Accelerated Publications

Carbonyldiphosphonate, a Selective Inhibitor of Mammalian DNA Polymerase δ†

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ABSTRACT: Twenty-three pyrophosphate analogues were screened as inhibitors of proliferating cell nuclear antigen independent DNA polymerase δ (pol δ) derived from calf thymus. Carbonyldiphosphonate (COMDP), also known as α-oxomethylenediphosphonate, inhibited pol δ with a potency (K_i = 1.8 μM) 20 times greater than that displayed for DNA polymerase α (pol α) derived from the same tissue. Characterization of the mechanism of inhibition of pol δ indicated that COMDP competed with the dNTP specified by the template and was not competitive with the template·primer. In the case of pol α, COMDP did not compete with either the dNTP or the polynucleotide substrate. COMDP inhibited the 3′→5′ exonuclease activity of pol δ weakly, displaying an IC_{50} greater than 1 mM.

Mammalian DNA polymerases α (pol α)[1] and δ (pol δ) are distinct, aphidicolin-sensitive DNA polymerases (Fry & Loeb, 1986; Focher et al., 1989; Wong et al., 1989). Both pol α and pol δ have been postulated to be essential for cellular DNA replication, working in a coordinated fashion in the synthesis of lagging and leading strands, respectively, at the replication fork (Focher et al., 1988a; Downey et al., 1988).

A role of pol α in replicative DNA synthesis is firmly established (Fry & Loeb, 1986), whereas a role for pol δ is not. In an effort to more precisely dissect the structure and function of pol δ, we have sought to develop inhibitor probes specifically targeted to it. Our first step was to survey the anti-pol δ activity of inhibitors of other DNA polymerases (Talanian et al., 1989). Among the nucleotide, nucleoside, purine base, aphidicolin, and pyrophosphate (PP_i) analogues tested, only PP_i and its isostere (difluoromethylene)diphosphonate displayed selectivity for pol δ relative to pol α. Prompted by the latter observation, we surveyed a wider series of PP_i analogues and have found one, carbonyldiphosphonate (COMDP), which inhibits proliferating cell nuclear antigen (PCNA) independent pol δ derived from calf thymus (Focher et al., 1989) with micromolar range potency and greater than 20-fold selectivity relative to pol α derived from the same source. Here we report the analysis of the mechanism of COMDP action on pol δ, and we describe previously unreported spectroscopic properties of the compound and a modified method for its synthesis.

Materials and Methods

Materials. Poly(dA), poly(dC), oligo(dT) [$(d)T_{12-18}$], oligo(dG) [$(d)G_{12-18}$], and Sephadex G-25 were from Pharmacia. [^{3}H]dTTP, [^{3}H]dGTP, and [^{32}P]dCTP were from New England Nuclear.

Inhibitor Syntheses. All solvents and reagents were analytical grade. NMR spectra were measured on a Bruker WP-270 SY or JEOL FX 90 Q spectrometer. NMR samples were ca. 5% w/v in D_2O. Chemical shifts are reported relative to external TMS [^{1}H, ^{13}C (benzene reference, δ 128)], external 85% H_3PO_4 (^{31}P), and external $CFCl_3$ (^{19}F). Coupling constants are reported in hertz. IR spectra were measured with a Beckman Acculab 2 spectrometer. Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses (C, H, N, Na) were performed by Galbraith Laboratories, and all agreed to ±0.4% of calculated values.

The following compounds were prepared as described: **1**, **6**, and **17** (McKenna et al., 1989a); **3** and **13–15** (McKenna et al., 1987); **7**, **12**, **16**, and **20–23** (McKenna et al., 1988); **5** (McKenna & Levy, 1989); **10** and **11** (McKenna et al., 1989b). **2** was prepared by titration of the corresponding acid (McKenna et al., 1987) with NaOH (Ye & McKenna, unpublished results).

Pyridinium Fluorochlorophosphonoacetate (**9**) (*Exemplary Procedure*). The triethyl ester (McKenna & Khawli, 1986) was refluxed in concentrated HCl (6 h). Evaporation (<1 mmHg, 60 °C) left a residue, which was treated with excess pyridine. The resulting suspension was stored at −20 °C overnight, and the product was filtered, washed with cold MeOH, recrystallized from MeOH, and dried overnight (<1 mmHg, 60 °C), giving **9** (84%) as white crystals: mp 159–160 °C dec; NMR ^{31}P δ 4.8 (d, $^2J_{PF}$ 80); NMR ^{19}F δ −129.2 (s, $^2J_{FP}$ 81); NMR ^{13}C δ 100.7 (dd, $^1J_{CF}$ 263, $^1J_{CP}$ 161, CFCl), 169.3 (dd, $^2J_{CF}$ 24, $^2J_{CP}$ 7, CO). Anal. ($C_7H_8O_5ClFNP$) C, H, N.

Pyridinium bromofluorophosphonoacetate (**8**): 87%, mp 152–153 °C dec; NMR ^{31}P δ 4.4 (d, $^2J_{PF}$ 76); NMR ^{19}F δ

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[1] Abbreviations: pol α, DNA polymerase α; pol δ, DNA polymerase δ; PP_i, pyrophosphate; PCNA, proliferating cell nuclear antigen; BSA, bovine serum albumin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; $NaBH_4$, sodium borohydride. Inhibitor acronyms are explained in Figure 1 and Table I.

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−132.6 (d, $^2J_{FP}$ 76); NMR ^{13}C δ 93.1 (dd, $^1J_{CF}$ 270, $^1J_{CP}$ 157, CFBr), 169.8 (dd, $^2J_{CF}$ 24, $^2J_{CP}$ 7, CO). Anal. ($C_7H_8O_5$-BrFNP) C, H, N.

Pyridinium 2-chloro-2-phosphonopropionate (**18**): 81%, mp 186–187 °C dec; NMR ^{31}P δ 12.6 (q, $^3J_{PH}$ 13); NMR ^{13}C δ 24.4 (q, $^1J_{CH}$ 132, CH_3), 65.1 [dq, $^1J_{CP}$ 133, $^2J_{CH}$ 4, C-(CH_3)Cl], 173.2 (d, $^2J_{CP}$ 5, CO). Anal. ($C_8H_{11}O_5ClNP$) C, H, N.

Pyridinium 2-bromo-2-phosphopropionate (**19**): 83%, mp 174–175 °C dec; NMR ^{31}P δ 12.8 (q, $^3J_{PH}$ 13); NMR ^{13}C δ 25.3 (q, $^1J_{CH}$ 133, CH_3), 55.1 [dq, $^1J_{CP}$ 132, $^2J_{CH}$ 4, C-(CH_3)Br], 173.1 (d, $^2J_{CP}$ 5, CO). Anal. ($C_8H_{11}O_5BrNP$) C, H, N.

Sodium α-oxomethylenediphosphonate (carbonyldiphosphonate) (**4**) was prepared by a modification of the method of Quimby et al. (1967). Tetraisopropyl (dichloromethylene)diphosphonate (6.0 g, 14.5 mmol) was refluxed in 12 N HCl (2 h), giving (dichloromethylene)diphosphonic acid (rotary evaporation), which was dissolved in 14.5% aqueous NaOH (30 mL) and refluxed (2 h). Crude **4** was precipitated (60 mL of MeOH), recrystallized from aqueous MeOH, and air-dried, giving a pale yellow powder: mp >200 °C; NMR ^{31}P δ 0.4 (s) [Quimby et al. (1967), δ 0.0 in H_2O]; NMR ^{13}C δ 245.2 (t, $^1J_{CP}$ 119); IR (nujol mull) 1612 cm^{-1} [Quimby et al. (1967), 1612 cm^{-1}]. Anal. ($CP_2O_7Na_4 \cdot 2.5H_2O$) C, H, Na.

Enzymes. Calf thymus DNA polymerase δ was provided by Drs. Federico Focher and Ulrich Hübscher. Two preparations were used: One, designated HAP-δ, was purified through step III, hydroxyapatite chromatography (Focher et al., 1988b). The second, designated FPLC-δ, was purified through step V, FPLC MonoS chromatography (Focher et al., 1989). FPLC-δ, a form of pol δ which is not stimulated by PCNA, contained four major peptides and was used in the analysis of inhibitor mechanism and assay of exonuclease activity. Calf thymus DNA polymerase α was prepared by immunoaffinity chromatography, as described (Chang et al., 1984). We distinguished pol α and pol δ by the sensitivity of the former but not the latter to potent inhibition by butylphenyl-dGTP (Lee et al., 1985) and by the presence of a DNA primase activity associated with the former and a 3′→5′ exonuclease activity with the latter. Terminal deoxynucleotidyltransferase was from New England Nuclear.

Exonuclease Substrates. The 3′-terminal end of $(dT)_{12-18}$ was labeled by addition of radiolabeled dNTPs catalyzed by terminal deoxynucleotidyltransferase, as described (Roychoudhury & Wu, 1980). The products were $(dT)_{12-18} \cdot ([^{32}P]dC)_{0.33}$ and $(dT)_{12-18} \cdot ([^3H]dT)_{0.3}$. The oligonucleotides were hybridized to poly(dA) [base ratio 10:1 (dA):(dT)] for use as exonuclease substrates.

Enzyme Assays. Unless noted otherwise, assays of pol α and pol δ using poly(dA)·oligo(dT) contained the following in a final volume of 25 μL: 75 mM K^+HEPES (pH 7.5), 1.25 mM DTT, 20% (v/v) glycerol, 10 mM $MgCl_2$, 10 mM KCl, 250 μg mL^{-1} BSA, 2 mM GMP, 20 μg mL^{-1} poly(dA)·$(dT)_{12-18}$ (base ratio 10:1), 10 μM [^{3}H]dTTP (2.26 Ci $mmol^{-1}$), and 0.005–0.02 unit of enzyme. In some experiments, poly(dC)·$(dG)_{12-18}$ (base ratio 10:1) and 10 μM [^{3}H]dGTP (2.26 Ci $mmol^{-1}$) were substituted respectively for poly(dA)·oligo(dT) and [^{3}H]dTTP. Samples were incubated 30 min at 37 °C, and the trichloroacetic acid precipitable radioactivity was determined as described (Neville & Brown, 1972).

Assays of pol α using activated DNA contained the following in 25 μL: 20 mM potassium phosphate (pH 7.2), 0.1 mM EDTA, 4 mM DTT, 250 μg mL^{-1} BSA, 10 mM $MgCl_2$, 50 μM each of dATP, dGTP, and dCTP, 20 μM [^{3}H]dTTP (0.6 Ci $mmol^{-1}$), 10 μg of DNase-treated calf thymus DNA (Worthington), and 0.1–0.3 unit of enzyme. Incubations were at 37 °C for 30 min. Assays of pol δ using activated DNA included the following in 25 μL: 75 mM K^+HEPES (pH 7.5), 1.25 mM DTT, 20% (v/v) glycerol, 10 mM $MgCl_2$, 100 mM KCl, 250 μg mL^{-1} BSA, 2 mM GMP, 25 μM each of dATP, dGTP, and dCTP, [^{3}H]dTTP (2.26 Ci $mmol^{-1}$), and 0.005–0.02 unit of enzyme. Incubations were at 37 °C for 60 min.

Assay of the 3′→5′ exonuclease activity of pol δ were identical with those of the polymerase activity using poly(dA)·oligo(dT) as the template·primer, except that poly(dA)·$(dT)_{12-18} \cdot ([^{32}P]dC)_{0.33}$ or poly(dA)·$(dT)_{12-18} \cdot ([^3H]dT)_{0.3}$ was substituted for poly(dA)·oligo(dT), and [^{3}H]dTTP and GMP were omitted. Reactions using the mismatched substrate required about 0.075 polymerase unit of enzyme and 12-min incubation. Reactions using the properly paired substrate required about 0.5 polymerase unit of enzyme and 20-min incubation. Reactions were terminated by addition of 25 μL of 200 mM EDTA. DNA was prepared for scintillation counting as described (LaDuca et al., 1986).

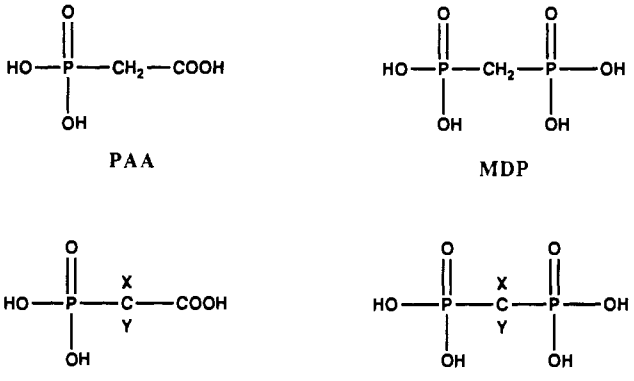
Results

Assay of Pol α and δ Activities. Poly(dA)·oligo(dT) is the template·primer preferred by pol δ (Focher et al., 1988b) and was used whenever possible in assessing inhibition action. Poly(dA)·oligo(dT) and the conditions for assaying pol δ also are effective for pol α, giving a level of DNA synthesis comparable to that directed by activated DNA under appropriate conditions (Talanian, unpublished results). All polymerase assays using poly(dA)·oligo(dT) included 2 mM GMP to inhibit the 3′→5′ exonuclease activity of pol δ (Byrnes et al., 1977), which was necessary to ensure linearity of the synthetic reactions at the low dNTP concentrations used in some experiments.

Screen of Methylenediphosphonic Acid and Phosphonoacetic Acid Derivatives as Inhibitors of Pol α and Pol δ Using Poly(dA)·Oligo(dT) as the Template·Primer. Twenty-three methylenediphosphonic acid (MDP) and phosphonoacetic acid (PAA) derivatives (Figure 1), many of which have been examined for their capacity to inhibit viral and mammalian DNA polymerases (McKenna et al., 1987; Öberg, 1989), were tested as inhibitors of pol α and HAP-δ in assays employing poly(dA)·oligo(dT) (Table I). The most potent inhibitors were the monohalogenated PAA analogues FPAA (**1**), BrPAA (**2**), and ClPAA (**3**), none of which displayed significant selectivity for either enzyme. Two compounds, COMDP (**4**) and FBrMDP (**7**), displayed significant selectivity for pol δ.

Potencies of Selected Compounds as Inhibitors of Pol α and Pol δ Assayed with Activated DNA as the Template·Primer. The template dependencies of the five most potent inhibitors from the above screen were tested by measuring their IC_{50} values as inhibitors of pol α and pol δ assayed with activated DNA. The results are summarized in Table II. Each compound was a weaker inhibitor of both enzymes under these conditions than in the analogous experiments using poly(dA)·oligo(dT). Further, with activated DNA, all five compounds inhibited pol δ with greater potencies than pol α. Because of its unique combination of potency and selectivity for pol δ, and the apparent template independence of that selectivity, COMDP was chosen for further study.

Patterns of Inhibition of Pol α and δ by COMDP. Figure 2 presents Lineweaver–Burk analyses of COMDP inhibition of pol α and FPLC-δ assayed with poly(dA)·oligo(dT), as a function of dTTP concentration. The results indicated that,



1, X=F, Y=H
2, X=Br, Y=H
3, X=Cl, Y=H
5, XY= =O
6, X=F, Y=F
8, X=F, Y=Br
9, X=F, Y=Cl
13, X=Cl, Y=Cl
14, X=Cl, Y=Br
15, X=Br, Y=Br
17, X=F, Y=Me
18, X=Cl, Y=Me
19, X=Br, Y=Me

4, XY= =O
7, X=F, Y=Br
10, X=F, Y=H
11, X=F, Y=F
12, X=F, Y=Cl
16, X=Cl, Y=Br
20, X=Cl, Y=H
21, X=Cl, Y=Cl
22, X=Br, Y=H
23, X=Br Y=Br

FIGURE 1: Structures of phosphonoacetic acid (PAA), methylenediphosphonic acid (MDP), and analogues of each.

Table I: Inhibition by Methylenediphosphonic Acid and Phosphonoacetic Acid Analogues of DNA Polymerases α and δ Assayed with Poly(dA)·Oligo(dT)[a]

no.	acronym	IC_{50} (μM)[b] pol α	pol δ
1	FPAA	1.2	0.9
2	BrPAA	8	3
3	ClPAA	5	4
4	COMDP	60	6
5	COPAA	22	22
6	F_2PAA	300	200
7	FBrMDP	>2000	400
8	FBrPAA	1000	900
9	FClPAA	2000	900
10	FMDP	1000	1000
11	F_2MDP	>1000	1000
12	FClMDP	1000	2000
13	Cl_2PAA	2000	2000
14	ClBrPAA	2000	2000
15	Br_2PAA	2000	2000
16	ClBrMDP	I	2000
17	FPPA	2000	>2000
18	ClPPA	>2000	>2000
19	BrPPA	I	>2000
20	ClMDP	I	>2000
21	Cl_2MDP	I	>2000
22	BrMDP	I	>2000
23	Br_2MDP	I	>2000

[a] DNA polymerase assays using poly(dA)·oligo(dT) were performed as described under Materials and Methods. [b] IC_{50} values were determined on the basis of concentration vs enzyme activity curves of up to eight points. Dicyclohexylamine and pyridine, the bases forming the counterions of several inhibitors, had no inhibitory effect at 5 mM on either enzyme (results not shown). I = inactive at 2 mM, the highest concentration tested.

with respect to dTTP, COMDP inhibited pol α uncompetitively and pol δ competitively. From the data of these plots we calculated K_i values for COMDP of 40 ± 5 μM for pol α and 1.8 ± 0.3 μM for pol δ (see legend to Figure 2). Experiments analogous to those of Figure 2 were performed to determine whether COMDP inhibition of pol α or pol δ is sensitive to template·primer concentration. The results, which are not shown, clearly demonstrated that for either enzyme

Table II: Inhibition of Methylenediphosphonic Acid and Phosphonoacetic Acid Analogues of DNA Polymerases α and δ Assayed with Activated DNA[a]

no.	acronym	IC_{50} (μM)[b] pol α	pol δ
1	FPAA	20	2
2	BrPAA	160	20
3	ClPAA	120	70
4	COMDP	300	40
5	COPAA	170	50

[a] DNA polymerase assays using activated DNA were performed as described under Materials and Methods. [b] IC_{50} values were determined on the basis of concentration vs enzyme activity curves of up to eight points.

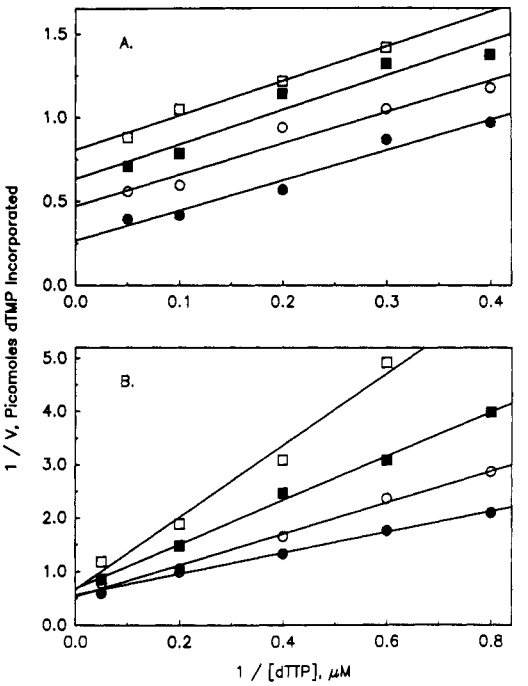


FIGURE 2: Inhibition of DNA polymerases α and δ by COMDP as a function of dTTP concentration. Enzymes were assayed as described under Materials and Methods, except that dTTP concentrations were varied as indicated. (A) DNA polymerase α, assayed in the presence of either 0 (●), 15 (○), 30 (■), or 60 μM (□) COMDP. (B) DNA polymerase δ, assayed in the presence of either 0 (●), 1 (○), 2 (■), or 4 μM (□) COMDP. Lines were fitted by least-squares regression. As appropriate, the slopes and x and y axis intercepts calculated from the equations of these lines were used to generate slope and intercept replots (not shown), which were likewise used to calculate K_i values. For pol α, the y axis intercept replot gave a K_i of 36 μM, and the x axis intercept replot gave a value of 45 μM. For pol δ, the slope replot indicated a K_i value of 1.5 μM, and the x axis intercept replot gave a value of 2.1 μM.

COMDP is not competitive with template·primer.

COMDP Competes Only with the dNTP Specified by the Template. With poly(dA)·oligo(dT) as the template·primer, only dTTP competes with COMDP; addition of a high concentration (800 μM) of either dATP, dGTP, or dCTP had little effect on its potency. With poly(dC)·oligo(dG) as the template·primer [a template·primer combination which directs a level of synthesis by poly δ about 20% of that directed by poly(dA)·oligo(dT)], COMDP was competitive only with dGTP; in this condition, the addition of 800 μM dATP, dCTP, or dTTP had little effect on its potency (results not shown).

Preincubation with COMDP Does Not Inactivate Pol δ. We observed that COMDP loses inhibitory activity in Tris buffer (50% after 1 month at −20 °C), whereas it is stable in HEPES buffer. The latter observation and consideration of the structure of COMDP suggested that its carbonyl group might

bind pol δ by forming a Schiff base with a primary amino group on the surface of the enzyme. Such an intermediate may be relatively stable and thus may not readily dissociate. To test that possibility, we examined the effect of exposing the enzyme to COMDP prior to assessment of its polymerase activity. FPLC-δ was incubated for 45 min at 37 °C with 8 μM COMDP in a poly(dA)·oligo(dT) polymerase reaction mix lacking only dTTP. The drug was then separated from the enzyme by Sephadex G-25 chromatography, and its activity as well as that of mock-treated enzyme was measured. The results of this experiment indicated that preincubation with COMDP had no effect on recovery of enzyme activity. We conclude that, under the polymerase assay conditions used in the experiments described here, COMDP does not form a demonstrably stable covalent complex with pol δ.

Effect of COMDP on the 3′→5′ Exonuclease Activity of Pol δ. We assayed the 3′→5′ exonuclease activity of FPLC-δ by measuring its ability to catalyze excision of the primer termini of the template·primers poly(dA)·$(dT)_{12-18}$·($[^{32}P]dC)_{0.33}$ and poly(dA)·$(dT)_{12-18}$·($[^{3}H]dT)_{0.3}$. Exonuclease activity measured on the mismatched primer terminus was 15–20% that of polymerase activity measured under standard conditions; with the properly base paired 3′-OH terminus, it was 2.5–4% that of polymerase activity. COMDP inhibited the exonuclease activity weakly, displaying IC_{50} values of >1 mM with both template·primers (results not shown).

Discussion

The long-range goal of the work presented in this paper has been to develop a selective small molecule inhibitor of pol δ which can be exploited to probe the structure, mechanism, and cellular function of the enzyme, and to distinguish its activity from that of other mammalian DNA polymerases, particularly pol α. COMDP represents a promising prototype for the development of an ideal pol δ inhibitor. In order to establish the generality of COMDP as a probe of mammalian pol δ, it will be important to determine its effect on PCNA-dependent calf thymus pol δ, as well as on pol δ derived from other tissues. In assays using poly(dA)·oligo(dT), COMDP is a respectably potent inhibitor of PCNA-independent calf thymus pol δ (K_i = 1.8 μM; Figure 2) and has significant selectivity, displaying a potency for pol δ over 20 times that for calf thymus pol α (Figure 2). Both the apparent potency and selectivity of the compound were decreased when activated DNA rather than poly(dA)·oligo(dT) was used as the template·primer. The compound appears to inhibit pol δ by mimicking a component of a 2′-deoxyribonucleoside 5′-triphosphate. The latter property is significant for two reasons. First, it constitutes strong evidence that the primary target for COMDP is the pol δ active site. Second, it suggests a rational basis for structural modification of COMDP toward the development of more potent and selective inhibitors of pol δ.

COMDP apparently inhibits pol α and pol δ by different mechanisms. The compound does not appear to mimic the template·primer with either enzyme, and in the case of pol α, its capacity to mimic a dNTP, if it exists at all, is likely masked by a "nonspecific" drug–protein interaction independent of the active site. With respect to pol δ, COMDP appears to act primarily by binding at a site within the dNTP binding domain of the enzyme. Given the structure of COMDP and its analogy with PP_i, we hypothesize that it binds pol δ at a part of the active site that reacts with the 5′-moiety of the incoming dNTP. This hypothesis might be tested by synthesizing and examining the structure–activity relationships of nucleotidyl derivatives of COMDP, for example, a dNTP in which the β–γ phosphoanhydride oxygen is replaced by a carbonyl group. The 3′→5′ exonuclease activity of pol δ, relative to the polymerase activity, is resistant to inhibition by COMDP. The latter observation suggests that the COMDP-induced effect on the polymerization of dNTPs has little impact on the action of the exonuclease-specific site of pol δ, and it argues that the polymerase and exonuclease sites are structurally distinct.

As indicated under Results, the structure of COMDP and the loss of potency it suffered in a Tris buffer suggested that COMDP action on pol δ might involve the formation of a stable Schiff base with an amino acid residue critical to the function of the dNTP binding domain. Although our ability to reverse the inhibitory effect of COMDP by Sephadex dialysis argued against stable adduct formation, we investigated this posssibility in an additional set of experiments. We asked whether $NaBH_4$ promoted irreversible inactivation of pol δ by COMDP; the experiments were modeled after those of Basu and Modak (1987) in which $NaBH_4$ was used to effect irreversible inactivation of *Escherichia coli* DNA polymerase I with pyridoxal phosphate. The results indicated that $NaBH_4$ promoted COMDP-dependent inactivation of pol δ. However, inactivation was not dependent on the presence of poly(dA)·oligo(dT) and was not prevented by excess dTTP. The latter observation suggested that Schiff base formation at the active site, if it occurred, was not the sole basis for COMDP inactivation of pol δ in the presence of $NaBH_4$. If the nucleotidyl derivatives of COMDP proposed above can be synthesized, they will be used to reexamine the possibility of pol δ active site labeling.

COMDP has a critical shortcoming as a discriminator of pol δ in vivo; it is charged and therefore likely penetrates intact cell membranes poorly if at all. Consistent with the notion of poor uptake is the observation of Stenberg (1981) that 500 μM COMDP had no effect on mammalian cell macromolecule synthesis. The charge of COMPD may limit its use as a probe of the in vivo function of pol δ to measurement of its capacity to inhibit macromolecule synthesis in isolated nuclei or in permeabilized cells. Experiments of the latter type are in progress.

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Articles

Template-Primer Activity of 5-(Hydroxymethyl)uracil-Containing DNA for Prokaryotic and Eukaryotic DNA and RNA Polymerases†

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ABSTRACT: We have utilized *Bacillus subtilis* phage SPO-1 DNA as a model of irradiated DNA. In this phage, all thymine (Thy) residues are replaced by 5-(hydroxymethyl)uracil (5HmUra), which is a known irradiation-induced derivative of DNA Thy. SPO-1 phage is naturally devoid of other such irradiation-induced DNA lesions. DNase I activated SPO-1 phage DNA served as well as, or even better than, the control DNAs (*Bacillus subtilis* DNA and calf thymus DNA) as a template-primer for *Escherichia coli*, *Micrococcus luteus*, and human HL-60 cell DNA polymerases. Futhermore, the template activity of SPO-1 phage DNA was also superior when transcription with *E. coli* RNA polymerase was investigated. The results reported here indicated that the replacement of Thy by 5HmUra is not deleterious to template and primer functions during DNA or RNA synthesis.

Three interesting situations are known when DNA Thy may partially or fully be replaced by 5HmUra: (i) 5HmUra is the natural constituent of DNA in certain *Bacillus subtilis* phages and dinoflagellates (Kallen et al., 1962; Okubo et al., 1964). (ii) 5HmUra is a known transmutation product in DNA of tritiated Thy (Teebor et al., 1984). It can also be induced by ionizing irradiation or other oxidative attack (Lewis et al., 1978; Frenkel et al., 1985; Frenkel & Chrzan, 1987; Teebor, et al., 1988). (iii) Finally, 5HmUra can be introduced into DNA from exogenous 5HmdUrd during semiconservative DNA replication (Matthes et al., 1979; Kahilainen et al., 1985; Kaufman, 1986; Boorstein et al., 1987; Vilpo & Vilpo, 1988).

We have investigated the biochemical consequences which might result if DNA Thy is replaced by 5HmUra. The experiments described in this report focus on the template-primer activity of 5HmUra-containing DNA. We have chosen *Bacillus subtilis* phage SPO-1 DNA for the experimental model, since all DNA Thy residues in this species are replaced naturally by 5HmUra (Kallen et al., 1962; Okubo et al., 1964). The results reported here demonstrate that the presence of 5HmUra does not compromise the template-primer functions of DNA; SPO-1 phage DNA is a good template-primer for various DNA and RNA polymerases when compared to the host (*Bacillus subtilis*) or calf thymus DNA.

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Experimental Procedures

Materials

Nonradioactive triphosphates were purchased from Calbiochem-Behring or from Sigma. [8-^{3}H]dATP (13.1 Ci/mmol) and [5-^{3}H]UTP (11.0 Ci/mmol) were from Amersham. *Escherichia coli* DNA polymerase I (Kornberg polymerase, endonuclease free; EC 2.7.7.7), *E. coli* RNA polymerase (EC 2.7.7.6), *Hin*dIII-digested λ phage DNA (molecular weight markers), and aphidicolin were purchased from Boehringer Mannheim. Sigma was the supplier of *Micrococcus luteus* DNA polymerase (EC 2.7.7.7), pancreatic DNase I (EC 3.1.21.1), and highly polymerized calf thymus DNA.

Methods

Template-Primers. Bacillus subtilis and its phage SPO-1 (catalog no. 27370-B1) were obtained from the American Type Culture Collection (Rockville, MD). The phage was cultured

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