Active Site Studies of Human Immunodeficiency Virus Reverse Transcriptase

Laura L. W. Mitchell and Barry S. Cooperman*

Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323

Received February 11, 1992; Revised Manuscript Received May 26, 1992

ABSTRACT: The active site of human immunodeficiency virus reverse transcriptase (HIV1-RT) was probed using three group-specific reagents: phenylglyoxal (PG), *N*-ethylmaleimide (NEM), and pyridoxal 5′-phosphate (PLP). The inactivation of HIV1-RT by arginine-specific PG was found to be completely protected against by adding primer-template. The potential active site arginine was localized to position 277 in the primary structure, suggesting that the polymerase domain of the enzyme should be considered as extending at least this far from the N terminus. The sulfhydryl-modifying reagent NEM completely inhibits NY5–HIV1-RT, which contains a cysteine at position 162, and such inhibition is protected against by primer-template. However, it does not strongly inhibit LAV–HIV1-RT, in which C162 is replaced by S162, indicating that while C162 may be at or near the active site or interact allosterically with primer-template, it is not essential for activity. The lysine-specific reagent PLP was found to be a noncompetitive inhibitor with respect to both primer-template [poly(rA)·oligo(dT)] and dTTP. The latter result differentiates HIV1-RT from other RTs, for which PLP has been shown to be a competitive inhibitor with respect to dTTP.

Understanding the catalytic mechanism of human immunodeficiency virus reverse transcriptase (HIV1-RT)[1] is a major goal of current work on this enzyme, both from the fundamental point of view and in the hope that such understanding will lead to the design of inhibitors that could be effective in the treatment of AIDS patients (Jacobo-Molina & Arnold, 1991). An important part of this effort is the identification of the active sites for both the RNA-dependent DNA polymerase and RNase H activities.

In the absence of an X-ray structure of a substrate (or substrate analogue) complex of HIV1-RT, efforts to identify potential active site residues have relied principally on two approaches: sequence alignment with other RTs and DNA polymerases, in the expectation that conserved residues will be functional (Johnson et al., 1986; Jacobo-Molina & Arnold, 1991), and site-specific mutagenesis, with the sites chosen most often based on the sequence alignment results [as summarized in Jacobo-Molina and Arnold (1991)]. Although several likely active site residues have been identified by these two approaches, the significant differences in the properties of HIV1-RT compared with other DNA polymerases, as well as the only modest degree of sequence homology in this family of enzymes, make it likely that the chemical modification approach, with its direct probing of active site structure, could also make a valuable contribution.

In the specific case of *Escherichia coli* DNA polymerase I, comparison of the results of chemical modification studies (Basu & Modak, 1987; Basu et al., 1988a; Rush & Konigsberg, 1990) with the crystal structure (Ollis et al., 1985) demonstrates clearly the value of this approach, carefully applied, in identifying active site residues. Chemical modification studies have also been used to identify potential active site residues in MoMuLV-RT (Basu et al., 1988b; Nanduri & Modak, 1990; Tirumalai et al., 1991; Reddy et al., 1991) and, in three papers published after this work was underway, in HIV1-RT (Basu et al., 1989, 1992; Sobol et al., 1991).

In this paper we use three group-specific reagents, phenylglyoxal (PG), *N*-ethylmaleimide (NEM), and pyridoxal 5′-phosphate (PLP), to identify potential active site residues within the amino acid sequence of HIV1-RT.

EXPERIMENTAL PROCEDURES

Materials

HIV1-RT was generously provided by Dr. Jasbir Seehra of Genetics Institute from a recombinant clone transformed into *E. coli* (Huber et al., 1989). Protein derived from two isolates, NY5 and LAV, was used in our experiments. All experiments were performed with both HIV1-RTs; results were similar except where noted. Some protein preparations needed further purification, and these were run on a size exclusion Spherogel TSK4000 column with an isocratic gradient of 50 mM Hepes (pH 7.0) with a flow rate of 0.7 mL/min. The following proteins were used as molecular weight standards: carbonic anhydrase (45 000), BSA (65 000), phosphorylase *b* (97 000), and β-galactosidase (116 000). Active HIV1-RT eluted at 11–13 min, with an apparent molecular weight of 110 000, in reasonable accord with the expected heterodimer (66/51) weight of 117 000 (Jacobo-Molina & Arnold, 1991), and was resolved from both higher and lower molecular weight contaminants. After addition of 0.25 mM DTT and 10% glycerol (final concentrations), the purified protein was stored at −20 °C, under which conditions its activity was stable for 2–4 months.

Poly(rA) (400–600 nt), oligo(dT) (12–18), preannealed poly(rA)·(dT12–18) (1:1 molar ratio), and dTTP were obtained from Pharmacia. All kinetic studies were determined using the preannealed primer-template, which was calculated [assuming 500 nt of poly(rA) and 15 nt of oligo(dT)] to have

* Author to whom correspondence should be addressed.

[1] Abbreviations: ACN, acetonitrile; AIDS, acquired immunodeficiency syndrome; AMV, avian myeloblastosis virus; BSA, bovine serum albumin; DEPC, diethyl pyrocarbonate; EIAV, equine infectious anemia virus; FIV, feline immunodeficiency virus; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; MoMuLV, Moloney murine leukemia virus; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PG, phenylglyoxal; PLP, pyridoxal 5′-phosphate; RLV, Rauscher murine leukemia virus; RT, reverse transcriptase; SIV, simian immunodeficiency virus; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

0006-2960/92/0431-7707$03.00/0

a molecular weight of 169 000. Otherwise, primer-template was prepared by combining equimolar amounts of poly(rA) and oligo(dT) in 1 mL of DEPC-treated water to a concentration of 3.5 μM. Concentrations were determined assuming 1 A_{260} unit equals 40 mg of polynucleotide (Sambrook et al., 1989). The solution was heated to 85 °C for 10 min and, after cooling, was stored in aliquots at −80 °C.

^{3}H-Labeled RNA–DNA hybrid for the RNase H assay was kindly supplied by Dr. Parimi Muralikrishna of this laboratory. RNA for the hybrid was prepared by extracting ^{3}H-labeled ribosomal rRNA from 50S subunits prepared from *E. coli* grown in the presence of [^{3}H-*methyl*]-*S*-adenosylmethionine (Smith, 1990). The DNA was prepared by *Hpa*II digestion of pK3535, which contains DNA coding for rRNA (Smith, 1990). Thirty-five micrograms of cDNA was hybridized with 35 μg of RNA by combining and heating to 85 °C for 15 min, followed by incubation at 55 °C for 60 min. The hybrid had a specific activity of 3000 cpm/μg.

[7-^{14}C]PG and [*methyl*-^{3}H]dTTP were obtained from Amersham. [2,3-^{14}C]NEM was obtained from Amersham and NEN Du Pont. Pyridoxal diacetate, 4-pyridoxic acid, 2-methyl-3-hydroxy-4-(hydroxymethyl)-5-(pyridylmethyl) phosphate and 2,4-dimethyl-3-hydroxy-5-(pyridylmethyl) phosphate were obtained as gifts from Dr. Jack Baldwin (Merck, West Point, PA). All other reagents were of reagent grade from Sigma and used without further purification, except for HPLC solvents which were of HPLC grade from Fisher and filtered twice before using. All solutions were made up with DEPC-treated water and then filter-sterilized to reduce RNase contaminants.

Methods

Polymerase Assay. Assay mixtures were composed of 20 mM Hepes (pH 7.8), 100 mM KCl, 10 mM $MgCl_2$, 10 μg of BSA, 1 mM DTT, 0.5 μg (30 nM) of poly(rA)·oligo(dT), 20 μM [*methyl*-^{3}H]dTTP (0.5 Ci/mol), and 10 μL of glycerol in 100 μL of total volume. Mixtures were incubated at 37 °C, and reactions were commenced by addition of enzyme (0.1–0.5 μg). After 30 min, reaction was quenched by addition of 1 mL of 10% (w/v) trichloroacetic acid (TCA) containing 10 mM sodium pyrophosphate. Samples were allowed to sit on ice for 10 min, filtered onto Whatman GF/C filters, washed with cold 10% TCA, cold water, and cold ethanol, dried, and counted. Efficiency of counting was approximately 40%. A sample to which no enzyme had been added served as a control and was subtracted from the others as background.

RNase H Assay. RNase H activity was assayed following the method of Basu et al. (1989). The reaction mixture (100 μL) contained 20 mM Hepes (pH 7.8), 1 mM $MgCl_2$, 100 mM KCl, 10 μg of BSA, and 1 μg of ^{3}H-labeled DNA:RNA hybrid. Reaction was initiated by the addition of 1 μg of RT. Following incubation at 37 °C for 60 min, the reaction was quenched by the addition of 1 mL of ice-cold 10% TCA containing 10 mM sodium pyrophosphate and maintained at 4 °C for 20 min. Following centrifugation, the radioactivity remaining in the supernatant was determined.

Inactivation of Polymerase Activity by PG. RT (10 μg) was incubated at 29 °C with 5 mM PG in 20 mM Hepes (pH 7.8) and 10% glycerol in a total volume of 40 μL, ±poly(rA)·oligo(dT) (5 μg), ±dTTP (200 μM). PG was omitted in the corresponding control reactions. One-microliter aliquots were removed and diluted 1:100 into the standard assay mixture at various times, and pseudo-first-order rates of activity loss were determined.

Inactivation of Polymerase Activity by NEM. The procedure employed was identical to the PG inactivation studies except that 5 mM NEM was added in place of PG. The concentration of DTT in the assay was increased to 2 mM to assist in quenching NEM. Control reactions lacked added NEM.

Inhibition of Polymerase Activity by PLP. PLP (0–80 μM) was added directly to the assay mixture (as above) with various final concentrations of poly(rA)·oligo(dT) and dTTP. After a 5-min preincubation at 37 °C, the assay was commenced by addition of dTTP. All procedures involving PLP were carried out in the dark to minimize its photodecomposition. A standard assay [20 μM dTTP, 0.5 μg of poly(rA)·oligo(dT), and no PLP] was run every day to correct for slight day-to-day variations. All other samples were normalized to this value, which varied ±9% over the course of our studies.

Covalent Incorporation of PG. RT (30–100 μg) was incubated with PG, ±5 μg of poly(rA)·oligo(dT), in a total volume of 100 μL, as above, except that [7-^{14}C]PG (1.8 Ci/mol) was used. At various times protein aliquots (10–15 μg) were removed into 10% formic acid (150 μL). Simultaneously, another aliquot (1 μg) was removed for an enzymatic activity assay. Acetic acid (1.5 mL) was added to the acid-quenched sample, and after 15 min on ice, precipitated polynucleotides were removed by centrifugation. The clear supernatant was injected onto a C-18 reversed-phase HPLC column (Synchropak RP-P). The column was then washed with 35% ACN containing 0.1% TFA for 20 min at 1.0 mL/min to remove excess reagent, followed by a gradient of 35–60% ACN in 10 min. The protein peak eluted at 28 min with an approximate yield of 65%. The protein amount was estimated from peak area at 214 nm (Buck et al., 1989). The conversion of peak area to protein amount was verified using the protein assay of Schaffner and Weissman (1973). An average molecular weight of 58 000 was used to calculate nanomoles of subunit.

Covalent Incorporation of NEM. RT (50 μg) was incubated with 5 mM [2,3-^{14}C]NEM (sp act. = 1.8 Ci/mol) in a volume of 40 μL under the following conditions: 20 mM Hepes (pH 7.8), 10% glycerol, 29 °C, ±100 μM dTTP, ±5 μg of poly(rA)·oligo(dT). At various times aliquots were removed and quenched by addition of an equal volume of 100 mM DTT. Samples were then treated as described for PG incorporation.

Trypsin Proteolysis and Peptide Purification. Samples for proteolysis were prepared by incubating 150–300 μg of RT with 5 mM [7-^{14}C]PG (sp act. = 22–60 Ci/mol) in 20 mM Hepes (pH 7.8), ±10 μg of poly(rA)·oligo(dT), for 1 h at 29 °C (total volume, 100 μL). Incubation was followed by overnight dialysis (Pierce microdialyzer) at 4 °C against several changes of trypsin buffer [10 mM Hepes, 45 mM borate (pH 8.0)]. Control experiments demonstrate that these conditions effectively limit further PG incorporation into RT. Following dialysis a small aliquot was injected onto an HPLC as described above to determine the extent of PG labeling of RT. Trypsin was then added (20% by weight of RT) to the remainder of the sample. Proteolysis was allowed to proceed for 6 h at 37 °C and quenched by quick freezing. Peptide specifically labeled by [^{14}C]PG was purified in a series of three chromatographic steps: (1) C-18 reversed-phase RP-HPLC column; (2) Supelco LC-3DP column, which has phenyl groups as part of its solid-phase support; (3) microbore capillary C-8 column (ABI).

Peptide Sequencing. Purified specifically labeled peptide was subjected to automated Edman sequencing, using an ABI Model 473A sequencer in the Department of Pathology at the

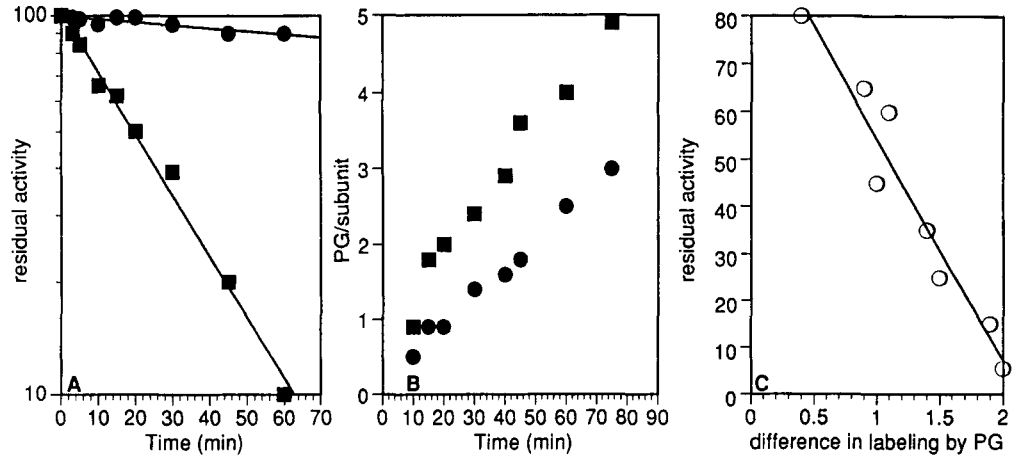

FIGURE 1: PG reaction with HIV1-RT. (A) Inactivation of HIV1-RT by PG in the presence (●) and absence (■) of poly(rA)·oligo(dT). HIV1-RT was incubated with 5 mM PG in the presence and absence of poly(rA)·oligo(dT) (300 nM), with removal of aliquots for assay at indicated times as described under Experimental Procedures. Residual activity is shown as a semilog plot vs time. (B) Incorporation of PG vs time in the presence (●) and absence (■) of poly(rA)·oligo(dT). HIV1-RT was incubated with [7-^{14}C]PG (1.8 Ci/mol, 5 mM) in the presence and absence of poly(rA)·oligo(dT) (300 nM) and incorporation level was determined as described under Experimental Procedures. (C) Difference in PG incorporation vs residual activity. Residual activity measurements are plotted vs PG incorporation/subunit [no poly(rA)·oligo(dT)] – PG incorporation/subunit [+poly(rA)·oligo(dT)].

University of Pennsylvania. The radioactivity in aliquots taken following each Edman cycle was determined.

RESULTS

Inactivation of HIV1-RT by PG. Incubation of HIV1-RT with PG leads to full inactivation of enzyme with a second-order rate constant for the inactivation of 7.2 min^{-1} M^{-1} (29 °C, pH 7.8). The presence of poly(rA)·oligo(dT) (300 nM–14 times K_m, see below) provides virtually full protection against inactivation (Figure 1A). In contrast, the presence of dTTP (200 μM—18 times K_m, see below) afforded no protection (data not shown). The presence of both substrates together results in a slight loss of activity similar to the loss observed in the presence of poly(rA)·oligo(dT) alone. Essentially identical results were obtained for both NY5–HIV1-RT and LAV–HIV1-RT.

Incorporation of PG. PG inactivation of HIV1-RT is accompanied by covalent incorporation of PG into RT (Figure 1B), and such incorporation is reduced in the presence of poly(rA)·oligo(dT). At full inactivation the protected sample incorporated approximately 3 PG/subunit, while the unprotected sample incorporated approximately 5 PG/subunit. Since PG reacts with arginine in a 2:1 stoichiometry (Takahashi, 1968), this difference (Figure 1C) is consistent with the modification of a single arginine being responsible for HIV1-RT inactivation. Essentially identical results were obtained for both NY5–HIV1-RT and for LAV–HIV1-RT.

Identification of a Specifically Labeled Peptide. HIV1-RT labeled with [7-^{14}C]PG in the presence and absence of poly(rA)·oligo(dT) was digested with trypsin. Borate was included in the dialysis and digestion buffers to stabilize the 1:1 PG–arginine adduct (Werber et al., 1975). In the absence of added borate, much lower yields of PG-labeled peptide were recovered, due to the instability of the PG–arginine adduct at neutral pH (Takahashi, 1968; Bond et al., 1980).

The resulting peptides were partially resolved by reversed-phase HPLC using a C-18 column (Figure 2). One peak of radioactivity was consistently found to be reduced when incorporation was carried out in the presence of poly(rA)·oligo(dT). This peak was purified by two additional chromatographic steps, one using a diisophenyl column (Figure 3A) and one using a microbore capillary C-8 column (Figure 3B). Sequencing the final peak obtained via automated Edman

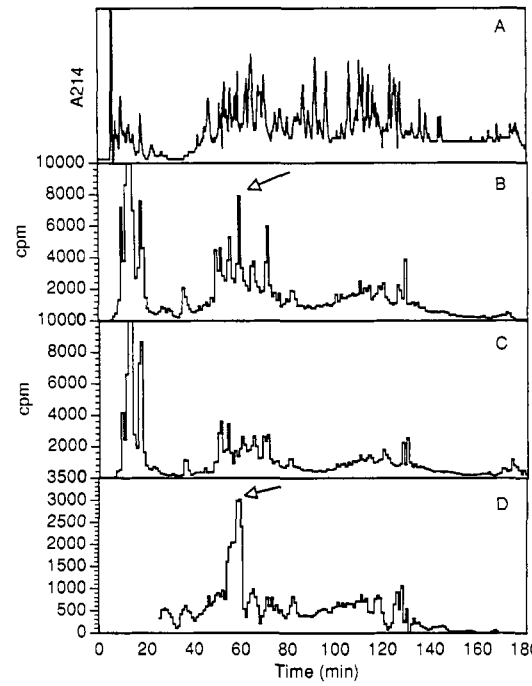

FIGURE 2: RP-HPLC analysis of trypsin proteolysis of [^{14}C]PG-labeled HIV1-RT. Analysis was carried out on a C-18 RP-HPLC column. Solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in ACN. Gradient employed: 20 min at 0% B, 30 min 0–10% B, 30 min 10–13% B, 80 min 13–40% B, 20 min 40–90% B. Flow rate: 0.7 mL/min. One-minute fractions were collected and counted. (A) Chromatogram (214-nm monitoring) for proteolyzed HIV1-RT labeled in the absence of poly(rA)·oligo(dT). Protein labeled in the presence of poly(rA)·oligo(dT) gave essentially the same result. (B) Radioactivity profile for HIV1-RT labeled in the absence of poly(rA)·oligo(dT). (C) Radioactivity profile for HIV1-RT labeled in the presence of poly(rA)·oligo(dT). (D) Difference radioactivity profile for (panel B – panel C), using boxcar averaging (n = 3). This experiment was repeated five times, and in each case the radioactive peak at 62 min (arrow) was found to be specifically decreased in the presence of poly(rA)·oligo(dT).

degradation cleanly gave the sequence IYPGIK (Table I). From the amino acid analyses the maximum contamination with a second peptide could not have exceeded 5%. This sequence corresponds to residues 270–275 in HIV1-RT (Table IV). Although it contains no arginine, there is an arginine at position 277, two beyond the last residue sequenced. The

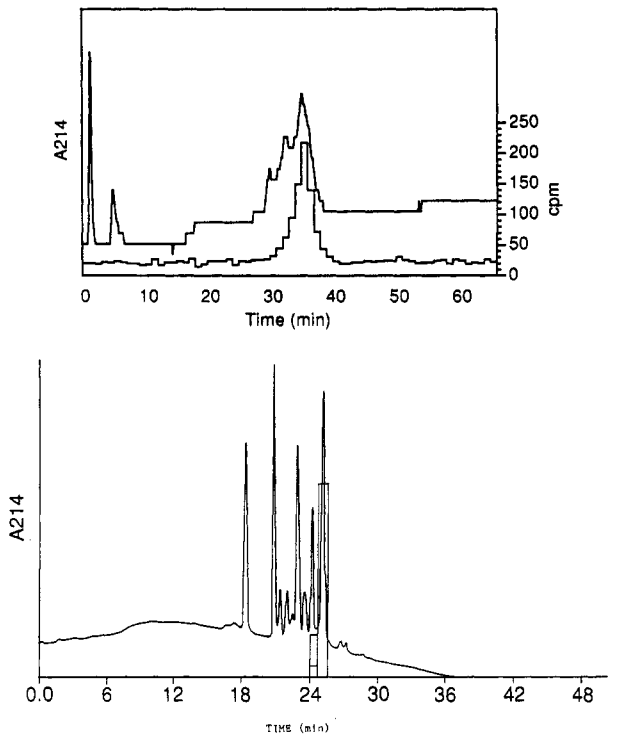

FIGURE 3: Purification of the peptide specifically labeled by [^{14}C]-PG. (A, top) Fractions corresponding to the specifically labeled peak in Figure 2 (61–64 min) were pooled, lyophilized, dissolved in 0.1% TFA, and applied to an LC 3DP column. Solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in ACN. Gradient employed: 5 min at 0% B, 60 min 0–20% B. Flow rate: 0.7 mL/min. One-minute fractions were collected and counted. (B, bottom) Fractions corresponding to the radioactive peak in panel A were pooled, lyophilized, dissolved in 0.1% TFA, and applied to a microbore capillary C-8 column. The histogram indicates radioactivity, which was only found in fractions 24 and 25. Solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in ACN. Gradient employed: 0–70% B in 35 min. Flow rate: 0.7 mL/min.

Table I: Specifically Labeled Peptide: Sequence, Yield, and Radioactivity[a]

cycle	residue	yield 1	yield 2	cpm1	cpm2
1	isoleucine	16	6.4	0	0
2	tyrosine	17	16	0	0
3	proline	17	18	0	0
4	glycine	25	19	0	0
5	isoleucine	23	19	0	0
6	lysine	19	14	0	0
7	X			0	0
8	X			55	60
9	X			nd	0

[a] Results of two independent experiments are shown. For each, the peptide appplied contained 16.5 pmol of [^{14}C]PG. Yields are shown in picomoles. Radioactivity is background subtracted. nd, not determined.

large reduction in yield following cycle 6 may be due to the loss of a lysine in this cycle, since the peptide adheres to the sequencing membrane via positive charges.

Also shown in Table I is the radioactivity released in each Edman cycle. Radioactivity was only found in the eighth cycle of sequencing, consistent with the labeling of Arg 277. Thus, although the yield of the sequencing reaction was too low to be reliable past the sixth cycle, the higher sensitivity afforded by the radioactive label provides strong evidence for assignment of the labeled residue as Arg 277.

One potential pitfall in this type of experiment is that one might be sequencing a mixture of two peptides, one present in high stoichiometry but not radioactively labeled and one present in low stoichiometry but containing the radioactive label, which would lead to an improper assignment of the site of PG modification. This is not a problem in the present case since the stoichiometry of [^{14}C]PG introduced into the Edman procedure was 16.5 pmol which, as seen from the results in Table I, corresponds about 1:1 with the amount of peptide sequenced. The fully purified, labeled peptide would be expected to contain one PG, since, as noted above, the borate buffers utilized in its isolation stabilize the monoadduct of PG to arginine rather than the diadduct.

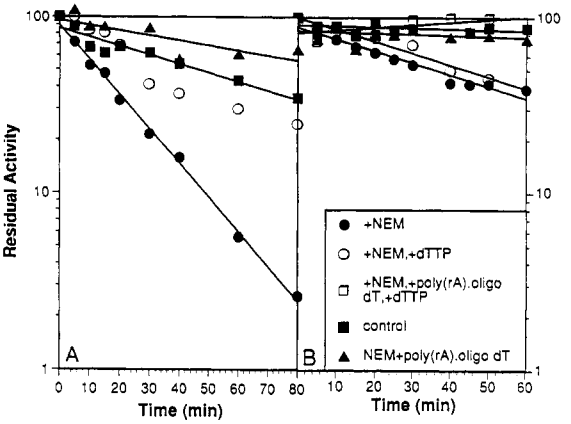

FIGURE 4: NEM inactivation of HIV1-RT. The effects of NEM on the polymerase activities of (A) NY5–HIV1-RT and (B) LAV–HIV1-RT vs time are shown as semilog plots.

Table II: [^{14}C]NEM Incorporation into HIV1-RT

addition	incorporation[a] into NY5	incorporation[a] into LAV
5 mM NEM	1.70	0.68
+ 250 nM PTe	0.90	0.73
+ 50 mM dTTP	nd	0.78
+ 250 nM PTe + 50 mM dTTP	0.78	0.76

[a] Moles per mole of RT, calculated for a molecular weight of 57 000. Samples were incubated for 60 min at 29 °C. Incorporation levels were determined as described under Experimental Procedures.

Q269 precedes I270 at the N terminus of the specifically labeled peptide, and cleavage at a Gln–Ile linkage is unexpected for trypsin. This result may reflect contaminants of the commercial preparations of trypsin we employed, as has been suggested previously (Gonzalez & Cooperman, 1986). The failure of trypsin to cleave following K275 may be due to steric hindrance by the modified arginine.

Inactivation of HIV1-RT by NEM. In contrast to the results obtained with PG, the effect of NEM on HIV1-RT activity depended upon which source of enzyme (from NY5 or LAV) was employed. NY5–HIV RT was fully inactivated by NEM in a reaction following pseudo-first-order kinetics, with a second-order rate constant of 9.3 M^{-1} min^{-1} (29 °C, pH 7.8; Figure 4A). dTTP (200 μM) provides some protection from inactivation by NEM. After an initial rate of inactivation close to that found with NEM alone, inactivation levels off at about 40% residual activity. Poly(rA)·oligo(dT) (300 nM) and poly(rA)·oligo(dT) plus dTTP confer almost complete protection. Under the same conditions, LAV–HIV1-RT was only partially inactivated by NEM, again leveling off at about 40% residual activity (Figure 4B). Poly(rA)·oligo(dT) (300 nM) or poly(rA)·oligo(dT) plus dTTP protect against such partial inactivation, but dTTP alone does not.

Incorporation of NEM. Results measuring the incorporation of NEM into HIV1-RT prepared from NY5 and LAV are listed in Table II. These results show, first, that one more NEM/subunit incorporates into unprotected NY5–HIV1-

Table III: Amino Acid Sequence Differences between NY5–HIV1-RT[a] and LAV–HIV1-RT[b]

position	NY5	LAV	position	NY5	LAV
−1	Met		358	Lys	Arg
102	Gln	Lys	376	Ala	Thr
122	Lys	Glu	400	Ala	Thr
162	Cys	Ser	435	Ile	Val
214	Phe	Leu	447	Asn	Ser
272	Ala	Pro	460	Asp	Asn
293	Val	Ile	468	Pro	Thr
357	Met	Thr	519	Ser	Asn

[a] Adachi et al. (1986) and J. Seehra, personal communication. [b] Ratner et al. (1985).

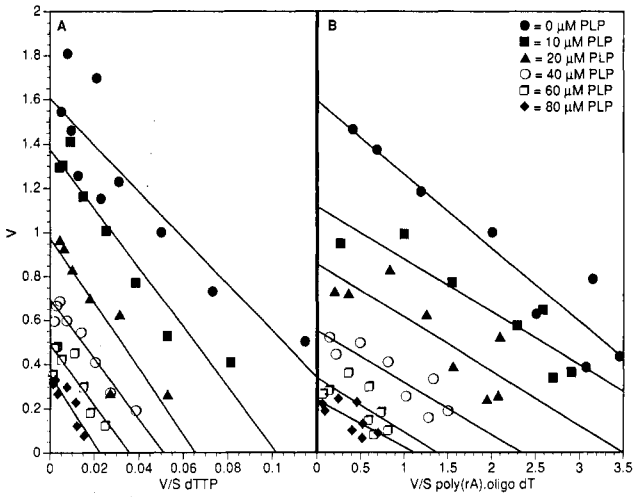

FIGURE 5: PLP inhibition of HIV1-RT. (A) Inhibition varying dTTP; (B) inhibition varying poly(rA)·oligo(dT). Concentrations of added PLP are indicated. Both sets of data are graphed in Eadie–Hofstee form.

RT than into LAV–HIV1-RT and, second, that addition of poly(rA)·oligo(dT) [or poly(rA)·oligo(dT) plus dTTP] reduces incorporation into NY5–HIV1-RT by approximately 1 NEM/subunit, while having no effect on incorporation into LAV–HIV1-RT. Comparison of the two HIV1-RT sequences shows changes in 16 of 561 amino acids (Table III), the majority of which are conservative. Most pertinently, Cys 162 in NY5–HIV1-RT is Ser 162 in LAV–HIV-RT. Coupled with the results presented in Figure 4 and Table II, this strongly suggests that NEM modification of Cys 162 is responsible for inactivation of NY5–HIV1-RT.

PLP Inhibition of HIV1-RT. The results presented in Figure 5 in Eadie–Hofstee form demonstrate that PLP is a noncompetitive inhibitor of HIV1-RT with respect to both dTTP (Figure 5A) and poly(rA)·oligo(dT) (Figure 5B). K_ms for dTTP and poly(rA)·oligo(dT) were determined to be 11.3 μM and 21 nM, respectively. These values are quite similar to those reported earlier by Majumder et al. (1989), 18 μM and 24 nM, respectively. Secondary plots of $1/V_{max,app}$ vs [PLP] are shown in Figure 6. The plot for which dTTP was varied showed a linear dependence on [PLP], indicating a single K_i of 20 μM, but the plot for which PTe was varied showed a slight concavity, possibly indicating two different PLP sites of similar intrinsic affinity. Curve fitting, to a model assuming that RT is inactive with either one or two bound PLPs and noncompetitive binding of PLP with respect to either poly(rA)·oligo(dT) or dTTP, yielded two K_i values, 37 and 53 μM.

Several PLP derivatives, including pyridoxal, pyridoxine, pyridoxal diacetate, 4-pyridoxic acid, 2-methyl-3-hydroxy-4-(hydroxymethyl)-5-(pyridylmethyl) phosphate and 2,4-di-

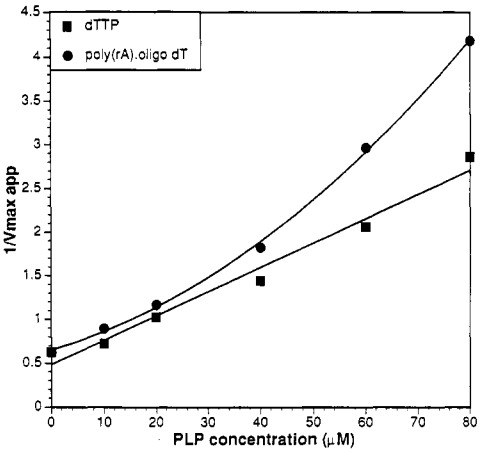

FIGURE 6: Secondary plots of $1/V_{max}$ vs [PLP] from the data in Figure 5. Lines drawn are for a K_i of 20 μM (dTTP) or two K_is equal to 37 and 53 μM [poly(rA)·oligo(dT)].

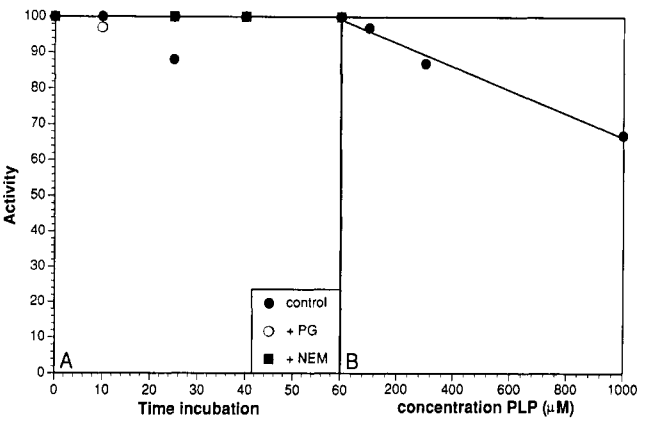

FIGURE 7: Effects of chemical modification on RNase H activity. (A) Effects on RNase H activity of incubation with PG (10 mM) or NEM (10 mM). (B) Effects on RNase H activity of preincubation (5 min) with different PLP concentrations.

methyl-3-hydroxy-5-(pyridylmethyl) phosphate, showed little or no inhibition of HIV1-RT activity at concentrations up to 1 mM, providing strong evidence for the structural specificity of PLP inhibition of HIV1-RT. Such structural specificity is typical of PLP inhibition of RTs (Modak, 1976a; Papas et al., 1977; Modak & Dumaswala, 1981).

Effects of PLP, PG, and NEM on RNase H Activity. In contrast to their effects on polymerase activity, neither PLP nor PG nor NEM was a potent inhibitor of the RNase H activity of HIV1-RT (Figure 7). Such differential sensitivity of the two enzyme activities toward various inhibitors has been found previously for HIV1-RT (Hizi et al., 1991) as well as for other RTs (Modak, 1976b; Gorecki & Panet, 1978; Srivastava & Modak, 1980), although Hizi et al. (1991, 1992), using a protocol somewhat different from that utilized here, do report greater sensitivity of RNase H activity toward NEM (and other sulfhydryl reagents) than we find.

DISCUSSION

The use of group-specific reagents can be a powerful tool in identifying active site residues. Such reagents often react more rapidly with residues at the active site than with residues located elsewhere in the enzyme molecule, due perhaps to enhanced solvent accessibility or to activation of active site side chains as nucleophiles. As a result, judicious choice of reaction conditions can result in specific labeling of an active site residue in high yield without excessive amounts of nonspecific labeling elsewhere, which simplifies identification

of the specifically labeled residue. In using group-specific reagents to identify potential active site residues, two necessary criteria that must be met are that modification leads to full inactivation and that substrates (or substrate analogues) protect against such inactivation. An inherent limitation of the chemical modification approach, which is particularly evident in our current results, is that there is no applicable criterion that is sufficient to prove that a modified residue is essential for activity, or even present at the active site (the same limitation applies to site-specific mutagenesis studies), and this limitation must be borne in mind in interpreting our results. For example, if there is an allosteric linkage between the active site and a residue remote from the active site, such that substrate binding inhibits chemical modification and chemical modification inhibits substrate binding, then modification of that residue can meet the two necessary criteria stated above without the residue being present at the active site.

A third necessary criterion that can be applied is that nonconservative site-specific mutation of the residue in question leads to enzyme inactivation. The many variant HIV-RTs that derive from clinical isolates can be considered in this light, with the caveat that changes in the sequence other than at the site of interest may also be affecting enzyme properties. In the case of our NEM studies, the results with the polymerase activity of LAV–HIV1-RT, which are similar to recent results of Hizi et al. (1991), also utilizing an HIV1-RT in which residue 162 is a serine rather than a cysteine (Hizi et al., 1988), clearly show that Cys 162 in NY5–HIV1-RT is not an essential residue. On the other hand, our results strongly imply that modification of this residue does lead to inactivation of polymerase and that its chemical reactivity is very much decreased when either poly(rA)·oligo(dT) or dTTP is bound to the enzyme. Whether this effect is direct, suggesting that Cys 162 is close to the active site, or allosteric, suggesting a conformational change in the enzyme on substrate binding, remains to be determined.

PG modification of Arg 277 meets the criteria of full inactivation and of substrate [poly(rA)·oligo(dT)] protection. In addition, Arg 277 is unusually reactive toward PG, a common feature of active site arginines [e.g., in inorganic pyrophosphatase (Cooperman & Chiu, 1973), ornithine decarboxylase (Marshall & Cohen, 1980), and DNA polymerase I (Mohan et al., 1988)]. The second-order rate constant for the inactivation of HIV1-RT is 7.2 M^{-1} min^{-1} at 29 °C, pH 7.8, some 5.5 times the corresponding rate constant of 1.3 M^{-1} min^{-1} for PG reaction with *N*-acetylarginine [calculated from the rate constant measured at 25 °C, pH 7.0 (0.15 M^{-1} min^{-1}; Cooperman & Chiu, 1973), assuming that the rate is proportional to OH^- concentration (Takahashi, 1968; Marshall & Cohen, 1980) and allowing a 40% increase in rate for a 4 °C rise in temperature]. Also, although there are 16 Arg residues per RT subunit, approximately 2 of the 5 PGs incorporated into HIV1-RT in the first 80 min of reaction (Figure 1) are incorporated into Arg 277. These results strongly implicate Arg 277 in primer-template binding to HIV1-RT, subject to the limitation discussed above.

Jacobo-Molina and Arnold (1991), extending an earlier analysis of Johnson et al. (1986), have divided the HIV1-RT sequence into three domains: the polymerization domain, the tether domain, and the RNase domain. The polymerase domain (residues P1–W266) and the RNase H domain (residues E438–I559) contain virtually all of the information required for these activities. The tether domain (residues A267–A437) is the most tolerant of amino acid substitution.

Table IV: RT Sequences Neighboring Arg 277 in HIV1-RT[a]

Isolate 255 295
HIV1P NDIQKLVGKLNWASQIYPGIKVRQLCKLLRGTKALTEVIPL
HIV16 NDIQKLVGKLNWASQIYPGIKVRQLCKLLRGTKALTEVIPL
HIV1B NDIQKLVGKLNWASQIYPGIKVRQLCKLLRGTKALTEVIPL
HIV15 NDIQKLVGKLNWASQIYPGIKVRQLCKLLRGTKALTEVIPL
HIV10 NDIQKLVGKLNWASQIYPGIKVRQLCKLLRGTKALTEVIPL
HIV1X NDIQKLVGKLNWASQIYPGIKVRQLCKLLRGTKALTEVIPL
HIV1N NDIQKLVGKLNWASQIYaGIKVRQLCKLLRGTKALTEVvPL
HIV1R NDIQKLVGKLNWASQIYaGIKVkQLCKLLRGTKALTEVvqL
HIV1A NDIQKLVGKLNWASQIYaGIKVkQLCKLLRGTKALTEVIPL
HIV1L NDIQKLVGKLNWASQIYaGIKVkQLCKLLRGTKALTEVIPL
HIV1E NDIQnLVerLNWASQIYPGIKVRQLCKLLRGTKALTEVIPL
HIV1M NDIQKLVGKLNWASQIYPGIKVkQLCKLLRGaKALTdivPL
HIV2 NDIQKLVGvLNWAaQlYPGIKtkhLCrLiRGkmtLTEevqw
SIV RT NelQKLaGKiNWASQaiPdIsikaLtnmmRGnqnLnstrqw
FIV RT NelQKLaGKiNWASQaiPdIsikaLtnmmRGnqnLnstrqw
EIAV RT NDvQKLmGnitWmSsgvPGltVkhiaattkGcleLnqkviw

[a] Small characters indicate nonconserved residue. Isolates are as listed in the Swiss-prot data bank. Comparisons were made using the Intelligenetics sequence program on the PennNET system at the University of Pennsylvania. Residue 277 is in boldface.

By this classification R277 falls outside the polymerization domain. However, it does lie between two highly conserved residues, P272 and L279. Further, as shown in Table IV, all of the sequenced HIV1-RT natural variants, as well as several closely related RTs, have either an Arg or a Lys in position 277, which is at least consistent with a positive charge in this position being important for binding of the negatively charged primer-template. Here it is also worth noting the conserved positive charge at R284 of HIV1-RT. Nanduri and Modak (1990) have aligned this residue with K329 of MoMuLV-RT, which they have placed at the primer-template binding site on the basis of chemical modification evidence.

Recent studies using photoaffinity labeling by oligo(dT)s have also provided interesting evidence for identifying the primer-template binding site of HIV-RT. Sobel et al. (1991) demonstrated that oligo$(dT)_{16}$ photoincorporates into HIV1-RT somewhere between residues 195 and 300. In a related study, Basu et al. (1992) presented strong evidence that oligo$(dT)_{15}$ photoincorporates into L289,T290 and L295,T296. These results, along with those presented herein, suggest that the polymerization domain might more properly be considered as extending past W266, perhaps as far as T296. Measuring the effects of site-specific mutations of residues between W266 and T296 on primer-template binding (e.g., by changing R277 and R284 to nonpositively charged residues) will clearly be of importance in further examining the validity of this suggestion.

PLP has long been known to inhibit RTs (Modak, 1976a,b). Since PLP inhibition is reversible, the rate at which equilibrium is established is an important consideration in selecting experimental conditions. In the present study, using conditions (preincubation with PLP for 5 min at 37 °C) that have been found to be sufficient to establish this equilibrium between PLP and other RTs, we find that PLP is a noncompetitive inhibitor with respect to primer-template, which is similar to results obtained with other RTs (Modak, 1976a; Papas et al., 1977; Modak & Dumaswala, 1981). However, our finding that PLP is also a noncompetitive inhibitor with respect to dTTP differs from earlier results with other RTs showing PLP to be a competitive inhibitor with respect to substrate dNTP, demonstrating a significant difference in this respect between HIV1-RT and other RTs.

Our results also contrast with those of Basu et al. (1989), who presented steady-state kinetic evidence that PLP was a

competitive inhibitor of HIV1-RT with respect to dGTP, using poly(rC)·oligo(dG) as primer-template. These workers found kinetic evidence for the involvement of two different PLP sites in inhibition, with the higher affinity site having a K_i of 125 μM. This value is 6-fold higher than the K_i of 20 μM we measure. Taking note of studies on other RTs showing the apparent K_i for PLP inhibition to be much lower for inhibition of poly(rA)·oligo(dT)-directed dT polymerization than for inhibition of poly(dC)·oligo(dG)-directed dG polymerization (Modak, 1976a; Modak & Dumaswala, 1981), one possibility for rationalizing our results with those of Basu et al. (1989) is that, in the presence of poly(rA)·oligo(dT), PLP binds to HIV1-RT in a noncompetitive manner via a high-affinity site that is not available in the presence of poly(rC)·oligo(dG). Differences in our experimental protocol from those of Basu et al., the details of which are not clear in their publication, might also be a factor.

In further work, Basu et al. (1989) showed that the presence of both poly(rC)·oligo(dG) and dGTP, but not of poly(rC)·oligo(dG) or dGTP alone, blocks $NaBH_4$-dependent PLP inactivation of HIV1-RT as well as $NaBH_4$-dependent irreversible covalent incorporation of PLP into Lys 263 and concluded, on the basis of these and the steady-state kinetic results, that K263 was likely to be part of the dGTP binding site. However, recent results of Hizi et al. (1991) call this conclusion into question. These workers showed that both HIV1-RT and HIV2-RT show similar inhibition by PLP, despite the fact that K263 is not conserved in HIV2-RT [it is a valine, instead (Table IV)].

In summary, our exploration of the active site of HIV1-RT with three group-specific reagents has (1) provided evidence for the involvement of R277 in primer-template binding, possibly as an essential residue, (2) shown that while NEM modification of C162 leads to inactivation of NY5 HIV1-RT and the chemical reactivity of C162 is drastically decreased in the presence of substrate, C162 is not essential for activity, and (3) demonstrated a high-affinity, inhibitory site for PLP binding that is not competitive with dTTP binding in the presence of poly(rA)·oligo(dT).

ADDED IN PROOF

Kohlstaedt et al. (1992) have recently published a 3.5-Å structure of HIV1-RT. In this structure C162 falls in the cleft of the palm and R277 is at the junction of the thumb and the palm. Within the precision of the current model, both of these positions could interact directly with bound primer-template (T. Steitz, private communication).

ACKNOWLEDGMENT

We express our appreciation to Dr. Jasbir Seehra (Genetics Institute) for gifts of HIV1-RT, Professor John Lambris (Pathology Department, University of Pennsylvania) for his help with sequencing studies, Dr. Parimi Muralikrishna for providing [^{3}H]RNA:DNA hybrid for the RNase H assay, and Nora Zuño for excellent technical assistance.

REFERENCES

Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A., & Martin, M. A. (1986) *J. Virol. 59*, 284–291.
Basu, A., & Modak, M. J. (1987) *Biochemistry 26*, 1704–1709.
Basu, S., Basu, A., & Modak, M. J. (1988a) *Biochemistry 27*, 6710–6716.
Basu, A., Nanduri, V. B., Gerard, G. F., & Modak, M. J. (1988b) *J. Biol. Chem. 263*, 1648–1653.
Basu, A., Tirumalai, R. S., & Modak, M. J. (1989) *J. Biol. Chem. 264*, 8746–8752.
Basu, A., Ahluwalia, K. K., Basu, S., & Modak, M. J. (1992) *Biochemistry 31*, 616–623.
Bond, M. W., Chiu, N. Y., & Cooperman, B. S. (1980) *Biochemistry 19*, 94–102.
Buck, M. A., Olah, T. A., Weitzmann, C. J., & Cooperman, B. S. (1989) *Anal. Biochem. 182*, 295–299.
Cooperman, B. S., & Chiu, N. Y. (1973) *Biochemistry 12*, 1676–1682.
Gonzalez, M. A., & Cooperman, B. S. (1986) *Biochemistry 25*, 7175–7185.
Gorecki, M., & Panet, A. (1978) *Biochemistry 17*, 2438–2442.
Hizi, A., McGill, C., & Hughes, S. H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1218–1222.
Hizi, A., Tal, R., Shaharabany, M., & Loya, S. (1991) *J. Biol. Chem. 266*, 6230–6239.
Hizi, A., Shaharabany, M., Tal, R., & Hughes, S. H. (1992) *J. Biol. Chem. 267*, 1293–1297.
Huber, H. E., McCoy, J. M., Seehra, J. S., & Richardson, C. C. (1989) *J. Biol. Chem. 264*, 4669–4678.
Jacobo-Molina, A., & Arnold, E. (1991) *Biochemistry 30*, 6351–6361.
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.
Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., & Steitz, T. A. (1992) *Science 256*, 1783–1790.
Majumdar, C., Stein, C. A., Cohen, J. S., Broder, S., & Wilson, S. H. (1989) *Biochemistry 28*, 1340–1346.
Marshall, M., & Cohen, P. P. (1980) *J. Biol. Chem. 255*, 7301–7305.
Modak, M. J. (1976a) *Biochemistry 15*, 3620–3626.
Modak, M. J. (1976b) *Biochem. Biophys. Res. Commun. 71*, 180–187.
Modak, M. J., & Dumaswala, U. J. (1981) *Biochim. Biophys. Acta 654*, 227–235.
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.
Pandey, V. N., Williams, K. R., Stone, K. L., & Modak, M. J. (1987) *Biochemistry 26*, 7744–7748.
Papas, T. S., Pry, T. W., & Marciani, D. J. (1977) *J. Biol. Chem. 252*, 1425–1430.
Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R., Jr., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C., & Wong-Staal, F. (1985) *Nature 313*, 277–284.
Reddy, G., Nanduri, V. B., Basu, A., & Modak, M. J. (1991) *Biochemistry 30*, 8195–8201.
Rush, J., & Konigsberg, W. H. (1990) *J. Biol. Chem. 265*, 4821–4827.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502–514.
Smith, J. (1990) Ph.D. thesis, University of Pennsylvania.
Sobol, R. W., Suhadolnik, R. J., Kumar, A., Lee, B. J., Hatfield, D. L., & Wilson, S. H. (1991) *Biochemistry 30*, 10623–10631.
Srivastava, A., & Modak, M. J. (1980) *J. Biol. Chem. 255*, 2000–2004.
Takahashi, K. (1968) *J. Biol. Chem. 263*, 6171–6179.
Tirumalai, R. S., & Modak, M. J. (1991) *Biochemistry 30*, 6436–6443.
Werber, M. M., Moldovan, M., & Sokolovsky, M. (1975) *Eur. J. Biochem. 53*, 207–216.