Biochemistry of Terminal Deoxynucleotidyltransferase: Identification, Characterization, Requirements, and Active-Site Involvement in the Catalysis of Associated Pyrophosphate Exchange and Pyrophosphorolytic Activity†

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ABSTRACT: Terminal deoxynucleotidyltransferase (TdT) has been found to catalyze both pyrophosphate exchange and pyrophosphorolysis reactions. Both reactions are strongly inhibited by antiserum to TdT. The reactions require the presence of a divalent cation, a single- or double-stranded oligomeric or polymeric DNA or RNA, and deoxyribonucleoside triphosphates (for PP_i exchange only). Of the three divalent cations tested, Mg^{2+} and Co^{2+} are equally effective, while Mn^{2+} neither is used for catalysis nor inhibits the Mg^{2+}-catalyzed reactions. Ribonucleoside triphosphates have been found to support the PP_i exchange reaction to a minor extent and have no inhibitory effect on the catalysis mediated by dNTPs. Inhibition studies, using SH group inhibitors, Zn chelator, and a substrate binding site specific reagent, revealed that PP_i exchange and pyrophosphorolysis reactions may be distinguished by differences in their sensitivity to inhibition by various reagents. While the PP_i exchange reaction is strongly inhibited by sulfhydryl reagents, *o*-phenanthroline, and pyridoxal phosphate, the pyrophosphorolysis reaction is insensitive to these reagents. In addition, the pyrophosphorolysis reaction is also found not to require a free 3′-OH terminus of a primer. This difference in the susceptibility of the two reactions indicates that discrete active-site structures exist in TdT which catalyze PP_i exchange and pyrophosphorolysis reactions.

Terminal deoxynucleotidyltransferase (TdT)[1] is a DNA polymerase unique in its ability to synthesize DNA in the absence of template direction. Some of the additional differences in the properties of TdT from other template-dependent DNA polymerases are (a) an affinity for ribonucleoside triphosphates in general and ATP in particular and (b) the highest affinity for Mn–dATP and yet the lowest turnover rate, resulting in the inhibition of polymerization of other dNTPs (Modak, 1978, 1979; Müller et al., 1978). In spite of extensive biochemical studies, the biological role of TdT has remained unknown. Nevertheless, TdT has been a very useful reagent in the preparation of oligo- and polydeoxynucleotides of defined sequences (Bollum, 1974) and has found important application in recombinant DNA technology. Certain similarities of catalysis between TdT and other replicative DNA polymerases permit the use of this relatively small enzyme as a model system in the study of dNTP binding, as well as in the determination of the role for intrinsic zinc in catalysis. With the exception of DNA polymerase β (Chang & Bollum, 1973; Wang et al., 1974; Tanabe et al., 1979), all of the known replicative DNA polymerases appear to be able to catalyze PP_i exchange and pyrophosphorolysis reactions (Loeb, 1974; Chang & Bollum, 1973; Seal & Loeb, 1976; Srivastava & Modak, 1980a). With respect to TdT, no information is available in the literature except for a footnote in a published paper (Kato et al., 1967) indicating the apparent absence of the PP_i exchange reaction. In our study of the substrate binding site on TdT using pyridoxal 5′-phosphate as a specific reagent [Modak, 1967a,b; M. J. Modak, unpublished experiments], we have examined the ability of purified TdT to catalyze PP_i exchange and pyrophosphorolysis reactions. We describe in this report the association of these two activities with TdT, conditions and requirements for their expression, and probable active-site involvement for catalysis of these activities.

Experimental Procedures

Ribo- and deoxyribonucleoside triphosphates, synthetic template–primers, and oligomeric primers were purchased from P-L Biochemicals. [^{3}H]dGTP (sp act. 8.4 Ci/mmol), [^{3}H]GTP (sp act. 17 Ci/mmol), and sodium [^{32}P]pyrophosphate (sp act. 1.6 Ci/mmol) were obtained from New England Nuclear. Phenylglyoxal was purchased from K & K Laboratories, *o*-phenanthroline from Matheson Coleman and Bell, and pyridoxal phosphate from Sigma Chemical Co. PEI–cellulose plates were purchased from VWR Scientific Inc. Near homogeneous preparations of TdT obtained from calf thymus extracts were purified as described before (Modak, 1979) and had a specific activity of 9600 units/mg with oligo$(dA)_{12-18}$, 20 μM dGTP, and Mn^{2+} as assay components in a Tris-buffered assay mixture. Substitution of Mg^{2+} in this system and increasing the dGTP concentration to 1 mM resulted in a specific activity of 40 000 units/mg. A partially purified batch of TdT (sp act. > 120 000 units/mg) prepared by Dr. R. Ratliff of Los Alamos Scientific Laboratories, Los Alamos, was kindly made available to us by Dr. Allen Silverstone. We have further purified this preparation by use of oligo(dT)–cellulose chromatography. Under our assay conditions the original and oligo(dT)-purified TdT had specific activities of 9000 and 10 000 units/mg, respectively. This enzyme was used in the identification of PP_i exchange and

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[1] Abbreviations used: TdT, terminal deoxynucleotidyltransferase; dNTPs, deoxynucleoside triphosphates; rNTPs, ribonucleoside triphosphates; DTT, dithiothreitol; Cl_3AcOH, trichloroacetic acid; PP_i, pyrophosphate; PG, phenylglyoxal; PyP, pyridoxal 5′-phosphate; *o*-phe, *o*-phenanthroline; NEM, *N*-ethylmaleimide; PCMB, *p*-(chloromercuri)-benzoate; AMV, avian myeloblastosis virus.

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pyrophosphorolysis activities and in antibody neutralization assays along with our preparation.

Antiserum to purified calf TdT, raised in mouse, rat, and rabbit against the same antigen, was generously provided by Dr. A. Silverstone of this institute. Two additional samples of anti-TdT were kindly donated by Dr. L. Loeb of the University of Washington, Seattle, and Dr. B. I. S. Srivastava of Roswell Park Memorial Institute, Buffalo. Activated DNA was prepared as described by Aposhian & Kornberg (1962).

Synthesis of the Dideoxy-Terminated Primer. Oligo-$(dA)_{12-18}$ primers ending with a dideoxyguanosine residue were synthesized in a final volume of 1.0 mL containing the following components: 50 mM Hepes buffer, pH 7.8, 1 mM dithiothreitol (DTT), 25 μg of oligo$(dA)_{12-18}$, 0.01% albumin, 20 μM dideoxy-GTP, 5 mM $MgCl_2$, 100 mM KCl, and 50 units of purified TdT. The reaction mixture was incubated at 37 °C for 30 min. The reaction was stopped by heating to 90 °C for 15 min in a hot water bath, and then the mixture was chilled immediately. Synthesis as monitored by [^{3}H]-dGMP incorporation with dideoxy-G-terminated oligo$(dA)_{12-18}$ was less than 1 pmol.

TdT Assays. Standard TdT assays contained, in a final volume of 100 μL, 50 mM Hepes buffer, pH 7.8, 1 mM DTT, 50 μg of bovine serum albumin, 0.5 μg of the primer, 20 μM [^{3}H]dGTP adjusted to a final specific activity of 1000 cpm/pmol, 5 mM $MgCl_2$, 100 mM KCl, and 1–5 units of TdT. The reaction mixtures were incubated at 37 °C for 15 min and the reactions terminated by the addition of 5% (w/v) trichloroacetic acid (Cl_3AcOH) containing 0.01 M sodium pyrophosphate. The acid-insoluble precipitate was collected on Whatman GF/B glass fiber filters, washed extensively with Cl_3AcOH containing sodium pyrophosphate, with water, and finally with ethanol, dried, and counted in a toluene-based scintillation fluid (Modak, 1976c).

Pyrophosphate Exchange and Pyrophosphorolysis Assays. Pyrophosphate exchange assays, in a final volume of 100 μL, contained 50 mM Hepes buffer, pH 7.8, 5 mM DTT, 2.5 μg of the template and/or primer, 1 mM sodium [^{32}P]pyrophosphate (80–200 dpm/pmol), 40 μM unlabeled substrate triphosphates, 2 mM $MgCl_2$, 20 mM KCl, and 5–10 units of TdT. Incubations at 37 °C were carried out for 60 min, and the reactions were terminated by the addition of 1 mL of 1 N perchloric acid (PCA) containing 50 mM sodium pyrophosphate followed by 100 μL of 10% activated charcoal (Norit). The tubes were kept at 0 °C for 10 min. The Norit was then transferred to GF/B glass fiber filters, washed extensively with water, and dried, and the radioactivity was determined by using a toluene-based scintillation fluid.

Pyrophosphorolysis assays were identical with pyrophosphate exchange assays except that the substrate triphosphates were omitted. The rest of the steps were the same as those described above.

Some of the experimental results were normalized for ready comparison since the experiments were carried out with different batches of enzyme preparations.

Isolation and Characterization of PP_i Exchange and Pyrophosphorolysis Reaction Products. The reactions were carried out as described above and stopped by the addition of 1 N PCA. After 10 min at 0 °C, the reaction mixture was centrifuged in an Eppendorf centrifuge at top speed for 2 min and 10 μL of the supernatant was directly spotted on a 0.1-mm PEI–cellulose F thin-layer chromatography (TLC) plate and chromatographed with 0.75 M potassium phosphate, pH 3.5, in the first dimension. Free [^{32}P]PP_i was detected and separated by cutting the plate, and the rest of the chromatogram was run in the second dimension with the same solvent along with nonradioactive standard markers. The products were detected and identified by autoradiography. The values obtained in this manner were quite comparable with those from the Norit adsorption assay method; for simplicity, the latter assay is therefore employed for all the experiments.

Alternatively, the reaction mixtures, after PCA addition and centrifugation, were loaded onto 0.5-cm 10% Norit columns separately and washed extensively of free PP_i with cold distilled water, pH 4.5, and the nucleotides were eluted with 50% ethanol–0.3 M ammonium hydroxide, lyophilized, dissolved in water, spotted, and analyzed on a PEI–cellulose plate in only one dimension. Results similar to those shown in Figure 2 are obtained except that some breakdown of the adsorbed product (into diphosphate) seemed to occur (data not shown).

Table I: Effect of TdT Antiserum on the Catalysis of Synthesis, Pyrophosphate Exchange, and Pyrophosphorolysis by TdT

enzyme prepn	preincubn with[a]	dilution	% act.[d] synthesis	% act.[d] PP_i exchange	% act.[d] pyrophosphorolysis
enzyme I[b]	normal rat serum	1:1 to 1:5	100	100	100
enzyme II[c]	normal rabbit serum	1:1 to 1:5	119	87	99
enzyme I	TdT antiserum I	1:5	26.7	26.2	37.9
		1:10	49.3	30.6	47.1
	TdT antiserum II	1:5	16.7	11.4	19.3
		1:10	25.8	22.9	24.6
	TdT antiserum III	1:5	11.0	11.6	16.8
	TdT antiserum IV	1:5	49.9	44.8	47.2
	TdT antiserum V	1:5	47.4	70.9	64.7
enzyme II	TdT antiserum I	1:5	27.1	43.2	39.6
		1:10	70.7	58.7	61.4
	TdT antiserum II	1:5	69.0	79.4	62.9
		1:10	81.9	90.5	78.6
	TdT antiserum V	1:5	39.2	30.6	37.6

[a] Preincubation was carried out with the enzyme fractions at 37 °C for 30 min with equal volumes of desired antiserum at proper dilution. The various antisera designations I–V are as follows: antiserum I was a rabbit anti-TdT donated by Dr. L. Loeb; antisera II–IV were anti-TdT prepared in mouse, rat, and rabbit, respectively, by Dr. A. Silverstone; antiserum V was a rabbit anti-TdT provided by Dr. B. I. S. Srivastava. [b] Enzyme I was isolated and purified as described by Modak (1979). [c] Enzyme II was purified by Dr. Ratliff and made available by Dr. Silverstone (see Experimental Procedures). [d] One hundred percent activity values for enzymes I and II for synthesis, PP_i exchange, and pyrophosphorolysis reaction were 800, 25, and 19 pmol, respectively.

Results

PP_i Exchange and Pyrophosphorolysis by TdT. Both reactions catalyzed by TdT were linear with time for at least 2 h (Figure 1), and reaction rates as a function of enzyme concentration were proportional (data not shown). Both reactions were inhibited to the same extent as the polymerization reaction when treated with anti-TdT serum, indicating that the catalysis of the enzymatic activities was effected by the same enzyme protein (Table I). Furthermore, the catalysis of these activities by an independently purified enzyme preparation and their subsequent inhibition, when challenged with several different antisera preparations, provided strong support for the association of these activities with TdT.

The reaction products of both reactions were determined to be dNTPs by TLC analysis of the products (Figure 2).

General Properties of PP_i Exchange and Pyrophosphorolysis Reactions. The ability of terminal deoxy-

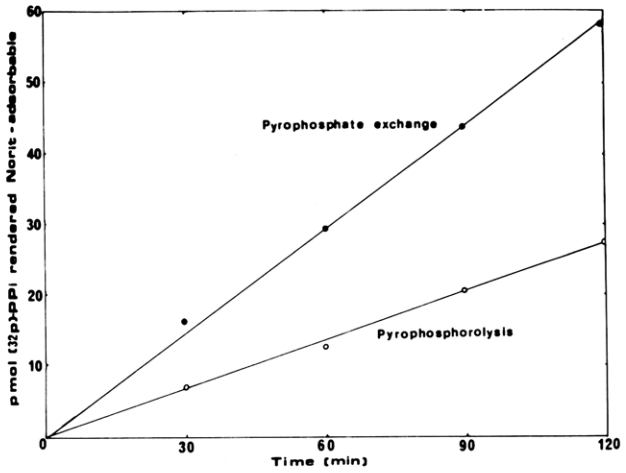

FIGURE 1: Kinetics of pyrophosphate exchange and pyrophosphorolysis catalyzed by TdT. Assays were performed by using oligo(dA)$_{\sim 15}$ as a primer as described under Experimental Procedures.

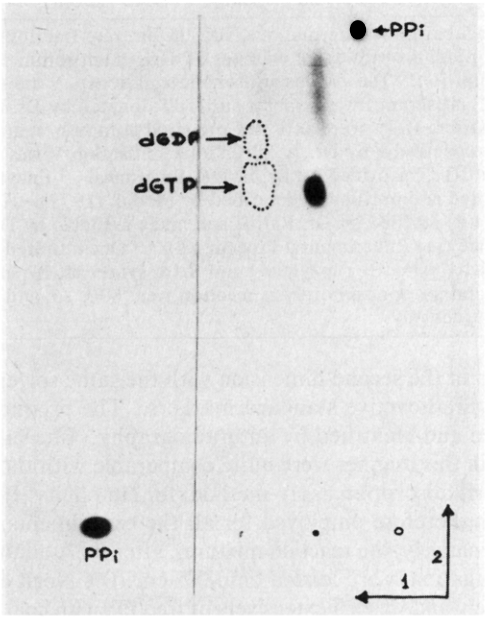

FIGURE 2: TLC separation and autoradiographic analysis of pyrophosphate exchange and pyrophosphorolysis reaction products. Preparation and analysis of the products were done as described under Experimental Procedures. The bulk of the free [^{32}P]PP$_i$ was removed in the first dimension, permitting an unambiguous detection of [^{32}P]dNTP product in the second dimension. The exposure timings for the first and second dimensions were 7 min and 48 h, respectively. The positions of standard markers are indicated.

Table II: Relative Magnitude of Synthesis, Pyrophosphate Exchange, and Pyrophosphorolysis Catalyzed by TdT Using Different Primers[a]

	pmol				
	[^{3}H]dGMP incorpd[b]			[^{32}P]PP$_i$ rendered Norit adsorbable	
primer	−PP$_i$	+PP$_i$	% inhibn	PP$_i$ exchange	pyrophosphorolysis
none				3.0	1.0
oligo(dA)	1357.2	143.9	89.4	55.4	25.8
activated DNA	137.9	11.7	91.5	37.2	30.6
poly(dT)	39.6	0.8	98.0	16.8	6.3
poly(dA-dT)	642.0	12.4	98.1	15.8	30.5

[a] Assays were done as described under Experimental Procedures. [b] Polymerization was monitored by using [^{3}H]dGTP as the sole substrate.

Table III: Primer Concentration Dependent Pyrophosphate Exchange and Pyrophosphorolysis Catalyzed by TdT[a]

	pmol	
oligo(dA) concn (μg)	PP$_i$ exchange	pyrophosphorolysis
0.0	<1	<1
0.25	<1	<1
0.5	<1	<1
1.0	6.2	5.6
1.25	8.6	7.5
2.5	48.2	23.1
5.0	55.2	25.5
7.5	57.7	26.2

[a] Assays were done as described under Experimental Procedures.

Table IV: Divalent Cation Utilization for Synthesis, Pyrophosphate Exchange, and Pyrophosphorolysis Catalyzed by TdT[a]

	pmol		
divalent cation	synthesis[b]	PP$_i$ exchange[b]	pyrophosphorolysis[c]
Mg^{2+}	492.8	63.4	44.1
Mn^{2+}	1726.1	<1	<1
Co^{2+}	32.8	48.9	32.8

[a] All assays were done as described under Experimental Procedures. [b] Optimal concentration of the metal ion for the synthetic reaction was determined to be 5 mM for Mg^{2+}, 1 mM for Mn^{2+}, and 2 mM for Co^{2+}, and the same concentration is used. [c] These reactions were carried out with the individual metal ion at 2 mM concentration.

nucleotidyltransferase to catalyze polymerization (synthesis), pyrophosphate exchange, and pyrophosphorolysis reactions using different primers is shown in Table II. Both reactions required a primer molecule for the expression of these activities; oligo(dA)$_{15}$ was the most effective. Polymeric primers, e.g., poly(dT) or poly(dA), appeared to be less effective for supporting these reactions. In most cases, the efficiency of the PP$_i$ exchange reaction was greater than that of pyrophosphorolysis. Indeed, in the PP$_i$ exchange reaction some pyrophosphorolytic reaction may occur (Deutscher & Kornberg, 1969; Seal & Loeb, 1976); we have not applied any correction to the PP$_i$ exchange numbers reported here. The ability of various primers to support PP$_i$ exchange and pyrophosphorolysis reactions did not vary significantly and did not seem to correlate with the relative utilization of these primers for DNA synthesis. However, the amount of primer required to detect both enzymatic activities was at least 10–20 times greater than that required for the polymerization reaction (Table III). It was interesting to note that the response of TdT to increasing primer concentration was sigmoidal with respect to catalysis of PP$_i$ exchange and pyrophosphorolysis, while such a response was not observed for the polymerization reaction. The implications, if any, of this finding are not apparent at the present time.

Metal Requirements. Both the PP$_i$ exchange and pyrophosphorolysis reactions require the presence of a divalent cation. Of the three different metal ions, Mg^{2+}, Mn^{2+}, and Co^{2+}, which are known to support the polymerization reaction, only Mg^{2+} and Co^{2+} were effectively used for the catalysis of PP$_i$ exchange and pyrophosphorolysis reactions (Table IV). Optimal concentrations of both of the divalent cations were found to be 2 mM. Manganese, which is an excellent activator

Table V: Relative Magnitude of Synthesis, Pyrophosphate Exchange, and Pyrophosphorolysis Catalyzed by TdT with Deoxy- and Ribotriphosphates

additions	pmol synthesis −PP_i	pmol synthesis +PP_i	pmol [^{32}P]PP_i rendered Norit adsorbable[c]
			24.9
dGTP	384.7	57.9 (15.1)[d]	56.2
rGTP	38.7	9.6 (24.8)	33.2
dGTP + GTP[a]	152.3	45.9 (30.1)	71.4
rGTP + dGTP[b]	33.5	5.5 (16.4)	71.4

[a] Synthesis was monitored by [^{3}H]dGTP incorporation in the presence of nonradioactive 40 μM rGTP. [b] Synthesis was monitored by [^{3}H]rGMP incorporation in the presence of 40 μM nonradioactive dGTP. [c] Only nonradioactive substrate triphosphates were included, and the assays were done as described under Experimental Procedures. [d] Figures in parentheses represent percent activity.

Table VI: Synthesis, Pyrophosphate Exchange, and Pyrophosphorolysis with RNA Primers Catalyzed by TdT[a]

primer	pmol synthesis	pmol PP_i exchange	pmol pyrophosphorolysis
AMV 70S RNA·4S tRNA	<1	22.3	26.6
poly(rA)·$(rU)_9$	<1	10.7	20.5
poly(rA)	<1	10.1	10.3
$(dT)_7$·rA	<2	16.6	14.0

[a] The assays were carried out as described under Experimental Procedures.

of dNTPs in the polymerization reactions, neither is utilized nor inhibits these activities. All further studies were carried out with only Mg^{2+} as the effective divalent cation.

Pyrophosphate Exchange Reaction with Deoxy- and Ribonucleoside Triphosphates. Since TdT is known to bind all four dNTPs and rNTPs, the effect of various concentrations of different nucleotides on the PP_i exchange was measured. Results clearly indicate that all four dNTPs, at high or low concentrations, supported the PP_i exchange reaction. rNTPs supported the PP_i exchange reaction, albeit to a much smaller extent (see Table V; data for only GTP and dGTP are shown). Since ribotriphosphates are known to be strong inhibitors of dNTP polymerization catalyzed by TdT (Modak, 1978, 1979), it was interesting to see if the PP_i exchange reaction using dNTPs was inhibited by rNTPs. It is most interesting to note that the presence of both dGTP and rGTP produced an additive effect as far as the extent of PP_i exchange reaction is concerned (Table V).

RNA as Primer for PP_i Exchange and Pyrophosphorolysis Reactions. TdT does not utilize RNA oligomers or polymers as primers for the addition of deoxynucleotides (Bollum, 1974; Sarin & Gallo, 1974). It was most unusual, therefore, to note that both synthetic and natural RNAs appeared to support the pyrophosphorolysis reaction (Table VI). There is probably no PP_i exchange reaction catalyzed in the presence of RNA primers as suggested by the almost identical values of PP_i exchange and pyrophosphorolysis. Thus, avian myeloblastosis virus (AMV) 70S RNA, poly(rA)·$(rU)_9$, and poly(rA) alone, as well as $(dT)_7$ with an rA terminus, could support the pyrophosphorolysis reaction, although there was a clear lack of synthesis on these primers. Studies to be detailed below that utilize primer termini which lack a 3′-OH and yet support pyrophosphorolysis also indicate the nonrequirement for a free 3′-OH terminus of a primer for this catalysis.

Table VII: Effect of Various Site-Specific Reagents on Synthesis, Pyrophosphate Exchange, and Pyrophosphorolysis Catalyzed by TdT

preincubn with[a]	concn of inhibitor	pmol[c] synthesis[b]	pmol[c] PP_i exchange[d]	pmol[c] pyrophosphorolysis
Hepes buffer alone (control)		1718.1 (100.0)[e]	57.5 (100.0)	25.3 (100.0)
PG	200 μM	1675.2 (97.5)	56.7 (98.6)	25.6 (101.0)
PP_i	500 μM	277.3 (16.1)	19.4 (33.8)	20.9 (83.0)
o-phe	1 mM	94.5 (5.5)	28.5 (49.6)	19.2 (75.9)
NEM	5 mM	65.3 (3.8)	21.0 (36.6)	17.5 (69.2)
PCMB	200 μM	29.2 (1.7)	9.6 (16.7)	17.4 (68.8)

[a] Enzyme fractions were preincubated with the inhibitors at 37 °C for 15 min. [b] Synthesis was measured in the absence of 1 mM PP_i. [c] Oligo$(dA)_{12-18}$ was used as a primer, and the assays were done as described under Experimental Procedures. [d] Values obtained are not corrected for the intrinsic pyrophosphorolysis reaction in this assay. [e] Figures in parentheses represent percent activity.

Response of PP_i Exchange and Pyrophosphorolysis Catalysis to Various Site-Specific Reagents. The reagents that selectively react with a specific structural component of an enzyme protein have been identified (Salvo et al., 1976; Modak, 1976a,b; Papas et al., 1977). Inhibition of TdT by sulfhydryl reagents [e.g., *p*-(chloromercuri)benzoate or *N*-ethylmaleimide] and *o*-phenanthroline indicated requirements for a reduced sulfhydryl group and the availability of intrinsic zinc in the enzyme protein, respectively, for catalysis to occur (Bollum, 1974). Our recent studies on the pyridoxal 5′-phosphate reaction with various DNA polymerases (Modak, 1976a–c), including TdT (M. J. Modak, unpublished experiments), have identified this compound to be reactive with the amino acid residue at or near the substrate binding site. Therefore, the effect of these compounds on the catalysis of various enzymatic activities associated with TdT may be used to infer the involvement of or requirement for a particular structural feature of TdT for the catalysis of a particular reaction. The results presented in Table VII show the response of synthesis, PP_i exchange, and pyrophosphorolysis to the treatment with pyridoxal 5′-phosphate, *o*-phenanthroline, and two sulfhydryl reagents. PP_i exchange and polymerization reactions exhibited susceptibility to all of the reagents tested, indicating a requirement for structural involvement of various regions of TdT protein for the catalysis. An arginine-specific compound, phenylglyoxal, which has been shown to be a template site specific reagent for replicative polymerases (Srivastava & Modak, 1980b), was also included in this study and was found to have no effect on any of the three activities expressed by TdT. In contrast, the pyrophosphorolysis reaction required none of the above structural components of TdT as judged by the insensitivity of this catalytic reaction to various reagents. In fact, the residual activity that we observe for the PP_i exchange reaction in the presence of various reagents might very well be due to pyrophosphorolysis activity and in that case true inhibition of the PP_i exchange reaction may be almost total. The resistance of the pyrophosphorolytic reaction to *o*-phenanthroline was indicative of the nonrequirement of intrinsic zinc in this function. This result was somewhat surprising since zinc in TdT has been implicated in the formation of a coordinate complex with a 3′-OH terminus of the primer which was a prerequisite for polymerization (Chang & Bollum, 1970). In order to confirm the nonrequirement of zinc for the pyrophosphorolysis reaction, we used a primer blocked at its

Table VIII: Effect of Primer Modification on Synthesis, Pyrophosphate Exchange, and Pyrophosphorolysis Catalyzed by TdT

primer	pmol		
	synthesis	PP_i exchange	pyrophosphorolysis
oligo(dA)$_{15}$	519.7 (100.0)[b]	67.6 (100.0)	50.2 (100.0)
oligo(dA-ddG)$_{16}$[a]	4.5 (0.7)	39.6 (58.6)	25.6 (51.9)

[a] Oligo(dA-ddG)$_{16}$ was synthesized as described under Experimental Procedures. [b] Figures in parentheses represent percent activity.

3′-OH end by incorporation of a dideoxy-GTP residue in a pyrophosphorolysis reaction to determine its ability to support the catalysis. Results presented in Table VIII show that a significant amount of the pyrophosphorolysis reaction continues in spite of the fact that the synthetic reaction is limited to only ~1% of the control value. These observations indeed confirm that a free 3′-OH group may not be required for the pyrophosphorolysis reaction.

Discussion

In this paper we demonstrate that PP_i exchange and pyrophosphorolysis reactions are catalyzed by TdT, in addition to the synthetic reaction. Concurrent inhibition of all the enzyme activities associated with two independently prepared TdT preparations and their inhibition when challenged with anti-TdT serum obtained from different sources confirm that these enzyme activities are indeed TdT associated. The presence of these activities in TdT strongly suggests that the mechanism of catalysis by TdT is similar to those of replicative polymerases and that the polymerization is not kinetically irreversible (Kato et al., 1967). Thus, with the exception of DNA polymerase β (Chang & Bollum, 1973; Wang et al., 1974; Tanabe et al., 1979), most of the DNA polymerizing enzymes are capable of catalyzing PP_i exchange and pyrophosphorolysis reactions.

A detailed characterization of and requirements for the catalysis of PP_i exchange and pyrophosphorolysis reactions revealed several interesting features and some unique properties of these reactions. Some of the salient features are summarized below. In spite of the known utilization of Mg^{2+}, Mn^{2+}, and Co^{2+} as cofactors for the synthetic reaction (Bollum, 1974; Marcus et al., 1976), Mn^{2+} is totally inactive in the catalysis of PP_i exchange and pyrophosphorolysis. Similarly, while Mg^{2+} and Co^{2+} exhibit significant differences in their effectiveness for the polymerization reaction, both divalent cations are equally effective in PP_i exchange and pyrophosphorolysis reactions. A variety of single- and double-stranded DNA primers were used for these reactions, and there does not appear to be any correlation between the extent of the relative ability of a particular primer to support DNA synthesis and its ability to support PP_i exchange and pyrophosphorolysis reactions.

Another interesting and quite unusual feature is the ability of various synthetic or natural RNAs to support the pyrophosphorolytic reaction although RNAs are not accepted as primers for the polymerization reaction (Bollum, 1974; Sarin & Gallo, 1974). There is no preference for a type of dNTP present during the catalysis of PP_i exchange; all four are equally effective. rNTPs, however, appear to be rather poor participants in the PP_i exchange reaction. Yet, when both rNTPs and dNTPs are present, they seem to exert an additive effect. For the polymerization reaction, the presence of a substrate binding site, a primer binding site, an intrinsic zinc, and reactive sulfhydryl groups on TdT is required (Bollum, 1974). Primer binding function is further proposed to require participation of intrinsic zinc (Chang & Bollum, 1970). Since various site-specific reagents have been known and identified, the analysis of the response of different catalytic reactions of TdT to these reagents was expected to provide valuable information on the function of each component in the catalytic process. While results of this study (Table VII) confirm that the participation of various sites is necessary for polymerization and the PP_i exchange reaction, pyrophosphorolysis is dependent on the presence of a primer alone, implying the requirement only for a primer binding site. The PP_i exchange reaction may be considered the equivalent of polymerization as far as the need for various sites is concerned. The resistance of pyrophosphorolysis to pyridoxal phosphate, a substrate binding site specific reagent, to PCMB and NEM, sulfhydryl reagents, and to *o*-phenanthroline, a zinc chelator, clearly indicates the independence of pyrophosphorolysis from some of the structural features of TdT. These characteristics of TdT are similar to those found for the catalysis of pyrophosphorolysis and RNase H reactions of AMV reverse transcriptase (Modak, 1976a; Srivastava & Modak, 1980a). In TdT, primer binding function has been proposed to require the formation of a coordinate complex between intrinsic zinc and the 3′-OH terminus of a primer. Since the pyrophosphorolysis reaction is insensitive to zinc chelation (Table VII), the binding of the enzyme to the primer may be considered a distinct process, most likely effected through a distinct primer binding site.

The recognition of a primer chain may be dependent upon its length, for an increase in the length of an oligomeric primer has been shown to result in a decreased K_m (indicating higher affinity) and an increased V_{max} (Chang & Bollum, 1970). Therefore, it appears that TdT may bind the primer in two ways: (a) through the intrinsic Zn–3′-OH of the primer terminus and (b) through the primer binding site which is independent of the 3′-OH of the primer. While both types of bindings may be necessary for polymerization, as well as for the PP_i exchange reaction, the pyrophosphorolysis reaction is independent of 3′-OH terminus binding. Further support for this contention may be found in the demonstration that a primer with a blocked 3′-OH terminus can effectively support pyrophosphorolysis but does not permit polymerization (Table VIII).

The ability of TdT to catalyze these varied reactions appears to be one of the highly conserved mechanistic features of many polymerase-type enzymes from various evolutionarily distant sources.

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Photoreactive Derivatives of Corticotropin. 1. Preparation and Characterization of 2,4-Dinitro-5-azidophenylsulfenyl Derivative of Corticotropin†

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ABSTRACT: A photoaffinity probe for corticotropin (ACTH) receptors was prepared by selective modification of the single tryptophan residue in ACTH. A new photoreactive agent, 2,4-dinitro-5-azidophenylsulfenyl chloride, was synthesized and used for introducing the photoreactive group into ACTH. 2,4-Dinitro-5-azidophenylsulfenyl-Trp^9-ACTH (DNAPS-ACTH) was also prepared by thiolysis of 2,4-dinitrophenylsulfenyl-Trp^9-ACTH to form 2-thiol-Trp^9-ACTH and reaction of this with 2,4-dinitro-5-azidofluorobenzene. DNAPS-ACTH was characterized by ultraviolet spectra, peptide mapping, and amino acid analysis. Covalent attachment of DNAPS-ACTH to a pituitary protein fraction FI by photolysis was demonstrated by ultraviolet absorption changes as well as by the use of tritiated DNAPS-ACTH.

Although corticotropin (ACTH)[1] was among the first polypeptide hormones to be utilized in direct binding studies (Lefkowitz et al., 1970), the identification and characterization of specific receptors for ACTH on its target tissues have proved to be a formidable task. In view of the strong tendency of ACTH to bind specifically to a variety of inert materials as well as nonreceptor components of the target tissue, fractionation of the plasma membrane components of the target cell on the basis of binding of the radioactive hormone may result in the isolation of nonreceptor components which are normally present in much higher concentrations than the physiologically relevant receptors. The covalent attachment of the hormone to the receptor under physiological conditions by affinity labeling appears to be a promising approach for the identification of receptors. Conventional chemical affinity labeling, however, is not suitable for this purpose, since the reactive group introduced into the hormone may be destroyed by water or reaction with other nucleophiles on the polypeptide prior to reaching the receptor. Furthermore, this method requires a nucleophilic group at the hormone binding region of the receptor. Both of these limitations can be overcome by the use of a photogenerated species for labeling the receptor (Bayley & Knowles, 1977).

For the identification of the receptor molecule by the method of photoaffinity labeling, it is necessary to perform two modifications on the polypeptide hormone. A suitable radioactive label as well as a photoreactive group must be introduced into the hormone molecule without significantly altering the affinity of the hormone for its physiological receptor. We have already described the successful preparation of specifically tritiated ACTH of high specific radioactivity (90 Ci/mmol) and full biological activity (Ramachandran & Behrens, 1977; Ramachandran et al., 1979). The ϵ-amino group of lysyl residues is the site that is generally modified for the introduction of a photoreactive group (Levy, 1973; Ji, 1977; Das et al., 1977) in peptides. In the case of ACTH, however, both the ϵ-amino group of lysine residues and the D-amino group at the N terminus are unsuitable for modification, since alteration of these sites lowers the biological potency considerably (Ramachandran, 1973). On the other hand, selective chemical modification of the single tryptophan residue in ACTH converts the hormone into a potent antagonist (Ramachandran & Lee, 1970a,b; Moyle et al., 1973). We have investigated the introduction of a photoreactive group at the 2 position of the indole moiety of the tryptophan residue. Previously, we prepared and characterized 2,4-dinitrophenylsulfenyl (DNPS), 2-nitro-4-carboxyphenylsulfenyl (NCPS), and 2-nitrocarbamidophenylsulfenyl (NCMPS)

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[1] Abbreviations used: ACTH, corticotropin; CM-cellulose, carboxymethylcellulose; DNAPS, 2,4-dinitro-5-azidophenylsulfenyl; DNPS, 2,4-dinitrophenylsulfenyl; NAPS, 2-nitro-4-azidophenylsulfenyl.

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