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Interaction of Fluorescently Labeled Dideoxynucleotides with HIV-1 Reverse Transcriptase†

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ABSTRACT: Succinylfluorescein-labeled dideoxyTTP has been used as a substrate for reverse transcriptase from HIV-1. On addition to the 3'-end of a primer molecule, there is a reduction of fluorescence yield of a factor of ca. 4. Release of a fluorescent DNA/DNA primer/template duplex from its complex with reverse transcriptase results in a reduction of fluorescence by a further factor of 2. The fluorescent nucleotide is incorporated somewhat less efficiently than 3'-azidoTMP and TMP, which show similar incorporation kinetics. Fluorescent chain-terminated primers have been used to investigate the interaction of normal and chain-terminated primer/template complexes with reverse transcriptase. The dissociation constant of a 36/18-mer was 0.65 nM, whereas that of the same complex after the addition of the fluorescent chain-terminating nucleotide to the primer was 3 nM at 25 °C. The rate of dissociation of the latter complex from the enzyme was 0.04 s^{-1}. This was decreased by a factor of ca. 10 at high concentrations (>200 μM) of the nucleotide triphosphate complementary to the next position of the template. The results obtained suggest that potent inhibition of reverse transcriptase activity in in vitro assays results from formation of a slowly dissociating complex between the enzyme and chain-terminated primer/template complexes. However, arguments are presented that lead to the conclusion that this is not the mode of inhibition in cells invaded by HIV. At the prevailing relative concentrations in this situation, chain termination resulting in incomplete transcription is likely to be the major factor.

A detailed knowledge of the structure of an enzyme and its catalytic mechanism is the basis for any approach to a rational design of potent and specific inhibitors. Crucial information can be provided by investigations of the dynamics and thermodynamics of the interactions of substrates with the protein. The availability of pure HIV-1 reverse transcriptase from bacterial expression systems (Tanese et al,. 1986; Larder et al., 1987; Hizi et al., 1988; Mous et al., 1988; Müller et al., 1989) has made this enzyme amenable to detailed biochemical and biophysical characterization. In the last few years, several studies examining the steady-state kinetics of the enzyme have been published (Cheng et al., 1987; Majumdar et al., 1988, 1989; Huber et al., 1989). While this method has the advantage that only catalytic amounts of enzyme are needed, it has the disadvantage that conclusions drawn concerning the mechanism are often indirect and that the rate and equilibrium constants of discrete steps in the mechanism cannot be determined. This limitation does not apply to studies using "substrate concentrations" of enzyme, which are now available.

Fluorescent substrate analogues are useful tools for investigation of enzyme–substrate interactions. Their use for kinetic studies often allows a more direct monitoring of certain steps in the catalytic process (e.g., binding of the substrate to the enzyme) than the more conventional biochemical approaches.

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Even fast processes are accessible to such "direct" observation by the use of rapid reaction techniques. In the work presented here, we show that a fluorescent chain-terminating nucleotide can be added to the 3′-end of a DNA primer in a template-directed manner in the presence of HIV-1 reverse transcriptase. The change in the fluorescent yield associated with this incorporation can be used as a signal for the interaction of both nucleoside triphosphates and primer/template duplexes with the enzyme.

Materials and Methods

Proteins. Recombinant HIV-1 reverse transcriptase (RT) was expressed in *Escherichia coli* and purified as described before (Müller et al., 1989). In the studies presented here, we used highly homogeneous preparations of the heterodimeric form of the enzyme resulting from coexpression of the 66- and 51-kDa subunits. RT concentration was routinely determined according to Bradford (1976) by use of a gravimetrically prepared solution of RT as standard. The specific activity using poly(rA)·$(dT)_{15}$ as primer/template was ca. 10000 units/mg. One unit catalyzes the incorporation of 1 nmol of TMP in 10 min at 37 °C.

T4 polynucleotide kinase was from New England Biolabs. Exonuclease III from *Escherichia coli* and bovine serum albumin (nuclease free) were purchased from Boehringer, Mannheim.

Nucleotides. Succinylfluorescein-labeled ddTTP (SFddTTTP) was from DuPont–New England Nuclear. [γ-^{32}P]ATP was obtained from Amersham; 3′-azidothymidine triphosphate (AZTTP) was kindly provided by D. Wahl. All other nucleotides were from Boehringer, Mannheim.

Oligodeoxynucleotides. Ologideoxynucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. Purification was done by HPLC reversed-phase chromatography on a Hypersil C18 column. After a first purification step using a gradient from 15 to 50% acetonitrile in 50 mM TEAA, dimethoxytrityl protecting groups were removed by treatment with 80% acetic acid. The deprotected oligodeoxynucleotides were further purified by use of the same column with a gradient from 7 to 14% acetonitrile.

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.

Fluorescence Measurements. Fluorescence measurements were done by use of an SLM Smart 8000 spectrofluorometer (Colora, Lorch) equipped with a PH-PC 9635 photomultiplier.

Reactions were performed in a buffer containing 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. The samples were excited at 500 nm, and emission intensity was measured at 532 nm (slit widths set at 2 nm). All experiments were done at 25 °C. Data were transferred to a personal computer and evaluated by use of the commercially available programs Enzfitter (Elsevier Biosoft) or Grafit (Erithacus Software Ltd.), which allow the user to enter his own equation for the fit procedure. The equation used to determine the dissociation constant between reverse transcriptase and fluorescent primer/template (as in Figure 4) was

$$F = F_{min} - \{(F_{max} - F_{min})[(E_o + P_T + K_1) - ((E_o + P_T + K_1)^2 - 4E_oP_T)^{1/2}]\}/2E_o$$

where F is the relative fluorescent intensity, F_{min} is the fluorescent intensity at the start of the titration, F_{max} is the fluorescent intensity at saturating concentrations of primer/template (P_T), E_o is the total concentration of reverse transcriptase, K_1 is the dissociation constant of the reverse transcriptase–primer/template complex. The value quoted for the dissociation constant was that obtained when F_{min}, F_{max}, and K_1 were allowed to vary to obtain the best fit.

In experiments in which fluorescent primer/template was displaced from its complex with reverse transcriptase by titration with unlabeled primer/template, experimental conditions were chosen that led to a simplification of the quantitative analysis of the data, since in the general case the solution of the set of equations describing the equilibrium for each species is the unwieldy solution of a cubic equation. The concentration of fluorescent primer/template complex was therefore chosen to be equal to that of reverse transcriptase, and the absolute concentrations were much higher than the dissociation constant obtained from the data shown in Figure 4. Thus, the titration starts essentially with a 1:1 complex between the two species, with negligible free concentrations in solution. Under these conditions, the expression describing the curve is reduced to the solution of a quadratic equation, which can be entered into one of the two programs mentioned above. Explicitly, the solution is

$$F = F_{max} - \{(F_{max} - F_{min})[(K_rE_o + K_rP_{T2}) - ((K_rE_o + K_rP_{T2})^2 - 4(K_r - 1)K_rE_oP_{T2})^{1/2}]\}/E_o(K_r - 1)$$

where F_{max} is now the relative fluorescence intensity at the beginning of the titration, F_{min} is the intensity at saturating concentrations of unlabeled primer/template (P_{T2}), and K_r is the relative affinity of the unlabeled and labeled primer/templates (i.e., $K_r = K_1/K_2$, where K_1 is the dissociation constant of labeled and K_2 is the dissociation constant of unlabeled primer/template). The values of K_r, F_{max}, and F_{min} were allowed to vary to obtain the best fit to the data.

Preparation of Fluorescence-Labeled Primer/Template. The 36-mer and 18-mer oligodeoxynucleotides were annealed in equimolar amounts (see above). Up to 2 nmol of the duplex was incubated in a reaction containing 4 nmol of SFddTTP, 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 20 μg/mL bovine serum albumin, and 150 pmol of purified HIV-1 reverse transcriptase. After 45 min at room temperature, the reaction mixture was extracted once with phenol, once with phenol–chloroform–isoamyl alcohol (30:29:1), and three times with water-saturated diethyl ether. Following gel filtration through Sephadex G-25, the material was concentrated by lyophilization in a speed-vac concentrator. Incorporation of the fluorescent nucleotide was checked by labeling an aliquot of the reaction product with ^{32}P by use of T4 polynucleotide kinase and [γ-^{32}P]ATP, according to standard procedures (Maniatis et al., 1982), and separating the radioactively labeled oligodeoxynucleotides on a denaturing polyacrylamide gel (15% acrylamide, 7 M urea). The concentration of the final product can be determined by completely digesting an aliquot with exonuclease III, which results in the reestablishment of the fluorescence properties of the free nucleotide. The concentration can then be calculated by comparison of the fluorescence intensity of such a preparation with a calibration curve prepared with the use of SFddTTP.

Prolonged incubation of the reaction mixtures for the labeling reaction at 37 °C resulted in, besides the elongation of the primer, partial addition of a fluorescent nucleotide to the template. This side reaction represents presumably a "blunt-end addition" that has been described for other DNA polymerases (Clark, 1988) and can be circumvented by adding SFddTMP to the free primer with terminal transferase as

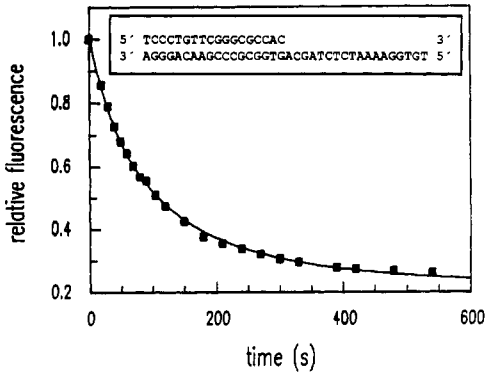

FIGURE 1: Incorporation of SFddTTP into a synthetic primer/template complex. Experimental conditions are described under Materials and Methods. The experiment shown here was done with final concentrations of 320 nM primer/template and 62.5 nM SFddTTP. The reaction was started by addition of a saturating amount of enzyme (2 μM). The oligodeoxynucleotide duplex used as primer/template is shown in the inset. The curve shows the best fit to a second-order rate equation.

described by Trainor and Jensen (1988) if only small amounts of fluorescent primer are needed.

Results and Discussion

Incorporation of SFddTTP into a Synthetic Primer/Template. It has been reported that succinylfluorescein-labeled dideoxythymidine triphosphate (SFddTTP) can serve as a substrate for the reverse transcriptase from avian myeloblastosis virus with an efficiency comparable to that of unmodified ddTTP (Prober et al., 1987). Using the synthetic oligodeoxynucleotide primer/template hybrid whose structure is shown in the inset of Figure 1, we investigated whether SFddTTP is also accepted as a substrate by the reverse transcriptase (RT) of HIV-1. Primer/template was 5′-labeled with ^{32}P according to standard procedures and incubated with SFddTTP and RT (see Materials and Methods for details). Analysis of the reaction product by denaturing gel electrophoresis revealed a characteristic shift in electrophoretic mobility of the primer oligodeoxynucleotide due to the addition of SFddTMP (data not shown).

As shown in Figure 1, a relatively slow change in fluorescence intensity occurs when SFddTTP is incubated with HIV-1 reverse transcriptase and the synthetic oligodeoxynucleotide primer/template duplex under polymerization conditions. The fluorescence intensity measured at 532 nm decreases to about 25% of the original value. Addition of exonuclease III at this stage and further incubation at 25 °C leads to the restoration of the original fluorescence intensity (data not shown), indicating that the change upon incubation with RT is caused by addition of SFddTMP to the primer.

It should be noted that the 4-fold decrease in fluorescence intensity seen on incorporating the fluorescent nucleotide into the primer molecule arises in a situation in which the fluorescent product is still bound to reverse transcriptase. As shown in experiments described below (Figures 4 and 5), there is a further decrease of a factor of 2 in fluorescence intensity on dissociation of the fluorescent primer/template from the enzyme. Thus, addition of SFddTMP to the 3′-end of the primer molecule in the primer/template complex free in solution here results in a reduction by a factor of 8 of the fluorescence yield. It has not yet been determined to what extent this is dependent on the exact nature of the primer/template or indeed on the state of hybridization.

A quantitative analysis of the time and concentration dependencies of the incorporation of the fluorescence chain

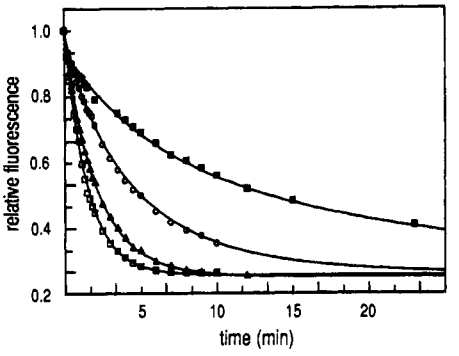

FIGURE 2: Incorporation of SFddTTP using different concentrations of primer/template. According to the conditions described under Materials and Methods with 62.5 nM SFddTTP and 1.1 μM RT as fixed concentrations, the primer/template concentration was varied (■, 80 nM; ○, 160 nM; ▲, 320 nM; □, 600 nM). Data were fitted to a second-order rate equation as in Figure 1.

terminator can be used to obtain the apparent second-order rate constant for the enzymatic process. The curve in Figure 3 shows a computer fit to the data by use of the rate equation for a second-order reaction. If we assume the following minimal mechanism for the incorporation

$$\text{RT-T/P} + \text{SFddNTP} \xrightarrow{K_a} \text{RT-T/P·SFddNTP} \xrightarrow{k_p} \text{RT-T/P-SFddNMP}$$

and furthermore assume the association reaction to be in rapid equilibrium, the apparent second-order rate constant for the incorporation is given by

$$k_{inc} = K_a k_p$$

if the concentration of fluorescent nucleotide is much less than $1/K_a$.

The fit shown in Figure 1 gives a value of 2.5×10^4 M^{-1} s^{-1} for k_{inc} for SFddTTP. This value can be compared with the k_{cat}/K_m value for the polymerization reaction, which can be calculated to be in the region of 5×10^4 M^{-1} s^{-1} for TTP by use of the specific activity of the heterodimer preparation used in these studies together with the published value for K_m (Cheng et al., 1987). Thus, the fluorescent dideoxynucleotide appears to be recognized and incorporated less well than a natural deoxynucleotide, but the sum of the two modifications (sugar and base) is not dramatic. It should be noted that the two constants that have been compared are not formally identical, since that calculated for the fluorescent analogue incorporates the true affinity of the nucleotide to the enzyme, whereas that calculated for TTP is from steady-state measurements and thus incorporates a K_m value. Independent evidence that the kinetics of incorporation are similar for TTP and the fluorescent analogue is mentioned below.

In Figure 2, the dependence of the rate of incorporation of fluorescent nucleotide on the concentration of reverse transcriptase–primer/template complex is shown (at constant fluorescent nucleotide concentration). The computer fits give values in the range of $(2–2.5) \times 10^4$ M^{-1} s^{-1} with no obvious tendency to saturate at up to 600 nM, suggesting that the dissociation constant of the nucleoside analogue triphosphate is at least 1 μM.

Figure 3 shows that in the presence of AZTTP the magnitude of the change in fluorescence is reduced, while the rate of the transient is increased. This effect can also be observed in the presence of the normal substrate, TTP. It is base specific and arises from competition between the two substrates. The competitive effect of AZTTP can be assessed quantitatively, as shown by the solid lines in Figure 3. The curves fitted to the data points lead to a value of k_{inc} for AZTTP of 1.2×10^5

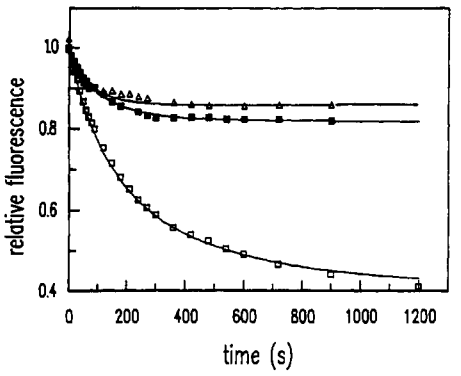

FIGURE 3: Competition of AZTTP with SFddTTP. Incorporation of SFddTTP was followed as described above (see Figures 1 and 2) except that different concentrations of AZTTP were present in the reaction mixtures. Concentrations used in this experiment were 100 nM primer/template, 150 nM SFddTTP, and 1 μM RT and 0 (□), 100 (■) or 150 (△) nM AZTTP. The curves show the best fits obtained by use of a numerical integration procedure (Runge–Kutta) combined with a least-squares regression.

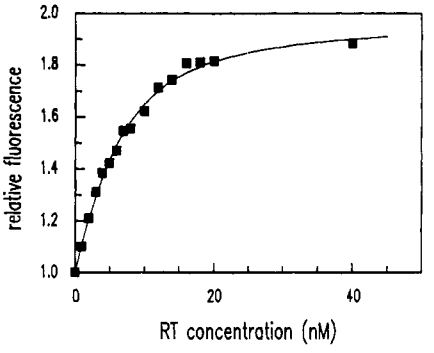

FIGURE 4: Titration of fluorescence-tagged primer/template with RT. Fluorescence-labeled primer/template (7 nM) was prepared as described under Materials and Methods and incubated with RT in a total volume of 0.5 mL of standard buffer at 25 °C. Each addition of enzyme was followed by a 1-min equilibration before the fluorescence signal was measured. The signal was seen to be stable after this period of time. The curve shows a fit obtained by nonlinear regression to the quadratic equation describing the binding equilibrium.

M^{-1} s^{-1}, which is slightly higher than that for the fluorescent chain terminator. TTP shows a quantitatively similar effect (data not shown). Thus, natural and chain-terminating nucleotides appear to be bound and incorporated with similar kinetics.

Interaction of RT with Template/Primer. As for other DNA polymerases, an ordered mechanism has been proposed for HIV-1 RT, with binding of primer/template to the enzyme preceding the binding of the first dNTP (Majumdar et al., 1989). A primer/template duplex labeled at the 3′-end of the primer with SFddTMP (referred to as 36/F19) can be prepared as described under Materials and Methods. It can then be used to examine its interaction with RT. Figure 4 shows the result of titrating RT to a solution of the fluorescence-tagged primer/template duplex. There is an increase of approximately 100% in the fluorescence intensity upon binding of RT. The dissociation constant obtained from the computer fit to the data is 3.2 nM. Titrations at lower concentrations, which should in principle lead to better determined parameters, were difficult because of noise on the very low fluorescence signals. However, a large number of experiments at different concentrations led to reproducible values of the dissociation constant, with an average value of 3 nM for different batches of both enzyme and primer/template. Similar dissociation constants have been found for complexes between the Klenow fragment of *E. coli* DNA polymerase I and small synthetic

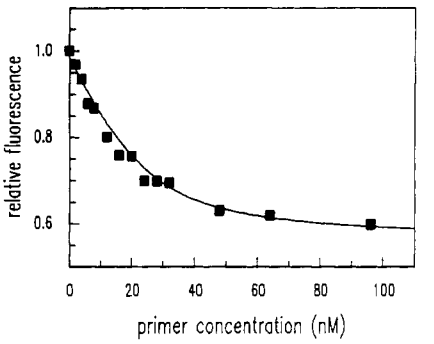

FIGURE 5: Displacement of 36/F19 from RT by unlabeled 36/18-mer. A preformed complex between RT and 36/F19 (each at a concentration of 30 nM) was titrated with the unlabeled form of the same primer/template. Each addition of 36/18-mer was followed by an equilibration period of 5 min before fluorescence intensity was measured. The curve shows the best fit to the quadratic equation describing the displacement under the assumption that essentially all of the fluorescent primer/template complex was bound to reverse transcriptase at the start of the titration.

primer/templates (5 nM; Kuchta et al., 1987) and between HIV-RT and a poly(rA)·oligo(dT) template (3 nM; Huber et al., 1989).

The fluorescent primer/template hybrid can also be used to determine the affinity of nonfluorescent hybrids. An example is shown in Figure 5. Here, the preformed complex between RT and the fluorescent primer/template is titrated with the unlabeled form (36/18-mer) of the identical oligodeoxynucleotide duplex. A quantitative analysis of the displacement leads to a value of ca. 0.65 nM for the dissociation constant of the nonlabeled duplex. Thus, the combined modifications in the sugar and base moieties lead to a ca. 5-fold reduction in the affinity of the primer/template duplex for RT. The dissociation constant for the commonly used primer/template hybrid poly(rA)·$(dT)_{15}$ was also determined in a similar fashion and found to have a value of 0.35 nM. Thus, there seems to be no major difference in the affinities of RNA/DNA and DNA/DNA hybrids for reverse transcriptase, although duplexes of identical sequences have not yet been compared.

The kinetics of interaction of primer/template with reverse transcriptase can also be studied by use of the fluorescence-tagged primers. The rate of dissociation can be determined as shown in Figure 6. Here, poly(rA)·$(dT)_{15}$ was used to displace a fluorescent primer/template from its complex with reverse transcriptase. Dissociation occurred at a rate of 0.04 s^{-1} and can be taken as representative of dissociation of the enzyme from the terminated 3′-end of DNA that has a substantial 5′-overhang in the template strand. The rate constant for association of 36/F19 with reverse transcriptase can be calculated to be ca. 10^7 M^{-1} s^{-1} from the dissociation rate and equilibrium constants.

Influence of Nucleotides on the RT–Primer/Template Complex. It has been reported earlier that a stable dead-end complex can be formed between mammalian DNA polymerase α, a terminated primer annealed to a template, and the next deoxynucleoside triphosphate complementary to the template (Fisher & Korn, 1981). Formation of such a dead-end complex has been suggested to be of importance for the potent inhibition of herpes simplex virus type 1 polymerase by acyclovir triphosphate (Reardon & Spector, 1989). It was thus of interest to examine the effect of deoxynucleoside triphosphates on the stability of the RT–primer/template complexes. When these experiments were performed, it was found that addition of dGTP to a preformed complex of RT and the

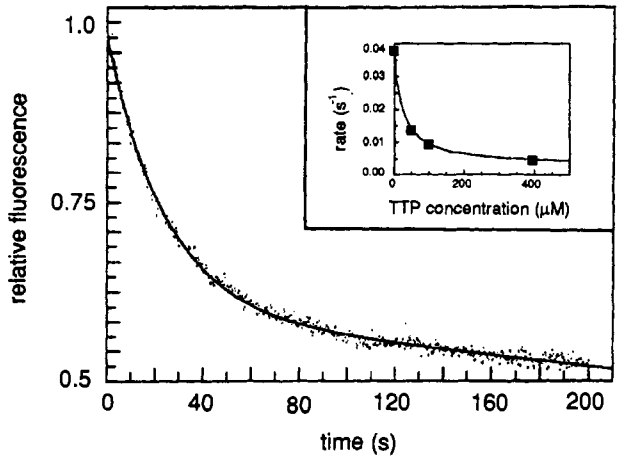

FIGURE 6: Dissociation of the RT–primer/template complex. A preformed complex of RT bound to a 36/F19 duplex (concentrations 50 and 30 nM, respectively; in this case adenine is the first unpaired base in the template) in standard assay buffer was mixed with a ca. 20-fold molar excess (respective to the primer) of poly(rA)·$(dT)_{15}$ by use of a SFA-12M Rapid Kinetics Accessory (Hi-Tech Scientific Ltd.) attached to the SLM fluorescence spectrophotometer. Data were collected in a Nicolet 310 digital oscilloscope, transferred to a personal computer, and fitted to a single-exponential model by use of the program Grafit. Under the conditions used in this experiment, bleaching of the fluorophore led to a slow monotonic decrease in fluorescence intensity. To correct for this, a linear slope was introduced into the fitting equation. The inset shows the dependence of the fitted rate constant on the concentration of TTP, which would be the next substrate in the absence of chain termination. The limited number of data points could be fitted to a hyperbola defined by an apparent dissociation constant for TTP of 23.8 μM and limiting rates of dissociation of 0.038 s^{-1} in the absence of TTP and 0.0027 s^{-1} at saturating TTP.

fluorescence labeled form of the oligodeoxynucleotide duplex led to a large change in the fluorescence yield. This could be used in a titration experiment (Figure 7A), which led to a value of 22 μM for the apparent dissociation constant of dGTP. This effect was base specific; i.e., titration with a deoxynucleoside triphosphate not complementary to the next position of the template (e.g., TTP) led only to minor changes in the opposite direction. Analogous experiments were performed with another oligodeoxynucleotide duplex, identical with the one shown in Figure 1 except that the base marked by an arrow was exchanged from cytosine to adenine. When this primer/template in its fluorescence-labeled form was bound to RT, titration with dGTP did not lead to a significant change in fluorescence. In contrast, addition of TTP now resulted in an increase of fluorescence intensity similar to the effect caused by dGTP in the experiment shown in Figure 7a. The apparent dissociation constant for TTP was ca. 10 μM.

Dideoxynucleotide triphosphate showed an effect that was similar to that of the corresponding dNTP, whereas ribonucleoside triphosphates and deoxynucleoside monophosphates led to a change of fluorescence intensity in the opposite direction (Figure 7B,C). Although these effects are also base specific, it is not yet clear whether dGTP and dGMP compete for the same binding site in the RT–primer/template complex. We can offer no explanation for the altered sign of the fluorescence change at present.

On the basis of these results, dissociation of the 36/F19–RT complex was monitored as described before with the modification that different concentrations of the next complementary dNTP were added. It was found that the stability of the RT–primer/template complex is increased in the presence of dNTP, as shown by the slower rate of dissociation of primer/template. The data presented in the inset of Figure 6

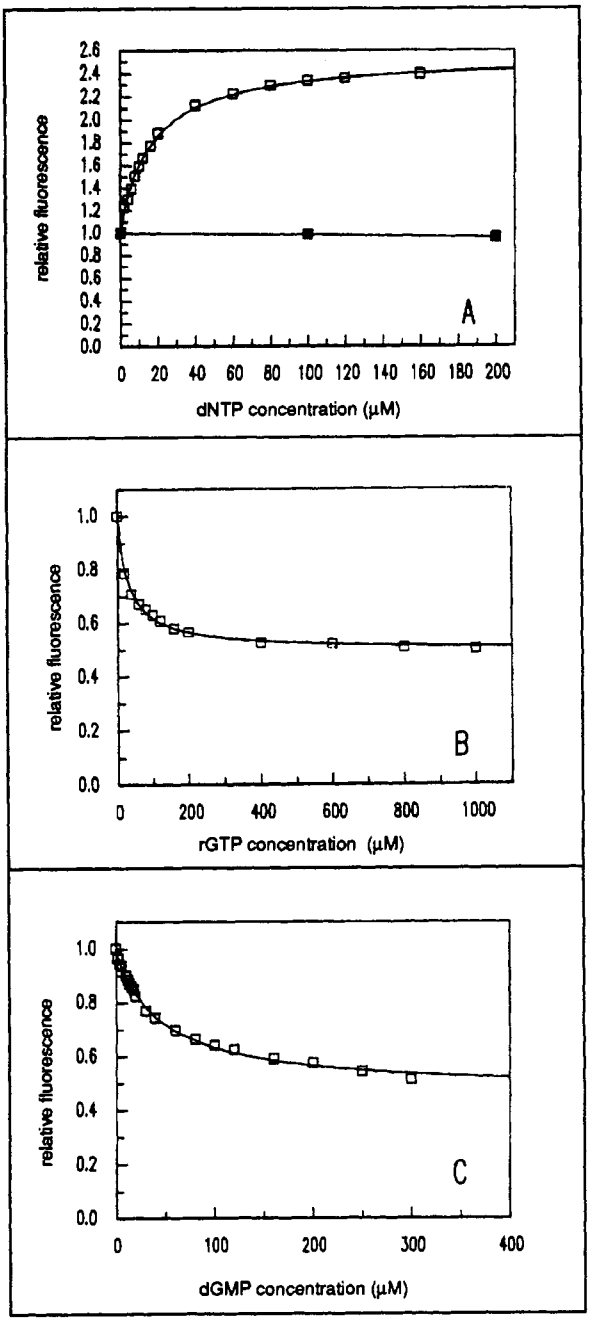

FIGURE 7: Influence of different nucleotides on the fluorescence of the RT–primer/template complex. The indicated nucleotides were titrated to preformed complexes between RT (100 nM) and the 36/F19 form (50 nM) of the oligodeoxynucleotide duplex shown in Figure 1. Panel A shows a comparison between the effect of dGTP (□) and TTP (■). Addition of dGMP (panel B) or rGTP (panel C) resulted in a change of fluorescence intensity in the opposite direction.

indicate a ca. 10-fold decrease of the dissociation rate saturating concentrations of TTP. Thus, the affinity of the enzyme for the primer/template is probably also increased by a factor of ca. 10 in the presence of high concentrations of TTP.

CONCLUSIONS

The results presented show that dideoxynucleotides containing a succinylfluorescein residue in the 5-position of the pyrimidine base can be efficiently added to a DNA primer in a template-directed manner in the presence of HIV-1 reverse transcriptase. Addition to the primer can be competed for by the corresponding deoxynucleoside triphosphate or by chain-terminating analogues of these. A quantitative analysis of the competitive effects shows that chain terminators such as 3′-

azidothymidine triphosphate or 2′,3′-dideoxythymidine triphosphate have no competitive advantage over the normal triphosphates. Thus, it seems unlikely that the potent inhibition seen by these analogues in vitro can be caused by this effect alone, at least with the relatively short templates normally used. This point is discussed in more detail below.

The fluorescent primer/template complexes obtained by addition of a fluorescent dideoxynucleotide to a primer can be used for monitoring the interaction of both primer/template and mononucleotides with reverse transcriptase. The dissociation constants for primer/template complexes were found to be in the nanomolar range in the absence of dideoxynucleoside triphosphates and are probably in the subnanomolar range in the presence of substantial concentrations (>100 μM) of the triphosphate of the nucleoside that would have been incorporated next in the absence of chain termination. The rates of dissociation of chain-terminated reverse transcriptase–primer/template complexes were in the region of 10^{-3} s^{-1} in the presence of deoxynucleotides. Together with a polymerization propagation rate of ca. 10 s^{-1} (the V_{max} value measured for our reverse transcriptase preparations leads to a value of ca. 2 s^{-1} for k_{cat} in the standard vitro assay using poly(rA)·$(dT)_{15}$ as primer/template and is assumed to represent a lower limit for the propagation rate; a value of ca. 10 s^{-1} has been reported for a heterotemplate by Huber et al. (1989)), this would lead to a "processivity" of ca. 10^4; i.e., the enzyme should dissociate approximately once every 10^4 bases during polymerization. Since the HIV genome is approximately 10 kB long, this processivity could be high enough to allow the enzyme to read the whole genome without dissociation. Experimental determination of RT processivity in vitro by other authors (Majumdar et al., 1988; Huber et al., 1989) has led to values of several hundred bases, but in these experiments processivity varied strongly with the primer/template system and the conditions used.

The slow rate of dissociation of reverse transcriptase from sites at the 3′-end of the growing DNA chain that still has a substantial template overhang but has been terminated with dideoxynucleotides and their derivatives appears to be the reason for the high potency of such analogues as reverse transcriptase inhibitors in vitro. In the typical assay for reverse transcriptase activity, there is a large excess of primer/template over enzyme. In this situation, three effects contribute to the inhibition seen. First, there is direct competitive inhibition with incorporation of the natural nucleotides. To be of significance, this effect would need concentrations of chain terminators that are comparable to those of the natural substrates, as shown by the results in Figure 3. In practice, substantial inhibition is seen at concentrations of terminators that are almost 3 orders of magnitude lower than substrate concentrations (50% inhibition of incorporation of TMP using poly(rA)·$(dT)_{15}$ as primer/template is seen at ca. 60 nM AZTTP in the presence of 20 μM TTP as substrate; D. Wahl, personal communication). Second, chain termination leads to removal of potential incorporation sites. This should be more noticeable in in vitro experiments than the simple competition effect, but the effect should still not be dramatic at the concentrations used due to the nature of the primer/template complexes used. Typically, these are poly(rA)·oligo(dT) duplexes with a base ratio of 1:1. Thus, only short stretches should be available for polymerization. Even if there are on average 10 potentially free sites on the template after the 3′-end of the primer, then assuming a similar efficiency of incorporation of natural nucleotide and chain terminator into the growing chain it is easy to show that for each stretch of 10 potential newly incorporated bases, the probability of chain termination is ca. 1% at a concentration of terminator that is 1000-fold lower than that of substrate. Thus, this effect should also lead to only weak inhibition under typical assay conditions. Third, the pool of enzyme available for polymerization will be progressively decreased by the formation of stable complexes between chain-terminated primer/template species, since these dissociate very slowly. It seems likely that the potent inhibition in vitro by the chain terminators arises largely via accumulation of such complexes, as has been demonstrated for herpes simplex DNA polymerase and acyclovir (Reardon & Spector, 1989).

It is of interest to consider which of the three factors mentioned above contribute to the inhibition of reverse transcription in vivo. Reverse transcriptase is present in HIV particles, probably at least in stoichiometric quantities with respect to viral RNA (Varmus & Swanstrom, 1984, and references given therein). The natural primer is a lysine tRNA (Wain-Hobson et al., 1985; Muesing et al., 1985), and the initiation complex, which appears to be present and preformed in virus particles, probably contains reverse transcriptase, $tRNA^{Lys}$, viral RNA, and the nucleocapsid protein p15, which appears to be essential for annealing of the tRNA stem to the primer binding site of the viral RNA (Barat et al., 1989). The exact details of the transcription mechanism are not understood, but a fundamental difference from the situation in vitro is apparent, namely, that there is not a large excess of primer/template over enzyme. It therefore seems unlikely that depletion of the pool of reverse transcriptase due to formation of stable complexes between terminated chains and enzyme contributes to the inhibition, since presumably each reverse transcriptase molecule must perform its function only once (or twice if the same molecule is used for both the minus- and plus-strand synthesis). However, the second effect mentioned above, i.e., prevention of completion of the complementary strand in either minus- or plus-strand synthesis, will be of much greater importance in terms of inhibition of transcription than with synthetic primer/templates where only the overall reduction of the amount of nucleotide incorporation is measured. This is because chain termination at any stage of minus- or plus-strand synthesis will lead to termination of transcription of the single-stranded RNA into double-stranded DNA and thus to inhibition of further steps in the replication cycle. The probability of such a break occuring, making the same assumptions as above and using a ratio of 1:1000 for the concentrations of chain terminator and natural nucleotide, is ca. 95% for 3000 bases incorporated, which is approximately the number of thymidines in one strand of the HIV proviral DNA. Thus, there may be similarly potent effects of chain terminators in vivo and in vitro, but for different reasons.

The latter arguments may be of importance in comparing the properties of chain terminators for chemotherapeutic purposes. Whereas the most important factor for potent inhibition in vitro with use of standard primer/template systems is probably the stability of the complex between reverse transcriptase and the terminated primer/template complex, it is possible that the most important factor in vivo is the rate of incorporation into the growing DNA chain. The latter can be tested in a direct manner with the assay described in this study (experiments shown in Figure 3). It should be borne in mind, however, that the additional factor of prodrug activation by cellular kinases also plays a decisive role in determining the suitability of a particular analogue for therapeutic purposes. Optimization of the properties of chain-terminating inhibitors as chemotherapeutic agents can only

be achieved when all of these factors are taken into consideration.

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Base- and Sequence-Dependent Binding of Aristololactam β-D-Glucoside to Deoxyribonucleic Acid

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ABSTRACT: The dependence on base-pair composition and sequence specificity of the (aristololactam β-D-glucoside)–DNA interaction was examined by spectrophotometric, spectrofluorometric, spectropolarimetric, thermal melting, thermodynamic, and viscometric studies. Binding of this alkaloid to various natural and synthetic DNAs was dependent upon the base composition and sequences of DNA. The binding parameters obtained from spectrophotometric analysis, according to an excluded-site model, indicated a relatively high affinity of the alkaloid binding to GC-rich DNA and alternating GC polymer. This affinity was further evidenced by the quenching of fluorescence intensity, decrease in quantum yield, and perturbations in circular dichroic spectrum. The alkaloid stabilized all DNAs against thermal denaturation. The temperature dependence of the binding constants was used to estimate the thermodynamic parameters involved in the complex formation of the alkaloid with various DNAs. The negative enthalpy and entropy change increased with increasing GC content of DNA and also compensated one another to produce a relatively small Gibbs free energy change. Viscometric studies showed that in the strong binding region the increase of contour length of DNA depended strongly on its base composition and sequence of bases, being larger for GC-rich DNA and alternating GC polymer. On the basis of these observations, it is concluded that the alkaloid binds to DNA by a mechanism of intercalation and exhibits considerable specificity toward alternating GC polymer.

During recent years there have been great advances in elucidating the factors that govern the affinity and specificity of binding of many naturally occurring and synthetic compounds to DNA. One important class of these compounds comprises those that bind to DNA by a mechanism of intercalation. Such compounds are important tools in molecular biology, and some are used for the treatment of cancer in man (Waring, 1981a,b; Wilson & Jones, 1982; Neidle & Waring, 1983; Neidle et al., 1987; Wilson, 1989).

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