Ferrate Oxidation of Murine Leukemia Virus Reverse Transcriptase: Identification of the Template-Primer Binding Domain†

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ABSTRACT: Treatment of murine leukemia virus reverse transcriptase (MuLV RT) with potassium ferrate, an oxidizing agent known to oxidize amino acids involved in phosphate binding domains of proteins, results in the irreversible inactivation of both the DNA polymerase and the RNase H activities. Significant protection from ferrate-mediated inactivation is observed in the presence of template-primer but not in the presence of substrate deoxynucleoside triphosphates. Furthermore, ferrate-treated enzyme loses template-primer binding activity as judged by UV-mediated cross-linking of radiolabeled DNA. Comparative tryptic peptide mapping by reverse-phase HPLC of native and ferrate-oxidized enzyme indicated the presence of two new peptides eluting at 38 and 57 min and a significant loss of a peptide eluting at 74 min. Purification, amino acid composition, and sequencing of these affected peptides revealed that they correspond to amino acid residues 285–295, 630–640, and 586–599, respectively, in the primary amino acid sequence of MuLV RT. These results indicate that the domains constituted by the above peptides are important for the template-primer binding function in MuLV RT. Peptide I is located in the polymerase domain whereas peptides II and III are located in the RNase H domain. Amino acid sequence analysis of peptides I and II suggested Lys-285 and Cys-635 as the probable sites of ferrate action.

Murine leukemia virus (MuLV)[1] reverse transcriptase (RT) is a single polypeptide chain containing both DNA polymerase and RNase H activities (Dickson et al., 1982). MuLV RT has been cloned and overexpressed in *Escherichia coli* (Kotewicz et al., 1985; Roth et al., 1985; Tanese et al., 1985). The biological and the enzymatic properties of both the viral and cloned reverse transcriptases have been extensively investigated and appear to be quite similar (Gerard & Grandgenett, 1975; Modak & Marcus, 1977; Modak & Srivastava, 1979; Srivastava & Modak, 1980a,b; Gerard, 1981; Kotewicz et al., 1985; Roth et al., 1985). The amino acid sequence of the reverse transcriptase has been deduced from the complete nucleotide sequence of a noninfectious proviral DNA (Shinnick et al., 1981), and the exact location of the genome segment coding for reverse transcriptase within the pol gene has been deciphered and confirmed by sequencing the amino and carboxy termini of the protein (Copeland et al., 1985). Cloning of MuLV RT gene in a high-expression vector system and the knowledge of the entire primary amino acid sequence of the enzyme protein have prompted us to undertake a detailed structure–function relationship study of this enzyme. Studies in our laboratory are aimed at defining the structure–function relationships in DNA polymerizing enzymes using various active-site-directed reagents. For example, pyridoxal phosphate, a substrate binding site directed reagent (Modak, 1976), has been successfully used to identify dNTP binding sites in a number of DNA polymerases. The locations of Lys-758 in *E. coli* DNA polymerase I (Basu et al., 1987), Lys-103 and Lys-421 in MuLV reverse transcriptase (Basu, A., et al., 1988), Lys-71 in rat β-polymerase (Basu et al., 1989a), and Lys-263 in HIV reverse transcriptase (Basu et al., 1989b) were identified as residues that are situated in, or near, the substrate binding domains of these enzymes. Similarly, with phenylglyoxal, a template-primer binding site directed reagent (Srivastava & Modak, 1980a), Arg-841 in *E. coli* DNA polymerase I has been identified as a residue essential for the DNA binding function (Mohan et al., 1988). Met-512 was also found to play some role in maintaining the template-primer binding function of DNA polymerase I (Klenow fragment), because its oxidation via ferrate treatment resulted in the loss of both polymerase and nuclease activities (Basu et al., 1987). We have now extended these studies to MuLV RT and have tentatively identified a number of residues which may play an important role in RT catalysis. For example, Lys-103 and Lys-329 have been identified as important residues required in substrate dNTP binding (Basu, A., et al., 1988, 1990) and template-primer binding function (Nanduri & Modak, 1990), respectively. In this paper, we report the ferrate-mediated inactivation of both polymerase and RNase H activities of MuLV RT. By means of tryptic peptide mapping and amino acid sequencing, the peptides containing ferrate-reactive sites have been identified and characterized. The possibility, that the residues contained in these peptides are important for the template-primer binding function, is discussed.

Materials and Methods

Materials

Synthetic template-primers, polynucleotides, oligonucleotides, and nonradioactive nucleotides were purchased from P-L Biochemicals. [^{3}H]ATP, dTTP, and other radiolabeled compounds were obtained from Amersham or New

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[1] Abbreviations: MuLV RT, murine leukemia virus reverse transcriptase; TPCK, L-1-(tosylamino)-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.

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England Nuclear. HPLC-grade water and acetonitrile were obtained from Fisher, while trifluoroacetic acid was purchased from Sigma. Trypsin (TPCK-treated) was obtained from Worthington.

Methods

Synthesis of Potassium Ferrate. Potassium ferrate was synthesized by the oxidation of ferric nitrate with alkaline hypochlorite solution and purified by precipitation with potassium hydroxide solution as described by Thompson et al. (1951).

Purification of MuLV RT. E. coli containing plasmid pB6B15.23 overproduces a complete MuLV RT with a molecular weight of approximately 80K (Roth et al., 1985). The enzyme was purified from cell extracts essentially as described by Roth et al. (1985). Briefly, the crude extract was chromatographed through DEAE-cellulose, phosphocellulose, and poly(ribouridylic acid)–agarose. The final enzyme preparations were >95% pure as judged by SDS–PAGE analysis. The specific activity of purified MuLV RT was 16 550 units/mg of protein (one unit is defined as the number of nanomoles of dTMP incorporated at 37 °C for 15 min using the standard assay described below).

Preparation of $[^3H](rA)_n{\cdot}(dT)_n$. $[^3H](rA)_n{\cdot}(dT)_n$ was synthesized by using $(dT)_n$ and *E. coli* RNA polymerase (Boehringer Mannheim). The reaction mixture (500 μL) contained 50 mM Tris-HCl, pH 7.8, 100 mM KCl, 8 mM $MgCl_2$, 30 μg of $(dT)_n$, 2 mM dithiothreitol, 100 μM ATP, 100 μCi of [^{3}H]ATP, and 50 units of *E. coli* RNA polymerase. The mixture was incubated at 37 °C for 1 h, after which the reaction was stopped by the addition of 10 mM EDTA and the product was extracted with an equal volume of phenol. The RNA·DNA hybrid contained in the aqueous phase was precipitated with 2.5 volumes of ethanol, and the pellet collected by centrifugation was washed with 70% ethanol and resuspended in 50 mM Tris-HCl, pH 7.8, containing 1 mM EDTA.

Preparation of $(rA)_n$–$[5'\text{-}^{32}P](dT)_{15}$. $(dT)_{15}$ was phosphorylated at the 5′-OH terminus with [γ-^{32}P]ATP by polynucleotide kinase as described by Maniatis et al. (1982) and was annealed to $(rA)_n$ in a nucleotide molar ratio of 1:1.

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

(B) RNase H Assay. The RNase H activity of MuLV RT was measured by using tritium-labeled $(rA)_n$ annealed to $(dT)_n$ as substrate [synthesis of $(rA)_n{\cdot}(dT)_n$ is described above]. 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 poly($[^3H](rA)_n{\cdot}(dT)_n$) (20 000 cpm), and 60 ng of enzyme. The reactions were carried out at 37 °C for 30 min, and the acid-insoluble counts were determined as described above for DNA polymerase assays.

Inactivation of MuLV RT by Potassium Ferrate Treatment. Ferrate reagent has a relatively short life in aqueous medium, and therefore a fresh solution prepared in 0.1 N NaOH was used as a source of ferrate ions. For dose response studies, 2 μg of MuLV RT in a 100-μL volume of buffered medium (50 mM phosphate buffer, pH 6.0, 1 mM $MnCl_2$, and 10% glycerol) was mixed with ferrate reagent (0–60 μM) and incubated at room temperature for 5 min. The concentration of ferrate was adjusted such that no more than 5-μL addition was required. This addition did not increase the pH of the medium. Aliquots from the ferrate-treated enzyme were withdrawn and diluted in 0.1% BSA, and DNA polymerase and RNase H activities were determined as described above. For protection studies, desired amounts of substrate triphosphate, activated DNA, or various synthetic template-primers were preincubated with the enzyme for 10 min on ice prior to the addition of ferrate.

Template-Primer Binding by MuLV RT. The ability of MuLV RT before and after ferrate treatment to bind template-primer was determined by UV-mediated cross-linking of radiolabeled template-primer to enzyme protein followed by SDS–polyacrylamide gel electrophoresis and autoradiography. For these experiments, $(rA)_n$ annealed to $[5'\text{-}^{32}P](dT)_{15}$ was used as labeled template-primer. The conditions for cross-linking of template-primer to MuLV RT 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 (120 000 cpm) in 50 μL of a solution containing 50 mM Hepes–KOH, pH 7.8, 50 mM KCl, 1 mM $MnCl_2$, 2 mM DTT, and 10% glycerol on ice for 15 min, followed by exposure to UV light for 15 min (3×10^4 ergs/mm^2). Samples were then boiled in the presence of 2% SDS and electrophoresed on an 8% SDS–polyacrylamide gel. The extent of cross-linking of template-primer to enzyme was quantitated by excising the radioactive bands and measuring Cerenkov counts associated with them.

Tryptic Peptide Mapping. For determining the site of ferrate action, we used 0.5–1.0 nmol of enzyme protein so that reproducible tryptic peptide maps could be generated. A large quantity of protein was needed in these experiments; hence, ferrate treatment was carried out by adding small aliquots of ferrate reagent to the enzyme (contained in 500 μL of the buffer) until 90% inactivation was achieved. The enzyme was then precipitated by addition of an equal volume of 20% trichloroacetic acid, and the precipitated protein was collected by centrifugation. The precipitate was then washed twice with 5% trichloroacetic acid followed by ether to remove the free acid. The precipitate was then suspended in 200 μL of 50 mM ammonium bicarbonate, pH 8, and was digested with trypsin (1:25 w/w ratio of trypsin to RT) at 37 °C for 16–20 hour. The tryptic peptides were acidified with trifluoroacetic acid (TFA) and injected onto a Vydac C-18 reverse-phase column (0.45 × 25 cm, 5-μm particle size, 300-Å pore size) that had been equilibrated 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 gradient: 0–40% B (0–90 min); 40–70% B (90–130 min). The flow rate was maintained at 0.7 mL/min. Peptides were simultaneously monitored at 215 and 278 nm. All the HPLC analyses were carried out on a Varian Vista 5500 HPLC system equipped with a polychrome 9060 diode array detector system.

Amino Acid Composition and Sequence Analyses. The peptide fractions of interest were collected and were individually repurified on a C-18 reverse-phase column equilibrated with 0.1% TFA using three different gradients as follows: 0–5% B (0–5 min) followed by 5–20% B (5–80 min) for peptide I; 0–15% B (0–10 min) followed by 15–30% B (10–85 min) for peptide II; and 0–20% B (0–5 min) followed by

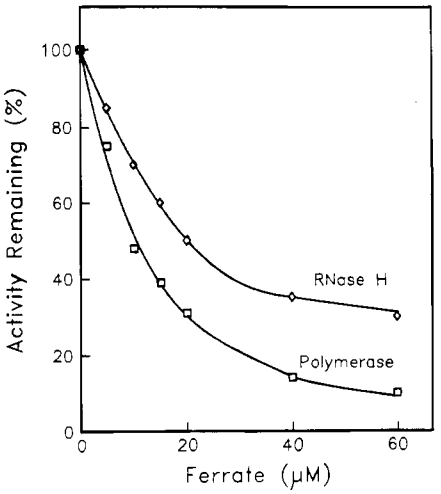

FIGURE 1: Effect of potassium ferrate on MuLV RT: inactivation of DNA polymerase and RNase H activities as a function of potassium ferrate concentration. MuLV RT (2 μg) was treated with ferrate at different concentrations as described under Methods. Aliquots of oxidized enzymes were removed to determine DNA polymerase (□) and RNase H (◇) activities.

20–80% B (5–105 min) for peptide III (74 min). For amino acid composition analysis, the purified peptides were lyophilized and 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 then converted to their phenylthiocarbamyl (PTC) derivatives and were analyzed by reverse-phase HPLC using two Nova-pak columns (0.45 × 15 cm) connected in series (Stone & Williams, 1986). Amino acid sequencing analysis was carried out at the protein chemistry facility of Yale University.

RESULTS

Inactivation of MuLV RT by Ferrate. A typical dose response of the addition of ferrate ion on the catalytic activity of MuLV RT is shown in Figure 1. It is clear from the figure that there is a progressive inactivation of both DNA polymerase and RNase H activities with the increase in ferrate ion concentration. In order to rule out the possibility that the enzyme inactivation by ferrate ion was due to reduced ferrate ion or its reactivity toward other components of the reaction mixture, ferrate reagent was preincubated for 5 min on ice (a) with 100-fold excess DTT and (b) with the reaction components devoid of enzyme. Enzyme protein and/or appropriate enzyme reaction components were then added, and the enzyme activity was determined. There was no inhibition of reverse transcriptase activity in either case (data not shown), indicating that the ferrate reaction with the enzyme protein was responsible for the observed inactivation.

Properties and Characterization of Ferrate-Mediated Inactivation. To determine the specificity of ferrate-mediated inactivation of MuLV RT, protective effects of substrate deoxynucleoside triphosphates (dNTPs) and template-primers on the enzyme inactivation were carried out. The presence of template-primers but not the substrate dNTPs protected both polymerase and RNase H activities from ferrate-mediated inactivation (Table I). As seen from Table I, both synthetic and natural template-primers significantly protected (60–90%) the enzyme from ferrate-mediated inactivation. Addition of single-stranded polynucleotides and oligonucleotides appears to provide partial protection, indicating that single-stranded DNA binding may not protect all of the sites oxidized by ferrate ions. Since organic hydroxyl groups have been known to react with ferrate ions (Steczko et al., 1983), it seemed likely

Table I: Effect of Addition of Substrate dTTP and Template-Primers on Ferrate-Mediated Inactivation of DNA Polymerase and RNase H Activities of MuLV RT[a]

addition	concn	DNA polymerase act. (%)[b]	RNase H act. (%)[c]
control (no ferrate)		100	100
none		8	20
dTTP	1 mM	26	18
	5 mM	19	22
$(rA)_n \cdot (dT)_{12-18}$	1 μg	52	ND
	2 μg	67	ND
	4 μg	82	75[d]
$(rC)_n \cdot (dG)_{12-18}$	4 μg	89	85[d]
$(dC)_n \cdot (dG)_{12-18}$	4 μg	50	ND
activated DNA	4 μg	90	ND
$(rA)_n$	4 μg	58	52
$(dT)_n$	4 μg	42	48
$(dT)_{12-18}$	4 μg	32	40

[a] Two micrograms of MuLV RT in a final volume of 100 μL containing 50 mM phosphate, pH 6.0, 1 mM $MnCl_2$, and 10% glycerol was treated with 5 μL of 1 mM ferrate (50 μM) as described under Methods, for 5 min at room temperature in the presence or absence of the indicated amount of dTTP or template-primers. For the enzyme assays, modified enzyme was diluted in 0.1% BSA, and aliquots representing 20 ng of enzyme were used in DNA polymerase assay and 60 ng of enzyme was used for RNase H assay (see Methods). [b] One hundred percent DNA polymerase activity represents 42 pmol of dTMP incorporation. [c] One hundred percent RNase H activity represents release of 14400 cpm from 0.5 μg of $[^3H](rA)_n \cdot (dT)_n$ substrate (20000 cpm). ND, not determined. [d] RNase H activity was determined after removing the template-primer by adsorbing it to DEAE-cellulose. The control and ferrate-treated enzymes were also treated in a similar manner as the template-protected enzyme. Recovery of protein from DEAE-cellulose was quantitated by monitoring the DNA polymerase activity.

Table II: Ferrate-Mediated Inactivation of MuLV RT: Effect of Template-Primer and 0.5 M Salt Addition[a]

addition	DNA polymer act. (%)
control (no ferrate)	100
control (+ferrate)	8
RT + $(rC)_n \cdot (dG)_{12-18}$ (4 μg) + ferrate	99
RT + 0.5 M NaCl	32
RT + 0.5 M NaCl + $(rC)_n \cdot (dG)_{12-18}$ (4 μg) + ferrate	43

[a] MuLV (2 μg) RT was modified by ferrate (50 μM) in the presence of the reagents shown above and assayed for activity under [^{3}H]dGTP as substrate (Modak & Marcus, 1977).

that the protection afforded by template-primers may be due to reactivity of the hydroxyl groups with ferrate. However, the fact that the presence of high concentrations of TTP did not show any protective effect (Table I) indicates that the reaction of ferrate with hydroxyl gruops is not the cause for the observed protective effect of template-primers. Further support for this interpretation was obtained by the observation that when MuLV RT was treated with ferrate ions in the presence of template-primer and 0.5 M NaCl, a condition which prevents the binding of DNA polymerases to DNA (Englund et al., 1969; Grandgenett & Rho, 1975; Abboud et al., 1978; DeVico et al., 1991), the protective effect of template-primer was abolished (Table II). These results indicate that the target of ferrate oxidation resides in the enzyme and that the targets are not accessible for reaction with ferrate when enzyme is present as enzyme-template–primer complex. A direct examination of ferrate-treated enzyme to bind to template-primer was also investigated. The extent of UV-mediated cross-linking of radiolabeled $(rA)_n \cdot (dT)_{15}$ to MuLV RT was determined and used as an indicator of template-primer binding. Results depicted in Figure 2 clearly show that the ferrate-treated enzyme loses its ability to bind

Table III: Amino Acid Composition Analysis of Peptides I, II, and III

	residues/mol of peptide					
	I		II		III	
amino acid residue	found	expected from sequence 286–295[a]	found	expected from sequence 630–640[a]	found	expected from sequence 586–599[a]
Asp	0	0	0.2	0	0	0
Glu	1.8	2.0	1.2	1.0	1.1	1.0
Ser	0.3	0	0.8	1.0	0.2	0
Gly	1.2	1.0	1.1	1.0	1.2	1.0
His	0	0	1.9	2.0	1.8	2.0
Arg	0.2	0	0	0	1.1	1.0
Thr	2.2	2.0	0.2	0	1.2	1.0
Ala	0.4	0	0.1	0	3.4	3.0
Pro	2.2	2.0	1.1	1.0	0	0
Tyr	0.3	0	0.1	0	1.8	2.0
Val	1.1	1.0	0	0	0.2	0
Met	1.0	1.0	0	0	0.3	0
Ile	0.2	0	2.3	2.0	1.9	2.0
Leu	0	0	0.9	1.0	0	0
Phe	0.1	0	0	0	1.0	1.0
Lys	1.8	1.0	0.9	1.0	0	0
Cys			0.9[b]	1.0		

[a] Numbers represent residues in the primary amino acid sequence of MuLV RT (Shinnick et al., 1981). [b] Cysteine was estimated as cysteic acid.

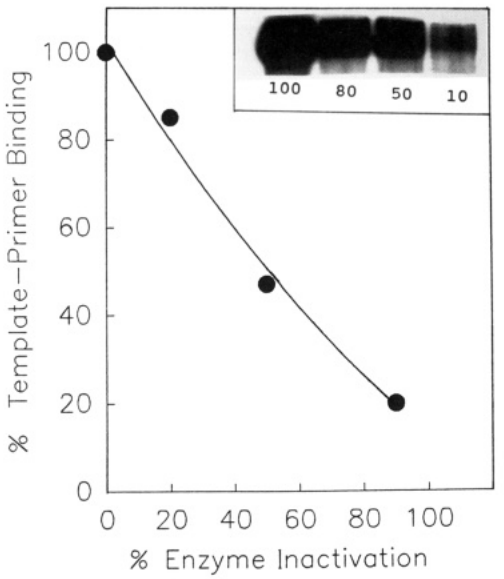

FIGURE 2: Template-primer binding by MuLV RT. MuLV RT inactivated to various extents by ferrate treatment was used to determine template-primer binding as described under Methods. The inset is an autoradiogram of an SDS–polyacrylamide gel showing results of UV-mediated cross-linking of ^{32}P-labeled template-primer to ferrate-treated enzyme. The values represent the percent DNA polymerase activity.

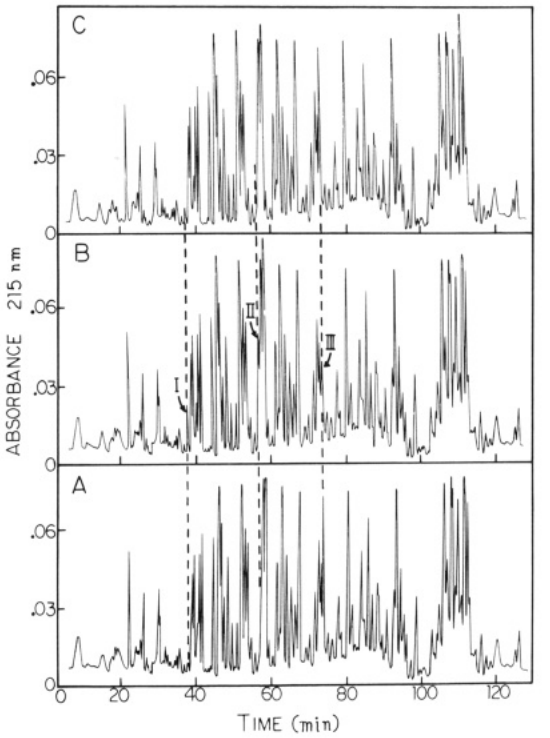

FIGURE 3: Reverse-phase HPLC separation of tryptic peptides derived from control, ferrate-inactivated, and template-primer-protected MuLV RT. One nanomole of MuLV RT was oxidized with ferrate in the presence and absence of template-primer. The tryptic peptides were loaded on a Vydac C-18 reverse-phase column equilibrated with 0.1% TFA and then eluted at a flow rate of 0.7 mL/min with increasing concentrations of buffer B (0.1% TFA in 70% acetonitrile): 0–90 min (0–40% B), 90–130 min (40–70% B). The three plots shown correspond to tryptic peptides obtained from untreated control enzyme (A), ferrate-oxidized enzyme (B), and enzyme oxidized by ferrate in the presence of template-primer (C). The positions of peptides I, II, and III are shown by vertical dashed lines.

to template-primer and that the loss in the ability of binding correlates well with the loss of enzyme activity.

Comparative Tryptic Maps of Native, Ferrate-Treated, and Template-Primer-Protected Enzyme. In order to locate the targets of ferrate action, we resorted to the comparative tryptic peptide mapping of native and ferrate-treated enzyme in the presence and absence of template-primer. Tryptic peptides from individual samples were resolved by reverse-phase HPLC using C-18 columns. From the comparison of tryptic peptides maps (Figure 3), it is evident that there are two new peptides eluting at 38 and 57 min and a significant reduction of peptide eluting at 74 min. The tryptic peptide map of the enzyme treated with ferrate in the presence of template-primer resembled that of the untreated enzyme.

Amino Acid Composition and Sequence Analyses of the Affected Peptides. An aliquot of each of the affected peptides was subjected to amino acid composition as described under Materials and Methods. The results indicated that the peptides eluting at 38, 57, and 74 min showed close correspondence to peptides constituted by amino acid residues 285–295, 630–640, and 586–599, respectively, in the primary amino acid sequence of MuLV RT (Table III). In order to further confirm the amino acid composition results and to identify the possible site of ferrate oxidation, peptides I and II were subjected to amino acid sequence analyses. Peptide III, which is present in sig-

Table IV: Amino Acid Sequence Analyses of Peptides I, II, and III

cycle	peptide I[a] residue		peptide II residue	peptide III residue
	N	$N+1$		
1	Glu	Lys	Leu	Tyr
2	Thr	Glu	Ser	Ala
3	Val	Thr	Ile	Phe
4	Met	Val	Ile	Ala
5	Gly	Met	His	Thr
6	Gln	Gly	*b*	Ala
7	Pro	Gln	Pro	His
8	Thr	Pro	Gly	Ile
9	Pro	Thr	His	His
10	Lys	Pro	Gln	Gly
11		Lys	Lys	Glu
12				Ile
13				Tyr
14				Arg

[a] Peptide I has a secondary sequence which has an additional Lys. The primary sequence (N) corresponds to residues 286–295 whereas the secondary sequence ($N+1$) corresponds to residues 285–295. Peptide II corresponds to residues 630–640, and peptide III corresponds to residues 586–599 in the primary amino acid sequence of MuLV RT. [b] Cysteine was detected as cysteic acid, indicating oxidation of this residue by ferrate ions.

nificantly reduced quantity in ferrate-treated enzyme, could not be included in such an analysis. Sequence analysis of peptide I yielded two identical sequences with an additional lysine residue in one of the two sequences (Table IV). Thus, one of the two sequences corresponded to residues 286–295 while the secondary sequence represented residues 285–295. Since residue 285 is a lysine residue which is followed by glutamic acid, the Lys-Glu bond in this peptide appears to be trypsin-resistant in ferrate-treated enzyme. Sequence analysis of peptide II (Table IV) clearly matches with residues 630–640 with the exception of Cys residue in cycle 6, indicating that Cys-635 is modified by the action of ferrate ion. Reexamination of amino acid composition analysis of peptide II further revealed the presence of a new absorption peak which elutes just before aspartic acid; this peak was identified as cysteic acid by comparing its retention time with that of standard cysteic acid (data not shown). The possible site of ferrate oxidation in peptide III, which was identified by its loss in the ferrate-treated enzyme, could not be determined.

Discussion

In this report, we show that the treatment of MuLV reverse transcriptase with ferrate ion results in the loss of both DNA polymerase and RNase H activities. Ferrate ion, which resembles phosphate anion by virtue of its steric and physicochemical properties, has been found to be a useful site-specific reagent for the identification of phosphoryl group recognition site(s) in a number of enzymes (Lee & Benisek, 1976, 1978; Rajababu & Axelrod, 1978; Steczko et al., 1979, 1983; Basu et al., 1987). Since the DNa polymerase class of enzymes are expected to contain sites which recognize phosphoryl groups, ferrate reagent was employed to identify the reactive sites. Further, by choosing the phosphate buffer as the medium to carry out inactivation, the chances of nonspecific interactions were greatly reduced. Treatment of MuLV RT with ferrate results in a progressive loss of both DNA polymerase and RNase H activities, and both activities are protected from inactivation if template-primers are included in the inactivation mixture. However, substrate deoxynucleoside triphosphates (dNTPs) alone do not protect the enzyme from inactivation. Earlier work has suggested that substrate dNTP binding in reverse transcriptases is dependent on the prior binding of enzyme to template-primer (Abraham & Modak, 1984; Basu, A., et al., 1988; Majumdar et al., 1988). Therefore, the nonprotective effects of dNTPs alone may not rule out ferrate ion effects on the residue involved in dNTP binding. However, the observation that the enzyme-template–primer complex, in which the dNTP binding pocket is freely accessible to dNTPs, is not inactivated by ferrate strongly suggests that ferrate ions do not oxidize residues involved in the dNTP binding process. Furthermore, the ferrate-mediated inactivation appears to be due to the inability of the oxidized enzyme to bind to template-primer as evidenced by the UV cross-linking experiments (Figure 2). These results indicate that the targets of ferrate oxidation may have an important function in template-primer binding. It is interesting to note that the mode of action of ferrate in MuLV RT is quite similar to that observed in *E. coli* DNA polymerase I (Basu et al., 1987). In both enzymes, polymerase and nuclease activities are equally affected by ferrate treatment, and the oxidation appears to affect the template-primer binding function.

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 (two-thirds) of reverse transcriptase should contain DNA polymerase activity and the C-terminal domain (one-third) should contain RNase H activity (Johnson et al., 1986). This was later independently confirmed by Tanese and Goff (1988) and Kotewicz et al. (1988), by cloning the two domains separately and showing the association of appropriate activities with the individual clones. These studies indicated that the two activities must utilize distinct and nonoverlapping active sites for nucleic acid binding and catalysis. Since template-primer binding is a prerequisite for both polymerase and RNase H activities and binding sites for the two activities appear to be contained in distinct domains, we expected at least two sites of oxidation (one in the N-terminal region and the other in the C-terminal region) in MuLV RT treated with ferrate reagent. Gross amino acid composition of control and ferrate-treated enzyme revealed no significant changes in amino acid composition (data not shown), suggesting that ferrate oxidation has not randomly affected or oxidized amino acid residues in MuLV RT. To identify the targets of ferrate action in the MuLV RT, we resorted to tryptic peptide mapping of native and ferrate-treated enzymes which revealed the presence of two new peptides eluting at 38 and 57 min and a consistent reduction of a peptide eluting at 76 min in the digest prepared from ferrate-treated enzyme. All three observed changes were restored to a standard pattern of the tryptic peptide map when the enzyme was protected with template-primer (Figure 3, panel A). This is in contrast to *E. coli* DNA polymerase I where only one of the five ferrate reactive sites was protected by DNA. Purification and amino acid composition of these affected peptides revealed that the peptide eluting at 38 min is located in the N-terminal domain of the enzyme and spans residues 285–295 in the primary amino acid sequence of MuLV RT. Peptides II and III eluting at 57 and 74 min are located in the C-terminal domain of the enzyme spanning amino acid residues 630–640 and 586–599, respectively. These results indicate that the regions containing peptides I, II, and III probably constitute template-primer binding domain of MuLV RT. Alternatively, one or more of these peptides may be important to maintain the structural integrity of template-primer binding domain and may not participate in catalytic function per se.

With regard to peptide I, the amino acid composition has shown that it is located in the N-terminal domain spanning residues 286–295. Amino acid sequence analysis of this

peptide has shown the presence of two overlapping sequences with a jagged N-terminus. The primary amino acid sequence of peptide I reads as Glu-Thr-Val-Met-Gly-Gln-Pro-Thr-Pro-Lys, and the secondary sequence is Lys-Glu-Thr-Val-Met-Gly-Gln-Pro-Thr-Pro-Lys. Therefore, the secondary sequence of peptide I contains an additional lysine residue at its N-terminus. The fact that the Lys-Glu bond in this sequence is not cleaved by trypsin seems quite unusual. Earlier, it was reported (Keil, 1982) that Lys–Asp and Lys–Pro bonds are somewhat resistant to trypsin digestion, but to our knowledge resistance of the Lys–Glu bond to trypsin digestion has not been reported. Furthermore, the absence of this peptide in the control digest suggests that trypsin cleavage at this site is not affected. Consequently, the resistance of the Lys–Glu bond to trypsin may be attributed to the probability of an altered peptide bond between these two residues as a result of ferrate treatment. If this scenario is correct, then the peptide bond between Lys-285 and Glu-286 may not withstand with peptide coupling manipulation required for sequencing, resulting in the significant loss of Lys-285 prior to Edman degradation. Therefore, it is likely that the primary peptide product from ferrate-oxidized enzyme may be a 11-residue peptide with Lys-285 at its N-terminal end. The importance of basic amino acids like lysines and arginines in the recognition of the phosphoryl group is well documented in literature, and the presence of a ferrate-sensitive lysine in such a region seems well justified. Another interesting feature of this lysine residue is the presence of Glu as the following residue. In the studies on the crystal structure of the *Eco*RI–DNA complex by Rosenberg and colleagues (McClarin et al., 1986), a similar feature, i.e., the presence of Arg-Glu at the DNA binding site, was noted. In the case of *Eco*RI, the amino acid residues that intereact directly with the bases are arranged in space so that there are alternating positive and negative charges. The presence of Arg-Glu pairs in the DNA binding cleft of *E. coli* DNA pol I was also noted by Mohan et al. (1988). Since lysines are functionally equivalent to arginines, the presence of the Lys-Glu pair in the DNA binding region could be a feature that MuLV reverse transcriptase shares with other DNA binding proteins like *Eco*RI and DNA polymerase I. Earlier studies carried out in our laboratory using 4-(oxoacetyl)phenoxyacetic acid (OAPA) had implicated Lys-329 as an important residue in the template-primer binding pocket of MuLV-RT (Nanduri & Modak, 1990). From these studies, it is quite apparent that multiple basic amino acid side chains are required for proper binding of template-primer. Moreover, the presence of basic amino acids in the DNA binding cleft would generate a positive electrostatic potential suitable for DNA binding as has been observed in the DNA binding cleft of *E. coli* DNA polymerase I (Ollis et al., 1985).

Amino acid sequence analysis of peptide II shows that Cys-635 is the probable target of ferrate oxidation since it was recovered as cysteic acid. Amino acid composition and sequence analysis of peptide III derived from the control enzyme, which is consistently missing or significantly decreased in the ferrate-treated enzyme, showed that this peptide contains many potential residues for ferrate oxidation, e.g., Tyr-586, Thr-590, His-592, His-594, and Tyr-598. However, since the peptide from ferrate-treated enzyme is not recovered, no definite site can be deduced. Since both peptides II and III are derived from the C-terminal domain, Cys-635 and one of the amino acids from peptide III could be important for template-primer/RNA–DNA binding required for RNase H activity. Recently, Repaske et al. (1989) have shown that Thr-582 and Asp-583 are important for RNase H activity in MuLV RT. Peptide III, which spans residues 586–599, is in close proximity of these residues, and therefore it is reasonable to expect that this peptide may form a part of the template-primer binding domain. The C-terminal domain in MuLV RT, besides containing the active site for RNase H, is also suggested to facilitate proper folding of the entire polypeptide into the active conformation and for stability of the enzyme (Gerard et al., 1986; Kotewicz et al., 1988). An insertional mutation around amino acid 578 in the C-terminal domain (mutant A 23; Tanese & Goff, 1988) abolishes both RNase H and DNA polymerase activities of MuLV RT, suggesting that some kind of interaction exists between RNase H and DNA polymerase domains. The sequence around Cys-635 is likely to contribute some function since it is conserved among retroviral RTs (Johnson et al., 1986). Recently, DeVico et al. (1991) have raised monospecific antibody against a synthetic peptide which represents the C-terminal sequence Val(536)-Asp(549) of HIV RT and have shown that this antibody directly competes for template-primer binding in HIV-1, HIV-2, EIAV, HTLV I, and AMV RTs. The above HIV1 RT sequence, against which the antibody was raised, is equivalent to the sequence Cys-(635)-Asp-653) of MuLV RT, which contains a part of the sequence of peptide II identified by us. All these results strongly indicate that the domain containing Cys-635 may play an important part in the template-primer binding function of retroviral RTs. Cys-635 lies in region 609–671 which by deletion has been shown to be nonessential for the DNA polymerase activity (Gerard et al., 1986). However, this does not mention the effect of deletion on RNase H activity, and hence the importance of Cys-635 in RNase H activity cannot be ruled out.

In conclusion, our studies have identified the domains that play an important role in the template-primer binding function of MuLV RT and have suggested probable amino acid residues in these domains which may be directly responsible for this function.

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Registry No. RT, 9068-38-6; DNA polymerase, 9012-90-2; RNase H, 9050-76-4; potassium ferrate, 39469-86-8.

REFERENCES

Abboud, M. M., Sim, W. J., Loeb, L. A., & Mildvan, A. S. (1978) *J. Biol. Chem. 253*, 3415–3421.

Abraham, K. I., & Modak, M. J. (1984) *Biochemistry 23*, 1176–1182.

Basu, A., & Modak, M. J. (1987) *Biochemistry 26*, 1704–1709.

Basu, A., Williams, K. R., & Modak, M. J. (1987) *J. Biol. Chem. 262*, 9601–9607.

Basu, A., Nanduri, V. B., Gerard, G. F., & Modak, M. J. (1988) *J. Biol. Chem. 263*, 1648–1653.

Basu, A., Kedar, P., Wilson, S. H., & Modak, M. J. (1989a) *Biochemistry 28*, 6305–6309.

Basu, A., Tirumalai, R. S., & Modak, M. J. (1989b) *J. Biol. Chem. 264*, 8746–8752.

Basu, A., Basu, S., & Modak, M. J. (1990) *J. Biol. Chem. 265*, 17162–17166.

Basu, S., Basu, A., & Modak, M. J. (1988) *Biochemistry 27*, 6710–6716.

Copeland, T. D., Gerard, G. F., Hixson, C. W., & Oroszlan, S. (1985) *Virlogy 143*, 676–679.

DeVico, A. L., Copeland, T. D., Oroszlan, S., Gallo, R. C., & Sarngadharan, M. G. (1991) *J. Biol. Chem. 266*, 6774–6779.
Dickson, C., Eisenman, H., Fan, E., Hunter, E., & Teich, N. (1982) in *Molecular Biology of Tumor Viruses: RNA Tumor Viruses* (Weiss, R. A., Teich, N. M., Varmus, H. E., & Coffin, J. M., Eds.) pp 513–648, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Englund, P. T., Kelly, R. B., & Kornberg, A. (1989) *J. Biol. Chem. 244*, 3095–3052.
Gerard, G. F. (1981) *Biochemistry 20*, 256–265.
Gerard, G. F., & Grandgenett, D. P. (1975) *J. Virol. 15*, 785–797.
Gerard, G. F., D'Alessio, J. H., Kotewicz, M. L., & Noon, M. C. (1986) *DNA 5*, 271–279.
Grandgenett, D. P., & Rho, H. M. (1975) *J. Virol. 15*, 526–533.
Johnson, M. S., McClure, M. A., Feng, D. F., Gray, J., & Doolittle, R. F. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7648–7652.
Keil, B. (1982) *Methods in protein sequence analysis* (Elzinga, M., Ed.) pp 291–304, Humana Press, Clifton, NJ.
Kotewicz, M. L., D'Alessio, J. M., Driftmier, K. M., Blodgett, K. P., & Gerard, G. F. (1985) *Gene 35*, 249–258.
Kotewicz, M. L., Sampson, C. M., D'Alessio, J. M., & Gerard, G. F. (1988) *Nucleic Acids Res. 116*, 265–277.
Lee, Y. M., & Benisek, W. F. (1976) *J. Biol. Chem. 251*, 1553–1560.
Lee, Y. M., & Benisek, W. F. (1978) *Arch. Biochem. Biophys. 188*, 31–36.
Majumdar, C., Stein, Cy A., Cohen, J. S., Broder, S., & Wilson, S. H. (1989) *Biochemistry 28*, 1340–1346.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
McClarin, J. A., Frederick, C. A., Wang, B.-C., Greene, P., Boyer, H. W., Grable, J., & Rosenberg, J. M. (1986) *Science 234*, 1526–1541.
Modak, M. J. (1976) *Biochemistry 15*, 3620–3626.
Modak, M. J., & Marcus, S. L. (1977) *J. Virol. 22*, 243–246.
Mohan, P. M., Basu, A., Basu, S., Abraham, K. I., & Modak, M. J. (1988) *Biochemistry 27*, 226–233.
Nanduri, V. B., & Modak, M. J. (1990) *Biochemistry 29*, 5258–5264.
Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G., & Steitz, T. A. (1985) *Nature 313*, 762–766.
Rajababu, C., & Axelrod, B. (1978) *Arch. Biochem. Biophys. 188*, 31–36.
Repaske, R., Hartley, J. W., Kavalick, M. F., O'Neill, R. R., & Austin, J. B. (1989) *J. Virol. 63*, 1460–1464.
Roth, M. J., Tanese, N., & Goff, S. P. (1985) *J. Biol. Chem. 260*, 9326–9335.
Shinnick, T. M., Lerner, R. A., & Sutcliff, J. G. (1981) *Nature 293*, 543–548.
Srivastava, A., & Modak, M. J. (1980a) *J. Biol. Chem. 255*, 917–921.
Srivastava, A., & Modak, M. J. (1980b) *J. Biol. Chem. 255*, 2000–2005.
Steczko, J., Walker, D. E., Hermodson, M., & Axelrod, B. (1979) *J. Biol. Chem. 254*, 3254–3258.
Steczko, J., Hermodson, M., Axelrod, B., & Dziembor-Kentzer, E. (1983) *J. Biol. Chem. 258*, 13248–13254.
Stone, K. L., & Williams, K. R. (1986) *J. Chromatogr. 359*, 203–212.
Tanese, N., & Goff, S. P. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1777–1781.
Tanese, N., Roth, M., & Goff, S. P. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4944–4948.
Thompson, G. W., Ockerman, L. T., & Schreyer, J. M. (1951) *J. Am. Chem. Soc. 73*, 1379–1381.

Structural Dynamics and Functional Domains of the Fur Protein†

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ABSTRACT: Proteolytic enzymes were used to detect metal-induced conformational changes in the ferric uptake regulation (Fur) protein of *Escherichia coli* K12. Metal binding results in enhanced cleavage of the N-terminal region of Fur by trypsin and chymotrypsin. Activation of both trypsinolysis sensitivity and DNA binding have similar metal ion specificity and concentration dependencies, suggesting that the conformational change detected is required for operator DNA binding. Isolation and characterization of biochemically generated fragments of Fur as well as other data indicate that the N-terminal region is necessary for the interaction of the repressor with DNA and that a C-terminal domain is sufficient for binding to metal ions.

Iron is essential for almost all forms of life (Neilands, 1972), but it is also a potentially toxic element because it can catalyze the formation of dangerously reactive hydroxyl radicals, which can damage virtually all cellular constituents (Halliwell, 1988). These two factors make it necessary to tightly regulate the intracellular concentration of iron. In all species studied, this regulation probably occurs at the level of uptake, since no mechanism to excrete iron is known (Neilands, 1990).

In many aerobic and facultative anaerobic microorganisms, iron uptake is accomplished by synthesis of ferric specific chelators called siderophores (Neilands, 1981). Siderophores can retrieve insoluble iron from the environment or the se-

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