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DNA SYNTHESIS OF WALKER-256 CARCINOSARCOMA NUCLEI *IN VITRO*

II. The Existence of TDP Phosphatase Activity and its Differences from TTP Phosphatase Activity[1,2]

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Abstract

Two forms of TDPase activity were found in Walker-256 cellular fractions: one was of cytoplasmic origin; the other was of nuclear origin. The former was insensitive to 0.2 mM ATP, while the latter was inhibited strongly by 0.2mM ATP. The type and extent of inhibition of TDPase and TTPase activities by ATP were different. The responses of TDPase and TTPase activities to Ca^{++} were different too. A purified TDPase preparation showed no detectable TTPase activity. It is likely that Walker-256 nuclear TDPase is a different enzyme from TTPase.

Introduction

Isolated nuclei from Walker-256 carcinosarcoma can be fractionated into nucleoplasmic, acidic protein, and DNA-histone complex (or deoxyribonucleoprotein, DNP) fractions according to Wang (1967). Ballal *et al.* (1970) showed that high concentration of ATP (2 mM) inhibited TTP incorporation into DNA by acidic protein fraction when either native DNA or denatured DNA were used as template. However, ATP stimulates TTP incorporation into DNA by using acidic protein fraction as enzyme source and DNP fraction as template.

(1) This is part of Y.-H. Lin's Ph. D. dissertation done at Department of Chemistry, UCLA, L. A. California, U. S. A. during his on leave from Institute of Botany, Academia Sinica, Taipei, Taiwan, R. O. C.

(2) Paper No. 156 of the Scientific Journal Series, Institute of Botany, Academia Sinica.

(3) The following abbreviations are used: ATP, adenosine 5′-triphosphate; TTP, thymidine 5′-triphosphate; TDP, thymidine 5′-diphosphate; TTPase, TTP phosphatase; TDPase, TDP phosphatase.

Other nucleoside triphosphates can not replace ATP for this stimulating effect. They also showed that ^{3}H-TTP can be hydrolyzed to thymidine by crude nuclear extract. ATP as well as other nucleoside triphoshates inhibits this hydrolysis. This suggests that the stimulating effect of ATP for TTP incorporation into DNA may be partially explained in terms of its inhibitory effect to the hydrolysis of TTP by dephosphorylating enzyme(s). So far, few papers concerning the enzymatic hydrolysis of TMP, TDP, or TTP in higher animal cells have been published. In the first paper of this series (Lin and Smith, 1974), we reported the existence of TTPase activity in Walker-256 tumor nuclei.

Here we present evidence showing the existence of TDPase activity and differences between this enzyme and TTPase.

Materials and Methods

Preparation of β-^{32}P-TDP

This was done mainly according to that described by Moffat (1967). β-^{32}P-TDP was prepared by condensing thymidine monophosphate morpholidate (TMPM) with ^{32}Pi under rigorously anhydrous conditions and passing the resulting mixture over a DEAE-cellulose column and eluting with a triethylamine-bicarbonate gradient. This separated the various components in the reaction mixture, *i.e.* TMPM, ^{32}Pi, and the desired product β-^{32}P-TDD. An absorbance measurement was made to determine the concentration of the β-^{32}P-TDP solution. An aliquot was counted on a filter paper disc by using 10 ml PPO/POPOP toluene in a Beckman liquid scintillation counter (Model LS-100). The average specific activity obtained was 1.75×10^{6} cpm/μmole TDP.

Isolation of Walker-256 carcinosarcoma nuclei

Female Sprague-Dawley rats were used. Isolation of Walker-256 carcinosarcoma nuclei was done according to Lin and Smith (1974).

Preparation of crude nuclear extract and different cytoplasmic and nuclear fractions

This was also done by the procedure of Lin and Smith (1974).

Assay procedure of TDPase activity

The reaction mixture contained in a total volume of 0.25 ml 0.1 M imidazole buffer pH 7.4; 0.4 mM TDP; 100,000 cpm of β-^{32}P-TDP, and a suitable amount of enzyme solution. The reaction mixture was incubated at 37°C for 15 min and the reaction was terminated by placing the incubation tube in boiling water for 3 min. Water was added, so that the total volume was 1.0 ml and the tube was centrifuged in a clinical centrifuge. An aliquot of 0.5 ml of the supernatant was withdrawn and placed in another tube

containing 0.5 ml water. The ^{32}Pi released in enzymatic reaction was then precipitated according to the procedure of Sugino and Miyoshi (1964). The precipitate was dissolved in 0.5 ml ammonia/acetone (1:1, v/v) and a 100 μl aliquot was counted on a filter paper disc in 10 ml PPO/POPOP toluene in a Beckman liquid scintillation counter (Model LS-100).

Assay procedure of TTPase activity

This was done according to that described by Lin and Smith (1974).

Results

Locality

The data of Table 1 suggest that there are at least two forms of TDP

Table 1. *Effect of ATP on TDP phosphatase activity of Walker-256 cellular fractions*

Reaction mixture contained 0.1 M imidazole buffer (pH 7.4), 2 mM $MgCl_2$, 0.2 mM ATP (if included), 0.4 mM TDP, ca. 100,000 cpm β-^{32}P-TDP, and enzyme solution in a total volume of 0.25 ml. Each value shown was the average of duplicate trials. Error limits were about 10%.

Fraction	Location	Pi released (n mole/tube)		% inhibition
		−ATP	+ATP	
Sucrose (0.25 M)	Cytoplasmic	10.8	9.2	15
Sucrose (2.2 M)	Cytoplasmic	7.0	7.9	-14
NaCl Extract I	Nuclear	1.2	0.2	83
NaCl Extract II	Nuclear	2.0	0.2	90
Acidic Protein	Nuclear	2.8	0.1	96

Table 2. *Effect of ATP on TTPase activity of Walker-256 cellular fractions*

The reaction mixture contained 0.1 M imidazole buffer (pH 7.4), 2 mM $MgCl_2$, 2 mM ATP (if included), 0.1 mM TTP, ca. 250,000 cpm γ-^{32}P-TTP, and enzyme solution in a final volume of 0.25 ml. Each value was the average of duplicate trials. Error limits were around 7%.

Fraction	Location	Pi released (n mole/tube)		% inhibition
		−ATP	+ATP	
Sucrose (0.25 M)	Cytoplasmic	59.1	33.9	42
Sucrose (2.2 M)	Cytoplasmic	32.9	7.6	77
NaCl Extract I (frozen)	Nuclear	7.5	3.1	59
NaCl Extract I (refrigerated)	Nuclear	7.5	3.1	59
NaCl Extract II (frozen)	Nuclear	8.2	0.9	89
NaCl Extract II (refrigerated)	Nuclear	14.0	3.5	75
Acidic Protein	Nuclear	5.3	0.4	92

phosphatase: one is of cytoplasmic origin; the other of nuclear origin. The former was not affected by ATP, while the latter was affected. By comparison, the results of Table 2 do not show clear difference between cytoplasmic and nuclear TTP phosphatase activities as far as ATP effect is concerned.

pH and buffer dependence of the enzyme

The TDPase activity is very dependent on the pH and buffer of the reaction mixture (Klein and Smith). A spectrum of pH ranging over four pH units, 6–10, was examined and maximal activity was found to reside in the area of pH 7.4 through pH 7.8 (Fig. 1). Two activity peaks are present, one in the imidazole and the other in the Tris buffer. Imidazole was selected as the buffer because previous knowledge and experimental evidence demonstrated Tris presence had the effect of hydrolyzing the substrate. The increase in activity around pH 6.6 has been demonstrated several times but an explanation is lacking.

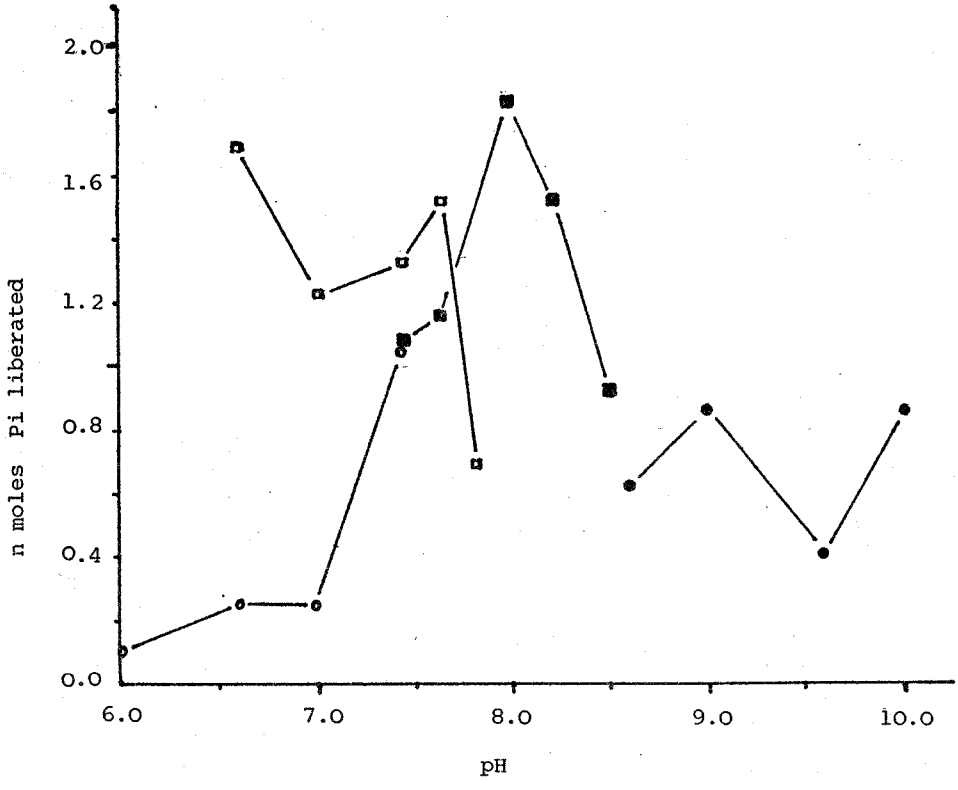

Fig. 1. pH and buffer dependence of TDPase activity. The reaction mixture contained a volume of 0.25 ml 0.1 M buffer, 2 mM $MgCl_2$, 0.4 mM TDP, ca. 100,000 cpm β-^{32}P-TDP, and NaCl extract II as enzyme solution. □–□–□, 0.1 M imidazole; ■–■–■, 0.1 M Tris-HCl; ○–○–○, 0.1 M phosphate; ●–●–●, 0.1 M glycin.

Conditions used to stabilize the enzyme

Both inhibition activity of ATP and total enzymatic activity decrease rapidly if the following prerequisites are not met.

1) The enzyme must be stored in a medium containing 20% glycerol.
2) The presence of a reducing agent in the storage mixture and in all solutions containing the enzyme. 2-Mercaptoethanol proved more efficient in retaining both aspects of activity than did dithiothreitol (Table 3).

Table 3. *Effect of reducing agents on both TDPase activity and its inhibition by ATP*

Reaction conditions were the same as those described in Table 1. NaCl extract II was used as enzyme source.

Addition	Pi released (n mole/tube)		% inhibition
	−ATP	+ATP	
No reducing agent	0.30	0.29	3
2-Mercaptoethanol (1 mM)	0.47	0.17	64
Dithiothreitol (1 mM)	0.42	0.29	31

Distinction between TTP and TDP phosphatase activities

Calcium ion at about 1 mM was found to stimulate TDPase activity in Walker-256 nuclear fractions (Klein and Smith). However, Ca^{++} at same concentration did not stimulate TTPase activity at all. The observation is reproducible, using several different nuclear preparations. The ATP sensitivity of TDPase activity is extremely acute. An inhibitor to substrate concentration ratio of 1:1 caused an inhibition of 95% of the original activity. But, an inhibitor to substrate concentration ratio of higher than 20:1 was needed in order to get same per cent inhibition of TTPase activity in the acidic protein fraction.

Discussion

A purified TDPase preparation was obtained from Walker-256 nuclear extract by ammonium sulfate precipitation, followed by DEAE-cellulose column chromatography, ammonium sulfate precipitation again, then by Sephadex G-200 column filtration procedure (Klein and Smith). This purified TDPase preparation showed no detectable TTPase activity. The finding, as well as the results which have already been shown, supports the suggestion that in Walker-256 nuclei there are two enzyme entities: one is responsible for TDPase activity and the other for TTPase activity.

The strong inhibition of TDPase by ATP may have close relation with enzyme regulation *in vivo*. The contribution of ATP effect on TDPase to DNA synthesis in intact cells remains to be examined.

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Walker-256 惡性瘤細胞核之去氧核酸的合成

II. 胸腺核苷5′-二磷酸水解酵素活性之存在以及它和胸腺核苷5′-三磷酸水解酵素活性兩者間之差別

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在 Walker-256 細胞抽取液可以發現兩種胸腺核苷 5′-二磷酸水解酵素 (TDPase) 之活性：一種來自細胞質；另一種來自細胞核。前者對於 0.2mM ATP 不敏感，而後者被 0.2mM ATP 强烈地抑制。TDPase 和 TTPase 兩種活性被 ATP 抑制的情形和程度有差別。TDPase 和 TTPase 兩種活性對於 Ca^{++} 的反應也不同。經過相當純化後的 TDPase 製備液並未具備可檢出的 TTPase 活性。所以非常可能地 Walker-256 細胞核內之 TDPase 是和 TTPase 不同分子的另一種酵素。