Sulphydryl groups in the template-primer-binding domain of murine leukaemia virus reverse transcriptase

Identification and functional analysis of cysteine-90

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Treatment of murine leukaemia virus reverse transcriptase with benzophenone 4-maleimide inactivates DNA polymerase activity, but has no effect on the RNAase H function. Kinetic measurements indicated that benzophenone 4-maleimide is a competitive inhibitor with respect to template–primer binding, but is non-competitive with respect to dNTP binding. Enzyme modified with benzophenone 4-maleimide cannot bind template–primer or primer alone, as judged by u.v.-mediated cross-linking of radiolabelled substrates. Of the eight cysteine residues in murine leukaemia virus reverse transcriptase, only two were modified by benzophenone 4-maleimide, which were identified as Cys-90 and Cys-310 by comparative tryptic-peptide mapping and amino acid composition analysis. Inclusion of template–

primer or primer alone in the modification mixture protected only Cys-90 from modification by benzophenone 4-maleimide. To investigate the role of Cys-90 in detail, we converted it to alanine by site-directed mutagenesis. The mutant enzyme, however, exhibited no loss either of DNA polymerase or of RNAase H activity. These results indicate that Cys-90 is located in a domain of murine leukaemia virus reverse transcriptase that binds template–primer, but may not have a direct role in the enzymic function of the enzyme. Ala-90 mutant murine leukaemia virus reverse transcriptase is at least 10-fold more susceptible to heat inactivation than is the wild-type enzyme, which suggests that Cys-90 in murine leukaemia virus reverse transcriptase may play a role in maintaining structural integrity.

INTRODUCTION

Early events in the retroviral life cycle are dependent on the virus-associated enzyme reverse transcriptase (RT), which copies the viral RNA genome to form a double-stranded proviral DNA (for a review, see Varmus and Swanstrom, 1982). The RT associated with murine leukaemia virus (MuLV) has been purified and has been shown to be a monomer of relative molecular mass about 70 000–80 000 (Gerard and Grandgenett, 1975; Verma, 1975). The primary amino acid sequence of MuLV-RT was deduced from the complete nucleotide sequence of the proviral DNA (Shinnick et al., 1981). The gene coding for MuLV-RT has been cloned into a number of expression vectors (Kotewicz et al., 1985; Roth et al., 1985), and the protein has been purified to homogeneity. The cloned protein, of M_r 71 000, enables the study of structure–function relationships in this class of enzymes.

There is a significant primary sequence similarity amongst all the known retroviral RTs (Johnson et al., 1986). By comparison of RT sequences with other DNA polymerases and with RNAase Hs, it was suggested that in all RTs the DNA polymerase and RNAase H domains are distinct from each other and joined together by tether sequences. Although all RTs characterized so far are multifunctional enzymes and have a similar domain organization, they differ significantly in their subunit organization, catalytic properties and patterns of inhibition by different drugs (Gerard and Grandgenett, 1980; Verma, 1981; Hizi et al., 1988). The enzyme from one system, therefore, may not always serve as a model for other systems. The three-dimensional structure of human immunodeficiency virus-1 (HIV1)-RT has been deciphered recently (Kohlstaedt et al., 1992), but, due to significant differences between HIV1-RT and

MuLV-RT, the structure of HIV1-RT cannot be extrapolated directly to MuLV-RT. MuLV-RT is active as a monomer, and the catalytic domains (DNA polymerase and RNAase H) are totally independent of each other (Kotewicz et al., 1988; Tanese and Goff, 1988; Hizi et al., 1990). However, HIV1-RT is active only as a dimer (Basu et al., 1992b), and there is a significant interaction between the two catalytic domains (Hizi et al., 1988; Prasad and Goff, 1989; Hizi et al., 1991).

In an attempt to understand the enzymic mechanisms and structure–function relationships of MuLV-RT, our laboratory has, over the past few years, carried out chemical modification studies, u.v.-mediated cross-linking of substrates and site-directed mutagenesis to identify domains and residues that are important for catalysis. For example, covalent modification with pyridoxal phosphate (A. Basu et al., 1988) and subsequent site-directed mutagenesis (Basu et al., 1990) has revealed Lys-103 to be one of the residues that are involved in substrate dNTP binding. U.v.-mediated cross-linking with oligo(dT)₈ has enabled us to identify residues 72–80 as forming a part of the primer-binding domain (Tirumalai and Modak, 1991). Ferrate ion-mediated oxidation of MuLV-RT has revealed a number of domains that are important for the template–primer binding function of this enzyme (Reddy et al., 1991).

All known RTs, except those of HIV1 and HIV2, are relatively rich in cysteine residues; MuLV-RT has eight cysteines, while avian myeloblastosis virus (AMV)-RT has twelve in the β -subunit and eight in the α -subunit (Weiss et al., 1985; Myers et al., 1990). It has also been well-documented that all RTs require reduced sulphydryl groups for their DNA polymerase and/or RNAase H activities (Gorecki and Panet, 1978; Parnaik and Das, 1981; Srivastava et al., 1983; Hizi et al.,

Abbreviations used: BP-MAL, benzophenone 4-maleimide; MuLV, murine leukaemia virus; AMV, avian myeloblastosis virus; HIV, human immunodeficiency virus; RT, reverse transcriptase; DTT, dithiothreitol; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

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1991, 1992). However, identification of sulphhydryl groups and the elucidation of the importance of individual sulphhydryl groups in the process of catalysis has not been reported. In this study, we have used a sulphhydryl-group-specific bifunctional reagent, benzophenone 4-maleimide (BP-MAL), to identify cysteine residues in MuLV-RT, and to show that Cys-90 and Cys-310 are selectively modified by BP-MAL. A detailed characterization of the chemically modified enzyme implied that the domain containing Cys-90 was important in binding template-primer. Site-directed mutagenesis of Cys-90, however, indicated no direct role for cysteine in the catalysis. This paper gives the results of this investigation and discusses possible implications for the role of Cys-90 in MuLV-RT.

MATERIALS AND METHODS

All synthetic template–primers and non-radioactive NTPs were obtained from PL Biochemicals. Trifluoroacetic acid (TFA), formamide and dithiothreitol (DTT) were from Sigma. Acetonitrile and h.p.l.c.-grade water were from Fisher Scientific Co. Trypsin (Tos-Phe-CH₂Cl-treated) was obtained from Worthington. BP-MAL was obtained from Molecular Probes (Junction City, OR, U.S.A.). All other chemicals were of analytical grade. MuLV-RT was purified essentially as described previously (Roth et al., 1985) from an *Escherichia coli* strain containing the plasmid pRT601, which codes for an RT of M_r 71 000. [³H](rA)_n·(dT)_n was synthesized and purified as described previously (Reddy et al., 1991).

Enzyme assays

DNA polymerase assay

The DNA polymerase activity of MuLV-RT was determined using synthetic template–primers as described previously (A. Basu et al., 1988).

RNAase H assay

The RNAase H activity of MuLV-RT was measured by using tritium-labelled (rA)_n·(dT)_n as the substrate. The reaction mixture contained 50 mM Hepes/KOH, pH 7.8, 1 mM DTT, 20 mM NaCl, 10 µg of BSA, 1 mM MnCl₂, 0.5 µg of [³H](rA)_n·(dT)_n (specific radioactivity 20 000 c.p.m.) and 100 ng of enzyme in a final volume of 100 µl. The reactions were carried out at 37 °C for 30 min, and acid-insoluble counts were determined.

Inactivation of MuLV-RT with BP-MAL

All the reactions were carried out in the dark as BP-MAL is a photolabile reagent. MuLV-RT (adjusted to 140 µg/ml) in 50 mM Hepes/KOH, pH 7.8, 20 mM NaCl and 10% (v/v) glycerol was treated with different concentrations of BP-MAL (added from stock solutions in dimethylformamide) at 4 °C for various times. The concentrations of BP-MAL in stock solutions were adjusted so that the final concentration of dimethylformamide in the reaction mixture was not more than 4%. The reactions were quenched by the addition of 10 mM DTT (final concentration) and, after a further incubation of 15 min on ice, small aliquots were assayed for DNA polymerase and for RNAase H activities. For protection experiments, various amounts of different substrates were incubated with the enzyme for 15 min on ice before adding BP-MAL.

Cross-linking studies

To examine whether the modification of MuLV-RT by BP-MAL led to its inability to bind to template–primer, the extent of u.v.-mediated cross-linking of native and BP-MAL-modified enzyme to radiolabelled template–primer was determined. For cross-linking, 2 µg of the enzyme was incubated on ice for 15 min with 0.5 µg (0.5 µCi) of labelled template–primer {poly(dA)·[³²P](dT)₁₅} or of primer {5′[³²P](dT)₁₅} in 30 µl of buffer containing 50 mM Hepes/KOH, pH 7.8, 20 mM NaCl, 10% (v/v) glycerol, 1 mM DTT and 1 mM EDTA. The reaction mixture was then exposed to u.v. irradiation at a dose of 4×10^{-4} J/mm² in a spectrolinker XL-1000 u.v. cross-linker (manufactured by Spectronics Corporation, Chestnut Hill, MA, U.S.A.). The u.v.-cross-linked enzyme species were analysed by SDS/PAGE. To measure the extent of cross-linking the radioactive bands were excised and dried, and the associated radioactivity was quantified by liquid-scintillation spectroscopy.

Protein chemistry of BP-MAL-modified MuLV-RT

For peptide analyses, 2 nmol of the enzyme was modified with BP-MAL as described above. The protein was precipitated with 10% (v/v) trichloroacetic acid (TCA) and collected by centrifugation at 10 000 g for 10 min at 4 °C. The precipitate was washed with 5% (v/v) cold TCA (until there was no trace of BP-MAL in the washings), water and alcohol, and finally was dried under vacuum. The pellet was solubilized in 25 µl of 8 M urea and then diluted to 200 µl with 100 mM ammonium bicarbonate, pH 8.0. Trypsin (Tos-Phe-CH₂Cl-treated) was then added at a protein:trypsin ratio of 50:1, and the mixture incubated at 37 °C. After 2 h, another aliquot of trypsin (50:1 protein:trypsin) was added and the incubation continued overnight. The tryptic digests were directly loaded on to a Vydac C-18 reverse-phase column (0.45 cm × 25 cm, 5 µm particle size, 30 nm pore size) that had been pre-equilibrated with 0.1% TFA (solvent A). Peptides were eluted by increasing the concentration of 70% (v/v) acetonitrile containing 0.1% TFA (solvent B) as described previously (Basu et al., 1988). The peptides of interest were dried in vacuum, redissolved in 200 µl of 0.1% TFA and further purified on a Vydac C-18 column using shallow gradients of solvent B for peptide elution (see the legend to Figure 4 for details).

Amino acid analysis

Purified peptides were hydrolysed in 6 M HCl containing 0.2% phenol for 16 h at 115 °C in a Waters Pico-tag work-station. The resulting amino acids were converted to their phenylthiocarbamyl derivatives with phenyl isothiocyanate and analysed by h.p.l.c. using Waters Nova-pak columns (two 0.45 cm × 15 cm columns attached in series) by the method described by Stone and Williams (1986).

Site-directed mutagenesis

Mutagenesis of codon-90 of MuLV-RT from cysteine to alanine was done using PCR. Two oligonucleotides corresponding to nucleotides 257–278 and 1089–1108 in the MuLV-RT sequence were synthesized chemically and used as primers for the PCR. The first oligonucleotide is a 22meric product with the sequence 5′-TACTGGTACCCGCCCGAGTCCC-3′ that contained the desired mutation (underlined). It also contains a naturally occurring *Kpn*I site (GGTACC). The second oligonucleotide, 5′-GTAGCCTGCTTCTCGTCGAC-3′, was 811 bases downstream from the 3′ end of the first oligonucleotide and is complementary

to the coding sequence. Plasmid pB6B15.23 containing the entire sequence of MuLV-RT (Roth et al., 1985) was linearized by digesting with *Bgl*II and 20 cycles of PCR were performed using the above oligonucleotides. The amplified DNA (850 bp) was purified, cleaved with *Kpn*I and *Sal*I and cloned into the expression vector pB6B15.23. The entire inserted region was sequenced by the dideoxy method to confirm that no change in the MuLV-RT sequence had occurred except for the desired mutation. The mutant protein was expressed and purified as described previously (Basu et al., 1990).

Table 1 Effect of BP-MAL on MuLV-RT: a dose-response study

MuLV RT (1 μ g) present in a reaction mixture containing 50 mM Tris/HCl, pH 8, 1 mM $MnCl_2$, 10% (v/v) glycerol and 20 mM NaCl was treated with BP-MAL to achieve the final concentration indicated in a volume of 50 μ l. After 10 min at 4 $^{\circ}C$, an equal volume of 20 mM DTT was added to each tube. A tube that received dimethylformamide (DMF) at a final concentration of 4% serves as a control for solvent effects (2 mM BP-MAL would give a 4% DMF effect). An aliquot corresponding to 20 ng of enzyme was used to determine DNA polymerase activity with $(rA)_n \cdot (dT)_{15}$ as a template-primer and $[^3H]dTTP$ as a substrate. An aliquot of enzyme of 100 ng was used for RNAase H activity assays. An RNAase H activity of 100% corresponds to a release of 10500 c.p.m. when 0.5 μ g of $[^3H](rA)_n \cdot (dT)_n$ is used as a substrate (20000 c.p.m.).

[BP-MAL] (mM)	DNA polymerase activity (pmol of dTMP incorporated)		RNAase H activity (%)
	15 min	30 min	
None	102	210	100
None (+ 4% DMF)	100	195	95
0.25	78	151	90
0.5	36	68	90
1.0	7	11	85
2.0	2	3	70

Table 2 Properties of BP-MAL inactivation of MuLV-RT

MuLV-RT (1 μ g) was treated with 0.5 mM BP-MAL for the desired period of time as described in the legend to Table 1. Treatment with BP-MAL was terminated by the addition of an equal volume of 20 mM DTT. For zero-time controls, DTT addition preceded the addition of BP-MAL. An aliquot representing the amount of enzyme indicated was then tested for DNA polymerase activity using $(rA)_n \cdot (dT)_{15}$ as a template-primer and $[^3H]dTTP$ as a substrate.

Time of BP-MAL treatment (min)	Enzyme per assay (ng)	DNA polymerase activity (pmol of dTMP/ 15 min)
0	20	105
5	20	70
10	20	38
20	20	18
0	40	198
20	40	34
0	100	420
20	100	72
20	40* + 2 μ g $(rA)_n \cdot (dT)_{15}$	41
20	40* + 100 mM DTT	45

* An enzyme aliquot (40 ng) was mixed with the amount of template primer or DTT indicated, allowed to stand on ice for 60 min and then assayed with the standard reaction mixture.

Table 3 Effect of dNTP and template-primer on BP-MAL-mediated inactivation of MuLV-RT

MuLV-RT (1 μ g) was modified with BP-MAL (0.5 mM) in 50 μ l of reaction mixture containing 50 mM Tris/HCl, pH 7.8, 20 mM NaCl, 10% (v/v) glycerol and 1 mM $MnCl_2$, as described in the Materials and methods section. For protection experiments, the amounts of substrate indicated were added to the reaction mixture before the addition of BP-MAL. BP-MAL-mediated inactivation was quenched by the addition of equal volume of 20 mM DTT and, after 10 min over ice, aliquots representing 20 ng of MuLV-RT were assayed for DNA polymerase activity, as described in the Materials and methods section. Abbreviation used: Enz, enzyme.

Additions	Activity (pmol of dTMP/ 15 min)	Activity (%)
Enz (control)	98	100
Enz + BP-MAL	17	17
Enz + TTP (1 mM)	140	100
Enz + TTP (1 mM) + BP-MAL	27	19
Enz + $(rA)_n \cdot (dT)_{15}$ (3 μ g)	160	100
Enz + $(rA)_n \cdot (dT)_{15}$ (3 μ g) + BP-MAL	112	70
Enz + $(rA)_n$ (3 μ g)	105	100
Enz + $(rA)_n$ (3 μ g) + BP-MAL	56	50
Enz + $(dT)_{15}$ (1 μ g)	110	100
Enz + $(dT)_{15}$ (1 μ g) + BP-MAL	82	75

Figure 1 Lineweaver-Burk plot analysis of BP-MAL-mediated inactivation of MuLV-RT

(a) An aliquot of MuLV-RT (20–40 ng) was treated with the desired concentration of BP-MAL in the presence of increasing concentrations of $(rA)_n \cdot (dT)_{15}$ and with a fixed concentration of dTTP (100 μ M). (b) MuLV-RT was treated with BP-MAL in the presence of increasing concentrations of dTTP with a fixed concentration (2 μ g) of $(rA)_n \cdot (dT)_{15}$. In all cases the initial rates of polymerization (dTTP incorporation) were determined, and the results are presented in the form of double reciprocal plots. (Δ) No BP-MAL (control); ($\circ$) 0.2 mM BP-MAL; ($\square$) 0.5 mM BP-MAL.

RESULTS

Inactivation of MuLV-RT with BP-MAL

Modification of MuLV-RT with BP-MAL, a highly specific sulphhydryl-modifying reagent, results in the loss of DNA polymerase activity of the enzyme (Table 1). As can be seen from Table 1, nearly complete inactivation of DNA polymerase activity

Figure 2 U.v.-mediated cross-linking of MuLV-RT to template-primer and to primer

MuLV-RT (2 μ g) was inactivated to about 95% by treatment with 0.5 mM BP-MAL for 30 min. The control and modified enzyme were cross-linked to radiolabelled substrates using u.v. energy, and the extent of cross-linking was measured as described in the Materials and methods section. Lanes 1 and 2 represent cross-linking of $(rA)_n \cdot [(^{32}P)dT]_{15}$ to control and to modified enzyme respectively; lanes 3 and 4 represent cross-linking of $5'[(^{32}P)dT]_{15}$ to control and to modified enzyme.

Figure 3 Purification of BP-MAL-modified peptides

(a) Fractions eluting at around 110 min and 119 min (Figure 4) were pooled, lyophilized, redissolved in 0.1% TFA (buffer A) and loaded on a Vydac C-18 column pre-equilibrated with buffer A. Peptides were eluted at a flow rate of 0.7 ml/min with increasing concentrations of buffer B [0.1% TFA in 70% (v/v) acetonitrile]: 0–10 min (0–45% buffer B), and 10–100 min (45–60% buffer B). BP-MAL-modified peptides are indicated by arrows. (b) Shows the characteristic absorption spectrum of the BP-MAL-modified peptides.

of MuLV-RT occurs with 1 mM BP-MAL, while its RNAse H activity was affected minimally. A time-course of inactivation of MuLV-RT with BP-MAL showed a progressive inactivation of the enzyme with time (Table 2). Furthermore, inactivation appears to be irreversible, as judged by the observations that (1)

no recovery of enzyme activity is seen when BP-MAL-treated enzyme was diluted 100-fold with reaction mixture lacking BP-MAL, and (2) treatment of BP-MAL-treated enzyme with 200-fold molar excess of DTT (even when incubated overnight on ice) did not result in the reactivation of enzyme (results not shown). It should also be noted that the addition of template-primer, which protects from BP-MAL inactivation (Table 3), does not reactivate previously inactivated enzyme (Table 2). The pattern of inhibition observed with BP-MAL was quite similar to that observed with *N*-ethylmaleimide on AMV-RT (Gorecki and Panet, 1978) and on MuLV-RT (Hizi et al., 1991).

Mode of inactivation

Studies with various sulphydryl reagents have suggested that the polymerase activity of AMV-RT is dependent on the presence of reduced sulphydryl groups. Competition experiments with substrate dNTP or with template-primer (Gorecki and Panet, 1978; Parnaik and Das, 1981) and direct substrate-binding assays (Srivastava et al., 1983) have suggested that the cysteine sulphydryl groups of AMV-RT are involved in the process of template-primer binding. Similar studies were carried out to determine the mode of action of BP-MAL on MuLV-RT. Lineweaver-Burk analysis of competition between $(rA)_n \cdot (dT)_{15}$ and inhibitor, as well as dNTP and inhibitor, is shown in Figure 1. The results indicate clearly that BP-MAL is competing directly with the template-primer-binding site of MuLV-RT. To emphasize this point further, the protective effects of various substrates of the DNA polymerization reaction on BP-MAL-mediated inactivation were measured. The results, shown in Table 3, show clearly that polynucleotides, but not dNTPs, protect the DNA polymerase activity against BP-MAL. The most important observation is that the extent of protection by oligo(dT)₁₅ alone is as high as that offered by template-primer. Earlier studies have shown that oligonucleotides do compete for the natural tRNA primer-binding site of reverse transcriptases (Sobol et al., 1991; Tirumalai and Modak, 1991; Basu et al., 1992a). Thus the results above indicate that the BP-MAL-sensitive sulphydryl residue(s) are probably located in the primer-binding site of the enzyme.

U.v.-mediated cross-linking of BP-MAL-modified enzyme to template-primer

U.v.-mediated cross-linking of substrate to enzyme has been shown to be a very sensitive assay procedure to measure the template-primer-binding function of DNA polymerases (S. Basu et al., 1988). To determine if the template-primer binding in BP-MAL-modified enzyme is affected, we used the above procedure. The results are shown in Figure 2. As can be seen from Figure 2, cross-linking both of template-primer as well as of primer alone to the enzyme decreases upon BP-MAL modification. The decrease in the extent of cross-linking of primer $[(^{32}P)dT]_{15}$ to the enzyme correlates very well with the loss of DNA polymerase activity; however, cross-linking of $(rA)_n \cdot [(^{32}P)dT]_{15}$ is not similarly correlated. The reason for this is poorly understood, but it could be due to a partial cross-linking of the template-primer to the RNAse H domain, which is not affected by BP-MAL.

Comparative tryptic maps of native and BP-MAL-treated enzyme in the presence or in the absence of template-primer

Comparative tryptic-peptide mapping by h.p.l.c. of native and of modified enzyme has been used to identify important domains in a number of DNA polymerases (Basu and Modak, 1987; A. Basu

Figure 4 Separation of tryptic peptides of MuLV-RT by reverse-phase h.p.l.c.

MuLV-RT (1 nmol) was modified by BP-MAL under various conditions, digested with trypsin and the resulting peptides were resolved on a Vydac C-18 column, as described in the Materials and methods section. The peptides were monitored both at 215 nm and at 278 nm simultaneously, but only the scans monitored at 278 nm are shown here for clarity. The plots shown here represent tryptic maps obtained from control enzyme (a), enzyme treated with BP-MAL (b), and enzyme treated with BP-MAL in the presence of template-primer (c). BP-MAL-modified peptides (labelled 1 and 2) are indicated by arrows.

et al., 1988, 1989; S. Basu et al., 1988; Mohan et al., 1988). Thus, to identify the domain(s) in the enzyme molecule containing the BP-MAL-reactive cysteine residue(s) that interacts with the template-primer, we resorted to comparative tryptic-peptide mapping. Figure 4 shows the reverse-phase tryptic-peptide maps, on a Vydac C-18 reverse-phase column, of native enzyme, BP-MAL-modified enzyme and enzyme modified with BP-MAL in the presence of template-primer or of primer alone. The BP-MAL-modified peptides were located by the appearance of new peptides in the map of BP-MAL-modified enzyme and by their characteristic absorption spectra (see Figure 3b). Of the two BP-MAL-modified peptides, only peptide 2, eluting after about 119 min, was found to be specifically protected from BP-MAL when the modification was carried out in the presence of template-primer or primer alone (Figure 4c). To characterize the BP-MAL-modified peptides, both peptides 1 and 2 (Figure 4) were purified further by rechromatography on a C-18 column using a shallow gradient of acetonitrile (Figure 3), and subjected to amino acid composition analysis. The amino acid composition of peptides 1 and 2, as shown in Table 4, revealed them to span residues 302–311 and 81–102 respectively, in the primary amino acid sequence of MuLV-RT (Copeland et al., 1985). Both the peptides contain one cysteine residue and, since maleimide has high specificity towards sulphhydryl groups at neutral pH (Partis et al., 1983), we conclude that Cys-310 in peptide 1 and Cys-90 in peptide 2 are the sites of modification. The high specificity of the maleimide group of BP-MAL towards cysteine residues has been noted in a number of other proteins. For example, Cys-98

of troponin and Cys-190 of tropomyosin (Tao et al., 1986), and Cys-697 of myosin (Agarwal et al., 1991) were found to be modified specifically by BP-MAL. Since only peptide 2, containing Cys-90, was specifically protected by template-primer from BP-MAL modification, we conclude that Cys-90 lies in a domain that is important in binding template-primer.

Site-directed mutagenesis of Cys-90

The above results have indicated that the domain containing Cys-90 is important in binding primer in MuLV-RT. Reduced cysteine residues are known to be involved directly in binding nucleotides in tubulin (Little and Luduena, 1987) and in the gene 5 protein of fd phage (Paradiso and Konigsberg, 1982). Thus, to see if the -SH group of Cys-90 in MuLV-RT is directly involved in nucleotide binding, we mutated Cys-90 to Ala by site-directed mutagenesis. The mutant protein was purified and its identity checked by Western-blot analysis using a monoclonal antibody raised against pure viral enzyme (the antibody was a generous gift from Dr. S. Goff of Columbia University, New York), as described previously (Basu et al., 1990). We then compared the overall catalytic activities of the wild-type and of the mutant enzyme. It is apparent from Table 5 that the two enzymes are equally active. There was, however, a slight but consistent increase in the specific activity of the DNA polymerase activity of the mutant enzyme as measured in three different isolates. The Cys-90 mutation results do not confirm the results obtained using chemical modification of this residue.

Table 4 Amino acid composition of peptides 1 and 2

See Figure 4. The numbers given represent residues in the primary amino acid sequence of MuLV-RT (Copeland et al., 1985).

Amino acid residue	Residues/mol of peptide			
	Peptide 1		Peptide 2	
	Found	Expected from sequence 302–311	Found	Expected from sequence 81–102
Cys	—*	1	—*	1
Asp	0	0	1.5	2
Thr	0.8	1	0.9	1
Ser	0.2	0	0.8	1
Glu	0.7	1	1.9	2
Pro	0.1	0	4.2	4
Gly	2.1	2	1.2	1
Ala	1.1	1	0.1	0
Val	0.2	0	2.1	2
Met	0.1	0	0	0
Ile	0	0	0.8	1
Leu	1.1	1	4.8	5
Tyr	0	0	0	0
Phe	1.9	2	0.2	0
His	0	0	0	0
Lys	0	0	1.1	1
Arg	1.1	1	0	0
Trp	—*	0	—*	1

* Cys and Trp residues were not determined.

Table 5 DNA polymerase and RNAase H activity of wild-type and mutant (Cys-90→Ala) MuLV-RT

DNA polymerase and RNAase H activities were assayed as described in the Materials and methods section. Activities of the wild-type enzyme were taken as 100%. A DNA polymerase activity of 100% represents the incorporation of about 100 pmol of TMP into (rA)₂·(dT)₁₅ in 15 min, catalysed by 20 ng of the enzyme. An RNAase H activity of 100% represents the release of about 11 000 c.p.m. from [³H](rA)₂·(dT)_n (0.5 µg; 20 000 c.p.m.) by 100 ng of MuLV-RT in 30 min. The values shown are the averages of three different assays.

Enzyme	Activity (%)	
	Polymerase	RNAase H
Wild-type	100	100
Mutant (Cys-90→Ala)	112	98

Heat stability

Determination of the stability of enzymes at elevated temperatures is commonly used to study the structural differences between related proteins (Hizi and Joklik, 1977; Hizi et al., 1991) and between mutant proteins (Polesky et al., 1990; Loya et al., 1992). Since the mutation of Cys-90 to Ala did not change the catalytic activity of MuLV-RT significantly, we tried to determine whether this mutation resulted in any structural change. Therefore, heat denaturation patterns of wild-type and of Cys-90→Ala mutant enzyme were analysed. As can be seen from Figure 5, there is a significant difference in the heat stability between the wild-type enzyme and the mutant enzyme, as judged by the DNA

Figure 5 Heat stability patterns of the wild-type and Cys-90→Ala mutant MuLV-RTs

Wild-type and mutant RTs were incubated at 50 °C in 100 µl of buffer containing 20 mM Tris/HCl, pH 7.8, 20 mM NaCl, 1 mM MnCl₂, 10% (v/v) glycerol, 100 µg of BSA and 2 mM DTT. After the periods of time indicated aliquots were removed and assayed for DNA polymerase activity. (□) Wild-type enzyme; (○) Cys-90→Ala mutant enzyme.

polymerase activity. Under the experimental conditions used, the half-life of the wild-type enzyme at 50 °C was about 210 min, while that of the mutant enzyme was only 20 min.

DISCUSSION

Studies so far have shown that all the RTs except HIV-RT are sulphhydryl-requiring enzymes (Gerard and Grandgenett, 1980; Verma, 1981; Goff, 1990). To map the important cysteine residues in MuLV-RT, we have used a cysteine-specific reagent, BP-MAL. This reagent was selected for a number of reasons. BP-MAL, unlike *N*-ethylmaleimide, has been shown to react rapidly and with high specificity with sulphhydryl residues in a number of proteins (Tao et al., 1986; Rajasekharan et al., 1987; Agarwal et al., 1991). This reagent has a high molar extinction coefficient (ϵ_{280} 13 000 M⁻¹·cm⁻¹) (Tao et al., 1985) and therefore provides a useful tag to monitor the site of labelling. Finally, the benzophenone moiety is a photoreactive group that can be used to cross-link and so identify residue(s) within 0.3–0.4 nm of the sulphhydryl groups. The detailed analysis presented here shows that BP-MAL reacts with two cysteine residues in MuLV-RT, and inactivates the DNA polymerase activity of MuLV-RT. The enzyme inactivation is attributed further to the inability of the BP-MAL-modified enzyme to bind to the template–primer and, more specifically, to the primer moiety (Figure 4). Furthermore, the presence of template–primer in the BP-MAL-modification reaction has also been found to reduce competitively BP-MAL binding as well as to reduce enzyme inactivation, suggesting a common site of interaction for template–primer and for BP-MAL. The identification of Cys-90 and of Cys-310 in MuLV-RT as the sites for BP-MAL action and the protection only of Cys-90 by template–primer, suggests strongly that the domain containing Cys-90 either forms part of the primer-binding region or is in close proximity to this region. This interpretation is also consistent with our earlier observations that the site of cross-linking of an oligomeric dT primer to MuLV-RT is in a peptide that spans residues 72–80 (Tirumalai and Modak, 1991).

It is important to point out that the domain containing Cys-90 in MuLV-RT is part of a unique secondary structure that is composed of two polypeptide double helices on both sides of an anti-parallel sequential dimer (Gerard et al., 1986). Such structures have been proposed to be involved in nucleic-acid binding in many proteins (Jurnak, 1985). In addition, the proximity of this domain to Lys-103, which has been implicated in dNTP binding (A. Basu et al., 1988, 1990), would be favourable for phosphodiester-bond formation. The proximity of the dNTP- and primer-binding domains has been well-documented in the case of *E. coli* DNA polymerase I (Basu and Modak, 1987; Catalano et al., 1990; Polesky et al., 1990; Rush and Konigsberg, 1990; Yadav et al., 1992).

To determine whether Cys-90 is directly involved we mutated this residue to alanine and compared the catalytic properties of the mutant enzyme with that of the wild-type enzyme. The Cys-90→Ala mutation had no effect on the DNA polymerase activity nor on the RNAase H activity (Table 5). Thus, the results from mutation do not confirm the conclusion drawn from the results of chemical modification. Taken together, these results suggest that the side-chain of Cys-90 may not directly interact with the polynucleotide substrate, but may be located within its binding domain. The enzyme inactivation following chemical modification may be explained as follows. The addition of a bulky benzophenone moiety to Cys-90, which is in the molecular proximity of the primer-binding domain, can render the enzyme inactive by physically interfering with the binding of the primer. The primer-cross-linking studies support this idea (Figure 2).

It is possible that Cys-90 and other cysteine residues in MuLV-RT may have some role in maintaining the overall structural integrity of the enzyme. To test this hypothesis, the thermal stability of the mutant enzyme was compared with that of the wild-type enzyme. The wild-type enzyme was found to be about 10-fold more stable than the mutant enzyme at 50 °C. It is not clear at present how a single mutation can affect the heat stability so markedly. A drastic change in the heat-stability patterns of the wild-type and mutant proteins has also been observed for HIV-1 RT (Loya et al., 1992).

The role of Cys-310, which reacts with BP-MAL as efficiently as does Cys-90, is not very clear, but its location in the three-dimensional structure may be inferred to be distal to the template-primer- and substrate-binding domains. In summary, Cys-90 in MuLV-RT appears to be in or near the active-site cleft, but it does not participate in the catalytic function. It appears that Cys-90 of MuLV-RT and possibly other cysteine residues in this class of enzymes may be required to maintain the structural integrity of the polymerase domain of the enzyme molecule.

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