

# Human immunodeficiency virus reverse transcriptase substrate-induced conformational changes and the mechanism of inhibition by nonnucleoside inhibitors

KATRIN RITTINGER<sup>†</sup>, GILLES DIVITA<sup>‡</sup>, AND ROGER S. GOODY<sup>†</sup>

<sup>†</sup>Max-Planck-Institut für Molekulare Physiologie, Abteilung Physikalische Biochemie, Rheinlanddamm 201, 44139 Dortmund, Germany; and <sup>‡</sup>Max-Planck-Institut für Medizinische Forschung, Abteilung Biophysik, Jahnstrasse 29, 69120 Heidelberg, Germany

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**ABSTRACT** A combination of transient kinetic and equilibrium titration methods has been used to show that both primer/template and nucleotide binding to human immunodeficiency virus type 1 (HIV-1) reverse transcriptase are two-step processes. In both cases, after initial formation of relatively weakly bound states, isomerization reactions lead to tightly bound states. In the case of deoxynucleotide binding to the reverse transcriptase–primer/template complex, the second step in the interaction is rate-limiting in the overall reaction during processive polymerization. Discrimination against incorrect nucleotides occurs both in the initial weak binding and in the second step but is purely kinetic in the second step (as opposed to thermodynamic in the first step). Nonnucleoside inhibitors have a relatively small effect on nucleotide-binding steps (overall affinity is reduced by a factor of *ca.* 10), while the affinity of the primer/template duplex is increased by at least a factor of 10. The major effect of nonnucleoside inhibitors is on the chemical step (nucleotide transfer).

Recent x-ray crystallographic work on human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) has led to detailed information on its structure in several states. Among these are inhibitor complexes (1, 2) and a complex with double-stranded DNA (3, 4). Progress on delineating the reaction mechanism of the RNA- and DNA-dependent polymerase activity has also been made, and the results of kinetic studies lead to a model for the kinetic mechanism of nucleotide incorporation. This model involves, in addition to the obligatory chemical step in which a deoxynucleotide is added to the end of the growing DNA chain, two-step binding mechanisms of both nucleic acid [an RNA-DNA or DNA-DNA duplex, referred to here as primer/template (or p/t in complexes)] and deoxynucleoside triphosphates (dNTPs). To complement the structural studies, it is important to give this model a firm quantitative basis, both with respect to understanding the polymerization mechanism in detail and as a basis for understanding the mechanisms of action of various inhibitor types and to stimulate ideas on new inhibitors that could be of therapeutic importance in AIDS. We present here results that demonstrate and quantitate the two-step binding mechanisms of nucleic acids and dNTPs and, in combination with other results (5), identify the chemical step as the point at which nonnucleoside inhibitors (NNIs) exert their major effect.

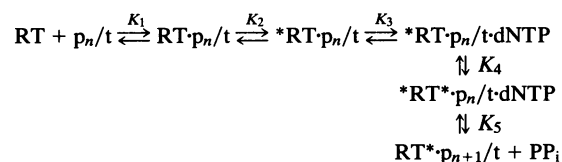
## MATERIALS AND METHODS

RT from HIV-1 was prepared from the previously described coexpression plasmid in *Escherichia coli* (6). Oligonucleotides and fluorescently labeled oligonucleotides were prepared as described (7). Oligonucleotides terminated at the 3' end with

a dideoxynucleotide were prepared by using RT to catalyze the transfer of the last nucleotide. NNIs (O-TIBO and Nevirapine, whose chemical names are in the abbreviations footnote) were gifts of A. Mertens (Boehringer Mannheim). Fluorescence titrations using oligonucleotides were performed as described (7, 8) on an SLM Smart 8000 spectrofluorometer. Fluorescence titrations of nucleotide binding were performed on preformed complexes of RT and primer/template. Stopped-flow experiments were performed with a double-mixing apparatus (High Tech Scientific, Salisbury, England) as described (8). The standard buffer contained 50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 50 mM KCl, and 1 mM dithiothreitol. Data fitting was done with the program GRAFIT (Erithacus Software, London).

## RESULTS AND DISCUSSION

A hypothetical model for the mechanism of RT resulting from previous transient kinetic experiments on primer/template binding and DNA chain elongation by RT can be summarized as shown in Scheme I, which is a model for the kinetic mechanism of the polymerase reaction in which the asterisks distinguish structural states of the system that otherwise have the same composition.



### Scheme I

Two important features of this scheme cannot be regarded as firmly established on the basis of earlier work—namely, the two-step binding of primer/template and the two-step binding of dNTP. Strong evidence in support of the former comes from the experiment shown in Fig. 1. As previously described, the kinetics of interaction of primer/template with RT can be studied by using fluorescently labeled primer/template (7, 8), and in stopped flow experiments, as shown in Fig. 1 *Upper*, biphasic kinetic curves are obtained. This has been interpreted to be due to two-step binding of primer/template to RT (8), but there remains some doubt as to whether this behavior arises only because of the presence of the fluorophore. The results shown in Fig. 1 *Lower* provide strong evidence that the suggested two-step binding mechanism is correct. As shown, a plot of the pseudo-first-order rate constant for the initial association of primer/template with RT (derived from the fast phase in experiments such as that in Fig. 1 *Upper*) against the

**Abbreviations:** RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1; O-TIBO, (+)-(S)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5-*jk*][1,4]-benzodiazepine (*1H*)-one; Nevirapine (BI-RG 587), 11-cyclopropyl-5,11-dihydro-4-methyl-6*H*-dipyrido[3,2-*b*:2',3'-*e*](1,4)diazepin-6-one; NNI, nonnucleoside inhibitor.

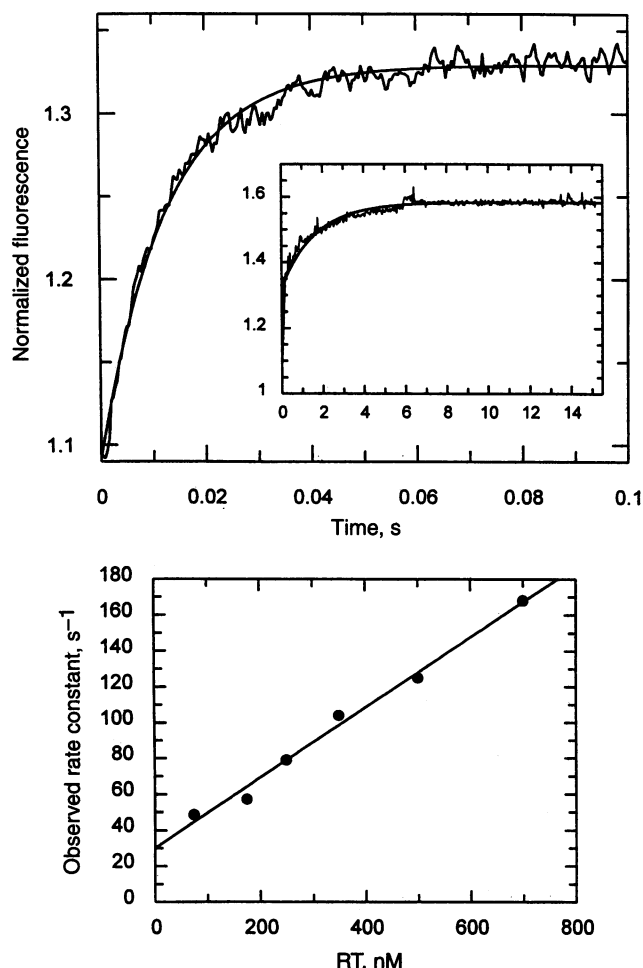


FIG. 1. Kinetics of binding of a fluorescently labeled primer/template (0.020  $\mu$ M) to HIV-1 RT (0.25  $\mu$ M). The following synthetic oligonucleotide primer/template (19-mer/16-mer) was used:

5'-TCCCTGTTTCGGGCGCCACT\*  
 3'-AGGACAAAGCCCGCGGTGATGATCTCTAAAGGTGT-5'.

T\* is the succinylfluorescein derivative of thymidine used previously (7, 8). Excitation was at 500 nm, and emission was detected via a cut-off filter (530 nm). (Upper) Typical stopped-flow result showing the first phase of the reaction, with the slower second phase shown in the *Inset*. The smooth lines show the results of fitting a biexponential function to the data. (Lower) Secondary plot of the dependence of the fitted pseudo-first-order rate constant for the first phase on the RT concentration.

enzyme concentration is linear up to concentrations of 0.7  $\mu$ M and rates of *ca.* 170  $s^{-1}$ . There is a clear intercept with the y axis of the graph at 30  $s^{-1}$ , and this is the value of the reverse rate constant ( $k_{-1}$ ) of the process being observed. This is a factor of 3000 faster than the dissociation rate measured by displacement of the preformed RT-p/t complex (0.009  $s^{-1}$ ; measured as in ref. 7). The forward rate constant ( $k_{+1}$ ) is given by the slope of the line and is  $2.0 \times 10^8 M^{-1}s^{-1}$ , so that the equilibrium constant for this process can be calculated to be  $6.5 \times 10^6 M^{-1}$ , which is a factor of *ca.* 50 lower than the affinity measured in direct titrations under identical conditions ( $3.0 \times 10^8 M^{-1}$ ; measured as described in refs. 7 and 8). The discrepancy is explained by the occurrence of the second step in the binding; by using the values of 0.5  $s^{-1}$  for the slow phase seen in Fig. 1 *Upper* and the rate of 0.009  $s^{-1}$  for the rate of dissociation of the primer/template from its preformed complex with RT, an overall affinity of  $3.6 \times 10^8 M^{-1}$  can be calculated, in excellent agreement with the equilibrium mea-

surements. Thus, there is convincing evidence for the two-step mechanism of primer/template binding to RT.

A large number of NNIs of HIV-1 RT, which are also in several cases potent inhibitors of HIV-1 replication, have now been identified (e.g., refs. 9–11), but their mechanism of action remains obscure. The effect of the NNI O-TIBO on the binding kinetics of primer/template has been described (8). The three rate constants that could be measured were all modified in the direction of tighter binding ( $k_{+1}$  and  $k_{+2}$  are increased, while  $k_{-2}$  is decreased); in keeping with this, we have now shown by direct titration of RT and primer/template in the presence of O-TIBO that the affinity is increased to the point where it could not be measured accurately in an equilibrium titration and appeared to be significantly greater than  $10^9 M^{-1}$  (data not shown). Conversely, the affinity of O-TIBO to the RT-p/t complex must be higher than to the free enzyme. From these results, it is clear that inhibition of RT activity by such compounds is not due to competition with binding to primer/template, and the previously observed inhibition pattern ("uncompetitive," ref. 12) with respect to primer/template can be explained by combining the observation of tighter binding of inhibitor to the enzyme-substrate complex with the further observation that the polymerization reaction is then strongly inhibited. This does not define at this point whether this inhibition is due to an effect on a step involved with binding of the second substrate, dNTP, or to an effect on the chemical step (transfer of dNMP to the end of the primer molecule).

The structural change involved in the second step in primer/template binding cannot be defined on the basis of present evidence. However, the recent determination of the structure of RT without bound primer/template or inhibitor molecules (13) may provide a clue to this. The domain structure of the enzymatically active 66-kDa subunit of RT has been compared to the appearance of a right hand, with fingers, thumb, palm, and connection domains (1). In the structures of the "apo" enzyme determined by the Harrison and Arnold group (13, 15), it is seen that the thumb domain is much closer to the fingers domain than in the RT-p/t or RT-NNI structures, so that a double-stranded nucleic acid could not bind in the groove between these two domains. This leads to the idea (15) that the second step identified in this and earlier work corresponds to the opening of the cleft after initial binding of the single-stranded region of the primer/template, although it is not clear how this mechanism can be reconciled with the known spectrum of primer/template substrates used by RT *in vivo* and *in vitro*.

The suggested two-step binding mechanism for dNTPs (14) was tested in the experiment shown in Fig. 2 *Upper*. Examination of Scheme I led to an experiment aimed at measuring  $K_4$ , which is based on the fact that the effective affinity of dNTP in an experiment in which the chemical step is prevented from occurring should be higher than that arising from transient kinetic experiments in which the rate constant of addition of a nucleotide to the end of a primer molecule is measured as a function of dNTP concentration, as in the work of Kati *et al.* (14). The difference between the two affinities would provide a measure of  $K_4$ . With use of a template/primer pair in which the primer molecule cannot be extended because it is terminated by a dideoxy nucleotide, protein (tryptophan) fluorescence was used to monitor the binding of dTTP. As shown in Fig. 2 *Upper*, saturation occurred in a concentration range that was surprisingly low in comparison with that seen for saturation of the incorporation rate. A  $K_d$  value of 0.018  $\mu$ M was obtained, which is  $>2$  orders of magnitude lower than the  $K_d$  obtained (5  $\mu$ M) in the quenched-flow experiments of Kati *et al.* (14). This result represents a striking confirmation of the kinetic mechanism suggested by Kati *et al.* and, moreover, allows a value to be assigned to  $K_4$ , which is calculated to be *ca.* 280.  $K_3$  is equated with  $1/K_d$  from the quenched-flow experiments and thus has a value of  $2 \times 10^5 M^{-1}$  from the work

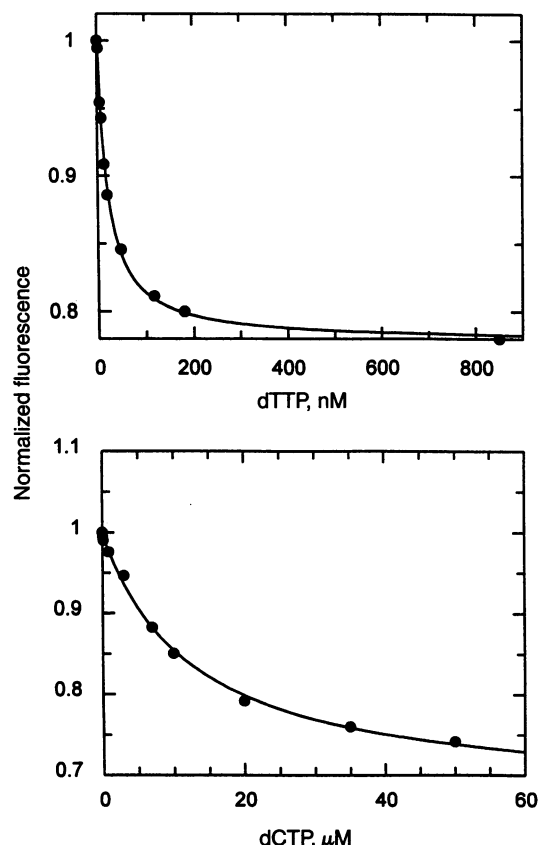


FIG. 2. Fluorescence titrations of nucleotide binding. A dideoxy-terminated primer/template ( $0.06 \mu\text{M}$ ) bound to RT ( $0.04 \mu\text{M}$ ) was titrated with correct (dTTP) or incorrect (dCTP) nucleotides while monitoring protein fluorescence (excited at 290 nm and observed at 340 nm). (Upper) Titration with the next correct nucleotide (dTTP), giving a  $K_d = 0.0176 \mu\text{M}$ . (Lower) Titration with an incorrect nucleotide (dCTP), giving a  $K_d = 12.6 \mu\text{M}$  for the C-A mismatch. In each panel the decrease in protein fluorescence was fitted to a quadratic equation to obtain the  $K_d$ . The following synthetic oligonucleotide was used:

5'-TCCCTGTTTCGGGCGCCACGdd  
3'-AGGACAAGCCCGCGGTGCAGATCTCTAAAAGGTGT-5'.

of Kati *et al.* (14). Similar results were obtained for other nucleotide pairs.

The data presented here on the binding of dNTPs were obtained with a substrate analog, the dideoxy-terminated primer molecule in the primer/template complex. It is possible that the affinity of the triphosphate in the presence of a natural 3' end is different from that seen here. However, it seems unlikely that the modification would result in a higher affinity of the second substrate. We have also used other methods of terminating the primer molecule for such studies. In particular, cordycepin (3'-deoxyadenosine) is very useful for this purpose, since it can be used for large-scale chemical synthesis of terminated primers. Nucleotide titrations with primer/template pairs containing cordycepin-terminated primers gave identical affinities to those obtained with dideoxynucleotide-terminated molecules (data not shown), suggesting that the detailed chemistry of the terminal sugar residue is not of importance for the binding steps of the nucleotide substrate.

We (7) and others (14) have already presented results suggesting that the binding of primer/template complex is tighter in the ternary RT·p/t·dNTP complex, as shown by the reduction of the rate of dissociation of primer/template from such a complex, and this emphasizes the different properties of the nucleotide-induced state. The nature of the conformational change occurring in the second step in nucleotide

binding is at present unclear. One interesting possibility is that translocation of the primer/template complex takes place at this stage, rather than with the chemical step. The results presented here allow the design of experiments aimed at resolving this question. Comparison of the crystal structure of the complex  $\text{RT} \cdot \text{p/t}$ , formed on mixing RT with primer/template, with that of  $\text{RT} \cdot \text{p/t} \cdot \text{dNTP}$ , which could be formed as a stable complex by mixing RT, primer/template in which the primer is terminated by a 2',3'-dideoxynucleotide or other chain terminator, and a dNTP, could decide this question (i.e., at which stage translocation takes place) as well as clarify other structural changes between the  $\text{RT}$  and  $\text{RT}^*$  states.

Fig. 2 Lower shows the results of titrating the "wrong" (i.e., mismatched) deoxynucleotide to the chain-terminated  $\text{RT} \cdot \text{p/t}$  complex. It is clear that the affinity is dramatically reduced in comparison with that of the conjugate deoxynucleotide ( $K_d = 12.6 \mu\text{M}$  for dCTP instead of dTTP). However, the  $K_d$  value is still considerably lower than that obtained in the quenched-flow kinetic experiments of Kati *et al.* (1.2 mM; ref. 14), suggesting that even in the mismatched situation, the second step in the mechanism occurs with an equilibrium constant that is large (calculated to be 95). The work of Kati *et al.* (14) has shown that the effective rate of nucleotide transfer is also inhibited, typically being reduced by a factor of 10 or more. Since the present results (summarized in Table 1) at first sight suggest that  $K_4$  is hardly affected in the mismatch reaction, we conclude that the individual rate constants of step 4 (i.e., the forward and reverse rate constants) are affected (reduced) in a similar manner. Thus, discrimination at step 4 is of a kinetic nature, whereas it is thermodynamic in step 3. This point is of fundamental interest, since thermodynamic control is limited by the thermodynamics of the overall reaction catalyzed by the enzyme, whereas there is no principal limitation on the extent of kinetic control.

The experiments shown in Fig. 3 were performed to help determine which steps in the mechanism are influenced by the NNIs O-TIBO and Nevirapine. Similar titrations to those shown in Fig. 2 were performed in the presence of  $3 \mu\text{M}$  O-TIBO (Fig. 3 Upper) or  $3 \mu\text{M}$  Nevirapine (Fig. 3 Lower). Surprisingly, the protein fluorescence is enhanced on binding of dNTP in the presence of O-TIBO, and there is an increase in the  $K_d$  for dTTP by a factor of ca. 5 (to  $0.105 \mu\text{M}$ ), which could be due to an effect on  $K_3$ ,  $K_4$ , or both. Increasing the concentration of O-TIBO had no further effect on the affinity, so that one possible envisaged explanation for the O-TIBO inhibition mechanism (inhibition of step 4) does not seem to apply, or at least not to account for the potent inhibition seen. The remaining possibility is an effect on the chemical step. Powerful inhibition of step 5 without significant inhibition of step 4 would result in step 5 becoming rate-controlling in the overall reaction. If, as appears to be the case with NNIs in general, there is residual activity even at saturating inhibitor concentration, it should be possible to measure the effective  $K_d$  for dNTP in the polymerization reaction by using the quenched-flow method even in the presence of the inhibitor. The apparent  $K_d$  for dNTP in an experiment of this sort is determined by the overall affinity of the nucleotide up to the complex preceding the rate-limiting step. In the absence of NNI, since step 4 is the rate-limiting step, only step 3 contrib-

Table 1. Equilibrium constants for nucleotide binding

Nucleotide	$K_{\text{overall}}^*$ $\mu\text{M}$	$1/K_3^\dagger$ $\mu\text{M}$	$K_4^\ddagger$	$k_4^\ddagger$ $\text{s}^{-1}$
dTTP (correct)	0.018	5	280	30
dCTP (incorrect)	12.6	1200	95	0.5

\*Based on the fluorescence titration in this report.  $K_{\text{overall}} = 1/K_3(1 + K_4)$ .

$^\dagger$ From rapid-quench single-turnover measurements (14).

$^\ddagger$ Dimensionless; calculated from  $K_{\text{overall}}$ ,  $1/K_3$ , and  $k_4$ .

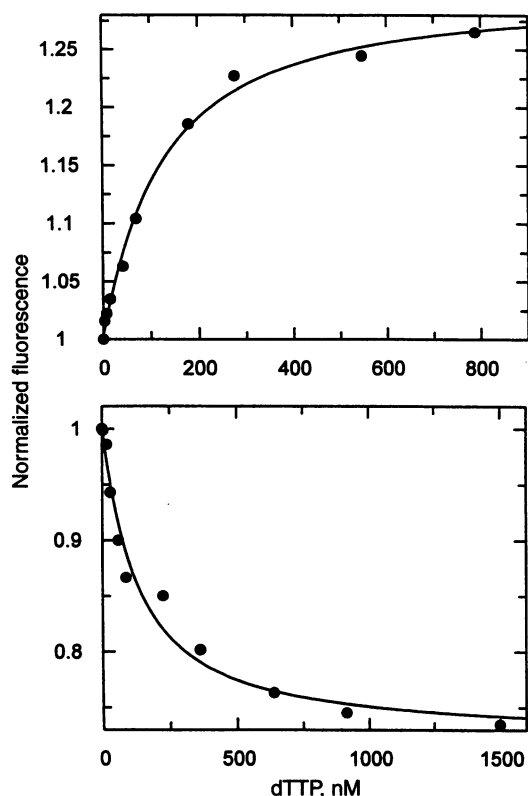


FIG. 3. Direct measurement of nucleotide binding in the presence of NNIs. Fluorescence titrations to measure nucleotide binding affinity in the presence of NNIs were performed with 0.04  $\mu$ M RT and 0.06  $\mu$ M DNA dideoxy-terminated primer/template under the conditions described in Fig. 2. (Upper) Titration in the presence of 3  $\mu$ M O-TIBO, giving a  $K_d = 0.105 \mu$ M. (Lower) Titration in the presence of 3  $\mu$ M Nevirapine, giving a  $K_d = 0.106 \mu$ M. In each figure, the change in protein fluorescence was fitted to the appropriate form of the quadratic equation to obtain the  $K_d$ . It is of interest to note that the direction of the fluorescence change was opposite in the presence of the two inhibitors.

utes, and  $K_d$  is equal to  $1/K_3$ . If the chemical step becomes rate controlling in the presence of NNI, the  $K_d$  should be determined by the product of  $K_3$  and  $K_4$  [or more exactly by  $K_3(1+K_4)$ ] in Scheme I. With the  $K_d$  value obtained in the presence of O-TIBO in the titration experiment (Fig. 3), we would expect a value of 0.105  $\mu$ M in the presence of O-TIBO for the quench experiment on the rate of nucleotide addition to the end of the primer. Spence *et al.* (5) have shown that in the presence of a NNI, the  $K_d$  value from a quench experiment on the rate of nucleotide incorporation is reduced from the typical value of 5  $\mu$ M in the absence of a NNI to 0.130  $\mu$ M in its presence. This is in perfect agreement with the prediction that arises from the model shown in Scheme I when using the data concerning step 4 obtained in the present work and assuming that the chemical step is strongly inhibited by the NNI. This agreement also confirms the similarity in the affinity of dNTPs to complexes of RT with primer/templates having natural (i.e., 2'-deoxynucleoside) or unnatural (2',3'-dideoxynucleoside) 3' ends.

The results discussed underline the interaction between the substrate and NNI binding sites on RT and, taken together with results from pre-steady-state studies on nucleotide incorporation (5), provide strong evidence that the chemical step itself is inhibited by the presence of such an inhibitor. It is

known from the crystal structure of RT complexed with NNIs that important interactions occur with Tyr-181 and Tyr-188 (1, 2). These residues flank the essential aspartates Asp-185 and Asp-186, which are situated on a turn between the two antiparallel  $\beta$ -strands in which the tyrosines are located. These aspartates are thought to be directly involved in the catalytic mechanism. Thus, relatively small changes in the positions of these residues caused by NNI binding to Tyr-181 and Tyr-188 could easily have a dramatic effect on the rate of the catalytic step without affecting nucleotide binding significantly.

The interaction between the nucleotide and NNI binding sites could have important implications for therapies based upon the coadministration of multiple drugs. It also means that a derivative combining the structural properties of a NNI with those of a dNTP could be an extremely potent RT inhibitor, under the assumption that the two parts of the molecule were linked in such a way that the nucleotide and nonnucleoside moieties could simultaneously reach their respective binding sites.

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