0014-4754/94/010023-06$1.50 + 0.20

Research Articles

Steady-state kinetic studies with the polysulfonate U-9843, an HIV reverse transcriptase inhibitor

I. W. Althaus, J. J. Chou[a], A. J. Gonzales[b], R. J. LeMay, M. R. Deibel, K.-C. Chou, F. J. Kezdy, D. L. Romero, R. C. Thomas, P. A. Aristoff, W. G. Tarpley and F. Reusser[c]

The Upjohn Company, Kalamazoo (Michigan 49001, USA)
Received 6 October 1993; accepted 12 October 1993

Abstract. The tetramer of ethylenesulfonic acid (U-9843) is a potent inhibitor of HIV-1 RT* and possesses excellent antiviral activity at nontoxic doses in HIV-1 infected lymphocytes grown in tissue culture. Kinetic studies of the HIV-1 RT-catalyzed RNA-directed DNA polymerase activity were carried out in order to determine if the inhibitor interacts with the template:primer or the deoxyribonucleotide triphosphate (dNTP) binding sites of the polymerase. Michaelis-Menten kinetics, which are based on the establishment of a rapid equilibrium between the enzyme and its substrates, proved inadequate for the analysis of the experimental data. The data were thus analyzed using steady-state Briggs-Haldane kinetics assuming that the template:primer binds to the enzyme first, followed by the binding of the dNTP and that the polymerase is a processive enzyme. Based on these assumptions, a velocity equation was derived which allows the calculation of all the specific forward and backward rate constants for the reactions occurring between the enzyme, its substrates and the inhibitor. The calculated rate constants are in agreement with this model and the results indicated that U-9843 acts as a noncompetitive inhibitor with respect to both the template:primer and dNTP binding sites. Hence, U-9843 exhibits the same binding affinity for the free enzyme as for the enzyme-substrate complexes and must inhibit the RT polymerase by interacting with a site distinct from the substrate binding sites. Thus, U-9843 appears to impair an event occurring after the formation of the enzyme-substrate complexes, which involves either an event leading up to the formation of the phosphoester bond, the formation of the ester bond itself or translocation of the enzyme relative to its template:primer following the formation of the ester bond.
Key words. HIV RT; polysulfonate; inhibitor; steady-state kinetics.

Several polysulfates and polysulfonates have been identified as potent inhibitors of the RNA-directed DNA polymerase of HIV RT* and have shown antiviral activity in HIV-1 infected lymphocytes grown in tissue culture[1–12]. We have previously reported that U-9843, the tetramer of ethylenesulfonic acid, is a potent inhibitor of the RNA and DNA-directed DNA polymerase and RNase H activities of HIV-1 RT[12]. The compound also inhibits other RT enzyme species including the ones isolated from HIV-2, AMV and MLV retroviruses. Kinetic inhibition studies were carried out to establish if U-9843 interacts with the substrate binding sites of the RNA-directed DNA polymerase function of the HIV-1 RT enzyme. At that time the kinetic data were analyzed using Michaelis-Menten kinetics which are based on an equilibrium system and the analysis led us to conclude that U-9843 acts uncompetitively to noncompetitively with respect to the template:primer binding sites in response to increasing concentrations of the inhibitor. This posed a somewhat problematic scenario in that such a system would require that the inhibitor binds very inefficiently to the free enzyme but quite efficiently to the enzyme-substrate complexes at low U-9843 concentrations, while at higher inhibitor concentrations it would bind with equal affinity to the free enzyme and the enzyme-substrate complexes. This problem was resolved in that we applied steady-state Briggs-Haldane kinetics rather than Michaelis-Menten kinetics to analyze the data. The equations developed allow for the calculation of the specific forward and backward rate constants for the essential reactions occurring between the enzyme, its substrates and the inhibitor.

Materials and methods

The expression of HIV-1 RT and its purification have been described[13]. The enzyme was devoid of *Escherichia coli* RNase H activity and consisted of p51/p66 heterodimers as evidenced by gel electrophoresis.

The synthetic template:primers poly (rA), oligo $(dT)_{10}$, poly (rC), and oligo $(dG)_{10}$ were purchased from Pharmacia. α-[^{35}S]-labeled dTTP and dGTP were purchased from Dupont NEN. Nonidet P-40 was purchased from Sigma.

The standard reaction mixtures for the HIV-1 RT RNA directed DNA polymerase assay contained 20 mM

dithiothreitol, 60 mM NaCl, 0.05% Nonidet P-40, 10 mM $MgCl_2$, 50 mM Tris HCl, pH 8.3, 10 µM of the cognate α-[^{35}S]-labeled deoxyribonucleotide-5′-triphosphate (final specific activity 1 Ci/mmol), 10 µg/ml of RNA template [poly (rA) or poly (rC)], 5 µg/ml of the appropriate primer $(dT)_{10}$ or $(dG)_{10}$ and 0.16 µg of purified HIV RT. The total volume of the reaction mixtures was 50 µl. The samples were incubated at 37 °C for 15 min. The reactions were terminated by the addition of equal volumes of 10% trichloroacetic acid. Incorporation of radiolabeled precursor was determined by collecting the precipitates on glass fiber filters, drying, and counting the samples.

As mentioned in the introduction, the kinetic data were initially analyzed using Michaelis-Menten kinetics which are based on a rapid equilibrium system. This analysis yielded ambiguous results[12]. The reason for this inadequacy resides in the fact that the enzyme needs to form an initiation complex with the two respective substrates before elongation of the primer can begin. Therefore, no immediate equilibrium is established between the reactants involved. The experimental data were thus expanded and then analyzed using steady-state Briggs-Haldane kinetics. In this latter case the enzyme-substrate complex does not need to be in equilibrium with the enzyme and its substrate. However, shortly after initiation of the reaction, enzyme-substrate complex is formed at the same rate as it dissociates. Steady-state schemes including all the reaction steps to be considered here, yield very complex velocity equations which are impractical to solve. For these reasons the general steady-state kinetic system used in this study was simplified as detailed in figure 1. The steady-state kinetics were limited to the reactions occurring between the enzyme and the substrates and rapid equilibrium kinetics were applied to the interactions pertaining to the inhibitor and the enzyme or the various enzyme-substrate complexes. Moreover, an ordered mechanism was assumed, whereby the template:primer complex binds first to the enzyme, followed by the addition of dNTP[14,15]. The polymerase is a processive enzyme and, after the addition of the first nucleotide, translocation occurs along the template, resulting in the incorporation of further nucleotides into the growing chain[14]. Under these conditions the formation of the phosphoester bond can be considered as irreversible as the reverse reaction occurs at an extremely low rate. In addition, the dissociation of the enzyme-product complex into its components is also negligible during the initial reaction phase. Thus, the enzyme-product does not differ from the initial enzyme-template:primer complex in that the former shuttles back to the enzyme-template:primer state and this reaction rate constant is equal to k_{cat} or k_{2p}, representing the turnover number (fig. 1). The pertinent rate are equilibrium constants and the symbols for the enzyme, the inhibitor and substrates used in this paper are defined in

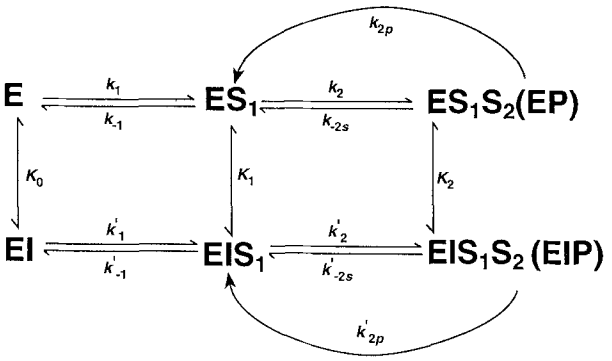


Figure 1. Steady-state reaction scheme for HIV RT. E = enzyme; S_1 = template:primer; S_2 = dNTP; K_0, K_1, K_2 = equilibrium constants between the inhibitor [I], the enzyme and its substrates; EP = enzyme-product complex; EIP = enzyme-inhibitor-product complex.

figure 1. The quaternary enzyme-inhibitor-template:primer-dNTP complex ought to be non-productive and $k'_{2p} \rightarrow 0$.

Thus, the HIV RT catalyzed system considered here consists of two substrates, S_1, representing the template:primer, S_2, representing the dNTP, and an inhibitor I. The reactions between the enzyme (E) and the low molecular weight inhibitor (I) are deemed as diffusion controlled reactions[16–18]. Hence, the conversions between E and EI, ES_1, and EIS_1, and ES_1S_2 and EIS_1S_2 occur at a much faster rate than the interconversions between the enzyme and its substrates. Thus, although the whole system is a steady-state one, there is an equilibrium between the inhibitor and the enzyme and the enzyme-substrate complexes[19]. The entire system can be expressed as shown in figure 1 where the equilibrium constants or K_is defined as K_0, K_1, and K_2, respectively, are given by

$$K_0 = \frac{[E][I]}{[EI]}, \quad K_1 = \frac{[ES_1][I]}{[EIS_1]}, \quad K_2 = \frac{[ES_1S_2][I]}{[EIS_1S_2]} \tag{1}$$

Using the apparent rate constants method[19], the mechanism expressed in figure 1 can be reduced to the one shown in figure 2. According to Chou's graphic rules of enzyme kinetics[20,21], the mechanism depicted in figure 2 can be expressed by a directed graph G (fig. 3a) and the transformed graph G† (fig. 3b) where $E_1 = E + EI$, $E_2 = ES_1 + EIS_1$, $E_3 = ES_1S_2 + EIS_1S_2$ and

$$\begin{cases} k_{12} = \dfrac{(k_{+1}K_0 + k'_{+1}[I])[S_1]}{K_0 + [I]} \\ k_{21} = \dfrac{k_{-1}K_1 + k'_{-1}[I]}{K_1 + [I]} \\ k_{23} = \dfrac{(k_{+2}K_1 + k'_{+2}[I])[S_2]}{K_1 + [I]} \\ k_{32} = \dfrac{(k_{-2s} + k_{+2p})K_2 + (k'_{-2s} + k'_{+2p})[I]}{K_2 + [I]} \end{cases} \tag{2}$$

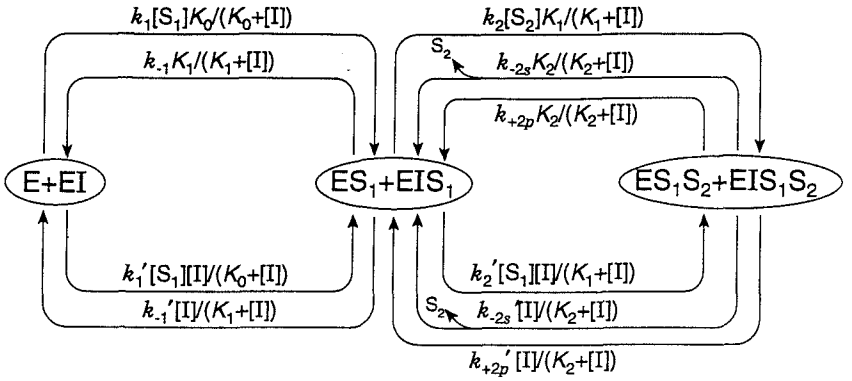
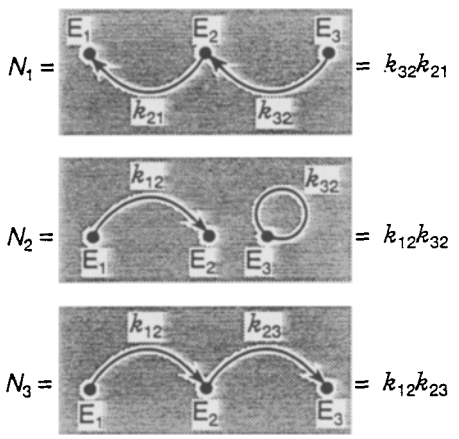


Figure 2. The enzyme-catalyzed mechanism derived from figure 1 by means of the apparent rate constant method[19].

According to Chou's graphic rule 2[20,21], the concentration of the mth enzyme species is given by

$$[E_m] = \frac{N_m}{\sum_{i=1}^{n} N_i} e_0, \quad (m = 1, 2, \ldots, n) \tag{3}$$

where e_0 is the total concentration of all enzyme species and



(4)

Substitution of equation **4** into equation **3** yields

$$[E_3] = \frac{k_{12}k_{23}e_0}{k_{32}k_{21} + k_{12}k_{32} + k_{12}k_{23}} \tag{5}$$

and the rate of product formation is (see fig. 1)

$$\frac{d[P]}{dt} = \frac{k_{12}k_{23}(k_{+2p}K_2 + k'_{+2p}[I])/(K_2 + [I])}{k_{32}k_{21} + k_{12}k_{32} + k_{12}k_{23}} e_0 \tag{6}$$

Results

The enzymatic kinetic studies with U-9843 were performed with synthetic homopolymeric template:primers to determine the type of inhibition pattern on the RNA-directed DNA polymerase activity of HIV-1 RT with respect to the template:primer and dNTP binding sites. This activity was first studied in the presence of varied concentrations of the template:primer poly (rA):$(dT)_{10}$ and fixed concentrations of dTTP, and vice versa in the

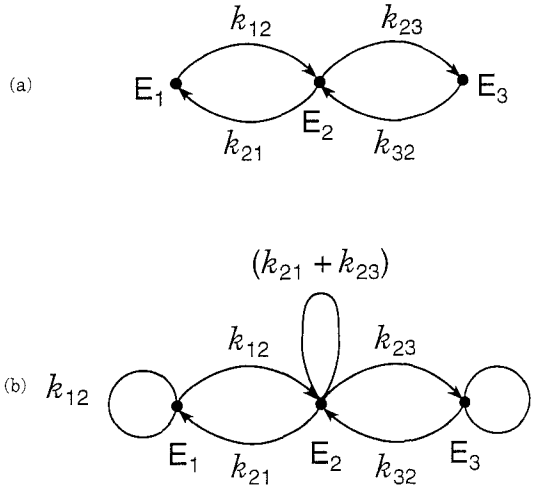


Figure 3. The graphical expression of the mechanism shown in figure 2. *a* The digraph G, and *b* its transformed graph $G^{\dagger}$[20,21], where $E_1 = E + EI$, $E_2 = ES_1 + EIS_1$, $E_3 = ES_1S_2 + EIS_1S_2$ and k_{12}, k_{21}, k_{23}, and k_{32} are given by equation 2.

presence of varied concentrations of dTTP and fixed concentrations of poly (rA):$(dT)_{10}$. Three inhibitor concentrations were used in addition to controls containing no U-9843. The analysis of the data was done via computer applying the modified steady-state kinetics described under 'Materials and methods'. The experimental results are given in table 1 and the calculated forward and backward reaction rates and equilibrium constants are shown in figure 4. These constants were derived by fitting the experimental data to equation **6**. We note that the calculated forward reaction rate constant k_1 for the formation of the enzyme-poly (rA):$(dT)_{10}$ complex was $4.4 \times 10^4\ M^{-1}\ sec^{-1}$ and the rate constant k_{-1} for the reverse reaction was $0.3\ sec^{-1}$ in the absence of the inhibitor. The k_{-1}/k_1 ratio amounts thus to 6.8 μM of template:primer. Published estimates for this ratio range from 0.6 nM to 3 μM of template:primer although these cited values were calculated under the assumption that the system is at equilibrium[22–24]. In the presence of U-9843, the

Table 1. Inhibition of HIV-1 RT poly (rA):$(dT)_{10}$-directed poly(dT) synthesis by U-9843

U-9843 (μM)	Poly(rA):$(dT)_{10}$ (μM)	dTTP (μM)	$d[P]/dt$ (μM × 10^{-6} sec^{-1})
0	0.75	153	3264
0.5	0.75	153	2844
0.875	0.75	153	1879
1.25	0.75	153	990
0	1.50	153	5113
0.5	1.50	153	3340
0.875	1.50	153	2738
1.25	1.50	153	1967
0	3.00	153	7829
0.5	3.00	153	5445
0.875	3.00	153	3746
1.25	3.00	153	2845
0	4.50	153	9982
0.5	4.50	153	6221
0.875	4.50	153	4543
1.25	4.50	153	3238
0	7.5	3.85	1460
0.25	7.5	3.85	1287
0.5	7.5	3.85	1157
1	7.5	3.85	960
0	7.5	7.7	2662
0.25	7.5	7.7	1937
0.5	7.5	7.7	1775
1	7.5	7.7	1098
0	7.5	15.2	4806
0.25	7.5	15.2	3235
0.5	7.5	15.2	3000
1	7.5	15.2	1800
0	7.5	38.5	8268
0.25	7.5	38.5	6162
0.5	7.5	38.5	4400
1	7.5	38.5	3158

Enzyme = 0.0274 μM, $d[P]/dt$ = μM × 10^{-6} of dTMP incorporated per sec.

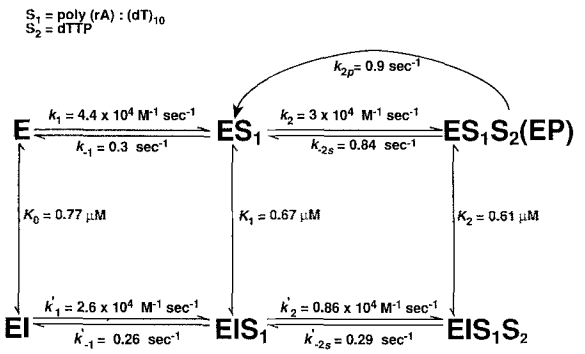


Figure 4. Inhibition of poly (rA):$(dT)_{10}$-directed poly (dT) synthesis by U-9843; steady-state kinetic parameters.

corresponding constants were reduced to $k'_1 = 2.6 \times 10^4$ M^{-1} sec^{-1} and $k'_{-1} = 0.26$ sec^{-1}. The forward and backward rate constants for the formation and dissociation of the ternary enzyme-poly (rA):$(dT)_{10}$-dTTP complex were $k_2 = 3 \times 10^4$ M^{-1} sec^{-1} and $k_{-2s} = 0.84$ sec^{-1}. The corresponding rate constants in the presence of the inhibitor U-9843 were 0.86×10^4 M^{-1} sec^{-1} for k'_2, assessing the formation of the enzyme-U-9843-poly (rA):$(dT)_{10}$-dTTP complex and 0.29 sec^{-1} for k'_{-2s}, assessing its dissociation. The translocation rate or turnover number k_{2p} for the enzyme was 0.9 sec^{-1} for the control reaction and essentially 0 for k'_{2p}, the corresponding value for the enzyme-inhibitor-template:primer-dNTP complex. The value for k_{2p} is somewhat lower than the one reported by Reardon[15] (14 sec^{-1}) but in good agreement with the one calculated by Anderson and Coleman[25] (1.2 sec^{-1}). Moreover, the inhibition constants or K_is calculated for the enzyme (K_0), the enzyme-template:primer (K_1) and the enzyme-template:primer-dTTP (K_2) complexes with the inhibitor were 0.77, 0.67, and 0.61 μM of U-9843, respectively. The overall fitting error was 0.5×10^{-5}. It is apparent that the values for the equilibrium constants or K_is are of the same magnitude and this indicates that U-9843 acts as a noncompetitive inhibitor with respect to both the poly (rA):$(dT)_{10}$ and the dTTP binding sites of the enzyme.

Analogous experiments were carried out in the presence of varied amounts of the homopolymeric template:primer poly (rC):$(dG)_{10}$ and a constant concentration of dGTP or vice versa in the presence of varied concentrations of dGTP and a constant amount of the cognate template:primer. The experimental results are shown in table 2 and the calculated rate constants and equilibrium constants are reported in figure 5. The forward rate constant k_1 for the association of the RT-poly (rC):$(dG)_{10}$ complex was 5.9×10^4 M^{-1} sec^{-1} and its dissociation rate constant k_{-1} was 0.39 sec^{-1}. In the presence of the inhibitor, k'_1, the forward reaction rate for the formation of the enzyme-U-9843-template:primer complex was 2.5×10^4 M^{-1} sec^{-1} and the value for k'_{-1}, the reverse reaction, was 0.23 sec^{-1}. In addition, the rate constant k_2 for the formation of enzyme-template:primer-dGTP complex was 2.8×10^4 M^{-1} sec^{-1} and the rate constant k_{-2s} for the reverse reaction was 0.82 sec^{-1}. In the presence of the inhibitor the rate constant k'_2 assessing the association of the enzyme-U-9843-template:primer-dGTP complex was 0.77×10^4 M^{-1} sec^{-1} and k'_{-2s}, the corresponding dissociation rate constant was 0.27 sec^{-1}. The turnover number or translocation rate k_{2p} was 0.4 sec^{-1} for the control reaction in this system and k'_{2p}, the analogous value for the enzyme-U-9843-template:primer-dNTP complex was essentially 0. The equilibrium constants were 0.47 μM of U-9843 for K_0, the enzyme-U-9843 complex, 0.45 μM for K_1, the enzyme-U-9843-poly (rC):$(dG)_{10}$ complex and 0.31 μM for K_2, the enzyme-U-9843-template:primer-dGTP complex. The overall fitting error was 1×10^{-4}. The K_is are of equal value and indicate that the inhibitor acts as a noncompetitive

Table 2. Inhibition of HIV-1 RT poly (rC):$(dG)_{10}$-directed poly (dG) synthesis by U-9843

U-9843 (μM)	Poly(rC):$(dG)_{10}$ (μM)	dGTP (μM)	$d[P]/dt$ (μM × 10^{-6} sec^{-1})
0	0.45	73	561
0.25	0.45	73	439
0.5	0.45	73	231
1	0.45	73	92
0	0.75	73	1061
0.25	0.75	73	793
0.5	0.75	73	445
1	0.75	73	238
0	1.50	73	1826
0.25	1.50	73	1232
0.5	1.50	73	646
1	1.50	73	353
0	3	73	2973
0.25	3	73	2013
0.5	3	73	1159
1	3	73	598
0	6	73	3684
0.25	6	73	2427
0.5	6	73	1813
1	6	73	887
0	7.5	3.65	1506
0.25	7.5	3.65	764
0.5	7.5	3.65	514
1	7.5	3.65	296
0	7.5	7.3	2725
0.25	7.5	7.3	1667
0.5	7.5	7.3	1126
1	7.5	7.3	668
0	7.5	14.8	3378
0.25	7.5	14.8	2047
0.5	7.5	14.8	1464
1	7.5	14.8	794
0	7.5	36.5	4766
0.25	7.5	36.5	2861
0.5	7.5	36.5	2023
1	7.5	36.5	1337

Enzyme = 0.0274 μM, $d[P]/dt$ = μM × 10^{-6} of dGMP incorporated per sec.

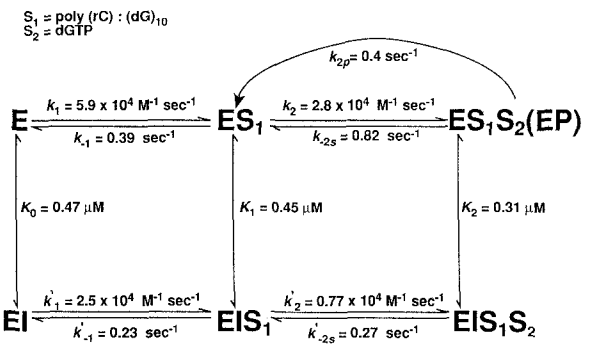


Figure 5. Inhibition of poly (rC):$(dG)_{10}$-directed poly (dG) synthesis by U-9843; steady-state kinetic parameters.

inhibitor with respect to the poly (rC):$(dG)_{10}$ and dGTP binding sites. Moreover, the K_1 and K_2 values calculated for the poly (rC):$(dG)_{10}$-catalyzed DNA polymerase system are of the same magnitude as the ones obtained for the poly (rA):$(dT)_{10}$-catalyzed system demonstrating that the potency of U-9843 is independent of the base composition of the template:primers.

Discussion

The inhibition kinetics of U-9843 on the RNA-directed DNA polymerase domain of HIV-1 RT was studied with respect to the nucleic acid and dNTP binding sites using synthetic homopolymeric template:primers. The analysis of the experimental results was carried out using somewhat modified steady-state Briggs-Haldane kinetics as detailed under 'Materials and methods'. These kinetics take into consideration that 1) the reaction is ordered in that the template:primer binds first to the enzyme and is followed by the addition of dNTP; 2) the enzyme-product complex elongated by one base does not dissociate into free enzyme and product but is recycled back to the enzyme-template:primer state by the process of translocation and the polymerase is, therefore, processive; 3) the formation of the phosphoester bond and the concomitant release of pyrophosphate is irreversible; and 4) the binding of the inhibitor to the free enzyme or the various enzyme-substrate complexes follows rapid equilibrium kinetics. The results obtained are consistent with this model. Equation **6** allows the calculation of the essential rate constants and equilibrium constants as it relates the amount of product formed by the RT enzyme to its two substrates, the template:primer and the dNTP, and the inhibitor. The steady-state Briggs-Haldane kinetic analysis applied here shows that the system is indeed a steady-state one and not an equilibrium system for the following reasons: The K_i values or equilibrium constants K_0, K_1, K_2 are of similar magnitude for all the enzyme-U-9843 and enzyme-U-9843-substrate complexes regardless of whether the reaction was directed by either poly (rA):$(dT)_{10}$ or poly(rC):$(dG)_{10}$, and these values are independent of the inhibitor concentration. Earlier analyses, using Michaelis-Menten kinetics, incorrectly suggested that U-9843, at low concentrations acts as an uncompetitive inhibitor with respect to the nucleic acid binding site of the enzyme. The steady-state analysis presented here, now clearly shows that U-9843 acts as a noncompetitive inhibitor with respect to both the template:primer and dNTP binding sites and that this inhibition pattern is independent of the U-9843 concentration. Moreover, the results show that U-9843 interacts with a site distinct from either the template:primer and the dNTP binding sites. Therefore, U-9843 appears to impair an event leading up to the formation of the phosphoester bond, the formation of the ester bond per se, or the event of translocation of the enzyme relative

to the template:primer following the formation of the ester bond.

Acknowledgments. We thank J. D. Baker for the drawing of some of the figures and J. K. Zelenock for editing the manuscript.

* Abbreviations used: HIV-1 RT, human immunodeficiency virus type 1 reverse transcriptase (EC 2.7.7.49); HIV-2 RT, human immunodeficiency virus type 2 reverse transcriptase; RNase H, ribonuclease H (EC 3.1.26.4); AMV, avian myoblastoma virus; MLV, murine leukemia virus.

[a] Present address: Department of Physics, University of Michigan, Ann Arbor (MI 48109, USA).

[b] Present address: Curriculum for Toxicology, University of North Carolina, Chapel Hill (NC 27599, USA).

[c] To whom correspondence should be addressed.

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