Subunit-selective Mutagenesis of Glu-89 Residue in Human Immunodeficiency Virus Reverse Transcriptase

CONTRIBUTION OF p66 AND p51 SUBUNITS TO NUCLEOSIDE ANALOG SENSITIVITY, DIVALENT CATION PREFERENCE, AND STEADY STATE KINETIC PROPERTIES*

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The E89G alteration in the human immunodeficiency virus type 1 reverse transcriptase has been shown to confer resistance to nucleoside analogs and a loss of magnesium cation preference (Prasad, V. R., Lowy, L., De Los Santos, T., Chiang, L., and Goff, S. P. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11363-11367. The wild type reverse transcriptase heterodimer, chimeric reverse transcriptases that contain the E89G alteration in one of the subunits (p66/p51m* and p66/p51w*), and the mutant enzyme (p66/p51m) were prepared. Analysis of steady state kinetic parameters showed that the mutant enzyme (p66/p51m) displayed a higher Vmax, a higher Kfm for 2',3'-dideoxythymidine triphosphate, and a higher Kfm for 2',3'-dideoxyximidine triphosphate than the wild type enzyme. The increased Km and Vmax values were observed only when a heterodimer contained the alteration in the p66 subunit. Tests for divalent cation requirement showed that only the dimers containing the wild type p66 (p66/p51w* and p66/p51m) displayed a preference for magnesium. Our results indicate that p66 plays a dominant role in deoxynucleotide triphosphate substrate recognition (Kfm), nucleoside analog sensitivity (Kfm), and magnesium preference. However, the increased Vmax displayed by the mutant enzyme (p66/p51m) appeared to be determined by both of the subunits.

Human immunodeficiency virus (HIV-1) reverse transcriptase (RT) is a key viral target in therapy for acquired immune deficiency syndrome (AIDS). All of the presently approved drugs for therapy, 2',3'-dideoxy-3'-azidothymidine, 2',3'-dideoxynosine, and 2',3'-dideoxyctydylidine, are RT inhibitors. The emergence of drug-resistant variants of HIV-1 in patients receiving long term therapy (1-4) has highlighted the need for a better understanding of the structure/function of the catalytic active site of RT. Previously we described the isolation of a nucleoside analog-resistant variant of HIV-1 RT (p66/p51m, E89G) expressed in bacteria that displayed decreased sensitivity to inhibition by 2',3'-dideoxy derivatives of all four nucleoside triphosphates, 3'-azido-3'-deoxythymidine triphosphate, and phosphonoformic acid (PFA, foscarnet) (5). Unlike the wild type enzyme with a characteristic preference for magnesium, the mutant enzyme was equally active in the presence of magnesium or manganese.

HIV-1 RT is a heterodimer consisting of two polypeptides, p66 and p51. The p51, derived by proteolytic processing of p69, shares its amino acid sequence with the amino-terminal part of p66. As revealed by the x-ray crystal structure of HIV-1 RT heterodimer (7,8), the three-dimensional structure of the p51 molecule is different from that of p66, despite the sequence identity. The single catalytic site on the heterodimer RT is thought to be located on p66, and a majority of the points of contact with the template-primer DNA are in the p66 chain. Previous studies have shown that the p66/p51 heterodimer is the most active form, p66/p66 homodimers are about a third as active as heterodimers, and p51 is least active (9,10). Subunit-selective mutagenesis of highly conserved residues of HIV-1 RT to determine the contribution of p51 subunit to the catalytic activity of the heterodimer enzyme (9,10) indicated that Asp-110, Asp-185, and Asp-186 of p66 are crucial to catalytic activity while those on p51 subunit are not. As our mutant carries the alteration on both of the polypeptides, we employed subunit-selective mutagenesis to examine the contribution of each Gly-89 residue in the mutant RT heterodimer to nucleoside analog resistance and altered divalent cation preference. Our results show that the deoxynucleotide triphosphate utilization (Kfm), nucleoside analog resistance (Kfm), and the loss of divalent cation preference map to the p66 polypeptide. An increase in Vmax observed for the E89G heterodimer RT, however, appears to be contributed to by both p51 and p66 polypeptides.

EXPERIMENTAL PROCEDURES

Bacteria and Plasmids—The Escherichia coli strain, M15::pDML1, and the HIV-1 RT expression plasmids pRT, pRT6H-PROT (11), and pHRTR51 (12), all of which contain RT sequences derived from a variant of HIV,,,,, were a gift of Dr. Stuart Le Grice, Case Western Reserve University. The constructs pRTm*, pHRTR51m*, and pRT6H-PROTM* used in this work are derivatives of Le Grice vectors, and contain RT sequences from the molecular clone HIVmexa.

Construction of Reverse Transcriptase Expression Vectors—Two types of RT expression plasmids are employed. One set of plasmids, pRT and pHRTR51, allows the expression of p66 or p51 subunits separately. The p51 encoded by the pHRTR51 contains an amino-terminal hexahistidine extension to facilitate purification via Ni2+-nitrilotriacetic acid chromatography. The second type, pRT6H-PROT, a double cassette expression construct pRT6H-PROT (13) and its genetic variant pDdTTP-1 (containing the E89G alteration) (5) as templates. All PCR reactions were carried out with Vent DNA polymerase (New England Biolabs, Inc.) to minimize undesired mutations. The reactions were carried out in an Eppendorf thermal cycler (Microcycler E) in a 50-pl reaction containing.

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1 The abbreviations used are: HIV, human immunodeficiency virus; RT, reverse transcriptase; PFA, phosphonoformic acid; PCR, polymerase chain reaction; dTTP, 2',3'-dideoxythymidine triphosphate.

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Mapping Enzyme Properties of HIV-1 RT to p66 and p51 Subunits

20 mM Tris-Cl, pH 8.8 (at 25 °C), 10 mM KCl, 10 mM (NH₄)₂SO₄, 4 mM MgSO₄, 0.1% Triton X-100, 20 mM Tris-Cl, pH 8.8 (at 37 °C), 100 mM dithiothreitol, 80 mM KCl, 6 mM MgCl₂, 0.96 μM (3'-OH ends) poly(rA) (Pharmacia Biotech Inc.), and 1.0 μM (3'-OH ends) oligo(dT) (Sigma). The enzyme concentration in the reaction mixture was 1 ng/μl, and the dTTP substrate concentration was varied as indicated in the reciprocal plots (Figs. 2 and 3).

The kinetic data were evaluated by plotting reciprocal initial velocities against reciprocal variable substrate concentrations, and the data were fitted to the appropriate rate equations using the Fortran programs of Cleland (15).

**RESULTS**

**RT Inhibition by ddTTP**—The ES9G alteration did not alter the competitive nature of ddTTP inhibition found in the wild type RT facilitating comparisons between the drug-resistant and drug-sensitive enzymes (Fig. 1). Table I shows that the  could be mutated (66°/51°) for ddTTP is 4.3 times higher than that for the wild type heterodimer. However, this is compensated for by an increased V₅₀° for the mutant as seen from comparable catalytic efficiencies (V₅₀°/K₅₀°) for the two enzymes (Table I). The K₅₀° for interaction of ddTTP with the mutant is higher (21-fold) than that of the wild type as expected for a drug-resistant enzyme (Table I). Thus, it appears that the mutant shows a decreased affinity for ddTTP while retaining the ability to utilize dTTP substrate at wild type efficiencies as indicated by the V₅₀°/K₅₀°.

The analysis of the chimeric RT heterodimers revealed that, in terms of K₅₀° and K₅₀° values, the chimeric enzyme containing p66° (p66°/p51°) is similar to the wild type enzyme (p66°/p51°), while the chimeric enzyme with p66° (p66°/p51°) behaves similar to the mutant heterodimer (p66°/p51°). For example, the magnitude of increase in K₅₀° for the chimera p66°/p51° is approximately the same as that observed for the mutant enzyme p66°/p51° (Table I). The other chimera p66°/p51°, however, displayed a K₅₀° value (2.3 nm) that was closer to that of p66°/p51° enzyme (3.0 nm). Thus, the ES9G alteration, in order to confer resistance to nucleoside analogs such as ddTTP, must be located near the p66 peptide. When present only on the p51 polypeptide, the ES9G alteration does not have a significant influence on the K₅₀° of the RT. The fact that an increase in K₅₀° is displayed by both mutant p66°/p51° and the chimera p66°/p51° indicates that the K₅₀° also segregates with the p66 polypeptide providing biochemical support to the notion that the deoxynucleotide triphosphate substrate binding site resides on the p66. However, a different pattern is observed with respect to enzyme velocity. An increase of 3.9-fold in the V₅₀° of p66°/p51° (10.8 pmol/min for p66°/p51° versus 2.8 pmol/min for p66°/p51°) does not appear to be solely associated with the p66 polypeptide. The chimera containing the mutant p66° (p66°/p51°) shows an increase of 1.5-fold, while the chimera p66°/p51° shows a 2.4-fold increase in V₅₀°. Thus, the increase in V₅₀° of the mutant p66°/p51° appears to be due to both p66° and p51° polypeptides.
Mapping Enzyme Properties of HIV-1 RT to p66 and p51 Subunits

RT Inhibition by Phosphonoformic Acid—In agreement with previous reports, the inhibition of HIV-1 RT by phosphonoformic acid was found to be noncompetitive (16) (Fig. 3 and Table II). The inhibition constant derived from the intercept of Lineeweaver-Burk plots (K_i = 0.13 ± 0.02 μM) for the wild type HIV-1 RT approximates the published value of 0.4 μM (16). The behavior of the wild type and the mutant enzymes with respect to phosphonoformic acid inhibition are similar to the pattern observed for ddTTP inhibition; the mutant enzyme displays an increase in K_m (3.3-fold) and V_max (2.8-fold) for dTTP and an increased K_i (both K_i and K_m are higher for the mutant heterodimer, see Table II) for PFA inhibition.

The analysis of the chimeric heterodimers for PFA inhibition corroborates the conclusions of the ddTTP inhibition studies. The increased K_m, dTTP, and K_i(PFA) (K_i slope) values of the mutant p66°/p51° segregate with the p66° polypeptide. Thus, resistance to PFA of the variant p66°/p51° requires the alteration to be present on the p66 chain of heterodimer RT, and p51 does not play a role in PFA resistance. As observed in the previous experiment, however, the increase in V_max displayed by the mutant enzyme (p66°/p51°) appears to result from the presence of either p51° or p66° subunit alone in a chimeric dimer, although the greatest increase is obtained when both of the polypeptides carry the alteration. The increase was greater (2.1-fold) when the alteration was present on the p51 polypeptide alone (p66°/p51°) as opposed to when it was present on the p66 alone (1.3-fold increase; p66°/p51°). Taken together, the results are suggestive of an important role for p51° in the increased V_max of the mutant enzyme (p66°/p51°).

Divalent Cation Preference—To investigate whether the divalent cation preference of RT maps to a particular subunit, we tested the four RT preparations for RNA-dependent DNA polymerase activity in magnesium or manganese mixtures (Fig. 4). The levels of enzyme activity observed in Mg²⁺ buffers are consistent with the enzyme velocity measurements presented in Tables I and II (Fig. 4A). The unusual ability of the E89G mutant to utilize either Mg²⁺ or Mn²⁺ at comparable efficiencies was exploited to examine the role of the two E89G residues in the loss of divalent cation preference. The chimeric enzyme containing the wild type p66 chain, p66°/p51°, retained a preference for Mg²⁺ cation like the wild type enzyme (the Mg²⁺/Mn²⁺ ratios were 4.98 for p66°/p51° and 7.53 for p66°/p51°), while
Mapping Enzyme Properties of HIV-1 RT to p66 and p51 Subunits

Fig. 3. Noncompetitive inhibition by PFA. Double-reciprocal plots are shown, as in Fig. 2, for the four heterodimeric RT preparations. A, p66\textsuperscript{m}/p51\textsuperscript{m}; B, p66\textsuperscript{m}/p51\textsuperscript{w}; C, p66\textsuperscript{w}/p51\textsuperscript{m}; and D, p66\textsuperscript{w}/p51\textsuperscript{w}. The inhibitor concentrations for A and B from the top are 0.0, 0.1, and 0.25 \(\mu\text{M}\), and the inhibitor concentrations for C and D from the top are 0.0, 3, and 6 \(\mu\text{M}\). 1/\(V\), [pmol dTTP incorporated min/16 N1-l].

**DISCUSSION**

The availability of an enzymatically active mutant enzyme displaying drug resistance and a loss of divalent cation preference (5) prompted us to test the role of the two polypeptides of HIV-1 RT in enzyme function. The present work has shown that the mutant enzyme, when compared with the wild type, displays a higher \(V_{\text{max}}\), a higher \(K_m\) for dTTP, and higher \(K_i\) values for both ddTTP and PFA. These results also suggest that the mechanism underlying the resistance involves modifications in the enzyme/substrate interactions as well as enzyme...
Velocity to help discriminate the dTTP substrate from the analog ddTTP.

Our studies with the chimeric heterodimers show that the increased Kᵰ values for both ddTTP and PFA are only observed in dimers containing the mutant p66, thus establishing that the E89G residue must be located on the p66 polypeptide chain to exert its influence on the drug sensitivity of HIV-1 RT heterodimer. When the E89G alteration is only present on the p51 molecule, the resulting heterodimer is as sensitive to ddTTP and PFA as the wild type enzyme. Thus the residue Gly-89 of p51 polypeptide does not influence nucleoside analog resistance. Furthermore, the Mg²⁺ preference of wild type RT was shared by the chimera containing the wild type p66 (p66ʷ/p51ʷ), and the lack of preference for magnesium is shared by the mutant RT and the chimeric RT containing the mutant p66 (p66ʷ/p51ʷ). The determinants that establish specificity for divalent cations thus appear to be located on the p66 molecule. The precise role of p66 in forming the divalent cation binding pocket is currently not understood. The fact that the divalent cation preference maps to p66 may indicate the presence of the divalent cation contact sites on p66 or an allosteric effect determined by residues located on the p66 molecule.

Previous efforts to determine the role of the two subunits in catalysis employed subunit-selective mutagenesis of the highly conserved aspartic acid residues at positions 185 and 186 (9) or at position 110 (10). This triad of aspartic acid residues is thought to play a key role in catalytic function (7) since (i) these residues are evolutionarily conserved among various retroviral reverse transcriptases (17–19); (ii) mutagenesis studies had earlier shown them to be critical for the RNA-dependent DNA polymerase activity of reverse transcriptase (20); and (iii) the crystal structure reveals that the aspartate residues of p66 are in the vicinity of the active site. Subunit-selective mutagenesis of aspartate residues demonstrated that the aspartate residues on p66 are essential for enzyme activity, while those on p51 are not. Thus it has been concluded that the catalytic function of RT resides in p66 and that the p51 subunit may play accessory roles. While the residues Asp-110, Asp-185, and Asp-186 of p51 may not contribute to RT function, a possibility that other residues of p51 could play important roles in some aspect of RT function has not been ruled out. It has been proposed that p51 may stabilize the enzyme, contribute to the efficiency of polymerase reaction, or allosterically modify the p66 molecule (9, 10). In the present work, the increased Kᵰ phenotype displayed by the mutant heterodimer segregated with the mutant p66 polypeptide providing additional evidence that the p66 plays a major role in catalysis. However, the increased Vₘₐₓ of the mutant was clearly not a sole function of mutant p66 polypeptide. The chimeric enzyme consisting of mutant p51 displayed a Vₘₐₓ value higher than both wild type heterodimer and the chimeric dimer p66ʷ/p51ʷ in two separate experiments (Tables I and II). This suggests that p51 plays, if not a greater, an equally important role as p66 subunit in contributing to the increased Vₘₐₓ phenotype displayed by the mutant heterodimer. Although the mechanism by which the E89G alteration influences the Vₘₐₓ of the enzymatic reaction is not clear, it appears that the presence of the alteration on both of the subunits had an additive effect on Vₘₐₓ. The X-ray crystal structure of HIV-1 RT heterodimer complexed with double-strandedDNA (?) shows that the 89th residue is located in the palm domain of each subunit. In the p66 subunit, the Glu-89 residue forms a part of the "template grip" and appears to make contact with the template DNA strand very close to the primer terminus. Thus the effect of the E89G alteration in the p66 polypeptide on Kᵰ and Kᵰ can be readily appreciated. However, the Glu-89 residue in p51 is located in a region away from the deoxynucleotide triphosphate binding site, distal to the interface between the two subunits. The effect of E89G on Vₘₐₓ, therefore, is likely to be manifested by allosteric effects. Additional studies are needed to establish whether p51 simply maximizes the enzyme reaction velocity by stabilizing the catalytic site on the p66 or actually contributes to the catalytic function of the heterodimer.

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FIG. 4. Divalent cation preference of heterodimer RTs. The RNA-dependent DNA polymerase activity was measured in both magnesium- and manganese-containing mixtures (see text). A shows the picomoles of dNMP incorporated by each heterodimer in the presence of magnesium or manganese as indicated. The closed boxes represent activities in Mg²⁺, and the open boxes represent those in Mn²⁺. B, the ratio of picomoles of dNMP incorporated in Mg²⁺ mixtures versus that in Mn²⁺ mixtures. The broken line marks the ratio of 1 expected for equal levels of activities with the two divalent cations. The numbers on the x axis are as follows: 1, p66ʷ/p51ʷ; 2, p66ʷ/p51ʷ; 3, p66ʷ/p51ʷ; and 4, p66ʷ/p51ʷ.
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