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INVESTIGATION OF THE STEREOCHEMICAL COURSE OF DNA SYNTHESIS CATALYSED BY HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REVERSE TRANSCRIPTASE

Sam Hopkins+, Phillip A. Furman+, and George R. Painter*

+Divisions of Virology and *Organic Chemistry,
Wellcome Research Laboratories
3030 Cornwallis Road
Research Triangle Park, North Carolina 27709

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The S_P-isomer of thymidine 5'-O-(1-thiotriphosphate) is used as substrate by HIV-1 reverse transcriptase. The absolute configuration of the internucleotide linkage in the oligonucleotide product was identified by ^{31}P NMR spectroscopy to be the R_P-isomer, indicating that incorporation of dTTPαS into the oligonucleotide proceeded with inversion of configuration at the α-phosphorus. The mechanistic implications of these observations are discussed.

The development of 3'-azido-3'-deoxythymidine (Retrovir™, AZT) as the first clinically approved drug for the treatment of HIV-1 infection has focused attention on the virally encoded reverse transcriptase as a primary target for the design of antiviral chemotherapeutic agents (1). The enzyme catalyses DNA synthesis by an ordered mechanism wherein template:primer binding precedes the binding of deoxynucleoside triphosphate (2). The overall mechanism of polymerization occurs in two kinetically distinct phases in which the initial binding of substrates and the first incorporation event are distinguishable from successive rounds of processive DNA synthesis. Purified HIV-1 reverse transcriptase performs many of the same functions associated with other retroviral reverse transcriptases including; 1) processive DNA synthesis to a level comparable with other known replicative polymerases, 2) intermolecular strand switching, consistent with the requirement for the reverse transcriptase to perform strand jumps during replication, and 3) limited strand displacement synthesis (3).

In the present study we have examined the stereochemical course of HIV-1 reverse transcriptase catalyzed DNA polymerization by following the incorporation of dTTPαS into a single-stranded oligodeoxynucleotide. Deoxynucleoside 5'-O-(1-thiotriphosphates) with defined stereochemistry around the α-phosphorus have been used as probes to monitor the stereochemical course of catalysis of E. coli DNA polymerase I (4), T4, and T7 DNA polymerases (5,6), and avian myeloblastosis virus reverse transcriptase (7). In each case, incorporation of the nucleotide analog

The abbreviations used are: dTTPαS, thymidine 5'-O-(1-thiotriphosphate); HEPES, (N-2-Hydroxyethylpiperazine-N'-2-Ethane sulfonic acid; HIV-1, Human Immunodeficiency Virus Type 1; RT, reverse transcriptase.

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proceeded with complete inversion of configuration about the α-phosphorus. The simplest interpretation is that the reaction proceeds by an SN_2 mechanism which involves nucleophilic attack by the 3'-hydroxyl group of the primer terminus on the α-phosphorus of the incoming deoxynucleoside triphosphate without the formation of a covalent enzyme-bound intermediate. Before the role of electronic and stereochemical factors in the design of effective enzyme inhibitors of HIV-1 transcription can be determined, it must be established if HIV-1 reverse transcriptase, like all other DNA polymerases studied to date, proceeds by an SN_2 type reaction mechanism.

MATERIALS AND METHODS

All radioactive materials and authentic S_p-isomer of dTTPαS were purchased from Du Pont Company, Biotechnology Systems, Wilmington, Delaware. Organic solvents, buffers and salts were reagent grade from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin. Polydeoxyribonucleotides, polyribonucleotides and ion-exchange resins were purchased from Pharmacia LKB Biotechnology, Piscataway, New Jersey. Deoxyribonuclease I was a product of Sigma Chemical Company, St. Louis, Missouri. RNase H was purchased from Promega Biological Research Products, Madison, Wisconsin. Avian myeloblastosis virus reverse transcriptase was from Boehringer Mannheim Biochemicals, Indianapolis Indiana. HIV reverse transcriptase was produced in E. coli , and purified by means of immunoaffinity chromatography(8,9). The enzyme was greater than 95% pure as judged by denaturing polyacrylamide gel electrophoresis and consisted of approximately equal molar proportions of p51 and p66.

Synthesis of the diastereomers of dTTPαS: (R,S) dTTPαS was synthesized by the method of Ludwig and Eckstein (10). The material was greater than 99% pure as judged by analytical reverse phase HPLC.

^{31}P NMR data collection: ^{31}P NMR spectra were acquired at 121.42 MHz in a 5-mm tunable, broadband probe on a Varian VXR-300 NMR spectrometer operating in the pulsed Fourier transform mode. Proton decoupling was achieved using the WALTZ pulse sequence (11). The temperature of the probe was maintained at 25 °C by equilibration with purified gaseous nitrogen of the appropriate temperature. ^{31}P chemical shifts are reported relative to external 85% phosphoric acid. Typical ^{31}P acquisition parameters are: spectral window, 10 kHz; 90° pulse width, 7.8 ms; delay between pulses, 1.2 s; 500-600 accumulations.

Synthesis of poly(thymidine 5'-phosphorothioate) by HIV-1 reverse transcriptase: HIV-1 reverse transcriptase (10mg) was added to 1 ml of a solution of 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 6 mM poly(rA)$_{500}$: oligo(dT)$_{10}$ (primer to template ratio = 10:1), and 40 mM (R,S) dTTPαS. The reaction was maintained at 37 °C for 24 h. The extent of incorporation of the analog was monitored in a parallel reaction containing the authentic S_p-isomer of [^{35}S]-dTTPαS. Reaction aliquots were taken at 30 minute intervals and assayed (12). Products from the cold reaction were separated using FPLC Superose-12 gel filtration chromatography with H_2O as the eluent. Fractions containing nucleotides were evaporated to dryness, redissolved in 50mM HEPES buffer (pH 7.5), 5mM $MgCl_2$, 50% D_2O, and analysed by NMR. Fractions containing polynucleotide material were pooled, evaporated to dryness, and was redissolved in 20 mM HEPES buffer (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT. This solution was preincubated at 37 °C for 15 minutes. RNase H (250 units) was added and the mixture was incubated for 4 hours at 37 °C. The extent of RNA digestion was monitored by size exclusion FPLC. After 4 hours the reaction was made 1% in SDS and extracted three times with chloroform: isoamyl alcohol (5:2). Aqueous phase products were separated by FPLC Superose-12 chromotography with H_2O as the eluent. Fractions containing DNA were pooled, evaporated to dryness, dissolved in 50 mM HEPES buffer (pH 7.5), 5 mM $MgCl_2$, 50% D_2O, and analysed by ^{31}P NMR.

Incorporation of [^{35}S] dTTPαS by HIV-1 reverse transcriptase: HIV-1 reverse transcriptase (1mg) was added to a reaction containing 50 mM HEPES buffer (pH 7.5), 5 mM $MgCl_2$, and 20 mg/ml poly $(rA)_{500}$: $oligo(dT)_{16}$ (1:1 molar ratio) in a volume of 100 µl. The R_p- or S_p-isomer of dTTPαS, specific activity 1355 Ci/mMol or 1320 Ci/mMol respectively, was added to a final concentration of 1 mM. Aliquots (5 ml) were removed from the reaction at 10 minute intervals, and analysed for product(12).

Synthesis of poly(thymidine 5'-phosphorothioate) by avian myeloblastosis virus reverse transcriptase: Avian myeloblastosis virus reverse transcriptase (2,000 units) was added to a solution of 0.5 ml containing 50 mM HEPES buffer (pH 7.5), 8 mM $MgCl_2$, 40 mM KCl, 2 mM DTT, 6 mM poly $(rA)_{500}$: $oligo(dT)_{10}$ (primer to template ratio equals10:1), and 40 mM (S) dTTPαS. The reaction was terminated after 15 h at 37 °C. Incorporation of the analog was monitored in a seperate reaction with the authentic S_p-isomer of [^{35}S]-dTTPαS. Aliquots were taken and assayed for product (12). Polynucleotides were isolated and analysed as described previously in the text.

RESULTS AND DISCUSSION

The direct incorporation of dTTPαS into a growing DNA polymer was monitored by following the incorporation of the optically pure [^{35}S]-labeled isomers of dTTPαS into poly $(rA)_{500}$: $oligo(dT)_{10}$. Only the Sp-isomer of dTTPαS was a substrate for the HIV-1 reverse transcriptase (Figure 1). The reactions were followed until all of the S_p-isomer was consumed and then continued for an additional 2 hours. At no time was incorporation of the R_p-isomer detected.

Assignment of the absolute configurations of the isomers of dTTPαS was made by ^{31}P chemical shifts. The a-thiophosphate region of the ^{31}P NMR spectrum of chemically synthesized (R,S)-dTTPαS (Figure 2A) contained two doublets appearing at 45.88 ppm and 45.41 ppm. These resonances were shifted downfield relative to the α-phosphorus resonance of thymidine 5'-triphosphate, which appeared at -10.11 ppm, due to replacement of a non-bridging oxygen with sulfur. Comparison with the ^{31}P NMR spectrum of authentic (S)-dTTPαS (Figure 2B) permited the assignment of the doublet centered at 45.88 ppm to the Sp-isomer and the doublet centered at 45.41 ppm to the R_p isomer. The ^{31}P NMR spectrum of the resolved fraction of unconsumed (R,S) dTTPαS isolated from a reaction containing poly $(rA)_{500}$: $oligo(dT)_{10}$, limiting amounts of (R,S) dTTPαS and HIV-1 reverse transcriptase is shown in Figure 2C. The only detectable alpha phosphorus resonance was

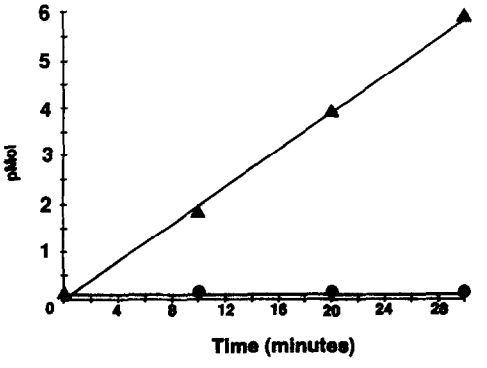

Figure 1. The time course of incorporation of the S_p(▲) and R_p(●) stereoisomers of dTTPαS.

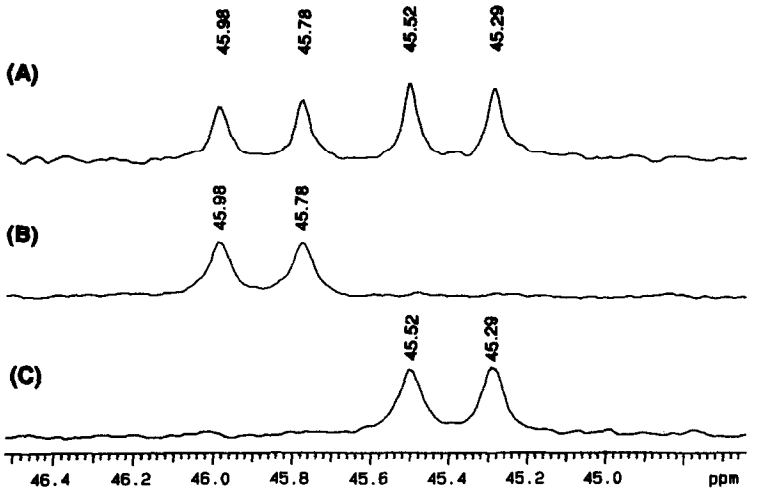

Figure 2. ^{31}P NMR spectra of the a-thiophosphate regions of (A) (R,S)-dTTPαS, (B) the authentic S_p-isomer of dTTPαS and (C) the unconsumed fraction of (R,S)-dTTPαS isolated from a reaction containing HIV-1 reverse transcriptase, poly(rA): oligo(dT), and limiting amounts of (R,S)-dTTPαS. Spectra were obtained in an aqueous solution of 50 mM HEPES buffer (pH 7.5), 5mM $MgCl_2$ containing 50% D_2O.

centered at 45.41 ppm, confirming that only the S_p-isomer was consumed in the reaction.

Identification of the absolute configuration of the phosphorothioate internucleotide linkage of the oligonucleotide product has generally been established by relying upon the preference of snake venom phosphodiesterase to hydrolize polymers with the R_p rather than the S_p configuration (4,5,6,). However, the assignment of the absolute configuration of a phosphorothioate internucleotide linkage can be made on the basis of ^{31}P NMR chemical shift (7). By comparing the ^{31}P NMR chemical shift of the phosphorotrhioate internucleotide linkage formed by avian myeloblastosis virus reverse transcriptase to the ^{31}P NMR chemical shifts of the R_p- and S_p-isomers of chemically synthesized 5'-O-thymidyl 3'-O-thymidyl phosphothioate, Bartlett and Eckstein have shown the ^{31}P NMR chemical shift of the R_p-isomer to be 55.9 ppm and the S_p-isomer to be 55.6 ppm (7). We isolated the polynucleotide product from reactions containing poly(rA):oligo(dT), excess (S) dTTPαS, and either HIV-1 or avian myeloblastosis virus reverse transcriptase and analysed the products by ^{31}P NMR. The spectra were identical with a single downfield resonance at 55.9 ppm.

We have demonstrated that the HIV-1 reverse transcriptase incorporates only the S_p-isomer of dTTPαS, and that the reaction proceeds with inversion of configuration at the α-phosphorus. This suggests that the catalytic process occurs by an in line SN_2 mechanism. Such a mechanism consists of a single nucleophillic attack by the 3'-hydroxyl of the DNA primer terminus on the α-phosphorus of the incomming deoxynucleoside triphosphate without formation of a covalent enzyme-bound intermediete (4,5,6,7). The reaction is proposed to progress through a trigonal bipyramidal transition state complex where the leaving group, pyrophosphate, and the 3'-hydroxyl group of the growing DNA polymer occupy apical positions. Although the results presented here do not exclude the possibility of the reaction proceeding by an SN_1 mechanism with total facial selection, we feel that this is an unlikely alternative.

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