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Allosteric Inhibition of Human Immunodeficiency Virus Type 1 Reverse Transcriptase by Tetrahydroimidazo[4,5,1-*jk*][1,4] benzodiazepin-2(1*H*)-one and -thione Compounds

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SUMMARY

The reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) is present in virions and infected cells as an heterodimer (p66/p51). A new class of potent and selective HIV-1 inhibitors, the tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione (TIBO) derivatives, were found to exert their antiviral activity by interacting with monomeric HIV-1 RT (p66) in a way different from that of previously studied RT inhibitors such as azidothymidine 5′-triphosphate. Upon examination of the kinetic properties of the heterodimeric HIV-1 RT and its inhibition by TIBO compounds, a positive cooperativity between the subunits of the enzyme with regard to the 2′-deoxynucleoside 5′-triphosphates and the template/primer was observed. The cooperativity with respect to the template/primer may result from a progressive dimerization in the presence of increasing concentrations of the template/primer, a process referred to as polysteric linkage. Because the cooperativity of p66/p51 was abolished in the presence of TIBO, these compounds behave as allosteric inhibitors.

RT (deoxynucleoside triphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.49) is a key enzyme in the replication of HIV-1, the causative agent of acquired immune deficiency syndrome. This enzyme is responsible for copying the single-stranded RNA viral genome to a double-stranded DNA intermediate, which is then integrated into host cell DNA. During the process of reverse transcription, RT displays multiple functions, including RNA-directed DNA polymerization, RNase H activity, and DNA-directed DNA polymerase activity.

HIV-1 RT has been found to occur as an heterodimer, consisting of two tightly associated chains of 66 and 51 kDa (1). The DNA-polymerizing and RNase H activities of HIV-1 RT are thought to reside in the amino- and carboxyl-terminal positions of the p66 chain, respectively. The p51 chain lacks RNase H activity (2, 3).

The TIBO derivatives, which have recently been shown to be very potent and selective inhibitors of HIV-1 replication *in vitro* (4), inhibit monomeric HIV-1 RT p66 in a way different from that of other known RT inhibitors (5). Here, we report studies on the kinetic behavior of the heterodimeric form of RT (p66/p51), the form believed to be present in virions and infected cells. We describe the allosteric properties of the heterodimeric enzyme and the allosteric inhibition of the enzyme by TIBO compounds.

Materials and Methods

Compounds. The origin of the prototype TIBO compound R82150, (+)-(*S*)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)-imidazo-[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-thione, has been described previously (4). Stock solutions of the compound were prepared in dimethylsulfoxide. Final dimethylsulfoxide concentrations in the RT and binding assays were <1%.

RT assay. The recombinant HIV-1 RT preparation used in our RT and binding assays was kindly provided by P. J. Barr from Chiron Corporation (Emeryville, CA). It was derived from an HIV-1 (SF2 strain) *pol* gene fragment coding for the RT (Pro-156–Leu-715) and expressed in yeast (6). Final concentration in the RT assay was 72 ng/ml (1.1 nM). Incorporation rate was estimated at 30 pmol of dTMP/hr in the poly(A)·oligo(dT)-directed assay (specific activity, 0.15 sec^{-1}) and 10 pmol of dGMP/hr in the poly(C)·oligo(dG)-directed assay (specific activity, 0.05 sec^{-1}). Specific activity was defined as the amount (micromoles) of incorporated ^{3}H-labeled deoxynucleotide per second and per micromole of enzyme at 37°.

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ABBREVIATIONS: RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1; AZT-TP, 3′-azido-2′,3′-dideoxythymidine 5′-triphosphate; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione.

Recombinant p51 was obtained from the same source and consisted of the amino acids Pro-156 to Phe-595. Used at a final concentration of 0.5 μM, under the same reaction conditions, the reaction rate was only one tenth as fast, compared with that of p66 (specific activity, 10^{-5} sec^{-1}), with poly(C)·oligo(dG) as the template primer. RT samples (p66 and p51) were divided into aliquots and stored at −80° until used. Stability under storage conditions was proven by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The p66 remained stable at 4°, but p51 was completely degraded within a few weeks when stored at 4°. Binding and kinetic experiments were done with freshly thawed p66 and p51 aliquots.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the RT samples revealed that p51 was >95% pure, whereas the p66 preparation showed a minor band (10%) at p53. This may correspond to a degradation product of p66. The monomeric, dimeric, and heterodimeric forms of HIV-1 RT are assumed to exist in equilibrium, depending on temperature, concentration of subunits, and salt concentration. The affinity of p51 and p66 to form heterodimers seems much higher than the affinity of p51 or p66 to form homodimers (6, 7). A p66 or p51 preparation is likely to exist in a monomer/homodimer equilibrium, shifted toward the monomers, whereas a p66/p51 equimolar preparation is likely to consist predominantly of heterodimers.

The standard RT reaction mixture contained 50 mM Tris·HCl, pH 8.1, 10 mM $MgCl_2$, 100 mM KCl, 2.2 mM dithiothreitol, and 0.05% (w/v) Triton X-100. The template/primer poly(C)·oligo(dG_{12-18}) was used at a concentration of 65 μg/ml. Templates and primers were purchased from Pharmacia (Uppsala, Sweden). ^{3}H-Labeled dGTP, dissolved in ethanol/water (1:1), was obtained from Amersham (Buckinghamshire, UK) and used at a concentration of 2.5 μM. Specific activity was 15.6 Ci/mmol. Ethanol was removed from [^{3}H]dGTP by Speed-Vac evaporation (Speed-Vac; Savant, Farmingdale, NY), to avoid interference from ethanol in the kinetic studies.

After addition of varying concentrations of inhibitors and enzyme, the reaction mixture was incubated for 1 hr at 37°. The extent of incorporation was determined by a standard trichloroacetic acid precipitation procedure, using Whatman GF/C glass fiber filter papers (Whatman, Maidstone, UK) and liquid scintillation counting the scintillation liquid was Ready Protein; (Beckman, Fullerton, CA).

Kinetic studies were performed with varying substrate (dGTP) and template/primer [poly(C)·oligo(dG)] concentrations. The incorporation rate of ^{3}H-labeled nucleotide was linear for at least 1 hr. Studies were performed under steady state conditions (i.e., the amount of substrate incorporated was <2.5% of the amount available in the reaction mixture) in 15-min assays. Analysis of experimental data (optimal fitting of the data points) was performed with a nonlinear regression analysis software program (ENZFITTER; Elsevier-Biosoft, Cambridge, UK), based on the Hill equation. We found by mathematical fitting analysis that efficiency of fitting of the data was higher with the Hill equation than with the Michaelis-Menten equation.

Binding assay. Binding of the 2′-deoxynucleoside 5′-triphosphates, [^{3}H]dGTP, and [^{3}H]AZT-TP (Moravek, Brea, CA) to HIV-1 RT was studied by equilibrium dialysis. The experiments were performed in 200-μl micro-cells with cellulose dialysis membranes (Diachema) (both from Dianorm, München, Germany) with a molecular weight cut-off of 5000. Before use, the membranes were rinsed and washed with distilled water, followed by conditioning in the dialyzing buffer. The dialyzing buffer (50 mM Tris·HCl, 100 mM NaCl, 2 mM dithiothreitol, 10 mM $MgCl_2$) was similar to the RT reaction buffer. Equilibrium for [^{3}H]dGTP was reached after 90 min at 37°.

The left chamber of each cell was filled with 140 μl of dialyzing buffer, containing the enzyme preparation p66 (0.25 μM) or p66/p51 (equimolar, 0.25 μM each). The right chamber of each cell was filled with 140 μl of dialyzing buffer, containing a dilution series of [^{3}H]dGTP or [^{3}H]AZT-TP. A complementary template, poly(C) or poly(A), was present in both cells, at a final concentration of 40 μg/ml. After equilibrium had been reached, 10 μl of each chamber were counted in 5 ml of scintillation liquid (Ready Safe; Beckman), in a liquid scintillation counter. Results were corrected for adsorption of tritiated compound to the Teflon cell wall or cellulose dialyzing membrane.

Under the experimental conditions used (2 hr at 37°), >70% of the RT activity was recovered from equilibrated micro-cells. The small loss could be accounted for by osmotic dilution and/or adsorption.

Results

Specific activities of different enzyme preparations. In order to study the kinetic behavior of HIV-1 heterodimer p66/p51, we compared the specific activities of different enzyme preparations in a poly(C)/oligo(dG)-directed RT assay, with or without Triton X-100 (Table 1). Triton X-100, a nonionic detergent, has been routinely used as a reaction mixture component in RT activity assays since it was included in the endogenous RT reactions (8) to disrupt virion particles.

We observed a 10-fold decrease in RT activity of p66 in the absence of Triton X-100, in a homopolymer-directed assay, whereas the low RT activity of p51 was not influenced by the detergent. A heterodimer preparation, consisting of an equimolar concentration of p51 and p66, showed similar RT activity as p66 in the presence of Triton X-100 (Table 1). In the absence of Triton X-100 p66 displayed 70% of the RT activity of the heterodimer.

Bearing in mind the likely negative impact of a detergent on the quaternary structure of proteins, we investigated steady state kinetic behavior of the heterodimer in the presence and absence of Triton X-100 (Fig. 1). In the presence of 0.05% Triton X-100 p66/p51 displayed Michaelis-Menten kinetic characteristics (K_m, 1.56 μg/ml), whereas in the absence of Triton X-100 a sigmoidal curve was obtained if RT activity was plotted as a function of the poly(C)·oligo(dG) concentration.

Positive cooperativity between subunits in p66/p51 as shown by kinetic studies. Under steady state conditions (a 15-min RT assay), in a reaction mixture without Triton X-100, the kinetic behavior of an equimolar preparation of p51 and p66 was studied by varying substrate (dGTP) and template/primer [poly(C)·oligo(dG)] concentrations. Results are summarized in Table 2. With regard to the substrate, the Hill coefficient (n_H) for p66/p51 and p66 was 1.36 and 1.1, respectively. With respect to the template/primer, n_H for p66/p51 and p66 was 1.8 and 0.99, respectively.

Another parameter of cooperativity, the ratio of substrate concentrations corresponding to 75% and 10% of the reaction velocity ($[S]_{0.75}/[S]_{0.10}$), pointed to the same conclusion. Whereas the cooperativity index for p66 was estimated at 5 and 10 for substrate and template, the indices increased to 32

TABLE 1

Specific activity of different enzyme preparations

Specific activity is expressed as μmol of incorporated [^{3}H]dGMP/sec/μmol of enzyme, at 37°. Values are mean ± standard deviation.

Enzyme[a]	Specific activity (10^{-3})	
	With Triton X-100 (0.05%)	Without Triton X-100
	μmol/sec/μmol	
p66	47 ± 10	4.5 ± 1.14
p51	0.0104 ± 0.01	0.013 ± 0.001
p66/p51	51 ± 10	6.7 ± 0.84

[a] Monomeric p66 in equilibrium with the homodimer p66/p66, monomeric p51 in equilibrium with the homodimer p51/51, or an equimolar concentration of p66 and p51 in equilibrium with the heterodimer p66/p51.

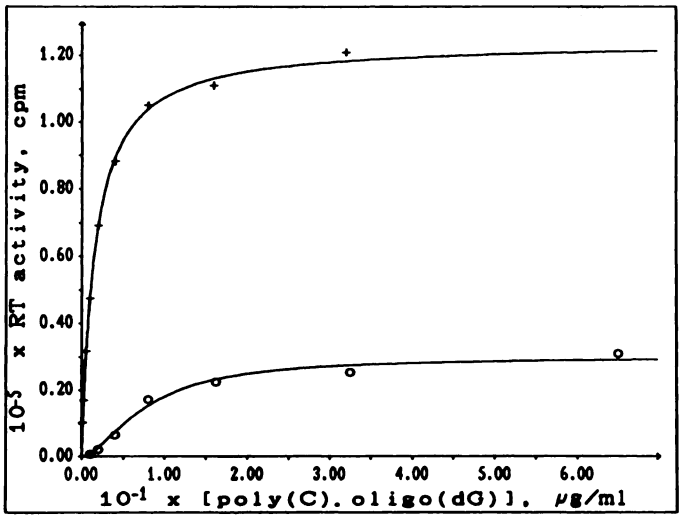

Fig. 1. Effect of Triton X-100 on RT activity. Heterodimeric RT activity, measured in the presence (+) or absence (○) of 0.05% Triton X-100, is plotted against increasing concentrations of the template/primer. The sigmoidal curve (○) is characterized by a Hill value of 1.7, a K value of 31 µg/ml, and a V_{max} value of 29,900 cpm; the hyperbolic curve (+) is defined by a K_m value of 1.56 µg/ml and a V_{max} value of 124,000 cpm.

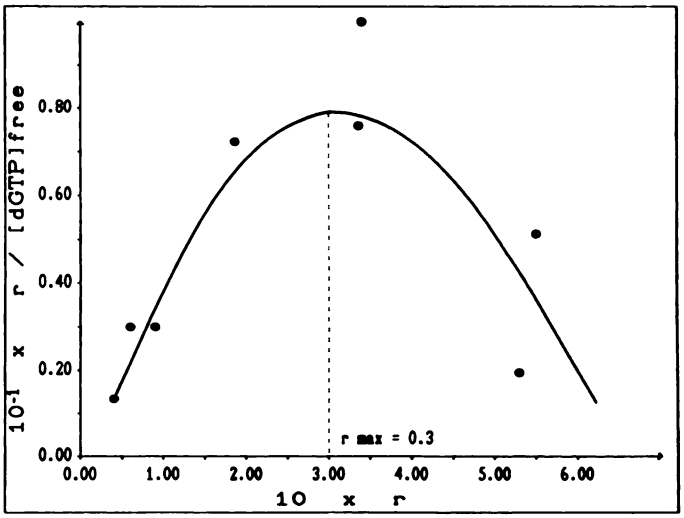

Fig. 2. Scatchard plot for the binding of [^{3}H]dGTP to heterodimeric p66/p51. The ratio of enzyme saturation (r) to free substrate concentration is plotted against saturation. This produces a curve with a maximum at a saturation of 0.3. From the formula $n_H = (1 - r_{max})^{-1}$, a Hill value of 1.4 is calculated.

TABLE 2

Allosteric properties of heterodimeric p66/p51 HIV-1 RT

Data represent mean values ± standard deviations for at least two (p66) or five (p66/p51) separate experiments

Enzyme	Substrate[a]		Template/primer[b]	
	n_H	K	n_H	K
		µM		µg/ml
p66	1.11 ± 0.05	3.2 ± 0.3	0.99 ± 0.02	11.7 ± 3
p66/p51	1.36 ± 0.05	3.56 ± 0.7	1.8 ± 0.38	27.4 ± 8

[a] Hill coefficients (n_H) and K values calculated with a nonlinear regression analysis software program from kinetic studies with varying substrate dGTP concentrations.
[b] Hill values and K values calculated with a nonlinear regresison analysis software program from kinetic studies with varying template/primer [poly(C)·oligo(dG)] concentrations.

and 16, respectively, when the heterodimer was used as the enzyme.

Positive cooperativity between subunits in HIV-1 RT as shown by equilibrium dialysis binding assays. Under the conditions used, dGTP showed positive cooperativity in binding to heterodimeric p66/p51 in the presence of complementary poly(C). A Scatchard plot of the experimental data is shown in Fig. 2. Whereas the binding of dGTP to p66 proved linear in the Scatchard plot (K_d, 0.116 µM; data not shown), the heterodimer preparation showed a curve with a maximum at $r = 0.3$. This is indicative of positive cooperativity. According to Dahlquist (9), a quantitative estimation of n_H can be obtained from the equation $n = (1 - r_{max})^{-1}$, where r_{max} stands for saturation at the maximum of the plot. From our data a n_H value of 1.4 could be calculated, which corresponds to the value obtained in kinetic studies.

Similar data were obtained when the binding of [^{3}H]AZT-TP to p66 and p66/p51 was measured. The binding of AZT-TP to p66 was linear in the Scatchard plot (K_d, 0.09 µM). For the heterodimer preparation, the Scatchard plot showed a curve with a maximum at $r = 0.4$ (data not shown).

Studies with both [^{3}H]dGTP and [^{3}H]AZT-TP pointed to the importance of a complementary template. For instance, no binding of AZT-TP was found when poly(C) was used as a template. These data are in keeping with the observations of Majumdar *et al.* (10) on the ordered reaction pathway for the RT-directed DNA synthesis and the observations of Basu *et al.* (11), who found that the 2′-deoxynucleoside 5′-triphosphate binding site functions only when the template/primer has bound to the enzyme.

Analysis of positive cooperativity. The heterodimeric p66/p51 is assumed to be in equilibrium with the monomeric forms p66 and p51. According to a recent report (10), only dimeric RT has enzymatic activity. This suggests that a shift to the right of the equilibrium p66 + p51 ⇄ p66/p51 may increase RT activity.

When such a shift was facilitated in a kinetic RT assay by increasing the p51 concentration 10-fold, compared with the p66 concentration, the sigmoidal relationship between RT activity and template/primer concentration observed with an equimolar p66/p51 preparation was replaced by a hyperbolic relationship (Fig. 3). The positive cooperativity obtained by increasing template/primer concentration was counteracted by increasing the p51 concentration. This indicates that the template/primer induces positive cooperativity through dimerization. The increased stability of RT in the presence of template/primer (12) may be due to dimerization as well.

The positive cooperativity with regard to substrate was not influenced by a 10-fold increase in the p51 concentration. The Hill coefficient remained at 1.36 (Fig. 4). This cooperativity should, therefore, be interpreted in terms of mutual interference of the two substrate binding sites on the heterodimer.

TIBO compounds as allosteric inhibitors. The effect of the TIBO derivative R82150, an inhibitor of HIV-1 RT, on the kinetic behavior of p66/p51 is presented in Figs. 5 and 6. With respect to both the substrate (dGTP) and template/primer [poly(C)·oligo(dG)], cooperativity of p66/p51 was abolished at a R82150 concentration of 1.4 µM. Hill coefficients decreased proportionally with increasing inhibitor concentration (data not shown). K values increased (substrate) or decreased (template), as indicated (Figs. 5 and 6).

A comparison of the IC_{50} values (concentrations that inhibit

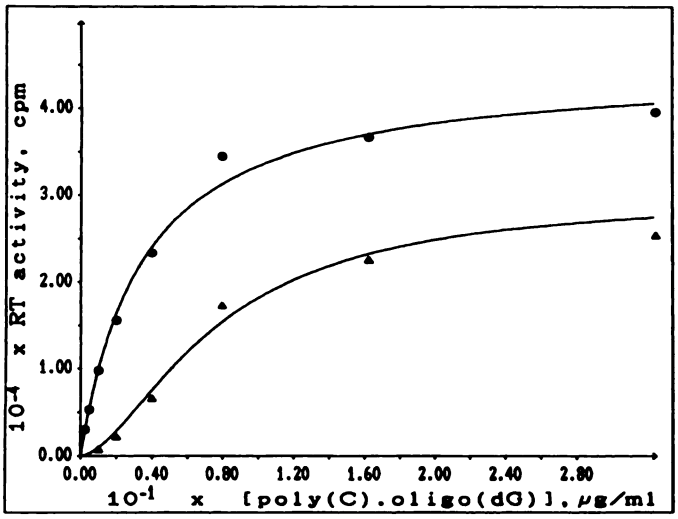

Fig. 3. Positive cooperativity of heterodimeric p66/p51 with regard to template/primer poly(C)·oligo(dG) is counteracted by increasing the p51 concentration. Heterodimeric RT activity is plotted against increasing concentrations of poly(C)·oligo(dG). A heterodimer preparation, consisting of 1.1 nM p51 and p66, resulted in a sigmoidal plot (▲) (n_H = 1.7, K = 31 μg/ml, V_{max} = 30,000 cpm), whereas a 10-fold excess in p51 concentration (11 nM), compared with the p66 concentration (1.1 nM), resulted in a hyperbolic plot (●) (K_m = 3.5 μg/ml, V_{max} = 45,000 cpm).

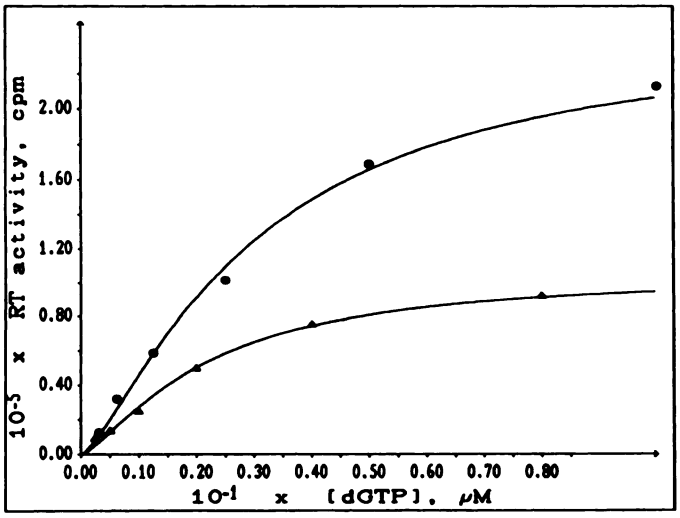

Fig. 4. Positive cooperativity of heterodimeric p66/p51 with respect to substrate dGTP is not influenced by excess p51 concentration. Heterodimeric RT activity is plotted against increasing concentrations of dGTP. Mathematical fitting analysis (with the Hill equation) of the kinetic data for a heterodimeric preparation, consisting of 1.1 nM p51 and 1.1 nM p66, gave a sigmoidal plot (▲) (n_H = 1.36, K = 2.9 μM, V_{max} = 106,797 cpm). A 10-fold excess in p51 concentration (11 nM), compared with the p66 concentration (1.1 nM), also resulted in a sigmoidal plot (●) (n_H = 1.37, K = 4.3 μM, V_{max} = 244,000 cpm).

RT activity by 50%) of R82150 for the different enzyme preparations, with or without 0.05% Triton X-100, is presented in Table 3. Poly(C)·oligo(dG)$_{12-18}$ was used as template/primer. The IC_{50} of R82150 for the heterodimer was between those for p66 and p51. As demonstrated previously (5), the IC_{50} of TIBO is not dependent on reaction velocity. Thus, the 2-fold decrease in IC_{50} noted upon omission of Triton X-100 from the reaction mixture indicates that Triton X-100 somehow counteracts the effect of R82150 on RT.

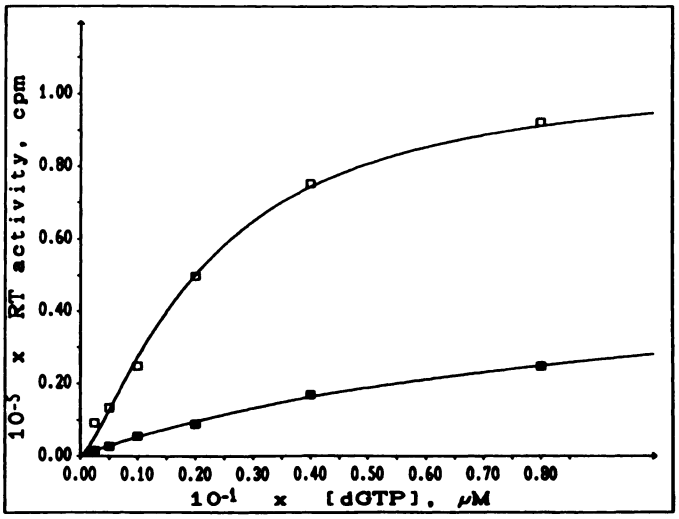

Fig. 5. Allosteric inactivation of heterodimeric p66/p51 by R82150, with respect to the substrate dGTP. The sigmoidal plot (□) obtained with p66/p51 (n_H = 1.36, K = 2.9 μM, V_{max} = 106,797 cpm) becomes hyperbolic (■) upon addition of 1.4 μM R82150 (n_H = 0.96, K = 9.8 μM, V_{max} = 58,000 cpm). The plots were obtained based on the Hill equation, following the same nonlinear regression analysis program.

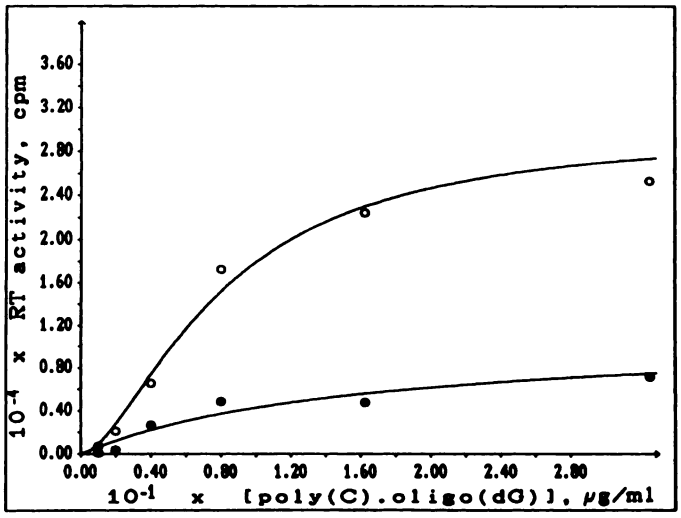

Fig. 6. Allosteric inactivation of heterodimeric p66/p51 by R82150, with respect to template/primer poly(C)·oligo(dG). The sigmoidal plot (○) obtained with p66/p51 (n_H = 1.7, K = 31 μg/ml, V_{max} = 30,000 cpm) becomes hyperbolic (●) upon addition of 1.4 μM R82150 (n_H = 0.99, K = 15.8 μg/ml, V_{max} = 11,150 cpm). The plots were obtained based on the Hill equation, following the same nonlinear regression analysis program.

Discussion

Although the RT complex is of paramount importance in the HIV replicative cycle and the target of several compounds with established antiviral efficacy, much needs to be elucidated about this enzyme. The lack of RT crystals, suitable for high resolution X-ray diffraction, is part of the problem. Targeting the RT with specific inhibitors is another approach toward resolving the mode of enzymatic action. TIBO derivatives have proven to be very potent inhibitors of HIV-1 replication in cell culture (4). The compounds are targeted at the HIV-1 RT, but their mode of interaction with the enzyme is different from that of presently known RT inhibitors (5). In a previous study

TABLE 3
Inhibition of RT activity in R82150 in the absence or presence of Triton X-100

Enzyme	IC_{50}[a]	
	Triton X-100 (0.05%)	No Triton X-100
p66 (1.1 nM)[b]	0.35 ± 0.03	0.22 ± 0.04
p51 (0.5 μM)	0.58 ± 0.15	0.31 ± 0.015
p66/p51 (1.1 nM)	0.49 ± 0.1	0.25 ± 0.01

[a] Concentrations (average values ± standard deviations) of R82150 that inhibit RT activity by 50%, using poly(C)·oligo(dG) as template/primer and dGTP as substrate.
[b] Concentrations of the enzyme, as used in the RT assays, are indicated in parentheses.

we focused on the interaction of TIBO with the recombinant monomeric p66 form of RT. We have now examined the mode of TIBO interaction with the heterodimeric p66/p51, which represents the RT form present in HIV virions (1) and HIV-infected cells (13).

When Triton X-100, a nonionic detergent, was omitted from the RT reaction mixture, the velocity of the enzyme showed sigmoidality when plotted as a function of the substrate (dGTP) or template/primer [poly(C)/oligo(dG)] concentration. Positive cooperativity was noted in kinetic studies with respect to both substrate and template/primer. This positive cooperativity for dGTP binding to p66/p51 was independently confirmed by equilibrium dialysis.

Whereas cooperativity with respect to the substrate was independent of the p66/p51 ratio, a 10-fold excess in p51 concentration, relative to the p66 concentration, abolished cooperativity with respect to the template/primer. Positive cooperativity with respect to the template/primer can be explained by a progressive dimerization by increasing concentrations of the template/primer. This process has been referred to as polysteric linkage (14). The Hill coefficient of 1.8 suggests two template/primer binding sites in the heterodimer.

With respect to the substrate (dGTP), positive cooperativity did not depend on the p66/p51 ratio. The Hill coefficient of 1.4 suggests two interactive substrate binding sites in the heterodimer. In the kinetic and binding studies, cooperativity for substrate (dGTP) was assessed in the presence of a saturating template concentration. This saturating template concentration shifts, according to our finding of polysteric linkage, the equilibrium of monomeric and heterodimeric enzyme forms toward the heterodimers. The equilibrium as such, although difficult to assess at nanomolar enzyme concentrations, is presently under study.

The decrease in cooperativity ($n_4 = 1$) noted in the presence of TIBO points to allosteric inhibition. As to the template/primer, the polysteric linkage could be counteracted by increasing TIBO inhibition, because TIBO has been found to show an uncompetitive type of inhibition with respect to the template/primer (5). As to the substrate, the TIBO effect on cooperativity could be explained according to the sequential interaction model of allosteric proteins (15), with a general formula of *R T*, where *R* stands for relaxed and *T* for tense state. If TIBO binding to RT is promoting a tense state, our data can be readily interpreted in terms of allosteric inhibition.

Allosteric properties have been observed with DNA polymerase α (16, 17) and III (18), with respect to template/primer. As for DNA polymerase III, the dimeric nature of the holoenzyme has been associated with the polymerization process in the replication fork. Evidence for allosterism in *in vitro* DNA synthesis on RNA templates by *Escherichia coli* DNA polymerase I and Mason-Pfizer monkey virus RT has been shown previously (19).

Majumdar *et al.* (10) described RT activity as an ordered bireactant-biproduct system. The binding of template/primer is followed by the binding of complementary 2′-deoxynucleoside 5′-triphosphate. This finding, together with studies by Basu *et al.* (11) and our own binding studies, suggests a single substrate binding site per subunit, the conformation of which is changed upon template/primer binding.

TIBO inhibition of RT shows a marked template preference, and this inhibition is of the uncompetitive type (5). Template/primer binding seems to affect substrate binding in the same way as it affects the binding of TIBO. Here we reported two new types of interaction. Dimerization is promoted by template/primer binding (polysteric linkage), and a positive cooperativity results from an interaction between the two substrate binding sites in the heterodimer. TIBO interferes with this cooperative effect between the two substrate binding sites, possibly by binding in the neighborhood of the substrate binding site.

In additional experiments, we found a similar allosteric kinetic behavior (with respect to the substrate dGTP) for the virion (HIV-1 III_B)-derived RT, when examined in an endogenous RT assay (data not shown).

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