Human Immunodeficiency Virus Reverse Transcriptase

SUBSTRATE AND INHIBITOR KINETICS WITH THYMIDINE 5'-TRIPHOSPHATE AND 3'-AZIDO-3'-DEOXYTHYMIDINE 5'-TRIPHOSPHATE*

John E. Heardon‡ and Wayne H. Miller

From the Experimental Therapy Division, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

3'-Azido-3'-deoxythymidine 5'-triphosphate (AZTTP) was an efficient substrate for the human immunodeficiency virus 1 reverse transcriptase. It was incorporated into both homopolymer and defined sequence DNA-primed RNA templates and DNA-primed DNA templates. The substrate and inhibitor kinetics of both AZTTP and dTTP were dependent on the template-primer and reaction conditions used. dTMP was incorporated into poly(rA)-oligo(dT) and into a defined sequence DNA-primed RNA template (when the other three 2'-deoxynucleoside 5'-triphosphates were present) as a conventional substrate, with steady-state $K_m$ values of 5–10 $\mu$M. The results suggest that the reverse transcriptase was capable of processive DNA polymerization on these DNA-primed RNA templates.

In contrast, in the absence of the other three 2'-deoxynucleoside 5'-triphosphates, the time course for incorporation of dTMP into the same defined sequence DNA-primed RNA template was biphasic. A burst of product formation was observed followed by a slow steady-state rate with a $K_m$ value of 0.082 $\mu$M. AZTMP incorporation into poly(rA)-oligo(dT) and into the defined sequence DNA-primed RNA template produced similar biphasic time courses and steady-state $K_m$ values. These results were consistent with rate-limiting dissociation of the polymerase-template-primer complex after "forced" termination of polymerization.

AZTMP and dTTP were both incorporated into the homopolymer DNA-primed DNA template, poly(dA)-oligo(dT), and a defined sequence DNA-primed DNA template as conventional substrates. Their $K_m$ values were similar (2–10 $\mu$M). The absence of biphasic time courses suggested that dissociation of the DNA-primed DNA templates from the enzyme, after forced termination, was not rate-limiting. This was consistent with a more distributive mode of DNA polymerization.

With the defined sequence template-primers and poly(dA)-oligo(dT), $K_v$ values for both dTTP and AZTTP were comparable to their $K_m$ values. Thus, AZTTP appeared to be a simple competitive substrate-inhibitor with respect to dTTP. AZTTP inhibition of dTMP incorporation into poly(rA)-oligo(dT) was linear at low concentrations (0–100 nM) of AZTTP ($K_i = 35$ nM) but became hyperbolic (decreasing potency) at concentrations of AZTTP above this range. A mechanism for this nonlinear inhibition is discussed.

Human immunodeficiency virus, the etiologic agent of acquired immunodeficiency syndrome (1, 2), contains a RNA-directed DNA polymerase (reverse transcriptase; EC 2.7.7.49) (3, 4). To date, the only approved clinical treatment for acquired immunodeficiency syndrome is the nucleoside analog 3'-azido-3'-deoxythymidine (Retrovir®, zidovudine, AZT) (5, 6). The most likely mechanism of antiviral action of AZT is inhibition of the viral reverse transcriptase by AZT triphosphate (AZTTP) (7). AZT has been shown to be a substrate for the cellular thymidine kinase, and the monophosphorylated form is converted to the triphosphate by other cellular kinases (8). Incorporation of AZTMP residues into DNA has been demonstrated (7), suggesting that AZTTP acts as an alternate substrate-inhibitor of reverse transcriptase. Since AZTTP does not contain a 3'-hydroxyl group, incorporation of AZTTP into DNA results in chain termination.

DNA synthesis catalyzed by reverse transcriptase occurs via an ordered mechanism for substrate binding, which is consistent with other DNA polymerases (9). Template-primer binding precedes binding of 2'-deoxynucleotide 5'-triphosphate, and on the synthetic homopolymer template-primer, poly(rA)-oligo(dT), the polymerization catalyzed by reverse transcriptase is processive (9, 25). $K_v$ values for AZTTP inhibition of human immunodeficiency virus reverse transcriptase from 2 to 120 nM have been reported (using poly(rA)-oligo(dT) as the template-primer) (7, 10–15).

In the present study, the substrate-inhibitor kinetic properties of dTTP and AZTTP were evaluated. Both homopolymer-template-primer and template-primers of defined sequence (in which the first nucleotide encoded by the template is dTMP) were used in the study. The results suggest that reverse transcriptase-catalyzed polymerization on RNA templates is processive whereas on DNA templates it is more distributive in nature. Forced termination of processive synthesis with RNA templates (i.e. termination after incorporation of an obligate chain terminator) resulted in rate-limiting dissociation of the template-primer from the enzyme. The slow rate of dissociation of the reverse transcriptase-template-primer complex accounted for the potent inhibition of the enzyme by AZTTP.

EXPERIMENTAL PROCEDURES

Materials—[methyl-3H]dTTP (20.4 Ci/mmol) was purchased from Du Pont-New England Nuclear. [methyl-3H]AZT (33 Ci/mmol) was purchased from Moravek Biochemicals Inc., Brea, CA; pyruvate kinase and phosphoenolpyruvate were from Boehringer Mannheim. Ultrapure deoxynucleoside 5'-triphosphates, (dA)$_{100}$, (rA)$_{100}$

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Experimental Therapy Div., Wellcome Research Laboratories, 3030 Cornwallis Rd., Research Triangle Park, NC 27709. Tel.: 919-248-4599.

1 The abbreviations used are: AZT, 3'-azido-3'-deoxythymidine; AZTMP, 3'-azido-3'-deoxythymidine 5'-monophosphate; AZTTP, 3'-azido-3'-deoxythymidine 5'-triphosphate; dNTP, 2'-deoxynucleoside 5'-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

20302
oligo(dT)\(_{18}\), and oligo(dT)\(_{12}\) were from Pharmacia LKB Biotechnology Inc. Plasmid pGEM4z and the in vitro transcription reagents were from Promega Biotec, Madison, WI. Restriction enzyme Smal was from Bethesda Research Laboratories, Bethesda, MD. The 44- and 21-mer oligonucleotides were prepared by Dr. Phillip A. Furman of these laboratories.

Preparation of RNA 44-mer Template—Plasmid pGEM4z was digested with the restriction enzyme Smal according to directions supplied by the manufacturer. Completion of the reaction was determined by gel electrophoresis. The digested plasmid was used as the template for T7 RNA polymerase in vitro transcription. The reagents and instructions provided by the manufacturer were used. All manipulations were performed under strict RNase-free conditions (diethyl pyrocarbonate-treated water). A sample of the RNA was radiolabeled to evaluate its purity by gel electrophoresis. The RNA was dephosphorylated using calf intestinal alkaline phosphatase (18) and then radiolabeled with T4 polynucleotide kinase and [\(\gamma^{32}P\)]ATP (19). Gel electrophoretic analysis showed the RNA to consist of 49-, 43-, and 44-mer. This material was used without further purification for kinetic studies (Scheme 1).

Template-Primer Annealing—Concentrations of oligonucleotides were estimated by their UV absorbance at 260 nm. To prepare r44:d21-mer (Scheme 1), equal molar amounts of the two oligonucleotides were combined in 10 mM Tris-HCl, pH 8, 0.5 mM EDTA, heated to 90°C for 1 min, placed at 50°C, and allowed to cool slowly over 30 min. The d44:d21-mer was prepared in the same manner. To prepare poly(rA)-oligo(dT) and poly(dA)-oligo(dT), a 2:1 molar ratio (primer to template) was combined in 10 mM Tris-HCl, pH 8, 0.5 mM EDTA at 50°C.

Enzyme Assays—Unless otherwise indicated, reaction mixtures for kinetic studies using either poly(rA)-oligo(dT) or poly(dA)-oligo(dT) contained buffer A (50 mM Tris-HCl, pH 7.8, 5 mM MgCl\(_2\), 0.025% Triton X-100), 2 \(\mu\)M template-primer, reverse transcriptase, and nucleotides in a total volume of 100–150 \(\mu\)l. Reaction mixtures for assays using either the r44:d21-mer or d44:d21-mer contained buffer A, 50 mM KCl, 2 \(\mu\)M template-primer, reverse transcriptase, and nucleotides in a total volume of 100–200 \(\mu\)l. Reaction mixtures, equilibrated at 37°C, were initiated with enzyme. Five to seven samples (15 or 30 \(\mu\)l) were removed during the course of the assay and spotted onto DE81 paper. The paper was washed, dried, and counted as described previously (20). The same results were obtained when samples were quenched with EDTA prior to spotting on DE81 paper. The EDTA quench was used as indicated in the text, when short reaction times were measured. The concentration of KCl required for optimal activity was dependent on the nature of the template-primer. These concentrations were determined here to be zero when poly(rA)-oligo(dT) and poly(dA)-oligo(dT) were combined with the r44:d21-mer and d44:d21-mer (data not shown). In all cases, K\(_{\text{m}}\) values for the different template-primers were <100 nM. K\(_{\text{m}}\) and V\(_{\text{max}}\) values were determined from linear steady-state velocities using the computer programs of Cleland (21).

Synthesis of [methyl-\(^3H\)]AZTTP—[methyl-\(^3H\)]AZT, 0.3 \(\mu\)mol (10 mCi), in 1 ml of water, was applied to a PrepSep C\(_18\) column (Fisher Scientific). After washing the column with 4 ml of 10 mM ammonium phosphate, pH 5.5, in 5% methanol to remove impurities, the [\(^3H\)]AZT was eluted with 2 ml of 7.5 mM ammonium phosphate in 25% methanol. The fractions containing [\(^3H\)]AZT were combined and concentrated under vacuum to 0.5 ml.

[\(^3H\)]AZTTP was synthesized using thymidine kinase from herpes simplex virus type 2, which was cloned, expressed in E. coli, and partially purified by Dick Gaillard, Steve Short, and Paul Ray from these laboratories. The reaction mixture (0.55 ml) contained 0.4 ml of the monophosphate synthesis reaction, 0.2 units of varicella zoster virus thymidine/thymidylic kinase (units based on \(\mu\)mol/min of thymidine phosphorylation), 40 IU of rabbit muscle pyruvate kinase, and 17 mM phosphoenolpyruvate. The reaction was complete after 3 days at 37°C. The reaction mixture was adjusted to pH 3 with phosphoric acid, and the insoluble protein was removed by centrifugation (6). The supernatant was applied to a Hammon Microsorb C\(_8\), high pressure liquid chromatography column (15 cm L \(\times\) 4.6 mm, inner diameter), which had been equilibrated with 20 mM ammonium phosphate, pH 3.5. [\(^3H\)]AZTTP was eluted with a gradient of 0–20% acetonitrile in water. Fractions containing [\(^3H\)]AZTTP were combined and dried under vacuum.

The purified [\(^3H\)]AZTTP contained approximately 98% AZTTP and 1% AZT diphosphate. Purity was determined by separation on TLC, and the distribution of radioactivity was determined by scanning the plates using a Bio-Scan system 200 imaging TLC scanner. Mono-, di-, and triphosphates were separated on cellulose-polyethyleneimine plastic backed TLC plates developed in 0.4 M LiCl, 0.4 M formic acid. R\(_f\) values were: monophosphate, 0.74; diphosphate, 0.41; triphosphate, 0.12. A small sample of [\(^3H\)]AZTTP was converted to [\(^3H\)]AZT with alkaline phosphatase and examined by silica gel TLC developed in chloroform/methanol (9:1). Radioactivity was detected only in the spot with R\(_f\) corresponding to AZT. R\(_f\) values were AZT, 0.57; thymidine, 0.38; 3'-amino-3'-deoxythymidine, 0.28; thymine, 0.41.

Theory—A kinetic scheme consistent with ordered binding of substrates and processive polymerization has been reported (9). A simplified kinetic model in which dissociation of the template-primer occurs after every nucleotide incorporation event is shown in Scheme 2, in which TP represents the template-primer, dNTP is the nucleotide encoded by the template, and TP\(_{+1}\) represents the template after incorporation of the required dNMP. In the presence of saturating template-primer, under conditions in which the rate constants for dissociation of the template-primer from the enzyme, k\(_{\text{off}}\), and k\(_{\text{a}}\), are much slower than k\(_{\text{d}}\), the Michaelis constant for dNTP is

\[
K_{\text{m}} = \frac{k_{\text{d}}(k_{\text{a}} + k_{\text{d}})}{k_{\text{a}}}
\]

When dissociation of the template-primer is rate determining (i.e. forced termination of processive polymerization), then k\(_{\text{off}}\) = k\(_{\text{d}}\). Under processive polymerization conditions in the presence of all required dNTPs, dissociation of the template-primer (TP\(_{+1}\)), k\(_{\text{d}}\), is not on the reaction pathway, and k\(_{\text{off}}\) = k\(_{\text{d}}\). Under these conditions, the Michaelis constant for dNTP is

\[
K_{\text{m}} = \frac{k_{\text{d}} + k_{\text{a}}}{k_{\text{a}}}
\]

In both cases (i.e. k\(_{\text{off}}\) = k\(_{\text{d}}\) and k\(_{\text{off}}\) = k\(_{\text{d}}\)), k\(_{\text{a}}/K_{\text{m}}\) > 1.

The results of this study are presented in two sets of experiments. The first set of experiments was designed to determine the effect of template-primer concentration on the rate of synthesis of poly(rA) .oligo(dT) and poly(dA) .oligo(dT) in the presence of dNMP in reaction mixtures of saturating template-primer. The second set of experiments was designed to determine the effect of template-primer concentration on the rate of synthesis of poly(rA) .oligo(dT) and poly(dA) .oligo(dT) in the presence of dNMP in reaction mixtures of limiting template-primer.
was diluted into dithiothreitol-free buffer immediately prior to use.

The $K_m$ value for dTTP was sensitive to buffer additives. In the presence of either bovine serum albumin, 0.025% Triton X-100, or no additive, the $K_m$ values for dTTP (with poly(rA)-oligo(dT) as the template-primer) were 2.5, 6.3, and 12 $\mu$M, respectively. Similar $K_m$ values determined under these different conditions have been reported (15, 25). The data presented here were obtained with buffer containing 0.025% Triton X-100.

**Substrate Kinetics on RNA Templates**—Reverse transcriptase-catalyzed incorporation of dTMP (in the absence of the other dNTPs) into the r44:d21-mer (Scheme 1) was biphasic. A burst of product formation was observed followed by a slow steady-state phase with a rate of 0.025 s$^{-1}$ (Fig. 1A). The $K_m$ value for the steady-state incorporation of dTMP was 0.082 $\mu$M. The amplitude of the burst for dTMP incorporation increased linearly with increasing concentrations of reverse transcriptase. From the slope of a replot of the amplitude of the burst versus the amount of reverse transcriptase added, an active site concentration of 0.26 $\mu$M was obtained (Fig. 1A). This concentration was used in the calculation of a turnover number of 2.7 s$^{-1}$ with the standard template-primer, poly(rA)-oligo(dT). This value is in good agreement with the reported value of 3 s$^{-1}$ (9, 24). The burst of product formation was not dependent upon preincubation of the template-primer with the enzyme, suggesting that template-primer binding to the enzyme was not the slow step. When the other three dNTPs were included in the reaction mixture with dTTP, no burst of product formation was observed (Fig. 1A), and the $K_m$ for dTTP was 10 $\mu$M. AZTTP was also incorporated into the r44.d21-mer in a biphasic time course (Fig. 1B). The amplitude of the burst for AZTTP incorporation increased linearly with increasing concentrations of reverse transcriptase. From the slope of a replot of the amplitude of the burst versus the amount of reverse transcriptase added, an active site concentration of 0.26 $\mu$M was once again obtained (Fig. 1B). The steady-state kinetic constants for dTTP and AZTTP incorporation into the r44:d21-mer are shown in Table I.

When reverse transcriptase was incubated with poly(rA)-oligo(dT), dTTP, and AZTTP, product formation versus time plots were linear and intersected at the origin (data not shown). Double-reciprocal plots at each fixed concentration of poly(rA)-oligo(dT) were linear, and the lines converged to the left of the ordinate, consistent with a sequential mechanism for substrate binding (9). The kinetic constants were: $K_m$(poly(rA)-oligo(dT)) = 0.059 $\mu$M, $K_m$(dTTP) = 0.025 $\mu$M, $K_m$(AZTTP) = 0.3 $\mu$M, and $k_{cat}$ = 2.7 s$^{-1}$. When AZTTP was the substrate, a burst of product formation was observed followed by a linear steady-state rate. As seen with the r44:d21-mer, the rapid initial incorporation increased linearly with increasing concentrations of reverse transcriptase (data not shown). The rapid initial phase of the biphasic time course for the incorporation of AZTTP was observable at a reduced temperature (Fig. 2). Similar data were obtained from studies on the incorporation of both AZTTP and dTTP (in the absence of the other dNTPs) into the r44:d21-mer, described above. The steady-state kinetic constants for dTTP and AZTTP incorporation into poly(rA)-oligo(dT) are shown in Table I.

**Substrate Kinetics on DNA Templates**—In contrast to the results obtained with the r44:d21-mer, when the d44:d21-mer was the template-primer neither dTTP nor AZTTP showed a burst of product formation. Similar results were obtained with poly(dA)-oligo(dT). The kinetic constants for dTTP and AZTTP incorporation into the d44:d21-mer and poly(dA)-oligo(dT) are shown in Table II.

**Inhibition Kinetics**—The data describing AZTTP inhibi-

---

**Table I.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (dTTP)</th>
<th>$K_m$ (AZTTP)</th>
<th>$K_m$ (dATP)</th>
<th>$K_m$ (dGTP)</th>
<th>$K_m$ (dCTP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTTP</td>
<td>10 $\mu$M</td>
<td>100 $\mu$M</td>
<td>100 $\mu$M</td>
<td>100 $\mu$M</td>
<td>100 $\mu$M</td>
</tr>
<tr>
<td>AZTTP</td>
<td>100 $\mu$M</td>
<td>100 $\mu$M</td>
<td>100 $\mu$M</td>
<td>100 $\mu$M</td>
<td>100 $\mu$M</td>
</tr>
</tbody>
</table>

**Table II.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (dTTP)</th>
<th>$K_m$ (AZTTP)</th>
<th>$K_m$ (dATP)</th>
<th>$K_m$ (dGTP)</th>
<th>$K_m$ (dCTP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTTP</td>
<td>2.5 $\mu$M</td>
<td>2.5 $\mu$M</td>
<td>2.5 $\mu$M</td>
<td>2.5 $\mu$M</td>
<td>2.5 $\mu$M</td>
</tr>
<tr>
<td>AZTTP</td>
<td>2.5 $\mu$M</td>
<td>2.5 $\mu$M</td>
<td>2.5 $\mu$M</td>
<td>2.5 $\mu$M</td>
<td>2.5 $\mu$M</td>
</tr>
</tbody>
</table>

**Fig. 1.**

**Fig. 2.**
### TABLE I

**Kinetic constants for reverse transcriptase on RNA templates**

<table>
<thead>
<tr>
<th>Template-primer</th>
<th>Substrate</th>
<th>Burst*</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (µM⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>r44:d21-mer</td>
<td>dTTP</td>
<td>Yes</td>
<td>0.082 ± 0.013</td>
<td>0.025 ± 0.0008</td>
<td>0.30</td>
</tr>
<tr>
<td>r44:d21-mer</td>
<td>dTTP(dNTPs)*</td>
<td>No</td>
<td>10 ± 2</td>
<td>1.9 ± 0.2</td>
<td>0.19</td>
</tr>
<tr>
<td>r44:d21-mer</td>
<td>AZTTP</td>
<td>Yes</td>
<td>0.19 ± 0.02</td>
<td>0.071 ± 0.002</td>
<td>0.97</td>
</tr>
<tr>
<td>Poly(rA)-oligo(dT)</td>
<td>dTTP</td>
<td>No</td>
<td>6.3 ± 0.8</td>
<td>2.7 ± 0.1</td>
<td>0.43</td>
</tr>
<tr>
<td>Poly(rA)-oligo(dT)</td>
<td>AZTTP</td>
<td>Yes</td>
<td>0.22 ± 0.01</td>
<td>0.059 ± 0.002</td>
<td>0.27</td>
</tr>
</tbody>
</table>

* Burst refers to the observation of a biphasic time course for product formation.

* Reaction mixtures contained 50 µM each dGTP, dATP, and dCTP.

### TABLE II

**Kinetic constants for reverse transcriptase on DNA templates**

<table>
<thead>
<tr>
<th>Template-primer</th>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (µM⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d44:d21-mer</td>
<td>dTTP</td>
<td>1.8 ± 0.1</td>
<td>0.68 ± 0.002</td>
<td>0.38</td>
</tr>
<tr>
<td>d44:d21-mer</td>
<td>dTTP(dNTP)*</td>
<td>2.5 ± 0.2</td>
<td>1.8 ± 0.06</td>
<td>0.72</td>
</tr>
<tr>
<td>d44:d21-mer</td>
<td>AZTTP</td>
<td>5.7 ± 0.5</td>
<td>0.55 ± 0.04</td>
<td>0.096</td>
</tr>
<tr>
<td>Poly(dA)-oligo(dT)</td>
<td>dTTP</td>
<td>7.4 ± 0.43</td>
<td>0.11 ± 0.004</td>
<td>0.015</td>
</tr>
<tr>
<td>Poly(dA)-oligo(dT)</td>
<td>AZTTP</td>
<td>9.3 ± 1</td>
<td>0.066 ± 0.008</td>
<td>0.0071</td>
</tr>
</tbody>
</table>

* Reaction mixtures contained 50 µM each dGTP, dATP, and dCTP.

**Fig. 3.** Lineweaver-Burke plot of AZTTP inhibition of dTTP incorporation into r44:d21-mer. Reaction mixtures (100 µl) contained buffer A, 50 mM KCl, 2 µM r44:d21-mer, dTTP (20.4 Ci/mmol), and AZTTP (0.0, 0.25, 0.5, and 1.0 µM). Inset, replot of slope versus [AZTTP] ($K_{AZTTP} = 0.16 ± 0.02$ µM).

### DISCUSSION

Early reports on the steady-state kinetics of reverse transcriptase suggested a processive mechanism for incorporation of dTMP into poly(rA)-oligo(dT) (9). However, when poly(dA)-oligo(dT) was used as the template-primer, the incorporation of dTMP catalyzed by reverse transcriptase appeared to be distributive (25). Similar contrasting kinetic behavior was observed in the present study for reverse transcriptase between RNA templates and DNA templates. During processive polymerization, the rate of template-primer dissociation from the enzyme is slow relative to the rate of translocation and binding of the next required nucleotide. Under these conditions, the rate-determining step is most likely the catalytic step or a precatalytic conformational change as is seen with *E. coli* DNA polymerase I (Klenow) (26). When the incorporation of a single nucleotide or obligate chain-terminating nucleotide analog into the r44:d21-mer was examined, the steady-state rate was determined by the rate of template-primer dissociation from the enzyme.

A kinetic mechanism describing the incorporation of dTMP (in the absence of the other dNTPs) into poly(rA)-oligo(dT) is shown in Scheme 2 (see “Experimental Procedures”). The results suggest that the first dTTP binds and is rapidly...
The kinetic data describing the incorporation of dTMP and AZTMP into the DNA-primed DNA templates were in sharp contrast to the results on the DNA-primed RNA templates. With both the d44:d21-mer and poly(dA)-oligo(dT), the kinetics of incorporation of both dTTP and AZTTP were linear, and no burst of product formation was observed. Furthermore, although a moderate increase in $k_{cat}$ for incorporation of dTMP into the d44:d21-mer was observed in the presence of the other three dNTPs, the $K_m$ value was essentially unchanged. These results suggest that polymerization on these template-primer is more distributive in nature.

The $K_m$ values for dTTP on the r44:d21-mer (Table III) were comparable with their respective $K_m$ values. These results support the interpretation that AZTTP is a simple competitive substrate-inhibitor with respect to dTTP. Similar results were obtained with the d44:d21-mer and poly(dA)-oligo(dT). With poly(dA)-oligo(dT), the $K_m$ value of 0.035 ± 0.004 μM was obtained from $k_{cat}/K_m$. As can been seen from Equations 1–3, the kinetic mechanism shown in Scheme 2, $k_{cat}/K_m$ is independent of a change in rate-determining step from $k_2$ to $k_3$ and $K_m/K_{cat}$ = $k_3/k_2$, consistent with the data presented here. Thus, the low $K_m$ values for AZTTP and dTTP (in the absence of the other dNTPs) result from the slow rate of template-primer dissociation from the enzyme.

Under conditions in which the rate of template-primer dissociation is slow relative to the other steps in the reaction pathway, if $k_3 > k_2$ (high commitment to catalysis), then in the steady-state $k_{cat} = k_3$, and $K_m = k_3/k_2$. From this, a $K_m$ value for AZTTP of 0.035 ± 0.004 μM was obtained from $-x = K_m(1 + [S]/K_m)$. These results suggest that polymerization on these template-primer is more distributive in nature.

The kinetics of incorporation of AZTMP into the r44:d21-mer were also consistent with Scheme 2. The similar values of $k_{cat}/K_m$ for both dTTP and AZTTP suggest that AZTTP is a very good substrate for reverse transcriptase. Further, the similar $k_{cat}$ values for both dTTP and AZTTP suggest that incorporation of an AZTMP residue onto the 3' primer terminus does not significantly affect the rate of dissociation of the template-primer from the enzyme. The kinetics of incorporation of dTMP into poly(rA)-oligo(dT) gave results similar to those for incorporation of dTMP into the r44:d21-mer.
was incorporated. Huber et al. (25) obtained similar results using poly(rA)-oligo(dT)$_{30}$. A similar gel electrophoresis assay was used here to examine the processivity with poly(rA)-oligo(dT)$_{30}$. A significant increase in processivity appeared to occur after the 4th dTMP residue incorporated (data not shown). If the rate constant for dissociation of poly(rA)-oligo(dT)$_{30}$ from the enzyme is greater after the first turnover than after subsequent turnovers, then the $K_m$ value for AZTTP would reflect this increased off-rate. In contrast, since the $K_m$ for AZTTP is off-rate dependent, the $K_m$ for AZTTP would depend on the number of dTMP incorporations prior to incorporation of AZTMP. If several dTMP residues were incorporated prior to incorporation of AZTMP, a $K_m$ value lower than the $K_m$ value might be expected. Further, as the ratio of AZTTP to dTTP increases, the probability of AZTTP incorporation during the initial turnover on a given template-primer increases. This would result in an increase in the apparent $K_m$ value, as seen here with poly(rA)-oligo(dT)$_{30}$, would be consistent with a dependence of the dissociation rate constant for the reverse transcriptase-poly(rA)-oligo(dT)$_{30}$ complex on the number of prior dTMP incorporation events.

The results presented here indicate that AZTTP is a simple competitive substrate-inhibitor with respect to dTTP and that the kinetic constants describing its incorporation into a template-primer and/or inhibition of dTTP incorporation are influenced greatly by the rate constant for dissociation of the reverse transcriptase-template-primer complex. The inhibition kinetics with the homopolymer template-primer poly(rA)-oligo(dT)$_{30}$ were more complex and may be indicative of a dependence of the template-primer dissociation rate on the extent of polymerization. A more complete presteady-state kinetic analysis should permit determination of the values for all of the rate constants in the kinetic pathway. The data presented here for dTMP incorporation into DNA-primed RNA templates is consistent with a kinetic mechanism similar to that described previously for E. coli DNA polymerase I (Klenow) (26).

Acknowledgments—We thank Dr. David Porter and Dr. Tom Spector for many helpful discussions throughout the course of this work and for many helpful criticisms of this manuscript. We also thank Dr. Tom Krentisky for his continued support and interest.

REFERENCES

Human immunodeficiency virus reverse transcriptase. Substrate and inhibitor kinetics with thymidine 5'-triphosphate and 3'-azido-3'-deoxythymidine 5'-triphosphate.

J E Reardon and W H Miller


Access the most updated version of this article at http://www.jbc.org/content/265/33/20302

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/33/20302.full.html#ref-list-1