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INHIBITOR STUDIES OF PHAGE T4 WILD-TYPE AND MUTANT DNA POLYMERASES. II. DIFFERENTIAL INHIBITION BY PYRIDOXAL 5′-PHOSPHATE*)

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Summary. — The sensitivities to pyridoxal 5′-phosphate of phage T4 wild-type and two ts mutant DNA polymerases, L98 (mutator) and CB121 (antimutator), were studied. The wild-type and the mutator enzyme we inhibited to an equal extent, while the antimutator enzyme was six times more sensitive. The mode of inhibition was competitive with the deoxynucleoside triphosphate substrates. The CB121 DNA polymerase had a three times lower affinity to its substrates but a twofold affinity to pyridoxal 5′-phosphate. The L98 enzyme had lower affinities to both the substrates and the inhibitor.

Key words: phage T4; *ts mutants*; *DNA polymerases*; *pyridoxal 5′-phosphate*

Introduction

A number of nucleotide-binding enzymes are inhibited by pyridoxa 5′-phosphate (Johnson and Deal, 1970; Piszkiewics and Smith, 1971; Greenwell *et al.*, 1973; Chen and Engel, 1974; Wimmer *et al.*, 1975; Frerman *et al.*, 1977); this is in particular the case with RNA and DNA polymerases (Nixon *et al.*, 1972; Bull *et al.*, 1975; Martial *et al.*, 1975; Modak, 1976a). Since the inhibition is alleviated by addition of nucleotide substrates, it is assumed that the inhibitor binds to the active site of the enzymes. In some cases the formation of a Schiff base between the aldehyde group of pyridoxal 5′-phosphate and the ε-amino group of a lysine residue was demonstrated (Piszkiewics and Smith, 1971; Bull *et al.*, 1975; Martial *et al.*, 1975; Frerman *et al.*, 1977). The effect of the inhibitor on DNA polymerases is not influenced by an excess of added amines (Martial *et al.*, 1975; Modak, 1976*a*, *b*) and is thus considerably more specific than its effect on other enzymes.

We studied the inhibition of phage T4 wild-type DNA polymerase and the DNA polymerases of the mutator mutant L98 the antimutator mutant CB121 by pyridoxal 5′-phosphate. Significant differences in the inhibitor affinities of the three enzymes and a strikingly enhanced sensitivity of the antimutator CB121 DNA polymerase were found.

*) Dedicated to Prof. K. Spies on the occasion of his sixtieth birthday.

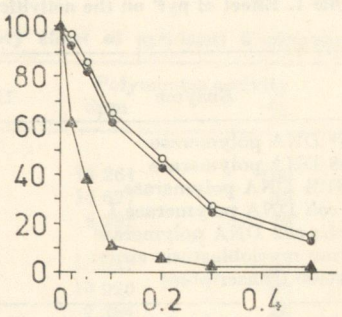

Fig. 1.
Dose effect curves of the interactions between pyP and DNA polymerases
● : T4+; ○ : L 98; ▲ : CB121
Abscissa: pyP concentration (mmol/l); ordinate: % enzyme activity

Materials and Methods

Bacteria and phage. Escherichia coli B and T4D wild-type phage originated from our collection. We are grateful to Dr. W. B. Wood (Pasadena) for the gift of the gene 43 (DNA polymerase) ts mutants L 98 (mutator) and CB121 (antimutator), as classified by Drake and Allen (1968).

Enzyme preparation. The DNA polymerase were isolated from phage-infected *E. coli* B cells as described (Schroeder and Jantschak, 1981), essentially following the procedure of Muzyczka *et al.* (1972). The 100-fold purified DNA polymerases were free of contaminating *E. coli* DNA polymerase I [tested with 1 mmol/l p-hydroxymercuribenzoate (Goulian *et al.*, 1968; Setlow, 1974)] and deoxynucleotide triphosphate - (dNTP-) degrading activities (Schroeder and Jantschak, 1981).

DNA polymerase assay. DNA polymerase activity was assayed according to Goulian *et al.* (1968) at 30° C in 120 µl of incubation mixtures containing 67 mmol/l Tris. HCl (pH 8.8), 16.6 mmo/l $(NH_4)_2SO_4$, 6.7 mmol/l $MgCl_2$, 6.7 µmol/l EDTA, 10 mmol/l 2-mercaptoethanol, 167 µg bovine serum albumin per ml, 33 µmol/l each of dATP, dCTP, dGTP and dTTP plus [^{3}H] dTTP, and 0.2 mmol/l denatured salmon sperm DNA (final concentrations). Pyridoxal 5′-phosphate (pyP) stock solutions were prepated in distilled water immediately before use. In experiments with the inhibitor, 10 µl pyP stock solution (in the controls: 10 µl H_2O) were added to 100 µl reaction buffer containing the other components. All reactions were started with 10 µl enzyme solution.

The deoxynucleoside 5′-triphosphates and pyridoxal 5′-phosphate were purchased from Boehringer, Mannheim; [^{3}H] dTTP was from the Radiochemical Centre, Amersham.

Results and Discussion

The dose-effect curves of the interaction between pyP and the three DNA polymerases (Fig. 1) show that the mutator L98 and the T4 wild-type enzyme were similarly sensitive to the inhibitor. The degree of inhibition is comparable to that of other DNA polymerases (Modak, 1976*a*). However, the antimutator CB121 DNA polymerase was about six times more sensitive to pyP than the other two enzymes. Table 1 shows the ID_{50} values and the residual activities of the enzymes at 0.1 and 0.5 mmol/l pyP along with available data for other polymerases.

Inhibition began immediately after adding the inhibitor (data not shown); thus, the inactive enzyme-pyP complex was formed rapidly. The effect of pyP was not influenced significantly by the presence of various Tris concentrations (Table 2).

To determine substrate and inhibitor affinities of the enzymes, the dependence of reaction rates on substrate concentration was measured. With

Table 1. Effect of pyP on the activities of three T4 DNA polymerases and DNA polymerases from other sources

Enzyme	ID_{50} pyP (mmol/l)	Residual activities (%) at 0.1 mmol/l pyP	0.5 mmol/l pyP
$T4^+$ DNA polymerase	0.16	61	13
L98 DNA polymerase	0.19	65	15
CB121 DNA polymerase	0.03	10	1
E. coli DNA polymerase I	—	—	49*
HeLa cell DNA polymerase	—	—	16*
Avian myeloblastosis virus reverse transcriptase	—	—	24*

* According to Modak (1976*a*).

denatured salmon sperm DNA used as the template, it was necessary to vary the concentrations of all four dNTPs simultaneously. This procedure appeared justified as the dependence of reaction rate on substrate concentration did not deviate visibly from Michaelis-Menten kinetics in the equimolar dNTP concentration range of 0.1—1 mmol/l applied here. During each elementary reaction step, only one of the four dNTP (the one complementary to the template) is accepted by the enzyme (Travaglini *et al.*, 1975). Therefore, the effective substrate concentration is that of the individual dNTP and not the sum of the four.

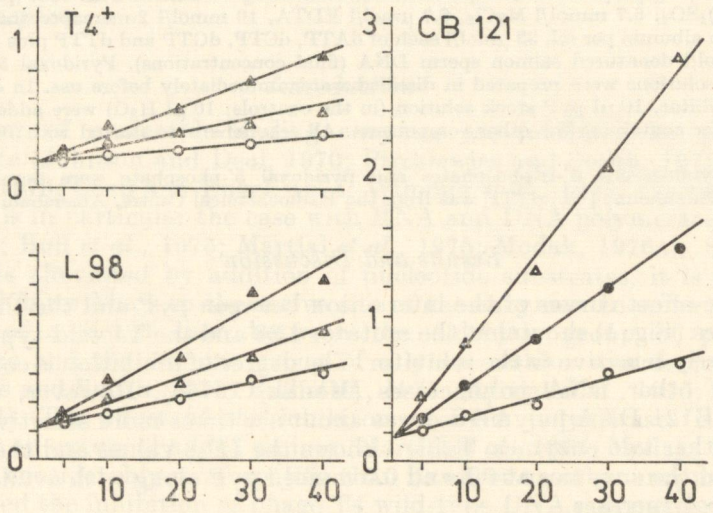

Fig. 2.
Double reciprocal plots of raction rate vs. substrate concentration in the presence of various concentrations of Pyp
Data from two ($T4^+$, CB121) or three (L98) experiments were brought into scale; 1/v (ordinates) is therefore dimensionless.
Abscissae: dNTP concentration (mmol/l)
pyP concentrations (mmol/l): ○ — 0, ● — 0.05, △ — 0.1, ▲ — 0.2

Table 2. Influence of buffer composition on the inhibitory effect of pyridoxal 5′-phosphate

Buffer*	pyP mmol/l	Polymerase activity dpm	%
A Standard assay buffer:			
67 Tris; 16.7 mmol/l $(NH_4)_2SO_4$	0	18 261	100
	0.1	12 677	69
	0.2	8 392	46
B 16.8 mmol/l Tris; 4.2 mmol/l $(NH_4)_2SO_4$;	0	15 067	100
16.6 mmol/l K_2SO_4	0.1	10 020	67
(25% of the Tris and $(NH_4)_2SO_4$ concentrations of buffer A)	0.2	7 384	49
C Borate buffer free of amines	0	18 313	100
33 mmol/l Na_3BO_3; 16.7 mmol/l K_2SO_4	0.1	11 261	62
	0.2	7 294	40

* pH and all other buffer components as described in Materials and Methods.

The kinetic constants refer to the stable incorporation of dNTPs which is actually the result of two reactions: the insertion of dNTP by the polymerase activity and the excision of a part of the newly incorporated 3′-terminal nucleotides by the polymerase-associated 3′-5′exonuclease activity (Gillin and Nossal, 1975). A comparison of kinetic data for stable incorporation with those for nucleotide utilization (the total of the incorporated and the removed dNTP) shows that the former are only a little higher than the latter (Gillin and Nossal, 1975, 1976; Schroeder and Jantschak, 1980). The exonuclease activities of the three DNA polymerases were less than half as sensitive to pyP than their polymerase activities (data not shown). The strong inhibition of the T4 DNA polymerase-associated 3′-5′ exonuclease activity by adriamycin (Goodman *et al.*, 1974) has been shown to be without influence on the polymerase reaction under synthesizing conditions (Goodman *et al.*, 1977). We assume, therefore, that the relatively weak inhibition of the exonuclease activity by pyP does not affect the stable incorporation of deoxynucleoside triphosphates.

Fig. 2 shows double reciprocal plots of reaction rates vs. substrate concentrations for the three DNA polymerases. They indicate a competitive mode of inhibition by pyP. The apparent kinetic constants K_m and K_i were determined from least squares fits of the reciprocal kinetic data (Table 3). Compared with the wild-type enzyme, L98 polymerase had a lower (apparent) affinity to both the substrates and the inhibitor. These effects compensate each other so that L98 DNA polymerase exhibited nearly the same sensitivity to pyP as the wild-type enzyme. The CB121 DNA polymerase had a three times increased K_m+ for dNTP and a twofold lower K_i for pyP, resulting in a sixfold pyP sensitivity of the CB121 enzyme compared with the other two DNA polymerases.

The competitive inhibition by pyP suggests that T4 DNA polymerase, like other enzymes reacting with pyP (Piszkiewics and Smith, 1971; Bull

Table 3. Apparent Michaelis and inhibitor constants of the three T4 DNA polymerases

DNA polymerase	K_m (mmol/l)	K_i (mmol/l)	$\frac{K_i}{K_m}$
T4+	27	70 ± 8	2.7
L98	46	140 ± 15	3.0
CB121	85	32 ± 4	0.4

et al., 1975; Martial *et al.*, 1975; Frerman *et al.*, 1977), contains a lysine residue in its active site, probably in the dNTP-binding subsite. Interestingly, the inhibitor affinity of the three DNA polymerases correlated with their accuracy of DNA replication: the mutator L98 DNA polymerase had the lowest affinity, that of the wild-type polymerase was intermediate, and the antimutator CB121 DNA polymerase displayed the highest affinity to pyP. Since pyP coenzyme binding is known to be stabilized by hydrophobic interactions (Janin and Chothia, 1978) changes in the hydrophobicity around the reactive lysine ε-amino group could be the reason for the different affinities to pyP of the T4 mutant enzymes. This tentative explanation is also supported by the fact that the sensitivities of the three enzymes to the aromatic chelating agent 1,10-phenanthroline rise in the same order as their sensitivities to pyP (Schroeder and Jantschak, 1978). On the other hand, CB121 DNA polymerase is less sensitive than L98 and T4 wild-type polymerase to the competitive dTTP analogue 3′-fluorothymidine 5′-triphosphate (Schroeder and Jantschak, 1980). Thus, CB121 antimutator DNA polymerase is not indiscriminately more sensitive to any kind of inhibitor. Of all eight inhibitors tested by us on T4 wild-type, L98 and CB121 DNA polymerases [in addition to pyP five template-binding drugs (Jantschak and Schroeder, 1980), 1,10-phenonanthroline (Schroeder and Jantschak, 1978) and 3′-fluorothymidine triphosphate (Schroeder and Jantschak, 1980)], pyP showed the highest selectivity for the antimutator CB121 DNA polymerase, inhibiting this enzyme six times more strongly than the wild-type and the L98 mutator T4 DNA polymerases.

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