Human Immunodeficiency Virus Reverse Transcriptase: Steady-State and Pre-Steady-State Kinetics of Nucleotide Incorporation

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ABSTRACT: Steady-state and pre-steady-state kinetic constants were determined for reverse transcriptase catalyzed incorporation of nucleotides and nucleotide analogues into defined-sequence DNA primed-RNA templates. 3′-Azido-3′-deoxythymidine 5′-triphosphate (AZTTP) was almost as efficient a substrate (k_{cat}/K_m) as dTTP for the enzyme. In contrast, the four 2′,3′-dideoxynucleoside 5′-triphosphates and 3′-deoxy-2′,3′-didehydrothymidine 5′-triphosphate (d4TTP) were 6–30-fold less efficient substrates of the enzyme. The k_{cat} values for all nucleotide analogues were similar, consistent with a kinetic model in which the steady-state rate-limiting step was dissociation of the template-primer from the enzyme [Reardon, J. E., & Miller, W. H. (1990) *J. Biol. Chem. 265*, 20302–20307]. The pre-steady-state kinetics of single-nucleotide incorporation were consistent with the kinetic model:

$$E + TP \overset{K_d^1}{\rightleftharpoons} E{\cdot}TP \overset{K_d^2}{\rightleftharpoons} E{\cdot}TP{\cdot}dNTP \xrightarrow{k_p} E{\cdot}TP_{n+1} \xrightarrow{k_{off}} E + TP_{n+1}$$

where E, TP, and dNTP represent reverse transcriptase, a defined-sequence DNA primed-RNA template, and 2′-deoxynucleoside 5′-triphosphate (or analogue), respectively. The dissociation constant (K_d^1) for template-primer binding was 10 nM, and the estimated rate constants for association and dissociation of the enzyme·template-primer complex were 4×10^6 M^{-1} s^{-1} and 0.04 s^{-1}, respectively. The dissociation constants (K_d^2) for dTTP, AZTTP, and 3′-deoxythymidine 5′-triphosphate (ddTTP) were 9, 11, and 4.6 μM, respectively. Thus, the differences in steady-state K_m values were not due to differences in binding of the nucleotide analogues to the enzyme. In contrast, the rate-limiting step during single-nucleotide incorporation (k_p) was sensitive to the structure of the nucleotide substrate. The values of k_p for dTTP, AZTTP, and ddTTP were 14, 5.4, and 0.41 s^{-1}, respectively. The K_m and k_{cat}/K_m values calculated from the values of K_d and k_p and the rate constants for dissociation of the enzyme·template-primer complexes (k_{off}) were in good agreement with the values obtained by steady-state kinetic analysis. Finally, there was no phosphorothioate elemental effect upon sulfur substitution at the α-phosphorus of dTTP. The results demonstrate that the substrate efficiency of nucleotide analogues was determined by the rate constant for phosphodiester bond formation or possibly by a conformational change preceding catalysis.

Human immunodeficiency virus encodes an RNA-directed DNA polymerase (reverse transcriptase; EC 2.7.7.49) that converts genomic viral RNA to double-stranded DNA (Rey et al., 1984; Hoffman et al., 1985). The essential role of reverse transcriptase in the retroviral life cycle has made it a primary target for development of therapeutic agents for treatment of acquired immunodeficiency syndrome (AIDS).[1] The first approved clinical treatment for AIDS was the nucleoside analogue 3′-azido-3′-deoxythymidine (RETROVIR, Zidovudine, AZT) (Mitsuya et al., 1985; Yarchoan et al., 1986). AZT is converted to the triphosphate by cellular kinases (Furman et al., 1986). AZTTP is a substrate for reverse transcriptase that competes with dTTP for incorporation into DNA. Incorporation of AZTMP into DNA results in chain termination (Furman et al., 1986; St. Clair et al., 1987).

A kinetic mechanism that explains the potent inhibition of reverse transcriptase by AZTTP and other obligate chain-terminating nucleotide analogues has been described (Reardon & Miller, 1990; Muller et al., 1991). The rate-determining step for AZTMP incorporation into the 3′-primer terminus is dissociation of the chain-terminated template-primer from the enzyme. The slow rate of dissociation of the template-primer from the enzyme leads to steady-state accumulation of the reverse transcriptase·chain-terminated template-primer complex and results in potent inhibition of the enzyme. Therefore, the steady-state inhibition of reverse transcriptase is not due to tight binding of AZTTP to the enzyme but to the slow dissociation of the chain-terminated template-primer product. This kinetic mechanism is characteristic of processive DNA polymerases (Bryant et al., 1983; Kuchta et al., 1987; Patel et al., 1991).

Many nucleoside triphosphate analogues have been tested as inhibitors of HIV reverse transcriptase (St. Clair et al., 1987; Cheng et al., 1987; Starnes & Cheng, 1988; White et al., 1989; Balzarini et al., 1991). Typically, the steady-state K_i value for a nucleoside triphosphate analogue is determined with a homopolymeric template-primer. Several factors complicate comparison of these steady-state K_i values for prediction of in vivo efficacy. First, the K_i value for an obligate chain-terminating nucleotide analogue is largely determined by the rate constant for dissociation of the enzyme·template-primer complex. Further, the rate constant for dissociation of the enzyme·template-primer complex depends on the template-primer sequence. Thus, the more processive reverse

[1] Abbreviations: AIDS, acquired immunodeficiency syndrome; AZT, 3′-azido-3′-deoxythymidine; AZTMP, 3′-azido-3′-deoxythymidine 5′-monophosphate; AZTTP, 3′-azido-3′-deoxythymidine 5′-triphosphate; dNTP, 2′-deoxynucleotide 5′-triphosphate; ddATP, 2′,3′-dideoxyadenosine 5′-triphosphate; ddCTP, 2′,3′-dideoxycytidine 5′-triphosphate; d4TTP, 3′-deoxy-2′,3′-didehydrothymidine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; HIV, human immunodeficiency virus; Tris, tris(hydroxymethyl)aminomethane.

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transcriptase is on a template-primer (i.e., the smaller the rate constant for dissociation of the enzyme·template-primer complex), the smaller the K_i value. For example, the K_i value for AZTTP inhibition of reverse transcriptase with the processive template-primer poly(rA)–oligo(dT) is 30 nM compared to 9.3 μM with the distributive template-primer poly(dA)–oligo(dT) (Reardon & Miller, 1990). Second, reverse transcriptase processively depends on salt concentration (Huber et al., 1989). Therefore, comparison of K_i values for inhibitors determined using different homopolymeric template-primers or with the same template-primer under different assay conditions is meaningless.

Because the in vivo ratio of reverse transcriptase to the $tRNA^{Lys3}$-primed genomic RNA is approximately 1:1 (Varmus & Swanstrom, 1984), inhibition of genome replication in vivo is not at steady-state. Therefore, the potent competitive inhibition by AZTTP observed at steady-state cannot contribute significantly to the antiviral effect. However, a single chain-termination event could theoretically prevent viral genome replication. Thus, the relative substrate efficiency of nucleotide analogues for reverse transcriptase may be a more reliable measurement of potential antiviral efficacy. The k_{cat}/K_m values for dTTP and AZTTP are similar, indicating that AZTTP is almost as efficient a substrate as dTTP for reverse transcriptase (Reardon & Miller, 1990). In the present study, steady-state kinetic constants were determined for a series of obligate chain-terminating nucleotide analogues. Differences in the steady-state K_m values were explained by analysis of the pre-steady-state kinetics of nucleotide incorporation.

Experimental Procedures

Materials. [*methyl*-^{3}H]dTTP, [8-^{3}H]dGTP, [5,5′-^{3}H]dCTP, [8-^{3}H(N)]dATP, [γ-^{32}P]ATP, dTTPαS (S_p isomer), and [α-^{35}S]dTTPαS (S_p isomer) were from Dupont New England Nuclear Research Products, Wilmington, DE; [5-^{3}H]AZTTP and [2,8-^{3}H]ddATP were from Moravek Biochemicals, Brea, CA; ultrapure 2′-deoxynucleoside 5′-triphosphates and ultrapure 2′,3′-dideoxynucleoside 5′-triphosphates were from Pharmacia Molecular Biology Products, Piscataway, NJ; the 21mer, 22mer, 23mer, and 24mer DNA oligonucleotides were from Midland Certified Reagent Co., Midland, TX; T4 polynucleotide kinase was from Bethesda Research Labs, Gaithersburg, MD; electrophoresis chemicals and buffers were from International Biotechnologies, New Haven, CT; DE81 paper and 2.5-cm disks were from Whatman, Clifton, NJ. The 44mer RNA template was prepared by in vitro transcription (Reardon & Miller, 1990). Reverse transcriptase from the HXB-2 strain of HIV, produced in *Escherichia coli* and purified by conventional chromatography, was kindly provided by Phillip A. Furman and Paul Ray of the Wellcome Research Laboratories. Reverse transcriptase concentrations were determined with $\epsilon_{279} = 334\,000\ M^{-1}\ cm^{-1}$.

Template-Primer Annealing. Concentrations of oligonucleotides were estimated by their UV absorbance at 260 nm with the following extinction coefficients (260 nm): r44mer, $\epsilon = 399\,000\ M^{-1}\ cm^{-1}$; d21mer, $\epsilon = 234\,000\ M^{-1}\ cm^{-1}$; d22mer, $\epsilon = 244\,000\ M^{-1}\ cm^{-1}$; d23mer, $\epsilon = 257\,000\ M^{-1}\ cm^{-1}$; d24mer, $\epsilon = 264\,000\ M^{-1}\ cm^{-1}$. Equimolar amounts of the r44mer and the primer of choice were combined in 10 mM Tris-HCl (pH 8) and 0.5 mM EDTA, heated to 90 °C for 3 min, and placed at 60 °C and allowed to cool slowly over 60 min. Annealed template-primers were stored at −20 °C. Radiolabeled (^{32}P) template-primers were annealed immediately prior to use.

Steady-State Enzyme Assays. Reaction mixtures (25 °C) for steady-state kinetic measurements contained 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 50 mM KCl, 0.025% Triton X-100, 1 μM template-primer, 2–20 nM HIV reverse transcriptase, and nucleotide substrates and/or inhibitors in a total volume of 100 μL. Aliquots (15 μL) were removed at five time points and spotted onto DE81 filters. The filters were washed with 125 mM Na_2HPO_4, and the amount of product formed was assessed by liquid scintillation counting in Ready Safe liquid scintilation fluid.

Rapid-Quench Experiments. Rapid-quench experiments were conducted using a Kin-Tec rapid-mixing apparatus (Johnson, 1986). Enzyme·template-primer complex (45 μL) in 50 mM Tris-HCl (pH 7.8), 50 mM KCl, 2.5 mM EDTA, and 0.025% Triton X-100 was rapidly mixed with the nucleotide substrate (45 μL) in 50 mM Tris-HCl (pH 7.8), 15 mM $MgCl_2$, 50 mM KCl, 2.5 mM EDTA, and 0.025% Triton X-100, and the reactions were then quenched with 0.3 M EDTA (final concentration) after time intervals of 5 ms or longer. When dTTPαS was the nucleotide substrate, 2 mM dithiothreitol was added to the reaction buffer, and 5 mM dithiothreitol was added to the EDTA quench solution. The method of measuring product formation was dependent on the radiolabeled substrate. When radiolabeled nucleotides were used, product formation was assessed by the DE81 filter binding assay. When radiolabeled template-primers were used, product formation was assessed by measuring the amount of substrate and product primers after separation by sequencing gel electrophoresis.

Gel Electrophoresis. Oligonucleotide primers were labeled at the 5′ end using T4 polynucleotide kinase and [γ-^{32}P]ATP according to the directions of the manufacturer. Samples for electrophoresis were heated to 90 °C for 3 min and then cooled rapidly on ice prior to electrophoresis in a 15% or 20% polyacrylamide/7 M urea gel at 85 constant W. After electrophoresis, Kodak XAR-2 film was exposed to the gel at −20 °C. The amount of substrate primer converted to product was determined by scanning the autoradiogram using a CAMAG electrophoresis scanner and/or by excising the radiolabeled substrate and product bands from the gel followed by liquid scintillation counting in Ready Safe liquid scintillation fluid. Reaction mixtures for determination of k_{cat} values for nucleotide analogues contained 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 50 mM KCl, 0.5 μM template-[5′-^{32}P]primer (3000 cpm/pmol), 15 nM reverse transcriptase, and the nucleotide concentration over 10 times its K_m value. Aliquots (5 μL) from the reaction mixtures were quenched with 5 μL of gel loading buffer (80% formamide, 50 mM Tris–borate, 1 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol).

Data Analysis. The nucleotide concentration dependence of the pre-steady-state burst rate for nucleotide incorporation was fitted to eq 1 where A was the amplitude of the burst, k_p

$$[\text{product}] = A\{1 - e^{-[k_p[S]/(K_d+[S])]t}\} + k_{ss}t \qquad (1)$$

was the first-order rate constant at saturating nucleotide substrate, [S] was the nucleotide concentration, K_d was the equilibrium dissociation constant for the nucleotide, and k_{ss} was the final steady-state rate.

Time courses at a fixed nucleotide concentration were fitted to

$$[\text{product}] = A[1 - e^{-k_{obs}t}] + k_{ss}t \qquad (2)$$

where A was the amplitude of the burst, k_{obs} was the observed first-order rate constant describing the burst, and k_{ss} was the final steady-state rate.

The equilibrium dissociation constant for reverse transcriptase binding to template-primer was determined by ti-

r44 mer 3'-CCC CUA GGA GAU CUC AGC UGG ACG UCC GUA CGU UCG AAC AGA GG-5'

d21 mer 5'-GGG GAT CCT CTA GAG TCG ACC-3'

d22 mer 5'-GGG GAT CCT CTA GAG TCG ACC T-3'

d23 mer 5'-GGG GAT CCT CTA GAG TCG ACC TG-3'

d24 mer 5'-GGG GAT CCT CTA GAG TCG ACC TGC-3'

FIGURE 1: Sequence of defined template-primers. The nomenclature refers to the type (RNA vs DNA) and the length of the oligonucleotide.

tration of the enzyme. The enzyme·template-primer complex concentrations, determined as a function of template-primer concentration, were fitted to eq 3 where [E·S] was the reverse

$$[E\cdot S] = 0.5(K_d + E_t + St) - 0.5\sqrt{(K_d + E_t + S_t)^2 - 4E_tS_t} \quad (3)$$

transcriptase·template-primer complex concentration, E_t was the total enzyme concentration, S_t was the total template-primer concentration, and K_d was the dissociation constant.

K_m, K_i, and V_{max} values were determined from linear steady-state velocities using the computer programs of Cleland (1979).

Theory. A simplified kinetic model for reverse transcriptase, where the concentration of template-primer is saturating and dissociation of the template-primer occurs after every nucleotide incorporation event, is shown in Scheme I where E·TP is the reverse transcriptase·template-primer complex, dNTP is the nucleotide encoded by the template, and TP_{n+1} is the template-primer after incorporation of the required dNMP.

Scheme I

$$E\cdot TP \overset{K_d}{\rightleftharpoons} E\cdot TP\cdot dNTP \xrightarrow{k_p} E\cdot TP_{n+1} \xrightarrow{k_{off}} E + TP_{n+1}$$

The steady-state rate-limiting step for single-nucleotide incorporation into defined-sequence template-primers is dissociation of the template-primer from the enzyme (i.e., $k_{cat} = k_{off}$) (Reardon & Miller, 1990). Under these conditions, the Michaelis constant for dNTP and k_{cat}/K_m are

$$K_m = k_{off}K_d/k_p \quad (4a)$$

$$k_{cat}/K_m = k_p/K_d \quad (4b)$$

RESULTS

Steady-State Kinetics of Nucleotide Incorporation. Steady-state kinetic constants were determined for incorporation of the four natural dNTP substrates into a series of related DNA-primed RNA templates (Figure 1). The steady-state rate-determining step, k_{cat} for single-nucleotide incorporation is dissociation of the enzyme·template-primer complex (Reardon & Miller, 1990). The rate constants for dissociation of the four related template-primers used in the present study from reverse transcriptase varied 8-fold (Table I). Rate constants for dissociation of template-primers from *E. coli* pol I (Klenow) and T7 DNA polymerase are also sequence-dependent (Kuchta et al., 1987; Patel et al., 1991). The similar k_{cat}/K_m values for the four natural dNTP substrates suggested that their binding to reverse transcriptase and their respective rates of bond formation were similar.

AZTTP competitively inhibits incorporation of dTMP into the r44:d21mer. The K_i value for AZTTP is equal to its K_m value as a substrate with this template-primer (Reardon & Miller, 1990).[2] Similarly, dTTP is a competitive inhibitor of AZTMP incorporation into the r44:d21 mer with a K_i value equal to its K_m value as a substrate. Thus, dTTP and AZTTP behave as classical competitive substrates with this template-primer. Therefore, determination of K_i values with a defined-sequence template-primer provided a simple method for obtaining K_m values for nonradiolabeled nucleoside triphosphate analogues. Once the K_m value was known, k_{cat} was determined using a gel electrophoresis–primer extension assay. This approach was used here to determine the substrate kinetic constants for a series of obligate chain-terminating nucleotide analogues. The k_{cat} and K_m values for AZTTP determined by this method agreed with those values obtained directly using [^{3}H]AZTTP (Table I). To further validate this method, the

Table I: Steady-State Kinetic Constants of Reverse Transcriptase[a]

template-primer	nucleotide	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
r44:d21	dTTP	0.035 ± 0.004[b]	0.025 ± 0.002[b]	0.71
	AZTTP	0.050 ± 0.004[b]	0.015 ± 0.002[b]	0.30
	AZTTP	0.057 ± 0.009[c]	0.025[d]	0.44
	ddTTP	0.24 ± 0.02[c]	0.012[d]	0.05
	d4TTP	0.24 ± 0.03[c]	0.032[d]	0.13
r44:d22	dGTP	0.057 ± 0.007[b]	0.047 ± 0.003[b]	0.82
	ddGTP	0.82 ± 0.04[c]	0.027[d]	0.03
r44:d23	dCTP	0.068 ± 0.007[b]	0.085 ± 0.006[b]	1.25
	ddCTP	0.20 ± 0.02[c]	0.042[d]	0.21
r44:d24	dATP	0.023 ± 0.004[b]	0.012 ± 0.001[b]	0.52
	ddATP	0.45 ± 0.08[b]	0.0072 ± 0.0007[b]	0.016
	ddATP	0.35 ± 0.05[c]	0.0090[d]	0.026

[a] Reactions were performed as described under Experimental Procedures. [b] Determined by direct measurement of [^{3}H]nucleotide incorporation. [c] K_i value for inhibition of [^{3}H]dNMP incorporation into the indicated template-primer. [d] Value determined using a gel electrophoresis–primer extension assay.

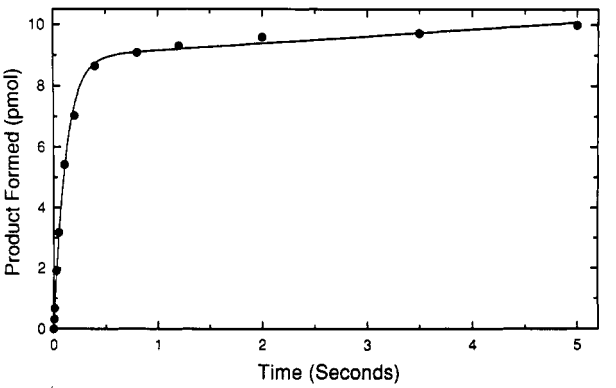

FIGURE 2: Pre-steady-state kinetics of dTMP incorporation into the r44:d21mer. A solution of reverse transcriptase (200 nM, 9 pmol) and r44:d21mer (500 nM) was mixed with 40 μM Mg^{2+}·[^{3}H]dTTP. The best fit (solid line) of the data to eq 2 gave a burst rate constant of 12 ± 2 s^{-1} and a steady-state rate of 0.23 ± 0.03 pmol s^{-1}.

[2] The steady-state K_m values reported here for incorporation of dTMP and AZTMP into the r44:d21mer are one-third the values reported previously (Reardon & Miller, 1990). The previous kinetic studies were conducted at 37 °C and employed reverse transcriptase derived from the mpRT4 plasmid, expressed in *E. coli*, and purified by immunoaffinity chromatography (Larder et al., 1987; Tisdale et al., 1988). The present study was conducted at 25 °C and used reverse transcriptase from the HXB-2 strain of HIV, that was expressed in *E. coli* and purified by conventional chromatographic methods. K_m values for dTMP incorporation into the r44:d21mer of 30–150 mM have been measured for HIV reverse transcriptases derived from several sources and purified by either conventional or immunoaffinity chromatography (unpublished data). In all cases, steady-state k_{cat} values were very similar when determined at 37 °C. Thus, the template-primer binding of reverse transcriptase was less influenced by small changes in amino acid sequence than was nucleotide binding and catalysis. This was not surprising considering the multitude of protein–nucleic acid interactions expected in the reverse transcriptase·template-primer binding domain.

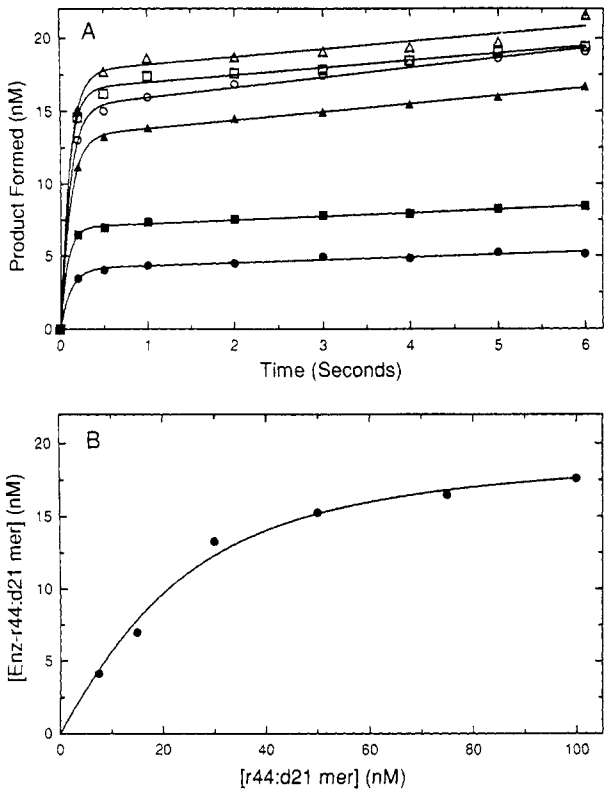

FIGURE 3: Measurement of the K_d for reverse transcriptase binding to the r44:d21mer. (A) Reverse transcriptase (40 nM, 1.8 pmol) was preequilibrated with increasing concentrations of [5′-^{32}P]r44:d21mer and was then mixed with 100 μM Mg^{2+}-[^{3}H]dTTP. The final r44:d21mer concentrations were (●) 7.5, (■) 15, (▲) 30, (○) 50, (□) 75, and (△) 100 nM. The data obtained at each r44:d21mer concentration were fit to eq 2 to determine the burst amplitude. (B) The burst amplitudes ([enzyme·r44:d21mer]) were plotted versus the total r44:d21mer concentrations. The best fit (solid line) of the data to eq 3 gave a K_d of 10 ± 1 nM and a total enzyme concentration of 20 ± 1 nM.

kinetic constants for a second nucleotide analogue, ddATP, were determined directly using [^{3}H]ddATP. The K_m and k_{cat} values determined directly agreed with those obtained from K_i measurements and primer extension assays.

Values of k_{cat} and K_m for AZTTP were similar to those for dTTP, indicating that AZTTP was almost as efficient a substrate for the enzyme (Table I). In contrast, k_{cat}/K_m values for the four 2′,3′-dideoxynucleotide analogues and d4TTP were $^1/_6$th to $^1/_{30}$th the value for the dNTP substrates. In all cases, the k_{cat} value for the nucleotide analogue was similar to that for the natural dNTP substrate. Thus, incorporation of a 2′,3′-dideoxynucleotide at the 3′-primer terminus did not significantly affect the rate of template-primer dissociation from the enzyme. The lower k_{cat}/K_m values for the 2′,3′-dideoxynucleotide analogues suggested either that their binding to reverse transcriptase was weaker and/or that they were incorporated at a slower rate than the corresponding dNTP substrates.

Pre-Steady-State Kinetics of dTTP Incorporation. The pre-steady-state kinetics of dTMP incorporation were examined to observe the first enzyme turnover. The time course for dTMP incorporation was biphasic (Figure 2). The amplitude of the initial burst of product formation, representing the initial turnover, was equal to 95% of the active-site concentration. The rapid initial incorporation of dTMP was followed by a slow phase with a linear rate of 0.23 ± 0.005 pmol/s. This corresponds to a rate constant of 0.026 s^{-1}, consistent with the steady-state k_{cat} value of 0.025 s^{-1} determined earlier.

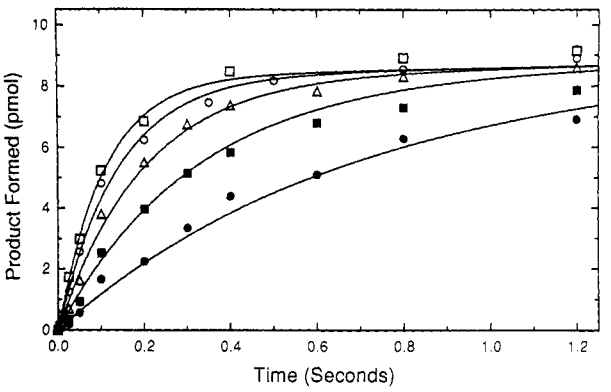

FIGURE 4: Pre-steady-state burst rate for dTMP incorporation into r44:d21mer: dTTP concentration dependence. Reverse transcriptase (200 nM, 9 pmol) was preequilibrated with r44:d21mer (500 nM) and was then mixed with increasing concentrations of Mg^{2+}-[^{3}H]dTTP. The final dTTP concentrations were (●) 1, (■) 2.5, (△) 5, (○) 10, and (□) 20 μM. The best fit (solid lines) of the data to eq 1 gave a dTTP K_d value of 9 ± 1 μM and maximum burst rate constant of 14 ± 2 s^{-1}.

Determination of K_d for Template-Primer Binding to Reverse Transcriptase. The equilibrium dissociation constant, K_d, for reverse transcriptase binding to template-primer was determined by titrating the enzyme with the r44:d21mer. The concentration of the enzyme·template-primer complex was determined by measuring the burst of product formation upon addition of dTTP (Figure 3A). This approach to determining the K_d value is possible because the rate constant for dissociation of the enzyme·template-primer complex is slow relative to bond formation (Patel et al., 1991). The K_d value of 10 ± 1 nM was obtained by plotting the fractional saturation of the burst of product formation (i.e., [enzyme·r44:d21mer]) versus the total r44:d21mer concentration (Figure 3B). Since the concentration of dTTP used to measure the burst amplitude is 1000× its steady-state K_m value, the postburst rate of product formation in Figure 3A will be governed by the association rate constant for the r44:d21mer until saturation of the enzyme is achieved. Thus, the slope of a plot of the postburst rate divided by the free enzyme concentration versus the concentration of free template-primer is the rate constant for r44:d21mer binding to reverse transcriptase. However, a valid estimate of the rate can be obtained only when there is a significant fraction of free enzyme remaining. Using the postburst rates for the 7.5, 15, 30, and 50 nM r44:d21mer concentrations (Figure 3A), a value of k_{on} for the r44:d21mer of 4 × 10^6 M^{-1} s^{-1} was obtained. From the K_d value of 10 nM and the k_{on} value of 4 × 10^6 M^{-1} s^{-1}, a value of k_{off} of 0.04 s^{-1} was calculated. The k_{cat} value for dCMP incorporation into the r44:d20mer (i.e., the dissociation rate constant for the r44:d21mer) was not determined. However, the k_{off} value of 0.04 s^{-1} calculated here is consistent with the values of 0.01–0.08 s^{-1} for the other template-primers used in this study.

Measurement of the Dissociation Constant for Nucleotide Binding. The dissociation constant, K_d, for dTTP was determined from the concentration dependence of the burst rate for incorporation of dTMP into the r44:d21mer. The data in Figure 4 were fit to eq 1 to give a K_d for dTTP of 9 ± 1 μM and a k_p value of 14 ± 2 s^{-1} (Scheme I). The satisfactory fit of the data to eq 1 was consistent with the kinetic model in Scheme I. The pre-steady-state kinetics of AZTMP and ddTMP incorporation were also examined (data not shown). The dissociation constant, K_d, and rate constant, k_p, for incorporation of AZTMP were 11 ± 1 μM and 5.4 ± 0.4 s^{-1}, respectively. The dissociation constant, K_d, and rate constant, k_p, for incorporation of ddTMP were 4.8 ± 0.6 μM and 0.41

Table II: Comparison of Kinetic Constants Determined by Steady-State and Pre-Steady-State Methods[a]

parameter	nucleotide substrate		
	dTTP	AZTTP	ddTTP
steady-state			
k_{cat} (s^{-1})	0.025 ± 0.002	0.015 ± 0.002	0.012
K_m (μM)	0.035 ± 0.004	0.050 ± 0.004	0.24 ± 0.02
k_{cat}/K_m (μM^{-1} s^{-1})	0.71	0.30	0.05
pre-steady-state			
k_p (s^{-1})	14 ± 2	5.4 ± 0.4	0.41 ± 0.04
K_d (μM)	9 ± 1	11 ± 1	4.8 ± 0.6
k_p/K_d (μM^{-1} s^{-1})	1.55	0.49	0.085
K_m^{calcd} (μM)	0.017	0.031	0.14

[a] Kinetic constants were determined as described in the text. Values for K_m^{calcd} were determined from K_d, k_p, and k_{cat} values according to eq 4. The ratio k_p/K_d is equal to k_{cat}/K_m on the basis of the kinetic model in Scheme I (Experimental Procedures).

± 0.04 s^{-1}, respectively. The K_d and k_p values determined by pre-steady-state kinetic analysis were used to calculate the steady-state kinetic constants (Table II).

Evaluation of the Phosphorothioate Elemental Effect. The phosphorothioate elemental effect of sulfur substitution at the α-phosphorus of dTTP (dTTPαS) on the pre-steady-state burst rate for nucleotide incorporation was examined. The rate constant for incorporation of 20 μM dTTPαS was 12 ± 2 s^{-1}, compared to a value of 11 ± 2 s^{-1} for 20 μM dTTP (data not shown). The rate-limiting chemical step for formation or hydrolysis of a phosphate ester bond shows a phosphorothioate elemental effect of 4–100 (Benkovic & Schray, 1971; Herschlag et al., 1991). The absence of a significant phosphorothioate elemental effect suggests that phosphodiester bond formation may not be rate-limiting during single-nucleotide incorporation catalyzed by reverse transcriptase.

Discussion

The steady-state kinetics of dTMP and AZTMP incorporation into a defined-sequence template-primer were examined in a previous study (Reardon & Miller, 1990). In the present study, the steady-state kinetic analysis has been extended to include all four natural dNTP substrates and a series of nucleotide analogues. The differences in K_m values for ddNTPs compared to dNTPs have been explained by determining the pre-steady-state kinetic constants for dTTP, AZTTP, and ddTTP. The results demonstrate that bond formation is not rate-limiting during steady-state turnover of the enzyme and confirm the earlier interpretation that k_{cat} is the rate constant for dissociation of the enzyme·template-primer complex. Slow dissociation of the enzyme·template-primer complex is a characteristic feature of all processive polymerases (Kuchta et al., 1987; McHenry, 1988, Patel et al., 1991).[3] This slow rate of dissociation results in steady-state accumulation of the enzyme·template-primer complex, allowing saturation of the enzyme to occur at very low concentrations of the nucleotide substrate. Thus, the low steady-state K_m values for the four naturally occurring nucleotide substrates are also diagnostic of a processive DNA polymerase.

The steady-state kinetic constants for AZTTP were very similar to those of dTTP and were consistent with the potent antiviral activity of AZT. In contrast, the 2′,3′-dideoxynucleotide analogues were 6–30-fold less efficient substrates than the parent nucleotides. This finding was surprising considering the similarity of the K_i values for AZTTP, ddTTP, and d4TTP for inhibition of dTMP incorporation into the homopolymeric template-primer poly(rA)-oligo(dT) (Starnes & Cheng, 1988; White et al., 1989). These results emphasize the importance of determining substrate kinetic constants on defined-sequence template-primers to accurately assess the potential efficacy of an antiviral agent at the nucleoside triphosphate level.

A kinetic mechanism for dTMP incorporation into the defined-sequence DNA primed-RNA template, r44:d21mer, is shown in Scheme II. The pre-steady-state kinetics of the reverse transcriptase polymerization reaction were very similar to those obtained by Kuchta et al. (1987) and Patel et al. (1991) for DNA polymerase I (Klenow) and T7 DNA polymerase, respectively. The K_d value for dTTP was 9 μM with reverse transcriptase, 18 μM with T7 DNA polymerase, and 5 μM for dATP with DNA polymerase I (Klenow). The most significant difference between the kinetics of reverse transcriptase and those of DNA polymerase I (Klenow) and T7 DNA polymerase is the magnitude of the rate-determining step for single-turnover incorporation of nucleotides. The rate constant measured here for reverse transcriptase of 14 s^{-1} is smaller than the values of 50 and 290 s^{-1} for DNA polymerase I (Klenow) and T7 DNA polymerase, respectively.

Scheme II

$$E + TP \underset{0.04\ s^{-1}}{\overset{4 \times 10^6\ M^{-1}\ s^{-1}}{\rightleftharpoons}} E{\cdot}TP \overset{K_d = 9\ \mu M}{\rightleftharpoons} E{\cdot}TP{\cdot}dNTP \xrightarrow{14\ s^{-1}}$$

$$E{\cdot}TP_{n+1} \xrightarrow{0.025\ s^{-1}} E + TP_{n+1}$$

The simplest interpretation of the pre-steady-state kinetic data is that catalysis is the rate-determining step for single-nucleotide incorporation. Alternatively, a conformational change preceding catalysis may be rate-determining. The kinetic mechanisms of *E. coli* Pol I (Klenow) and T7 DNA polymerase have been studied in detail (Kuchta et al., 1987, 1988; Dahlberg & Benkovic, 1991; Patel et al., 1991; Wong et al., 1991; Donlin et al., 1991). In both cases, the rate-determining step for single-nucleotide incorporation into defined-sequence DNA template-primers is a conformational change preceding catalysis. This conformational change is proposed to be integral to the mechanism by which DNA polymerases catalyze DNA replication with high fidelity (Wong et al., 1991). Evidence for a conformational change preceding catalysis is partially based on the finding that the rate-limiting chemical step in the nonenzymatic formation or breakage of a phosphotriester bond shows a phosphorothioate elemental effect of 50–100 (Benkovic & Schray, 1971). Thus, because nucleotide substrates containing an α-phosphothioate group are incorporated by *E. coli* Pol I (Klenow) and T7 DNA polymerase at only a modestly reduced rate (3–4-fold), catalysis may not be rate-determining. Similarly, in the present study, a phosphorothioate elemental effect of 1 was consistent with a non-rate-limiting chemistry step. However, Herschlag et al. (1991) recently determined the phosphorothioate effect for hydrolysis of the phosphate diester methyl dinitrophenyl phosphate to be 4. Therefore, only a small phosphorothioate effect might be expected if chemistry was rate-determining in the polymerization reaction. For this reason, the phosphorothioate elemental effect may not be a reliable diagnostic tool to determine whether chemistry is rate-limiting.

The results of the pre-steady-state kinetic studies of dTMP, AZTMP, and ddTMP incorporation provide insight into the observed differences in the steady-state K_m values. On the basis of the kinetic mechanism for reverse transcriptase shown in Scheme I, the kinetic constants determined by pre-steady-state kinetic analysis were in good agreement with the

[3] The possibility that k_{cat} is a slow conformational change preceding dissociation of the enzyme·template-primer complex cannot be ruled out by the present data.

steady-state values (Table II). The finding that all three nucleotide substrates bound similarly to reverse transcriptase is consistent with their structural similarity. The pre-steady-state data clearly demonstrate that the relative efficiency of steady-state incorporation of nucleotides into DNA is most influenced by the rate of phosphodiester bond formation or possibly a conformational change preceding catalysis.

The relative substrate efficiency of an obligate chain-terminating nucleotide analogue compared to the parent dNTP can be used to calculate a theoretical probability of chain termination and consequent antiviral efficacy. For example, the HIV genome (HXB-2 strain) is 9.7 kilobases in length and contains 2164 T, 3411 A, 2370 G, and 1773 C residues. If a single chain-termination event is sufficient to prevent replication of the first strand of the HIV genome, the probability of chain termination during replication will be dependent on the number of potential sites for chain termination, the relative substrate efficiency (k_{cat}/K_m) of the chain-terminating nucleotide analogue compared to the natural substrate, and the relative concentration of the two species in the infected cell. Thus, the percentage of successfully replicated genomes is given by

$$\% \text{ genome replication} = 100\left(1 - \frac{k_{cat}/K_m[\text{ddNTP}]}{k_{cat}/K_m[\text{dNTP}]}\right)^n \quad (5)$$

where n is the number of sites for substrate incorporation in the genome. Exposure of CEM cells to 0.05 μM d4T (IC_{50} = 0.15 μM) results in an intracellular d4TTP concentration of 0.064 μM (Mansuri et al., 1989; Ho & Hitchcock, 1989). Further, the d4TTP concentration was linearly dependent on the d4T concentration added to the cells. The concentration of dTTP in CEM cells is 60 μM (Kinahan et al., 1979; Grindey et al., 1979). Thus, at IC_{50} levels of d4T, ratios of [dNTP]/[ddNTP] of 300:1 are achieved. This dTTP:d4TTP concentration ratio is sufficient to inhibit genome replication by >80%. Similarly, the k_{cat}/K_m data reported here and the nucleotide pool data reported by others (Mitsuya & Broder, 1986; Cooney et al., 1986; Balzarini et al., 1987, 1988; Blakley et al., 1990; Johnson & Fridland, 1989; Johnson et al., 1988) suggest that at therapeutically relevant concentrations of the 2′,3′-dideoxynucleosides, the ratios of [dNTP]/[ddNTP] achieved in cells are sufficient to inhibit reverse transcription of the HIV genome and thus account for the observed in vitro antiviral effects.

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Identification of Peptides from the Adenine Binding Domains of ATP and AMP in Adenylate Kinase: Isolation of Photoaffinity-Labeled Peptides by Metal Chelate Chromatography†

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ABSTRACT: Photoaffinity labeling with azidoadenine nucleotides was used to identify peptides from the ATP and AMP binding domains on chicken muscle adenylate kinase. Competition binding studies and enzyme assays showed that the 8-azido analogues of Ap_4A and ATP modified only the $MgATP^{2-}$ site of adenylate kinase, whereas the 2-azido analogue of ADP modified the enzyme at both the ATP and AMP sites. The positions of the two nucleotide binding sites on the enzyme were deduced by isolating and sequencing the modified peptides. Photolabeled peptides were isolated by a new procedure that used metal chelate chromatography to affinity purify the photolabeled peptides prior to final purification by reverse-phase HPLC. The sequences of the peptides that were photolabeled with the 8-azido analogues corresponded to residues K28–L44, T153–K166, and T125–E135 of the chicken muscle enzyme. The residues that were present in both tryptic- and *Staphylococcus aureus* V-8 protease-generated versions of these peptides were assigned to the ATP binding domain on the basis of selective photoaffinity labeling with the 8-azidoadenine analogues. These peptides and an additional peptide corresponding to positions I110–K123 were photolabeled with 2-N_3ADP. Since I110–K123 was photolabeled by 2-N_3ADP but not by 8-N_3Ap_4A, it was assigned to the AMP binding domain.

Adenylate kinase (EC 2.7.4.3) catalyzes a phosphoryl exchange between $MgATP^{2-}$ and uncomplexed AMP. The enzyme contains two nucleotide binding sites that differ in their specificity (Noda, 1973) and possesses a glycine-rich region, thought to be a common motif for nucleotide phosphate binding (Fry et al., 1985). Because of these structural features and its relatively small size and ubiquitous nature, adenylate kinase is generally regarded as the prototype nucleotide binding protein. Consequently, a considerable amount of structural information is known about the enzyme from ligand binding studies (Hamada et al., 1979; Reinstein et al., 1990), comparative analysis of the primary structure (Fry et al., 1985; Schulz et al., 1986), X-ray crystallography (Schulz et al., 1974; Pai et al., 1977; Egner et al., 1987), and NMR (Fry et al., 1985, 1988). However, the assignment of the two nucleotide binding domains of adenylate kinase remains controversial [cf. Kim et al. (1989) and Tsai and Yan (1991)].

Photoaffinity labeling with azidonucleotide analogues provides a direct approach for elucidating nucleotide binding domains on proteins. Azidoadenine nucleotides bind in the adenine nucleotide binding domain of proteins, often serving as substrates for the enzymatic reaction. Photolysis with UV light activates the azido moiety which, if bound to the protein, will react within the binding domain. In theory, once a protein is photolabeled, the regions containing the nucleotide binding domain can be identified by isolating the modified peptide(s) (Potter & Haley, 1983). However, technical problems associated with recovery of modified peptides have hampered the broad application of photoaffinity labeling as a technique for identifying active-site peptides. The major hindrance is the lability of the labeled photoprobe during peptide separation by reverse-phase HPLC. The problem stems from inherent lability of both the N-glycosidic bond of the nucleotide and certain covalent bonds formed upon photoinsertion (Lewis et al., 1989; Haley, 1991). In fact, cases where photolabeled

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