Kinetics of Interaction of HIV Reverse Transcriptase with Primer/Template†

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ABSTRACT: Intrinsic protein fluorescence of reverse transcriptases from HIV-1 and HIV-2 provides a sensitive signal for monitoring the interaction of the enzymes with primer/template duplex molecules. K_d values for 18/36-mer DNA/DNA duplexes were found to be in the range of a few nanomolar (about 3 times higher for the enzyme from HIV-2 than for that from HIV-1). The quenching of protein fluorescence induced on binding primer/template, together with an increase in extrinsic fluorescence on interaction with primer/template containing a fluorescent nucleotide at the 3′-end of the primer, was used to investigate the kinetics of interaction with reverse transcriptase from HIV-1. The results can be explained in terms of a two-step binding model, with a rapid diffusion-limited initial association (k_{ass} = ca. 5×10^8 M^{-1} s^{-1}) followed by a slow isomerization step (k = ca. 0.5 s^{-1}). These (forward) rate constants are increased in the presence of a non-nucleoside inhibitor (S-TIBO) of HIV-1 reverse transcriptase, while the reverse rate constant for the second step is decreased, leading to an increase in affinity between the enzyme and primer/template by a factor of at least 10 when S-TIBO is bound. The results are discussed in terms of present knowledge of the structure of reverse transcriptase.

The availability of large amounts of reverse transcriptase from HIV-1[1] (e.g., Tanese et al., 1986; Larder et al., 1987a; Hizi et al., 1988; Müller et al., 1989) and HIV-2 (LeGrice et al., 1988; Müller et al., 1991a) allows a number of approaches to the investigation of the structure and mechanism of this enzyme. The structure of the heterodimeric form of the HIV-1 enzyme has recently been solved to a resolution of 3.5 Å (Kohlstaedt et al., 1992), and a number of studies have been directed toward aspects of the mechanism of interaction with its substrates (e.g., Majumdar et al., 1988; Huber et al., 1989; Müller et al., 1991b; Painter et al., 1991; Basu et al., 1992; Kati et al., 1992; Reardon, 1992) and with its natural primer, the human $tRNA^{Lys-3}$ (e.g., Barat et al., 1991; Kohlstaedt & Steitz, 1992; Sarih-Cottin et al., 1992; Weiss et al., 1992; Restle et al., 1993). A knowledge of the kinetic parameters for the interaction of the protein with nucleic acids is important for understanding both the detailed chemical mechanism of polymerization and the relatively complex overall mechanism of reverse transcription (Pelsika & Benkovic, 1992). We have previously used fluorescence-labeled nucleotides and oligonucleotides to investigate kinetic aspects of the interaction of RT with primer/template and nucleotides.

Here we show that intrinsic protein fluorescence can also be used as a signal for the interaction of RT with primer/template and extend the studies into the rapid kinetic domain using both protein and nucleotide analog fluorescence as signals. We show that the detailed kinetics of interaction of RT with primer/template are modified by the presence of non-nucleoside inhibitors.

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[1] Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV-1 and HIV-2, human immunodeficiency virus type 1 and type 2; RT, reverse transcriptase; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one; S-TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepine-2(1*H*)-thione; nevirapine (BIRG), 11-cyclopropyl-5,11-dihydro-4-methyl-6*H*-dipyrido[3,2-*b*:2′,3′-*e*]diazepin-6-one; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Proteins. Recombinant HIV reverse transcriptases were expressed in *Escherichia coli* and purified as described before (Müller et al., 1989, 1991a). Highly homogeneous preparations of the heterodimeric forms of the enzyme resulting from coexpression of the 66- and 51-kD subunits were used. Enzyme concentrations were routinely determined according to Bradford (1976) using a gravimetrically prepared solution of RT as standard.

The HIV-1 RT double mutant (Y181I, Y188L) was created by site-directed mutagenesis using the polymerase chain reaction. PCR was carried out according to standard protocols (Saiki et al., 1985). The resulting PCR fragment was cut with two restriction enzymes (*Eco*RV and *Xho*II) and recloned into plasmid pRT_{66} (Müller et al., 1989), giving pRT_{66}Y181I/Y188L. DNA sequence analysis confirmed that the desired mutations were present. For preparation of the mutant heterodimer, induced cells harboring this plasmid were mixed with cells harboring pRT_{51} (Müller et al., 1989) before lysis and protein purification. The resulting heterodimer thus had the double mutation only in the 66 kD subunit.

Nucleotides. Succinylfluorescein-labeled ddTTP (SFd-dTTP) was from Du Pont-New England Nuclear. (γ-^{32}P)ATP was obtained from Amersham.

Non-Nucleoside Inhibitor. S-TIBO was kindly provided by Dr. A. Mertens, Boehringer Mannheim.

Oligodeoxynucleotides. Oligodeoxynucleotides were synthesized on an Applied Biosystems 380 B DNA synthesizer and purified by standard procedures. Primer and template oligodeoxynucleotides were annealed by heating a mixture of both in 20 mM Tris-HCl pH 7.5 for 15 min at 70 °C, followed by cooling to room temperature over a period of ca. 2 h in a water bath.

Preparation of Fluorescence-Labeled Primer/Template. 36-mer and 18-mer oligodeoxynucleotides (for sequences see Figure 1) were labeled with a fluorescent thymidine derivative at the 3′-end of the primer as described (Müller et al., 1991b). The concentration of the resulting labeled 19/36-mer was

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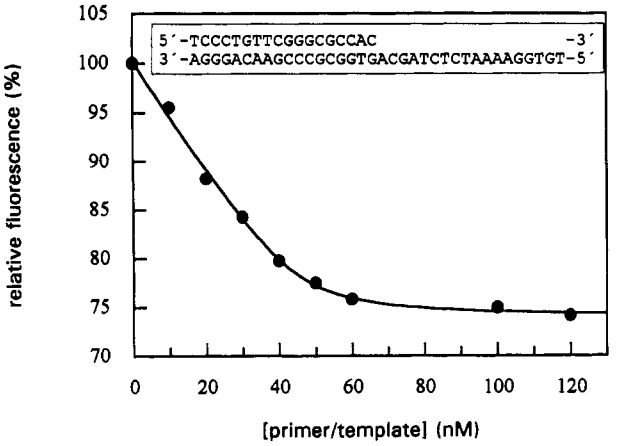

FIGURE 1: Titration of HIV-1 RT (45 nM) with an 18/36-mer primer/template (shown in the inset) using tryptophan fluorescence as a signal of binding (for conditions, see Materials and Methods). The curve shows the best fit of the data to the quadratic equation describing the binding of the primer/template to a single site in the heterodimeric form of the enzyme. The fit gives values of 1.8 nM for the K_d and 26% as the maximal quenching of fluorescence induced by binding.

determined by degradation with exonuclease III, followed by comparison of the fluorescence spectrum with that of the free labeled nucleoside triphosphate at known concentrations.

Fluorescence Titrations. Fluorescence titrations were performed using a SLM Smart 8000 spectrofluorometer (Colora, Lorch) equipped with a PH-PC 9635 photomultiplier. The buffer contained 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, and 1 mM dithiothreitol in a total volume of 0.5 mL. For tryptophan fluorescence, the samples were excited at 295 nm and the emission intensity was measured at 338 nm (slit widths 2 and 8 nm, respectively). All data were corrected for the background intensity of the buffer, for dilution, and for the inner filter effect (slight at the concentrations used). Measurements were made in a 0.5-cm-pathlength cuvette to minimize the filter effect, and the residual effect was estimated in control experiments using *N*-acetyltryptophanamide and increasing the oligonucleotide concentration up to 500 nM. Data were transferred to a personal computer and evaluated using the commercially available fitting program Grafit (Erithacus Software), which allows the user to define his own equations. A quadratic equation analogous to the one given by Müller et al. (1991b) describing the binding equilibrium was used for the fitting procedure. Values for the dissociation constant (K_d), the amplitude of the fluorescence change, and the enzyme concentration were allowed to vary during the fitting procedure. For experiments with fluorescent primer/template, excitation was at 500 nm and the emission intensity was measured at 532 nm (slit widths set at 2 and 8 nm for excitation and emission, respectively). All experiments were done at a temperature of 25 °C.

Rapid Kinetics. Experiments on the kinetics of association of RT with primer/template were performed using a stopped-flow apparatus (High Tech Scientific, Salisbury, England). Excitation of tryptophan fluorescence was at 295 nm using a Hg–Xe high-pressure arc lamp (100 W), and detection was through a filter with a cutoff at 320 nm. Excitation of fluorescein-labeled oligonucleotide fluorescence was at 500 nm, and detection was through a filter with a cutoff at 530 nm. Data were collected using an analog digital converter in an IBM PC-compatible computer and analyzed online using the software package provided by High Tech Scientific and offline using Grafit. The latter allowed fitting to single or multiple exponentials as well as to second-order kinetic equations.

RESULTS AND DISCUSSION

Fluorescence Titrations To Estimate the Affinity of Reverse Transcriptase for Primer/Template Complexes. The reverse transcriptases from HIV-1 and HIV-2 show typical tryptophan fluorescence characteristics, with an excitation maximum at 282 nm and an emission maximum at 338 nm. For titrations with oligonucleotides, which exhibit significant absorption at 282 nm, an excitation wavelength of 295 nm was used to minimize the inner filter effect.

The presence of a large number of tryptophans in HIV RTs (for HIV-1, 19 in the 66-kD subunit of the heterodimer and 18 in the 51-kD subunit) offers a potential signal for investigating the interaction of the enzymes with substrate and other ligand molecules, but the very number of these residues leads to the expectation that changes in the tryptophan fluorescence associated with individual events in the enzymatic mechanism should not be large. It was therefore somewhat surprising to find that a substantial quenching of tryptophan fluorescence occurs on interaction of a DNA/DNA primer/template molecule with RT from HIV-1 (Figure 1). This change in fluorescence (ca. 26% quenching) can be used to determine the affinity of primer/template complexes for the enzyme, and from the data shown in Figure 1, a value of 1.8 nM is obtained for the dissociation constant (K_d) for the 18/36-mer shown in the figure. A value of 0.6 nM was found for the same primer/template complex using a different method in which competition with a fluorescence-labeled primer/template molecule was used as an assay of binding (Müller et al., 1991b). The method used earlier was less direct, since it involved a competitive titration, so we regard the value obtained in the present work as more reliable.

The change in intrinsic fluorescence related to the binding of primer/template complexes to RT is of use not only for determining the affinity of the duplexes for the enzyme but also for determining the stoichiometry of binding and thus the fraction of RT active in binding to primer/template. This was found to vary somewhat from preparation to preparation, with the sample used for Figure 1 showing the highest binding activity (stoichiometry of 1.07:1 primer/template:heterodimer). The reason for the variation in the amount of "active" RT in different preparations is not clear, since all preparations appeared to be homogeneous as judged by SDS gel electrophoresis, and there was no obvious correlation with the specific activity of the enzyme as measured in a standard reverse transcriptase assay using poly(rA)·$(dT)_{15}$ as template and dTTP as nucleotide substrate.

Figure 2 shows titrations with a primer/template complex in which the primer is labeled with a fluorescent dideoxynucleotide at its 3′-end. In Figure 2A, the primer/template is added incrementally to a constant concentration of RT, and the decrease in tryptophan fluorescence is used as a signal for the interaction. This leads to a K_d value of 3.8 nM, suggesting that, as previously reported (Müller et al., 1991b), the presence of the modified nucleotide at the 3′-end of the primer leads to a moderate reduction of affinity for RT. The amplitude of fluorescence quenching is larger (35%) than that seen with unlabeled oligonucleotides. This is probably due to an interaction of the fluorescein group with the protein but not to radiationless fluorescence energy transfer, since a discrete emission peak arising from such an effect is not seen. In Figure 2B, RT was added incrementally to a constant concentration of primer/template, and the increase in nucleotide fluorescence was used to monitor complex formation. Evaluation of these data leads to a value of 2.4 nM for K_d, showing that both types of signal monitor the same process

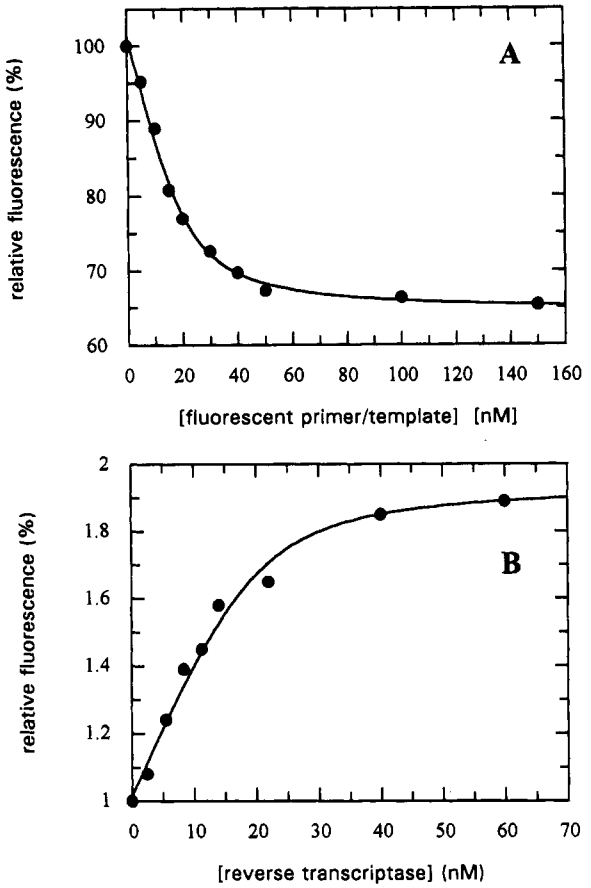

FIGURE 2: Titrations of HIV-1 RT (20 nM) with a fluoresent primer/template (19/36-mer corresponding to the primer/template shown in Figure 1 with an additional fluorescent 3′-deoxythymidine residue at the 3′-end of the primer [see Müller et al. (1991b)]). In Figure 2A, quenching of the intrinsic (tryptophan) fluorescence of the protein is used as the signal as the fluorescent primer/template concentration is increased. The best fit to the data was obtained with a K_d of 3.8 nM and an amplitude of the fluorescence change of 35%. In Figure 2B, RT is titrated against a constant concentration of fluorescent primer/template (20 nM) using the increase in fluorescence of the nucleoside derivative as a signal of binding (for conditions, see Materials and Methods). The best fit was obtained for a K_d value of 2.4 nM and a maximal fluorescent enhancement of 90%.

(on the equilibrium time scale) and that there is reasonable agreement between the results.

Similar titrations with poly(rA)·$(dT)_{15}$ led to a 33% quenching of tryptophan fluorescence and a K_d value of ca. 7 nM (Figure 3). This is similar to the K_d value of 11 nM obtained by Painter et al. (1991). It is not yet clear whether this lower affinity and higher amplitude of the fluorescence change when compared to the 18/36-mer primer/template used for Figure 1 is the result of using an RNA/DNA rather than a DNA/DNA duplex or whether it arises from the different nature of this primer/template (a polymeric template with, presumably, a statistical distribution of primer molecules along its length). It is also highly likely that there is some sequence dependence of both parameters, but this has not yet been tested for experimentally. However, the observation of increasing fluorescence quenching with increasing length of primer/template used indicated that more than 18 bases were implied in the RT primer/template interaction, consistent with modeling studies with the recently published RT structure (Kohlstaedt et al., 1992) and the estimation of the distance between the polymerase and RNase H sites (Kati et al., 1992; Gopalakrishnan et al., 1992). We have used a length of 18 nucleotides for the primer molecule to allow a comparison with earlier work using this length. However, it is clear that the manner of binding may depend on the length of the primer

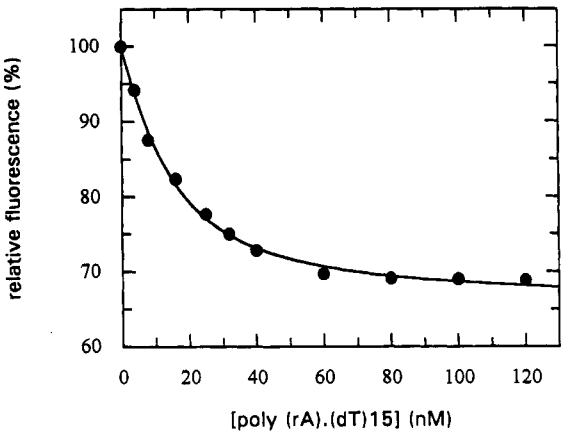

FIGURE 3: Titration of HIV-1 RT (20 nM) with poly(rA)·$(dT)^{15}$. The concentration of the primer/template refers to the concentration of primer molecules. The curve shows the fit for a K_d of 7 nM and an amplitude of 33% for the fluorescence quenching.

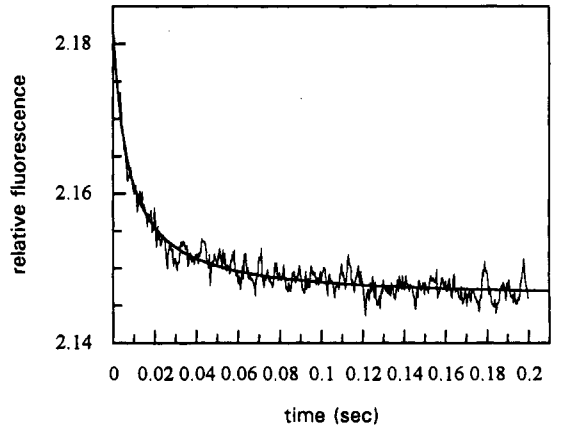

FIGURE 4: Kinetics of binding of primer/template (250 nM) to HIV-1 RT (250 nM). The two components were mixed in a stopped-flow appartus, and the protein fluorescence was monitored as a signal of binding. The data were analyzed as a single-step second-order reaction and gave a rate constant of 5×10^8 M^{-1} s^{-1} for the association rate constant. The fluorescence signal was stable after the initial rapid transient.

molecule, especially since the distance between the polymerase and RNase H active sites is thought to correspond to ca. 18 base pairs. We are presently examining the effects of composition (DNA/DNA, DNA/RNA, tRNA/RNA), length, degree of overhang, and type of overhang of the primer/template duplexes on their interaction with reverse transcriptase.

Kinetics of Interaction of Reverse Transcriptase with Primer/Template. The changes in both intrinsic protein fluorescence and nucleoside analog fluorescence described here can be used for transient kinetic experiments on the interaction of primer/template with RT. As shown in Figure 4, the quenching of tryptophan fluorescence occurs in a single step when the two components are mixed in a stopped-flow apparatus. The rate of this step is concentration dependent, and the results obtained can be fitted using a second-order rate constant of $3–5 \times 10^8$ M^{-1} s^{-1} for concentrations up to 450 nM of both reagents. If this rate constant is taken together with the rate constant for dissociation of a complex between RT and primer/template that we reported previously (4×10^{-2} s^{-1}; Müller et al., 1991b) to calculate the K_d value of the complex, a value of 10^{-10} M for the expected K_d is obtained. This is a factor of 10–20 lower than that measured by direct fluorescence titration (Figure 1), suggesting that a simple one-step binding mechanism is not sufficient to describe this interaction. This is confirmed by the following experiments.

When RT was mixed with the fluorescent primer/template complex in the stopped-flow apparatus, the increase in labeled primer/template fluorescence showed a time dependence which deviated markedly from a single exponential (Figure 5). The data could be well fitted to an equation with two exponential terms. Of the two phases, only the first (Figure 5B) was concentration dependent when the RT concentration was varied from 25 to 250 nM. The quantitative dependence of the rate constant can be explained by a second-order rate constant of ca. 3×10^8 M^{-1} s^{-1}, which suggests that it reflects the same process as that monitored by the tryptophan fluorescence. The second phase (see Figure 5A) was concentration independent in the range examined. The relative amplitudes of the two phases were also independent of concentration and was ca. 1:1.25 (fast:slow).

The kinetic results obtained can be explained on the basis of a two-step binding model for the interaction of RT with primer/template, as shown in Scheme I.

Scheme I

$$\mathrm{RT + P/T} \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} \mathrm{RT{\cdot}P/T} \underset{k_{-2}}{\overset{k_{+2}}{\leftrightarrow}} \mathrm{RT^*{\cdot}P/T}$$

The value of the second-order rate constant of 5×10^8 M^{-1} s^{-1} (from both types of experiment described above) is assigned to k_{+1} and that of the slow first-order rate constant (0.5 s^{-1}) seen with the fluorescent primer/template to k_{+2}. The slow rate of dissociation of primer/template (4×10^{-2} s^{-1}; Müller et al., 1991b) from its complex with RT cannot be assigned to k_{-1}, since this would mean that the value for the effective binding constant between the two species (given by $K_a = 1/K_d = K_1[1 + K_2]$) would be at least as large as K_1, which would be $5 \times 10^8/4 \times 10^{-2}$, i.e., ca. 10^{10} M^{-1}. Since this is much higher than the measured affinity, we assign the value of 4×10^{-2} M^{-1} s^{-1} to k_{-2}. We were not able to estimate k_{-1}, since it was not possible to measure the pseudo-first-order rate constant for the first step in the mechanism at concentrations that were low enough to allow an extrapolation to zero concentration. The value calculated for k_{-1} using the values obtained for the remaining rate constants in Scheme I and the measured affinity between RT and primer/template is ca. 5 s^{-1}. This would be consistent with the results obtained, since a value of the pseudo-first-order rate constant for the first phase as low as ca. 12 s^{-1} was obtained at the lowest usable concentration of RT in excess of fluorescent primer/template, placing an upper limit on the value of k_{-1}.

A feature of the model presented is that changes in both the tryptophan and the nucleotide analog fluorescence occur in the first step of the mechanism, while the second step leads to a change in the nucleotide but not in the tryptophan fluorescence. We have to consider whether the slow fluorescence transient seen with the fluorescent primer/template reflects a property of RT·primer/template complexes or whether it is an artifact due to the presence of the fluorescent group. While we cannot exclude the latter with certainty, we have already seen that the second-order rate constant measured using tryptophan fluorescence together with the slow rate of release of the primer/template would lead to a K_d value that is much lower (i.e., the affinity is much higher) than that seen from direct titrations if the binding were a simple one-step process. We therefore consider it justified to use the two-step model as the basis for explaining the results seen.

Interaction of HIV-2 and HIV-1 (Y181I, Y188L) Reverse Transcriptase with Primer/Template. Certain key experiments have been duplicated using HIV-2 RT, which shows fluorescence spectral characteristics similar to those of the

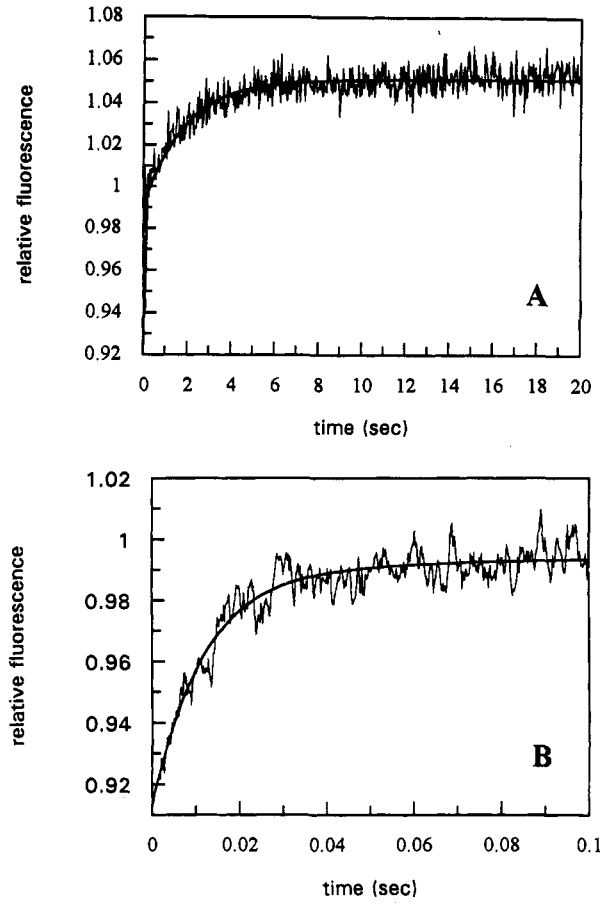

FIGURE 5: Kinetics of binding of fluorescent primer/template (25 nM; see caption to Figure 2) to HIV-1 RT (250 nM). The fluorescence of the 3'-terminal nucleoside analog was used as a signal of binding. In Figure 5A, the complete time course of the transient is shown. In Figure 5B, the first 100 ms of the curve shown in Figure 5A are expanded. The time course could be fit using a sum of two exponential terms with rate constants of 83.6 and 0.5 s^{-1} with relative amplitudes of 1:1.25.

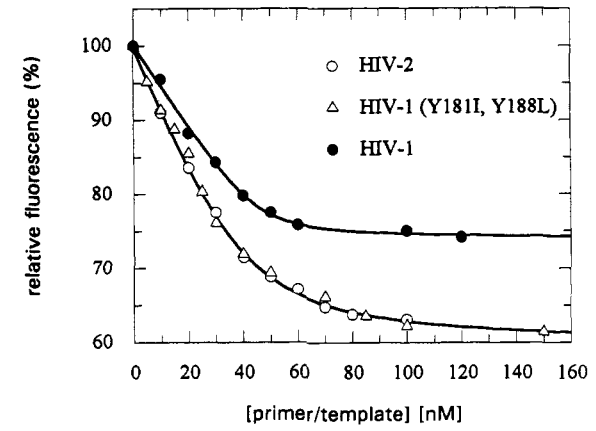

FIGURE 6: Titrations of primer/template against HIV-1, HIV-2, and an HIV-1 double mutant (Y181I, Y188L) RT using intrinsic protein fluorescence as a signal of binding. Protein concentrations were 45, 42.5, and 40 nM, respectively. The fitted K_d values are 1.8, 5.4, and 5.7 nM, respectively.

HIV-1 enzyme. Figure 6 shows a titration of the enzyme with primer/template. Two features are apparent from analysis of such experiments. Firstly, the affinity is lower than that of HIV-1 RT (K_d = 5.7 nM, cf. 1.8 nM from Figure 1). Secondly, the amplitude of the fluorescence change (40% quenching) is significantly higher than that for the HIV-1 enzyme (26%). We have also used a double mutant of HIV-1 RT (Shih et al., 1991), in which the tyrosine residues at positions 181 and 188 of the sequence are replaced by the

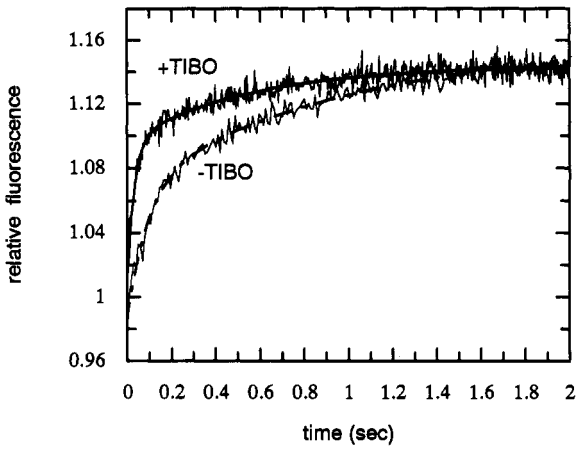

FIGURE 7: Kinetics of binding of fluorescent primer/template (16 nM) to HIV-1 RT (50 nM) in the absence and presence of S-TIBO (0.5 μM) using nucleoside analog fluorescence as a signal of binding. The curves were fitted as the sum of two exponentials, essentially approximating the first step as pseudo first order. Rate constants were 10 and 1.0 s^{-1}, repectively, for the two phases in the absence of S-TIBO (lower curve) and 31.9 and 1.9 s^{-1} in the presence of S-TIBO (upper curve). The relative amplitudes were 1:1.1 (fast: slow) in the absence of TIBO and 1:0.55 in its presence. The change in ratio was caused by a reduction in amplitude of the second phase by a factor of ca. 2.

residues which occur in the HIV-2 enzyme (isoleucine and leucine, respectively). This results in a protein that also binds primer/template with reduced affinity and with a larger fluorescence change than seen for the original HIV-1 sequence. The parameters are remarkably similar to those of HIV-2 RT. Titrations for all three species (i.e., HIV-1 RT, the HIV-2 version, and the HIV-1 double mutant) are shown in the same graph in Figure 6 to allow a direct comparison.

Effect of Non-Nucleoside Inhibitors of HIV-1 Reverse Transcriptase on the Interaction with Primer/Template. Fluorescent primer/template has also been used to examine the effect of non-nucleoside inhibitors of RT activity on the interaction with primer/template [for review, see Grob et al. (1992) and De Clerq (1992)]. In preliminary titration experiments, we could show that one of these substances (TIBO; Pauwels et al., 1990) did not compete with binding of primer/template. There was, in fact, some indication of stronger binding in the presence than in the absence of TIBO. For this reason, we examined the effect of the inhibitor on the kinetics of binding and dissociation of primer/template. Figure 7 shows the effect on the binding transient. As in the absence of TIBO, there is a biphasic approach to equilibrium. However, there were significant and reproducible differences under the two sets of conditions, as shown in Figure 7. The first phase was of similar amplitude but a factor of ca. 3 faster at 0.5 μM S-TIBO. The second phase was also faster but of smaller amplitude than in the absence of TIBO. This agrees with our observation of a slight quenching of fluorescence of the complex between fluorescent primer/template and RT on addition of TIBO. The rate constants for the two phases withthe particular preparation of RT used were 6.4×10^8 M^{-1} s^{-1} for k_{+1} in the presence of TIBO (2.2×10^8 M^{-1} s^{-1} in its absence) and 1.9 s^{-1} for k_{+2} in the presence of TIBO (1 s^{-1} in its absence). In addition, the rate of dissociation (measured as previously described by displacement of the fluorescent primer/template by poly(rA)·$(dT)_{15}$; Müller et al., 1991b) was reduced from 0.044 to 0.025 s^{-1} by the presence of TIBO. Thus, unless k_{-1} is increased by TIBO (which seems very unlikely in view of the effect on the forward rate constant), primer/template binds more strongly in the presence of the inhibitor. The exact magnitude of this effect cannot be determined using the presently available data, but an increase by a factor of ca. 3 for k_{+1} and changes by factors of ca. 2 for k_{+2} (increase) and k_{-1} (decrease) suggest that the affinity is at least 1 order of magnitude higher.

While the results obtained in the present work are not sufficient to define the mechanism of inhibition of RT by non-nucleoside analogs such as TIBO and nevirapine (or BI-RG-587; Merluzzi et al., 1990), they do help to explain some of the results seen in steady-state experiments concerning the inhibition by such substances. It has been reported that the inhibition pattern by TIBO and nevirapine with respect to primer/template is of the uncompetitive type in steady-state assays. This type of inhibition can arise if the affinity of substrate to the enzyme–inhibitor complex is higher than to the enzyme alone, but substrate cannot be processed in this complex. The evidence presented here suggests that there is indeed an increase in affinity between RT and primer/template when one of the non-nucleoside inhibitors is bound. The additional assumption we have to make is that reaction with the second substrate, a deoxynucleoside triphosphate, cannot occur in this complex. Whether this is due to lack of binding of the second substrate or to an effect on the catalytic step is not apparent from these results, but several reports suggest that the inhibition with respect to nucleoside triphosphate is noncompetitive (Frank et al., 1991; Goldman et al., 1991; Wu et al., 1991; Debyser et al., 1992; Dueweke et al., 1992). In conflict with this, our own results (Müller et al., unpublished results) suggest that there is competitive inhibition between binding of nucleotide and TIBO to the RT–primer/template complex, although it is not clear whether this effect is direct, i.e., whether their binding sites are completely or partially overlapping, or whether they bind to different sites which have a mutual effect on each other. We note that the site of binding of the BIRG inhibitors has been localized in the region of Tyr-181 and Tyr-188 by photoaffinity labeling (Cohen et al., 1991) and that these bracket Asp-185 and Asp-186, two highly conserved residues in polymerases which are considered to be directly involved in the enzymatic mechanism and which must therefore, according to these considerations, be near to the active site (Johnson et al., 1986; Larder et al., 1987b; Argos, 1988). The binding site for nevirapine has been located in the 3-D structure of RT from HIV-1 and is seen to be indeed in the region of Tyr-181 and Tyr-188 and neccessarily near to the conserved aspartate residues (Kohlstaedt et al., 1992). An influence of non-nucleoside inhibitors on the manner in which both primer/template and nucleotide substrate interact with the enzyme is thus understandable in general terms.

As reported in a previous section, replacement of Tyr-181 and Tyr-188 in HIV-1 RT by the HIV-2 residues isoleucine and leucine leads to a change in behavior of the enzyme toward primer/template to that which is typical of HIV-2 RT. The same mutations also lead to a dramatic loss in affinity of the enzyme for non-nucleoside inhibitors (Shih et al., 1991; Sardana et al., 1992; and our own results), in keeping with the observation that HIV-2 RT is not sensitive to non-nucleoside inhibitors of the TIBO or BIRG types. Thus, there seems to be an interplay between the detailed structure of the protein in the 180–190 region of the sequence and the interaction with primer/template. With the presently available information, a detailed interpretation of these effects cannot yet be made. This should be remedied as soon as the high-resolution structure of a complex between reverse transcriptase and a primer/template becomes available.

CONCLUSION

We conclude that the fluorescence techniques reported here can be used as a powerful tool to monitor the interaction of

RT with primer/template molecules. The relatively large change in intrinsic fluorescence is surprising in view of the large number of tryptophans in the enzymes (37 for HIV-1, 34 for HIV-2), suggesting that a large fraction of these are involved, directly or indirectly via conformational changes, with the interaction of the protein with the nucleic acid duplex. Reliable structural data on the interaction with primer/template are not yet available for HIV RT. In the recent contribution of Kohlstaedt et al. (1992), the possible mode of binding was investigated by computer docking studies, leading to a plausible binding mode, but experimental data will be needed to confirm and add substance to this model. In particular, it is possible that binding of primer/template induces relatively large structural changes in the protein, so the structure obtained in the absence of primer/template may not be a good starting point for docking studies. Against this argument, the fact that the structure was obtained with a non-nucleoside inhibitor (BIRG) bound to the enzyme could mean that a form of the enzyme which is correctly configured for binding to primer/template was used, since the work reported here can be interpreted to indicate that the form of RT induced by binding of inhibitor is similar to the primer/template binding form, since the affinity of the latter is increased by the presence of the inhibitor.

Application of the techniques described here, together with more detailed structural information, should lead to progress in understanding how RT interacts with its substrates and with inhibitors and eventually to such therapeutically important and related issues as the emergence of resistance to different classes of inhibitor and the low accuracy of RT as a DNA polymerase.

NOTE ADDED IN PROOF

The forthcoming 3-Å-resolution structure of HIV reverse transcriptase complexed with an 18/19-mer primer [E. Arnold, personal communication; Jacobo-Molina, A., et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* (in press)] confirms the previously suggested conformational change on binding of primer/template, which was based on the earlier determination of the structure of the protein both with and without bound DNA/DNA duplex at lower spatial resolution [Arnold, E., Jacobo-Molina, A., Nanni, R. G., Williams, R. L., Lu, X., Ding, J., Clarke, A. D., Jr., Zhang, A., Ferris, A. L., Clark, P., Hizi, A., & Hughes, S. H. (1991) *Nature 357*, 85–89]. It has now been interpreted as a movement of the p66 "thumb" domain (E. Arnold, personal communication).

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REFERENCES

Argos, P. (1988) *Nucleic Acids Res. 16*, 9909–9916.
Barat, C., Lullien, V., Schatz, O., Keith, G., Nugeyre, M. T., Gruninger-Leitch, F., Barre-Sinoussi, F., LeGrice, S. F., & Darlix, J. L. (1989) *EMBO J. 8*, 3279–3285.
Barat, C., Legrice, S. F. J., & Darlix, J. L. (1991) *Nucleic Acids Res. 19*, 751–757.
Basu, A., Ahluwalia, K. K., Basu, S., & Modak, M. J. (1992) *Biochemistry 31*, 616–623.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Cohen, K. A., Hopkins, J., Ingraham, R. H., Pargellis, C., Wu, J. C., Palladino, D. E. H., Kinkade, P., Warren, T. C., Rogers, S., Adams, J., Farina, P. R., & Grob, P. M. (1991) *J. Biol. Chem. 266*, 14670–14674.
Debyser, Z., Vandamme, A. M., Pauwels, R., Baba, M., Desmyter, J., & Declercq, E. (1992) *J. Biol. Chem. 267*, 11769–11776.
Dueweke, T. J., Kezdy, F. J., Waszak, G. A., Deibel, M. R., & Tarpley, W. G. (1992) *J. Biol. Chem. 267*, 27–30.
Frank, K. B., Noll, G. J., Connell, E. V., & Sim, I. S. (1991) *J. Biol. Chem. 266*, 14232–14236.
Goldman, M. E., Nunberg, J. H., O'Brien, J. A., Quintero, J. C., Schleif, W. A., Freund, K. F., Gaul, S. L., Saari, W. S., Wai, J. S., Hoffman, J. M., Anderson, P. S., Hupe, D. J., Emini, E. A., & Stern, A. M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6863–6867.
Gopalakrishnan, V., Peliska, J. A., & Benkovic, S. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10763–10767.
Grob, P. M., Wu, J. C., Cohen, K. A., Ingraham, R. H., Shih, C. K., Hargrave, K. D., Mctague, T. L., & Merluzzi, V. J. (1992) *AIDS Res. Hum. Retroviruses 8*, 145–152.
Hizi, A., McGill, C., & Hughes, S. H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1218–1222.
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.
Kati, W. M., Johnson, K. A., Jerva, L. F., & Anderson, K. S. (1992) *J. Biol. Chem. 267*, 25988–25997.
Kohlstaedt, L. A. & Steitz, T. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9652–9656.
Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., & Steitz, T. A. (1992) *Science 256*, 1783–1790.
Larder, B., Purifoy, D., Powell, K., & Darby, G. (1987a) *EMBO J. 6*, 3133–3137.
Larder, B. A., Purifoy, D. J., Powell, K. L., & Darby, G. (1987b) *Nature 327*, 716–717.
LeGrice, S. F. J., Zehnle, R., & Mous, J. (1988) *J. Virol. 62*, 2525–2529.
Majumdar, C., Abbotts, J., Broder, S., & Wilson, S. H. (1988) *J. Biol. Chem. 263*, 15657–15665.
Merluzzi, V. J., Hargrave, K. D., Labadia, M., Grozinger, K., Skoog, M., Wu, J. C., Shih, C. K., Eckner, K., Hattox, S., Adams, J., Rosehthal, A. S., Faanes, R., Eckner, R. J., Koup, R. A., & Sullivan, J. L. (1990) *Science 250*, 1411–1413.
Müller, B., Restle, T., Weiss, S., Gautel, M., Sczakiel, G., & Goody, R. S. (1989) *J. Biol. Chem. 264*, 13975–13978.
Müller, B., Restle, T., Kühnel, H., & Goody, R.S. (1991a) *J. Biol. Chem. 266*, 14709–14713.
Müller, B., Restle, T., Reinstein, J., & Goody, R. S. (1991b) *Biochemistry 30*, 3709–3715.
Painter, G. R., Wright, L. L., Hopkins, S., & Furman, P. A. (1991) *J. Biol. Chem. 266*, 19362–19368.
Pauwels, R., Andries, K., Desmyter, J., Schols, D., Kukla, M. J., Breslin, H. J., Raeymaeckers, A., VanGelder, J., Woestenborghs, R., Heykants, J., et al. (1990) *Nature 343*, 470–474.
Peliska, J. A., & Benkovic, S. J. (1992) *Science 258*, 1112–1118.
Reardon, J. E. (1992) *Biochemistry 31*, 4473–4479.
Restle, T., et al. (1993) *Biochemistry* (submitted for publication).
Saiki, R. K., Scharf, S. J., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., & Arnheim, N. (1985) *Science 230*, 1350–1354.
Sardana, V. V., Emini, E. A., Gotlib, L., Graham, D. J., Lineberger, D. W., Long, W. J., Schlabach, A. J., Wolfgang, J. A., & Condra, J. H. (1992) *J. Biol. Chem. 267*, 17526–17530.
Sarih-Cottin, L., Bordier, B., Musierforsyth, K., Andreola, M. L., Barr, P. J., & Litvak, S. (1992) *J. Mol. Biol. 226*, 1–6.
Shih, C. K., Rose, J. M., Hansen, G. L., Wu, J. C., Bacolla, A., & Griffin, J. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9878–9882.
Tanese, N., Sodroski, J., Haseltine, W. A., & Goff, S. P. (1986) *J. Virol. 59*, 743–745.
Weiss, S., Konig, B., Muller, H. J., Seidel, H., & Goody, R. S. (1992) *Gene 111*, 183–197.
Wu, J. C., Warren, T. C., Adams, J., Proudfoot, J., Skiles, J., Raghavan, P., Perry, C., Potocki, I., Farina, P. R., & Grob, P. M. (1991) *Biochemistry 30*, 2022–2026.