Conformational Changes of HIV Reverse Transcriptase Subunits on Formation of the Heterodimer: Correlation with k_{cat} and K_m†

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Received April 21, 1992; Revised Manuscript Received June 17, 1992

ABSTRACT: The reverse transcriptase (RT) from the human immunodeficiency virus (HIV) is initially expressed as a 66-kDa protein and is subsequently proteolytically processed in vivo to form a 66-kDa/51-kDa heterodimer. Comparison of circular dichroism spectra of the 66-kDa, 51-kDa, and heterodimeric forms of RT indicates that the conversion is accompanied by dramatic changes in subunit conformation. The mean residue ellipticity per subunit at 220 nm decreases from -10.7×10^3 deg cm^2 dmol^{-1} for the 66-kDa protein to -6×10^3 deg cm^2 dmol^{-1} for the heterodimer. The same loss of ellipticity is observed whether the heterodimer is produced by proteolysis or by mixing a separately-expressed cloned 51-kDa subunit with the 66-kDa protein. Comparison with the spectrum of the cloned 51-kDa protein suggests that much of the conformational change arises from formation of the 51-kDa subunit but substantial changes occur in the remaining 66-kDa subunit as well. A kinetic analysis was performed to correlate these conformational changes with changes in enzyme function. Application of an integrated Michaelis–Menten equation to the catalysis of poly(dT) formation using a d(pT)$_{20}$–poly(rA) primer-template shows that the k_{cat} for the heterodimer is approximately half that of the 66 kDa enzyme, decreasing from 2.9 to 1.2 nucleotides/s upon formation of the heterodimer. However, K_m values for the primer-template decrease from 0.54 to 0.12 μM upon heterodimer formation. Thus, k_{cat}/K_m is 2-fold larger for the heterodimer, giving it a distinct catalytic advantage at undersaturating concentrations of enzyme and primer-template. These kinetic data are compatible with a model in which half the polymerase active centers are inactivated on formation of the heterodimer, but this loss of catalytic potential is more than offset by an increase in affinity of the heterodimer for the primer-template.

Human immunodeficiency virus (HIV)[1] has been shown to be the cause of acquired immunodeficiency syndrome (AIDS) (Barré-Sinoussi et al., 1983; Gallo et al., 1984). Reverse transcriptase (RT)[1] is the sole retroviral enzyme required to convert the single-stranded RNA of the retroviral genome into double-stranded DNA suitable for integration into the host cell chromosome [reviewed in Vaishnav and Wong-Staal (1991)]. Because of its critical role in the life cycle of HIV, reverse transcriptase has been extensively characterized. It is known that the enzyme exists in vivo as a heterodimer consisting of a 66-kDa protein subunit and a 51 kDa subunit, the latter an N-terminal partial proteolysis fragment of the full-length gene product (diMarzo Veronese et al, 1986; Lightfoote et al., 1986). Pure, cloned 66-kDa subunit has been reported to have roughly half of the specific activity of the heterodimer (Lowe et al., 1988; Müller et al., 1989), while the isolated cloned 51-kDa N-terminal domain has been reported to have virtually no RT activity (Hansen et al., 1988; Tisdale et al., 1988; Müller et al., 1989). These activity phenomena may be related to the ability of the subunits to dimerize, since dissociation of the proteolytically formed heterodimer has never been observed, while the cloned 66-kDa subunit exists in solution in an equilibrium between monomer and dimer and the 51-kDa subunit exists in solution almost entirely as a monomer (Müller et al., 1989; Restle et al., 1990). An association constant for the 66-kDa/51-kDa heterodimer of 4.9×10^5 M^{-1} has been determined by measurement of the association of separately expressed cloned 66- and 51-kDa subunits by analytical ultracentrifugation (Becerra et al., 1991). On the other hand, attempts to determine an equilibrium constant for the proteolytically-produced heterodimer by measuring subunit dissociation were unsuccessful and suggested a lower limit for K_{assn} of 10^9 M^{-1} (Restle et al., 1990). The difference in the magnitude of the dissociation constants measured by these two separate methods may indicate that the cloned subunit, 15 residues shorter than the authentic 51-kDa subunit, and the 51-kDa subunits proteolytically produced in vivo were not functionally identical. We have used circular dichroism (CD) spectroscopy to investigate the conformational differences between a cloned 51-kDa subunit of 438 amino acid residues and the 66-kDa subunit, as well as conformational changes occurring when the 66-kDa homodimer is proteolyzed to the heterodimer. The formation of a fully-functional heterodimer is associated with significant conformational changes in the polypeptide backbones of both the 66-kDa monomer and the new 51-kDa monomer, signaled by changes in circular dichroism that are surprisingly large considering that both the homogeneous 66-kDa and the 66-kDa/51-kDa heterodimer forms of reverse transcriptase have rather similar enzyme activity. We have correlated the observed changes in conformation with changes in k_{cat}/K_m accompanying heterodimer formation, changes which give a distinct catalytic advantage to the heterodimer, but only at relatively low enzyme and primer-template concentrations.

MATERIALS AND METHODS

Cloning of Reverse Transcriptase. The DNA encoding the reverse transcriptase gene of HIV was provided by Drs.

† This work was supported by NIH Grant GM21919. S.F.A. is supported by a predoctoral fellowship from the Howard Hughes Medical Institute. This work is in partial fulfillment of the Ph.D. requirements for S.F.A.

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[1] Abbreviations: RT, reverse transcriptase; CD, circular dichroism; HIV, human immunodeficiency virus type 1; LTR, long terminal repeat; PBS, primer binding site.

0006-2960/92/0431-8221$03.00/0

T. C. Lin and William Konigsberg as a fragment cloned in the Bluescript plasmid. The fragment of the POL gene containing the structural gene for the reverse transcriptase was originally taken from the λBH10 plasmid containing the entire coding region of the HIV genome without the LTRs (Ratner et al., 1985). A fragment of the temporary vector, containing nucleotides 2130–3809 encoding amino acids Pro^{1} through Phe^{561} of reverse transcriptase (the in vivo C-terminus is Leu^{560}), was removed as an *Nde*I–*Xba*I fragment and cloned into the the polylinker of plasmid pAR3039 (Studier & Moffat, 1986) which had been cut with *Nde*I and *Spe*I (*Spe*I produces an end compatible with *Xba*I). The *Nde*I site in the polylinker of pAR3039 provided an in-frame AUG start codon. Downstream of the *Spe*I site in the same polylinker is a stop codon and a T7 RNA polymerase terminator. Overexpression of reverse transcriptase from this plasmid (pSART), under the control of a T7 RNA polymerase promoter, was achieved in *Escherichia coli* BL21(DE3) cells as described by Studier and Moffat (1986). Edman degradation of the N-terminus of the overproduced reverse transcriptase showed the N-terminal residue to be Pro (A. Y. Shamoo, unpublished data), indicating that the BL21(DE3) strain of *E. coli* removes the N-terminal methionine residue introduced by the insertion of the AUG start codon. This results in a polypeptide chain which differs from the enzyme produced in HIV-infected cells by a single additional Phe residue at the C-terminus.

The clone for the 51-kDa domain (pSCRT) was constructed by digestion of the plasmid pSART with *Kpn*I and religating the plasmid to introduce a frameshift, which results in the addition of the sequence HTKELEEMNK downstream of Gln^{428} before reaching a stop codon following the Lys codon at position 438 in our construct. The resulting 438 amino acid gene product therefore differs from the in vivo 51K protein at the C-terminus by 10 different amino acids produced by the cloning strategy. A 51-kDa construct with an authentic C-terminus, Phe^{440}, was generated using a PCR-generated DNA fragment, but the resultant protein when overproduced in *E. coli* was extremely susceptible to the *E. coli* proteases and was therefore difficult to isolate in a homogeneous form.

Purification of the 66-kDa and Heterodimeric Forms of Reverse Transcriptase. The *E. coli* [BL21 (DE3)] transformed with pSART was grown in M9 medium in 1.5-L flasks and induced at an OD of 0.75 with 0.3 g/L isopropyl thiogalactoside (IPTG). Four hours after induction, after an OD_{600} of 1.2–1.5 was reached, cells were pelleted by centrifugation at 4000 rpm (2700g) for 5 min and resuspended in 50 mL of buffer A (50 mM Tris-HCl, pH 8.0, 200 mM KCl, 1 mM EDTA, and 1 mM DTT). Cells were then sonicated on ice twice for 3 min and centrifuged for 15 min at 5000 rpm (3000*g*) at 4 °C. The supernatant was then placed on ice, a 10% poly(ethylenimine) solution (Aldrich) was added over 5 min to a final concentration of 0.8%, and the mixture was centrifuged for 15 min at 15 000 rpm. Solid ammonium sulfate was added to the supernatant on ice to 35% saturation and then centrifuged at 15 000 rpm (27000*g*) for 20 min. The pellet was discarded, and a second fractionation at 65% ammonium sulfate was carried out on the supernatant. The 65% pellet was resuspended in 20 mL of buffer B (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM BME, and 10% glycerol) including 50 mM NaCl and dialyzed against 1 L of the same buffer overnight. The dialysate was centrifuged to remove denatured proteins, loaded onto a 10 cm × 2 cm Trisacryl SP column (IBF Biotechnics), and washed with 50 mL of buffer B + 50 mM NaCl. A 200-mL gradient from 50 to 250 mM NaCl in buffer B was used to elute the RT from the column. Fractions containing RT were identified by SDS–PAGE on 10% acrylamide gels (Laemmli, 1970) stained with Coomassie Brilliant Blue. Fractions containing RT were pooled, diluted to 100 mM NaCl with buffer B, and loaded onto a 10 cm × 2 cm Cibacron Blue + CM column (Bio-Rad). The latter column was eluted with a gradient of 150 mM–2 M NaCl in buffer B. The fractions were monitored on a 10% acrylamide–SDS gel, and those containing the 66-kDa band only were pooled, concentrated to 0.5 mL in an Amicon ultrafiltration apparatus using a YM30 membrane, and loaded onto a 100 cm × 2.5 cm Sepharose 6B gel exclusion column (Pharmacia). The column was eluted at a rate of 10 mL/h with buffer B containing 1 M NaCl, and fractions containing protein were identified by SDS–PAGE as before. Before final pooling, fractions containing RT were assayed for reverse transcriptase activity as described below. The assay is also diagnostic for the presence of endonuclease contaminants. Fractions with reverse transcriptase activity and no endonuclease were pooled and concentrated by ultrafiltration on a YM30 membrane to ~30 μM. The sample was dialyzed overnight against buffer B + 150 mM NaCl. Protein concentrations were determined by assay with bicinchoninic acid (Pierce) or by the optical density at 280 nm on the basis of extinction coefficients of $\epsilon = 1.36 \times 10^5\ M^{-1}\ cm^{-1}$ for the 66-kDa protein and $1.23 \times 10^5\ M^{-1}\ cm^{-1}$ for the 51-kDa protein. The enzyme was stored at 4 °C in buffer B + 300 mM NaCl. The fraction of 66-kDa protein present in a preparation is highly dependent on the speed of purification. If the *E. coli* extract or other partially-purified fractions are stored for any length of time prior to the Trisacryl SP column, the resulting RT is largely heterodimer. Any remaining 66-kDa protein can be separated from the heterodimer by the Trisacryl SP column, and both fractions can then be further purified by the same protocol.

Purification of the Cloned 51-kDa Subunit. Cells were grown and harvested as described for the 66-kDa protein above, and purified by the same procedures through the dialysis step following ammonium sulfate precipitation. At an NaCl concentration of 50 mM, RT will bind to Trisacryl SP but not to DEAE-Sephadex, so a 10 cm × 2 cm DEAE column was used as a prefilter to remove several contaminating proteins from the dialysate during loading of the 51-kDa protein onto a 10 cm × 2 cm Trisacryl SP column. After the coupled columns were washed with buffer B + 50 mM NaCl, the DEAE prefilter was removed, and the Trisacryl SP column was eluted with a 50–250 mM NaCl gradient. Fractions containing 51-kDa protein, as identified by SDS–PAGE, were pooled, concentrated on an Amicon YM-30 ultrafiltration membrane, and loaded onto a 100 cm × 2.5 cm Sepharose 6B column. All subsequent purification steps were identical to those of 66-kDa protein.

SDS–PAGE of the three forms of reverse transcriptase, the 66-kDa/51-kDa heterodimer, the 66-kDa form of the enzyme, and the cloned 51-kDa subunit, is shown in Figure 1. These gels are stained with Coomassie Blue.

Assay for Reverse Transcriptase Activity. All reaction mixtures contained 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM DTT, and 6 mM $MgCl_2$. In assays for which a quantitative estimate of nuclease contamination was not necessary, 0.2 unit/μL RNasin ribonuclease inhibitor (Promega) was added. $d(pT)_{20}$ (Pharmacia) and poly(adenylic acid) (Sigma) were dissolved in TE buffer (10 mM Tris-HCl, pH 7.8, and 1 mM EDTA), and the concentrations of these stocks were calculated from the absorbance at 260 nm according to the standard extinction coefficients (Sambrook et al., 1990). A 10× stock solution of primer-template complex was created by diluting $d(pT)_{20}$ and poly(rA) stocks in TE buffer to a final

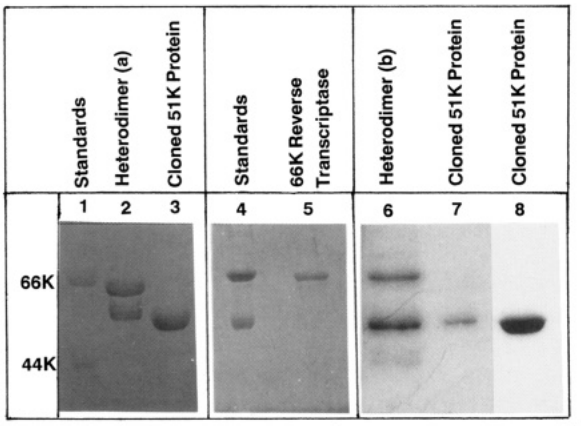

FIGURE 1: SDS–PAGE of the 66-kDa/51-kDa, the 66-kDa, and the cloned 51-kDa forms of HIV reverse transcriptase. Column 1, standards; column 2, heterodimer (preparation a); column 3, cloned 51-kDa protein, heavily loaded; column 4, standards; column 5, 66-kDa protein; column 6, heterodimer (preparation b); column 7, cloned 51-kDa protein, normally loaded; column 8, cloned 51-kDa protein, heavily loaded.

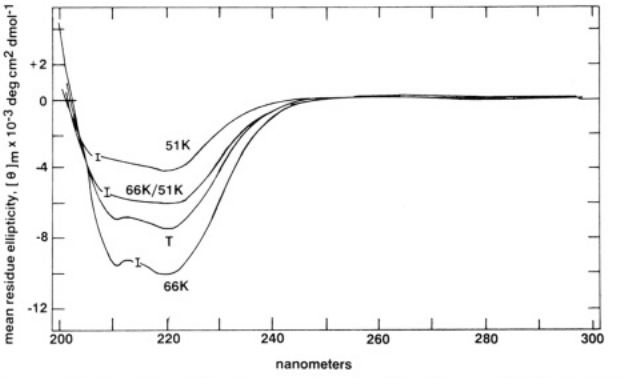

FIGURE 2: Circular dichroism spectra of various forms of HIV reverse transcriptase. Curves (lower to upper) represent pure 66-kDa enzyme, (T) theoretical curve calculated for the heterodimer (see text), protease-produced heterodimer, and cloned 51-kDa protein. All spectra were taken at 25 °C on samples containing 3 μM subunits in 10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM BME, 1 mM EDTA, and 10% glycerol. Mean residue ellipticities were calculated on the basis of a 561 amino acid 66-kDa subunit and a 438 amino acid 51-kDa subunit. The theoretical spectrum was calculated from the sum of the half-contributions of the 66- and 51-kDa spectra. Error bars indicate the amount of noise in the CD signal.

concentration of 10 μM each strand. The solution was heated to 80 °C for 5 min and slowly cooled to room temperature to anneal the primer to the template. Primer-template concentration was varied between 0.1 and 1.0 μM. The TTP concentration was 2.5 mM, to which 0.1 μCi of α-^{32}P-labeled TTP was added in a total volume of 20 μL. The subunit concentration of reverse transcriptase ranged from 20 to 100 nM (i.e., 10–50 nM heterodimer). The reaction was started by addition of either enzyme (from a stock solution of 5.85 μM) or primer-template, and the reaction was incubated for 3 min at 37 °C. The mixture was then spotted near one edge of an 8 in. × 10 in. piece of Whatman 3MM filter paper prespotted with 20 μL of 200 mM EDTA to quench the reaction. After the paper was allowed to dry for 10 min, it was hung in a TLC tank containing 59% saturated ammonium sulfate solution. The solvent was allowed to migrate to the top of the chromatogram, separating the unincorporated TTP from the reverse transcription products. On such chromatograms, any nuclease present results in a smeared band rather than clean separation of the transcripts. The chromatogram was air-dried and exposed to Fuji AR film overnight, and the resulting autoradiogram was used to locate the incorporated nucleotide spots. The products were then cut out, suspended in 9 mL of Opti-fluor scintillation fluid (Packard), and counted 3–5 times for 1 min each in a Packard Tri-Carb liquid scintillation spectrometer (Packard). The averaged fraction of total counts incorporated was multiplied by the concentration of TTP present to give the amount of nucleotide incorporated.

Circular Dichroism. Spectra of RT were taken using an AVIV DS60 spectropolarimeter at 25 °C in a cell of 0.2-cm path length. The slit width was 1.5 nm, and signal averaging was set to 1 s/point with sampling every 0.5 nm. All proteins were at a concentration of 3 μM subunits (i.e., 1.5 μM homo- or heterodimer) in 2.5 mL of buffer B + 100 mM NaCl. Time course experiments were performed under the same conditions, except the spectra were taken at 37 °C.

RESULTS

Circular Dichroism of the 66-kDa Homodimer, the 66-kDa/51-kDa Heterodimer, and the Cloned 51-kDa Forms of Reverse Transcriptase. Circular dichroism spectra of the 66-kDa, the 66-kDa/51-Da heterodimer resulting from enzymatic cleavage by *E. coli* protease, and the cloned 51-kDa forms of reverse transcriptase at identical concentrations of 3 μM subunits are shown in Figure 2. The 66-kDa form of reverse transcriptase is the most optically active, with a negative molar ellipticity of $\sim$–6.0 × 10^6 deg cm^2 dmol^{-1} at both 212 and 220 nm. For a protein of 561 amino acids, a negative molar ellipticity of this magnitude is only moderate, representing a mean residue ellipticity of $\sim$–10.7 × 10^3 deg cm^2 dmol^{-1} (Figure 2) compared to $\sim$–35 × 10^3 deg cm^2 dmol^{-1} for a largely α-helical protein (Woody, 1968; Saxena & Wetlaufer, 1971). While the CD spectrum of the 66-kDa protein does show a minimum near 222 nm suggesting the presence of α-helix, the total percent α-helix must be less than 50%. A calculation by the early Greenfield and Fasman (1969) approximation suggests $\sim$17% α-helix and $\sim$83% β-sheet with very little coil structure. The application of more recent methods of estimating secondary structure based on composite CD spectra of real proteins (Yang et al., 1986) suggests 23% α-helix, 10% β-sheet, 30% β-turns, and 37% coil for the 66-kDa form of RT.

In striking contrast to the CD spectrum of the 66-kDa protein, the CD spectrum of the cloned 51-kDa protein shows a maximum negative molar rotation of only –1.7 × 10^6 deg cm^2 dmol^{-1} at 222 nm, corresponding to a mean residue ellipticity of $\sim$–4 × 10^3 deg cm^2 dmol^{-1}. Such a dramatic decrease in ellipticity upon the removal of the C-terminal RNase H domain, 120 amino acids (123 amino acids in our construct), raises the issue of whether the cloned 51-kDa polypeptide simply does not fold properly. On the other hand, the heterodimer produced in vivo by proteolysis in *E. coli* and purified as described under Materials and Methods also shows a dramatic decrease in negative ellipticity in the near-ultraviolet (Figure 2). Molar rotation of this heterodimer is $\sim$–3.2 × 10^6 deg cm^2 dmol^{-1} at 222 nm or a mean residue ellipticity of $\sim$–6 × 10^3 deg cm^2 dmol^{-1}.

Thus, the proteolysis and removal of the RNase H domain from half the subunits of RT to produce the heterodimer are accompanied by a dramatic decrease in negative ellipticity. In fact, the decrease in negative ellipticity accompanying proteolysis is even greater than would have been predicted by the CD spectrum calculated for a 50:50 ratio of 66-kDa monomer and 51-kDa monomer by summing the half-contributions expected from the CD spectra of the isolated 66- and 51-kDa forms (theoretical curve in Figure 2). Thus, at the time of formation of the heterodimer, there are further adjustments of the conformation in addition to the dramatic changes

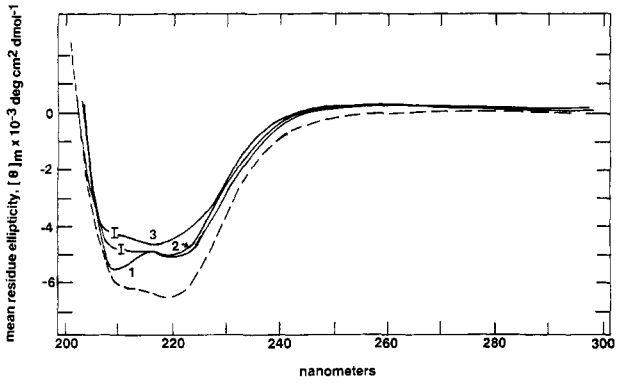

FIGURE 3: Changes in circular dichroism spectra during association of a 1:1 mixture of 66- and 51-kDa reverse transcriptase subunits. (1) Spectrum 10 min after mixing; (2) spectrum 20 min after mixing; (3) spectrum 2 h after mixing. All spectra were taken at 37 °C on a mixture of 1.5 μM 66-kDa and 1.5 μM 51-kDa subunits in 10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM BME, 1 mM EDTA, and 10% glycerol. The dashed line represents the theoretical curve expected from a 1:1 mixture of 66- and 51-kDa subunits at the moment of mixing. Error bars indicate the amount of noise in the CD signal.

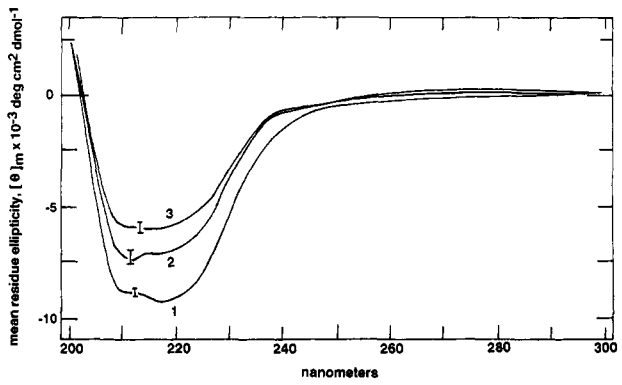

FIGURE 4: Changes in circular dichroism spectra produced by proteolysis of 66-kDa reverse transcriptase to heterodimer by trypsin. (1) 66-kDa protein before the addition of 20 μM trypsin; (2) 66-kDa protein after 14-min incubation; 20 μM trypsin was added to both the sample and the blank; the magnitude of the trypsin blank CD curve was ~10% that of the RT; (3) 66-kDa protein after 2-h incubation with trypsin. All spectra were taken at 37 °C with 10 μM protein, 10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM BME, 1 mM EDTA, and 10% glycerol. Error bars indicate the amount of noise in the CD signal.

associated with the creation of the 51-kDa monomer.

Changes in the Circular Dichroism Accompanying the Incubation of a 1:1 Mixture of 66-kDa and Cloned 51-kDa Forms of Reverse Transcriptase. The fact that conformational changes occur upon formation of the heterodimer in addition to those attributable solely to the conversion of one 66-kDa monomer to a 51-kDa monomer is supported by the observation of time-dependent changes in the CD spectrum of the protein subsequent to forming a 1:1 mixture of the 66- and 51-kDa forms of the protein. A time course for the CD spectrum of such a mixture is shown in Figure 3 at times of 10 min, 20 min, and 2 h after mixing. The control is the CD spectrum expected from a 1:1 mixture of the two forms of the enzyme.

Changes in the CD Spectrum of the 66-kDa Form of Reverse Transcriptase Induced by Proteolysis with Trypsin. While trypsin can potentially hydrolyze many sites within the reverse transcriptase polypeptide chain, brief treatment of the homogeneous 66-kDa form of the enzyme with trypsin results in the formation of a mixture of predominantly 66- and 51-kDa forms of the protein if digestion is not continued too long. Upon treatment of the homogeneous 66-kDa form of the protein with trypsin, the circular dichroism spectra show a very rapid decrease in negative ellipticity around 220 nm, and by 14 min, under the conditions employed for the experiment shown in Figure 4, the molar ellipticity has already decreased by ~25%. At 3 h, both the shape and magnitude of the CD spectrum have changed further toward those expected from the authentic heterodimer as pictured in Figure 2. We have not been able to take the trypsin-induced conversion to heterodimer to completion because extended incubation times result in proteolysis of multiple regions of the polypeptide.

Kinetic Analyses of the Activities of the 66-kDa Homodimer, the 66-kDa/51-kDA Heterodimer, the Cloned 51-kDa Enzyme, and 10:1 Mixtures of the 51- and 66-kDa Forms of Reverse Transcriptase. The circular dichroism studies presented above have established that there are substantial conformational differences between the several forms of reverse transcriptase. Thus, conversion of the 66-kDa homodimer to the heterodimer, the form which appears to be the relevant catalytic species in vivo, is associated with significant changes in conformation that appear to be greater than can be explained by the loss of a 123-residue C-terminal domain. Such a large apparent change in secondary structure might be expected to be associated with significant changes in the catalytic properties of the reverse transcriptase. In order to examine this possibility, we have carried out a Michaelis–Menten kinetic analysis of both the 66-kDa homodimer and the heterodimer forms of the enzyme as functions of variable primer-template and enzyme concentrations. The homogeneous cloned 51-kDa enzyme has very little activity, and complete kinetic analyses were not attempted. Data under specific conditions employing the cloned 51-kDa protein and mixtures of the cloned 51- and 66-kDa subunits will be presented below.

Since the concentrations of primer-template and protein employed in the standard assay conditions for reverse transcriptase in this as well as other studies in the literature result in undersaturation, i.e., failure to completely form the binary complex between enzyme and primer-template, an integrated form of the Michaelis–Menten equation must be used for the complete kinetic analysis. The treatment used in this study was one adapted from the method developed by Martin and Coleman (1981) for the similar situation applying to assays of T7 RNA polymerase activity. In this case, the overall steady-state kinetic mechanism assumed for the production of the RNA–DNA hybrid was

$$\text{enzyme} + \text{primer-template} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{enzyme–primer-template} + \text{NTPs} \xrightarrow{k_{cat}} \text{enzyme} + \text{extended DNA–RNA hybrid} \quad (1)$$

NTPs were at concentrations well above their estimated K_m's throughout, so k_{cat} can be considered a simple first-order rate constant. The exact steady-state solution of eq 1 is

$$V = 1/2k_{cat}\{D_T + E_T + K_m - [(D_T + E_T + K_m)^2 - 4E_TD_T]^{1/2}\} \quad (2)$$

The data were fit using a nonlinear least-squares minimization algorithm based on the Gauss–Newton method (Johnson & Frasier, 1985). The rate of TMP incorporation, nucleotides s^{-1} (V, the dependent variable), is fit to the independent variables total enzyme and primer-template concentrations (E_T and D_T, respectively) and to the fit parameters K_m and k_{cat}. The results of the analyses at three different concentrations of the homogeneous 66-kDa enzyme and four different concentrations of the heterodimer are shown in Figure 5. The vertical lines represent the overlapping error bars for one to three sets of multiple assays at each primer-template and enzyme concentration. The curves are those generated from

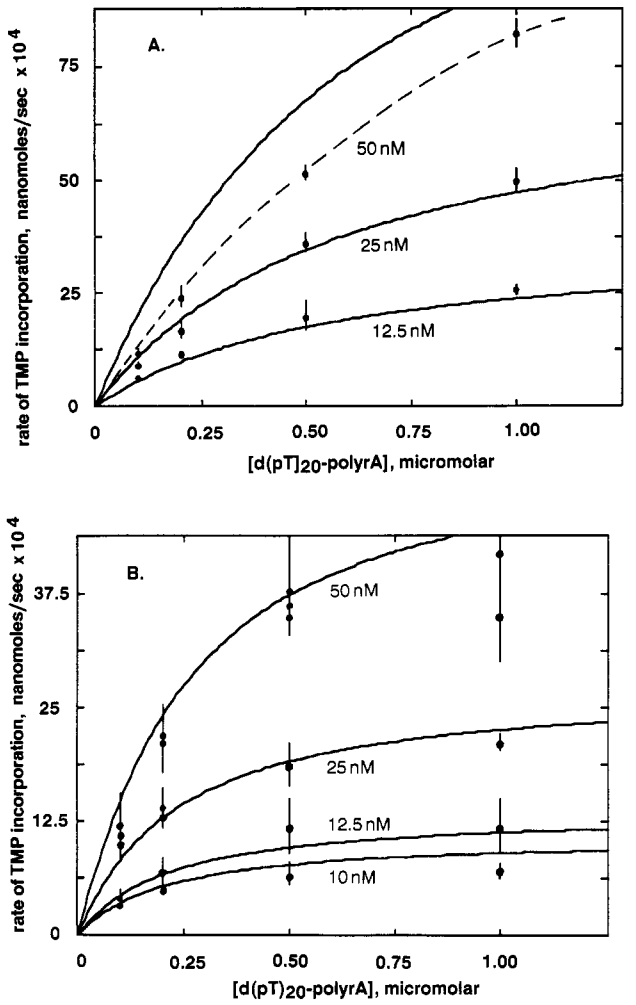

FIGURE 5: Rate of TMP incorporation vs primer-template concentration curves for (A) 66-kDa reverse transcriptase and (B) 66-kDa/51-kDa heterodimeric enzyme. The solid lines represent the best-fit simultaneous solution of the data at each enzyme concentration to the integrated Michaelis–Menten equation as determined by nonlinear least-squares minimization. Filled circles represent the actual average data points from observations in triplicate, and vertical error bars show the precision of the data at each point. The dashed line in (A) illustrates that the observed curve for 50 nM 66-kDa dimer (100 nM subunits) is below that predicted by the curve-fitting program applied to the two lower enzyme concentrations. All assays were performed in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM DTT, 6 mM $MgCl_2$, 0.2 unit/mL RNasin, 2.5 mM TTP, and 0.1 μCi of [α-^{32}P]-TTP for 3 min at 37 °C as detailed under Materials and Methods.

the pair of k_{cat} and K_m values that resulted in the best simultaneous fit of the data at all the enzyme concentrations (Table I). In the case of the 66-kDa enzyme, however, we consistently observed that the data for the lower enzyme concentrations were easily fit by one pair of k_{cat} and K_m values but these values consistently projected a higher than observed activity for the 66-kDa enzyme at the 100 nM concentration (Figure 5A). This nonlinearity, an apparent inhibition at high enzyme concentration, was not observed for the heterodimer (Figure 5B). We do not know the explanation for this phenomenon, but it is in the opposite direction to a possible activation that might be expected if there were an inactive monomer to active dimer formation over the protein concentration range employed for the kinetics (see Discussion).

The k_{cat} and K_m values in Table I show that the 66-kDa enzyme actually has a higher maximum velocity than the heterodimer, 2.94 vs. 1.22 TMP's incorporated s^{-1}. These numbers are expressed per dimer for both the 66-kDa enzyme and the heterodimer. The latter is known to contain one active site per dimer (LeGrice et al., 1991). If the active center on each 66-kDa subunit retains activity, then the k_{cat} values for the heterodimer and 66-kDa enzyme would be almost equivalent. The heterodimer shows more than a 4-fold decrease in K_m compared to pure 66-kDa; hence, at the concentration of enzyme and template usually employed for assay, any decrease in k_{cat} is more than offset by an increase in binding affinity so that the efficiency of the enzyme, k_{cat}/K_m, increases on conversion to the heterodimer. Thus, under the conditions of a typical assay, formation of the heterodimer would result in a 2–4-fold increase in specific activity. Examples of this activation from our own work using addition of the cloned 51-kDa subunit and trypsinolysis to form the heterodimer are shown in Table II.

Table I: Kinetic Constants of the 66K Homodimer and the 66K/51K Heterodimer Forms of Reverse Transcriptase from HIV

enzyme	k_{cat} (nucleotides s^{-1})	K_m (μM)	k_{cat}/K_m (nucleotides s^{-1} M^{-1} × 10^6)
66K homodimer[a]	2.94 (1.93–3.94)	0.54 (0.31–0.92)	5.4
66K/51K heterodimer[a]	1.22 (0.96–1.49)	0.12 (0.07–0.21)	10.2

[a] Conditions as described under Materials and Methods. The values of k_{cat} and K_m given are those derived from the best simultaneous fit of the families of activity vs primer-template curves given in Figure 5A,B. The numbers in parentheses are confidence intervals, approximately one standard deviation, and reflect the precision of the fit of all the experimental points, not necessarily the accuracy of the k_{cat} and K_m. The error bars in the velocity measurements which result in the precision range are shown in Figure 5. Because of the inhibition observed at 50 nM 66-kDa enzyme, this curve was not used for calculating kinetic constants.

Table II: Activation of the 66K Homodimer of Reverse Transcriptase on Formation of the Heterodimer

subunit composition	initial activity (nucleotides s^{-1})	activity at 2 h (nucleotides s^{-1})	activation factor (x-fold)
51K[a]	0.0003		
66K[a]	1.36		
10:1 (51K:66K)[b]	1.35	2.8	2.1
66K + trypsin[c]	0.68	1.0	1.4

[a] Present in the same concentrations in the assay mixture as used in the 10:1 (51K:66K) assay in row three. [b] 50 nM 66K enzyme was incubated with 500 nM 51K protein at 37 °C in 50 mM Tris-HCl, 6 mM $MgCl_2$, 10 mM DTT, 232 mM NaCl, pH 8, and 0.2 unit/L RNasin; aliquots were removed at various time points and assayed as described under Materials and Methods. [c] Conditions were as described in footnote *b* except that 20 μM trypsin was added rather than 500 nM 51K and the incubation temperature was 20 °C. The reaction mixture for the initial point contained trypsin inhibitor to stop the reaction. These conditions appear to lower the reaction velocity.

Activation of the homogeneous 66-kDa homodimer by addition of the cloned 51-kDa protein is not instantaneous, but requires significant incubation time to reach maximum activation. If excess 51-kDa protein is added, activation occurs more rapidly, and the example given in Table II contains a 10:1 ratio of 51-kDa/66-kDa subunits. Specific activity, expressed per 66-kDa subunit present, rises by 2.1-fold in 2 h for the experiment shown in Table II. The cloned 51-kDa protein has extremely low reverse transcriptase activity, ~0.0003 TMP incorporated s^{-1}, at the concentrations of 51-kDa protein present in the assay (Table II), and would by itself make a negligible contribution to the observed activity (row 1, Table II). The presence of heat-denatured BSA did not inhibit the 51-kDa subunit-mediated activation, and SDS–PAGE of the enzyme showed no change in intensity of the 66-kDa band, indicating that the 51-kDa protein was not contaminated by a protease. The increase in specific activity upon formation of heterodimer under our conditions is similar

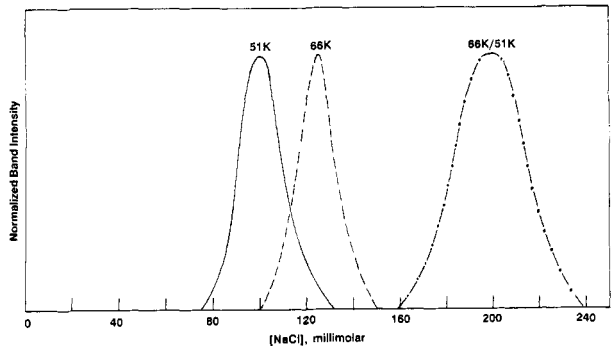

FIGURE 6: Salt dependence of reverse transcriptase binding to ssDNA columns. Column buffer is 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM BME, and 10% glycerol. The columns were eluted with a gradient of 0–250 mM NaCl, and fractions were assayed for RT by SDS–PAGE. Band intensities were normalized to the same scale and plotted as a function of NaCl concentration.

to those reported in the literature (Lowe et al., 1988; Müller et al., 1989; LeGrice & Grüninger-Leitch, 1990). We have also obtained activation by treating the 66-kDa homodimer with trypsin, and a typical experiment is shown in Table II. The degree of activation has not been as great as obtained by adding the homogeneous 51-kDa subunit, probably because of significant nonspecific proteolysis by trypsin.

Binding of the 51-kDa Protein, the 66-kDa Homodimer, and the 66-kDa/51-kDa Heterodimer to a ssDNA–Cellulose Column. The relative affinity for single-stranded nucleotides of the three forms of reverse transcriptase can be qualitatively analyzed by binding and elution of the homogeneous proteins from a ssDNA–cellulose column. All three forms of the RT protein bind to ssDNA–cellulose, and the elution profiles of the cloned 51-kDa subunit, the 66-kDa homodimer, and the 66-kDa/51-kDa heterodimer from separate DNA–cellulose columns are represented on one axis in Figure 6. While nonspecific, this experiment allows some qualitative conclusions. The cloned 51-kDa protein retains ssDNA binding affinity, eluting at 100 mM NaCl, rather similar to that of the 66-kDa form of the protein which elutes at 125 mM NaCl. In contrast, the heterodimer binds with substantially greater affinity to ssDNA, eluting at 200 mM NaCl (Figure 6). While we do not know how closely the K_m for the primer-template derived from the kinetic analyses presented above approaches the true dissociation constant, the differential binding of the 66-kDa homodimer and the heterodimer to ssDNA supports the postulate that one of the properties induced by heterodimer formation is a significantly greater affinity for the primer-template.

DISCUSSION

The reverse transcriptase from HIV as well as other retroviruses is a DNA polymerase which has the unusual properties of normally synthesizing DNA on RNA-primed RNA templates, on DNA-primed RNA templates, and on DNA-primed DNA templates. A C-terminal RNase H domain also allows the polymerase to processively remove the RNA from the RNA–DNA hybrid intermediate created by the first phases of the reverse transcription. While the coding sequence for the RT of HIV is included in a viral transcript directing the synthesis of a polyprotein, proteolysis of that polyprotein by the viral protease first produces an RT polypeptide of 66 kDa [see Goff (1990) for a review]. If the HIV protease cleavage site is used as the boundary between domains, the DNA polymerase domain can be considered to consist of the N-terminal 440 amino acids and the RNase H domain to consist of the C-terminal 120 residues (123 residues in our cloned enzyme). Although the isolated cloned 51-kDa protein, minus the RNase H domain, binds to DNA (Figure 6), it has orders of magnitude less DNA polymerase activity than the 66-kDa protein (Table II). Thus, the domain division is obviously somewhat artificial. As has been observed previously, the RNase H active site appears to be closely connected to the DNA polymerase active center and may be required for processivity of both catalytic functions (Furfine & Reardon, 1991).

While the 66-kDa enzyme can form a homodimer and is active in solution (Figure 5, Table II), compelling evidence demonstrates that the major species of RT in the virus is a heterodimer of 66- and 51-kDa subunits, the latter produced by an additional specific proteolytic cleavage which removes the 15-kDa C-terminal RNase H domain from one of the subunits (diMarzo Veronese et al., 1986; Lightfoote et al., 1986). Although several proteases are capable of cleaving the RT in the region of Gln^{428}–Phe^{440}, the amino acid sequence around the cleavage site suggests that the HIV-coded protease is the enzyme responsible for this cleavage in infected T-cells. Once formed, the heterodimer is relatively resistant to further proteolysis, even at the same peptide bond originally cleaved but located in the opposite 66-kDa monomer (Lowe et al., 1988).

The above phenomena suggest that there are likely to be conformational changes associated with the conversion of the 66-kDa homodimer into the heterodimer. In order to investigate this possibility, we carried out the CD studies shown here and correlated them with kinetic analyses of the different HIV reverse transcriptase species. While there is a dramatic change in the CD spectrum accompanying the conversion of the 66-kDa protein to the heterodimer (Figure 2), this is not associated with any remarkable change in the catalytic potential of the enzyme. In fact, the 66-kDa enzyme (assuming an equivalent, single-active-site dimer) has a significantly higher k_{cat} in the polymerase reaction than the heterodimer (Figure 5 and Table I). From the standpoint of catalytic potential, the real advantage of the heterodimer resides in the lower K_m and hence larger k_{cat}/K_m, a significant advantage at low concentrations of enzyme and primer-template, conditions which may apply in vivo. For the development of an initial integrated kinetic treatment, we have used the more readily available oligo(dT)–poly(rA) primer-template.

The conformational changes on formation of the heterodimer that cause such dramatic CD changes are not apparently associated with global rearrangements of the molecule, since such properties as DNA binding (Figure 6) and the catalytic activity are reasonably similar for both the 66-kDa enzyme and the heterodimer (Figures 5 and 6, Table II). If the various contributions to the ellipticity of the 66-kDa protein were considered to be relatively evenly distributed throughout the molecule, removal of a 15-kDa domain should decrease the optical activity of the remainder by roughly 23%. Instead, the minimum ellipticity of the 51-kDa subunit decreases by 74% compared to that of the 66-kDa subunit. The only way to explain such a decrease is by a decrease in the percentage of α-helix coupled with a large increase in the percentage of β-turn and/or coil, both of which can have significant positive ellipticity between 200 and 230 nm (Johnson, 1988, 1990). If the removal of the C-terminal 15-kDa polypeptide from one 66-kDa subunit results in a high ratio of β-turns and random-coil to α-helix, a spectrum similar to that of the heterodimer could result (Figure 2). It is possible that rather subtle changes in the precise conformations of extensive β-turns and coil structure could induce enough additional positive ellipticity in the 200–230-nm region to

account for what at first seems a very large difference in CD for a protein not undergoing major unfolding. The secondary structure of the 66-kDa protein only gives rise to moderate negative molar ellipticity in the near-ultraviolet; hence, less drastic alterations in structure can potentially produce a relatively large percentage change in ellipticity. The fact that our monomeric 51-kDa subunit was cloned and that the heterodimer was formed in vivo by the actions of *E. coli* proteases might lead to the suspicion that the cloned 51-kDa subunit does not fold properly, but the fact that the cloned protein retains nucleic acid binding and modest catalytic activities indicates that the important regions of the enzyme are still intact (Figure 6, Table II).

The magnitude of the ellipticity minimum for the heterodimeric enzyme is half that of the pure 66-kDa subunit. As the 51-kDa subunits retain some optical activity (Figure 2), a total negative ellipticity of greater than 50% would have been expected from the heterodimer. Thus, there must be additional changes of secondary structure in one or both of the subunits on formation of the heterodimer. There could be indirect conformational changes induced in the remaining 66-kDa subunit by the changes in the 51-kDa monomer. The minimum ellipticity is roughly the same for the heterodimer produced by proteolysis as it is for the heterodimer reconstituted from cloned subunits, so the final configuration of the subunits must, to a first approximation, be independent of whether the 51-kDa protein is produced in close proximity by the viral protease or added later. If there were no difference in the conformation of the heterodimer formed by these two pathways, the equilibrium constants for heterodimer association given by Restle et al. (1990) and Becerra et al. (1991) should have been similar, barring major differences in their cloned proteins. The cloned 51-kDa monomer employed by Becerra et al. (1991) has a slightly different C-terminus from that used by Restle et al. (1990), so it is possible the two heterodimers are not strictly comparable.

While it may be obvious that a major structural rearrangement is required for formation of the stable heterodimer, the underlying functional reason for the heterodimeric structure of the HIV RT is not. The RT from the murine leukemia virus is a monomer of ~80K, containing the polymerase and RNase H functions (Eisenman & Vogt, 1978; Kopchick et al., 1978; Hu et al., 1986; Tanese et al., 1986). In the avian retroviruses, the mature RT is an $\alpha\beta$ heterodimer in which the smaller α-subunit is a cleavage product resulting from the removal of the integrase domain which the β-subunit retains. Both the α- and β-subunits contain the RNase H subdomain [see Goff (1990) for a review]. Therefore, removal of a sterically unfavorable RNaseH domain is not required for all reverse transcriptases, and even formation of a dimer is not always necessary. It has been noted that the presence of the 51-kDa subunit protects the 66-kDa subunit to which it is bound from proteolytic processing (Lowe et al., 1988) but this is an insufficient explanation for the heterodimer, as mutation of a single residue in the protease cleavage region could accomplish the same result. Therefore, the heterodimeric structure of HIV RT must be optimal for some function, and the 51-kDa subunit must play an important role in that function.

Although it has been reported that the catalytic aspartate residues can be mutated in the 51-kDa subunit and the heterodimer retains full activity (LeGrice et al., 1991), our data point to a significant role for the 51-kDa subunit in binding and catalysis. At enzyme and template concentrations employed in vitro, the fraction of active enzyme or degree of saturation of the primer-template may be quite different than that present in vivo. We have therefore used a nonlinear curve-fitting program to fit data to the integrated form of the Michaelis–Menten equation in order to compare the catalytic potential of the 66-kDa enzyme and the heterodimer on the basis of their fundamental kinetic constants (Table I). Under these conditions, our kinetic constants are close to those extrapolated from more limited data (Majumdar et al., 1988; Huber et al., 1989; Reardon et al., 1990). While the 66-kDa protein, which is reported to be in equilibrium between dimer and monomer, has a higher k_{cat} than the heterodimer, the lowering of the K_m by a factor of 4 for the heterodimer, which results in a 2-fold increase in enzymatic efficiency at low primer-template and enzyme concentrations, shows that there is a catalytic advantage to the heterodimeric form of reverse transcriptase (Table I).

Since the isolated 51-kDa subunit is essentially devoid of RT activity, its role in the function of the heterodimer would not seem to be directly catalytic. In fact, complete kinetic analysis by the integrated Michaelis–Menten treatment presented here supports that conclusion, since a heterodimer containing a 51-kDa subunit has a k_{cat} approximately half that of the 66-kDa enzyme (Figure 5). A straightforward but not exclusive interpretation of the comparative kinetics of the 66-kDa and heterodimer forms of the HIV RT is that the heterodimer has lost half its active sites by inactivation of the 51-kDa monomer. This would assume either that a loosely-associated dimer of 66-kDa subunits leaves both polymerizing sites active or that the monomer itself is active. The alternate explanation for the relative activities of the 66-kDa and heterodimer forms of the enzyme, namely, that the heterodimer decreases the catalytic potential of the active centers (assuming the same number of active centers in both forms of the enzyme), seems less likely.

There has been conflicting evidence presented in the literature as to whether the 66-kDa monomer is active. Separation of monomers and dimers of the 66-kDa protein (overexpressed in *E. coli*) by HPLC size-exclusion chromatography has been reported (Müller et al., 1989; Restle et al., 1990). The monomer fraction was found to have ~2% the specific activity of the dimer fraction (Restle et al., 1990). On the other hand, it has been reported by Deibel et al. (1990) that while a 66-kDa monomer fraction isolated at dilute concentration (0.5 mg/mL or $\sim 10^{-5}$ M) was relatively inactive, a 66-kDa monomer isolated by denaturation of the heterodimer followed by refolding retained full activity. The K_d for the 66-kDa monomer–dimer equilibrium derived from the HPLC analysis has been reported as 4.3×10^{-6} M (Restle et al., 1990) and by equilibrium centrifugation as 2×10^{-6} M (Becerra et al., 1991). We have no direct evidence for the quaternary state of the 66-kDa protein at the concentrations used for the kinetics reported here (20–100 nM, expressed as subunit concentration), but if the K_d values given above were applied, most of the enzyme would be present as monomer.

Dissociation of the 66-kDa dimer has been reported to be slow (Restle et al., 1990). However, the stock solution of enzyme from which our assay dilutions were made was 5.85 μM, which would already have been 30–40% monomer on the basis of the above values of K_d. The kinetics and high activity of the 66-kDa protein do not show any evidence of an inactive monomer–active dimer equilibrium; however, a further variable could be the presence of the primer-template which could shift the equilibrium in favor of a more tightly-bound dimer. Gel retardation studies show that the 66-kDa, the cloned 51-kDa, and the 66-kDa 51 kDa heterodimer retain the primer-template hybrid by forming single complexes all of which migrate at the same speed in the gel (S. Anderson and J.

Coleman, unpublished data). Since the 66-kDa/51-kDa enzyme is almost certainly a dimer in the bound form, it is likely that the bound form of the other two enzymes is also the dimer. Gel retardation confirms that the 66- and 51-kDa forms of the enzyme bind more weakly to the primer-template. The inhibition of the transcription rate observed at high concentrations of the 66-kDa enzyme and not seen for the heterodimer could represent formation of a type of dimer that occludes one of the active centers.

The only advantage of the heterodimer that our analysis detects is a distinctly higher affinity for the primer-template (Figure 6, Table I). It thus seems possible that the 66-kDa/51-kDa subunit interaction produces a structural alteration in the nucleic acid binding surface that increases the favorable contacts with the primer-template. While the enhancement of potential catalytic function that this change induces does not appear dramatic, it could be a distinct advantage, depending on the concentrations of tRNA-primed viral genome and RT that apply in vivo. It is also possible that the affinity of the heterodimer for the natural primer, i.e., $tRNA^{Lys}$–PBS primer-template, is more enhanced than is the case for the $d(pT)_{20}$–poly(rA) primer-template. Some evidence indicates that RT enhances the annealing of $tRNA^{Lys}$ to the PBS RNA sequence (Barat et al., 1989; Anderson, unpublished data). The nucleocapsid protein may form a complex with RT which enhances the enzyme activity as suggested by some early studies (Sykora & Moelling, 1981), and also observed under some conditions when we add our cloned nucleocapsid protein (Fitzgerald & Coleman, 1991) to the RT reaction mix (Anderson and Fitzgerald, unpublished results). It is possible the two latter functions could be more dramatically dependent on creation of the heterodimer and require further investigation.

ACKNOWLEDGMENT

We are greatly indebted to Samuel H. Coleman for the construction of the pSCRT plasmid and to Gerald Koji Sonoda for the determination of the elution profiles for the three forms of reverse transcriptase from the single-stranded DNA–cellulose columns.

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