Catalytically Distinct Conformations of the Ribonuclease H of HIV-1 Reverse Transcriptase by Substrate Cleavage Patterns and Inhibition by Azidothymidylate and *N*-Ethylmaleimide†

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ABSTRACT: The RNase H activity of recombinant HIV-1 reverse transcriptase (RT) has been characterized with respect to inhibition by azidothymidylate (AZTMP) and *N*-ethylmaleimide (NEM) and to cleavage patterns using either poly(rA)/poly(dT) or poly(rG)/poly(dC) as model substrate and either Mg^{2+} or Mn^{2+} as divalent cation activator. The inhibitory potency of AZTMP and other nucleotide analogues was found to be dependent on both the composition of the substrate and the divalent cation. The enzyme was significantly more sensitive to AZTMP inhibition with poly(rG)/poly(dC) than with poly(rA)/poly(dT) as substrate and in Mn^{2+} than in Mg^{2+} with either substrate. Kinetic studies indicated that AZTMP is a competitive inhibitor with respect to the substrate in Mn^{2+} whereas it behaves as an uncompetitive inhibitor in Mg^{2+}. These results suggest that the enzyme may exist in two distinct forms depending on whether Mg^{2+} or Mn^{2+} is the divalent cation activator. Consistent with this suggestion is the alteration in the mode of cleavage of the substrate upon substitution of Mg^{2+} with Mn^{2+}. In Mg^{2+}, hydrolysis of poly(rA)/poly(dT) appears to be solely endonucleolytic, whereas in Mn^{2+}, hydrolysis is both endonucleolytic and exonucleolytic. With poly(rG)/poly(dC) as substrate, hydrolysis is both endonucleolytic and exonucleolytic in either Mg^{2+} or Mn^{2+}. There is a positive correlation between sensitivity to AZTMP and production of mononucleotides, suggesting that the exonuclease activity of RNase H is preferentially inhibited by AZTMP. The sensitivity of RNase H to inhibition by *N*-ethylmaleimide was also found to be markedly influenced by the substrate composition and the divalent cation activator, being most sensitive under conditions in which endonucleolytic activity predominates. These findings suggest that the RNase H activity of HIV-1 reverse transcriptase may undergo conformational changes upon interaction with RNA/DNA hybrids and that these changes are dependent on the divalent cation activator.

The reverse transcriptase (RT)[1] of HIV-1 has been and continues to be an important target for antiretroviral therapy (Mitsuya *et al.*, 1991; Hirsch & D'Aquila, 1993). The enzyme is essential for the infectivity of HIV-1 as it is required for the conversion of single-stranded genomic RNA to double-stranded proviral DNA (Goff, 1990; Arnold & Arnold, 1991). The protein has two distinct enzymatic activities: a DNA polymerase activity which can use either RNA or DNA as template and an RNase H activity which degrades the RNA moiety of DNA/RNA hybrids (Verma, 1977; Moelling *et al.*, 1971; Varmus & Swanstrom, 1984; Starnes & Cheng, 1989). The RNase H activity is believed to be required for several stages in viral replication, including the removal of viral genomic RNA following the synthesis of minus-strand DNA (Watson *et al.*, 1979) and of the tRNA that has functioned as primer for minus-strand synthesis (Omar & Faras, 1982). It is also responsible for the generation of the polypurine tract that primes the synthesis of plus-strand DNA as well as for its subsequent removal (Champoux *et al.*, 1984; Mitra *et al.*, 1982; Resnick *et al.*, 1984; Smith *et al.*, 1984).

Both the RNase H and the DNA polymerase activities of HIV-1 RT are essential for viral replication. Genetic analysis has shown that point mutations in the RNase H domain which selectively abolish RNase H activity block viral replication and attenuate viral infection (Repaske *et al.*, 1991; Tisdale *et al.*, 1991; Telesnitsky *et al.*, 1992). Thus, there is increasing interest in exploiting this enzymatic activity as a target for the development of antiretroviral agents. We have recently found that the RNase H activity of HIV-1 RT can be inhibited by AZTMP (Tan *et al.*, 1991), the major intracellular metabolite of AZT (Furman *et al.*, 1986). With poly(rA)/poly(dT) as substrate and Mg^{2+} as divalent cation activator, the IC_{50} for AZTMP is approximately 5 mM and approximately 50 μM when Mn^{2+} is substituted for Mg^{2+} (Tan *et al.*, 1991). Since AZTMP can reach millimolar concentrations intracellularly, we have proposed that AZTMP may play a role in the antiviral activity of AZT, *i.e.*, that the effectiveness of *AZT in vivo* may be due to inhibition of the RNase H activity by AZTMP in addition to inhibition of the DNA polymerase activity by AZTTP. In the present study we have used the synthetic homopolymers poly(rA)/poly(dT) and poly(rG)/poly(dC) as model substrates to further characterize the RNase H activity of HIV-1 RT with respect to endouclease

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[1] Abbreviations: RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1; RNase H, ribonuclease H; AZTMP, 3′-azido-3′-deoxythymidine 5′-phosphate; NH_2-TMP, 3′-amino-3′-deoxythymidine 5′-phosphate; ddNMP, 2′,3′-dideoxynucleoside 5′-monophosphate, pGpG, guanylyl(3′–5′)guanosine 5′-phosphate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide.

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and 3′–5′ exonuclease activities, to elucidate the mechanism of inhibition of RNase H activity by AZTMP, and to investigate the molecular basis for the potentiation of AZTMP inhibition by Mn^{2+}.

MATERIALS AND METHODS

Enzymes. Recombinant HIV-1 RT (Diebel *et al.*, 1990), expressed in *Escherichia coli*, was purified to apparent homogeneity as previously described (Tan *et al.*, 1991). T4 polynucleotide kinase was from Gibco-BRL. Phosphodiesterase I and *E. coli* RNA polymerase were from U.S. Biochemical Corp.

Polynucleotides. The RNase H substrates poly(rA)/poly(dT) and poly(rG)/poly(dT) and poly(rG)/poly(dC) were prepared as described by Starnes and Cheng (1989) using *E. coli* RNA polymerase. The RNA strand of each homopolymer duplex was labeled with [2,8-^{3}H]ATP, [8,5′-^{3}H]GTP, [α-^{32}P]-ATP, or [α-^{32}P]GTP (New England Nuclear).

Nucleotides. The nucleotide analogue 3′-azido-3′-deoxythymidine 5′-phosphate (AZTMP) was prepared as previously described (Tan *et al.*, 1991). The synthesis of 3′-amino-3′-deoxythymidine 5′-phosphate (NH_2-TMP) was achieved by catalytic reduction of AZTMP. An aqueous solution of AZTMP (175 mg, 0.5 mmol) was shaken with hydrogen gas in the presence of 10% palladium on charcoal (100 mg) at 40 lb psi and ambient temperature for 4 h using a Parr hydrogenation apparatus. The catalyst was removed by filtration, and the pH of the filtrate was adjusted to 6.8 prior to being charged onto a Dowex-1 ($HCOO^-$) column. The column was washed with water and eluted with a gradient of 0.2–4 M formic acid. Product-containing fractions were pooled, evaporated, and coevaporated with ethanol to dryness. The residue was taken up in water and freeze-dried to obtain 104 mg of the desired product (65% yield) which was chromatographically pure: UV λ_{max} (H_2O) 266 nm (ϵ 9.2 × 10^3). The purity of the product was confirmed by thin-layer chromatography on cellulose-coated plastic plates developed in 2-propanol/NH_4OH/H_2O, 7:1:2 (v/v/v); R_f AZTMP 0.7 and NH_2-TMP 0.5. 2′,3′-Dideoxynucleoside 5′-phosphates (ddNMP) were obtained from Pharmacia-LKB. Guanylyl-(3′–5′)guanosine (GpG) was from Calbiochem. [5′-^{32}P]pGpG was prepared by 5′-phosphorylation of GpG with T4 polynucleotide kinase and [γ-^{32}P]ATP (New England Nuclear). [5′-^{32}P]GMP was prepared by treatment of [5′-^{32}P]pGpG with phosphodiesterase I.

RNase H Assay. RNase H activity was determined in a final volume of 50 μL. Reaction mixtures contained 40 mM Tris-HCl, pH 8.3, 0.08 mg/mL bovine serum albumin, 3% glycerol, and 0.02% Nonidet P-40. For [^{3}H]-poly(rA)/poly-(dT) degradation, 400 nM substrate (as ribonucleotide), 2000–4000 cpm/pmol of AMP, was present in either 8 mM $MgCl_2$ or 4 mM $MnCl_2$. For [^{3}H]-poly(rG)/poly(dC) degradation, 400 nM substrate (as ribonucleotide), 2000–4000 cpm/pmol of GMP, was present in either 8 mM $MgCl_2$ and 50 mM KCl or 4 mM $MnCl_2$ and 120 mM KCl. The amount of HIV-1 RT varied depending on reaction conditions and is indicated in the figure legends. After incubation at 37 °C, the reaction was stopped by the addition of 150 μL of 10% trichloroacetic acid and 10 μg of salmon sperm DNA, followed by centrifugation at 15K rpm for 10 min. A 100-μL aliquot of the supernatant was transferred to scintillation solution for measuring acid-soluble radioactivity.

The concentration of inhibitor which results in 50% inhibition of activity (IC_{50}) was determined from Hill plots, $\log I = \log [(V_0 - V)/V]$. IC_{50} values were determined at approximately K_m concentrations of substrates. The concentrations of substrates used in Figure 1 were approximately 5–10 times K_m.

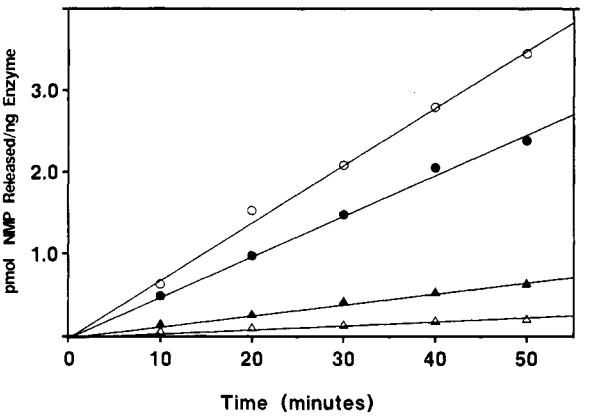

FIGURE 1: Effect of divalent cations on the rate of hydrolysis of poly(rA)/poly(dT) and poly(rG)/poly(dC). The hydrolysis of [^{3}H]-poly(rA)/poly(dT) (△, ▲) and [^{3}H]poly(rG)/poly(dC) (○, ●) was measured as a function of time in either 8 mM Mg^{2+} (open symbols) or 4 mM Mn^{2+} (solid symbols). The concentration of substrate was 400 nM in each case.

Inhibitor kinetics were determined in the standard RNase H assay except that the concentrations of [^{3}H]poly(rA)/poly-(dT) and [^{3}H]poly(rG)/poly(dC) were varied in the absence or presence of different concentrations of inhibitor. Experimental data were fitted to the Michaelis–Menten equation with a nonlinear regression program (Enzfitter) and are displayed as Lineweaver–Burk plots. K_i values of the inhibitors were determined by plotting $1/k_{cat}$ or K_m/k_{cat} *versus* inhibitor concentration and fitted with a linear regression program.

Product Analysis. The sizes of the products of RNase H degradation of [^{32}P]poly(rA)/poly(dT) and [^{32}P]poly(rG)/poly(dC) were determined by denaturing gel electrophoresis of the products of a standard reaction. At the indicated time points, a 5-μL aliquot of the reaction mixture was quenched with 5 μL of stop solution (80% formamide, 20 mM EDTA, and 0.02% bromophenol blue). After heating at 95 °C for 3 min, the products were separated by electrophoresis on a 12% polyacrylamide–8 M urea gel and located by autoradiography.

RESULTS

The Substrate Preference of the RNase H Activity of HIV-1 RT Is Affected by Divalent Cations. The effects of divalent cations (Mg^{2+} and Mn^{2+}) on the rates of hydrolysis of poly-(rA)/poly(dT) and poly(rG)/poly(dC) by the RNase H activity of HIV-1 RT are shown in Figure 1. In the presence of 8 mM Mg^{2+}, RNase H exhibits a 12-fold preference for poly(rG)/poly(dC) over poly(rA)/poly(dT) as substrate, whereas in the presence of 4 mM Mn^{2+} the preference for poly(rG)/poly(dC) is approximately 3.5-fold. Furthermore, RNase H activity is slightly more active in Mg^{2+} than in Mn^{2+} with poly(rG)/poly(dC) as substrate (1.4-fold), whereas it is 2-fold more active with Mn^{2+} than with Mg^{2+} as divalent cation when the substrate is poly(rA)/poly(dT). The assay was linear with enzyme concentration with both substrates in both Mn^{2+} and Mg^{2+}.

Effect of Substrate Composition and Divalent Cations on Inhibition of the RNase H Activity of HIV-1 RT by Nucleotides and Nucleotide Analogues. We have shown previously that the RNase H activity of HIV-1 RT, with poly-(rA)/poly(dT) as substrate, can be inhibited by millimolar concentrations of AZTMP when Mg^{2+} is the divalent cation

Table 1: Inhibition of the RNase H Activity of HIV-1 Reverse Transcriptase by Nucleotides and Nucleotide Analogues[a]

	IC_{50} in $MgCl_2$ (mM)		IC_{50} in $MnCl_2$ (mM)	
nucleotide	poly-[(rA)/(dT)]	poly-[(rG)/(dC)]	poly-[(rA)/(dT)]	poly-[(rG)/(dC)]
dAMP	>4.0	>4.0	1.5	>4.0
dGMP	>4.0	>4.0	3.6	>4.0
dCMP	>4.0	>4.0	>4.0	>4.0
dTMP	>4.0	>4.0	>4.0	>4.0
ddAMP	>4.0	1.2	0.22	0.53
ddGMP	>4.0	4.0	0.45	1.2
ddTMP	>4.0	1.8	>4.0	>4.0
AZTMP	>4.0	0.25	0.013	0.025
NH_2-TMP	4.0	0.94	0.075	0.10

[a] The concentration of nucleotide or nucleotide analogue that inhibits RNase H activity 50% (IC_{50}) was determined as described in Materials and Methods.

activator and that substitution of Mn^{2+} for Mg^{2+} increases the sensitivity of the enzyme to AZTMP, decreasing the IC_{50} from approximately 5 mM to 50 μM (Tan *et al.*, 1991). We further observed that, in the presence of Mn^{2+}, RNase H is sensitive to inhibition by dAMP and ddAMP but not by dCMP, dGMP, dTMP, ddTMP, or ribonucleotides. To define the structural requirements for inhibition of RNase H activity, we have determined the effects of both complementary and noncomplementary nucleotides and nucleotide analogues on RNase H activity with either poly(rA)/poly(dT) or poly(rG)/poly(dC) as substrates in the presence of either Mg^{2+} or Mn^{2+} (Table 1). We found that, with both substrates, the naturally occurring deoxynucleotides were rather ineffective inhibitors of RNase H activity in either Mg^{2+} or Mn^{2+}. The dideoxynucleotides were also ineffective inhibitors of RNase H in Mg^{2+}, whereas inhibition was potentiated in Mn^{2+}, particularly with poly(rA)/poly(dT) as substrate. 3′-Substitution of the dideoxynucleotides with an azido or amino group resulted in a greater inhibitory activity with poly(rG)/poly(dC) as substrate, and the substitution of Mn^{2+} for Mg^{2+} further potentiated the inhibition. However, the ability of the 3′-substituted dideoxynucleotides to inhibit RNase H activity with poly(rA)/poly(dT) as substrate was increased markedly by substitution of Mn^{2+} for Mg^{2+}. No inhibition was seen with ribonucleotides or with nucleosides, including AZT, in either Mg^{2+} or Mn^{2+} (data not shown).

These results suggest that the inhibition of the RNase H activity of HIV-1 RT by nucleotide analogues is dependent on the substrate composition. For both substrates, purine nucleotides were more effective inhibitors than pyrimidine nucleotides and no base specificity was apparent. The structure at the 2′-position of the sugar appears to be crucial since only 2′-deoxynucleotides were inhibitory. However, nucleotides with substituents at the 3′-position of the sugar were effective inhibitors, with $-N_3 > -NH_2 > -H > -OH$.

Kinetic Analysis of the Mechanism of Inhibition of RNase H Activity by AZTMP. In order to understand how AZTMP interacts with the RNase H of HIV-1 RT, we have determined the mechanism of AZTMP inhibition of RNase H activity by kinetic analysis under steady-state conditions. Initial rates of hydrolysis were measured over a wide range of substrate concentrations and at several AZTMP concentrations. As shown in Figure 2, with poly(rA)/poly(dT) as substrate and Mn^{2+} as divalent cation activator, AZTMP is a competitive inhibitor with respect to the substrate, suggesting that AZTMP binds at the active site of RNase H. The K_i was found to be 4 μM. In contrast, with poly(rG)/poly(dC) as substrate and Mg^{2+} as divalent cation activator, the pattern of AZTMP

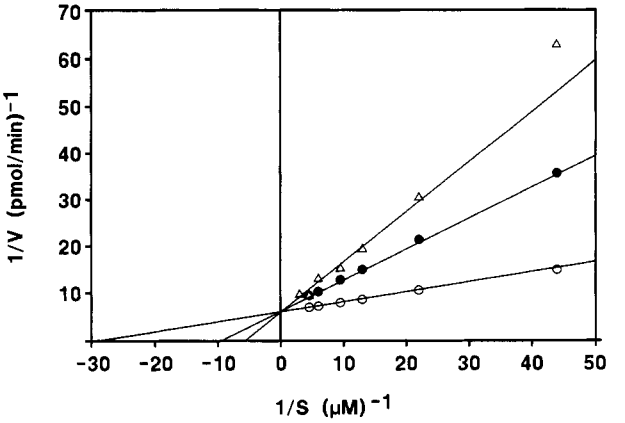

FIGURE 2: Pattern of inhibition of poly(rA)/poly(dT) hydrolysis by AZTMP in the presence of Mn^{2+}. Competitive inhibition of RNase H activity by AZTMP with [^{3}H]poly(rA)/poly(dT) as substrate and 4 mM Mn^{2+} as divalent cation is displayed as a double-reciprocal plot of the rate of nucleotide released by 10 ng of HIV-1 RT with no AZTMP (○), 12 μM AZTMP (●), and 13 μM AZTMP (△). The concentration of poly(rA)/poly(dT) varied from 23 to 221 nm. Incubation was at 37 °C for 10 min.

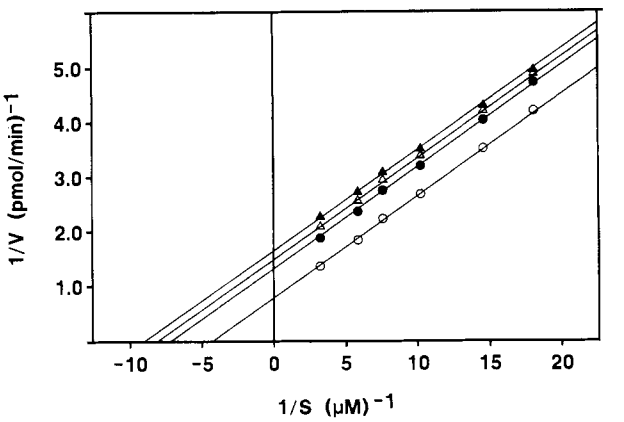

FIGURE 3: Pattern of inhibition of poly(rG)/poly(dC) hydrolysis by AZTMP in the presence of Mg^{2+}. Uncompetitive inhibition of RNase H activity by AZTMP with [^{3}H]poly(rG)/poly(dC) as substrate and 8 mM Mg^{2+} as divalent cation is displayed as a double-reciprocal plot of the rate of nucleotide released by 2.5 ng of HIV-1 RT with no AZTMP (○), 0.2 mM AZTMP (●), 0.25 mM AZTMP (△), and 0.3 mM AZTMP (▲). The concentration of poly(rG)/poly(dC) varied from 50 to 380 nM. Incubation was at 37 °C for 10 min.

inhibition is uncompetitive with respect to the substrate (Figure 3), with a K_i of 0.29 mM, suggesting that binding of the enzyme to the RNA/DNA hybrid is not affected by AZTMP in the presence of Mg^{2+} but that AZTMP binds to the enzyme–substrate complex. However, in the presence of Mn^{2+}, the pattern of inhibition of poly(rG)/poly(dC) hydrolysis is mixed (Figure 4), suggesting that AZTMP binds to both free enzyme and enzyme–substrate complex and competes with the substrate for binding to free enzyme in the presence of Mn^{2+}.

Effects of Divalent Metal Ions and Substrate Composition on the Sizes of Products Generated by RNase H. To determine the basis for the difference in the sensitivity of RNase H to inhibition by AZTMP with either poly(rA)/poly(dT) or poly(rG)/poly(dC) as substrate in the presence of Mg^{2+}, as well as the mechanism by which Mn^{2+} increases the sensitivity of the enzyme to AZTMP, the size distribution of products generated by RNase H with these substrates was determined by denaturing gel electrophoresis. As shown in Figure 5, the time-dependent size distribution of products generated by RNase H with poly(rA)/poly(dT) as substrate in the presence of Mg^{2+} differed markedly from that when Mn^{2+} was the divalent cation activator. In the presence of Mg^{2+} (Figure

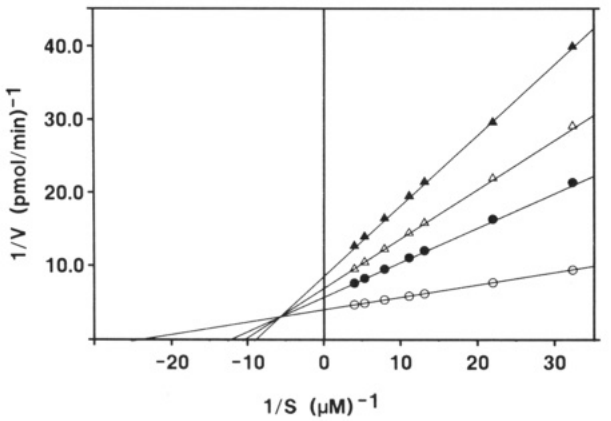

FIGURE 4: Pattern of inhibition of poly(rG)/poly(dC) hydrolysis by AZTMP in the presence of Mn^{2+}. Mixed inhibition of RNase H activity by AZTMP with [^{3}H]poly(rG)/poly(dC) as substrate and 4 mM Mn^{2+} as divalent cation is displayed as a double-reciprocal plot of the rate of nucleotide released by 5 ng of HIV-1 RT with no AZTMP (○), 23 μM AZTMP (●), 24 μM AZTMP (△), and 25 μM AZTMP (▲). The concentration of poly(rG)/poly(dC) varied from 31 to 249 nM. Incubation was for 10 min at 37 °C.

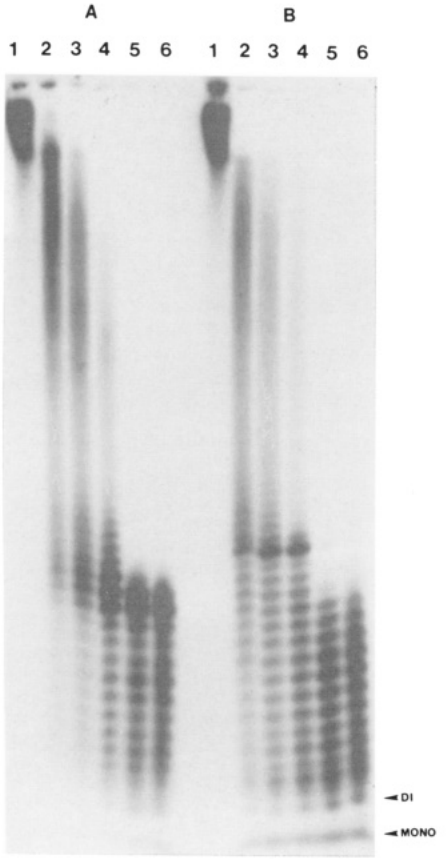

FIGURE 5: Sizes of the products of RNase H digestion of poly(rA)/poly(dT) as a function of time. Products were separated by denaturing gel electrophoresis as described in Materials and Methods. In panel A the divalent cation was 8 mM Mg^{2+} and the amount of HIV-1 RT was 20 ng. In panel B the divalent cation was 4 mM Mn^{2+} and the amount of HIV-1 RT was 20 ng. In both panels the concentration of [^{32}P]poly(rA)/poly(dT) was 70 nM and incubation at 37 °C was for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 60 min (lane 5), and 120 min (lane 6). The positions of the mono- and dinucleotide markers are indicated by arrows.

5A) there was an initial generation of large intermediates with an accumulation of oligonucleotides 14–18 nucleotides in length. This was followed by the appearance of smaller oligonucleotides 3–14 nucleotides in length after 20 min of incubation, which increased in quantity on further incubation, suggesting that the smaller oligonucleotides are generated by

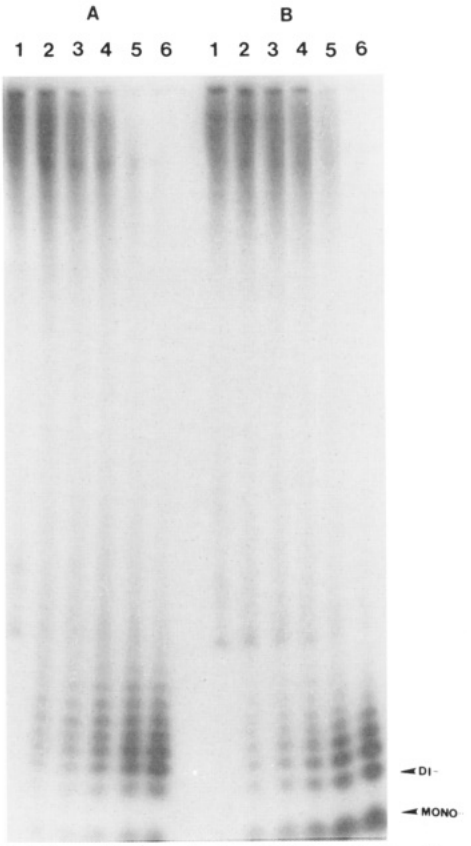

FIGURE 6: Sizes of the products of RNase H digestion of poly(rG)/poly(dC) as a function of time. Products were separated by denaturing gel electrophoresis as described in Materials and Methods. In panel A the divalent cation was 8 mM Mg^{2+} and in panel B it was 4 mM Mn^{2+}. In both panels 5 ng of HIV-1 RT were present and the concentration of [^{32}P]poly(rG)/poly(dC) was 200 nM. Incubation at 37 °C was for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 60 min (lane 5), and 120 min (lane 6). The positions of the mono- and dinucleotide markers are indicated by arrows.

the hydrolysis of larger intermediates. In contrast, in the presence of Mn^{2+} (Figure 5B) smaller oligonucleotides (1–18 nucleotides in length) were produced simultaneously with the larger oligonucleotides (>18 nucleotides in length) very early in the reaction. The most significant difference is that when Mg^{2+} was the divalent cation activator little or no mononucleotide was generated even after prolonged hydrolysis, whereas in the presence of Mn^{2+} mononucleotides were generated at the first time point taken, *i.e.*, within 5 min. These results suggest that endonuclease activity is primarily responsible for the digestion of poly(rA)/poly(dT) in the presence of Mg^{2+}, *i.e.*, for both the initial generation of the large oligonucleotides and the subsequent hydrolysis to a ladder of smaller RNA fragments, as evidenced by the lack of any detectable mononucleotide products with this divalent cation. In the presence of Mn^{2+} it appears that both endonuclease and exonuclease activities participate in the degradation of poly(rA)/poly(dT), since both large intermediates and smaller oligonucleotides (including mononucleotides) are produced very early in the course of the reaction. It is likely that the initial larger oligonucleotides are generated by an endonuclease activity whereas the smaller oligonucleotides are produced by subsequent exonucleolytic hydrolysis. These results further suggest that Mn^{2+} activates the exonuclease activity of RNase H with poly(rA)poly(dT) as substrate.

The size distribution of cleavage products of poly(rG)/poly(dC) generated by RNase H differed considerably from that of poly(rA)poly(dT) (Figure 6). In the presence of either

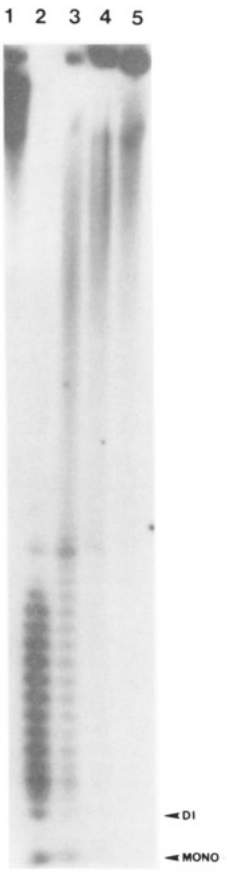

FIGURE 7: Effect of AZTMP on the sizes of products generated by RNase H with poly(rA)/poly(dT) as substrate in the presence of Mn^{2+}. HIV-1 RT (20 ng) was incubated with 120 nM [^{32}P]poly-(rA)/poly(dT) in the presence of 4 mM Mn^{2+} with no AZTMP (lanes 1 and 2), 30 μM AZTMP (lane 3), 35 μM AZTMP (lane 4), and 40 μM AZTMP (lane 5). Reactions were incubated at 37 °C for 0 min (lane 1) or 60 min (lanes 2–5). The positions of the mono- and dinucleotide markers are indicated by arrows.

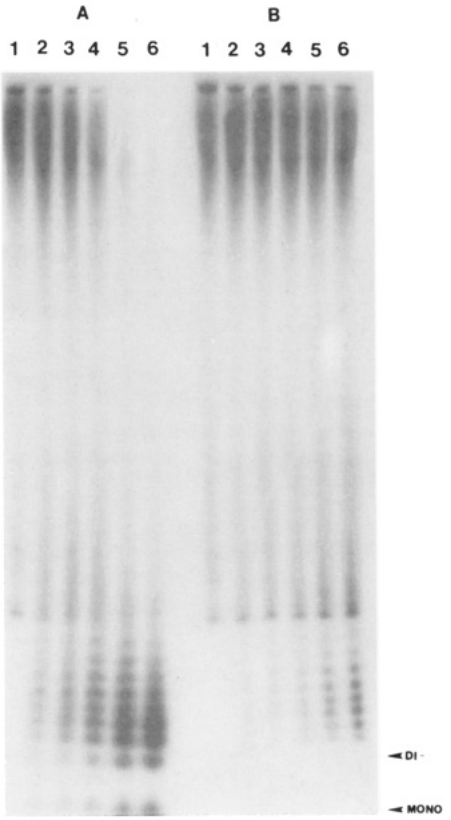

FIGURE 8: Effect of AZTMP on the sizes of products generated by RNase H with poly(rG)/poly(dC) as substrate in the presence of 8 mM Mg^{2+}. HIV-1 RT (5 ng) was incubated with 200 nM [^{32}P]-poly(rG)/poly(dC) in the absence of AZTMP (A) or in the presence of 2 mM AZTMP (B). Incubation at 37 °C was for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 60 min (lane 5), and 120 min (lane 6). The positions of the mono- and dinucleotide markers are indicated by arrows.

Mg^{2+} or Mn^{2+}, poly(rG)/poly(dC) was degraded to smaller oligonucleotides than was poly(rA)/poly(dT), with little or no generation of intermediates of chain length >10 nucleotides. With this substrate the most prominent products in the presence of Mg^{2+} (Figure 6A) were oligonucleotides 3–6 nucleotides in length, whereas with Mn^{2+} as divalent cation activator (Figure 6B) the major products at all time points were mono- and oligonucleotides 1–4 nucleotides in length. These results suggest that, with poly(rG)/poly(dC) as substrate, both endonuclease and exonuclease activities are active in the presence of Mg^{2+}. The most striking difference between the patterns of degradation of the two substrates was seen in the presence of Mg^{2+}, *i.e.*, mononucleotide products are generated with poly(rG)/poly(dC) as substrate but not with poly(rA)/poly(dT). For both substrates, exonuclease activity was preferentially increased when Mn^{2+} was substituted for Mg^{2+}.

Effects of AZTMP on Product Size. Analysis of the effects of AZTMP on the size distribution of digestion products demonstrated that, with poly(rA)/poly(dT) as substrate in Mn^{2+} (Figure 7), increasing concentrations of AZTMP resulted in the loss of both mononucleotide and oligonucleotide products, suggesting that AZTMP inhibits both exonuclease and endonuclease activity. With poly(rG)/poly(dC) as substrate in the presence of Mg^{2+} (Figure 8) mono-, di-, and trinucleotide products completely disappeared in the presence of 2 mM AZTMP, whereas larger oligonucleotides and polynucleotides were not significantly affected. These results suggest that AZTMP preferentially inhibits the exonuclease activity of RNase H. Consistent with this interpretation, the degradation of poly(rA)/poly(dT) in Mg^{2+}, which is endonucleolytic, was not inhibited by AZTMP except at very high concentrations (data not shown).

Effects of Divalent Cation and Substrate Composition on Inhibition of RNase H by N-Ethylmaleimide. *N*-Ethylmaleimide (NEM), a thiol-reactive reagent, has been shown to inhibit the RNase H activity of HIV-1 RT with poly(rA)/poly(dT) as substrate and Mg^{2+} as divalent cation activator (Hizi *et al.*, 1991, 1992). To determine whether NEM inhibition of RNase H activity is affected by either divalent cations or substrate composition, the effects of increasing concentrations of NEM on the hydrolysis of poly(rA)/poly-(dT) and poly(rG)/poly(dC) in either Mg^{2+} or Mn^{2+} were determined (Figure 9). With poly(rA)/poly(dT) as substrate, 0.1 mM NEM inhibited RNase H activity 50% when Mg^{2+} was the divalent cation activator, whereas a similar degree of inhibition required 0.7 mM NEM when Mn^{2+} was the divalent cation activator. The enzyme was much more resistant to NEM inhibition with poly(rG)/poly(dC) as substrate. In the presence of Mg^{2+}, the activity was 50% inhibited at approximately 4 mM NEM, and in the presence of Mn^{2+} the activity was completely resistant to NEM inhibition with this substrate up to a concentration of 5 mM. Thus, similar to AZTMP inhibition, the inhibition of RNase H activity by NEM is dependent on the nucleotide composition of the substrate and the divalent cation activator. However, in contrast to AZTMP, which is most inhibitory with poly(rG)/poly(dC) as substrate and Mn^{2+} as divalent cation activator,

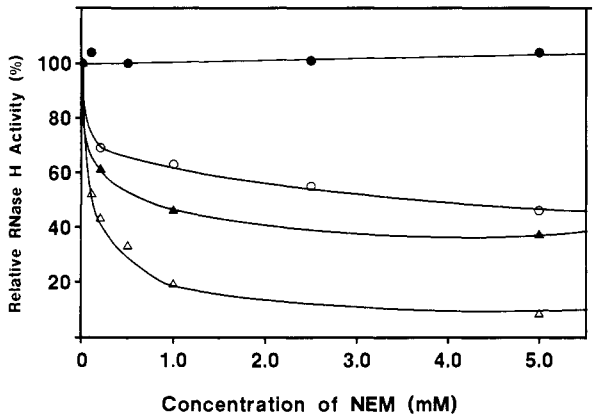

FIGURE 9: Effect of NEM on RNase H activity. RNase H activity was determined as described in Materials and Methods with either 60 nM [^{3}H]poly(rA)/poly(dT) and 20 ng of HIV-1 RT (△, ▲) or 80 nM [^{3}H]poly(rG)/poly(dC) and 5 ng of HIV-1 RT (○, ●) in the presence of 8 mM Mg^{2+} (open symbols) or 4 mM Mn^{2+} (solid symbols). The NEM concentration was varied as indicated.

NEM is most inhibitory with poly(rA)/poly(dT) as substrate in the presence of Mg^{2+}. Thus it appears that the poly(rA)/poly(dT)- and Mg^{2+}-dependent form of RNase H is sensitive to NEM but resistant to AZTMP, whereas the poly(rG)/poly(dC)- and Mn^{2+}-dependent form is sensitive to AZTMP but resistant to NEM.

DISCUSSION

The results presented in this paper can best be understood in terms of a model in which the RNase H domain of HIV-1 RT exists in at least two catalytically distinct conformations. Form A prefers poly(rG)/poly(dC) as substrate and Mg^{2+} as divalent cation and is sensitive to inactivation by NEM but resistant to inhibition by AZTMP, whereas form B hydrolyzes both poly(rG)/poly(dC) and poly(rA)/poly(dT) substrates, prefers Mn^{2+} as divalent cation, and is sensitive to inhibition by AZTMP but resistant to NEM. Form A acts primarily as an endonuclease with little or no production of mononucleotide products. Form B acts as both endonuclease and exonuclease, but primarily as an exonuclease after initial endonucleolytic cleavage.

Our results shed light on conflicting reports on the exo- and endonuclease activities of RNase H. Recent studies in which viral RNA segments annealed to oligodeoxynucleotides were used as substrates for the RNase H activity of HIV-1 RT have demonstrated that the initial cleavage of the viral RNA is carried out by an endonuclease activity, resulting in the generation of relatively large-sized intermediates. However, it is not clear whether the subsequent degradation of these intermediates to smaller fragments is catalyzed by an endonuclease or a 3′–5′ exonuclease. From the size distribution of the reaction products, Schatz *et al.* (1991) and Wohrl and Moelling (1991) have implicated a 3′–5′ exonuclease activity, based in part on the observation by Starnes and Cheng (1989) that the final products of degradation of poly(rG)/poly(dC) by the RNase H of HIV-1 RT mainly consist of tri-, di-, and mononucleotides. However, in a recent study, DeStefano *et al.* (1991) arrived at a different conclusion, *i.e.*, that the final cleavage products are generated by a partially processive 3′–5′ endonuclease activity. This was based on the observation that only a small amount of mononucleotide was generated, contrary to what one would have expected from the action of a 3′–5′ exonuclease activity. In the present study we have used synthetic homopolymeric RNA/DNA hybrids as substrates for the RNase H of HIV-1 RT. The use of RNA/DNA hybrids lacking unannealed RNA termini allows exonucleolytic activity to be detected without initial cleavage by an endonuclease activity, which could confuse the interpretation of the data. Furthermore, the use of uniformly labeled RNA strands increases the sensitivity of detection of 3′–5′ exonuclease activity, since radioactive nucleotides are always present at the termini.

The present study demonstrates that the RNase H activity of HIV-1 RT has 3′–5′ exonuclease activity in addition to endonuclease activity. Whether the RNase H functions as an endonuclease or an exonuclease depends on the base composition and/or structure of the RNA/DNA hybrid substrate, as well as on the divalent cation activator. In the presence of Mg^{2+}, the initial cleavage of poly(rA)/poly(dT), as well as the subsequent generation of smaller oligonucleotides, was carried out by an endonuclease activity, since little or no mononucleotide was generated even after exhaustive digestion. However, when Mn^{2+} was the divalent cation activator, although the initial cleavage was catalyzed by an endonuclease activity, further hydrolysis was carried out by a 3′–5′ exonuclease activity. In contrast to the hydrolysis of poly(rA)/poly(dT), both endonuclease and 3′–5′ exonuclease activities were involved in the digestion of poly(rG)/poly(dC), in the presence of either Mg^{2+} or Mn^{2+}, resulting in the generation of small oligonucleotides and mononucleotides. With either substrate, the 3′–5′ exonuclease activity was significantly more active in the presence of Mn^{2+} than in Mg^{2+}. These findings suggest that the RNase H of HIV-1 RT may exist in two conformations: one in which both the endonuclease and exonuclease are active and another in which only the endonuclease is active. They further suggest that the conformational state of the enzyme can be influenced by both the divalent cation activator and the composition and/or structure of the substrate. Recent studies on the crystal structure of HIV-1 RT at 7-Å resolution demonstrated significant movement of protein electron density in DNA-containing *versus* DNA-free RT, consistent with a substrate-induced conformational change (Arnold *et al.*, 1992).

The sensitivity of RNase H to inhibition by nucleotide analogues was also found to be influenced by substrate and divalent cation activator. In the presence of Mg^{2+}, RNase H was resistant to inhibition by AZTMP with poly(rA)/poly(dT) as substrate, whereas with poly(rG)/poly(dC) as substrate it was inhibited by concentrations of AZTMP (IC_{50} of 0.25 mM) which are considerably lower than what has been reported to be achievable intracellularly (Furman *et al.*, 1986). Substitution of Mn^{2+} for Mg^{2+} further potentiated AZTMP inhibition (IC_{50} of 10–20 μM) with both substrates. The positive correlation of the sensitivity of the RNase H activity of HIV-1 RT to inhibition by AZTMP with the appearance of mononucleotides among the reaction products and the disappearance of mononucleotide products in the presence of AZTMP suggest that the inhibition of RNase H activity is primarily due to inhibition of 3′–5′ exonuclease activity, although the endonuclease activity is also sensitive to inhibition by higher concentrations of AZTMP.

Analysis of the kinetics of inhibition with poly(rG)/poly(dC) as substrate and Mg^{2+} as divalent cation activator indicated that AZTMP is an uncompetitive inhibitor against the substrate, suggesting that AZTMP does not affect the binding of substrate to the enzyme but rather that the putative AZTMP binding site is affected by the binding of substrate, *i.e.*, that the binding of substrate in Mg^{2+} induces a conformational change in the enzyme which allows binding of AZTMP. In contrast, in the presence of Mn^{2+}, AZTMP is

a competitive inhibitor with poly(rA)/poly(dT) as substrate, suggesting that AZTMP binds at the active site of RNase H. The observation of mixed inhibition of poly(rG)/poly(dC) hydrolysis in the presence of Mn^{2+} also suggests that AZTMP competes with substrate for binding to free enzyme but that it also interacts with the enzyme–substrate complex. The different patterns of inhibition suggest that the form of the enzyme that interacts with AZTMP in Mg^{2+} is distinct from the one that interacts with this inhibitor in Mn^{2+}.

The sensitivity of RNase H to NEM was also found to be dependent on the substrate composition and divalent cation activator, although, in contrast to AZTMP, NEM inhibition was more pronounced in Mg^{2+} than in Mn^{2+}. Previous studies on the inhibition of the RNase H activity of HIV-1 RT by thiol-reactive reagents demonstrated that Cys^{280}, which is located in the polymerase domain of RT, although essential for the interaction of the enzyme with NEM, is not involved in catalysis (Hizi *et al.*, 1991, 1992). It was suggested that the chemical modification of Cys^{280} by NEM affects the structure of the protein such that RNase H activity is selectively abolished (Hizi *et al.*, 1992). The marked change in the sensitivity of RNase H to NEM when Mn^{2+} is substituted for Mg^{2+} is also consistent with the suggestion that the enzyme can exist in more than one conformation.

The response of the RNase H activity of HIV-1 RT to divalent cations is similar to that of highly purified calf thymus RNase HI, a four-subunit enzyme which was reported to exist in both Mg^{2+}- and Mn^{2+}-dependent forms (Busen, 1980). The Mn^{2+}-dependent activity was distinguished from the Mg^{2+}-dependent activity by requiring 4 times as much antibody to neutralize its activity. RNase HI was found to be more sensitive to inhibition by NEM in Mg^{2+} than in Mn^{2+}, and it was proposed that Mg^{2+} and Mn^{2+} stabilize different conformations of the enzyme. Two distinct RNase H activities have been partially purified from rat liver nuclei, one being Mg^{2+}-dependent and the other Mn^{2+}-dependent (Sawai *et al.*, 1978). The Mg^{2+}-dependent enzyme was estimated to be approximately 35 kDa, whereas the Mn^{2+}-dependent enzyme was estimated to be 150 kDa. Similar to both calf thymus RNase HI and the RNase H of HIV-1 RT, the Mn^{2+}-dependent enzyme was more resistant to inhibition by thiol-reactive reagents than the Mg^{2+}-dependent enzyme. More interestingly, the Mg^{2+}-dependent RNase H behaved as an endonuclease whereas the Mn^{2+}-dependent enzyme cleaved RNA/DNA hybrids exonucleolytically. Although the two RNase H activities in rat liver nuclei are physically separable, it is possible that they may be related, *i.e.*, the smaller polypeptide may be a proteolytic product of the larger protein.

In the present studies on the RNase H activity of HIV-1 RT, the use of synthetic homopolymer RNA/DNA hybrids as model substrates has allowed delineation of the effects of substrate composition and/or structure and divalent cation activator on the mechanism of RNA hydrolysis as well as the mechanisms of inhibition by nucleotide analogues. The finding that both substrate structure and metal activator can affect fundamental properties of the enzyme, possibly by affecting enzyme conformation, has provided some insight into the factors which may allow this versatile enzyme to carry out both specific cleavages of replication intermediates and the rapid, nonspecific degradation of viral RNA once its template function is fulfilled.

REFERENCES

Arnold, E., & Arnold, G. A. (1991) *Adv. Virus Res. 39*, 1–87.

Arnold, E., Jacobo-Molina, A., Nanni, R. G., Williams, R. L., Lu, X., Ding, J., Clark, A. D., Jr., Zhang, A., Ferris, A. L., Clark, P., Hizi, A., & Hughes, S. H. (1992) *Nature 357*, 85–89.

Busen, W. (1980) *J. Biol. Chem. 255*, 9434–9443.

Champoux, J. J., Gilboa, E., & Baltimore, D. (1984) *J. Virol. 49*, 686–691.

DeStefano, J. J., Buiser, R. G., Mallaber, L. M., Bambara, R. A., & Fay, P. J. (1991) *J. Biol. Chem. 266*, 24295–24301.

Diebel, M. R., Jr., McQuade, T. J., Brunner, D. P., & Tarpley, W. G. (1990) *AIDS Res. Hum. Retroviruses 6*, 329–340.

Furman, P. A., Fyfe, J. A., St. Clair, M. H., Weinhold, K., Rideout, J. L., Freeman, G. A., Lehrman, S. N., Bolognesi, D. P., Broder, S. A., Mitsuya, H., & Barry, D. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8333–8337.

Goff, S. P. (1990) *J. Acquired Immune Defic. Syndr. 3*, 817–831.

Hirsch, M. S., & D'Aquila, R. T. (1993) *N. Engl. J. Med. 328*, 1686–1695.

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.

Mitra, S. W., Chow, M., Champoux, J., & Baltimore, D. (1982) *J. Biol. Chem. 257*, 5983–5986.

Mitsuya, H. Yarchoan, R., Kageyama, S., & Broder, S. (1991) *FASEB J. 5*, 2369–2381.

Moelling, K., Bolognesi, D. P., Bauer, H., Busen, W., Plassmann, H. W., & Hausen, P. (1991) *Nature 234*, 240–243.

Omer, C. A., & Faras, A. J. (1982) *Cell 30*, 797–805.

Repaske, R., Hartley, J. W., Kavlick, M. F., O'Neill, R. R., & Austin, J. B. (1989) *J. Virol. 63*, 1460–1464.

Resnick, R., Omer, C. A., & Faras, A. J. (1984) *J. Virol. 51*, 813–821.

Sawai, Y., Unno, M., & Tsukada, K. (1978) *Biochem. Biophys. Res. Commun. 84*, 313–321.

Schatz, O., Mous, J., & Le Grice, S. F. J. (1990) *EMBO J. 9*, 1171–1176.

Smith, J. K., Cywinski, A., & Taylor, J. M. (1984) *J. Virol. 49*, 200–204.

Starnes, M. C., & Cheng, Y.-C. (1989) *J. Biol. Chem. 264*, 7073–7077.

Tan, C.-K., Civil, R., Mian, A. M., So, A. G., & Downey, K. M. (1991) *Biochemistry 30*, 4831–4835.

Telesnitsky, A., Blain, S. W., & Goff, S. P. (1992) *J. Virol. 66*, 615–622.

Tisdale, M., Schulze, T., Larder, B. A., & Moelling, K. (1991) *J. Gen. Virol. 72*, 59–66.

Varmus, H., & Swanstrom, R. (1984) in *RNA Tumor Viruses* (Weiss, R., Teich, N., Varmus, H., & Coffin, J., Eds.) 2nd ed., pp 369–512, Cold Spring Harbor Laboratory Publications, Cold Spring Harbor, NY.

Verma, I. M. (1977) *Biochim. Biophys. Acta 473*, 1–38.

Watson, K. F., Schendel, P. L., Rosok, M. J., & Ramsey, L. R. (1979) *Biochemistry 18*, 3210–3219.

Wohrl, B. M., & Moelling, K. (1991) *Biochemistry 29*, 10141–10147.