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Mechanism of Selective Inhibition of 3′ to 5′ Exonuclease Activity of *Escherichia coli* DNA Polymerase I by Nucleoside 5′-Monophosphates†

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ABSTRACT: The 3′ to 5′ exonuclease activity of *Escherichia coli* DNA polymerase I can be selectively inhibited by nucleoside 5′-monophosphates, whereas the DNA polymerase activity is not inhibited. The results of kinetic studies show that nucleotides containing a free 3′-hydroxy group and a 5′-phosphoryl group are competitive inhibitors of the 3′ to 5′ exonuclease. Previous studies by Huberman and Kornberg [Huberman, J., and Kornberg, A. (1970), *J. Biol. Chem. 245,* 5326] have demonstrated a binding site for nucleoside 5′-monophosphates on DNA polymerase I. The K_{dissoc} values for nucleoside 5′-monophosphates determined in that study are comparable to the K_i values determined in the present study, suggesting that the specific binding site for nucleoside 5′-monophosphates represents the inhibitor site of the 3′ to 5′ exonuclease activity. We propose that (1) the binding site for nucleoside 5′-monophosphates on DNA polymerase I may represent the product site of the 3′ to 5′ exonuclease activity, (2) the primer terminus site for the 3′ to 5′ exonuclease activity is distinct from the primer terminus site for the polymerase activity, and (3) nucleoside 5′-monophosphates bind at the primer terminus site for the 3′ to 5′ exonuclease activity.

The ability of bacterial and phage DNA polymerases to copy DNA templates in vivo and in vitro with such high fidelity has been ascribed in part to the associated 3′ to 5′ exonuclease activity of these DNA polymerases (Brutlag and Kornberg, 1972; Muzcyzka et al., 1972). The 3′ to 5′ exonuclease activity has a proof-reading function as it excises a mismatched nucleotide incorporated at the primer terminus prior to further chain extension, thereby helping to maintain replication fidelity. We have recently demonstrated that the proofreading 3′ to 5′ exonuclease activity of both *E. coli* DNA polymerase I and mammalian DNA polymerase δ can be selectively inhibited by nucleoside 5′-monophosphates, whereas the DNA polymerase activity is not inhibited (Byrnes et al., 1977). The present study was undertaken to elucidate the mechanism of this selective inhibition.

Materials and Methods

Unlabeled nucleosides, nucleotides, and nucleotide analogues were obtained from either P-L Biochemicals Inc. or Sigma Chemical Co. The purity of nucleoside 5′-monophosphates was examined by PEI-cellulose thin-layer chromatography as described by Cashel et al. (1969) and no contamination by nucleoside diphosphates or triphosphates was detectable (<0.1%). However, because of the high concentrations of nucleoside 5′-monophosphates used in the experiments, they were further purified: dAMP was purified by chromatography on Dowex-1 (chloride) as described by Cohen (1966) and TMP by chromatography on Dowex-1 (formate) as described by Hulbert and Furlong (1967). PEI-cellulose thin-layer chromatography plates were obtained from Brinkman Instruments. [^{3}H]dTTP and γ-[^{32}P]ATP were purchased from Amersham Corp. Poly(dA-dT) was purchased from Grand Island Biological Co. and was dialyzed before use against 0.01 M Tris-HCl[1] (pH 7.4), 0.06 M KCl. Oligo[d(T)$_4$] was obtained from Collaborative Research, Inc. Polynucleotide kinase was purchased from Boehringer Mannheim Corp. *E. coli* B (¾ log) was purchased from Grain Processing Corp. Calf thymus was purchased from Pel-Freez Biologicals Inc. *Hin*II and *Hin*III restriction endonuclease fragments of ^{32}P-labeled SV-40 DNA were the generous gift of Dr. Walter Scott.

DNA polymerase I was prepared from *E. coli* B according to Jovin et al. (1969) through step VII. Terminal deoxynucleotidyltransferase was prepared from calf thymus according to Bollum et al. (1974) through step 5′.

[^{3}H]poly[d(T)$_{50}$] was synthesized with terminal deoxynucleotidyltransferase using oligo[d(T)$_4$] as primer and [^{3}H]-dTTP (25–200 cpm/pmol) according to the method of Bollum (1968). The chain length of [^{3}H]poly[d(T)$_{50}$] was determined by enzymatic end-group labeling using γ-[^{32}P]ATP and

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[1] Abbreviations used are: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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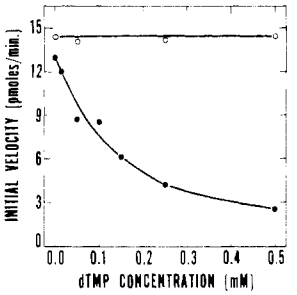

FIGURE 1: Effect of dTMP on the DNA polymerase and 3′ to 5′ exonuclease activities of DNA polymerase I. DNA polymerase and exonuclease activities were assayed separately as described under Materials and Methods except (1) in the DNA polymerase assay the concentration of poly(dA-dT) was 1.8 μM and (2) in the exonuclease assay the concentration of [^{3}H]poly[d(T)$_{50}$] was 2 μM. Initial velocities are expressed as picomoles of nucleotide incorporated per minute in the DNA polymerase assay (○-○) and as picomoles of nucleotide released per minute in the exonuclease assay (●-●).

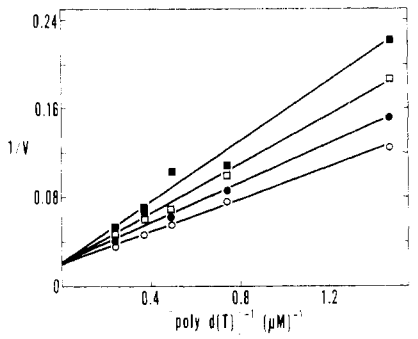

FIGURE 2: Plot of the reciprocal of initial velocity (V) vs. the reciprocal of [^{3}H]poly[d(T)$_{50}$] concentration (μM) at various fixed concentrations of dTMP. Initial velocities are expressed as picomoles of nucleotide released per minute. The concentrations of dTMP are: 0 (○-○), 0.044 mM (●-●), 0.088 mM (□-□), and 0.132 mM (■-■).

polynucleotide kinase according to Low et al. (1976) and by gel electrophoresis on 5% polyacrylamide gels containing 98% formamide according to Maniatis et al. (1975). *Hin*II and *Hin*III restriction endonuclease fragments of ^{32}P-labeled SV-40 DNA were used as markers.

Poly(dA-dT) labeled at the 3′-terminus with [^{3}H]dTMP was prepared as described previously (Byrnes et al., 1977).

DNA Polymerase Assay. The standard reaction mixture contained in a final volume of 0.3 mL: 67 mM potassium phosphate buffer (pH 7.5), 6.7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 33 μM dATP, 33 μM [^{3}H]dTTP (88 cpm/pmol), 20 μM poly(dA-dT), and 0.15 unit of DNA polymerase I diluted in 65 mM Tris-HCl (pH 7.8), 10 mM 2-mercaptoethanol, 0.2 M KCl, and 1 mg/mL bovine serum albumin. After incubation at 37 °C for 10 min, the reaction was stopped by the addition of 2 mL of cold 5% trichloroacetic acid containing 10 mM sodium pyrophosphate. The precipitate was collected, washed, and counted as previously described (Byrnes et al., 1976). One unit of DNA polymerase catalyzes the incorporation of 10 nmol total nucleotide in 30 min at 37 °C. Poly(dA-dT) concentrations are expressed as concentration of total nucleotide.

3′ to 5′ Exonuclease Assay. The standard reaction mixture contained in a final volume of 0.2 mL: 50 mM Hepes buffer (pH 7.4), 5 mM $MgCl_2$, 7.5 μM [^{3}H]poly[d(T)$_{50}$] (25–200 cpm/pmol), and 0.15 unit of 3′ to 5′ exonuclease diluted in 65 mM Tris-HCl (pH 7.8), 10 mM 2-mercaptoethanol, 0.2 M KCl, and 1 mg/mL bovine serum albumin. The reaction mixture was incubated at 37 °C, and 20-μL aliquots were applied to 2.4-cm circles of Whatman DE-81 paper after 0, 5, 10, 15, 20, and 30 min of incubation. The circles were washed, dried, and counted as described by Brutlag and Kornberg (1972). Initial velocities were determined from a least-squares fit of nucleotide hydrolyzed plotted vs. time. One unit of 3′ to 5′ exonuclease catalyzes the hydrolysis of 10 nmol of nucleotide in 30 min at 37 °C. The ratio of DNA polymerase activity to exonuclease activity is approximately 10:1. [^{3}H]poly[d(T)$_{50}$] concentrations are expressed as concentration of total nucleotide. In some experiments, poly(dA-dT) labeled at the 3′ terminus with [^{3}H]dTMP was used as substrate for measuring 3′ to 5′ exonuclease activity.

TABLE I: Inhibition of 3′ to 5′ Exonuclease Activity of DNA Polymerase I by Nucleotides and Nucleotide Analogues.[a]

Nucleoside, nucleotide, or nucleotide analogue added	Inhibition of 3′ to 5′ exonuclease act. (%)
5′-dAMP	69
5′-dGMP	58
5′-dTMP	46
5′-dCMP	19
5′-AMP	58
5′-*ara*-AMP	4
2′-*O*-Methyl-5′-AMP	0
3′-AMP	0
3′-Deoxy-5′-AMP	6
3′,5′-cAMP	0
3′,5′-TDP	0
3′-*O*-Acetyl-5′-AMP	0
Adenosine	0

[a] Exonuclease activity was assayed as described under Materials and Methods except that the concentration of [^{3}H]poly[d(T)$_{50}$] was 4 μM. Nucleosides, nucleotides, and nucleotide analogues were present at a concentration of 0.2 mM.

Results

Selective Inhibition of 3′ to 5′ Exonuclease Activity of DNA Polymerase. The effects of dTMP on the 3′ to 5′ exonuclease activity and the DNA polymerase activity of DNA polymerase I are shown in Figure 1. The addition of increasing concentrations of dTMP results in a progressive inhibition of 3′ to 5′ exonuclease activity, as measured by the release of [^{3}H]dTMP from [^{3}H]poly[d(T)$_{50}$]. At a dTMP concentration of 0.1 mM, the rate of [^{3}H]dTMP release was inhibited approximately 50%. No inhibition of DNA polymerase activity, as measured by the incorporation of [^{3}H]dTMP into poly(dA-dT), was observed over the same concentration range. Similar results were obtained with the other deoxynucleoside 5′-monophosphates [dAMP, dGMP, and dCMP (data not shown)], although the concentration of each nucleotide required to inhibit exonuclease activity 50% was different. In these experiments, the substrate for the exonuclease ([^{3}H]poly[d(T)$_{50}$]) and the primer/template for the DNA polymerase [poly(dA-dT)] were present at approximately half-saturating concentrations.

Structural Requirements for Inhibition of 3′ to 5′ Exonuclease Activity. As shown in Table I, only those nucleotides containing a 5′-phosphoryl group and a 3′-hydroxyl group are inhibitors of exonuclease activity. No inhibition was observed with adenosine, 3′-AMP or 3′,5′-cAMP. Analogues which lack a 3′-hydroxyl group such as 3′-deoxyadenosine 5′-monophosphate fail to inhibit the exonuclease activity. The inhibitory effect is markedly decreased when the 2′-hydroxyl group is inverted in the arabinose configuration, as in 5′-*ara*-AMP, or when the 2′-hydroxy group in the ribose configuration is

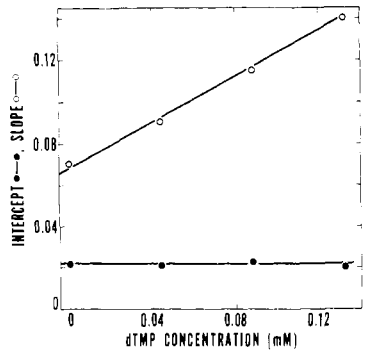

FIGURE 3: Replots of the slopes (○-○) and the intercepts (●-●) from Figure 2 as a function of dTMP concentration.

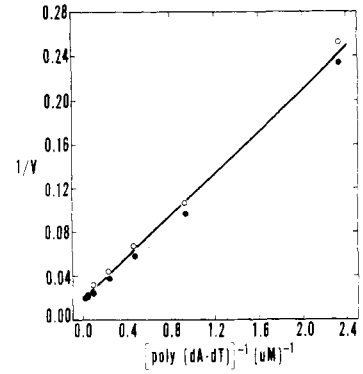

FIGURE 4: Plot of the reciprocal of initial velocity (V) vs. the reciprocal of the poly(dA-dT) concentration (μM) in the presence (●-●) and absence (○-○) of 0.33 mM dTMP. Initial velocities are expressed as picomoles of nucleotide incorporated per minute.

blocked with a methyl group such as 2′-*O*-methyl-5′-AMP. Analogues in which the 3′-hydroxyl group is blocked by a phosphoryl group such as 3′,5′-thymidine diphosphate or an acetyl group such as 3′-*O*-acetyl-5′-AMP also fail to inhibit the exonuclease activity.

Kinetics and Mechanism of Inhibition. Figure 2 shows the reciprocal of the initial rate of nucleotide released plotted as a function of the reciprocal of the substrate concentration at three different concentrations of the inhibitor dTMP. The inhibition pattern is consistent with competitive inhibition and suggests that nucleoside 5′-monophosphates bind at the active site of the 3′ to 5′ exonuclease. The inhibitor constant (K_i) for dTMP was found to be 0.13 mM from a replot of the slopes as a function of inhibitor concentration (Figure 3). The K_i for dTMP was also found to be 0.1 mM when poly(dA-dT) labeled at the 3′ terminus with [^{3}H]TMP was used as substrate for 3′ to 5′ exonuclease activity (data not shown). Since nucleoside 5′-monophosphates are the sole products of the exonuclease (Lehman and Richardson, 1964), the data are consistent with the hypothesis that nucleoside 5′-monophosphates are product inhibitors of exonuclease activity.

The primer terminus is a substrate for both the DNA polymerase and the 3′ to 5′ exonuclease activities of DNA polymerase I. We therefore investigated the effect of dTMP on DNA synthesis as a function of primer/template concentration. Figure 4 shows the reciprocal of the initial rate of nucleotide incorporation plotted as a function of the reciprocal of the substrate concentration with the primer/template poly(dA-dT) as the variable substrate, in the presence and absence of 0.33 mM dTMP. No inhibition of DNA polymerase activity by dTMP was observed.

Correlation between K_i and K_{dissoc} Values for Nucleoside 5′-Monophosphates. The K_i values for several other nucleoside 5′-monophosphates were determined as described in Figures 2 and 3 and are listed in Table II. Also listed are the dissociation constants (K_{dissoc}) for these nucleotides which were previously reported by Huberman and Kornberg (1970). Purine nucleotides, which have a greater affinity for binding to DNA polymerase than pyrimidine nucleotides, similarly have lower K_i values. *ara*-AMP which has a high K_{dissoc} has a correspondingly high K_i. The close correspondence between the K_i and K_{dissoc} values would suggest that the binding site on the enzyme for nucleoside 5′-monophosphates represents the inhibitor site of the 3′ to 5′ exonuclease activity.

TABLE II: K_i and K_{dissoc} Values of Nucleoside 5′-Monophosphates.[a]

Nucleotide	K_i (mM)	K_{dissoc} (mM)
5′-dAMP	0.022	0.018
5′-dGMP	0.058	0.027
5′-dTMP	0.132	0.088
5′-dCMP	0.167	0.134
5′-AMP	0.045	0.035
ara-AMP	0.91	>2.3

[a] K_i values were determined as described in the text. K_{dissoc} values were taken from the data of Huberman and Kornberg (1970).

Discussion

The present study demonstrates that nucleotides containing a free 3′-hydroxyl group and a 5′-phosphoryl group selectively inhibit the proofreading 3′ to 5′ exonuclease activity of *E. coli* DNA polymerase I, while the DNA polymerase activity is not inhibited. The kinetics of inhibition of exonuclease activity by nucleoside 5′-monophosphates are competitive, suggesting that the inhibitor binds at the active site of the enzyme. Since nucleoside 5′-monophosphates are products of the exonuclease, the data are consistent with the hypothesis that nucleoside 5′-monophosphates are product inhibitors of the exonuclease activity.

Previous studies by Huberman and Kornberg (1970) demonstrated a single binding site on DNA polymerase I for nucleoside 5′-monophosphates, distinct from the nucleoside triphosphate binding site. The dissociation constants for the deoxynucleoside monophosphates ranged from 0.01 to 0.1 mM. The K_i values for the nucleoside monophosphates determined in the present study correspond to the K_{dissoc} values previously obtained. Furthermore, the structural requirements for inhibitors of exonuclease activity are identical to the structural requirements for binding of these nucleotides to DNA polymerase I, i.e., a free 3′-hydroxyl group and a 5′-phosphoryl group. Thus, it would appear that the binding site on DNA polymerase I for nucleoside 5′-monophosphates is the inhibitor site of the 3′ to 5′ exonuclease activity.

The concentrations of nucleoside 5′-monophosphates required to inhibit exonuclease activity in the present study are much lower than those reported earlier in the study of Huberman and Kornberg (1970), in which it was found that neither DNA polymerase nor 3′ to 5′ exonuclease activity was inhibited by nucleoside 5′-monophosphates except at nucleotide concentrations of 50-fold greater than their K_{dissoc}. The reasons for the different results are not readily apparent; however, the assay conditions were quite different in the two studies. For example, in the present study poly[d(T)$_{50}$] was used as substrate for the 3′ to 5′ exonuclease, whereas in the previous study d(pTpT) was used as substrate. The apparent K_m for poly[d(T)$_{50}$] is 4 μM in total nucleotide or 0.08 μM in

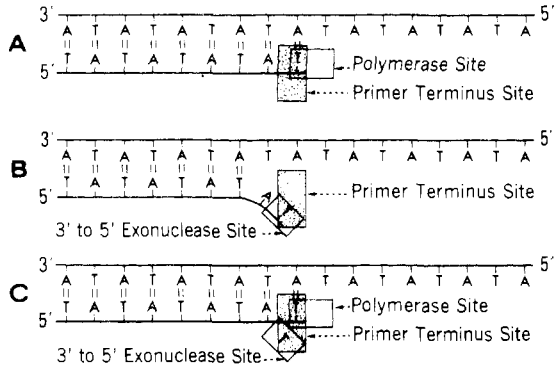

FIGURE 5: Schematic diagram of the primer terminus site of DNA polymerase I.

polynucleotide concentration. The apparent K_m for d(pTpT) is much higher; Lehman and Richardson (1964) reported a K_m for d(pTpT) of 0.34 mM, whereas Huberman and Kornberg (1970), using different assay conditions, reported a K_m of 15 mM. Thus, it would appear that longer polynucleotides are preferred substrates for the 3′ to 5′ exonuclease activity and that the conditions of assay can markedly affect the kinetic constants.

Huberman and Kornberg (1970) proposed that the nucleoside 5′-monophosphate binding site represents the site that binds the 3′ terminus of the primer/template because of the close correspondence between structural requirements for binding and for primer terminus function: a 3′-hydroxyl group is required for binding of a nucleoside 5′-monophosphate, for hydrolysis of the primer terminus by the 3′ to 5′ exonuclease activity, and for chain extension by the DNA polymerase activity. Although the primer terminus serves as a substrate for both the DNA polymerase and the 3′ to 5′ exonuclease activities of DNA polymerase I, we have observed that nucleoside 5′-monophosphates are competitive inhibitors of exonuclease activity but not of DNA polymerase activity when measured as a function of primer/template concentrations. These data suggest that the primer terminus site for hydrolysis is distinct from the primer terminus site for polymerization.

The observations of Brutlag and Kornberg (1972) are very relevant to the present studies. These investigators have shown that the primer terminus, when it is properly base-paired with the template, is a substrate for DNA polymerase activity but not for exonuclease activity. Conversely, when the primer terminus is single-stranded or frayed DNA, it is a substrate for exonuclease activity but not for DNA polymerase activity. Together with the results of the present study, these observations suggest that there are two distinct subsites in the primer terminus site of DNA polymerase I, one of which is the substrate site for DNA polymerase activity and the other the substrate site for the exonuclease activity. This is depicted schematically in Figure 5. As shown in panel A, when the primer terminus binds at the DNA polymerase site it allows polymerization to occur, whereas when the primer terminus occupies the exonuclease site (Panel B) it becomes a substrate for the hydrolytic reaction. The binding of a nucleoside 5′-monophosphate at the exonuclease site (Panel C) would prevent the binding of the primer terminus at this site and inhibit hydrolysis by exonuclease activity; however, the DNA polymerase activity would not be inhibited.

The lack of base specificity for the inhibition of exonuclease activity by nucleoside 5′-monophosphates is also consistent with the above model and further distinguishes the DNA polymerase site from the exonuclease site. As shown by Brutlag and Kornberg (1972), DNA polymerization requires that the primer terminus be properly base paired with the template. This suggests that there is a strict base specificity for binding at the polymerase site, requiring correct base pairing with the template. In contrast, there is broader base specificity for binding of the primer terminus at the exonuclease site, since any nucleotide, either paired or unpaired, at the primer terminus could bind at this site. Furthermore, the strict base specificity for binding at the polymerase site would prevent the binding of a mismatched primer terminus at this site and thus favor binding at the exonuclease site, where it would be exposed to hydrolytic attack by the exonuclease activity. This would provide a mechanism for rapid hydrolysis of mismatched nucleotides during DNA synthesis.

We have recently shown that selective inhibition of the proofreading 3′ to 5′ exonuclease activity of DNA polymerase I by nucleoside 5′-monophosphates results in an increased error frequency during DNA synthesis in vitro (Byrnes et al., 1977). It may be possible to take advantage of this system to elucidate the relative contributions of the DNA polymerase activity and the 3′ to 5′ exonuclease activity in maintaining the fidelity of DNA synthesis.

Acknowledgments

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