DIFFERENTIAL MODE OF INHIBITION OF TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE BY 3'-dATP, ATP, βaraATP AND αaraATP

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1. Introduction

Terminal deoxynucleotidyl transferase (TdT) catalyzes the primer-dependent but template-independent DNA polymerization of deoxynucleoside 5'-triphosphates; the enzyme was discovered and purified to homogeneity by Bollum [1,2]. The TdT is only present in thymus, in bone marrow [3] and in blood lymphoblasts of patients with acute lymphoblastic leukemia [4]. TdT has also been reported to be present in nonthymic cells [5], as well as in germinating wheat embryo [6]; for a critical re-examination of these observations an optimized TdT assay [2,7] and specific inhibitors are necessary. To establish these prerequisites proper experiments with purified TdT preparations must be performed.

In the present study it is shown that the activity of a 700-fold enriched TdT preparation from thymus is competitively inhibited by ATP, 3'-deoxyadenosine triphosphate (3'-dATP; cordycepin triphosphate) and 9-β-D-arabinofuranosyladenine 5'-triphosphate (βaraATP) with respect to both the natural substrate 2'-dATP (dATP) and the initiator oligo [d(pA)$_3$]. 9-α-D-Arabinofuranosyladenine 5'-triphosphate (αaraATP) was found to exert no inhibitory effect toward TdT.

2. Materials and methods

2.1. *Compounds*

The following materials were obtained d[^{14}C]ATP (spec. act. 52.8 mCi/mmol) from NEN, Boston, Mass.; poly(dA) from Miles, Elkhart, Ind.; βaraATP from P-L Biochemicals, Milwaukee and ATP from Boehringer Mannheim, Tutzing.

Poly (d[^{3}H]A) (spec. act. 5000 cpm/nmol) was synthesized as in [8] and 3'-dATP as in [9]. αAraATP was a gift of Dr L. L. Bennett, jr (Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Al).

2.2. *TdT purification*

TdT was prepared from calf thymus as in [2]; step VII with spec. act. 9400 units/mg protein (0.57 mg protein/ml) was used for the experiments. One unit of enzyme is equal to 1 mmol dATP incorporated into DNA per hour.

2.3. *TdT assay*

The enzyme was assayed in the following mixture (100 μl final vol.): 200 mM K-cacodylate (pH 7.2), 1 mM 2-mercaptoethanol, 4 mM $MgCl_2$, 20 μg/ml bovine serum albumin, varying amounts of d[^{14}C]ATP (spec. act. 300 cpm/nmol), 100 μM d(pA)$_3$ as initiator and 1000 enzyme units/ml. In some experiments unlabelled dATP and 10 μM d(p[^{3}H]A)$_3$ (spec. act. 5000 cpm/nmol) were used instead of d[^{14}C]ATP and d(pA)$_3$. After incubation at 35°C for 1 h, the polynucleotides in 80 μl aliquots were either collected on GF/C filters and processed as in [10] or analyzed by paper chromatography. Under the conditions described, the reaction kinetics was linear with time.

2.4. *Inhibition analysis*

In the kinetic experiments to determine the K_m for dATP, labeled dATP from 1–10 μM was added to the assays; in the case of determining K_m for the

initiator $d(pA)_3$, unlabeled oligonucleotide assays from 1–10 μM (and 200 μM labeled dATP) were used. The inhibitor constants (K_i) were calculated by the Lineweaver and Burk method [11] with the use of either a constant initiator concentration (100 μM $d(pA)_3$), varying substrate concentrations (2.5 μM, 5 μM or 10 μM dATP) and two different inhibitor concentrations (1.5 and 10 μM of the analogue) or a constant substrate concentration (200 μM labelled dATP), varying initiator concentrations (1 μM, 3 μM and 6 μM $d(pA)_3$) and two different inhibitor concentrations (1 μM and 5 μM of the triphosphate). Slopes and intercepts were calculated by linear regression analysis.

The chain length of oligo(d[^{3}H]A) was determined as in [9,12]; the strips were counted after treatment with 0.5 M NaCl [13]. Protein was determined by Lowry's method [14]. Both unlabeled and radioactive $d(pA)_3$ or $d(pA)_4$ were prepared by limited degradation of poly(dA) with DNase I [15].

3. Results

Under assay conditions, using 50 μM d[^{14}C]ATP as substrate and 100 μM $d(pA)_3$ as initiator, the following dATP analogues inhibit the TdT-mediated DNA synthesis (table 1): 3′-dATP, ATP and βaraATP. At 100 μM analogue, 3′-dATP reduces the incorporation rate by 64%, ATP by 22% and βaraATP by 55%. The inhibition caused by αaraATP is negligible.

The type of inhibition of the different analogues in the TdT reaction was studied by the Lineweaver and Burk method [11]. The inhibition kinetic constants were calculated either under constant substrate (dATP) concentrations and variation of the initiator $d(pA)_3$ concentration or in assays with $d(pA)_3$ as variable component and dATP as constant component. The concentrations of the variable components were chosen in the range of their K_m constant. The experiments revealed that the inhibition of 3′-dATP, ATP and βaraATP was of the competitive type, both with respect to the natural substrate dATP and to the initiator $d(pA)_3$. As a measure of the relative affinity of the enzyme for inhibitor and dATP or $d(pA)_3$ in competitive inhibition, the ratio K_i:K_m can be adopted [16]; the lower the value for K_i:K_m, the stronger is the inhibitory potency of a substance. The highest affinity towards TdT activity was observed with 3′-dATP as inhibitor in those assays containing substrate concentrations around the K_m value and high initiator concentrations; βaraATP and ATP affect TdT reaction to a lower extent (table 2). In case $d(pA)_3$ was the variable component (concentrations around K_m value) and dATP (at the high concentration of 200 μM) was constant component, the highest

Table 1
Inhibition of TdT by different dATP analogues

Analogue (μM)	Incorporation rate	
	(nmol/h)	(%)
–	83.4	100
3′-dATP, 100	30.0	36
ATP, 100	65.1	78
βaraATP, 100	37.5	45
αaraATP, 100	81.7	98

The experiments were performed with 50 μM d[^{14}C]ATP and 100 μM $d(pA)_3$

Table 2
Inhibition analysis of TdT

Analogue	K_m (μM) dATP	K_i (μM)	$\frac{K_i}{K_m}$	K_m (μM) $d(pA)_3$	K_i (μM)	$\frac{K_i}{K_m}$
3′-dATP	3.2	3.4	1.06	1.9	0.7	0.37
ATP	3.2	21.7	6.78	1.9	4.2	2.21
βaraATP	3.2	4.9	1.53	1.9	16.1	8.47

The inhibition studies shown in the left section were performed with 100 μM $d(pA)_3$ and d[^{14}C]ATP as variable component; for those in the right section a constant substrate concentration (200 μM d[^{14}C]ATP) and varying initiator concentrations were used. For further details, see section 2

affinity was observed with 3′-dATP; the affinities of ATP and βaraATP to the enzyme (table 2) are lower.

The finding indicating that 3′-dATP and ATP inhibit TdT reaction sensitively in a competitive way with respect to the initiator led us to suggest that these analogues act as chain terminators. To prove this assumption, d(p[^{3}H]A)$_3$ was incubated in the presence of the triphosphates dATP, 3′-dATP, ATP or βaraATP; the respective product was subsequently chromatographed to determine the chain length of the oligonucleotide. In the case of incubation of d(p[^{3}H]A)$_3$ with dATP (fig.1; left) 34% total radioactivity is found at the origin (R_F 0–0.5); at this position only oligomers with a chain length of > 10 dAMP moieties are found [12]. Only 7% radioactivity is found at the R_F value, characteristic for d(pA)$_3$. If the oligomer is incubated with 3′-dATP (fig.1; left) virtually no radioactivity is detected at R_F 0; 66% is recovered at the position of d(pA)$_3$ and 28% at d(pA)$_4$. In fig.1 (right) the radioactivity pattern of the product after incubation of d(p[^{3}H]A)$_3$ with ATP and βaraATP is shown. If incubated with ATP 32% radioactive product is identified as d(pA)$_4$, 65% total oligonucleotide did not serve as initiator and is found at d(pA)$_3$. In the case of incubation of d(pA)$_3$ with βaraATP a radioactivity pattern is found which shows no pronounced peak; this observation indicates that most of d(pA)$_3$ has undergone an enzymic reaction resulting in oligomer formation of not only high molecular weights but also of intermediate low molecular weights.

4. Discussion

The sensitivity of the purified TdT towards ATP (one substrate of the RNA polymerases), 3′-dATP (a selective inhibitor of RNA-synthesizing polymerases [9]), βaraATP (an inhibitor of DNA polymerase α and β; [17] and αaraATP (a selective inhibitor of DNA polymerase α [18]) in an assay with dATP and oligo-[d(pA)$_3$] was tested. Under the assay conditions used, αaraATP was without influence on TdT activity. The other 3 triphosphates were found to inhibit sensitively TdT-mediated DNA synthesis; the inhibition was of the competitive type both with respect to dATP and oligo[d(pA)$_3$]. While βaraATP does not function as chain terminator, 3′-dATP and ATP obviously inhibit the TdT reaction by reducing the initiator capacity for this enzyme. The conclusion that 3′-dATP and ATP act as chain terminator must be drawn from the experiments which revealed that after incubation of

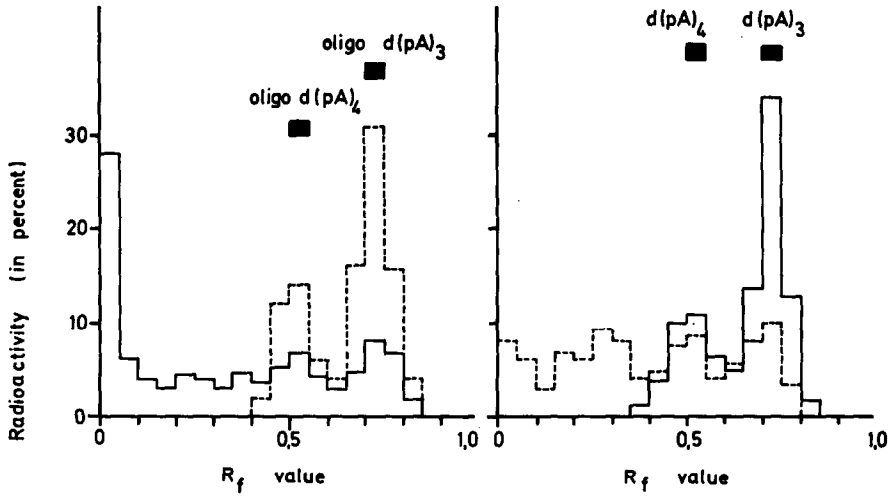

Fig.1. Chain length of oligo [d(pa)] after incubation with TdT and different nucleoside triphosphates. 10 μM d(p[^{3}H]A)$_3$ were added to the TdT reaction mixture and incubated in the presence of dATP, 3′-dATP, ATP or βaraATP at 100 μM. After incubation (1 h, 35°C) a 80 μl aliquot was analyzed by paper chromatography to determine the chain length of oligo [d(p[^{3}H]A)]. Radioactivity pattern of the product after incubation with dATP (——) and 3′-dATP (– – –) (left), or with ATP (——) and βaraATP (– – –) (right). The bars mark the positions of the authentic compounds.

oligo [d(pA)$_3$] with 3′-dATP or ATP only tetranucleotide products are formed.

These data demonstrated that 3′-dATP and ATP are 2 selective inhibitors for TdT; they are inactive in DNA-synthesizing systems both with DNA polymerase α or β as enzyme [9,19].

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