Reverse Transcriptase from Human Immunodeficiency Virus: A Single Template-Primer Binding Site Serves Two Physically Separable Catalytic Functions

Marc S. Krug‡ and Shelby L. Berger*

Section on Genes and Gene Products, Laboratory of Biochemistry, National Cancer Institute, Bethesda, Maryland 20892

Received May 8, 1991; Revised Manuscript Received August 13, 1991

ABSTRACT: The binding of substrates to recombinant reverse transcriptase from human immunodeficiency virus (HIV) and the natural enzyme from avian myeloblastosis virus (AMV) has been examined by analyzing both the ribonuclease H and the RNA-dependent DNA polymerase activities. With 3′-end-labeled globin mRNA hybridized to $(dT)_{15}$ as the substrate in the ribonuclease H reaction, the enzymes partially deadenylated the mRNA in a distributive manner. Under these conditions, there was a rapid initial burst followed by a prolonged, but much slower, steady-state rate. The biphasic reaction made possible determinations of kinetic constants as follows: values for K_m, K_D, and k_{cat} were, respectively, 27 nM, 11 nM, and 5×10^{-3} s^{-1} for the HIV enzyme and 30 nM, 9 nM, and 5×10^{-3} s^{-1}, respectively, for the avian enzyme. These constants were used to derive other parameters: The rate of association of the template-primer with reverse transcriptase was $\sim 2 \times 10^5$ M^{-1} s^{-1}, and the rate of dissociation was $\sim 2 \times 10^{-3}$ s^{-1}, regardless of the source of the enzyme. The rate of release of the product was essentially equivalent to the value of k_{cat} indicated above for each of the enzymes. The polymerase reaction was evaluated under processive conditions of synthesis; values of K_m and k_{cat} of ~ 6 nM and ~ 2.5 s^{-1}, respectively, for the human enzyme, and ~ 10 nM and ~ 2 s^{-1}, respectively, for the avian enzyme were observed. The interaction of substrates with HIV reverse transcriptase was characterized further with the aid of ribonucleoside–vanadyl complexes. These complexes inhibited the polymerase and ribonuclease H activities of the enzyme competitively with respect to globin mRNA·$(dT)_{15}$. Values of K_i ranging from 1 to 3 mM were obtained. With respect to deoxyribonucleoside triphosphate substrates in the polymerase reaction, mixed inhibition was observed. Deoxyribonucleoside triphosphates had no effect on kinetic parameters governing the ribonuclease H activity of the HIV enzyme but apparently facilitated the formation of active enzyme. These data fit a model in which one template-primer binding site serves both the polymerase and the ribonuclease H catalytic sites.

Reverse transcriptase is a key constituent in the life cycle of retroviruses. The enzyme catalyzes three reactions all of which are essential for viral replication: polymerization of a DNA strand complementary to viral genomic RNA templates; hydrolysis of the RNA strand of the DNA·RNA hybrid; and synthesis of the second DNA strand (Varmus & Swanstrom, 1982). The first and last of these activities are usually grouped together as the polymerase function of reverse transcriptase; the second activity is known as the associated ribonuclease H (RNase H). The polymerization and hydrolytic functions are interdependent. For example, first or (−) strand synthesis is primed by a specific tRNA (Verma et al., 1972; Taylor & Illmensee, 1975; Staskus et al., 1976) which is later removed by RNase H (Omer & Faras, 1982), while second or (+) strand DNA synthesis commences only after the RNase H activity has exposed (−) strand DNA for use as a template and generated the correct RNA primer (Champoux et al., 1984; Finston & Champoux, 1984; Resnick et al., 1984; Smith et al., 1984a,b). The polymerase has been the object of several mechanistic studies (Majumdar et al., 1988, 1989; Huber et al., 1989; Bebenek et al., 1989; Kedar et al., 1990; Mendelman et al., 1990; Reardon & Miller, 1990) and the target of numerous drug development projects aimed at finding a remedy for acquired immune deficiency disease (AIDS) (Mitsuya & Broder, 1987; Yarchoan et al., 1989; Take et al., 1989; White et al., 1989; Goldman et al., 1990; Nakane & Ono, 1990; Ono et al., 1990). In contrast, many aspects of RNase H catalysis remain to be explored and exploited.

An RNase H cleaves phosphodiester bonds in the RNA moiety of a DNA·RNA hybrid. RNase H activities associated with reverse transcriptases are endonucleases (Krug & Berger, 1989; Oyama et al., 1989); the products are oligoribonucleotides of varying sizes (Gerard, 1981; Starnes & Cheng, 1989), with 5′-phosphoryl and 3′-hydroxyl termini (Baltimore & Smoler, 1972; Keller & Crouch, 1972; Berkower et al., 1973). The enzymatic reaction is believed to be processive (Leis et al., 1973; Gerard, 1981; Hansen et al., 1988) because the rate of hydrolysis of labeled DNA·RNA hybrids was virtually unaffected by the subsequent addition of a large quantity of unlabeled hybrid. Although these data clearly indicate that multiple cleavage events occurred on a given substrate before the enzyme dissociated and initiated hydrolysis on a new molecule, the data do not provide firm evidence that the enzyme proceeds vectorially in either direction. However, directionality can be imposed on the system by using concomitant polymerization of deoxyribonucleoside triphosphates (dNTPs) to generate the DNA moiety of the hybrid substrate (Varmus & Swanstrom, 1982; Oyama et al., 1989; Schatz et al., 1990). Indeed, models in which there is a tight coupling between polymerization and hydrolysis of RNA have been proposed (Levin et al., 1988; Oyama et al., 1989; Schatz et al., 1990).

The presence of the RNase H and polymerase activities on the same polypeptide, as in Moloney murine leukemia virus (MMLV) (Grandgenett et al., 1972; Gerard & Grandgenett,

* Address correspondence to this author at Building 10, Room B1B38, NIH, Bethesda, MD 20892.

‡ Present address: University of Tennessee, 956 Court Ave., Room H300, Memphis, TN 38163.

1975), or in the same heterodimer, as in avian myeloblastosis virus (AMV) (Moelling et al., 1971) or human immunodeficiency virus (HIV) (Hansen et al., 1987), raised questions about the relationship between the two functions. Alignment of protein sequences from several reverse transcriptases with other DNA polymerases and with the ribonuclease H from *Escherichia coli* favored assigning the polymerase catalytic site of HIV reverse transcriptase to the amino-terminal two-thirds of the molecule and the RNase H catalytic site to the carboxyl-terminal region of the larger subunit (Johnson et al., 1986; Doolittle et al., 1989; Poch et al., 1989). Other reverse transcriptases appeared to be organized similarly on the basis of experiments involving linker insertion mutagenesis (Tanese & Goff, 1988; Hizi et al., 1989a,b; Prasad & Goff, 1989), point mutagenesis (Larder et al., 1987; Repaske et al., 1989; Schatz et al., 1989; Basu et al., 1990), deletion mutagenesis (Kotewicz et al., 1988; Levin et al., 1988; Prasad & Goff, 1989), and partial proteolytic cleavage (Lai & Verma, 1978; Hansen et al., 1988). However, attempts to define the region to which the template-primer binds by analyzing mutants (Kotewicz et al., 1988; Levin et al., 1988; Tanese & Goff, 1988; Hizi et al., 1989a,b; Repaske et al., 1989; Schatz et al., 1989; Basu et al., 1990; Mendelman et al., 1990), or evaluating chimeric reverse transcriptases (Hizi et al., 1989b), failed to distinguish between the two activities.

In this paper, we report binding constants and kinetic constants that describe the inteaction of the template-primer with HIV reverse transcriptase and, in more limited studies, the enzyme from AMV. We have also studied the effect of ribonucleoside–vanadyl complexes as inhibitors in both the polymerase and the RNase H reactions. Regardless of whether the RNase H activity or the RNA-dependent DNA polymerase activity was under scrutiny, the constants that characterize the binding of DNA·RNA hybrids or the inhibition with vanadyl complexes were essentially the same. Our results suggest that the two physically separable catalytic functions share a common template-primer binding site.

Materials and Methods

Materials. The sources of enzymes and nucleic acids were as follows: HIV reverse transcriptase, a generous gift from Dr. Samuel Wilson, National Cancer Institute; AMV reverse transcriptase, Molecular Genetic Resources (Tampa, FL); *E. coli* RNase H, poly(A) polymerase, T4 polynucleotide kinase, T4 RNA ligase, and rabbit globin mRNA containing ~25% ribosomal RNA on a molar basis, BRL (Life Technologies, Inc.); $(dT)_{15}$, Boehringer Mannheim; nucleosides and nucleoside triphosphates, Sigma; [α-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]dCTP (800 Ci/mmol), Amersham; and [5′-^{32}P]-cytidine 3′,5′-bisphosphate (3000 Ci/mmol), New England Nuclear. Drop dialysis was carried out with 13-mm type VM filters with a pore size of 0.05 μm (Millipore), whereas 2.4-cm GF/C glass fiber filters (Whatman) were used for collecting trichloroacetic acid precipitable radioactivity in several types of samples. Columns containing Sephadex G-50 (Pharmacia) for removal of unincorporated nucleoside triphosphates or pCp from macromolecules by spin chromatography were purchased from 5 Prime → 3 Prime, Inc. (West Chester, PA).

Preparation of 3′-End-Labeled Globin mRNA. Globin mRNA with an average of one to two [^{32}P]adenylate residues at the 3′ end was prepared in a volume of 100 μL of a buffer composed of 50 mM Tris-HCl at pH 8.0, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 250 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol, by incubating 0.25 μM globin mRNA with 1 μM [α-^{32}P]ATP (300 μCi) and 10 units of *E. coli* poly(A) polymerase at 37 °C for 15 min. Reactions were stopped by the addition of 5 μL of 10% sodium dodecyl sulfate (SDS) and 5 μL of proteinase K at 2 mg/mL and incubating the reactions at 37 °C for an additional 15 min. The labeled mRNA was purified by adding 100 μL of water and extracting the sample twice with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) and once with an equal volume of chloroform and drop-dialyzing the aqueous phase against water to remove buffer, unincorporated ATP, and other contaminants (Wallace, 1987a,b).

Globin mRNA was also end-labeled with pCp. The reaction was carried out for 2 h at 16 °C by mixing 50 μM ATP, 625 nM globin mRNA, and 825 nM cytidine [5′-^{32}P]bisphosphate with 0.4 μg/mL T4 RNA ligase (~10 μM) in 100 μL of buffer composed of 50 mM HEPES–KOH at pH 8.3, 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.1 mM EDTA. The reaction was terminated by inactivating the enzyme at 65 °C for 2 min. Unincorporated pCp and buffer were removed by G-50 Sephadex spin column chromatography. Yields of labeled mRNA were >95%.

Polymerase Assays. The polymerase activity of reverse transcriptases was measured with the stated concentrations of enzyme, globin mRNA, and [^{32}P]dNTPs in the presence of 2 μM $(dT)_{15}$ at 37 °C either in buffer for the HIV enzyme (50 mM Tris-HCl at pH 8.0, 5 mM $MgCl_2$, 75 mM KCl, 0.1 mM EDTA, and 2.5 mM dithiothreitol) or in buffer for the AMV enzyme (50 mM Tris-HCl at pH 8.3, 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, and 1 mM dithiothreitol). Aliquots were withdrawn at 1-min intervals and spotted onto glass fiber filters. Samples on filters were assayed for radioactivity either with a BioScan/QC 2000 counter or by measuring Cerenkov radiation in a Packard scintillation counter; they were subsequently washed with 5% trichloroacetic acid containing 20 mM sodium pyrophosphate and assayed again for radioactivity (Berger, 1987). Initial rates of incorporation of labeled precursors into DNA were determined from linear curves; the rates were proportional to the enzyme concentration. Data points represent the average of at least two independent determinations of the rate of the reaction. In all cases, stocks of deoxyribonucleoside triphosphates were prepared by adding an equimolar amount of $MgCl_2$ in order to compensate for binding of Mg^{2+} by dNTPs.

Ribonuclease H Assays. Measurements of the RNase H activity of reverse transcriptases were carried out at the same temperature and in the same buffers specified above for the polymerase assays essentially as described by Berger et al. (1983). *E. coli* RNase H was assayed similarly in a buffer composed of 50 mM Tris-HCl at pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, and 1 mM dithiothreitol. Briefly, reactions were initiated by adding prewarmed end-labeled globin mRNA at the stated concentration in the appropriate buffer to a prewarmed solution containing the stated concentration of enzyme mixed with 2 μM $(dT)_{15}$ primer, also in buffer. Labeled globin mRNA solutions with $(6–10) \times 10^5$ dpm (5–10 μCi/pmol) were brought to the stated concentration by the addition of unlabeled globin mRNA. Initial rates were determined by withdrawing four to eight aliquots during the indicated interval and determining acid-precipitable radioactivity remaining as described above for polymerization assays. Rates were linear and proportional to the enzyme concentration. Data points represent the average of at least two independent measurements of the rate of the reaction.

It should be pointed out that assays performed with mRNA[^{32}P]pA contain labeled rRNA as a contaminant. These nonpolyadenylated, but labeled molecules cannot bind $(dT)_{15}$ and are, therefore, not substrates for hydrolysis cata-

lyzed by RNase H (Krug & Berger, 1987). Thus, at completion of the reaction, ~25% of the acid-precipitable radioactivity persists, reflecting ~25% rRNA in the preparations. All rate calculations were corrected accordingly. These considerations do not pertain to mRNA[^{32}P]pCp because RNA ligase, the enzyme responsible for forming the adduct, exhibits a marked preference for labeling terminal poly(A) (England et al., 1980).

Preparation of Ribonucleoside–Vanadyl Complexes. Vanadyl complexes were synthesized as 200 mM stock solutions with the exception of those formed from guanosine and its derivatives; because of limited solubility, guanosine derivatives were prepared at 50 mM. In most cases, formation of a complex could be inferred from a change in color from gray-black to olive green, as the pH approached neutrality. When phosphorylated compounds were used as the starting materials, the final color was closer to blue-green. In all cases, the complexes were obtained by mixing equimolar amounts of vanadyl sulfate and the nucleosides or nucleoside derivatives and heating and titrating the solution as described for the preparation of vanadyl complexes with ribonucleosides (Berger & Birkenmeier, 1979). The standard equimolar mixture of all four ribonucleoside–vanadyl complexes was synthesized by mixing the four ribonucleosides with vanadyl sulfate before initiating complex formation. Where indicated, mixtures of vanadyl complexes were obtained by synthesizing individual complexes and subsequently combining them.

Curve Fitting. Binding data were fitted to rectangular hyperbolic saturation curves by nonlinear least-squares techniques with the aid of the computer program originally written by Yamaoka et al. (1981). Scatchard analyses were carried out by obtaining best-fit lines.

Wherever possible, kinetic data were analyzed by iterative nonlinear least-squares fitting of initial velocity against substrate concentration. In a few cases, data were not sufficient to permit hyperbolic fitting, but the qualitative results were, nevertheless, clear. With three exceptions in Figure 3, all of the curves in the kinetic analysis were drawn according to the parameters provided by the nonlinear least-squares approach.

Results

Scheme for Analysis of the Kinetics of RNase H Activity. Previous studies of the RNase H activity of reverse transcriptases were undertaken with large substrates, usually radioactive poly(A) hybridized to oligo(dT) or related heteroduplexes, which must be cleaved many times to produce the acid-soluble fragments used as a measure of catalysis (Leis et al., 1973; Gerard, 1981; Grandgenett et al., 1985; Hansen et al., 1988). To dissect this complex reaction, we devised a simpler assay. Globin mRNA was 3′-end-labeled by the addition of one to two [^{32}P]adenylate residues and hybridized to $(dT)_{15}$; upon incubation with RNase H, the poly(A) tail of the mRNA, after only one scission, is released as a radioactive, acid-soluble fragment. Thus, the loss of acid-precipitable material serves as an assay, as before, but because each substrate molecule has only an end-label, the enzyme must dissociate from the products of the first round of catalysis and reassociate with new labeled substrates before a second *recognizable* series of catalytic events can occur. In short, only one cleavage per poly(A) tail is counted even if more were to occur. The reaction is shown in Scheme I where E is the free enzyme, S the RNA·DNA hybrid substrate composed of globin mRNA[^{32}P]pA·$(dT)_{15}$, ES the enzyme–substrate complex, EP the enzyme–product complex in which a partially deadenylated mRNA is still bound to DNA, and P the dissociated RNA·DNA products. Two steps are diagrammed as irreversible: the step described by k_2 is a hydrolytic reaction which is thermodynamically unfavorable in the reverse direction, and that governed by k_3 is made irreversible by studying initial rates. It should be clear that the enzyme is behaving distributively with this substrate. If the RNase H were to cleave the poly(A) tail more than once, the additional cuts would not cause an increase in acid-soluble radioactivity and would, therefore, remain undetected. However, such limited processivity is of minor consequence because the poly(A) tails of globin mRNA are short (~20 residues), and, as we will show below, the rate-limiting step is the dissociation of the enzyme from the products. Thus, several cleavages per RNA molecule will have no effect on the reaction rate in this particular assay.

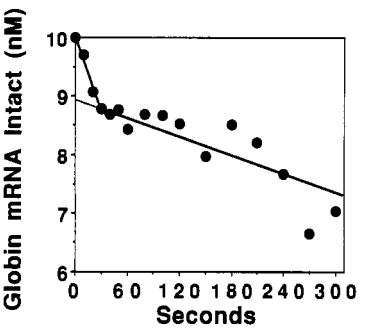

FIGURE 1: Time course of hydrolysis of 3′-end-labeled globin mRNA hybridized to $(dT)_{15}$ by HIV reverse transcriptase. Duplicate 20-μL incubation mixtures containing 1.6 μg/mL HIV reverse transcriptase (a preparation containing a 4/1 mixture of p66/p51 subunits) and 10 nM globin mRNA (600 000 dpm) were incubated for the stated time as described under Materials and Methods. All components were at 37 °C before initiation of the reaction. Aliquots of 1 μL were withdrawn for assay of acid-insoluble radioactivity. Each point represents the results from duplicate samples. The slower, steady-state rate has been extrapolated to the ordinate to indicate the amount of substrate consumed in the burst.

Scheme I

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E + P \qquad (1)$$

It is also necessary to consider the fate of the released, labeled oligo(A) fragment. The remnant of the poly(A) tail is measured as acid-soluble regardless of whether or not it remains bound to the primer. Because there is excess oligo(dT) in the reaction, rates of hydrolysis are unaffected.

Time Course of the Hydrolysis of Hybridized Globin mRNA by HIV Reverse Transcriptase. The kinetics of deadenylation of 3′-end-labeled mRNA hybridized to oligothymidylate are shown in Figure 1. The reaction was biphasic; a phase in which products were formed very rapidly was followed by a much slower steady-state rate. Since RNA is hydrolyzed during the rapid phase, i.e., the burst, k_1 and k_2 must be much larger than k_3. Under these conditions, the steady-state rate is governed by k_3 which, in turn, is equivalent to k_{cat}. The data suggest that the regeneration of free enzyme is rate-limiting. Figure 1 also displays the intersection on the ordinate of the curve representing the steady-state rate, after extrapolation to zero time. Because each active site turns over only once during the burst at saturating substrate concentration, the intercept on the ordinate, in units of cleaved substrate molecules, is equal to the number of active sites in the enzyme preparation. With one active site for RNase H per native reverse transcriptase molecule, the intercept is a direct measure of the number of active enzyme molecules in the preparation. This value is particularly instructive when recombinant enzymes are examined.

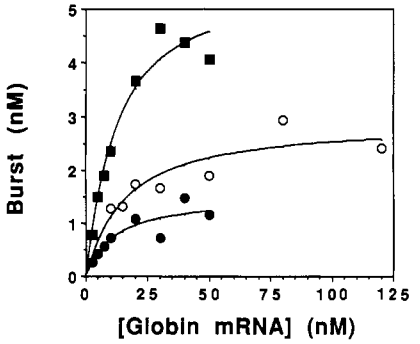

FIGURE 2: Analysis of the burst: The time course of hydrolysis of the stated concentration of 3'-end-labeled globin mRNA·$(dT)_{15}$ by the RNase H activity of 1.6 μg/mL HIV reverse transcriptase (●) or 160 units/mL AMV reverse transcriptase (■) was determined in duplicate 10-μL volumes as described in Figure 1 in order to ascertain the size of the burst. Burst size was also measured with globin mRNA[^{32}P]pCp in the presence of 100 μM each dNTP and 0.45 μg/mL HIV reverse transcriptase (○) using a preparation consisting solely of p66 subunits. In both cases, enzymatic rates of hydrolysis were determined by withdrawing 1.5-μL aliquots. The *curves* are theoretical extrapolations based on the values of K_D and the active-site concentrations obtained with the Scatchard method of analysis.

In vivo, the HIV enzyme is composed of two subunits, p66 and p51 (di Marzo Veronese et at., 1986). The p51 subunit is derived from p66 by removal of a 15-kDa fragment containing the catalytic domain for RNase H (Farmerie et al., 1987; Lowe et al., 1988; Mous et al., 1988; Müller et al., 1989). Thus, the heterodimer retains one RNase H catalytic site. In vivo, proteolysis of p66 is highly specific, whereas in vitro, the recombinant p51 subunit may not be identical to the native p51 subunit because an *E. coli* protease substitutes for the enzyme encoded in the HIV genome (Müller et al., 1989). Furthermore, preparations of recombinant reverse transcriptase used in this study invariably contained homodimers of p66. Because the recombinant homodimer has been shown to be indistinguishable from the native heterodimer in kinetic studies of the polymerase activity (Majumdar et al., 1989; Kedar et al., 1990), the assumption has been made that, regardless of the subunit structure of the enzyme, there is only one functional RNase H domain. This idea, supported by recent crystallographic data (Davies et al., 1991), appears to be borne out by the data presented below.

In Figure 1, the globin mRNA concentration was not saturating, and the burst size was less than the amount of active enzyme. Nevertheless, the burst is most readily visualized under conditions of limiting substrate, circumstances which maximize the percent change in labeled mRNA remaining rather than the total consumed during the burst. The size of the burst was evaluated as a function of the substrate concentration in the presence of a fixed amount of reverse transcriptase by extrapolating steady-state rates to zero time (see Figure 1). The experiment was performed with both the HIV reverse transcriptase and the enzyme from AMV (Figure 2). It is apparent that the size of the burst increases with increasing amounts of substrate until a limit imposed by the number of active sites in the preparation is achieved. The size of the burst, evidently, does not exceed the amount of enzyme. These relationships are made clearer by analyzing the same data using the Scatchard method. The amount of DNA·RNA hybrid bound to the enzyme is set equal to the size of the burst, and the free substrate concentration is calculated by subtracting the amount bound from the total. Then, by plotting bound/free on the ordinate against bound on the abscissa, one obtains a straight line with a slope equal to $-1/K_D$ and an intercept on the abscissa, n, equal to the concentration of active sites in the assay. From these values, one can infer that the amount of AMV RNase H activity is ~75% (n = 5.5 ± 0.2 nM) of that predicted from the number of units used (160 units/mL), the molecular weight, and the highest specific activity reported by the manufacturer, among many preparations; K_D is 9 ± 1 nM.

The analysis of the HIV enzyme was undertaken in greater detail, i.e., in the presence and absence of dNTPs for polymerization. Because RNase H activity and polymerase activity in vivo are interdependent and chronologically overlapping, the effect of concomitant polymerization on the burst was examined. However, in order to obtain meaningful data, globin mRNA was 3'-end-labeled with pCp, rather than with ATP, to prevent poly(A) tails released as acid-soluble fragments from priming synthesis of DNA either on mRNA or on oligo(dT) templates. The 3'-terminal phosphate on RNA blocked the extension of the poly(A) tails and thereby prevented fragments which were initially acid-soluble from regaining acid-precipitability (Berger et al., 1983; Krug & Berger, 1989). That mRNA[^{32}P]pCp is identical to mRNA[^{32}P]pA with respect to hydrolysis of RNA by RNase H has already been demonstrated (Krug & Berger, 1989).

Regardless of whether or not concomitant polymerization in the presence of all four deoxyribonucleoside triphosphates occurred, the hydrolysis of RNA by the RNase H of the HIV enzyme displayed an initial burst (Figure 2), with the same value of K_D (14 ± 3 nM in the presence of dNTPs compared to 11 ± 3 nM in their absence). However, the number of active sites in the preparation was dramatically changed; when dNTPs were present, the number of active sites (n = 2.9 ± 0.2 nM) was nearly equal to the value of the dimer concentration calculated from the molecular weight and the known amount of enzyme (0.45 μg/mL). In the absence of dNTPs (n = 1.5 ± 0.1 nM for 1.6 μg/mL enzyme), only one-sixth of the preparation seemed to be functional. These results suggest that dNTPs do not affect the binding of substrate to functional enzyme but do affect the ability of the enzyme to assume an active conformation for catalysis, perhaps by promoting dimerization. In this connection, it is interesting that high concentrations of ammonium phosphate are believed to stabilize the polymerase activity of HIV reverse transcriptase by facilitating formation of p66 dimers (Rowley et al., 1990) and that dNTPs may be responsible for the observation that p66 homodimers (which readily dissociate) and p66·p51 heterodimers (which appear to be stable) (Restle et al., 1990) are indistinguishable in the polymerization reaction. The results are also compatible with the view that one RNase H site per dimer is functional regardless of the subunit composition of the enzyme.

Steady-State Kinetics of RNA Hydrolysis and DNA Polymerization. Steady-state reactions were evaluated by measuring initial rates at a fixed concentration of enzyme in the presence of varying globin mRNA·oligo(dT) concentrations. Kinetic constants were obtained with the aid of double-reciprocal plots ($1/v$ against $1/S$) in which the intercept on the ordinate is a measure of $1/V_{max}$ and the intercept on the abscissa is $-1/K_m$. Representative examples of some aspects of these data are presented in Figure 3A,B (curves generated in the absence of inhibitors), and the constants derived from these examples are displayed in Table I.

The value of K_m for the two reverse transcriptases in this study was essentially the same. For the HIV enzyme, the value was 27 nM in the presence or absence of triphosphates, and that for the AMV enzyme was 30 nM in the absence of polymerization. In each instance, values of K_m were greater than

Table I: Kinetic Properties of Reverse Transcriptases and *E. coli* Ribonuclease H

kinetic parameter	ribonuclease H		
	HIV	AMV	*E. coli*
K_m (nM)	27 (26–33)[a]	30 (22–40)	250 (250–290)
K_D (nM)	11 ± 3[c]	9 ± 1[c]	ND[b]
k_{cat} (s^{-1})	5×10^{-3} [$(5–8) \times 10^{-3}$]	5×10^{-3}	6×10^{-2}
k_1 (M^{-1} s^{-1})	2×10^5 [$(2–3) \times 10^5$]	2×10^5	ND
k_{-1} (s^{-1})	2×10^{-3} [$(2–5) \times 10^{-3}$]	2×10^{-3}	ND
k_3 (s^{-1})	5×10^{-3} [$(5–8) \times 10^{-3}$]	5×10^{-3}	ND

kinetic parameter	RNA-dependent DNA polymerase	
	HIV	AMV
K_m for RNA·DNA[d] (nM)	6 (2–11)	10
K_m for dNTP[e] (μM)	24	ND
k_{cat} (s^{-1})	2 (1–2.5)	2

[a] Values in parentheses delineate the span of the experimental results. [b] ND, not determined. [c] Standard error. [d] RNA·DNA refers to globin mRNA·$(dT)_{15}$. [e] The value of K_m for dNTP refers to an equimolar mixture of all four deoxyribonucleoside triphosphates.

those for K_D (K_D = ~12.5 and 9 nM for the HIV and AMV enzymes, respectively), reflecting the effect of rate constants on the Michaelis constants. The hydrolysis of RNA in DNA·RNA hybrids catalyzed by the *E. coli* enzyme was also examined. Here, it is clear that the value of K_m is approximately 10-fold higher than that for either of the aforementioned reverse transcriptases, namely, ~250 nM (Table I).

The curves also provide a means for measuring turnover numbers. Since the concentration of enzymatically active reverse transcriptase can be determined from the size of the burst in the RNase H reaction, values of the turnover numbers ($V_{max}/[E]$) can be obtained. The results indicate a value of 5×10^{-3} s^{-1} for either the HIV or the AMV enzyme (Figure 3A,B and Table I). The existence of the burst also makes possible a determination of k_3; because k_3, the regeneration of free enzyme, is rate-limiting under the conditions described in Scheme I, k_3 is equal to $V_{max}/[E]$ for both the HIV- and AMV-associated RNase H activities.

The situation is more complex for the reaction catalyzed by *E. coli* RNase H. In this instance, there is no burst (data not shown), and steady-state rates appear throughout. Without a burst, the amount of enzyme in the preparation must be estimated from the number of units in the assay and the specific activity. Since the latter is not routinely provided by the supplier, our best estimate of k_{cat} is 6×10^{-2} s^{-1}, a value approximately 20-fold greater than that calculated for either reverse transcriptase associated RNase H activity (Table I).

The data in Figures 2 and 3 contain all of the information necessary for calculations of k_1 and k_{-1} in the RNase H reaction (Scheme I). The expression for the maximum velocity of the reaction is shown in eq 2 and that for K_m in eq 3. Given

$$V_{max} = \frac{k_2 k_3 [E]}{k_2 + k_3} \quad (2)$$

$$K_m = \frac{k_3(k_2 + k_{-1})}{k_1(k_2 + k_3)} \quad (3)$$

the existence of the burst, the rate-limiting step is known to be k_3. Whereas k_3 must be much smaller than the other rate constants in the forward direction, the relative magnitude of k_{-1} is not immediately apparent. However, after careful consideration of the nearly identical nature of the substrates and products, k_{-1} should be roughly equivalent to k_3 since the two rate constants describe highly similar reactions. Then, with $k_2 \gg k_{-1}$ and $k_2 \gg k_3$, the expressions simplify to V_{max}

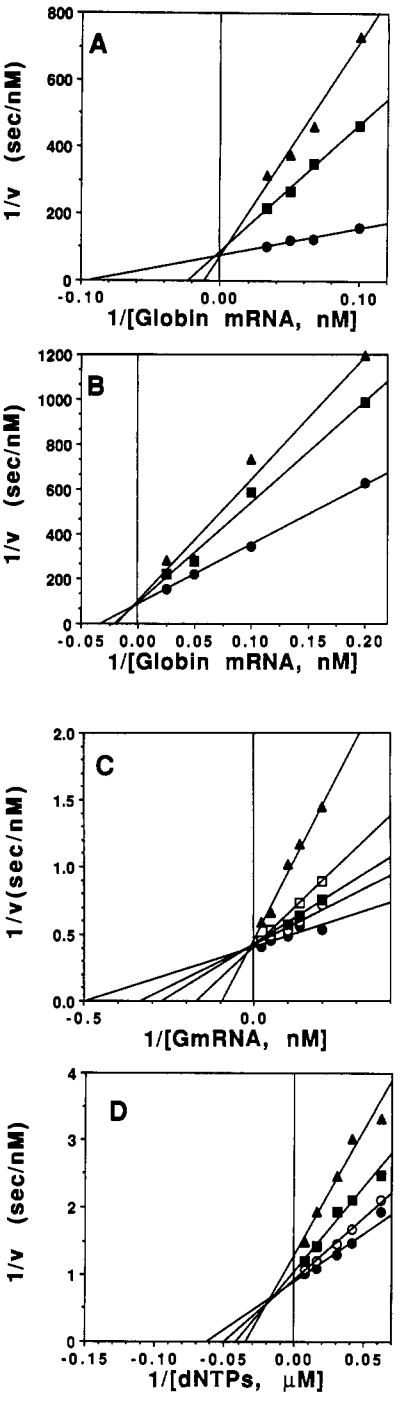

FIGURE 3: Inhibition of the polymerase and RNase H activities of reverse transcriptase by ribonucleoside–vanadyl complexes. RNase H reactions (panels A and B) were performed as described under Materials and Methods in 10 μL with globin mRNA at the stated concentrations, and either 53 units/mL AMV reverse transcriptase (A) or 1.6 μg/mL HIV reverse transcriptase (preparation described in Figure 1B). The standard mixture of vanadyl complexes was included as follows: (●) none; (○) 0.5 mM; (■) 1 mM; (□) 1.5 mM; (▲) 2.0 mM. Deoxyribonucleoside triphosphates were omitted. Reactions were assayed by acid precipitation of 1.5-μL aliquots. Points are derived from initial rates determined at least in duplicate. RNA-dependent DNA polymerase activities (panels C and D) were determined similarly in the presence of 16 μM dCTP and 64 μM each of dATP, dGTP, and TTP, together with the stated concentration of unlabeled globin mRNA and 1.3 μg/mL HIV reverse transcriptase (p66 subunits) (C) or with the stated concentration of dNTPs, 100 nM unlabeled globin mRNA, and a 3 μg/mL aliquot of a different preparation of the HIV enzyme (p66 subunits) (D). In both polymerase reactions, the initial rates of incorporation of 5 μCi of [α-^{32}P]dCTP into DNA were ascertained in the presence or absence of vanadyl complexes as noted for the RNase H reactions.

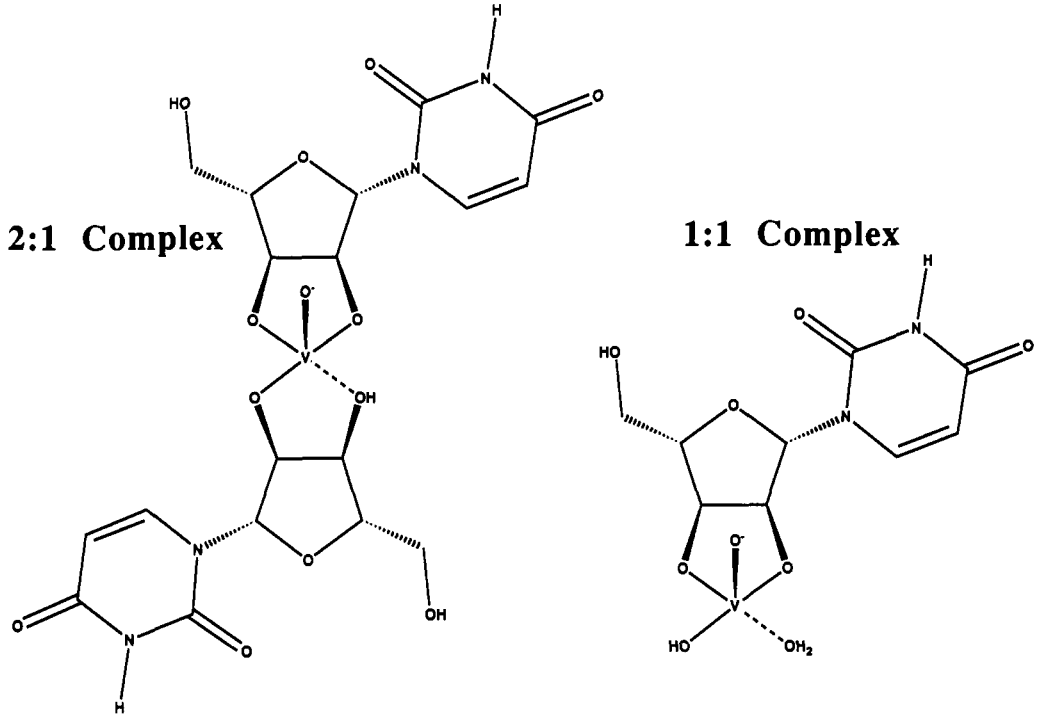

FIGURE 4: Proposed structures for uridine–vanadyl complexes. The structures shown are based on theoretical models of uridine complexed with the oxovanadium ion carried out by Lindquist et al. (1973) and nuclear magnetic resonance and electron paramagnetic resonance structural studies of adenosine 5′-triphosphate complexed with the oxovanadium ion carried out by Sakurai et al. (1982).

$= k_3[E]$ as indicated above, and $K_m = k_3/k_1$. Since K_m and k_3 have already been determined, k_1 is 2.0×10^5 M^{-1} s^{-1} for both the HIV and AMV RNase H activities. Furthermore, from K_D and k_1, the values for k_{-1} are 2×10^{-3} s^{-1} for both the HIV and AMV enzymes. As predicted, these values are similar to those for k_3.

The steady-state kinetics of polymerization have also been examined. The value of K_m for the DNA·RNA hybrid is 6 nM for the HIV enzyme (Table I and Figure 3C) and ~10 nM for the AMV enzyme (Table I). Although K_m is not a binding constant in the polymerization reaction, it is interesting that K_D for the RNase H reaction and K_m for the polymerase reaction are similar regardless of which of the reverse transcriptases is evaluated.

Inhibition of HIV Reverse Transcriptase with Ribonucleoside–Vanadyl Complexes. Ribonucleoside–vanadyl complexes are transition-state analogues of the cyclic 2′,3′-monophosphates that are generated by many ribonucleases during catalysis (Lienhard et al., 1971). Since ribonuclease H activities of all types digest DNA·RNA hybrids into oligoribonucleotides containing 5′-phosphates and 3′-hydroxyl groups (Baltimore & Smoler, 1972; Keller & Crouch, 1972; Berkower et al., 1973), it is highly unlikely that vanadyl complexes are related to any of the intermediates involved in catalysis. Nevertheless, concentrations of these complexes above 2 mM have been shown to inhibit the polymerization reaction of AMV reverse transcriptase (Puskas et al., 1982). It was of interest, therefore, to investigate the effect of these materials on RNase H activities of several types. Figure 4 shows two possible structures: a complex in which one oxovanadium ion interacts with one ribonucleoside (Lindquist et al., 1973) and a variation in which one oxovanadium ion is complexed to two ribonucleosides (Sakurai et al., 1982). In both cases, the end result is a small molecule relative to the DNA·RNA hybrid substrate of reverse transcriptase, but of a size comparable to deoxyribonucleoside triphosphate precursors, or their monophosphate intermediates which are incorporated into DNA.

The inhibition of the RNase H of HIV and AMV reverse transcriptase by the standard equimolar mixture of all four ribonucleoside–vanadyl complexes is illustrated in Figure 3A,B. In each case, the double-reciprocal plots of $1/v$ against $1/$[DNA·RNA] hybrid substrate intersect on or very near the ordinate, indicating an effect on K_m, but virtually none on V_{max}. Thus, vanadyl complexes inhibit RNase H activity by competing with the DNA·RNA hybrid substrate with a K_i value of 2–3 mM for each enzyme.

Vanadyl complexes were subsequently tested as inhibitors of the polymerase activity of HIV reverse transcriptase and were found to be competitive with the DNA·RNA hybrid substrate during the synthesis of globin cDNA (Figure 3C). Furthermore, the value of K_i (1–3 mM) was the same regardless of whether the reaction was the polymerization of DNA or the hydrolysis of RNA. However, when vanadyl complexes were tested with the deoxyribonucleoside triphosphates as the variable substrate in the polymerase reaction, there was mixed inhibition; both K_m and V_{max} were affected. The results suggest that vanadyl complexes do not interfere with the binding of the dNTP substrate to reverse transcriptase. Rather, they interact with a site on the enzyme normally occupied by the DNA·RNA hybrid during either hydrolysis of RNA or synthesis of (−) strand DNA. These data are, therefore, consistent with a single binding site for the template-primer.

Inhibition of AMV Reverse Transcriptase with Derivatives of Ribonucleoside–Vanadyl Complexes. The interaction of vanadyl complexes with reverse transcriptase was characterized further by investigating several modified complexes as well as related compounds, all of which were tested at 5 mM with globin mRNA·oligo(dT) and AMV reverse transcriptase. In these studies, the vanadyl complexes of guanosine, inosine, uridine, 5-iodouridine, and 3-deazauridine inhibited the RNase H function of the enzyme almost as well as the standard equimolar mixture of all four ribonucleoside–vanadyl complexes whereas vanadyl complexes made with adenosine, cytidine, a mixture of four ribonucleoside 5′-phosphates, or ATP

Table II: Inhibitors of the AMV Ribonuclease H Activity

substance	activity[a] (%)
vanadyl complexes of	
a mixture of the four ribonucleosides[b]	4
guanosine	20
uridine	30
5-iodouridine	30
3-deazauridine	20
inosine	20
a mixture of the four ribonucleoside 5′-monophosphates	100
adenosine	100
cytidine	100
adenosine 5′-triphosphate	100
neutralized vanadyl sulfate	100
a mixture of the four ribonucleosides	100
a mixture of the four nucleoside 2′,3′-monophosphates	100
a mixture of the four nucleoside cyclic 2′,3′-phosphates	100

[a] Hydroysis of 15 nM 3′-end-labeled globin mRNA·$(dT)_{15}$ in the presence or absence of 5 mM putative inhibitor was carried out for 5 min with 3.3 nM reverse transcriptase. Inhibition is reported as percent enzyme activity remaining. In the absence of additives, the enzyme activity is defined as 100%. [b] Referred to as the standard mixture.

did not inhibit (Table II). Furthermore, neutralized vanadyl sulfate, ribonucleosides, ribonucleoside 2′- and 3′-monophosphates (mixed isomers), and ribonucleoside cyclic 2′,3′-phosphates also did not inhibit the RNase H activity (Table II). These results indicate that the substituents at both the 5′ and 3′ positions of the ribonucleoside moiety are important. It is particularly interesting that the standard mixture of four vanadyl complexes was a better inhibitor than vanadyl complexes synthesized from only one ribonucleoside at a concentration equal to the sum of the concentrations of the four different complexes (Table II, the complexes made either with U or with G compared with the standard mixture). Complexes prepared from a mixture of nucleosides are more potent inhibitors than a reconstituted mixture of the individual complexes (data not shown). These results are in agreement with the model in which two ribonucleosides complex with one oxovanadium ion (Sakurai et al., 1982) (Figure 4) and suggest that the best inhibitors contain two different ribonucleosides within the same complex.

Discussion

The RNase H activities of HIV and AMV reverse transcriptases have been characterized and compared with the RNase H from *E. coli* with the use of a 3′-end-labeled mRNA·oligo(dT) hybrid, which generates a recognizable product after a single hydrolytic cleavage. With this substrate, the reaction is distributive: dissociation of the enzyme from product and reassociation with fresh substrate accompany virtually every catalytic event. Under these conditions, the reactions catalyzed by reverse transcriptases consisted of a burst followed by a much slower steady-state rate. These kinetics, which have been elegantly described by Laidler and Bunting (1973), allowed us to dissect the RNase H reaction. In contrast, when the same substrate was used to evaluate the reaction catalyzed by *E. coli* RNase H, a burst was not observed, and the K_m value, 250 nM, was much higher than that measured for reverse transcriptases (~30 nM). The *E. coli* enzyme was also characterized by Hogrefe et al. (1990) using substrates composed of four DNA·RNA base pairs flanked on both strands with short DNA·DNA hybrids. Again, products could be discerned after one cleavage per molecule, no burst was noted, and a K_m value of 4 μM was obtained. The combined results from the two laboratories highlight the differences between the viral enzymes and the simpler, cellular RNase H activities exemplified by the *E. coli* enzyme. Furthermore, viral RNase H activities, unlike the cellular enzymes, are highly sensitive to the length and/or structure of the DNA·RNA substrate, exhibiting no activity on short heteroduplexes that can be cleaved by *E. coli* RNase H (Leis et al., 1973). As shown below, the polymerase and RNase H functions of dimeric reverse transcriptases are not independent with respect to the handling of the DNA·RNA hybrid. Thus, concepts obtained from the study of cellular RNase H activities should be applied cautiously to reverse transcriptases.

We propose that the DNA·RNA hybrid binds to reverse transcriptase in a single site regardless of whether the enzyme is catalyzing the polymerization of DNA or the hydrolytic cleavage of RNA. This conclusion is based on two observations: (i) the agreement of several of our rate constants and K_D and K_m values for DNA·RNA hybrids with those of certain other laboratories, all of which studied the polymerase reaction (see below); and (ii) the similarity of the quantitative and qualitative effects of vanadyl complexes on both reactions. Other types of data agree with this interpretation: (i) Although the RNase H and polymerase catalytic sites of the HIV enzyme are separable by linker insertion mutagenesis, the combined results of the Goff and Hughes groups (Hizi et al., 1989a,b; Prasad & Goff, 1989) suggest that the majority of mutations alter both activities, presumably by influencing the binding of substrates to a common site. (ii) Isolated carboxyl termini bearing the RNase H catalytic site are probably not active. The presence of the p51 subunit of the HIV enzyme was essential for demonstrating the RNase H activity of the isolated p15 subunit (Hostomsky et al., 1991), and a p24 chymotryptic fragment of AMV reverse transcriptase was enriched for RNase H activity but was not rigorously free of intact or nearly intact subunits (Lai & Verma, 1978). Here, the implication is that the larger fragments bind the DNA·RNA hybrids and interact with smaller polypeptides containing only the RNase H catalytic site. (iii) Studies of DNA·RNA hybrids interacting with HIV reverse transcriptase using tryptophan fluorescence as a probe also revealed one class of energetically equivalent binding sites within the p51/p66 heterodimer (Painter et al., 1990).

Any evaluation of the kinetics of the uninhibited reactions catalyzed by reverse transcriptase requires a detailed comparison of our results with those of others. Such an analysis depends on the conclusions of the Wilson group (Majumdar et al., 1988) which showed that the template-primer binds to the enzyme first while template–products dissociate last; in the polymerase reaction only, the binding of dNTPs and the release of pyrophosphate intervene. Thus, the first and last steps should be described by the same constants if the same sites are used by the RNase H and polymerase functions. We cite three examples.

k_{cat} in Polymerase and RNase H Reactions Exhibiting a Burst. In our first example, we compare the value of k_{cat} in the RNase H reaction (called k_3 in Scheme I) to k_{cat} in the polymerase reaction (Reardon & Miller, 1990), with both studied under distributive conditions. In both reactions, a burst was observed. Since there is no evidence to support a modified enzyme intermediate, the burst is best interpreted in terms of a mechanism in which dissociation of products to regenerate free enzyme is rate-limiting. The values of k_{cat} ranged from 0.005 s^{-1} for the RNase H reaction to 0.025–0.071 s^{-1} for the polymerase reaction; the spread in the polymerization rates reflected the use of different substrates. We suggest that the similarity of these rate constants favors a model in which a

single template–product binding site serves both the polymerase and the RNase H functions.

k_{-1} in the Polymerase and RNase H Reactions. The rate of dissociation of template-primer from HIV reverse transcriptase (designated k_{-1} or k_{off}) provides a second example for which constants measured in the polymerase reaction can be compared to those in the RNase H reaction. Huber et al. (1989), studying the polymerase function, found that enzyme–hybrid complexes decayed with a half-life of 2–3 min; our value of k_{-1} for the RNase H reaction, (2–5) × 10^{-3} s^{-1}, was equivalent to a half-life of 2–6 min. The agreement of the measurements suggests that both describe dissociation from the same site on the enzyme.

k_1 and k_{-1} in the Polymerase and RNase Reactions. In the third example, k_1 was scrutinized. The Richardson group found that k_{on} (equivalent to k_1), in the absence of Mg^{2+}, was (1–2) × 10^6 M^{-1} s^{-1} for the polymerase, while we obtained a value of 2 × 10^5 M^{-1} s^{-1} for the RNase H reaction with a different substrate in the presence of Mg^{2+}. Once again, the similarity of the constants appears to support the concept of a single site in both reactions. In contrast, the results of Wilson and co-workers differed markedly. They (Majumdar et al., 1989) calculated k_{on} and k_{off} by studying the interaction of HIV reverse transcriptase with a thiophosphate-containing DNA primer, $Sd(C)_{28}$. The following values were obtained: k_{on}, 5.8 × 10^8 M^{-1} s^{-1}; k_{off}, 1.6 s^{-1}. We believe that the discrepancies of several hundredfold stem from oversimplification. The Wilson group used an expression for an enzymatic reaction in which a single substrate is converted into a single product to analyze the much more complex actual case of a processive reaction with a fixed order of binding substrates and releasing products; this approach produced an intermingling of terms derived from both distributive and processive synthesis. Since the parameters involving processivity dominated their expression, the resulting k_{on} and k_{off} calculated from it reflected translocation of the enzyme along the template during processive synthesis more closely than binding of substrates to free enzyme and release of products to regenerate free enzyme.

The Wilson group (Kedar et al., 1990) also measured k_{on} and k_{off} using an analysis that recognized the contribution of the second substrate, namely, TTP or AZTTP; the latter also served as an inhibitor. They began by writing a simplified expression for k_{off} to be used in the subsequent calculation of k_{on}. Several problems mar this approach: (i) The expression for k_{off} for oligo(dT) with TTP as the second substrate involved unproven assumptions about the relative rates of binding and release of TTP. Furthermore, it could not be solved. (ii) The same equation *could* be evaluated with AZTTP as the second substrate, but the derivation of k_{off} with TTP as the second substrate did not apply to AZTTP; the substitution of one substrate for another changed the reaction scheme from processive to distributive. Nevertheless, k_{off} calculated with AZTTP as a substrate was assumed to be equivalent to k_{off} with TTP. (iii) Further difficulties were encountered: (A) K_i for AZTTP as a competitive inhibitor was set equal to K_D, an assumption challenged by Reardon and Miller (1990) based on the details of polymerizing TMP into product. However, the belief that K_i is equivalent to K_D is systematically incorrect when the inhibitor is a competitive substrate. (B) The value of k_{cat} with AZTTP as a substrate was also uncertain, because it was not clear that the enzyme was turning over. Thus, the values substituted into a questionable equation for k_{off} were, themselves, problematic. (iv) K_m and K_D for poly(A)·oligo(dT) were assumed, rather than shown, to be identical. Thus, both parameters needed to calculate k_{on}, namely, k_{off} and K_D, were controversial. The value of k_{on} for oligo(dT) from their analysis, 2.8 × 10^8 M^{-1} s^{-1}, is very nearly equal to that obtained for $Sd(C)_{28}$, and the value of k_{off}, 39 s^{-1}, is also similar to Wilson's previous measurements. We suggest that this is a coincidence based on the inappropriate substitution of parameters measured during processive synthesis, for the most part, into equations derived for simple distributive reactions. In other words, the assumptions on which the analysis was based were not reevaluated when substrates were switched. The disagreements between the Wilson group and others serve to emphasize the extraordinary complexity of reverse transcriptase catalyzed reactions and the difficulties inherent in dissecting them.

Inhibition by Ribonucleoside–Vanadyl Complexes. Vanadyl complexes inhibited both the polymerase and the RNase H reactions competitively with respect to the template-primer. In the polymerase reaction, the inhibition was mixed with respect to dNTPs. These are the expected results when substrates add to an enzyme sequentially in a fixed order and when the inhibitor competes for a binding site with the first substrate. The data suggest that the same template-primer binding site is occupied by the complexes regardless of which reaction is in progress. This finding is important because it represents a different approach to the treatment of AIDS; unlike AZT or DDI which bind to the dNTP site, vanadyl complexes are targeted to the template-primer binding site. Furthermore, since vanadyl complexes appear to be complicated mixtures of two ribonucleosides interacting with one oxovanadium ion, the possibility exists for identifying the most potent forms and using them to design drugs.

Acknowledgments

We are grateful to Dr. John Westley of the University of Chicago for his critical evaluation of the data.

References

Baltimore, D., & Smoler, D. F. (1972) *J. Biol. Chem. 247*, 7282–7287.

Basu, A., Basu, S., & Modak, M. J. (1990) *J. Biol. Chem. 265*, 17162–17166.

Bebenek, K., Abbotts, J., Roberts, J. D., Wilson, S. H., & Kunkel, T. A. (1989) *J. Biol. Chem. 264*, 16948–16956.

Berger, S. L. (1987a) *Methods Enzymol. 152*, 49–54.

Berger, S. L., & Birkenmeier, C. S. (1979) *Biochemistry 18*, 5143–5149.

Berger, S. L., Wallace, D. M., Puskas, R. S., & Eschenfeldt, W. H. (1983) *Biochemistry 22*, 2365–2373.

Berkower, I., Leis, J., & Hurwitz, J. (1973) *J. Biol. Chem. 248*, 5914–5921.

Champoux, J. J., Gilboa, E., & Baltimore, D. (1984) *J. Virol. 49*, 686–691.

Davies, J. F., II, Hostomska, Z., Hostomsky, Z., Jordan, S. R., & Matthews, D. A. (1991) *Science 252*, 88–95.

di Marzo Veronese, F., Copeland, T. D., DeVico, A. L., Rahman, R., Oroszlan, S., Gallo, R. C., & Sarngadharan, M. G. (1986) *Science 231*, 1289–1291.

Doolittle, R. F., Feng, D.-F., Johnson, M. S., & McClure, M. A. (1989) *Q. Rev. Biol. 64*, 1–30.

England, T. E., Bruce, A. G., & Uhlenbeck, O. C. (1980) *Methods Enzymol. 65*, 65–74.

Farmerie, W. G., Loeb, D. D., Casavant, N. C., Hutchinson, C. A., III, Edgell, M. H., & Swanstrom, R. (1987) *Science 236*, 305–308.

Finston, W. I., & Champoux, J. J. (1984) *J. Virol. 51*, 26–33.

Gerard, G. F. (1981) *Biochemistry 20*, 256–265.

Gerard, G. F., & Grandgenett, D. P. (1975) *J. Virol. 15*, 785–797.

Goldman, M. E., Salituro, G. S., Bowen, J. A., Williamson, J. M., Zink, D. L., Schleif, W. A., & Emini, E. A. (1990) *Mol. Pharmacol. 38*, 20–25.

Grandgenett, D. P., Gerard, G. F., & Green, M. (1972) *J. Virol. 10*, 1136–1142.

Grandgenett, D. P., Quinn, T., Hippenmeyer, P. J., & Oroszlan, S. (1985) *J. Biol. Chem. 260*, 8243–8249.

Hansen, J., Schulze, T., & Moelling, K. (1987) *J. Biol. Chem. 262*, 12393–12396.

Hansen, J., Schulze, T., Mellert, V., & Moelling, K. (1988) *EMBO J. 7*, 239–243.

Hizi, A., Barber, A., & Hughes, S. H. (1989a) *Virology 170*, 326–329.

Hizi, A., Hughes, S. H., & Shaharabany, M. (1989b) *Virology 175*, 575–580.

Hogrefe, H. H., Hogrefe, R. I., Walder, R. Y., & Walder, J. A. (1990) *J. Biol. Chem. 265*, 5561–5566.

Hostomsky, Z., Hostomska, Z., Hudson, G. O., Moomaw, E., & Nodes, B. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1148–1152.

Huber, H. E., McCoy, J. M., Seehra, J. S., & Richardson, C. C. (1989) *J. Biol. Chem. 264*, 4669–4678.

Johnson, M. S., McClure, M. A., Feng, D.-F., Gray, J., & Doolittle, R. F. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7648–7652.

Kedar, P. S., Abbotts, J., Kovacs, T., Lesiak, K., Torrence, P., & Wilson, S. H. (1990) *Biochemistry 29*, 3603–3611.

Keller, W., & Crouch, R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3360–3364.

Kotewicz, M. L., Sampson, C. M., D'Alessio, J. M., & Gerard, G. F. (1988) *Nucleic Acids Res. 16*, 265–277.

Krug, M. S., & Berger, S. L. (1987) *Anal. Biochem. 153*, 315–323.

Krug, M. S., & Berger, S. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3539–3543.

Lai, M.-H., & Verma, I. M. (1978) *J. Virol. 25*, 652–663.

Laidler, K. J., & Bunting, P. S. (1973) *The Chemical Kinetics of Enzyme Action*, pp 185–187, Oxford University Press, London.

Larder, B. A., Purifoy, D. J. M., Powell, K. L., & Darby, G. (1987) *Nature 327*, 716–717.

Leis, J. P., Berkower, I., & Hurwitz, J. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 466–470.

Levin, J. G., Crouch, R. J., Post, K., Hu, S. C., McKelvin, D., Zweig, M., Court, D. L., & Gerwin, B. I. (1988) *J. Virol. 62*, 4376–4380.

Lienhard, G. E., Secemski, I. I., Koehler, K. A., & Lindquist, R. N. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 45–51.

Lindquist, R. N., Lynn, J. L., Jr., & Leinhard, G. E. (1973) *J. Am. Chem. Soc. 95*, 8762–8766.

Lowe, D. M., Aitken, A., Bradley, C., Darby, G. K., Larder, B. A., Powell, K. L., Purifoy, D. J. M., Tisdale, M., & Stammers, D. K. (1988) *Biochemistry 27*, 8884–8889.

Majumdar, C., Abbotts, J., Broder, S., & Wilson, S. H. (1988) *J. Biol. Chem. 263*, 15657–15665.

Majumdar, C., Stein, C. A., Cohen, J. S., Broder, S., & Wilson, S. H. (1989) *Biochemistry 28*, 1340–1346.

Mendelman, L. V., Petruska, J., & Goodman, M. F. (1990) *J. Biol. Chem. 265*, 2338–2346.

Mitsuya, H., & Broder, S. (1987) *Nature 325*, 773–778.

Moelling, K., Bolognesi, D. P., Bauer, H., Büsen, W., Plassmann, H. W., & Hansen, P. (1971) *Nature (London), New Biol. 234*, 240–243.

Mous, J., Heimer, E. P., & Le Grice, S. F. J. (1988) *J. Virol. 62*, 1433–1436.

Müller, B., Restle, T., Weiss, S., Gautel, M., Sczakiel, G., & Goody, R. S. (1989) *J. Biol. Chem. 264*, 13975–13978.

Nakane, H., & Ono, K. (1990) *Biochemistry 29*, 2841–2845.

Omer, C. A., & Faras, A. J. (1982) *Cell 30*, 797–805.

Ono, K., Nakane, H., Fukushima, M., Chermann, J.-C., & Barrê-Sinoussi, F. (1990) *Eur. J. Biochem. 190*, 469–476.

Oyama, F., Kikuchi, R., Crouch, R. J., & Uchida, T. (1989) *J. Biol. Chem. 264*, 18808–18817.

Painter, G. R., Wright, L. L., Hopkins, S., & Furman, P. A. (1990) *J. Cell. Biochem.*, S14D.

Poch, O., Sauvaget, I., Delarue, M., & Tordo, N. (1989) *EMBO J. 8*, 3867–3874.

Prasad, V. R., & Goff, S. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3104–3108.

Puskas, R. S., Manley, N. R., Wallace, D. M., & Berger, S. L. (1982) *Biochemistry 21*, 4602–4608.

Reardon, J. E., & Miller, W. H. (1990) *J. Biol. Chem. 265*, 20302–20307.

Repaske, R., Hartley, J. W., Kavlick, M. F., O'Neill, R. R., & Austin, J. B. (1989) *J. Virol. 63*, 1460–1464.

Resnick, R., Omer, C. A., & Faras, A. J. (1984) *J. Virol. 51*, 813–821.

Restle, T., Müller, B., & Goody, R. S. (1990) *J. Biol. Chem. 265*, 8986–8988.

Rowley, G. L., Ma, Q.-F., Bathurst, I. C., Barr, P. J., & Kenyon, G. L. (1990) *Biochem. Biophys. Res. Commun. 167*, 673–679.

Sakurai, H., Goda, T., & Shimomura, S. (1982) *Biochem. Biophys. Res. Commun. 108*, 474–478.

Schatz, O., Cromme, F. V., Grüninger-Leitch, F., & Le Grice, S. F. J. (1989) *FEBS Lett. 257*, 311–314.

Schatz, O., Mous, J., & Le Grice, S. F. J. (1990) *EMBO J. 9*, 1171–1176.

Schinazi, R. F., Eriksson, B. F. H., & Hughes, S. H. (1989) *Antimicrob. Agents Chemother. 33*, 115–117.

Smith, J. K., Cywinski, A., & Taylor, J. M. (1984a) *J. Virol. 49*, 200–204.

Smith, J. K., Cywinski, A., & Taylor, J. M. (1984b) *J. Virol. 51*, 314–319.

Starnes, M. C., & Cheng, Y.-C. (1989) *J. Biol. Chem. 264*, 7073–7077.

Staskus, K. A., Collett, M. S., & Faras, A. J. (1976) *Virology 71*, 162–168.

Take, Y., Inouye, Y., & Nakamura, S. (1989) *J. Antibiot. 42*, 107–115.

Tanese, N., & Goff, S. P. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1777–1781.

Taylor, J., & Illmensee, R. (1975) *J. Virol. 16*, 553–558.

Varmus, H., & Swanstrom, R. (1982) in *Molecular Biology of Tumor Viruses, RNA Tumor Viruses* (Weiss, R., Teich, N., Varmus, H., & Coffin, J., Eds.) 2nd ed., pp 369–512, Cold Spring Harbor Laboratory Publications, Cold Spring Harbor, NY.

Verma, I. M., Meuth, N. L., & Baltimore, D. (1972) *J. Virol. 10*, 622–627.

Wallace, D. M. (1987a) *Methods Enzymol. 152*, 33–40.

Wallace, D. M. (1987b) *Methods Enzymol. 152*, 41–48.
White, E. L., Parker, W. B., Macy, L. J., Shaddix, S. C., McCaleb, G., Secrist, J. A., III, Vince, R., & Shannon, W. M. (1989) *Biochem. Biophys. Res. Commun. 161*, 393–398.
Yamaoka, K., Tanigawara, Y., Nakagawa, T., & Uno, T. (1981) *J. Pharmacobio-Dyn. 4*, 879–885.
Yarchoan, R., Mitsuya, H., & Broder, S. (1989) *Am. J. Med. 87*, 191–200.

Localization of a Polynucleotide Binding Region in the HIV-1 Reverse Transcriptase: Implications for Primer Binding†

Robert W. Sobol,‡ Robert J. Suhadolnik,*,‡ Amalendra Kumar,§ Byeong Jae Lee,∥ Dolph L. Hatfield,∥ and Samuel H. Wilson§

Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140, and Laboratories of Biochemistry and of Experimental Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Received June 11, 1991; Revised Manuscript Received August 13, 1991

ABSTRACT: Properties of primer recognition by purified human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) p66 homodimer have been investigated. Earlier studies had shown that RNA-directed DNA synthesis catalyzed by HIV-1 RT proceeds by an ordered mechanism in which template–primer combines with the free enzyme to form the first complex in the reaction scheme, and it was also shown that primer alone is a competitive inhibitor of template–primer. In this study, enzyme–primer binding has been further characterized utilizing $pd(T)_8$ and $pd(T)_{16}$ as model primers and UV cross-linking to covalently trap the enzyme–primer complexes. Competition experiments with several authentic primers, including $tRNA_3^{Lys}$, indicate that $pd(T)_n$ binds to the kinetically significant primer binding site of RT. Salt reversal experiments suggested that the free energy of $pd(T)_n$ binding to RT has a large nonelectrostatic component. Binding of $pd(T)_n$ to p66-RT is not affected by dNTPs and does not require the presence of template. The site of UV cross-linking of $pd(T)_{16}$ was localized to the NH_2-terminal half of p66 by use of V8 protease hydrolysis and microsequencing. Our results indicate that a polynucleotide binding site is in close proximity to residues in the peptide comprising amino acids 195~300. This region could be either a single-stranded template or single-stranded primer binding site; however, we have documented the specificity of binding with oligonucleotides that act as primer in the in vitro DNA synthesis reaction. Therefore, this $d(T)_{16}$ binding site may be part of a primer-binding groove within the HIV-1 reverse transcriptase.

Human immunodeficiency virus type 1 (HIV-1)[1] is a retrovirus widely considered to be the etiologic agent of acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Popovic et al., 1984). As with all retroviruses, an essential feature of HIV-1 replication is reverse transcription of the plus-strand RNA genome into DNA, a process requiring the RNA-dependent DNA polymerase, known as reverse transcriptase (E.C. 2.7.7.7) (Temin & Mizutani, 1970; Baltimore, 1970). Retroviral reverse transcriptases initiate DNA synthesis in vivo from the 3′-hydroxyl of a host cell tRNA annealed to its complementary region, or primer binding site, around 650 residues from the 5′-end of the viral genome (Panet et al., 1975). Previous work by Litvak and co-workers (Litvak & Araya, 1982; Sarih et al., 1988) and Haseltine et al. (1977) demonstrated specific binding of primer $tRNA^{Trp}$ by avian myeloblastosis virus (AMV) reverse transcriptase (RT); the AMV RT holoenzyme also is able to promote positioning of $tRNA^{Trp}$ at the primer binding site of the retroviral RNA genome (Litvak & Araya, 1982; Sarih et al., 1988). A recombinant HIV-1 RT expressed in yeast is able to form a specific complex with bovine $tRNA^{Lys}$, and this was with a higher binding affinity than for the control species $tRNA^{Trp}$ (Sallafranque-Andreola et al., 1989). Additionally, a recombinant HIV-1 RT expressed in *Escherichia coli* specifically binds $tRNA_3^{Lys}$, as demonstrated in Northwestern binding assays and gel mobility shift assays (Barat et al., 1989).

Kinetic studies of HIV-1 RT have indicated that the reaction pathway for DNA synthesis is ordered, with template–primer and free enzyme combining to form the first complex in the reaction sequence (Majumdar et al., 1988; Huber et al., 1989). Through the use of substrate analogues for dNTP and primer, further evidence was obtained for the formation of this template–primer/enzyme complex in the pathway, and it was found that the free enzyme can bind primer in the absence

† This study was supported in part by awards from the NIH Intramural AIDS Targeted Antiviral Program (S.H.W. and D.L.H.), NIH Grant PO1-CA29545 (R.J.S.), and a federal work-study award (R. W.S.).

* To whom correspondence should be addressed.
‡ Temple University School of Medicine.
§ Laboratory of Biochemistry, NCI, NIH.
∥ Laboratory of Experimental Carcinogenesis, NCI, NIH.

[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; AMV, avian myeloblastosis virus; SSB, ssDNA binding protein from *E. coli*; DTT, dithiothreitol; $Sd(C)_{28}$, 28-residue oligodeoxycytidylate with sulfur substituted at a nonbridge oxygen of each phosphate atom; $d(C)_{19-24}$, mixture of 19–24-residue-long normal oligodeoxycytidylate; $d(T)_8$ and $d(T)_{16}$, oligomers of deoxythymidylate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NP40, Nonidet P-40; hnRNP, heterogenous nuclear ribonucleoprotein; dNTP, deoxynucleoside triphosphate; TNP-ATP, 2′,3′-di-*O*-(2,4,6-trinitrophenyl)adenosine triphosphate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; MuLV, murine leukemia virus; PVDF, poly(vinylidene difluoride).

0006-2960/91/0430-10623$02.50/0