Site-directed Mutagenesis of Moloney Murine Leukemia Virus Reverse Transcriptase

DEMONSTRATION OF LYSINE 103 IN THE NUCLEOTIDE BINDING SITE*

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Retroviral reverse transcriptases catalyze the synthesis of double-stranded DNA of encapsidated genomic RNA through a complex process (Gilboa et al., 1979). Reverse transcriptases purified from a number of retroviruses exhibit two basic activities, a DNA polymerase and an RNase activity (Verma, 1975; Gerard and Grandgenett, 1975). The DNA polymerase activity catalyzes the formation of a phosphodiester bond between the incoming dNTP and the 3' end of a primer and can copy either RNA or DNA templates. The RNase activity cleaves the 3' to the incoming dNTP and the 3' end of a primer and the processive mode of DNA synthesis. These observations suggest that only Lys103 and not Lys421 is the catalytically important residue that is involved in the binding of substrate dNTP in Moloney murine leukemia virus reverse transcriptase.

Lys103 and Lys421 of Moloney murine leukemia virus reverse transcriptase have been implicated in the dNTP binding function as judged by their reactivity to a substrate binding site-directed reagent, pyridoxal 5'-phosphate (Basu, A., Nanduri, V. B., Gerard, G. F., and Modak, M. J. (1988) J. Biol. Chem. 263, 1648-1653). To assess the true catalytic importance of the individual lysine residues in Moloney murine leukemia virus reverse transcriptase, we mutated Lys103 and Lys421 to leucine and alanine, respectively. Analysis of the mutant enzymes revealed that mutation at the 103 position had a drastic effect on the DNA polymerase activity whereas the 421 mutation had no effect. Both mutants exhibited normal RNase H activity as well as the ability to bind to DNA or RNA templates as judged by UV-mediated cross-linking of the enzyme to the template primers. The enzyme with mutation at codon 421 (Lys→Ala) exhibited properties that were indistinguishable from the wild type with respect to its mode of catalysis, i.e. preference of template primer and divalent metal ion, RNA- or DNA-dependent DNA polymerase activity, RNase H activity, and the processive mode of DNA synthesis. These observations suggest that only Lys103 and not Lys421 is the catalytically important residue that is involved in the binding of substrate dNTP in Moloney murine leukemia virus reverse transcriptase.

Materials and Methods

Restriction endonucleases and DNA modifying enzymes were obtained from Bethesda Research Laboratories. Oligonucleotide primers...
for DNA sequencing and mutagenesis were obtained from Genetic Designs and purified by high pressure liquid chromatography using the Zorbax oligo column from Du Pont-New England Nuclear. Reagents for M13 sequencing were purchased from New England Biolabs, and radioactive nucleotides [α-32P]dATP (800 Ci/mmol) and [α-32P]dCTP (800 Ci/mmol) were from Amersham Corp. Decoy oligonucleotides, (rA)$_n$ and (dA)$_n$, were obtained from Pharmacia LKB Biotechnology Inc. Poly(rA) and poly(dA) primed with oligo(dT)$_{12-18}$ in a 1:1 molar nucleotide ratio were used as template primers for most of the experiments. Anti-rabbit IgG conjugated with alkaline phosphatase was from Sigma. The rabbit antibody against purified MuLV-RT was a generous gift from Dr. Monica Roth of Robert Wood Johnson Medical School of this university. All other chemicals were of reagent grade.

**Site-directed Mutagenesis**

The expression plasmid pB6B15.23 (Roth et al., 1985), which contains the entire Moloney murine leukemia virus reverse transcriptase gene sequence, was a kind gift from Dr. Stephen Goff of Columbia University. Mutagenesis of lysines at positions 103 and 421 was carried out according to the method of Roth et al. (1985). A 1.9-kilobase KpnI-BglII fragment was isolated and subcloned in M13mp19 digested with KpnI and HincII. The recombinant M13mp19 virus (with insert) was used to infect an Escherichia coli dut--ung- strain (CJ236) to produce template DNA containing uracil residues. The synthetic oligonucleotide 5'-CTGTTAAGCTTACAGGACT-3', which introduced nucleotide changes in the wild-type sequence, was used as the primer for the generation of the Lys$^{103}$ to leucine mutation, whereas the oligonucleotide 5'-ACTGACAGCGGATGCAG-3' was used to mutate Lys$^{421}$ to alanine. The desired mutants were identified directly by dideoxy sequencing (Sanger et al., 1977). A 1.1-kilobase KpnI-BglII fragment containing the appropriate mutations was cloned into the pB6B15.23 KpnI-BglII-digested vector. The mutant protein were then expressed by the protocol described earlier (Roth et al., 1985).

**Enzyme Assays**

**DNA Polymerase Assay**—DNA-dependent DNA polymerase activity of partially purified RT preparations was determined using (rA)$_n$- (dT)$_n$ in a 1:1 molar nucleotide ratio and dTTP as template primer and substrate, respectively. The reaction mixture, in a final volume of 100 μl, contained 50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 10 μg of bovine serum albumin, 1 mM MnCl$_2$, 60 mM NaCl, 0.5 μg of (rA)$_n$- (dT)$_n$, 20 μM dTTP, 0.5 μCi of [α-32P]dTTP, and 2 μl of partially purified or 20 ng of purified enzyme. After a 20-min incubation at 37 °C, reactions were terminated by the addition of 5% trichloroacetic acid containing 10 mM EDTA. Acid-insoluble material was collected on GF/B glass fiber filters, and enzymatic activity was determined by scintillation spectroscopy. For processivity studies, 1-30 mM enzyme was preincubated with the appropriate template primer for 5 min in 45 μl of 50 mM Tris-HCl, pH 8.0, 60 mM KCl, 1 mM dithiothreitol, and 0.05% Nonidet P-40 (buffer A). Reactions were started by the addition of MgCl$_2$/MnCl$_2$ and dNTP in 5 μl of buffer A to give a final concentration of 10 mM MgCl$_2$ or 1 mM MnCl$_2$ and 100 μM dNTP. Reactions were stopped at regular intervals with p of 0.5 M EDTA. All incubations were carried out at 37 °C. Reaction products were analyzed by polyacrylamide gel electrophoresis in the presence of 7 M urea as described by Huber et al. (1989).

**RNase H Assay**—RNase H activity in the partially purified enzyme preparations was measured by using tritiated (rA)$_n$- (dT)$_n$ as substrate. The reaction mixture in a final volume of 100 μl contained 50 mM Hepes-KOH, pH 7.8, 1 mM dithiothreitol, 20 mM NaCl, 10 μg of bovine serum albumin, 1 mM MnCl$_2$, 0.5 μg of (H)tritiated (rA)$_n$- (dT)$_n$ (20,000 cpm), and 50 ng of the enzyme protein. The reactions were carried out at 37 °C for 30 min, and the acid-insoluble counts were determined as described for the DNA polymerase assay. The reaction products were separated by polyacrylamide gel electrophoresis in the presence of 7 M urea as described by Huber et al. (1989).

**Purification of Reverse Transcriptase**—Five hundred-milliliter cultures of the wild type and two mutants were grown, and enzyme induction and purification were carried out essentially as described by Roth et al. (1985). Proteins obtained after two column chromatography steps, DEAE-cellulose and phosphocellulose, were used for enzyme characterization as we found to be 90% pure at this stage. In the case of mutant 103, which showed no polymerase activity in the crude lysate, the reverse transcriptase protein was identified by Western blot (immunoblot) analysis of individual column fractions. The fractions that showed maximum reverse transcriptase protein were pooled and used for further studies.

**Western Blotting**—Equal amounts of protein after DEAE fractionation of wild-type and mutant forms of reverse transcriptase were denatured and fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The fractionated proteins were transferred electrophoretically onto Immobilon-P membranes and reacted with polyclonal antibody raised in rabbits against pure MuLV-RT (Roth et al., 1985). Identification of the bands reacting with the MuLV-RT antibody was accomplished by reaction with an anti-rabbit antibody conjugated with alkaline phosphatase.

**Template Primer Binding Assay**—The ability of the mutant enzymes to bind to template primer was examined by determining the extent of UV-mediated cross-linking of the enzyme protein with radiolabeled template primer. The protocol for cross-linking of the enzyme protein to template primer was essentially as described earlier (Basu, S., et al., 1988). The primer (dT)$_n$, labeled at the 5' end by polynucleotide kinase, was annealed with (dA)$_n$. The resulting template primer, (dA)$_n$- (dT)$_n$ (1 μg, 0.2 μCi), was mixed with 2 μg of the enzyme and then exposed to UV irradiation for 15 min. The extent of enzyme cross-linked to template primer was determined by SDS-polyacrylamide gel electrophoresis followed by autoradiography (Basu, S., et al., 1988). We have chosen the template primer (dA)$_n$- (dT)$_n$ for the binding studies even though it is utilized poorly by MuLV-RT for synthesis (Modak and Marcus, 1977a). This avoided the ambiguity associated with the use of (rA)$_n$- (dT)$_n$, which can serve as template primer as well as a substrate for RNase H activity. The observation that the catalysis of (rA)$_n$. (dT)$_n$-directed cleavage by RNase H is essentially identical to (dA)$_n$. (dT)$_n$ (Modak and Marcus, 1977b). The cross-linking studies (see Fig. 1B) provide direct proof of binding of this template primer to MuLV-RT.

**RESULTS AND DISCUSSION**

We have shown previously that Lys$^{103}$ and Lys$^{421}$ of MuLV-RT are specific targets of reaction by PLP, a substrate binding site-directed reagent for DNA polymerases; this finding implied involvement of both lysines in the process of substrate dNTP binding (Roth et al., 1985). To determine the importance of the individual lysine residues, we mutated Lys$^{103}$ to leucine and Lys$^{421}$ to alanine. The selection of leucine and alanine as substitutions for lysine was based on the fact that these are uncharged residues and have small side chains. Therefore, these substitutions are expected to have a minimal effect on the secondary structure of mutant RTs.

Enzyme proteins were purified from lysates of bacterial cultures expressing either the wild-type or mutant proteins as described under "Materials and Methods." Fig. 1A shows a Western blot analysis of bacterial extracts expressing the wild-type and mutant forms of MuLV-RT. A 71-kDa protein cross-reacting with polyclonal antibody against pure MuLV-RT was detected in all extracts, indicating that the wild-type and mutant clones were both expressing RT. Several bands representing lower molecular weight species were also seen to cross-react with the antibody in all three instances. These were probably the breakdown products of the RT protein in E. coli extracts.

Since the mutants were able to express full-length RT protein, the next step was to determine the polymerase and RNase H activities associated with the protein. Mutant 421 showed no change in polymerase activity whereas mutant 103 showed only 1% of the activity expressed by wild-type RT (Table I). Analysis of the RNase H activity indicated that both mutants had nucleolytic activity comparable with that of the wild-type enzyme.

**Characterization of Lys$^{103}$ Mutant of MuLV-RT**—The results described above show clearly that mutation of lysine 103 in MuLV-RT severely affects polymerase activity. Since the polymerase reaction requires binding of both template primer and substrate dNTPs, it was essential to demonstrate whether the binding of only one or both components was affected in the mutant enzyme. In MuLV-RT, substrate dNTP binding only occurs subsequent to the binding of template primer to
Lys\textsuperscript{103} in the dNTP Binding Site of MuLV Reverse Transcriptase

**FIG. 1.** Immunoblotting and UV-mediated cross-linking of (dA)\textsubscript{2}-[^{32}P](dT)\textsubscript{15} to wild-type and mutant forms of MuLV-RT. A, wild-type and mutant forms of MuLV-RT were analyzed on 8% SDS-polyacrylamide gel and immunoblotted with anti MuLV-RT antibody. Lane 1, wild type; lane 2, Lys\textsuperscript{103} mutant; lane 3, Lys\textsuperscript{421} mutant. B, wild-type and mutant MuLV-RTs were cross-linked to (dA)\textsubscript{2}-[^{32}P](dT)\textsubscript{15} by UV irradiation and analyzed by polyacrylamide gel electrophoresis as described under "Materials and Methods." Lane 1, E. coli DNA polymerase 1, large fragment, taken as control; lane 2, heat-inactivated MuLV-RT; lane 3, wild type; lane 4, Lys\textsuperscript{103} mutant; lane 5, Lys\textsuperscript{421} mutant MuLV-RT.

**TABLE I**
DNA polymerase and RNase H activities of wild-type and mutant RTs

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>RNase H</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin. sequence</td>
<td>New sequence</td>
<td>%</td>
</tr>
<tr>
<td>Wild-type</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Lys\textsuperscript{103} mutant</td>
<td>95</td>
<td>LPVKRPRTN</td>
</tr>
<tr>
<td>Lys\textsuperscript{421} mutant</td>
<td>98</td>
<td>AVLTDEAGK</td>
</tr>
</tbody>
</table>

The amino acids underlined indicate the positions of mutation.

The enzyme (Basu, A., et al., 1988). Therefore, we attempted to determine the extent of template primer binding by the mutant and the wild-type enzyme. We used both an indirect and direct approach to accomplish this goal. In the indirect approach, inactivation of RT-associated RNase H activity by inclusion of DNA template primer is used to indicate template primer binding by RT (Modak and Marcuse, 1977a). In the direct approach, binding of the DNA template primer to the enzyme is measured via UV-mediated cross-linking of the enzyme to template primer. Table II summarizes the results of the RNase H experiment. The RNase H activity of all three enzymes exhibited similar sensitivity to the addition of calf thymus-activated DNA or (dA)\textsubscript{2}-(dT)\textsubscript{15}, indicating that DNA, which is not a substrate for RNase H, was able to bind to the enzyme via the polymerase domain and displace it from the RNase H-substrate complex (Modak and Marcuse, 1977b). Furthermore, the ability of the mutant enzymes to bind to template primer is also demonstrated by comparing the efficiency with which wild-type and mutant enzymes cross-link to labeled (dA)\textsubscript{2}-(dT)\textsubscript{15}, where dT\textsubscript{15} carries the label at its 5' end (Fig. 1B). The extent of cross-linking of mutant 103 to (dA)\textsubscript{2}-(dT)\textsubscript{15} was equivalent to that of the wild-type enzyme. The choice of (dA)\textsubscript{2}, as a template in these studies rules out the possibility that the enzyme binds to the template primer via the RNase H domain. These results indicate that the Lys\textsuperscript{103} mutant retains its ability to bind to DNA, and hence the observed loss in polymerase activity must be due to its inability to bind to substrate dNTP, as reported earlier (Basu, A., et al., 1988).

The two functional domains, i.e. the polymerase and the RNase H domains of reverse transcriptases, have been predicted (Johnson et al., 1986) and have been demonstrated to reside in separate regions of the enzyme (Tanese and Goff, 1988; Kotewicz et al., 1988). The DNA polymerase domain resides in the N-terminal region whereas, the RNase H domain is contained in the C-terminal region of the protein. Thus, our observation that the mutation of Lys\textsuperscript{103}, which lies in the N-terminal region of the enzyme, has a drastic effect on polymerase activity without affecting RNase H activity is consistent with the two-domain constitution of RT. In addition, computer analysis of the amino acid sequences has revealed that Lys\textsuperscript{103} is conserved in all retroviral reverse transcriptases, but Lys\textsuperscript{421} is not (Johnson et al., 1986; Doolittle et al., 1989). Thus, our recent mutagenesis studies and previous chemical modification studies (Basu, A., et al., 1988) show clearly that Lys\textsuperscript{103} is one of the most important residues in the polymerase active site and that it is required for the dNTP binding activity of MuLV-RT.

**Characterization of Lys\textsuperscript{103} Mutant of MuLV-RT—Chemical modification of MuLV-RT with pyridoxal 5'-phosphate in the presence and absence of substrate dNTP had indicated clearly that Lys\textsuperscript{103} and Lys\textsuperscript{421} were the targets of inhibitor action (Basu, A., et al., 1988). The present mutagenesis results, however, indicate that Lys\textsuperscript{421} has no apparent role in polymerase or RNase H reactions. In order to establish if mutation of this lysine has altered any other property of the enzyme, we carried out a detailed analysis of the template primer preferences, rates of reaction, and kinetic constants for both template and substrates. We found no significant difference between the properties of the Lys\textsuperscript{421} mutant enzyme and the wild-type enzyme (data not shown).

Previously, we had also noted the reactivity of two distinct lysine residues in the PLP-mediated inactivation of E. coli DNA polymerase I. One of the lysines in pol I was required for substrate dNTP binding (Basu and Modak, 1987) whereas the 2nd lysine was required for the processive mode of synthesis (Basu, S., et al., 1988). We therefore considered the possibility that lysine 421 in MuLV-RT, which does not appear to be involved in the substrate binding per se, may influence the enzyme-template primer interaction in a manner analogous to that found for E. coli DNA polymerase I (Basu, S., et al., 1988). Modification of Lys\textsuperscript{421} in the E. coli

**TABLE II**
Effect of natural and synthetic DNA on RNase H activity of wild-type and mutant RTS

<table>
<thead>
<tr>
<th>DNA\textsuperscript{a}</th>
<th>Wild type</th>
<th>Lys\textsuperscript{103} mutant</th>
<th>Lys\textsuperscript{421} mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated DNA</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1 mg</td>
<td>77</td>
<td>90</td>
<td>75</td>
</tr>
<tr>
<td>5 mg</td>
<td>60</td>
<td>58</td>
<td>67</td>
</tr>
<tr>
<td>(dA)\textsubscript{2}-(dT)\textsubscript{15}</td>
<td>62</td>
<td>60</td>
<td>63</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Activated calf thymus DNA or (dA)\textsubscript{2}-(dT)\textsubscript{15} (1:1 molar ratio) at indicated concentrations was added as a challenger DNA in the reaction mixture prior to the addition of the enzyme.
pol I enzyme was shown to result in the loss of the processive mode of DNA synthesis by the modified polymerase. Therefore, the mode of DNA synthesis on single-stranded template with respect to processivity and template sequence-dependent "strong stops" by the wild-type and mutant MuLV-RT were compared. Processivity on single-stranded templates was measured by the extension of labeled primers. Poly(rA) primed with 5' [32P]-labeled oligo(dT)15 was extended with reverse transcriptase, and products were monitored with increasing time (Fig. 2, panel I) and with increasing enzyme primed with 5' [32P]-labeled oligo(dT)15, was extended with increasing time (Fig. 2, panel I) and with increasing enzyme. Furthermore, the pattern of distinct DNA sequence-dependent stops observed during replication of single-stranded DNA (Abbotts et al., 1988; Huber et al., 1989) also seems to be unaffected by the mutation of Lys452.

The results presented here indicate that Lys452 apparently has no catalytic role in the DNA polymerase activity of MuLV-RT. One possible explanation for the reactivity of both Lys103 and Lys452 to pyridoxal 5'-phosphate and their protection by the substrate dNTP may be the molecular proximity of the 2 residues in the three-dimensional structure of RT. In such a situation, the presence of substrate dNTP in the active site pocket may block Lys452 sterically and prevent its modification by PLP.

In conclusion, the mutagenesis studies reported here show clearly that lysine 103 is catalytically important and is probably involved in the substrate binding process in MuLV-RT.

Acknowledgments—We thank Stephen Goff of Columbia University College of Physicians and Surgeons for the generous gift of the plasmid used in these studies. We also thank Catherine Joyce of Yale University for helpful advice regarding the mutagenesis protocols.

REFERENCES

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FIG. 2. Mode of DNA synthesis on single-stranded templates by wild-type and mutant MuLV RT. Panel I, reverse transcriptase (5.6 nM) was preincubated with 80 nM (rA)50-32P(dT)15, and the reaction was started by the addition of TTP and Mn++ (see "Materials and Methods"). Aliquots were withdrawn at various times and products analyzed on an 8% urea-polyacrylamide gel. Lanes 1–3 represents synthesis at 2.5, 5, and 10 min. Panel II, reactions of 1.5 nM (lane 1) and 2.8 nM (lane 2) reverse transcriptase with 56 nM (rA)50-32P(dT)15 were initiated as described above. Reactions were stopped after 10 min and products analyzed on an 8% urea-acylamide gel. Panel III, Reactions with 20 nM reverse transcriptase and 150 nM M13 DNA to which a 5' 32P-labeled 15-mer had been annealed were initiated as described under "Materials and Methods." DNA products at different times were analyzed as above. Lanes 1–3 represent 10, 20, and 30 min. a represents wild-type RT, b, Lys452 mutant.

**Lys<sup>103</sup> in the dNTP Binding Site of MuLV Reverse Transcriptase**
Site-directed mutagenesis of Moloney murine leukemia virus reverse transcriptase. Demonstration of lysine 103 in the nucleotide binding site.

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