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Lysine-329 of Murine Leukemia Virus Reverse Transcriptase: Possible Involvement in the Template-Primer Binding Function†

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ABSTRACT: Treatment of murine leukemia virus reverse transcriptase (MuLV RT) with 4-(oxoacetyl)-phenoxyacetic acid (OAPA) results in the loss of DNA polymerase as well as template-primer binding activity but has no effect on the RT-associated RNase-H activity. Binding stoichiometry revealed that approximately 3 mol of OAPA bound per mole of enzyme, when complete enzyme activation occurred. However, in the presence of template-primer, OAPA does not abolish polymerase activity and 2 mol of OAPA remains bound to 1 mol of enzyme. This observation suggests that only one OAPA reactive site is responsible for the loss of polymerase activity. This site was located on a single tryptic peptide by comparing the maps of the native enzyme and the enzyme treated with OAPA in the presence and absence of template-primer. The appearance of a new peptide peak eluting at 125 min from a C-18 reverse-phase column was consistently noted in the tryptic digest of enzyme treated with OAPA. This peak was absent in tryptic peptides made from the control enzyme or the enzyme protein that was treated with OAPA in the presence of activated DNA or synthetic template-primers. Amino acid composition and sequence analyses of this peptide revealed that it spanned residues 312-342 in the primary amino acid sequence of MuLV RT. Since this peptide does not contain arginine residues and Lys-329 exhibited resistance to tryptic digestion, we conclude that Lys-329 is the target of OAPA action. The correlation of the loss of enzyme activity with modification of Lys-329 by OAPA with concomitant loss of template-primer binding activity strongly implicates Lys-329 in the template-primer binding function of MuLV RT.

Moloney murine leukemia virus reverse transcriptase (MuLV RT)[1] is a single polypeptide enzyme, with a molecular mass of approximately 80 000 daltons, which expresses both polymerase and RNase-H activities (Dickson et al., 1982). The complete amino acid sequence of MuLV RT has been deduced from the nucleotide sequence of a noninfectious proviral DNA (Shinnick et al., 1981) and the exact location

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[1] Abbreviations: MuLV, Moloney murine leukemia virus; RT, reverse transcriptase; PLP, pyridoxal 5'-phosphate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; HPLC, high-performance liquid chromatography; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TP, template-primer; OAPA, 4-(oxoacetyl)phenoxyacetic acid; HIV, human immunodeficiency virus; BLV, bovine leukemia virus; RSV, Rous sarcoma virus; HTLV, human T-cell leukemia virus; act. DNA, activated deoxyribonucleic acid.

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of the genome segment coding for RT has been deciphered and confirmed by sequencing amino and carboxy terminal residues (Copeland et al., 1985). The gene for MuLV reverse transcriptase has been cloned and overexpressed in *Escherichia coli* (Kotewicz et al., 1985; Roth et al., 1985). The availability of relatively large amounts of enzyme and the knowledge of the primary amino acid sequence of RT have now made the study of the structure–function relationship of this enzyme feasible. For the past few years, our laboratory has been defining the structure–function relationships in various DNA polymerases, utilizing different active-site-directed reagents. We have defined the structural domains of DNA polymerases that are responsible for the binding of substrate dNTPs and template-primers. Thus, using pyridoxal phosphate (PLP) a substrate dNTP binding-site-directed reagent (Modak 1976), we have been able to define the nucleotide-binding pockets of various DNA polymerases. For example, we have identified Lys-758 in *E. coli* polymerase I (Basu & Modak, 1987), Lys-103 and Lys-421 in MuLV RT (Basu et al., 1988), Lys-263 in HIV RT (Basu et al., 1989a), and Lys-71 in rat β-polymerase (Basu et al., 1989b) using PLP. Similarly, potassium ferrate, a site-directed oxidizing reagent, which inactivates both *E. coli* polymerase I (Basu et al., 1987) and MuLV RT (Nanduri et al., 1988), was used to identify the important residues that are essential for maintaining the integrity of these enzymes. We reported earlier that phenylglyoxal is a specific inhibitor of a variety of DNA polymerases including reverse transcriptases and that its reaction at the template-primer binding site was responsible for the enzyme inactivation (Srivastava & Modak, 1980). By use of *E. coli* DNA polymerase I, the target of phenylglyoxal was shown to be Arg-841 (Mohan et al., 1988). We then extended the phenylglyoxal studies to MuLV RT in order to determine the phenylglyoxal reactive site(s) and thereby identify amino acid residue(s) that may be implicated in the template-primer binding function. Furthermore, their location in the primary amino acid sequence of MuLV RT is likely to be coincident with the template-primer binding domain of this enzyme. The results indicated specificity in the inhibitory action of phenylglyoxal on the activity of MuLV RT as described before. However, the binding site of phenylglyoxal to enzyme protein could not be identified because of the instability of the phenylglyoxal–protein adduct (data not shown). Simultaneously, we were examining the properties of a newly synthesized phenylglyoxal analogue, 4-(oxoacetyl)phenoxyacetic acid (OAPA), which exhibited inhibitory properties similar to that of phenylglyoxal. Both of these compounds have been reported to form stable adducts with enzyme proteins (Takahashi, 1968; Duerksen & Wilkinson, 1987). When the effect of OAPA on MULV RT was examined, it was found to inhibit the polymerase activity with inhibitory characteristics quite similar to those of phenylglyoxal. In this communication, we describe the characteristics of OAPA-mediated inactivation of MuLV RT and show that modification of Lys-329 by OAPA correlates very well with the loss of polymerase activity, as well as template-primer binding function. Thus, Lys-329 appears to be an important residue, possibly involved in the template-primer binding function of MuLV RT.

Materials and Methods

All the nonradioactive dNTPs, calf thymus DNA, template-primers, oligonucleotides, and polynucleotides were purchased from P-L Biochemicals. Radioactive nucleotides were the products of NEN. Trypsin (TPCK) was obtained from Worthington. HPLC-grade water and acetonitrile were obtained from Fisher. Iodoacetamide, *p*-hydroxyacetophenone, and selenium dioxide were purchased from Sigma. Activated DNA was prepared by treatment of native calf thymus DNA with DNase I for 20 min, essentially as described by Maniatis et al. (1982). The digestion time of 20 min was found to produce activated DNA that gave maximum activity with MuLV RT. This DNA was used in all protection experiments.

Synthesis of 4-(Oxoacetyl)phenoxyacetic Acid (OAPA). Synthesis of OAPA was carried out according to the method described by Duerksen and Wilkinson (1987). Briefly, *p*-hydroxyacetophenone was treated with iodoacetamide to form *p*-acetylphenoxyacetamide, followed by oxidation of the amide group to form *p*-acetylphenoxyacetic acid. Oxidation of *p*-acetylphenoxyacetic acid with selenium dioxide resulted in the formation of 4-(oxoacetyl)phenoxyacetic acid crystals. The crystals of OAPA thus formed were collected and stored at −20 °C. OAPA was dissolved in 50% ethanol and was used for these studies.

Purification of MuLV RT. MuLV RT was purified from *E. coli* cells containing the plasmid pB6B15.23 by the procedure of Roth et al. (1985). Briefly, the cells were disrupted with lysozyme followed by NP-40 treatment. The clarified extract was then chromatographed through DEAE-cellulose, phosphocellulose, and poly(ribouridylic acid)–agarose. The final enzyme preparation was >95% pure as judged by SDS–PAGE analysis. In addition, some quantity of purified MuLV RT was generously donated by Dr. G. Gerard of Bethesda Research Laboratories.

Enzyme Assays

DNA Polymerase Assay. DNA polymerase activity of MuLV RT was determined by using synthetic template-primers as described earlier (Modak & Marcus, 1977). The reaction mixture, in a final volume of 100 μL contained the following: 50 mM Hepes–KOH, pH 7.8, 1 mM $MnCl_2$, 1 mM DTT, 10 μg of BSA, 100 mM KCl, 0.5 μg of $(rA)_n \cdot (dT)_{12-18}$, 20 μM dTTP, 1 μCi of [^{3}H]dTTP, and 20 ng of enzyme. After a 15-min incubation at 37 °C, the reaction was terminated by the addition of 5% trichloroacetic acid (TCA) containing 10 mM pyrophosphate. Acid-insoluble material was collected on GF/B glass fiber filters and radioactivity was determined by scintillation spectroscopy.

RNase-H Assays. RNase-H activity was measured by the release of radioactivity from [^{3}H]$(rA)_n$ annealed to $(dT)_n$. The reaction mixture in a final volume of 100 μL contained the following: 50 mM Hepes–KOH, pH 7.8, 1 mM $MnCl_2$, 20 mM NaCl, 1 mM DTT, 10 μg of BSA, 0.5 μg of [^{3}H]-$(rA)_n \cdot (dT)_n$, and 60 ng of enzyme. The reaction was carried out at 37 °C for 30 min and the acid-insoluble counts were determined as described above for the DNA polymerase assays.

Modification of MuLV RT with OAPA. MuLV RT (2 μg) was incubated with desired amounts of OAPA in a final volume of 25 μL containing 50 mM Hepes-KOH, pH 7.8, 1 mM DTT, and 20% glycerol at 37 °C for 60 min. At the end of the incubation, aliquots from the reaction mixture were diluted with 0.1% BSA and the polymerase and the RNase-H activities were determined as described above. For protection studies, desired amounts of substrate nucleotides and template-primers were preincubated with the enzyme for 5 min on ice, prior to the addition of OAPA. After an additional hour of incubation at 37 °C, aliquots were withdrawn and diluted with 0.1% BSA, and the polymerase and RNase-H activities were determined.

UV-Mediated Cross-Linking of Template-Primer to MuLV RT. Synthetic template-primer $(rA)_n \cdot (dT)_{12-18}$ was labeled with [^{32}P]TTP at its 3′-terminus by β-polymerase. The ability of MuLV RT to bind template-primer was determined by

measuring the extent of UV-mediated cross-linking of RT to labeled template-primer. The conditions for cross-linking of template-primer to enzyme were essentially as described earlier (Basu S. et al., 1988). Briefly, 2 μg of enzyme was incubated with 0.5 μg of labeled template-primer (~150000 cpm) on ice for 15 min followed by expossure to UV radiation for 15 min. The samples were denatured by heating at 70 °C for 5 min and treated with phosphodiesterase and micrococcal nuclease simultaneously for 2 h. Samples were then mixed with protein-solubilizing solution and electrophoresed on 8% SDS-polyacrylamide gels. The extent of cross-linking was quantitated by excising the radioactive bands and measuring Cerenkov counts associated with them.

Stoichiometry of OAPA Bound to MuLV RT. A spectrophotometric method was used to determine the stoichiometry of OAPA bound to RT since OAPA has a high extinction coefficient (ϵ_{283} = 16000 M^{-1} cm^{-1}). A standard curve of concentration of OAPA vs absorbance at 282 nm was generated, using a Shimadzu UV-160U spectrophotometer. Two nanomoles of RT was incubated with 3 mM OAPA in the presence and absence of template-primer for 1 h at 37 °C, and aliquots were withdrawn to determine the polymerase activity. The template-primer was then removed from the enzyme by adsorbing to DEAE-cellulose. The enzyme was then precipitated with TCA (to a final concentration of 10%), followed by extensive washing with 5% TCA to remove unreacted OAPA. A similar procedure was carried out with native enzyme as a control. Both the control and modified enzymes were then dissolved in 8 M urea and the increase in absorbance at 282 nm in the modified enzyme was used to determine the moles of OAPA bound to a mole of enzyme protein in the presence and absence of template-primer.

Protease Digestion and HPLC Mapping of Peptides. In order to locate the site of OAPA reactivity, tryptic peptide mapping of native, OAPA-modified, and template-primer-protected RT was carried out. For this purpose, 1 nmol of RT was modified with OAPA in the presence and absence of template-primer as described above. After incubation at 37 °C for 60 min, protein was precipitated by the addition of TCA (final concentration to 10%). The precipitate was washed twice with 5% TCA followed by water and finally dried with ether. The precipitate was then suspended in 50 mM ammonium bicarbonate (pH 8), and trypsin was added at a protein to trypsin ratio of 25:1. After 6 h of digestion at 37 °C, the tryptic peptides were acidified with TFA (pH 2) and were directly injected onto a C-18 reverse-phase column (0.45 × 25 cm, 5-μm particle size and 300-Å pore size) that had been preequilibrated with 0.1% TFA (solvent A). Elution of the peptides was effected by increasing the concentration of solvent B (70% acetonitrile in 0.1% TFA) with the following time schedule: 0–40% B (0–90 min), 40–70% B (90–130 min). Flow rate was maintained at 0.7 mL/min. All the HPLC analyses were carried out on a Varian Vista 5500 HPLC system equipped with a Polychrome 9060 diode array detector system. Peptides were monitored simultaneously at 215 and 282 nm.

Amino Acid Composition and Peptide Sequence Analysis. The peptide of interest was hydrolyzed in 6 N HCl containing 0.2% phenol for 16 h at 115 °C in a Waters picotag work station. The resulting amino acids were converted to their phenylthiocarbamyl derivatives and were analyzed on two Waters Nova pak columns (0.45 × 15 cm) connected in series (Stone & Williams, 1986). Amino acid sequence analysis was carried out at the protein chemistry facility at Yale University.

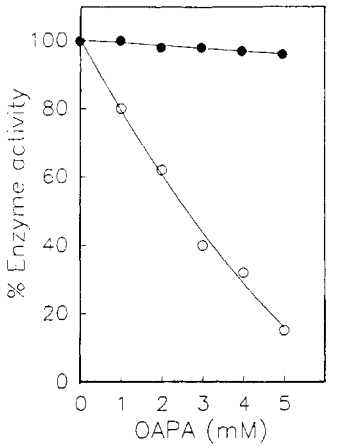

FIGURE 1: Effect of OAPA on MuLV RT. Inactivation of MuLV RT as a function of OAPA concentration. MuLV RT (2 μg) was treated with the desired concentration of OAPA as described under Materials and Methods. Aliquots of modified enzyme were removed to determine DNA polymerase (○) and RNase-H activities (●).

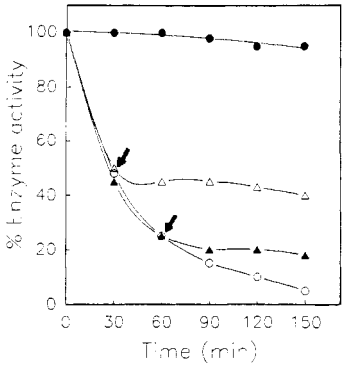

FIGURE 2: Time course of inactivation of MuLV RT by OAPA. MuLV RT (200 μg/mL) was incubated with OAPA (3 mM) and aliquots were removed at various time intervals to determine the polymerase activity (○). The arrows indicate the addition of $(rA)_n$ (200 μg/mL) to the ongoing reaction. At indicated times, aliquots were withdrawn to determine the polymerase activity (▲). Polymerase activity (●) in the absence of OAPA is also shown.

RESULTS

Inactivation of MuLV RT by OAPA. Preincubation of MuLV RT with increasing concentrations of OAPA at 37 °C for 1 h resulted in the loss of DNA polymerase activity in a dose-dependent manner (Figure 1), while RNase-H activity remained essentially unaffected. A typical time course of inactivation of polymerase using a fixed concentration of OAPA (3 mM) is shown in Figure 2. A linear time-dependent inactivation of polymerase activity was also observed.

Protection Studies. In order to determine the specificity of OAPA, the protective effects of dNTPs and various template-primers on enzyme inactivation were examined. As seen from Table I, both natural and synthetic template-primers protect the enzyme from OAPA-mediated inactivation, whereas substrate dNTPs do not have any protective effect. Addition of various template-primers, including activated DNA and single-stranded polynucleotides, significantly protects the enzyme from OAPA-mediated inactivation, whereas oligonucleotides do not show the same level of protection. Furthermore, addition of poly(rA) to the inactivation mixture instantly protects the enzyme from further OAPA-mediated inactivation (Figure 2). These results suggest that an OAPA-reactive site(s) is likely to be involved in the binding of template or template-primer by MuLV RT.

Effect of OAPA Treatment on the Binding of MuLV RT to Template-Primer. In order to directly determine the ability

Table I: Effect of Addition of Substrates and Template-Primers on OAPA-Mediated Inactivation of DNA Polymerase Activity of MuLV RT[a]

additions	concn	DNA polymerase act.[b] (%)
control (no additions)		100
OAPA	3 mM	10
$(rA)_n$	80 μg/mL	95
$(dT)_n$	80 μg/mL	76
$(rA)_n \cdot (dT)_{12-18}$	80 μg/mL	81
$(rC)_n \cdot (dG)_{12-18}$	80 μg/mL	86
activated DNA	80 μg/mL	85
$(rA)_9$	80 μg/mL	31
$(dT)_{10}$	80 μg/mL	42
TTP/dGTP/dCTP	5 mM	16

[a] MuLV RT (2 μg) was incubated with various templates, primers, and substrate dNTPs at indicated concentrations in a final reaction volume of 25 μL for 5 min on ice. OAPA was then added to individual tubes (except control) to a final concentration of 3 mM and further incubation was continued at 37 °C for 1 h. An aliquot from individual tubes was then diluted with 0.1% bovine serum albumin and 20 ng of equivalent enzyme protein was used to determine DNA polymerase activity as described under Materials and Methods. Results are those obtained from a typical experiment. [b] One hundred precent DNA polymerase activity represents 45 pmol of dTMP incorporation (15 min)$^{-1}$ (20 ng of enzyme protein)$^{-1}$.

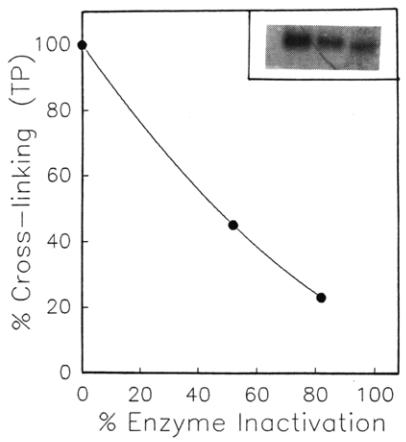

FIGURE 3: Effect of OAPA on the template-primer binding function of MuLV RT. MuLV RT (200 μg/mL) was incubated with OAPA (3 mM) in a reaction mixture containing 50 mM Hepes, pH 7.8, and 20% glycerol. At various time intervals (0, 30, 60 min), 5 μg of enzyme was withdrawn from the reaction mixture and a small aliquot representing 20 ng of enzyme was used for determining the DNA polymerase activity. Three micrograms of RT was used for determining the template-primer binding function by UV-mediated cross-linking to the enzyme as described under Materials and Methods. For quantitation of cross-linking of TP to enzyme, appropriate radioactive bands were excised from the gel and radioactivity was determined. The inset shows the autoradiographs of UV-cross-linked TP to enzyme. One hundred percent cross-linking is equal to 18 000 cpm.

of OAPA-modified enzyme to bind to template-primer, we used the protocol of UV-mediated cross-linking of radiolabeled template-primer to MuLV RT as a template-primer binding assay. Results shown in Figure 3 clearly indicate that the degree of enzyme inactivation by OAPA treatment correlates well with the loss of template-primer binding activity (decrease in the ability to cross-link to labeled template-primer). Thus, an OAPA-reactive site(s) appears to be essential for the interaction of MuLV RT with template-primer.

Effect of High Salt on the Protection of MuLV by Template-Primer from OAPA-Mediated Inactivation. The interaction of enzyme with template-primer has been shown to occur through ionic bonding, and therefore, an increase in the ionic strength of the medium results in the dissociation of the enzyme–template-primer complex (Englund et al., 1969). To

Table II: OAPA-Mediated Inactivation of MuLV RT: Effect of Activated DNA and 0.5 M Salt[a]

addition	TMP incorporated (pmol)	DNA polymerase act.[b] (%)
MuLV RT	42	100
MuLV RT + OAPA	4	10
MuLV RT + 0.5 M NaCl	41	98
MuLV RT + 0.5 M NaCl + OAPA	13	31
MuLV RT + 0.5 M NaCl + act. DNA	45	107
MuLV RT + 0.5 M NaCl + act. DNA + OAPA	15	35

[a] MuLV-RT (2 μg) was modified with 3 mM OAPA in a final volume of 50 μL in the presence or absence of 2 μg of activated DNA and 0.5 M NaCl where indicated. After 1 h at 37 °C, an aliquot from each reaction mixture was withdrawn and diluted with 0.1% bovine serum albumin solution. An aliquot of dilute enzyme representing 20 ng was then assayed for DNA polymerase activity. [b] One hundred percent DNA polymerase activity corresponds to 42 pmol dTMP incorporated/15 min.

Table III: Stoichiometry of OAPA Binding to MuLV RT[a]

addition	DNA polymerase act.[b] (%)	mol of OAPA/ mol of RT
MuLV RT (no OAPA)	100	none
MuLV RT + OAPA	10	2.9 ± 0.3
MuLV RT + act. DNA + OAPA	85	1.8 ± 0.4
MuLV RT + 0.5 M NaCl + act. DNA + OAPA	22	2.7 ± 0.2

[a] MuLV RT (2 nmol, 160 μg) was treated with 3 mM OAPA in a 400-μL reaction mixture containing 50 mM Hepes–KOH (pH 7.8), 1 mM DTT, and 20% glycerol. One hundred micrograms of activated DNA and 0.5 M NaCl were present in the indicated reaction mixture. After 1 h of incubation at 37 °C, 2-μL aliquots were withdrawn from the individual reaction mixtures to determine the DNA polymerase activity. The remainders of the reaction mixtures were then adjusted to 0.25 M NaCl and mixed with DEAE-cellulose to adsorb DNA. The enzyme protein (which does not bind to DEAE-cellulose under these conditions) was recovered by centrifugation and precipitated by the addition of trichloroacetic acid to a final concentration of 10%. Precipitated protein was extensively washed with 5% TCA and distilled water and finally dissolved in 8 M urea. The absorbance of the clear solutions was determined at 282 nm and the amount of OAPA present in individual samples was calculated by using the extinction coefficient value (ϵ_{282} = 16 000 M^{-1} cm^{-1}) as described under Materials and Methods. The ratio of mol OAPA/mol MuLV RT is an average of three experiments. [b] One hundred percent polymerase activity corresponds to 50 pmol of dTMP incorporated (15 min)$^{-1}$ (20 ng of enzyme protein)$^{-1}$.

determine that the action of OAPA is on the enzyme protein and not due to its interaction with polynucleotide templates, the effect of addition of 0.5 M NaCl on the ability of template-primer to protect the enzyme from OAPA-mediated inactivation was examined. Activated DNA, which MuLV RT uses as a template-primer quite efficiently, and which protects MuLV RT from OAPA inactivation, was used as a representative template-primer. Results presented in Tables II and III clearly indicate that, in the presence of high salt, addition of activated DNA affords little protection to the enzyme from OAPA-mediated inactivation. These results provide further support to the suggestion that OAPA is probably a template-primer binding-site-specific reagent for MuLV RT.

Stoichiometry of OAPA Binding to MuLV RT. OAPA has been reported to be an arginine-specific reagent and its inhibitory effect on diverse enzymes has been attributed to modification of selective arginines that are implicated in enzyme catalysis (Duerksen & Wilkinson, 1987). In order to determine the number of OAPA-reactive sites, which would presumably be arginine residues, the binding stoichiometry

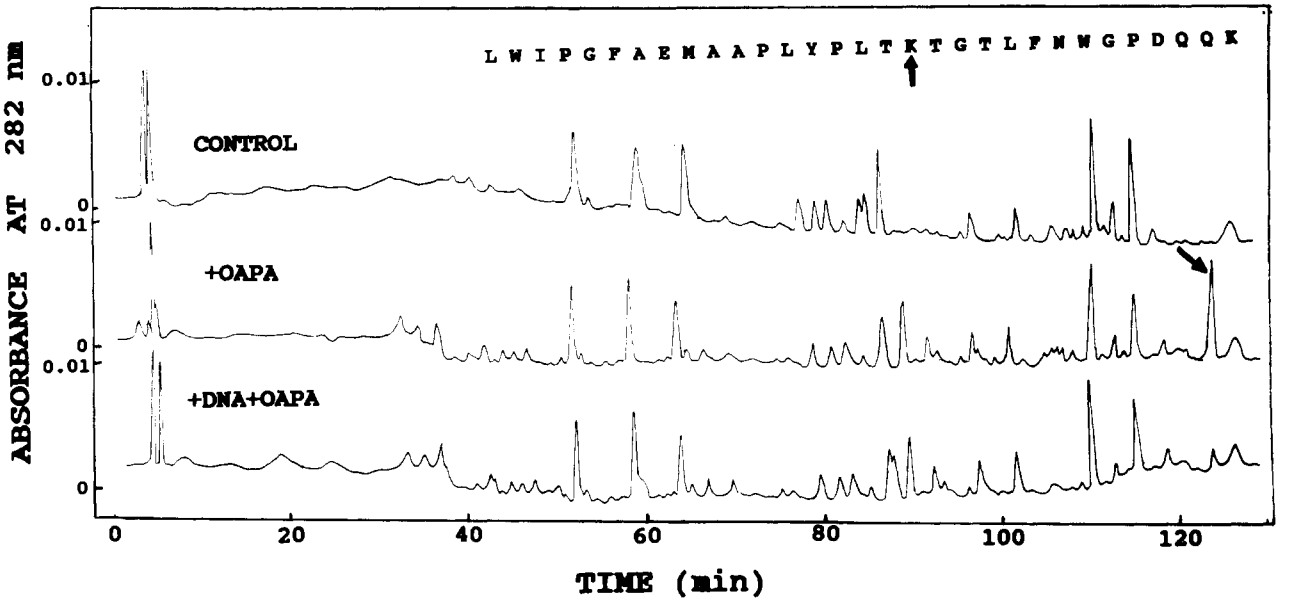

FIGURE 4: Reverse-phase HPLC separation of tryptic peptides derived from control and OAPA-modified MuLV RT in the presence and absence of template primer. MuLV RT (1 nmol) was modified with OAPA in the presence and absence of template-primer. Tryptic digestion of modified and native enzymes was carried out in 50 mM ammonium bicarbonate, pH 8.0, for 6 h at 37 °C. The tryptic peptides were loaded on a Vydac C-18 reverse-phase column equilibrated with 0.1% TFA (solvent A) and peptides were then eluted at a flow rate of 0.7 mL/min with increasing concentrations of solvent B (0.1% TFA in 70% acetonitrile) with the following time schedule: 0–90 min (0–40% B), 90–130 min (40–70% acetonitrile); 0–90 min (0–40% B), 90–130 min (40–70% B). For simplicity the peptide maps monitored at 282 nm have been shown. The arrow shows the position of peptide eluting at 125 min, which is the target of OAPA modification. The inset shows the amino acid sequence of the OAPA-modified peptide.

of OAPA to MuLV RT was determined by using three different conditions as follows: (a) when there is complete inactivation of enzyme activity by OAPA treatment, (b) when OAPA-mediated inactivation is prevented by the addition of activated DNA, and (c) when OAPA and DNA are present together with 0.5 M NaCl in the inactivation mixture. Results of binding stoichiometry under these conditions are shown in Table III. It is clear that the binding of 3 mol of OAPA/mol of MuLV RT results in nearly complete inactivation. However, when enzyme inactivation by OAPA is prevented by the addition of activated DNA, 2 mol of OAPA is still bound to the enzyme. Therefore, the presence of activated DNA appears to protect the reactivity of a single site in MULV RT as well as inactivation of the enzyme. The role of DNA in preventing reactivity of only a single site is further confirmed by the observation that in the presence of salt, which blocks the binding of DNA to enzyme, approximately 3 mol of OAPA is bound per mole of enzyme.

Tryptic Peptide Mapping of OAPA-Modified Enzyme by HPLC. The results of the peptide mapping are shown in Figure 4. In the OAPA-modified enzyme, a peptide eluting at 125 min was consistently noted while this peptide was clearly absent in digests of control as well as activated-DNA-protected RT. These results indicate that the peptide appearing at 125 min probably contains a modification due to OAPA reactivity. This new peptide has a very high absorbance at 282 nm as seen from Figure 4 [OAPA has an extinction coefficient of 16 000 M^{-1} cm^{-1} at 283 nm (Duerksen & Wilkinson, 1987)], indicating that OAPA is contained in the new peptide.

Since stoichiometric studies had indicated that 3 mol of OAPA was bound per mole of enzyme, we had expected to see three distinct peptides with an altered elution pattern, only one of them being protected from OAPA reactivity in the presence of template-primer. Repeated attempts and alterations in elution conditions for the HPLC column (e.g., the use of a phosphate buffer gradient in place of the standard TFA–acetonitrile system) have failed to show more than one new peptide. The reason for not observing all of the OAPA-modified peptides is not known. However, the fact that no loss of peptide species in the digest of OAPA-treated enzyme when compared to that of native enzyme is observed suggests that the undetected peptides may be highly hydrophobic in character and thus do not elute from HPLC columns as distinct species. Alternatively, modified peptides may have lost OAPA during tryptic peptide mapping (which had to be carried out at neutral pH). Nevertheless, the single OAPA-reactive peptide that we have observed seems to be fully protected in the presence of template-primer and therefore must contain the catalytically important residue that is reactive to OAPA.

Table IV: Amino Acid Composition Analysis of Peptide Eluting at 125 min

amino acid residue	residues/mol of peptide: found[a]	residues/mol of peptide: expected from sequence 312–342[b]	amino acid residue	residues/mol of peptide: found[a]	residues/mol of peptide: expected from sequence 312–342[b]
Cys			Met	0.6	1
Asp	2.3	2	Ile	0.6	1
Thr	3.8	3	Leu	3.9	4
Ser	0.2		Tyr	0.6	1
Glu	3.4	3	Phe	2.6	2
Pro	3.9	4	His	0.3	
Gly	2.8	3	Lys	1.2[c]	2
Ala	2.5	3	Arg	0.4	
Val	0.4		Trp	ND[d]	2

[a] This is a typical amino acid composition analysis run of modified peptide. [b] Represents amino acid residues in the primary sequence of MuLV RT (Shinnick et al., 1981). [c] Absence of one lysine residue in the composition indicates that one of the two lysines (Lys-329) was resistant to tryptic digestion. [d] ND, not determined.

Amino Acid Composition and Sequence Analysis of the New Peptide. The amino acid composition of the peptide eluting at 125 min from OAPA-modified RT is shown in Table

IV. The composition of this peptide matches with amino acid residues 312–342 present in the primary structure of MuLV RT (Shinnick et al., 1981). This analysis was further confirmed by sequencing the first 13 N-terminal amino acids. The fact that the 30 amino acid sequence that we have identified by composition studies contains a trypsin-resistant lysine (Lys-329) strongly suggests that Lys-329 is the target of OAPA reactivity.

Discussion

In this article we report the use of 4-(oxoacetyl)phenoxyacetic acid, an analogue of phenylglyoxal, to identify the amino acid residue that may reside in the template-primer binding domain of MuLV RT. Phenylglyoxal has been shown to be a template-primer binding-site-directed reagent in *E. coli* DNA polymerase I and Rauscher leukemia virus RT (Srivastava & Modak, 1980) and has been used to identify Arg-841 in *E. coli* DNA polymerase I as an essential residue in template-primer binding function (Mohan et al., 1988).

Our present studies indicate that OAPA has properties similar to that of phenylglyoxal, which suggests that OAPA acts as a template-primer binding-site-directed reagent (Table I). The specificity of OAPA toward the template-primer binding function of MuLV RT has been indicated by the observation that protection of polymerase activity can be achieved by the inclusion of template-primer or template polynucleotides but not by oligonucleotides or substrate dNTPs. Similarly, addition of polynucleotide to an ongoing inactivation mixture instantly protects the enzyme from further inactivation (Figure 2). It is interesting to note that both template-primers and templates [such as poly(A) or poly(dT)] by themselves provide protection from OAPA inactivation while oligomeric nucleotide primers or substrate dNTPs are ineffective. These results appear to suggest that OAPA-reactive site(s) may be important in recognition and binding of polynucleotide templates. The affinity and binding of polynucleotides to RTs has been well documented (Abrell et al., 1972; Srivastava, 1973; Marcus et al., 1974; Erickson, 1975). The loss of template-primer binding ability of OAPA-treated enzyme (Figure 2) is consistent with the implication of an OAPA-reactive site in binding function. Further support for this notion is found in the observation that in the presence of high ionic strength (which does not permit binding of enzyme to template primer) template-primer provides no protection from OAPA (Table II). The stoichiometry of OAPA binding to enzyme under these conditions (Table III) further indicates that there is a single OAPA-reactive site that is responsible for the observed loss of template-primer binding and enzyme activity. A note of caution, however, may be appropriate here. Our studies clearly show that modification of a single OAPA-reactive site, which was subsequently identified as Lys-329, significantly weakens the interaction of enzyme with templates. No direct demonstration of the involvement of Lys-329 in the binding of template has been achieved. Site-directed mutagenesis of this residue may provide direct proof for the role of this lysine in catalysis by MuLV RT.

Binding of OAPA to native MuLV RT amounted to 3 mol/mol of enzyme whereas in the presence of activated DNA binding of OAPA was reduced to 2 mol/mol of enzyme. Therefore, when tryptic peptide maps from control and modified enzymes were compared, we expected to see at least three modified (as judged by their altered elution patterns as well as high absorbance at 282 nm) peptide peaks representing three sites of OAPA reactivity in the modified enzyme. Furthermore, modification in the presence of DNA should selectively eliminate the appearance of one of the three peptides (as expected from the stoichiometry). However, results consistently showed the presence of only one modified peptide in OAPA-treated samples and appearance of this peptide was indeed blocked by inclusion of activated DNA in the modification mixture. While these results clearly show that the peptide that we have identified contains a catalytically or functionally important site/residue, two other catalytically unimportant peptides containing OAPA-reactive sites have eluded detection. The possible reason for not obtaining all three modified peptides is briefly discussed under Results.

Amino acid composition and partial sequence analysis of the OAPA-modified peptide permitted identification and possible location of the OAPA-reactive site within the peptide. On the basis of composition analysis (Table IV) the peptide was tentatively assigned to encompass residues 312–342 in the primary amino acid sequence of MuLV RT. This assignment was confirmed by sequencing the first 13 residues. Since glyoxal-containing reagents have been known for their high perference for arginine residues (Takahashi, 1968; Duerksen & Wilkinson, 1987), the absence of an arginine residue in this peptide was quite surprising. Nevertheless, the presence of trypsin-resistant lysine in the OAPA-modified peptide strongly suggested that Lys-329 within this peptide is the site of OAPA modification. Therefore, the lysine residue detected in the composition analysis probably represents the trypsin cleavage site, while the OAPA-modified lysine has eluded detection (Basu et al., 1988). If Lys-329 was not modified, one would have expected tryptic cleavage at that site. One other explanation for the trypsin resistance of Lys-329 in this peptide may be related to or postulated by the absence of this lysine in the MuLV RT used in the present investigation, via its mutation to threonine. This requires only a single base substitution in the lysine codon (AAA to ACA). The composition analysis does indicate the presence of an extra threonine. Such a scenario, however, seems unlikely since identical results were obtained with enzyme protein purified from a clone produced by Dr. Gerard of Bethesda Research Laboratories (data not shown). This was the source of enzyme in our previous study on PLP sensitivity of MuLV RT (Basu et al., 1988). Since lysine is the amino acid most closely related to arginine and some OAPA reactivity toward lysine has been demonstrated in ubiquitin (Duerksen-Hughes et al., 1987), we conclude that Lys-329 is the site of OAPA action. The fact that many nucleotide-binding enzymes and proteins do utilize specific lysine and/or arginine residues in their nucleotide-binding reactions makes this assignment reasonable.

A computer-aided comparison of amino acid sequences of different reverse transcriptases and that of *E. coli* RNase-H has led to the proposal that the N-terminal domain of reverse transcriptase should contain DNA polymerase activity and the C-terminal domain should contain RNase-H activity (Johnson et al., 1986). This concept was later confirmed independently by Tanese and Goff (1988) and Gerard and his colleagues (Kotewicz et al., 1988) by cloning the two domains separately and showing the association of the appropriate activities with the individual clones. These results indicate that the two activities must utilize distinct and nonoverlapping active sites for nucleic binding and catalysis. The results presented in this paper demonstrate that modification of Lys-329 in the N-terminal domain of MuLV RT abolishes the polymerase activity without affecting the RNase-H activity (Figure 2). Extension and alignment of amino acid sequences from Johnson et al. (1986) show that Lys-329 is conserved in BLV

RT and there is a homologus substitution by arginine in HIV, HTLV, and RSV RTs as shown:

FCRLWIPGFAEMAAPLYP LT	K TGTLFNWGPDQQK	MuLV RT	(Shinnick et al., 1981)
WVSKGTPTLRQPLHSLYGALQ	R HTDPRDQIYL	HTLV-1 RT	(Seiki et. al., 1983)
WVSRGTPTTRRPLQLLYSSL	K GIDDFRAIIQL	BLV RT	(Rice et al., 1985)
WLRPALGIPPRLMGPFYEQL	R GSDPNEAREWN	RSV RT	(Schwartz et al., 1983)
W ASQIYPGIKVRQLCKLL	R GTKALTEVIPL	HIV RT	(Ratner et al., 1985)

The above sequence comparison also indicates that several residues (*) around Lys-329 are conserved, and therefore, this region may be generally important in reverse transcriptases. Our results indicate that the function of this region is likely to be in the binding of template-primers. Conserved lysine/arginine may be important in ionic bonding with the phosphate backbone of template-primer, while the adjacent tyrosine and leucine residues may be involved in hydrophobic interactions with bases in the template-primer. In conclusion, we have identified Lys-329 as an important residue in MuLV RT that most probably functions in template-primer binding.

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