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Incorporation of 4-Thiothymidine Into DNA by the Klenow Fragment and HIV-1 Reverse Transcriptase

Tata Venkata S. Rao, Martin T. Haber, Jane M. Sayer* and Donald M. Jerina

Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

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Abstract—The 5′-triphosphate of 4-thiothymidine (4S–TTP) is an excellent substrate for the Klenow fragment of *Escherichia coli* DNA polymerase I and HIV-1 reverse transcriptase with values of k_{cat}/K_m within a factor of ~3 of those for TTP. A large UV change ($\Delta\epsilon = -9770$ M^{-1} cm^{-1} at 340 nm) associated with incorporation of 4S–TMP into nucleic acid duplexes makes possible a rapid, continuous spectrophotometric assay of the reaction progress. © 2000 Published by Elsevier Science Ltd.

The kinetics of nucleotide incorporation into DNA duplexes have been extensively studied in attempts to understand the mechanism of action of DNA polymerases.[1] Recently, the role of hydrogen bonding and base pairing in the fidelity of DNA polymerization has been debated.[2] A nucleotide containing a difluorotoluene shape analogue of thymine, which lacks the ability to form any of the hydrogen bonds found normally in DNA base pairs, is enzymatically incorporated into DNA with good kinetics and high fidelity.[3]

4-Thiothymidine triphosphate[4] (4S–TTP) is incorporated into DNA by *Escherichia Coli* DNA polymerase I,[5] and synthetic oligonucleotides containing 4S–T have been used as probes of protein–DNA contacts.[6] Substitution of one oxygen of TTP by sulfur to form 4S–TTP may result in modification of one of the two hydrogen bonds found in the product dA-T base pair (Fig. 1), since the 1.68 Å length of the C–S double bond in 4S–T is significantly greater than the 1.23 Å length of the corresponding C–O double bond of thymidine.[7] Incorporation of one or two 4S–T's into a short oligonucleotide duplex has little effect on the melting temperature or circular dichroism spectra, consistent with only minimal effects on duplex DNA structure.[4b] Raman spectroscopy of oligonucleotide duplexes containing 4S–T indicates that the strength of the hydrogen bonds to 4S–T is similar to that of normal Watson–Crick hydrogen bonds.[6b] There is other evidence supporting the existence of N–H···S hydrogen bonds[8] in general, but the exact orientation of the dA-(4S–T) base pair in a modified DNA duplex has not been determined.

Substitution of sulfur for oxygen shifts the UV absorbance maximum from 267 nm for TTP to 335 nm for 4S–TTP, well removed from the background absorbance of DNA. Furthermore, incorporation of 4S–TTP into a DNA template–primer duplex results in significant hypochromicity (Fig. 2), which forms the basis for a convenient, continuous non-radiochemical assay for DNA polymerization.

The observed molar absorbance decrease at 340 nm is $\Delta\epsilon = 9770$ M^{-1} cm^{-1}.[10] Incubations with duplexes designed to incorporate 2, 3 and 4 residues[11] of 4S–T showed that $\Delta\epsilon$ increased linearly with the number of 4S–T residues incorporated with a slope of 9770 ± 150 M^{-1} cm^{-1}. This linearity is consistent with our previously reported results with normal nucleotides.[12] However, the value of the hypochromicity ($\Delta\epsilon$) at 340 nm is much larger than the value of $\Delta\epsilon$ at 275 nm[12] for TTP, where the large background absorbance of the DNA template-primer also interferes with the assay. For 4S–T, the hypochromicity at 340 nm is much larger than its hypochromicity at 260 nm.[6c]

Under *processive* conditions,[1] multiple nucleotides are incorporated into the DNA homopolymer duplex $p(dA)_{40-60}.p(dT)_{20}$. In order to determine the average number of nucleotides incorporated into the product, the template-primer duplex was incubated with 4S–TTP and Klenow fragment until no more UV change was observed. The double-stranded product was isolated by

*Corresponding author. Tel.: +1-301-496-9893; fax: +1-301-402-0008; e-mail: jmsayer@helix.nih.gov

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precipitation with ethanol at 0 °C to remove excess 4S–TTP. Comparison of the product absorbance at 260 and 335 nm allowed calculation of the number of 4S–T residues incorporated. The most notable difference between 4S–TTP and TTP was a low overall incorporation (only 4.7 ± 1.0 nucleotides) of 4S–TTP by intact Klenow fragment. In contrast, an average of approximately 35 thymidine residues are incorporated by intact Klenow fragment into this template-primer, as measured by filter binding assay of ^{3}H-TTP incorporation.[12] (Note that the 20-mer primer presumably binds randomly to template (average length 50 nucleotides), so that the overhang is random unless the primer can change its position on the template.) For 4S–TTP the relative rates of incorporation and exonucleolytic proofreading apparently become approximately equal after incorporation of only a few 4S–T residues. In contrast, the incorporation rate is always much faster than the exonuclease rate for the natural substrate TTP, so that thymidine is incorporated until the end of the homopolymer duplex (or slightly beyond due to slippage). The mechanism resulting in lower incorporation of 4S–T into the duplex homopolymers by intact Klenow fragment is unknown, and may involve a decrease in the incorporation rate or an increase in the exonuclease rate with multiple, contiguous 4S–T residues, or a combination of both effects. With exonuclease-free Klenow fragment, approximately 37 4S–TTP residues were incorporated into DNA after an overnight reaction, comparable to the result with intact Klenow fragment and TTP. In general, a processivity of 20–25[1e] is observed for Klenow fragment depending on the template-primer.

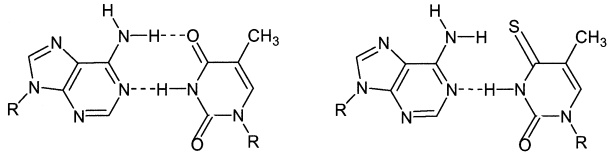

Figure 1. Base paired structures of dA-T and dA-(4S–T).

Kinetic parameters for the *processive* incorporation of 4S–TTP and TTP into the homopolymers are given in Table 1. These results are consistent with the reported k_{cat} for TTP of 3.8 s^{-1} obtained with the homopolymer, poly(dA)$_{1000}$(T)$_{10}$.[1d] Sulfur substitution decreases K_m by 3.6-fold while decreasing k_{cat} only slightly. The specificity constant k_{cat}/K_mshows that under these conditions 4S–TTP is a somewhat better substrate than TTP for Klenow fragment.

Results of the steady-state kinetics measurements under *non-processive* conditions[1] where a single nucleotide is incorporated into a DNA 9/20-mer template-primer by Klenow fragment or into a RNA/DNA template-primer by HIV-1 reverse transcriptase (RT) are given in Table 2. With Klenow fragment, both k_{cat} and K_m increase with 4S–TTP, resulting in no change in k_{cat}/K_m relative to that of TTP. The observed, small increase in K_m may reflect somewhat poorer binding of the altered substrate by the binary complex of enzyme and DNA duplex. The increase in k_{cat} suggests that the rate of dissociation of the oligonucleotide product from the enzyme is increased upon incorporation of 4S–T relative to T, since this dissociation step is known to be rate limiting for the non-processive reaction of the normal nucleoside triphosphates with this same duplex.[1c]

Table 1. Steady-state parameters for DNA polymerization by Klenow fragment under processive conditions with the homopolymer template-primer duplex, p(dA)$_{40-60}$·p(dT)$_{20}$[a]

Substrate	K_m (μM)	k_{cat} (s^{-1})	10^{-5} k_{cat}/K_m (M^{-1} s^{-1})
TTP	5.0	3.0	6.0
4S–TTP	1.4	2.3	15.7

[a]Kinetic measurements were conducted[13] with Klenow fragment using 1 μM p(dA)$_{40-60}$·p(dT)$_{20}$ as template primer. Assays using 0.2–15 μM ^{3}H–TTP (2.0 Ci/mmol) as substrate were done by radiochemical filter binding assay.[15] Assays using 0.5–50 μM 4S–TTP as substrate were conducted by following the change in absorbance at 340 nm in microspectrophotometric cells.

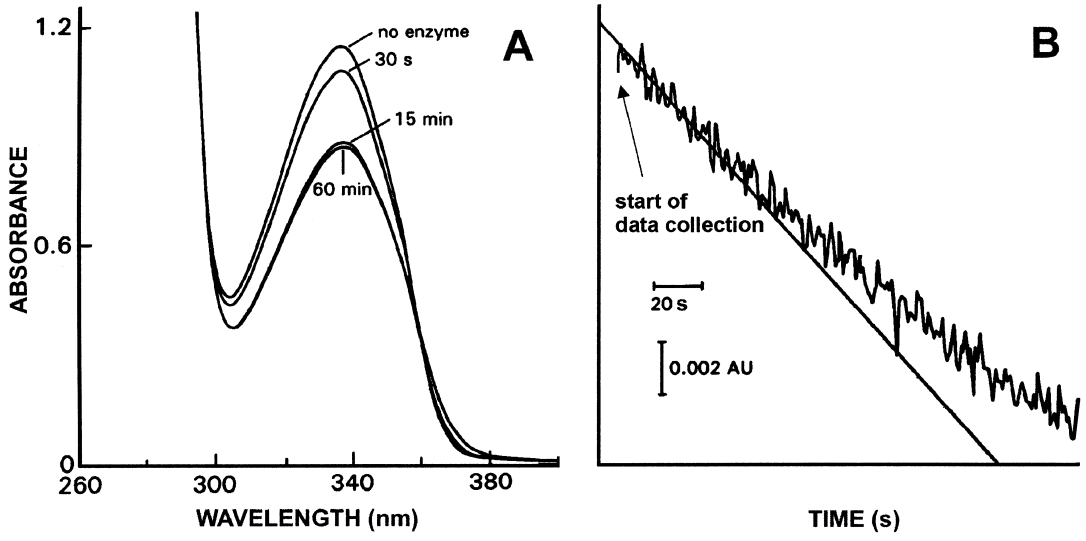

Figure 2. UV spectral change (A) and time course (B) for incorporation of one 4S–TTP into a 9/20-mer DNA duplex[9] by the Klenow fragment. Reaction conditions:[13] (A) 52 μM 4S–TTP, 25 μM duplex and 40 nM exonuclease-free Klenow fragment, and (B) 5 μM 4S–TTP, 10 μM duplex and 10 nM Klenow fragment.

Table 2. Steady-state parameters for DNA polymerization by Klenow fragment and HIV-1 reverse transcriptase (RT) under non-processive conditions with short template-primer duplexes

Enzyme	Substrate	K_m (μM)	k_{cat} (s^{-1})	$10^{-5}\ k_{cat}/K_m$ ($M^{-1}\ s^{-1}$)
Klenow fragment[a]	TTP	0.51	0.37	7.3
Klenow fragment	4S–TTP	1.82	1.73	9.5
HIV-1 RT[b]	TTP	0.36	1.02	28.0
HIV-1 RT	4S–TTP	0.50	0.44	8.8

[a]Spectrophotometric assays with 10 nM Klenow fragment were conducted[13] with 10 μM DNA template-primer[9] and 2.5–75 μM 4S–TTP. Radiochemical assays with 4 nM Klenow fragment were conducted under the same conditions with 10 μM DNA template-primer and 0.2–15 μM ^{3}H-TTP (7.2 Ci/mmol). The rate of thymidine incorporation was determined with the radiochemical filter binding assay.[15]
[b]Spectrophotometric assays with RT were carried out under conditions similar to those described[16] in 50 mM Tris buffer, pH 7.4, containing 50 mM NaCl and 10 mM $MgCl_2$ with 5 μM DNA-9-mer (primer)/RNA-20-mer (5′-AAACCCUUGGACGGCUGCGA-OH, template), 0.25–25 μM 4S–TTP and 14 nM HIV-1 RT. Radiochemical assays were conducted under the same conditions with 5 μM DNA-9-mer/RNA-20-mer, 0.1–20 μM ^{3}H-TTP (18 Ci/mmol) and 1.0 nM HIV-1 RT. HIV-1 RT (MW 110000 Da) consisting of equimolar amounts of subunits p66 and p51 was a gift from Dr. Samuel Wilson.

To investigate further the nature of the rate determining step for incorporation of 4S–TTP, pre-steady-state kinetics of the reaction were measured on a msec time scale.[14] With related DNA duplexes, nucleotide incorporation by the Klenow fragment exhibits a burst[1c,1d] with a rate of 50 s^{-1}, which is much faster than product release. This fast burst step is assigned to a conformational change in the enzyme that occurs after substrate binding and before phosphodiester bond formation, which is even faster.[1f] A second slow conformational change follows the bond-formation step before product release.[1g] The reaction of 4S–TTP showed no burst, and proceeded with an initial incorporation rate of about 2–3 s^{-1}, which is comparable to the rate of turnover in the steady state. A reasonable interpretation is that dissociation of the oligonucleotide product is not appreciably slower than the preceding step(s) in the catalytic process for the Klenow fragment with 4S–TTP as substrate. Thus with 4S–TTP as substrate, there is no evidence for rate limiting dissociation of the oligonucleotide product from the enzyme, and either the conformational changes or bond formation may be at least partially rate limiting.

With HIV-1 RT, comparison of 4S–TTP with TTP indicates little or no change in K_m and a small decrease in k_{cat} with a resultant decrease in k_{cat}/K_m (Table 2). The decrease in k_{cat} suggests a decrease in the dissociation rate for the modified substrate, since product release is rate limiting for non-processive kinetics of TTP with this polymerase also.[17] However, this kinetic scheme may not apply to the 4S–TTP substrate (see above).

In summary, the chromophoric substrate 4S–TTP is well suited for use in a rapid, continuous optical assay to replace slow and time intensive single-point radiochemical assays for polymerase-mediated replication reactions in many applications such as enzyme purification and high throughput screening. Oligonucleotide templates or nucleoside triphosphates containing the fluorescent analogue 2-aminopurine have also been used in continuous DNA polymerase assays.[18] We observed little change (<4-fold for K_m and ⩽3-fold for k_{cat}/K_m) in the steady-state kinetic parameters for enzymatic incorporation of 4S–TTP relative to TTP into a DNA duplexes under both processive and non-processive conditions. This is expected since X-ray crystallographic studies of the polymerase tertiary complex indicate that the polymerase has little interaction with the major groove side of the DNA at the insertion site.[19] The total extent of incorporation of 4–ST into a homopolymer primer by intact Klenow fragment was much lower than that of T. This observation is presumably a result of a slowing of the polymerization relative to the exonuclease rate in the presence of the modified oligonucleotide, since similar extents of incorporation of 4–ST and T were observed with *exo*-Klenow fragment where no exonuclease activity is present. Pre-steady-state kinetics of incorporation of a single nucleotide residue into a synthetic template-primer by Klenow fragment under conditions that give a rapid burst of T incorporation[1c,1d] provided no evidence for such a burst with 4–ST. Thus, in contrast with the normal oligonucleotide, a step other than dissociation of the 4–ST modified oligonucleotide duplex from the enzyme may be rate determining.

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4. (a) 4-Thiothymidine was prepared as described in ref 4b except that the hydroxyl groups were protected as the *tert*-butyldimethylsilyl derivatives rather than the acetates. After deprotection with *tert*-butylammonium fluoride, 4-thiothymidine was converted to the 5′-*O*-triphosphate as described in ref 4c. The product was purified by HPLC on an ion-exchange column (SynchroPak AX100, 21.2×250 mm) eluted with a two step gradient at a flow rate of 8 mL/min. The first step is from 0 to 60% B in 15 min followed by increasing B to 100% in 5 min (solvent A, 0.025 M Et_3N–H_2CO_3, pH 7.5; solvent B, 0.5 M Et_3N–H_2CO_3, pH 7.5). The HPLC peak eluting at 25 min was collected, and the aqueous solution was concentrated

at ambient temperature. Periodically, the solution pH was checked and maintained at 7.5. Methanol was added to the syrup and evaporation continued. The resulting syrup was dissolved in a minimum volume of 0.1 M Et_3N–H_2CO_3, pH 7.5, and chromatographed on a reverse phase Hamilton PRP-1 column (7.0 × 305 mm) by ramping the gradient from 100% A to 100% B in 30 min (solvent A: 0.1 M Et_3N–H_2CO_3; solvent B: 50:50 Et_3N–H_2CO_3:CH_3CN) in order to remove residual inorganic pyrophosphate. The peak eluting at 10 min was collected, and the solvent was carefully evaporated at ambient temperature. The triphosphate was characterized by ^{31}P NMR (D_2O, phosphoric acid external standard): δ −14.8, −2.95 and 2.2 ppm. Purity was confirmed by TLC as described in ref 4c. Concentrations of 4S–TTP were determined spectrophotometrically at 335 nm, $\epsilon = 22\,300\ M^{-1}\ cm^{-1}$ (ref 4d). (b) Connolly, B. A.; Newman, P. C. *Nucleic Acids Res*. **1989**, *17*, 4957. (c) Kovacs, T.; Otvos, L. *Tetrahedron Lett*. **1988**, *29*, 4525. (d) Fox, J. J.; Von Praag, D.; Wempen, I.; Doerr, I .L.; Cheong, L.; Knoll, J. E.; Eidinoff, M. L.; Bendich, A.; Brown, G. B. *J. Am. Chem. Soc*. **1959**, *81*, 178.

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9. Equimolar amounts of the template strand DNA 20-mer (5′-AAACCCTTGGACGGCTGCGA-OH) and primer strand DNA 9-mer (5′-TCGCAGCCG-OH) were mixed and annealed as described in ref 1c. Oligonucleotide concentrations were estimated based on $A_{260\ nm}$ using the program Oligo (National Biosciences, Plymouth, MN).

10. The value of Δε was obtained by incubating 25 μM template-primer (DNA-9-mer/DNA-20-mer) and excess 4S–TTP with exonuclease-free Klenow fragment (Unites States Biochemical) under standard buffer conditions (ref. 13) and allowing the reaction to go to completion. Exonuclease-free Klenow fragment was used since with intact Klenow fragment the total absorbance change was smaller and changes in absorbance were observed to reverse direction with prolonged reaction times, indicating slow hydrolysis of the product.

11. The following three template DNA strands were used: 5′-AAACCCTTGAACGGCTGCGA-OH, 5′-AAACCCTTAAACGGCTGCGA-OH, and 5′-AAACCCTAAAACGGCTGCGA-OH, which when annealed with primer 9-mer in the final template-primer duplex contain sites for incorporation of 2, 3 and 4 (thio)thymidines, respectively, opposite to the underlined A's.

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13. Reactions were conducted in 50 mM Na MOPS buffer (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT and 0.5 mM EGTA, as used for previous kinetic experiments with Klenow fragment and similar template-primer duplexes (ref 1c). Kinetic parameters (25 °C) were obtained by fitting the Michaelis–Menten equation to initial rates measured at several triphosphate concentrations using the program Enzfitter (Biosoft, Cambridge, UK).

14. (a) Stopped-flow spectrometers from Kin-Tek Instruments (ref 14b), with two identical loading syringes (actuated by either nitrogen gas pressure or a stepping motor) leading to a rapid (∼1.6 ms) mixing chamber connected to a stop syringe, were used. The stop chamber was irradiated by a variable wavelength monochromatic light source set to 340 nm and monitored by a photodiode connected to a computer. Syringe A contained 4 μM Klenow fragment and 20 μM DNA-9-mer/DNA-20-mer (ref 9) in MOPS buffer. Syringe B contained 5–60 μM 4S–TTP in MOPS buffer. Data were collected for time periods ranging from 0.1 s to up to 10 s. The results of several runs at each substrate concentration were averaged. (b) Johnson, K. A. *Methods Enzymol*. **1986,** *134*, 677.

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