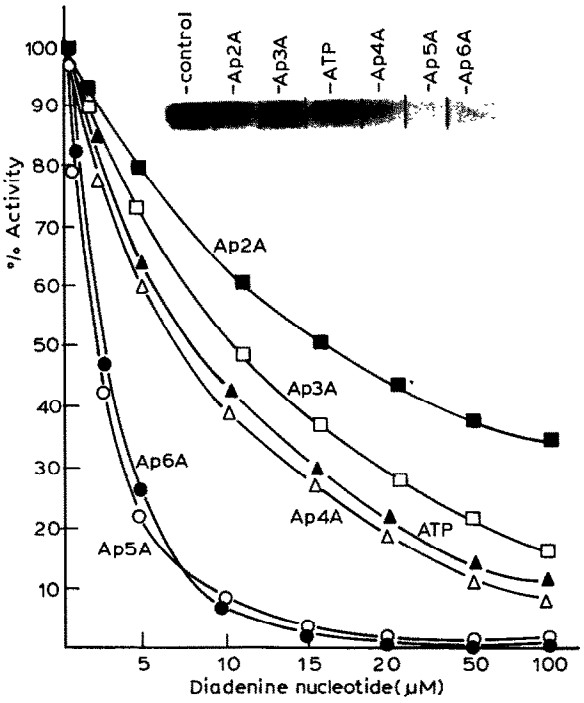

Fig.1. A dose-response pattern of ATP and diadenosine phosphates on the activity of TdT. Various dinucleotide analogues were present at the indicated concentrations in standard assay mixtures with Mn^{2+} as a divalent cation and activated DNA as the primer. Incubations were carried out for 15 min and acid-insoluble counts were determined. 100% activity represents incorporation of 350 pmol [^{3}H]dGTP per 15 min. (Inset) Autoradiogram of SDS-polyacrylamide gel showing UV-mediated cross-linking of [α-^{32}P]dTTP to TdT in the presence of 5 μM of the indicated inhibitor.

Therefore, in all subsequent studies, a homogeneous preparation of 45 kDa TdT was used as a test enzyme.

3.2. *Mechanism of inhibition of TdT by diadenosine phosphates*

Since ATP is known to be a competitive inhibitor of TdT with respect to substrate dNTPs, and ATP inhibits affinity labeling of TdT with substrate dNTP [13], we examined the effect of various diadenosine phosphates on the extent of UV-mediated cross-linking of substrate dNTP to TdT. The results presented as an inset in fig.1 clearly indicate that all diadenosine phosphates significantly inhibit cross-linking of dTTP to TdT, strongly implying a competition with substrate dNTPs. A systematic kinetic analysis of the inhibitory action of these compounds was then carried out and the results are presented in the form of double-reciprocal plots of velocity vs substrate concentration (fig.2). A competitive mode of inhibition by all of the diadenosine phosphates is quite apparent from these plots. The inhibition constants obtained with individual diadenosine phosphate analogues are listed in table 1. These constants (K_i) do confirm that Ap5A and Ap6A are superior inhibitors of TdT, compared to the remaining analogues. When similar analyses were carried out with varying primer concentrations, non-competitive plots were clearly noted (fig.3) except for Ap5A which exhibited competitive plots (data for Ap2A and Ap3A not shown). Thus, Ap5A seems to be the only dinucleotide that shows competitive inhibition with both substrate dNTP and primer DNA.

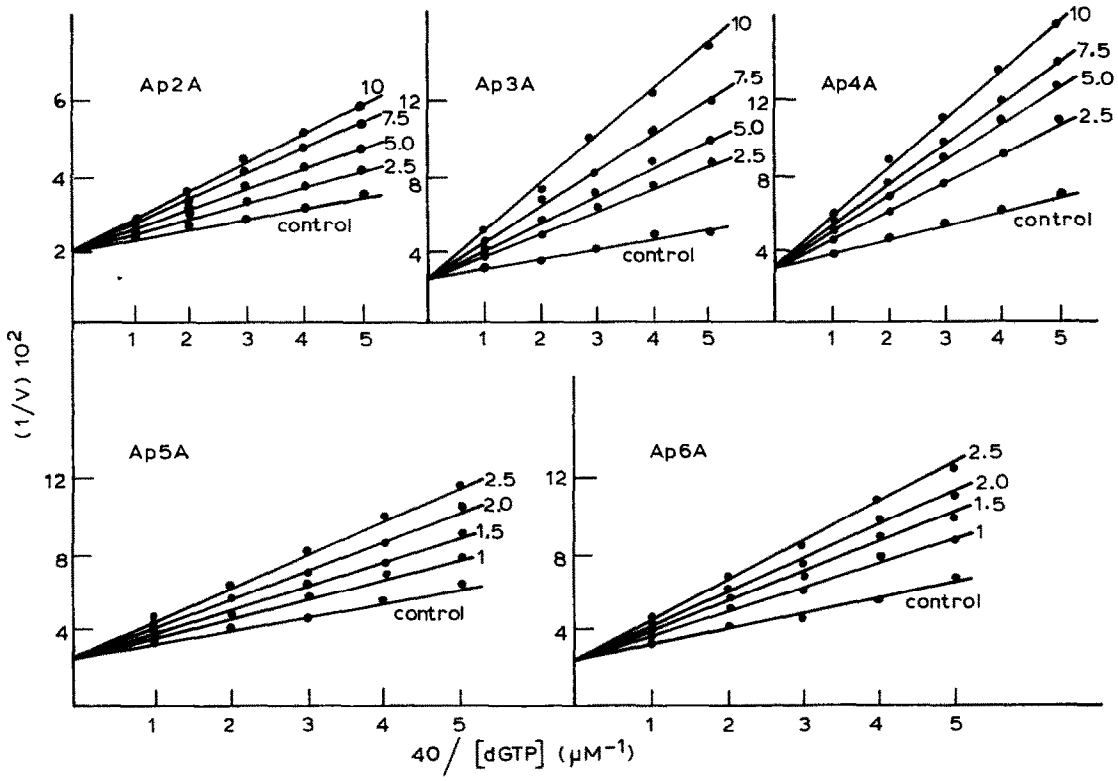

Fig.2. Kinetic analysis of diadenosine phosphate-mediated inhibition of TdT. Double-reciprocal plots with changing substrate concentration: TdT activity was measuring using indicated dGTP concentration in the presence or absence of desired inhibitor concentration (μM). Velocity vs substrate concentration as a function of inhibitor concentration is presented in double-reciprocal plots.

Table 1

Inhibitor constants (K_i) of various diadenine nucleotides

Inhibitor	K_i ($\times 10^{-6}$ M)
ATP	5.0
Ap2A	6.5
Ap3A	5.7
Ap4A	4.8
Ap5A	1.5
Ap6A	1.3

The value of K_i for each inhibitor was obtained from the data shown in fig.2. Both Dixon plots and replots of slopes vs inhibitor concentrations were used in the calculation of K_i. The values shown above are the averages of three sets of experiments. The value of K_i for ATP was taken from [8]

3.3. *Protective effect of substrate and primer against diadenosine phosphate-mediated inhibition of TdT*

To confirm that Ap5A-mediated inhibition of TdT was indeed competitive with both substrate and primer, the effect of increasing substrate and primer concentrations individually on the extent of inhibition of TdT catalysis was examined. The results summarized in table 2 show that an increase in substrate concentration decreases the extent of inhibition of TdT by all the diadenosine phosphates. In fact, the inhibitory effect of all diadenosine phosphates, with the exception of Ap5A, could be fully reversed. Increasing the primer concentration had no effect on the degree of inhibition of TdT by these compounds, again with the exception of Ap5A. The inhibition effected by Ap5A could be reduced (but not fully reversed) by both substrate and primer.

4. DISCUSSION

The recognition of ribonucleotides and particularly ATP as a substrate analogue (of dNTP) is a unique property of TdT amongst DNA polymerases [8,10]. Various analogues of ATP have also been found to be effective inhibitors of TdT catalysis. For example, 8-azido ATP and its photolysed product [9], Ara-ATP [14], dialdehyde ATP [15], ethano-ATP [16] and Ap4A [17] have been shown to compete with dNTP for binding to TdT. The present study shows that diadenosine nucleotides, where the two adenosine moieties are linked 5′-5′ via a chain of phosphates in an anhydride linkage, are also recognized as substrate analogues by TdT. All these compounds exhibited classical competitive kinetics with substrate dNTP and also blocked the cross-linking of substrate dNTP to TdT (fig.1). The studies reported herein are performed with synthetic diadenosine phosphates and their presence in higher eukaryotes is not known, with the exception of Ap4A. The

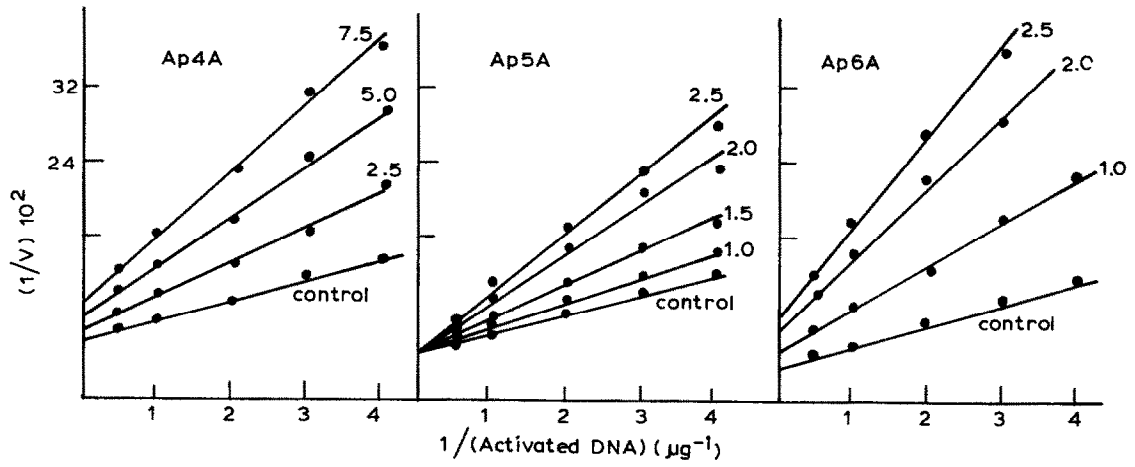

Fig.3. Kinetic analysis of diadenosine phosphate-mediated inhibition of TdT: effect of changing primer concentration. Experimental details were identical to those described in fig.2 except that primer and inhibitor concentrations were varied as shown.

Table 2

Effect of increasing substrate and primer concentration on the extent of TdT inhibition by various adenine dinucleotides

Substrate (μM)	Primer (μg)	% activity in the presence of dinucleotide (5 μM)						
		None (control)	NAD or NADP	Ap2A	Ap3A	Ap4A	Ap5A	Ap6A
20	0.25	100	100	80	60	40	4	4
50	0.25	100	100	100	88	85	30	65
100	0.25	100	100	100	100	100	56	100
200	0.25	100	100	100	100	100	56	100
20	0.25	100	100	80	60	40	4	4
20	0.5	100	100	79	61	40	25	8
20	1.5	100	100	80	60	39	48	6
20	5.0	100	100	80	60	39	54	6

5 μM of desired dinucleotide analogue was used in a standard reaction mixture containing increasing concentrations of substrate and fixed concentration of primer; or varying concentrations of primer and fixed concentration of substrate as indicated. The results are expressed as % TdT activity with respect to their controls

presence and levels of Ap4A were reported to be directly linked to the replication cycle of eukaryotic cells [18]. Intracellular generation of Ap4A and similar purine dinucleotide phosphates could conceivably occur as a result of the breakdown of the CAP structure of eukaryotic mRNAs. The only other commonly found intracellular dinucleotides are the two coenzymes, NAD and NADP. Both of these coenzymes, however, have no effect on TdT catalysis (not shown). Thus, in spite of the uncertainty regarding the physiological significance of diadenosine phosphates, these compounds seem to be excellent biochemical reagents in the study of TdT catalysis. While the target of the majority of these analogues is the substrate-binding site in TdT, one of them, Ap5A, is unusual as judged by its reactivity at both substrate- and primer-binding domains in TdT.

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