THE ISOLATION OF A HIGH MOLECULAR WEIGHT TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE FROM CALF THYMUS*

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SUMMARY: A new terminal deoxynucleotidyl transferase (TDT)[1] has been isolated from calf thymus of higher molecular weight than that originally isolated by Bollum (1962). The enzymes are probably metabolically related as one or the other is found depending on the purification procedure. However a direct conversion from one to the other has not been achieved. Although most of the enzymatic properties are very similar in contrast the new TDT also has the ability to use a template. This suggests that TDT's may be proteolytic degradation products of template-requiring DNA polymerases. Thus the finding of TDT's in a variety of rapidly metabolizing cells could be due to the uncovering of proteolytic activity rather than the synthesis of a new class of template-independent polymerases.

INTRODUCTION

Terminal deoxynucleotidyl transferase (TDT)[1] catalyses the primer-dependent but template independent polymerization of deoxynucleoside 5'-triphosphates. First detected in calf thymus extracts and purified by Bollum (Bollum, 1962), similar activities have recently been reported in various normal and malignant human cells grown in culture (Srivastava, 1974) and isolated from the whole blood cells of patients with acute lymphocytic leukemia (McCaffrey et al., 1973) or with chronic myelogenous leukemia in

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[1] In this paper TDT1 refers to the enzyme originally isolated by Bollum and TDT2 to the higher molecular weight enzyme described here. Other abbreviations used are: CCC DNA for covalently-closed circular DNA, OC DNA for open circular DNA, pOHMB for p-Hydroxymercuribenzoate, NEM for N-ethylmaleimide and SDS for sodium dodecyl sulfate. pT_{4-5} refers to a mixture of pT_4 and pT_5, dNTP to the deoxynucleoside triphosphates.

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blast crisis (Sarin and Gallo, 1974) and from the periferal blood leukocytes of a patient with chronic myeloblastic leukemia (Bhattacharyya, 1975). Although the in vivo function of TDT is unknown, speculation has centred on its apparent relation to the thymus, suggesting a function in the immune system (Baltimore, 1974). Its use as a diagnostic tool for certain leukemic states has also been proposed (Sarin and Gallo, 1974).

This communication describes the isolation and characterization of a second TDT from calf thymus, distinct from Bollum's enzyme, with a higher molecular weight. However, they may be related in that depending upon the experimental conditions used for purification either TDT1 or TDT2 (but not both) is found.

MATERIALS AND METHODS

The isolation of TDT2 uses many of the purification steps described by Bollum (Bollum, 1968) with the order rearranged to facilitate the purification. At no stage was a low pH (i.e. <7.0) incubation as described by Bollum included. TDT activity was measured (Bollum, 1968) at 37 °C with pT_{4-5} as primer in reaction mixtures containing 40 mM NaCadodylate pH 7.0, 1 mM dithiothreitol, 1 mM dNTP and either 10 mM $MgCl_2$ or 1 mM $CoCl_2$. DNA polymerase (which copurifies with TDT during the early stages of purification and can be used as a marker for TDT) was detected by an ethidium fluorescence assay (Morgan and Pulleyblank, 1974). The reaction mixture contained 50 mM KP_i pH 7.5, 1 mM of each dNTP, 10 mM $MgCl_2$, and 1 A_{260} of heat-denatured calf thymus DNA. 10 µl samples were added to 2 ml of the high pH ethidium assay mixture. The product is mostly covalently-linked complementary DNA as indicated by the return of fluorescence after a heating and cooling cycle.

The modified procedure is as follows: 450 g of calf thymus were suspended in 50 mM Tris HCl pH 7.5, 100 mM NaCl, 1 mM EDTA and disrupted using a Colloid Mill (Gifford-Wood, Hudson, New York). After filtration through cheesecloth and removal of chromatin by centrifugation (23,000g, 20 minutes) TDT was precipitated by the addition of solid $(NH_4)SO_4$ to 50% saturation and

centrifuged at 8,000 x g for 15 minutes. The pellet was suspended in 0.2 M KP_i pH 7.5, 1 mM 2-mercaptoethanol (Buffer B) and the ionic strength lowered to that of Buffer B with the addition of 50 mM KP_i pH 7.5, 1 mM 2-mercaptoethanol. Nucleic acid was removed with DEAE cellulose equilibrated in Buffer B (Bollum, 1968) by a batchwise procedure. After the removal of absorbant by filtration, the filtrate was diluted 1:4 with 1 mM 2-mercaptoethanol and protein absorbed to phosphocellulose (500 ml packed volume) for chromatography (Bollum, 1968).

Fractions with DNA polymerase activity were concentrated, resuspended in 0.1 M KP_i pH 7.5, 1 mM 2-mercaptoethanol and after dialysis against the same buffer, chromatographed on Sephadex G-100 (Bollum, 1968). TDT activity was pooled and chromatographed over a single-stranded calf thymus DNA-agarose column (Schaller et al., 1972) and eluted with a linear KCl gradient in 25 mM KP_i pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 5% (v/v) glycerol (TDT2 activity eluted as a single peak at ~65 mM KCl). Subsequent purifications over Sephadex G-100 with this same buffer were at various KCl concentrations. For comparative purposes TDT1 was isolated by the normal procedure up to and including Sephadex G-100 chromatography (Bollum, 1968). One unit of TDT activity incorporates 1 nmole of dNTP per hour at 37°C.

Heat denaturation of TDT2 was performed at 50°C in 25 mM KP_i pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 50% (v/v) glycerol. Endonuclease activity was measured fluorometrically (Morgan and Pulleyblank, 1974) with PM2 CCC DNA as substrate in 30 mM KP_i pH 7.5, 10 mM $MgCl_2$, 1 A_{260} PM2 DNA at 37°C. Exonuclease activity was also measured fluorometrically with heat denatured calf thymus DNA as substrate in a reaction mixture containing 20 mM KP_i pH 7.5, 10 mM $MgCl_2$, 1 A_{260} DNA. One unit hydrolyses 1 nmole of DNA phosphate per hour at 37°C.

SDS polyacrylamide gel electrophoresis was performed as described by Weber and Osborn, 1969.

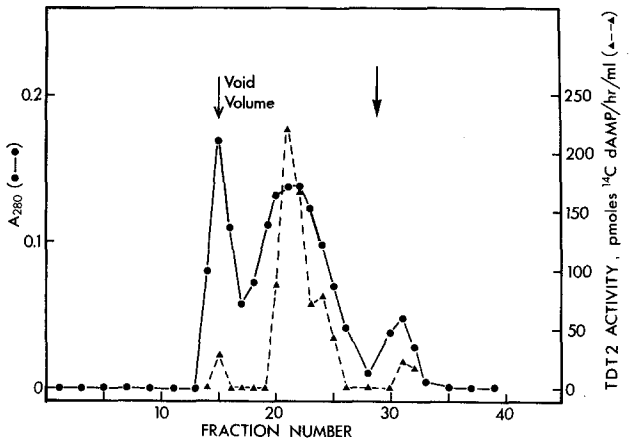

Figure 1. Chromatography of TDT2 activity over Sephadex G-100 in 25 mM KP_i pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 5% glycerol, 0.5 M KCl. The heavy arrow indicates the elution position of TDT1. Both activities were assayed as described in Materials and Methods with Mg^{++} dATP as substrate.

RESULTS

During attempts to isolate TDT1 a second, distinct TDT activity was isolated when the purification procedure was modified as described in Materials and Methods. With this procedure all the TDT was of a higher molecular weight than TDT1 (32,360 daltons, Chang and Bollum, 1971).

Figure 1 shows the elution profile of this activity on Sephadex G-100. The excluded peak is DNA and the elution position of TDT1 is marked by the arrow. This new activity has a much higher molecular weight than TDT1 and its elution position is not changed by the addition of KCl to the eluting buffer up to 1.0 M or increasing the buffer concentration to 0.1 M KP_i. Calibration of the G-100 column shows the activity to be included to a slightly greater extent than *E. coli* DNA polymerase 1 (105,000 daltons) but eluting well before bovine serum albumin (68,000 daltons). SDS polyacrylamide gel electrophoresis of TDT2 (figure 2) shows essentially one peak of 79,000 daltons with *E. coli* RNA polymerase subunits as markers. Contamina-

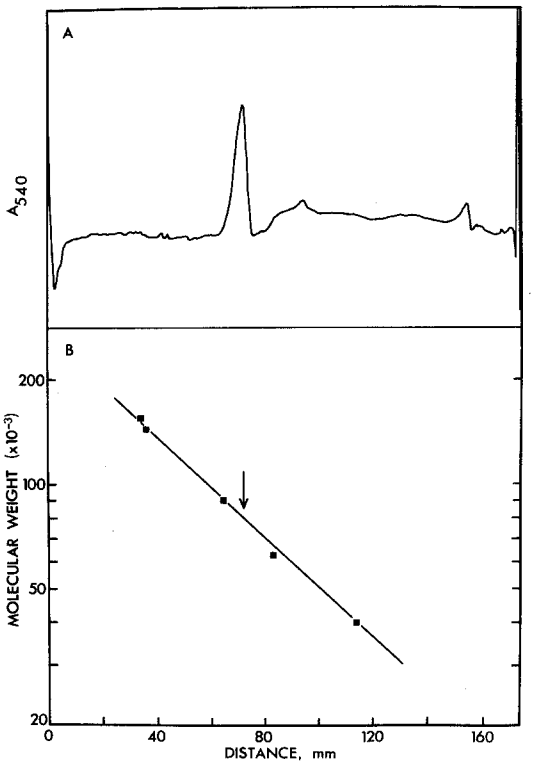

Figure 2. A. Tracing of a SDS polyacrylamide gel of the TDT2 activity isolated by Sephadex G-100 chromatography in Figure 1 deliberately incompletely destained (see Results). B. Determination of the molecular weight of the main protein band of A (indicated by the arrow) with E. coli RNA polymerase subunits as internal standards.

tion by TDT1 should give a band at 26,500 daltons (β subunit, Chang and Bollum, 1971); however even when the gel was deliberately not completely destained, no discernable band at the expected position was detectable. These data suggest that TDT2 is a single polypeptide of 79,000 daltons distinct from TDT1.

We have been able to obtain fractions with a specific activity of 10,000 units/mg (with dATP as substrate in the presence of Mg^{++}) which also suggests that this activity is not due to a slight contamination with TDT1

(which is not visible in SDS gels and would have to chromatograph anomalously on G-100). Although this activity can be repeatedly chromatographed on G-100, eluting at the same position as in figure 1, it appears unstable, losing activity rapidly upon manipulation. The TDT2 used in the subsequent experiments was stable stored in 50% (v/v) glycerol at -20°C for 1 year.

TABLE 1

Substrate Specificity of TDT2

dNTP	nmoles dNTP incorporated per mg protein per hour at 37°C	
	Mg^{++}	Co^{++}
dATP	245 (1.00)	276 (1.13)
dGTP	400 (1.63)	564 (2.30)
dCTP	32 (0.13)	3525 (14.39)
dTTP	25 (0.10)	4033 (16.46)

TDT activity was assayed as described in Materials and Methods with $^{pT}4\text{-}5$ (50 μM final concentration) as primer. Incorporation relative to Mg^{++} dATP (1.00) given in brackets. All values are the average of four determinations.

Some properties of TDT2 are summarized in Tables 1 and 2. With respect to substrate specificity TDT2 resembles TDT1 preferring purine dNTPs with Mg^{++} present and pyrimidine dNTPs with Co^{++} present. However unlike TDT1 there seems to be slightly better polymerization with Co^{++} even with purine dNTPs. Table 2 compares some properties of TDT2 with TDT1. Both activities depend on exogenously added primer and are inhibited by NEM, pOHMB and SDS to about the same extent when these are included in the reaction mixture.

TABLE 2

COMPARISON OF THE PROPERTIES OF TDT1 AND TDT2

Conditions *	TDT2 nmoles dNTP incorporated/ mg protein/hour	%	TDT1 nmoles dNTP incorporated/ mg protein/hour	%
Complete reaction mixture	245	100	1,878	100
Primer omitted	0	0	0	0
Primer omitted + activated calf thymus DNA	329	134	90	5
Complete + 0.01% SDS	27	11	97	6
Complete + 0.1% Triton X-100	188	77	1,407	75
Complete + 5 mM NEM	20	8	428	23
Complete + 0.1 mM pOHMB	189	77	1,214	65
DNA polymerase activity	43.3	-	0	-
pH optimum (cacodylate buffer)	7.5	-	7.0	-
Cacodylate replaced by Tris HCl or Tris Acetate pH 7.5	12	5	94	5
S Value	4-5		3.65	

* Assay conditions as outlined in Materials and Methods with Mg^{++} and dATP and 50 μM pT_{4-5} as primer. Activated calf thymus DNA (Aposhian and Kornberg, 1962) was substituted for pT_{4-5} as primer at 120 μM. Most values are the average of four determinations. Conditions for sedimentation and markers are given in Chang and Bollum (1971). DNA polymerase activity was assayed by the fluorescence assay described in Materials and Methods.

The presence of Triton X-100 has a similar effect on both activities (Chang and Bollum, 1971; Kato et al., 1967). The use of Tris buffer at pH 7.5 drastically reduces the observed activity for both TDT1 and TDT2. The two enzymes have different pH optima (7.5 for TDT2 compared to 7.0 for TDT1)

and consistent with the molecular weights TDT2 sediments at 4-5S, whereas TDT1 sediments at 3.65S.

TDT and DNA polymerase activities were assayed by two procedures, either the incorporation of radiolabelled dNTPs into TCA insoluble material or the ethidium fluorescence assay. The latter is particularly useful for confirming DNA polymerase in the presence of any possible TDT. Even in the presence of all 4 dNTPs, the product of TDT is a random polymer which shows about 5% the fluorescence of duplex DNA under the high pH ethidium fluorescence assay (Morgan and Pulleyblank, 1974). This small amount of fluorescence disappears after a heat denaturation step. Using denatured calf thymus DNA as template and primer for DNA polymerase the product is a hairpin duplex and the increase in fluorescence of samples with time as duplex is formed is paralleled by the increase in fluorescence with time after heat denaturation, (Kornberg, 1974; Coulter et al., 1974). Although TDT activity was barely detectable in crude extracts after centrifuging down the chromatin, in reconstruction experiments when purified TDT1 or TDT2 were added back to the extracts at a level of about 4 mg/ml of protein from the crude extract in the reaction mixture, TDT1 was completely inhibited and TDT2 inhibited to the extent of 50%. The high levels of RNA present probably bind the enzymes making any quantitative assays impossible. However depending on the purification procedure TDT activity was observed to appear over a period of weeks in certain fractions stored in ice. In a procedure closely resembling Bollum's (1974) except that the ammonium sulfate precipitation (0-45% saturation fraction) preceded the phosphocellulose absorption (at pH 7.6 rather than 6.5) the hydroxylapatite column gave a peak of DNA polymerase lacking TDT. After two months of storage in the eluting buffer (~0.25 M KP_i pH 7.6, 1 mM 2-mercaptoethanol), it had developed a high level of TDT activity with a lower level of DNA polymerase activity. Attempts to accelerate the conversion of the remaining polymerase to TDT using widely varying levels of trypsin, chymotrypsin and pronase

were unsuccessful. The lower pH of 6.5, conditions used in certain steps of Bollum's procedure, is known to favor the action of cathepsins (Fruton, 1960), and could account for the production of TDT1 in one case and TDT2 in the other.

TDT2 was also found to have template guided DNA polymerase activity (Table 2) which was not due to contaminating DNA polymerase α which is well separated from TDT2 on Sephadex G-100. The TDT2 was rerun on Sephadex G-100 and DNA polymerase activity still chromatographed with the TDT2 although there was now no DNA polymerase activity in the excluded portion of the column. The DNA polymerase was readily distinguished from TDT using the ethidium fluorescence assay as above. The TDT activity was sensitive to mercurials as is DNA polymerase α (Weissbach, 1975), and thus is not DNA polymerase β. It's molecular weight excludes DNA polymerase γ.

The most purified TDT2 contained trace amounts of endonuclease (for TDT2 at 60 μg/ml PM2 CCC DNA was converted to OC DNA in 5 minutes whereas 1 ng/ml of pancreatic DNase took 10 minutes) which showed the same activity profile on Sephadex G-100 as did the TDT2. Also exonuclease activity was detectable at 13 units/mg of protein. The heat inactivation profiles were different for the 3 activities, TDT2 being completely inactivated by 5 minutes incubation at 50°C; the exonuclease was >90% inactivated after 30 minutes at 50°C and endonuclease <10%. If the endonuclease and exonuclease are contaminants they occur at very low levels assuming similar specific activities to other known enzymes.

DISCUSSION

In contrast to the two TDT activities previously reported with identical molecular weights, (Marcus et al., 1976), TDT1 and TDT2 are distinctly different proteins as determined by their physical properties. Their TDT activity varies in a parallel fashion under a variety of conditions. The higher molecular weight of TDT2 and its ability to act both as a DNA polymerase and TDT, the latter activity arising on storage of semi-purified fractions

of calf thymus DNA polymerase, are highly suggestive that TDTs are derived from DNA polymerases by proteolysis. It remains to be shown that a purified DNA polymerase can be converted to a TDT. Chang and Bollum (1972) reported that antibody directed against DNA polymerase α (6S-8S DNA polymerase) does not cross-react with TDT1 although inhibition of DNA polymerase activity from a variety of mammalian sources was observed. Kung et al., (1976) have recently demonstrated that anti-TDT1 antibody does not inhibit DNA polymerase α activity. However no firm conclusions can be made from these studies as to the relatedness of the proteins since the polymerase antigenic determinants may have been modified or removed by proteolysis. We suggest that the presence of TDT may be artifactual, and a physiological role such as generating antibody diversity (Baltimore, 1974) would seem unlikely.

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