The Y181C Substitution in 3'-Azido-3'-deoxythymidine-resistant Human Immunodeficiency Virus, Type 1, Reverse Transcriptase Suppresses the ATP-mediated Repair of the 3'-Azido-3'-deoxythymidine 5'-Monophosphate-terminated Primer*

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Boulbaba Selmi‡§, Jérôme Deval‡§, Karine Alvarez‡, Joëlle Boretto‡, Simon Sarfati, Catherine Guerreiro, and Bruno Canard‡**

From the 3CNRS and Universités d'Aix-Marseille I and II, UMR 6098, Architecture et Fonction des Macromolécules Biologiques, ESIL-Casé 925, 163 Avenue de Luminy, 13288 Marseille Cedex 09 and the Institut Pasteur, 28 Rue du Docteur Roux, 75724 Paris Cedex 15, France

Resistance to zidovudine (3'-azido-3'-deoxythymidine, AZT) by the human immunodeficiency virus, type 1, requires multiple amino acid substitutions such as D67N/K70R/T215F/K219Q in the viral reverse transcriptase (RT). In this background of AZT resistance, additional “suppressive” substitutions such as Y181C restore sensitivity to AZT. In order to characterize the mechanism of this AZT resistance suppression, the Y181C substitution was introduced into both wild-type and AZT-resistant reverse transcriptase. The introduction of the Y181C substitution suppresses the increased repair (or unblocking) of the AZTMP-terminated primer provided by the AZT resistance substitutions in RT using either DNA or RNA templates, independently from the RT RNase H activity. Contrary to wild-type RT, the low level of unblocking activity is not due to inhibition by the next correct nucleotide binding to the RT/AZTMP-terminated primer complex. When Y181C is added to the AZT resistance substitutions, ATP binds with less affinity to the AZTMP-terminated primer-RT binary complex. These results provide an insight into one possible molecular mechanism of re-sensitization of AZT-resistant viruses by suppressive substitutions.

The replication of the human immunodeficiency virus (HIV) depends on its own DNA polymerase, the viral reverse transcriptase (RT) (1). This enzyme is a major target for drugs acting as inhibitors of retroviral replication. During the HIV replication cycle, RT exhibits several activities such as RNA- and DNA-dependent polymerization, DNA-dependent RNA cleavage (RNase H), strand transfer, and strand displacement (2–5). Because dNTPs are natural substrates for RT, nucleoside analogues are currently used in vitro, in clinical trials, and in the clinic to inhibit retroviral replication through DNA chain termination (6, 7).

3'-Azido-3'-deoxythymidine (AZT, zidovudine) is such an analogue and was the first drug approved against HIV-1 (8). However, HIV-1 mutants escaping AZT inhibition were isolated soon after the introduction of AZT in the clinic (9). Genotypic analyses of these viruses have shown that a typical pattern of amino acid substitutions occurred in the pol gene encoding the viral RT. Resistance to AZT is acquired through the selection of specific substitutions such as M41L, D67N, K70R, L210W, T215F or T215Y, and K219Q in the RT molecule. These mutations, termed “TAMs” for thymidine-associated mutations, can confer >100-fold resistance to HIV-1 against AZT and d4T (stavudine) (7, 9). The first phenotypic difference between wild-type and AZT-resistant RT to be identified in vitro was the increased processivity of RNA-directed DNA synthesis catalyzed by the D67N/K70R/T215F/K219Q RT compared with that of wild-type RT (10). Canard et al. (11) have shown that AZT-resistant RT binds in a different manner to AZTMP-terminated DNA chains than wild-type RT, suggesting that AZT resistance might be an RT-mediated repair mechanism. Subsequently, it was shown that one AZT resistance mechanism involves repair of the AZTMP-terminated primer either by ATP (12) or inorganic pyrophosphate (13). These repair reactions have in common the nucleophilic attack of a hydroxyl group from either the γ-phosphate of ATP or PPi on the terminal phosphodiesther bond of the AZTMP-terminated DNA primer. The product of this repair reaction is a free 3'-OH group allowing DNA synthesis to resume.

A class of non-nucleoside RT inhibitors (NNRTIs), such as nevirapine, inhibits HIV-1 by a different mechanism than chain termination (14, 15). Thus, NNRTIs are effective against AZT-resistant mutant viruses (16). Nevirapine is a potent, highly specific inhibitor that binds to HIV-1 RT by a mechanism that is noncompetitive with respect to primer, template, initiation tRNA<sup>poly</sup>, and nucleotide (17, 18). This inhibitor binds to conserved residues tyrosine 181 and 188 of the p66 subunit of RT in a hydrophobic pocket adjacent to the polymerase-active site. The Nevirapine-binding site is defined by two β-sheets from amino acid residues 100–110 and 180–190, respectively (18–20). In contrast to AZT resistance, high level resistance to Nevirapine can be conferred by a single mutation at codon 181 (Tyr to Cys) (21). Introduction of this change into either the recombinant enzyme or virus increases the median inhibitory concentration (IC<sub>50</sub>) 100-fold for Nevirapine (22).
While conferring resistance to NNRTI, however, the Y181C substitution in an AZT-resistance background (D67N/K70R/T215Y/K219Q) suppresses AZT resistance, i.e. restores AZT sensitivity to wild-type levels (21). These types of substitutions conferring resistance to a drug while annihilating resistance to another drug have been termed “suppressive” substitutions. Among the most clinically relevant are L74V, M184V, or Y181C, which suppress AZT resistance while conferring resistance to didanosine, lamivudine, and nevirapine, respectively (21, 23, 24). Sensitization to AZT by M184V is the only substitution for which suppression has been studied at the enzyme level. AZT sensitization is observed both in the presence and the absence of the mutations that confer resistance to AZT and affects repair of the AZTMP-terminated primer (25, 26).

A detailed mechanistic view of drug resistance suppression is thus lacking. Understanding the mechanisms of drug resistance and drug resistance suppression may provide a basis for a rational combination of drugs of sustained efficacy (6, 25, 27).

We have introduced the Y181C substitution into both wild-type and AZT-resistant RT, purified the corresponding enzymes, and studied their biochemical properties toward AZT resistance. In this report, we show that the Y181C substitution does not change nucleotide selectivity in a manner consistent with AZT resistance suppression, but suppresses repair of the AZTMP-terminated DNA chain by ATP and PP.

EXPERIMENTAL PROCEDURES

Proteins, Reagents, Expression, and Purification of Enzymes—Wild-type and AZT-resistant (D67N/K70R/T215Y/K219Q) RT gene constructs have been described (28). Both plasmids were used to construct mutant RT genes bearing Y181C and D67N/K70R/Y181C/T215Y/K219Q/Y181C substitutions by using synthetic oligonucleotides and a strategy described previously (29). The RNaseH + version of the four RTs studied here was obtained after introduction of the E478Q substitution using the same approach (30). All constructs were verified by restriction enzyme analysis and nucleotide sequencing to confirm that no unwanted changes were introduced. Recombinant RTs were coexpressed with the HIV-1 protease in Escherichia coli to obtain p66/p51 heterodimers, which were later purified to homogeneity by using affinity chromatography as described (29). RTs were assayed by active-site titration as described before (29, 31). DNA oligonucleotides were obtained from Invitrogen. Oligonucleotides were 5'-32P-labeled using T4 polynucleotide kinase (New England Biolabs). All γ32P-labeled adenosine 5'-triphosphate, 2'-deoxy, and 2',3'-dideoxynucleoside 5'-triphosphates were purchased from Amersham Biosciences. AZT 5'-triphosphate was synthesized and purified as described (28). Bacteriophage MS2 genomic RNA was purchased from Roche Applied Science.

Pre-steady State Kinetics of a Single Nucleotide Incorporation into DNA—Pre-steady state kinetics were performed using AZTTP and dTTP in conjunction with wild-type, AZT-resistant RT, and AZT-resistant Y181C as described (31). The DNA/DNA primer-template used for the rapid reactions was a 5'-labeled 21-mer primer (5'-ATACTTATCACATTATCC-3') annealed to a 31-mer template (5'-TATTGTCTAGTTATATTATTTTTTTATTTTTTTTTGGATA-3') for the incorporation of dTTP and AZTTP. The formation of product (P) over time was fitted with a burst equation 1,

\[
P(t) = A(1 - \exp(-k_{app}t)) + k_{1}t
\]  

where A is the amplitude of the burst, k_{app} is the apparent kinetic constant of formation of the phosphodiester bond, and k_{1} is the kinetic constant of the steady state, linear phase. Although curve fitting was better with an equation containing two exponential terms (32, 33) than with Equation 1, either equation gave the same dependence of k_{app} on dNTP concentration, described by the following hyperbolic Equation 2,

\[
k_{app} = k_{amp}(dNTP/K_{a} + dNTP)
\]  

where K_{a} and k_{amp} are the equilibrium and the catalytic rate constant of the dNTPase reaction, respectively. K_{a} and k_{amp} were determined from curve-fitting using the Kaleidagraph software. These constants were used to calculate preference and resistance as described previously (34, 35).

Pyrophosphorolysis and ATP-mediated Repair Assays—The primer-template system used for pyrophosphorolysis and ATP-mediated repair assays was a 5'-32P-end-labeled 21-mer DNA primer (5'-ATACTTATCACATTATCC-3') annealed to a 31-mer (5'-TTGGGCCGATCGG CATAGTTAAGATG-3') DNA template. The single template A specifying a single thymidine insertion site 1 or 4 bases away (RNA and DNA template, respectively) from the 3' end of the primer is shown in boldface. DNA polymerization was initiated by the addition of wild-type or mutant RT (100 nM) in RT buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.05% Triton X-100) and nucleotides (25 μM each of dATP, dCTP, and dGTP) and data were fitted to a hyperbola to obtain the IC50 for each primer and template combination. The template A specifying the first possible thymidine insertion site was 10 bases away from the 3' end of the primer. Primers were added with wild-type or mutants RT (100 nM) in RT buffer, and polymerization was initiated by the addition of nucleotide mixture (50 μM each of dATP, dCTP, and dGTP) and 10 μM analogue-TP (AZTTP) in 6 mM MgCl₂ for 15 min at 37 °C. The repair reaction was started by adding dTTP to reach a final concentration of 50 μM in the presence of either RT, as indicated. Aliquots were withdrawn during the time course of the reaction and products were analyzed by denaturing gel electrophoresis (28). The % repair of blocked primer is the ratio ×100 of extension products larger than 22 or 25 nucleotides over those larger than 21 or 24 nucleotides (for RNA or DNA template, respectively). Values were calculated as averages from three independent experiments and differed by less than 10%.

ATP-mediated Repair Assays on a Primed Bacteriophage MS2 RNA Template—The primer-template system used for ATP-mediated repair assays was a 5'-32P-end-labeled 21-mer DNA primer (5'-AGCTACG- CGAGGAGGCTCGC-3') annealed to a MS2 single-stranded RNA template (GenBank accession number V00642). The template A specifying the first possible thymidine insertion site was 10 bases away from the 3' end of the primer. Primers were added with wild-type or mutants RT (100 nM) in RT buffer, and polymerization was initiated by the addition of nucleotide mixture (50 μM each of dATP, dCTP, and dGTP) and 10 μM analogue-TP (AZTTP) in 6 mM MgCl₂ for 15 min at 37 °C. The repair reaction was started by adding dTTP to reach a final concentration of 50 μM in the presence of ATP, as indicated. Aliquots were withdrawn during the time course of the reaction, and products were analyzed by denaturing gel electrophoresis (28). The % repair of blocked primer is the ratio ×100 of extension products larger than 31 nucleotides over those larger than 30 nucleotides. Values were calculated as averages from three independent experiments and differed by less than 10%.

ATP-mediated Repair in the Presence of the Next Correct Nucleotide dGTP—The primer-template system used for ATP-mediated repair assays was a 5'-32P-enabeled 24-mer DNA primer (5'-ATACCTTTAACA TATCATGATCGGCCA-3') annealed to a 35-mer (5'-GTCGCGTGTGCG CGCGATACATGTTAAGATG-3') DNA template (25 μM). The single template A specifying a single thymidine insertion site 1 base away from the 3' end of the primer is shown in boldface. The AZTMP-terminated primer-DNA template was synthesized by the addition of wild-type or mutant RT (10 nM) and 5 μM analogue-TP (AZT-TP) for 15 min at 37 °C in RT buffer. The repair reaction was started by the addition of ATP (3.2 mM), dTTP, dCTP, and dATP (25 μM each), and the next correct nucleotide dGTP to reach various final concentrations (5, 25, 100, 500, 750, and 1000 μM). Aliquots were withdrawn during the time course of the reaction, and the reaction was stopped with EDTA/formamide. The rate of repair was determined during the linear phase of the reaction (between 0 and 60 min), using denaturing gel electrophoresis analysis. Under all conditions tested, the 15-min time point was found to be in the linear phase of the initial reaction rate. The percentage of inhibition was calculated at this time point for all next correct nucleotide concentrations. The percent inhibition relative to the uninhibited reaction was plotted against the concentration of dGTP, and data were fitted to a hyperbola to obtain the IC₅₀ for each enzyme. Values were calculated as averages from two independent experiments.

Pre-steady State Kinetics of ATP-mediated Repair of the AZT-terminated Primer—The primer-template system used for this study was an AZTMP-terminated 22-mer DNA primer (5'-ATACCTTTAA CATTATCGGTATTAGT-3') annealed to a 35-mer (5'-TTGGGCCGATCGG CATAGTTAAGTAAGATG-3') DNA template. The AZTMP-terminated primer was prepared by the addition of a terminal AZTMP (25 μM) described above). The addition of ATP (3.2 mM) to the reaction mixture initiated the repair, and products were analyzed by denaturing gel electrophoresis. The concentration dependence was fitted to a Michaelis-Menten model to estimate the K_m and V_max for the reaction. When ATP is the only substrate, the reaction rate is expressed as

\[
V = \frac{V_{m}[S]}{K_{m} + [S]}
\]  

where V is the reaction rate, V_m is the maximum reaction rate, [S] is the substrate concentration, and K_m is the Michaelis constant.
Y181C substitution in RT is a major resistance determinant of AZT-resistant RTs. When Y181C is introduced into an RT bearing the AZT resistance substitutions D67N/K70R/T215Y/K219Q, AZT resistance is lost, indicating that the presence of Y181C antagonizes the RT-mediated mechanism of AZT resistance. One AZT resistance mechanism is elucidated at the nucleotide level; the AZTMP-terminated primer was examined comparatively using RT bearing substitutions Y181C and AZT-resistant/Y181C RT, the 2.8-fold difference in nucleotide incorporation efficiencies is reduced (compare $k_{pol}/K_a$ for AZTTP to $k_{pol}/K_d$ for AZTMP, leading to a 1.28-fold preference for AZTTP). These data indicate that AZT-resistant/Y181C has lost the preference for AZTTP and is thus becoming resistant, not sensitive, to AZTTP. This increase in AZTTP resistance is 1.80/0.86 = 2.1-fold. As neither relative affinities nor incorporation efficiencies vary in a manner consistent with suppression, we conclude that AZTTP re-sensitization through nucleotide selectivity is not involved in the Y181C-mediated AZT resistance suppression.

The repair of the AZTMP-terminated primer was examined. ATP- and PPγ-mediated Repair of an AZTMP-terminated Primer Annealed to RNA Templates—Removal of AZTMP from a chain-terminated primer is one mechanism of AZT resistance (12, 13). Either PPγ or ATP can serve as the unblocking agent once DNA synthesis is terminated by AZTMP. In this reaction, RT uses the PPγ or the γ-phosphate of a nucleoside 5′-triphosphate to unblock the chain-terminated DNA, which can be extended again by canonical dNTPs. When the phosphate donor is PPγ, the reaction is pyrophosphorylization, the reversal of the polymerization reaction. The role of ATP in AZT resistance mediated by TAMs is increasingly well documented and established, whereas the role of PPγ, in AZT resistance is unclear. Indeed, AZT-resistant RT was reported to exhibit the same (40), a higher (13, 28), or a lower (12, 41, 42) pyrophosphorylization activity than wild-type RT.

The effect of PPγ and ATP in the repair reaction was first examined sequentially. In this assay, a 5′-32P-labeled DNA primer (21-mer) was annealed to an RNA template (31-mer) containing a single dTMP (or AZMP) insertion site immediately adjacent to the 3′ end of the primer, and AZTTP and RT are added to the reaction. Once DNA synthesis is terminated by the insertion of AZTMP, either PPγ or ATP is added together with dTTP to the reaction. To mimic conditions found under physiological conditions, millimolar concentrations of ATP are generally used (12). Extension products are diagnostically significant for the repair reaction. Fig. 1A shows the results of an experiment performed with ATP (three molar) as the unblocking agent. In this case, ATP unblocks the DNA chain with the concomitant

| TABLE I | Y181C and Suppression of Zidovudine Resistance |
|---------|---------------------------------------------|
| Wild-type RT | AZT-resistant RT | AZT-resistant/Y181C RT |
| $K_a$ (µM) | $k_{pol}$ (s⁻¹) | $K_d$ (µM⁻¹ s⁻¹) |
| dTTP | AZTTP | dTTP | AZTTP | dTTP | AZTTP |
| 17.5 ± 1.7 | 16.9 | 46.7 | 7.9 | 78.6 | 4.0 |
| 12.8 ± 1.9 | 1.9 | 8.5 | 4.0 | 3.7 | 1.9 |
| 0.75 ± 0.15 | 0.115 | 1.8 | 0.5 | 0.09 | 0.115 |
| Preference for AZTTP | 2.4 | 2.8 | 1.28 | 1.5 |
| Resistance | 0.96 | 1.5 | 1.38 | 1.25 |

$K_a$ and $k_{pol}$ were determined as described under "Experimental Procedures." S.D. was < 20%.

The kinetic analysis is summarized in Table I. AZTTP binds to wild-type RT ($K_d = 7.1$ µM) with a 2.5-fold greater affinity than dTTP ($K_d = 17.5$ µM). Catalytic rate constants ($k_{pol}$) for both substrates (dTTP and AZTTP) are comparable (13.2 and 12.8 s⁻¹, respectively). The ratio of nucleotide incorporation efficiencies (1.8/0.75) indicates that wild-type RT actually prefers 2.4-fold AZTTP relative to dTTP.

As shown in Table I, the AZT-resistant RT binds to AZTTP ($K_d = 7.9$ µM) with a 6-fold better affinity than that observed for dTTP ($K_d = 46.7$ µM). The catalytic rate constant ($k_{pol}$) for dTTP obtained by AZT-resistant RT is 8.5 s⁻¹, which is over 2-fold higher than that for AZTTP ($k_{pol} = 4$ s⁻¹). AZT-resistant RT also exhibits a higher overall incorporation efficiency ($k_{pol}/K_d$ for AZTTP (0.5 s⁻¹µM⁻¹) than for dTTP (0.18 s⁻¹µM⁻¹)). Thus, similarly to wild-type RT, AZT-resistant RT does not discriminate AZTTP and still prefers 2.8-fold AZTTP relative to dTTP. Both relative affinities (as judged by $K_d$ values for dTTP and AZTTP) and incorporation efficiencies do not support a resistance mechanism involving discrimination, a long known result in accordance with others (36, 39, 40).

In the case of AZT-resistant/Y181C RT, catalytic rate constants obtained for dTTP and AZTTP are quite similar (3.7 and 1.9 s⁻¹µM⁻¹, respectively). When Y181C is added to the AZT-resistant background, a 2.3-fold increase in the affinity to AZTTP is observed over dTTP. Thus, AZT-resistant/Y181C RT reduces the ratio of $K_d$/dTTP/$K_d$/AZTTP observed using AZT-resistant RT (6-fold) to that of wild-type RT (2.3 for AZT-resistant/Y181C and 2.3-fold for wild-type). This change in relative affinity is not consistent with the suppression of resistance. By using AZT-resistant/Y181C RT, the 2.8-fold difference in nucleotide incorporation efficiencies is reduced (compare $k_{pol}/K_a$ for AZTTP to $k_{pol}/K_d$ for AZTMP, leading to a 1.28-fold preference for AZTTP). These data indicate that AZT-resistant/Y181C has lost the preference for AZTTP and is thus becoming resistant, not sensitive, to AZTTP. This increase in AZTTP resistance is 1.80/0.86 = 2.1-fold. As neither relative affinities nor incorporation efficiencies vary in a manner consistent with suppression, we conclude that AZTTP re-sensitization through nucleotide selectivity is not involved in the Y181C-mediated AZT resistance suppression.

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release of AppppAZT which is not detected under these experimental conditions (12). The 3'-OH group of the primer DNA is now free and can be extended by dTTP present in the reaction up to a product corresponding to the length of the RNA template. Quantitative results extracted from Fig. 1A are presented graphically in Fig. 1B. AZT-resistant RT shows a higher (2.6-fold) ability to unblock the AZTMP-terminated primer than wild-type RT. These results are in agreement with those previously observed by others (12). When present in either AZT-resistant or wild-type RT, the Y181C substitution decreases (about 2-fold) the ability of each respective RT to unblock the AZTMP-terminated primer. We conclude that the Y181C substitution exhibits a suppressive effect on ATP-mediated repair of the AZTMP-terminated primer by RT.

Similar experiments were conducted using PPi as the unblocking agent, and results are presented in Fig. 1C. RT-mediated repair of the AZTMP-terminated primer using 150 μM PPi proceeds with a much faster rate than when 3.2 mM ATP are used as the unblocking reagent (compare Fig. 1B to C). However, unlike when ATP is used as the unblocking agent, AZT-resistant RT does not exhibit an increased PPi-mediated repair of the AZTMP-terminated primer relative to wild-type RT (Fig. 1C, see also the discussion section). In either AZT-resistant or wild-type RT, though, the presence of the Y181C substitution decreases pyrophosphorolysis of the AZTMP-terminated primer.

In order to verify if these results could be reproduced under different experimental conditions, a DNA primer was annealed to a single-stranded RNA molecule provided by the RNA genome of bacteriophage MS2. This primer-RNA template was used to monitor RT-mediated repair of the AZTMP-terminated primer under conditions described previously (see “Experimental Procedures”). The first AZTMP-insertion site is located 10 bases away from the 3'-end of the primer. Chain termination is

![Fig. 1. Unblocking of incorporated AZTMP by wild-type and variant RT using a DNA/RNA primer-template.](image-url)
first achieved by the addition of a mix of nucleotides in which dTTP had been replaced by AZTTP. The repair reaction is then started by the addition of ATP (3.2 mM), in the presence of dTTP. As shown in Fig. 1D, a significant amount (2-fold) of rescued DNA polymerization products are observed in the reaction catalyzed by AZT-resistant RT relative to wild type. The presence of Y181C decreases the ability to unblock the AZTMP-terminated primer either for wild-type or AZT-resistant RT. These results agree with those observed using the DNA/RNA primer-template system (Fig. 1B).

Therefore, we conclude that under these experimental conditions, ATP-mediated repair is more efficient than PPi-mediated repair. Introduction of the Y181C substitution into either wild-type RT or a D67N/K70R/T215F/K219Q substitution background in RT diminishes both ATP- and PPi-mediated repair of the AZTMP-terminated primer annealed to RNA templates.

**Suppression of ATP-mediated Repair Is Independent from RNase H Activity**—The fact that RNase H activity resides on the same polypeptide chain as the polymerase activity suggests a coupling between degradation of the genomic RNA and synthesis of DNA in the first step of reverse transcription (43, 44). It was shown that some RNA cleavage occurs regardless of the position of the 3' end of the annealed DNA oligonucleotide, confirming that not all cleavage sites are directed by binding of the polymerase to a primer (45). In order to avoid the RNA template cleavage during DNA polymerization, it was of interest to test the ATP-mediated repair of AZTMP-terminated primer by RT in which the RNase H activity is absent. The substitution E478Q in RT is known to inactivate its RNase H activity (30). This substitution was introduced in the RT genes investigated here, the corresponding enzymes purified, checked for their inability to cleave RNA on RNA/DNA duplexes, and used to study repair of the AZTMP-terminated primer. The primer-template system used is a 5'-32P-labeled DNA primer (21-mer) annealed to RNA template (31-mer) containing a single dTMP (or AZTMP) insertion site under conditions described above. Fig. 1E shows the results of such an experiment. Addition of ATP (3.2 mM) and dTTP in the reaction medium induces an increase in the rate of the ATP-mediated repair of AZTMP-terminated primer by the AZT-resistant RT/RNase H relative to wild-type RT/RNase H-. This increase is similar to the increase observed using RNase H+-wild-type and AZT-Res RTs (Fig. 1B). The presence of the AZT-resistant/Y181C/RNase H--substitutions suppresses the ATP-mediated repair activity toward AZTMP-terminated primers to a level comparable with that of wild type (RNase H-). The wild-type, AZT-resistant/Y181C and Y181C RTs altered in their RNase H domains show about a 2-fold increase in the rate of the ATP-mediated repair of AZTMP-terminated primer relative to the corresponding RTs unaltered in their RNase H domains. Much like in the case of the latter RTs (Fig. 1, A and B), the Y181C substitution confers the lowest repair activity to RT. Therefore, we conclude RNase H activity does not seem to be involved in suppression of the ATP-mediated repair of the AZTMP-terminated primer.

**ATP- and PPi-mediated Repair of an AZTMP-terminated Primer Annealed to a DNA Template**—Because HIV-1 replication involves both a RNA-dependent and a DNA-dependent DNA polymerization, experiments similar to those described above were conducted by using a DNA template. The results are presented in Fig. 2. By using ATP as the unblocking agent, AZT-resistant RT shows a higher (1.7-fold) ability to unblock the AZTMP-terminated primer than wild-type RT (Fig. 2A), in agreement with results from others (12). When tested with AZT-resistant/Y181C RT, a lower ability of ~58 and 30% to unblock the terminal AZTMP was observed relative to AZT-resistant and wild-type RT, respectively. As a control, reverse transcriptase bearing Y181C was tested under these experimental conditions. The Y181C substitution alone decreases the ability to unblock the AZTMP relative to AZT-resistant/Y181C and AZT-resistant RT. Similar experiments were conducted using PPi, as the unblocking agent (Fig. 2B). Much like the results obtained using RNA templates, the AZT-resistant RT showed a slightly decreased pyrophosphorolytic repair activity toward the AZTMP-terminated primer relative to wild-type RT. The presence of the Y181C substitution increases this tendency, confirming the role of the Y181C substitution in impeding the repair reaction. We conclude that the Y181C substitution exhibits a suppressive effect on ATP-mediated repair of the AZTMP-terminated primer by RT on both RNA and DNA templates.

**AZT Resistance Suppression by Y181C in the Presence of the Next Correct Nucleotide**—The increase in ATP-mediated repair by AZT-resistant RT relative to wild type is due to the alteration of biochemical properties that are pre-existing in wild-type RT (12). In addition to facilitating repair per se, AZT resistance substitutions decrease the propensity of RT to make a dead-end complex (DEC) unable to repair the AZTMP-terminated primer (12). The DEC involves the next complementary dNTP binding to RT in complex with the analogue-terminated primer (12). By impeding the formation of the DEC, AZT resistance substitutions favor excision of terminal ddNMP by

![Fig. 2. Pyrophosphate and ATP-mediated unblocking of incorporated AZTMP by wild-type and variant RT on DNA/DNA primer-template.](image)
ATP to yield Apppp(ddN) and a 3'-OH terminated primer. Thus, the influence of the next correct nucleotide on repair correlates with the presence or absence of the DEC.

The effect of Y181C on repair in the presence of the next correct nucleotide was investigated using wild-type, AZT-resistant, and AZT-resistant/Y181C RT. In this assay, AZTMP is first added at the 3'-terminus of a DNA primer using RT. The RT-mediated repair reaction of this AZTMP-terminated primer is then started by the addition of ATP (3.2 mM), dTTP, dCTP, dATP (25 μM each), and the next correct nucleotide dGTP at various final concentrations (5, 25, 100, 250, 500, 750, and 1000 μM). dGTP binds to the RT/AZTMP-terminated primer complex and inhibits ATP-mediated repair.

The results are presented in Fig. 3, which shows both the repair efficiencies of RT and the inhibition of repair by dGTP. Wild-type RT is inhibited by increasing amounts of dGTP, with a repair IC_{50} of 62 ± 8 μM (Fig. 3, A and B). Fig. 3A shows that AZT-resistant RT repairs more efficiently the AZTMP-terminated primer than the other RTs, in accordance with our previous results. But in addition, dGTP has a very weak effect on repair inhibition (IC_{50} = 818 ± 86 μM, Fig. 3, A and B). We conclude that next correct nucleotide concentration is not relevant to explain suppression of repair, raising the possibility that Y181C impedes DEC formation.

Kinetics of ATP-mediated Repair of AZT-terminated Primer—
ATP, as well as other NTPs, is a substrate of the repair reaction. It binds to the RT/AZTMP-terminated primer complex and reacts with the terminal AZTMP to yield AppppAZT (12). The influence of Y181C on the kinetics of binding of ATP to this complex was thus examined. In this experiment, the primer-template-RT complex is made, and the rate of the unblocking reaction is measured as a function of ATP concentration, giving access to steady state kinetic parameters K_{m(ATP)} and k_{cat(ATP)}.

The effects of the Y181C substitution were examined in an AZT-resistant background. Results are presented in Fig. 4 which shows a gel analysis of the removal of the terminal AZTMP by 3.2 mM ATP using wild-type, AZT-resistant, and AZT-resistant/Y181C RT (Fig. 4, A–C, respectively). AZT-resistant RT (Fig. 4B) possesses a greater ability to remove the
tation of experiments performed as described in "Experimental Procedures." Denaturing gel electrophoresis analysis and autoradiography of the reaction products generated over time by 3.2 mM ATP are shown with wild-type RT (A), AZT-resistant RT (B), and AZT-resistant/Y181C RT (C). The relative amount of unblocked AZTMP-terminated primer (indicated as −AZTMP) was quantitated as the amount of products smaller in size than the initial AZTMP-terminated primer divided by the total amount of radioactive band products. D, graphic representation of the quantification of experiments performed as described in A–C, using ATP concentrations of 0.25, 0.5, 1, 2, 3.2, 5, 6, and 15 mM. Wild-type RT, AZT-resistant RT, and AZT-resistant/Y181C RT are labeled as WT-RT (○), Res-RT (□), Sup-RT (○), respectively.

Steady state kinetics parameters for the ATP-mediated AZTMP-terminated primer unblocking reaction by HIV-RT.

| RT                |  
|-------------------|
|                   | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ | Effect |
|-------------------|-----------------|-------|----------------------|--------|
| Wild-type RT      | 5.5             | 1.1   | 0.048                | 1      |
| AZT-Res RT        | 29.1            | 2.3   | 0.124                | 2.6    |
| AZT-Res/Y181C RT  | 20.1            | 4.1   | 0.049                | 1      |

$^a$ $k_{\text{cat}}$ and $K_m$ were determined from Fig. 4, as described under “Experimental Procedures.” S.D. was <20%.

II. The AZT resistance substitutions increase 5.2-fold the apparent rate constant $k_{\text{cat}}$ of the unblocking reaction, from 5.5 to 29 $10^{-5}$ s$^{-1}$ for wild-type and AZT-resistant RT, respectively. $K_m(\text{ATP})$, is slightly affected from 1.1 to 2.3 mM for wild-type and AZT-resistant RT, respectively. The values of $K_m(\text{ATP}) = 1.1 \text{ mM}$ compares well with the value of 1.6 ms obtained by others (46).

The presence of the Y181C substitution has a detrimental effect on the unblocking reaction at the levels of both unblocking rate and affinity of ATP for the complex poised for the unblocking reaction. The apparent rate constant $k_{\text{cat}}$ is decreased from 29 to 20 $10^{-5}$ s$^{-1}$, whereas the affinity for ATP is further decreased as judged by an increase in $K_m(\text{ATP})$, up to 4.1 mM. These constants result in an overall efficiency of the unblocking reaction of 0.048, 0.124, and 0.049 s$^{-1}$mM$^{-1}$ for wild-type, AZT-resistant, and AZT-resistant/Y181C RT, respectively. The AZT resistance substitutions increase 2.6-fold the ability to remove the terminal AZTMP, and the presence of Y181C in the AZT resistance background restores an unblocking efficiency of 0.048 s$^{-1}$mM$^{-1}$ nearly identical to that of wild-type RT (0.049 s$^{-1}$mM$^{-1}$).

To summarize and conclude our study, the introduction in RT of the Y181C substitution in an AZT resistance substitution background does not re-sensitize RT to AZTTP through a nucleotide selection mechanism. Instead, the presence of Y181C decreases the unblocking activity provided by the AZT resistance substitutions on DNA and RNA templates, independently from the RT RNase H activity. The suppressive effect on repair is not due to inhibition by the next correct nucleotide but at least in part to a decreased affinity of the binary complex for the pyrophosphate donor ATP.

**DISCUSSION**

Drug resistance is a common problem encountered during antiviral chemotherapies, and in this context, suppressor mutations have become increasingly important. Indeed, the presence of suppressor mutations might allow the use of a rational combination of drugs ensuring sustained treatment efficacy (6). It is therefore important to understand the precise mechanism of resistance suppression because it may improve the design of second generation nucleoside analogues and their use in the clinic.

Among suppressive substitutions, those of AZT resistance are best documented. The mechanism of AZT resistance provides a test ground to study possible suppression mechanisms at play in the RT active site. Suppressive mutations were identified early in the development of antiretroviral drugs, and their potential importance is recognized immediately. AZT was the first drug to induce a specific pattern of resistance substitutions in RT, and the subsequent addition of the Y181C substitution into an AZT resistance background was the first suppressive substitution identified (21). Subsequently, other AZT resistance suppressor mutations were documented, such as K65R, L74V, L100I, M184V, or A114S (6, 21, 23, 24, 27, 47–52). The M184V substitution in HIV-1 RT causes resistance to lamivudine and increases the sensitivity of the virus to AZT both in the presence or the absence of mutations conferring...
resistance to AZT (25, 26). However, a complete understanding of the mechanisms of drug resistance has long been awaited in order to understand, in turn, how mechanisms of suppression could function.

Drug resistance by HIV RT can arise by two basic mechanisms. First, the interaction of the drug with RT can be altered by amino acid substitutions specifying resistance. In the case of NRTIs, whether the binding affinity or the catalytic rate of incorporation into DNA can be altered (35, 36, 38, 53). In the case of AZT monotherapy, however, resistance occurs by a second type of mechanism. The specific set of substitutions M184V/L+/D67N/K70R/L210W/T215F or T215Y/R219Q does not alter significantly either the binding constant $K_d$ of AZTTP for AZT-resistant RT or the catalytic rate constant $k_{cat}$ of incorporation of AZTTP into DNA relative to wild-type RT. Instead, these substitutions facilitate the catalytic removal of the terminal AZTTP by AZT-resistant RT relative to wild-type RT. This repair reaction requires a pyrophosphate donor under the form of pyrophosphate or a nucleoside 5’-triphosphate. In an attempt to understand further the molecular basis of this enhanced reaction, Meyer et al. (12) have shown that RT-mediated repair is enhanced at two levels in AZT-resistant RT. First, the catalytic removal of the terminal AZTMP is enhanced through a better ATP affinity provided mainly by the 41 and 215 substitutions. Second, these substitutions decrease the formation of the DEC unable to excise the terminal AZTMP (12, 28, 54, 55).

In vivo, the nature of the preferred pyrophosphate donor in the repair reaction in RT-mediated AZT resistance is unknown. We have found that the difference in $K_p$-dependent repair activity between wild-type and AZT-resistant RT varies with enzyme preparation as well as with experimental conditions, and the reason for this is unknown. Nucleotide-dependent repair seems more robust under a variety of experimental conditions; the AZT-resistant RT is always more efficient than wild-type RT to repair the AZTMP-terminated primer, in agreement with others (12, 41, 56). In our study, although repair mediated by 150 $\mu$M PP is much more powerful that repair mediated by 3.2 mM ATP (compare Fig. 1, B to C), the difference in repair efficiency between wild-type and AZT-resistant RT argues strongly in favor of a better significance of NTP-mediated repair rather PP$_p$-mediated repair in vivo. Also of interest is the fact that $K_m$(ATP), values reported here (1.1 to 4.1 mM) are close to values of NTP concentrations found in cells, making the nucleotide-dependent repair reaction likely to play an important role in vivo.

In this context, the Y181C substitution could mediate different effects on the AZT resistance mechanism. Y181C could mediate an enhanced AZTMP incorporation relative to that of dTTP, re-sensitizing the virus to AZT despite an enhanced repair brought by the AZT resistance mutations. Our results show that Y181C has no effect in this direction. Instead, Y181C present in this AZT resistance background provides a 2-fold resistance to AZTTP. We interpret these results as a confirmation that nucleotide selectivity is irrelevant in the process of nucleoside resistance when high levels of repair of the terminated primer pre-exist in RT (57).

Our results show that Y181C influences the ATP-mediated repair of the AZTMP-terminated DNA primer. We further show that this effect is independent from the nature of the template (RNA or DNA) and does not depend on the presence of an intact RNase H active site. Instead, our results show that Y181C counteracts the enhanced repair reaction exhibited by AZT-resistant RT and restores low repair levels comparable with wild type.

What is the precise mechanism of the Y181C-mediated suppression of repair? There are several steps in the repair reaction that could potentially be altered by the presence of the Y181C substitution. The repair reaction depends on the formation of a binary complex made of RT and AZTMP-terminated DNA primer-template. A pyrophosphate donor then adds to make a ternary complex able to engage in the repair reaction. Meyer et al. (12) have discovered the existence of a second inactive form of RT with its primer-template. Our present results are in agreement with repair IC$_50$ of 110 ± 40 and 230 ± 40 $\mu$M for DEC reported by Meyer et al. (12) in the case of wild-type and AZT-resistant RT, respectively. However, we show here that the presence of Y181C in the AZT resistance substitution background does not influence the binding of the next correct nucleotide in a manner consistent with AZT resistance suppression. Indeed, we find that Y181C, in the context of AZT resistance substitutions, decreases the binding efficiency of the pyrophosphate donor ATP.

AZT resistance substitutions, and more specifically T215F or T215Y, induce long range conformational changes at the RT active site able to displace key amino acids up to 1.2 Å (58). Ren et al. (58) also suggested that Y181C disrupts these long range conformational changes. As we found that the Y181C substitution affects ATP binding ($K_m$, effect) to the AZT-resistant RT active site, our data agree with this suggestion in a manner consistent with AZT resistance suppression. However, the crystallographic model of Ren et al. (58) did not include either primer-template or nucleotide. In the latter ligands, being able to induce some conformational change (59), care must be taken in the interpretation of our data. For other suppressive substitutions, Götte et al. (25) have shown that the presence of the M184V substitution impairs pyrophosphorolysis, whereas Arion et al. (52) have shown that A114S has a direct effect on pyrophosphorolysis of the RT-mediated AZTMP-terminated DNA chain.

In conclusion, we have shown that the Y181C substitution mainly decreases the apparent rate of the pyrophosphorolysis reaction via the affinity of the primer-template-AZT-resistant RT complex for ATP. Our results do not exclude that Y181C mediates other effects consistent with AZT resistance suppression. It is interesting to note that AZT resistance mutations promote a 2–3-fold unblocking activity by using purified RT (Figs. 1 and 3, values ranging from 2- to 4-fold have been reported elsewhere (12, 26, 56, 60)). Due to the reiterative process of chain termination after repair, this modest increase may translate into a >100-fold AZT resistance in infected cells. Much like in the case of AZT resistance, the modest suppressive effect observed here (Figs. 1 and 2 and Table II) may also translate into a larger effect in the infected cell. If correct, this hypothesis would be consistent with our results showing that the apparently increased AZTTP discrimination by AZT-resistant/Y181C is irrelevant relative to the suppression of repair by this enzyme. In other words, for “naturally” highly repaired chain terminators such as AZTTP, repair would be the key parameter involved in resistance and suppression of resistance. It would be of interest to characterize further the mechanisms involved in other AZT resistance-suppressive substitutions, such as K65R, L74V, L100I, A114S, or M184V. This knowledge should aid the design of novel nucleoside analogues of increased efficacy as well as protocols for the optimal combination of nucleoside drugs.

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