3′-Azido-3′-deoxythymidine-resistant Mutants of DNA Polymerase β Identified by in Vivo Selection*

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We developed an in vivo selection to identify 3′-azido-3′-deoxythymidine (AZT)-resistant mutants of rat DNA polymerase β (pol β). The selection utilizes pol β’s ability to substitute for Escherichia coli DNA polymerase I (pol I) in the SC18-12 strain, which lacks active pol I. pol β allows SC18-12 cells to grow, but they depend on pol β activity, so inhibition of pol β by AZT kills them. We screened a library of randomly mutated pol β cDNA for complementation of the pol I defect in the presence of AZT, and identified AZT-resistant mutants. We purified two enzymes with nonconservative mutations in the palm domain of the polymerase. The substitutions D246V and R253M result in reductions in the steady-state catalytic efficiency (Kcat/Km) of AZT-TP incorporation. The efficiency of dTTP incorporation was unchanged for the D246V enzyme, indicating that the substantial decrease in AZT-TP incorporation is responsible for its drug resistance. The R253M enzyme exhibits significantly higher Kcat/dTTP and Kcat/dTTP values, implying that the incorporation reaction is altered. These are the first pol β mutants demonstrated to exhibit AZT resistance in vitro. The locations of the Asp-246 and Arg-253 side chains indicate that substrate specificity is influenced by residues distant from the nucleotide-binding pocket.

Efficient and accurate synthesis of DNA, during replication and repair, is essential to the integrity of any genome. Template-directed synthesis requires that a polymerase select the appropriate deoxynucleotide triphosphate (dNTP) and exclude incorrect bases. The interaction between a polymerase and substrates must therefore be highly specific, yet flexible, in order to maintain sequence fidelity. The smallest of the eukaryotic enzymes that accomplish this reaction is DNA polymerase β (pol β), a mammalian polymerase which fills short gaps in DNA (1). pol β has been implicated in base excision repair (2, 3) and meiosis (4). pol β is highly suitable for structure-function studies of the molecular mechanism of DNA synthesis due to the availability of information on the polymerase-DNA-substrate ternary complex (5, 6).

DNA synthesis by pol β can be compromised by the nucleoside analog drug AZT, which closely resembles a normal substrate. AZT-triphosphate (AZT-TP) presents a normal thymine moiety which may form a Watson-Crick base pair with adenine, but has a modified sugar ring that results in chain termination. pol β incorporates AZT into DNA in vitro (7) implying that pol β is not able to distinguish perfectly between the drug and the natural nucleotide substrate. This susceptibility of pol β makes AZT resistance a useful probe of enzyme-substrate interactions involved in DNA synthesis.

The molecular basis for polymerase substrate specificity, and specifically AZT discrimination, is not well understood. The clinical problem of AZT-resistant HIV has been attributed to mutations in HIV reverse transcriptase. However, the mutant reverse transcriptase enzymes have exhibited little or no change in AZT-TP incorporation in vitro, despite their ability to confer AZT resistance in vivo (8). The absence of an apparent in vitro phenotype makes these enzymes troublesome subjects for mechanistic studies of substrate specificity.

In order to investigate substrate specificity and drug resistance in pol β, we have developed a system for the identification of AZT-resistant forms of pol β, using in vivo complementation of polymerase-deficient Escherichia coli. The recA718polA12 E. coli strain carries a temperature-sensitive mutation in the pol A gene, rendering DNA polymerase I (pol I) inactive above 37 °C (9). Expression of rat pol β in these cells restores their ability to conduct DNA replication and repair at a nonpermissive temperature, indicating that pol β is able to substitute for E. coli pol I (10). Because growth of these cells at a nonpermissive temperature depends on the activity of pol β, any inhibitor of pol β is lethal to the cells. At 37°C in the presence of drug, cells expressing drug-sensitive pol β will die, while the cells expressing drug-resistant mutants will survive. The survival of a cell in the presence of AZT depends on the ability of pol β to distinguish between deoxynucleotide triphosphates (dNTPs) and very similar molecules. This creates selective pressure for mutant polymerases with strict substrate specificity.

In vivo selection is a powerful strategy for the identification of drug-resistant mutants. The ability to screen large numbers of clones makes it possible to seek drug resistance mutations among a library of randomly mutated clones. We report here a selection for AZT resistance mutations in pol β. We show that AZT can prevent wild type pol β from complementing the pol I defect, allowing selection for drug-resistant mutants. We have exploited this system to select clones expressing drug-resistant pol β variants. We have identified AZT-resistant mutants carrying single amino acid changes in locations distributed around the enzyme.

We describe two mutant enzymes identified by this selection which carry nonconservative substitutions in the palm domain causing dramatic increases in AZT resistance in vitro. The drug resistance of these mutant enzymes correlates with a drop in the steady state turnover number for AZT-TP incorporation relative to dTTP incorporation. Both enzymes carry a single amino acid substitution that is not near the active site or

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1 The abbreviations used are: pol, polymerase; AZT-TP, 3′-azido-3′-deoxythymidine triphosphate; HIV, human immunodeficiency virus; IPTG, isopropyl-1-thio-β-D-galactopyranoside; NA, nutrient agar; NB, nutrient broth.

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nucleotide binding pocket, suggesting that substrate specificity is influenced by residues distant from the active site.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Media—The strain used for isolating AZT-resistant mutants is known as SC18-12 and has the genotype recA718 polA12 uvrA55 trplE65 lon-11 sul A1 (11). The strain BL21 DE3 was used for protein expression and has genotype F−ompT hsdSBRΔBmB− gal dcm (DE3).

Nutrient agar (NA) was Difco nutrient agar with 5 g/liter NaCl. Nutrient Broth (NB) and LB broth were prepared according to manufacturer’s instructions (Difco).

AZT Survival of SC18-12 Cells Expressing pol β—The ability of SC18-12 cells expressing WT pol β to survive a range of AZT concentrations was measured in order to identify an AZT concentration which would kill the cells when they depend on pol β but not when pol I is active. SC18-12 cells were transformed by electroporation with the IPTG-inducible expression vector pBS2, a derivative of pHSG576 (12) containing the pol β cDNA. Transformants were allowed to recover at 30 °C for 2 h. Transformation efficiency was determined by plating serially diluted cells on NA 30 °C. Cells were kept overnight at 4 °C (to allow transformation efficiency plates to grow and be counted) then plated at approximately 400 colony forming units/plate on NA with chloramphenicol (30 μg/mL) and tetracycline (12.5 μg/mL), 1 mM IPTG, and varying concentrations of AZT (Sigma). Duplicate plates were incubated at 30 and 37 °C. The number of colonies formed on each drug concentration was divided by the number formed in the absence of drug to give the surviving fraction.

Construction of a Mutated pol β cDNA Library—In order to generate a pool of mutant pol β enzymes, the rat pol β cDNA was amplified, with primers designed to anneal immediately upstream and downstream of the gene, under mutagenic polymerase chain reaction conditions expected to produce mostly single base changes (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 50 μM dNTPs, 30 pmol of each primer, 5 units of Taq polymerase cycle parameters; 1 min, 94 °C; 2 min, 55 °C; 3 min, 72 °C for 30 cycles) (13). The mutated polymerase chain reaction product was subcloned into the pBlueScript SK+ vector and the pBS2 vector.

Selection of AZT-resistant Clones—Drug-resistant mutants were selected out of the pool of random mutations on the basis of their ability to grow in the presence of AZT. SC18-12 cells were transformed by electroporation with the mutated pol β cDNA library. Transformants were allowed to recover at 30 °C for 2 h. Transformation efficiency was determined on NA 30 °C. Cells were diluted in saline and plated at approximately 400 colony forming units/plate on NA with chloramphenicol (30 μg/mL), 1 mM IPTG, and 600 nM AZT (Sigma). Duplicate plates were incubated at 30 and 37 °C. Two equivalent plates were incubated at 30 °C (permissive temperature) to determine the total number of colony forming units screened.

The surviving colonies were isolated on NA with antibiotics. The pBS2 plasmid was isolated from each of these strains and sequenced to allow transformation efficiency plates to grow and be counted) then plated at approximately 400 colony forming units/plate on NA with antibiotics, 1 mM IPTG, and 600 nM AZT (Sigma). Plates were incubated for 2 days. Transformation efficiency was determined by plating on increasing concentrations of AZT. Plates were incubated at 37 °C for 2 days. The number of colonies formed on each drug concentration was divided by the number formed in the absence of drug to give the surviving fraction.

AZT Survival of Drug-resistant Strains—The AZT resistance phenotype of the confirmed mutants was quantified by plating on increasing concentrations of AZT. Plasmids identified as carrying drug resistance after electroporation with the mutated pol β cDNA were electroporated back into SC18-12 cells and asceptically grown on rich medium with antibiotics, 1 mM IPTG, and 600 nM AZT where indicated. Plates were incubated for 2 h. Transformation efficiency was determined by plating on increasing concentrations of AZT. Plates were incubated at 37 °C for 2 days. The number of colonies formed on each drug concentration was divided by the number formed in the absence of drug to give the surviving fraction.

Primer Extension Assay—The ability of each enzyme to incorporate dATP into activated calf thymus DNA. Reaction conditions were 50 mM Tris, pH 8.4, 20 mM MgCl₂, 100 mM NaCl, 200 μg/ml bovine serum albumin, 20 μM dithiothreitol, 50 μM dNTPs, and 100 ng of DNA polymerase I in 20 μL. Reactions were initiated at 37 °C for 15 min and then stopped with EDTA. Reactions were spotted onto GFA filters, which were washed twice in 22.5 mM NaPP₃, 8.5% concentrated perchloric acid, twice in 22.5 mg/ml NaPP₃, 8% concentrated hydrochloric acid, and then in 95% EtOH. Filters were dried and counted in a scintillation counter.

RESULTS

AZT Prevents Heterologous Complementation by pol β—WT rat pol β is capable of substituting for the E. coli enzyme DNA polymerase I in recA718polA12 cells, restoring the cells’ ability to grow on rich medium at 37 °C (10). We examined whether AZT would interfere with functional complementation of the polymerase defect of recA718polA12 cells by pol β. A representative rotary streak assay is shown in Fig. 1A. On the top row
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Fig. 1. A, rotary streak assay. SC18-12 cells or SC18-12 cells expressing WT pol β were grown on NA at 30 or 37 °C in the presence of antibiotics and IPTG, and with 600 nM AZT where indicated. Cells were streaked by drawing an inoculating loop slowly across the radius of the plate while spinning it on a plating wheel, creating a cell density gradient from the center to the perimeter of the plate. Typical results are shown. B, AZT survival curve. SC-18 cells expressing WT pol β were grown on NA at 30 (□) or 37 °C (○) in the presence of antibiotics and IPTG, and varying concentrations of AZT. The number of colonies formed on each drug concentration was divided by the number formed in the absence of drug to give the surviving fraction. Results are representative of three independent experiments.

are plates grown at 30 °C, a permissive temperature at which pol I is active. The SC18-12 cells grow well at 30 °C with or without expression of WT pol β. The addition of 600 nM AZT to the medium (upper right) resulted in streaks that were somewhat sparse and translucent in appearance, but did not prevent the streak from growing out to the edge of the plate, where the cells are least dense. At 37 °C (bottom row, Fig. 1A), SC18-12 cells are unable to form a rotary streak unless they express WT pol β. In the presence of AZT (bottom right), SC18-12 cells expressing WT pol β were unable to form a streak, demonstrating that pol β was unable to complement the growth defect of SC18-12 cells in the presence of AZT. These results indicate that AZT is toxic to the cells when they are dependent on pol β activity. At 30 °C, when pol I is functional, SC18-12 cells are not prevented by AZT from forming a rotary streak (top right), demonstrating that the drug at this concent-

tration is not lethal to the cells when they can rely on bacterial polymerases and do not require pol β.

In order to identify the range of AZT concentrations that would affect pol β but not bacterial polymerases, we measured the ability of the cells to grow on increasing concentrations of AZT (Fig. 1B). The surviving fraction (ratio of colonies formed on a given drug concentration to colonies formed in the absence of drug) drops off sharply at 400–500 nM AZT. Those colonies that do form in the presence of AZT (at all concentrations measured) are usually smaller than normal, and appear translucent rather than opaque white, and irregularly shaped rather than round. The presence of 600 nM AZT in the medium is sufficient to kill virtually all pol β dependent growth, without affecting colony formation at 30 °C. Therefore, we conclude that drug-resistant pol β mutants can be identified by selecting for ability to complement the recA718polA12 growth defect in the presence of 600 nM AZT.

Selection of Drug-resistant Mutants—We selected AZT-resistant mutants from a pool of plasmids carrying the randomly mutated pol β cDNA (see “Experimental Procedures”). Of approximately 66,000 mutant colonies screened, 171 grew at 37 °C on nutrient agar containing 600 nM AZT. When the pol β expressing plasmids were rescued from these colonies and sequenced, the majority were found to have more than one mutation, and were put aside. In order to confirm that the AZT-resistant phenotype resulted from expression of mutant pol β, rather than a cellular mutation, we transformed each of the mutant plasmids that carried a single mutation back into SC18-12 cells. Of those that carried only one predicted amino acid substitution, nine were consistently able to confer resistance to AZT on recA718polA12 cells when re-transformed into these cells. For further characterization, we chose two highly drug-resistant mutants, D246V and R253M.

Location of Mutations in the Protein Structure—The pol β protein is composed of two domains: the 8-kDa domain, which contains the N terminus of the protein and has deoxyribophosphatase activity (17), and the 31-kDa domain, which can perform the incorporation reaction alone. The 31-kDa domain can be further subdivided into fingers, palm, and thumb domains by analogy to the shape of a hand. As in other polymerases, the active site is defined by two Mg2+ ions which participate in the catalytic reaction; these atoms are held in place by three conserved acidic residues (5, 18, 19). In the case of pol β, the Mg2+ ions are coordinated by the residues Asp-190, Asp-192, and Glu-154, and the active site is defined by two Mg2+ ions and bound nucleotide. Replacement of the Arg residue at position 253 with Met (using the program WHATIF) does not introduce any obvious steric clash with neighboring side chains, but does eliminate a hydrogen bond with Glu-154 and another with the backbone at Asp-226. The side chain of Asp-246 is solvent exposed in this structure. Both Arg-253 and Asp-246 are conserved in the rat, human, and Xenopus pol β enzymes (22), while most residues in the 240–253 loop are not, which is consistent with the suggestion that these residues have an important role in pol β function.

AZT-resistant Heterologous Complementation by D246V and R253M Mutant Enzymes—To characterize more fully the AZT resistance phenotype of mutant enzymes, we tested their ability to substitute for pol I in the presence and absence of AZT. The D246V and R253M mutant enzymes were able to comple-
ment the growth defect of SC18-12 cells as well as wild type in the absence of drug (data not shown). In the presence of AZT, cells expressing pol β with the mutation D246V or R253M were substantially more drug resistant than cells expressing WT pol β. Fig. 3A shows typical results of a rotary streak assay of SC18-12 cells expressing WT and mutant pol β. The D246V substitution conferred a moderate improvement in ability to grow on AZT, forming a partial streak. The R253M mutation allowed the cells to form a streak on AZT extending out to the perimeter of the plate. For a quantitative measure of AZT resistance, we determined the surviving fraction of cells expressing WT and mutant pol β when plated on increasing concentrations of AZT. Both pol β R253M and D246V enabled SC18-12 cells to form colonies on AZT just as readily as on no drug, up to the drug concentration that is tolerated by the bacterial polymerases (Fig. 3B). In addition, the colonies formed by cells expressing pol β R253M appeared round and opaque, and otherwise hearty, unlike the colonies formed by cells depending on pol β WT or D246V, which were irregularly shaped and translucent.

Mutants That Complement pol I Defect in the Presence of AZT Exhibit Reduced AZT-TP Incorporation in Vitro—To determine whether the D246V and R253M enzymes are resistant to AZT in vitro, we purified the mutant proteins as described under “Experimental Procedures.” We found that both enzymes were as active as wild type, as measured by their ability to incorporate dNTPs into activated DNA (data not shown).

We used a steady-state primer extension assay to ask whether the in vivo drug-resistant phenotypes of the D246V and R253M mutants were due to increased specificity for dTTP over AZT-TP over AZT-TP. All three pol β enzymes did extend the primer by one AZT-TP molecule, although the reaction was much slower than dTTP incorporation (Fig. 4A). In steady state conditions, the AZT-TP incorporation reaction reaches saturation in about 35 min, while dTTP incorporation saturates after 2 min. We measured the reaction rate (incorporation as a function of time during the linear phase of the reaction, Fig. 4B) at eight substrate concentrations for each enzyme to determine the steady state constants for AZT-TP and dTTP incorporation by WT and mutant pol β enzymes. Fig. 4C shows typical Michaelis-Menten plots from which $K_m$ and $V_{max}$ are derived. The WT pol β enzyme has a 10-fold higher $K_m$ and 10-fold lower $V_{max}$ value for AZT-TP incorporation as compared with dTTP (Table I). The catalytic efficiency $K_{cat}/K_m$ is therefore nearly 2 orders of magnitude lower for AZT-TP incorporation than dTTP. This substantial difference in catalytic efficiency explains why pol β preferentially incorporates dTTP.

The $K_m$ (AZT-TP) of both mutants was only slightly higher than that of WT. The most significant change in these reactions was in $K_{cat}$ (AZT-TP), which decreased almost an order of magnitude for D246V and 3-fold for R253M compared with WT. This produced a decrease in catalytic efficiency ($K_{cat}/K_m$) of AZT-TP one order of magnitude below WT for D246V, and 3.5-fold for R253M. The steady-state constants of the D246V mutant enzyme for dTTP incorporation were essentially the same as wild type (Table I), implying that the reaction with natural substrate was unaffected by the mutation. While R253M was equally competent at dTTP incorporation as measured by catalytic efficiency, it exhibited a 7-fold higher $K_m$ and 4-fold higher $K_{cat}$. For this enzyme, the incorporation of dTTP is not compromised, but apparently proceeds via a kinetically altered pathway.

The in vivo phenotypes, where R253M is the strongest cannot be explained by the kinetics of the AZT reaction alone. The drug-resistance phenotype seems to correlate well with the
The ratio of turnover numbers $K_{cat}(dTTP)/K_{cat}(AZTTP)$. This ratio is increased from 11.5 for wild type to 52.2 in the moderately resistant D246V and 116 in the strong phenotype mutant R253M.

We performed a gel mobility shift assay to determine whether the mutant enzymes had an altered affinity for primer-template DNA (Fig. 5). The $K_D$ for DNA of WT pol β was $15.4 \pm 6.5$ nM, compared with $3.0 \pm 1.1$ nM for D246V and $12.7 \pm 2.1$ nM for R253M. Therefore, the R253M enzyme appears to interact with primer-template DNA with an affinity
Experimental Procedures.

Evidence that pol β expression of pol β E. coli et al., in addition confirms the findings of Bouayadi et al. (7) and HIV-reverse transcriptase, it clearly -sensitized mammalian cells in culture to AZT. Although incorporation of AZT-TP by pol β is inefficient compared with pol α (7) and HIV-inverse transcriptase, it clearly can be lethal to cells, which may be clinically important when AZT is used therapeutically.

We exploited the AZT sensitivity exhibited by SC18-12 cells when depending on pol β to select AZT-resistant mutants of pol β. This approach allowed us to identify drug-resistant mutants within a large pool containing random mutations. These pol β mutants present an opportunity to examine the biochemical mechanisms of AZT resistance.

The two mutants characterized, D246V and R253M, discriminate more effectively than WT pol β against AZT-TP. Both mutants incorporate AZT-TP less efficiently than WT, but are still proficient in dTTP incorporation. These pol β mutants are the first AZT-resistant polymerases to demonstrate significantly reduced incorporation of AZT-TP in steady state. The drug-resistance phenotype of pol β D246V is explained by the 10-fold reduction in catalytic efficiency of AZT-TP incorporation. In the absence of any changes to the dTTP reaction, this enzyme is presumably 10 times better than WT at discriminating against AZT. The R253M mutant, however, probably owes its drug resistance to the combination of moderately less efficient AZT-TP incorporation and a simultaneous perturbation of the dTTP incorporation reaction. The increase in K_m and K_cat for dTTP implies the reaction pathway is altered, possibly by a change in rate-limiting step, while remaining about equally efficient. The K_m values for D246V were similar to WT, and for R253M the value of K_m for dTTP increased so that K_m(dTTP) is similar to K_m(AZT-TP), implying that the difference in K_m for dTTP and AZT-TP does not determine the enzyme’s ability to discriminate between the two substrates and exclude AZT-TP. While steady-state K_m is not necessarily equal to the substrate binding constant, these results are consistent with a scenario in which substrate binding affinity does not control drug resistance. For the three enzymes examined here, AZT resistance in vivo increases with the ratio K_cat(dTTP)/K_cat(AZT-TP), in agreement with the suggestion that turnover number is the critical parameter.

The 5-fold decrease in K_m(DNA) exhibited by D246V represents a modest increase in DNA binding affinity. The difference is small, but raises the possibility that tighter binding to primer-template DNA increases discrimination against AZT-TP, possibly by tightening the steric of the binding pocket so as to exclude a larger than normal substrate.

The identification of these mutants implies that single amino acid substitutions in pol β are capable of improving its discrimination against AZT-TP as a substrate. The retained efficiency of dTTP incorporation implies that the improved specificity has occurred at little or no cost to the enzyme.

Molecular Mechanisms of Substrate Specificity—In theory, the most straightforward mechanism for a mutation to affect AZT incorporation would be a mutation that discouraged AZT-TP binding through a change in a residue that contacts incoming dNTP molecules, or the protrusion of a new side chain into the binding pocket. This would lead to the prediction that AZT resistance mutations would arise in the amino acids that surround the bound nucleotide in the enzyme-substrate complex. However, the D246V and R253M mutations are located on a loop in the palm domain at a distance from the nucleotide binding pocket which makes it highly implausible that they participate directly in nucleotide binding. These mutations demonstrate that substrate selection is influenced by amino acids which are not in direct contact with the substrate. Their effect on nucleotide interactions could be explained by a more subtle mechanism, in which disruption of the side chain interactions that hold the palm domain together has an indirect impact on the geometry of the active site, or influences the dynamic abilities of the protein.

Any changes in the palm domain caused by the D246V and R253M mutations could affect the geometry of the active site by altering the enzyme’s contacts with the Mg2 ions, the primer-template DNA, or the incoming substrate itself. Crystal structures have indicated that pol β positions nucleotide substrates through interactions with all three components of the molecule: the base moiety pairs with the template base, the phosphate groups coordinate the catalytic Mg2 ions, and the ribose ring contacts the protein side chains and backbone (5, 21). In crystal soaking experiments, Pelletier et al. (23) found that AZT-TP could enter the nucleotide binding pocket, but that steric clash of the azide group with the protein backbone at residues 271–274 forced the nucleotide into a distorted conformation in

**Table I**

| pol β | AZT-TP incorporation | dTTP incorporation |
|------|----------------------|--------------------|
|      | K_m (μM) | K_m (s⁻¹) | K_cat/K_m | K_m (μM) | K_m (s⁻¹) | K_cat/K_m | K_m (μM) |
| WT   | 199 ± 23 | 0.065 ± 0.002 | 3.31 x 10⁻⁴ | 2.26 ± 0.8 | 0.75 ± 0.06 | 3.12 x 10⁻⁴ | 11.5 |
| D246V| 292 ± 56 | 0.0092 ± 0.0007 | 3.66 x 10⁻⁵ | 29.1 ± 5.4 | 0.48 ± 0.02 | 1.63 x 10⁻⁵ | 52.2 |
| R253M| 233 ± 50 | 0.024 ± 0.002 | 9.70 x 10⁻⁵ | 176.5 ± 66 | 2.78 ± 1.2 | 1.58 x 10⁻⁵ | 115.8 |

**FIG. 5**. Gel mobility shift assay. End-labeled 16:45 DNA at 0.1 nm was incubated with indicated protein concentrations as described under “Experimental Procedures.” The gel shown is an assay of the R253M protein and is representative of three independent determinations. Fraction bound was determined by Phosphorimage quantitation of the gel. The dissociation constant K_m was derived from Sigmaplot fitting of the fraction bound versus protein concentration as described under “Experimental Procedures.”
which not only the sugar ring but the base and the phosphates are positioned incorrectly. Therefore, although only the sugar ring of AZT-TP differs from the natural substrate, a change in the contacts with the other groups of the molecule could exacerbate the already poor fit of AZT-TP in the binding pocket.

Pol β is known to undergo conformational changes during the binding and catalysis cycle (24, 6). These conformational changes, or the transitions between them, may require stabilization by various side chain interactions. The β-sheet surface of the palm domain includes residues that contact the DNA and nucleotide substrates, and the three essential Asp residues that coordinate the Mg$^{2+}$ ions as well. Immediately adjacent to Arg-253 lies a potentially crucial interaction between Asp-256, one of the catalytic aspartates, and Arg-254. In the crystal structure of pol β bound to gapped DNA alone (1bpx), this interaction includes two apparent hydrogen bonds between the amino and carboxyl groups. In the "closed" structure, in which pol β is bound to DNA and ddCTP (1bpy), the bidentate interaction is reduced to a single H-bond and Arg-254 forms a new interaction with the backbone at Ile-225. Moreover, in the structure of pol β bound to a non-gapped primer-template (1bpf), Arg-254 H-bonds instead to the 3' end of the primer. The different contacts with catalytically essential moieties formed by the Arg-254 side chain in different binding states suggests that Arg-254 may be involved in the stabilization of the different conformations of the enzyme or the transitions between them. This role could be analogous to that proposed by Sawaya et al. (6) for Arg-258, which also interacts with one of the catalytic Asp residues.

Arg-253 forms hydrogen bonds with the side chain of Glu-154 and the backbone at Asp-226 (5, 21), making it part of an intricate network of H-bonds and hydrophobic contacts that hold the palm domain together. A possible explanation for the R253M phenotype is that the elimination of these H-bonds, combined with the steric and electrostatic effect of the introduction of a Met residue, displaces the neighboring Arg-254 residue. This could then disturb catalytic geometry by influencing the positioning of the primer terminus, or the Asp-256 side chain. In addition, if the Arg-254 side chain normally cycles during the various binding events between various contacts with the primer, protein backbone, and Asp-256, then a change in the position of this residue could alter the kinetics of the conformational changes.

The effect of the D246V mutation is more difficult to rationalize. Asp-246, as a charged residue, may also participate in palm domain intramolecular interactions, although its role is not apparent from the crystal structures. The 240–253 loop sticks out from the palm domain in the crystal structure so that the side chains are mostly solvent exposed, and Asp-246 is located at the tip of the loop. One possible explanation is that the tip of the loop is flexible, and does not assume a physiologically relevant position in crystallization conditions. If the loop moved a bit in either direction, Asp-246 and the surrounding residues could be closely associated with either the palm or N-terminal domains, possibly forming a hinge-like region. Mutation of the hinge between the palm and C-terminal domains has been found to reduce fidelity by interfering with conformational changes during incorporation, demonstrating the importance of the dynamic properties of the enzyme (25, 26).

Another intriguing, although speculative, possibility is that the loop is the site of protein-protein interactions. The loop could be the site of regulatory contacts, such as the interaction with AP endonuclease during BER (27) or the binding of XRCC1 which prevents pol β from engaging in strand displacement (28). In that case, the loop could be seen as a molecular switch affecting the enzyme’s catalytic and binding capacities, and the mutation could cause the switch to “stick.” A switch trapped in a on, off, or halfway position could freeze the enzyme in an inappropriate conformation. A distortion of this nature could explain why such a mutation could affect pol β function even in E. coli, where the normal protein regulators are probably absent, and in vitro.

These mutants demonstrate that changes in residues at some distance from the nucleotide binding pocket can have a profound impact on substrate selection by pol β. Similarly, while some clinical isolates of AZT-resistant reverse transcriptase mutants have been found to carry mutations in the nucleotide binding pocket, others are mutated in residues not believed to interact with nucleotide substrates (29–31). Therefore, remote control of substrate choice by amino acids distant from the binding pocket may be a general property of DNA polymerases. These intramolecular interactions may be responsible for much polymerase substrate specificity, including sequence fidelity.

Implications for Therapeutic Use of AZT—That pol β is able to incorporate lethal amounts of AZT in bacterial cells raises the possibility that pol β contributes to the genotoxic effects of AZT. Cells in culture have been shown to up-regulate pol β activity and down-regulate other polymerases in response to methotrexate and during induced apoptosis (32, 33). This indicates that tumors subjected to DNA-damaging drugs may increase pol β activity in an attempt to repair their DNA, while shutting down DNA synthesis by other polymerases. If AZT or other drugs commonly used in combination with AZT trigger this response, pol β may be the most active native polymerase in many cells during antiviral therapy, and the ability of these cells to survive AZT treatment with intact genomes could depend on the ability of pol β to avoid incorporating AZT into DNA. Therefore the genotoxicity of antiviral therapy may be affected by pol β substrate specificity.

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