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Reverse transcriptase incorporation of 1,5-anhydrohexitol nucleotides

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ABSTRACT

Several reverse transcriptases were studied for their ability to accept anhydrohexitol triphosphates, having a conformationally restricted six-membered ring, as substrate for template-directed synthesis of HNA. It was found that AMV, M-MLV, M-MLV (H^-), RAV2 and HIV-1 reverse transcriptases were able to recognise the anhydrohexitol triphosphate as substrate and to efficiently catalyse the incorporation of one non-natural anhydrohexitol nucleotide opposite a natural complementary nucleotide. However, only the dimeric enzymes, the RAV2 and HIV-1 reverse transcriptases, seemed to be able to further extend the primer with another anhydrohexitol building block. Subsequently, several HIV-1 mutants (4×AZT, 4×AZT/L100I, L74V, M184V and K65A) were likewise analysed, resulting in selection of K65A and, in particular, M184V as the most succesful mutant HIV-1 reverse transcriptases capable of elongating a DNA primer with several 1,5-anhydrohexitol adenines in an efficient way. Results of kinetic experiments in the presence of this enzyme revealed that incorporation of one anhydrohexitol nucleotide of adenine or thymine gave an increased (for 1,5-anhydrohexitol-ATP) and a slightly decreased (for 1,5-anhydrohexitol-TTP) K_m value in comparison to that of their natural counterparts. However, no more than four analogues could be inserted under the experimental conditions required for selective incorporation. Investigation of incorporation of the altritol anhydrohexitol nucleotide of adenine in the presence of M184V and Vent (exo^-) DNA polymerase proved that an adjacent hydroxyl group on C3 of 1,5-anhydrohexitol-ATP has a detrimental effect on the substrate activity of the six-ring analogue. These results could be rationalised based on the X-ray structure of HIV-1 reverse transcriptase.

INTRODUCTION

Reverse transcriptases (RT) play a defining role in the retrovirus life cycle. The enzyme is responsible for the synthesis of a double-stranded DNA copy from the single-stranded RNA genome, which is inserted into the host genome to establish the integrated provirus (1). In an effort to obtain detailed structural information about these enzymes and to gain considerable understanding of their biochemical mechanism, a sizeable number of crystal structures of RTs have been determined (2–6). Although these X-ray structures have provided useful information regarding the tertiary folding pattern and general location of the various amino acids, a detailed mapping of the substrate-binding site is still missing. With the exception of the three acidic residues (Asp110, Asp185 and Asp186), which are presumed to interact with the dNTP substrate by binding to the divalent cation required for the polymerase reaction, no other residue has been shown experimentally to participate in the substrate binding function of human immunodeficiency virus type 1 (HIV-1) RT. To expand our knowledge in this field, a lot of mutational studies have been performed (7–15) to gain an insight into the influence of altered amino acid residues on fidelity of DNA synthesis. In particular, residues belonging to the dNTP-binding pocket (amino acids at positions 65, 72, 113, 115, 151, 183, 184 and 219) have been investigated extensively. Since it is, however, not easy to translate structural data into functional properties, an interesting strategy is to focus on the difference in substrate specificity of analogues of natural nucleoside triphosphates between several RTs. In this regard, triphosphate building blocks modified in their base part (16–20), sugar part (21–25) or triphosphate moiety (26) have been studied in primers, templates (18–20,27) or as triphosphate building blocks (17–27) as substrates for HIV-1 (17–25), AMV (16,22,26,27) and M-MLV RTs (16,22). Concerning triphosphate building blocks with an altered base moiety, several researchers (18–20,27,28) established that for most RTs the size and shape complementarity of the base part of a dNTP is responsible for the high fidelity during DNA replication, rather than the ability of dNTPs to hydrogen bond to the template nucleotide. Concerning the sugar part, it is proven, on the one hand, that elongation cannot be accomplished in the absence of a 3′-OH group (22). On the other hand, it has been shown that the presence of a 3′-OH group does not automatically lead to extension. Also, some conformational flexibility is proven to be indispensable for polymerisation (21–25).

Studies of this kind, investigating interactions between the polymerase-binding site and triphoshates, are extremely useful to ascertain the mechanism of substrate binding, providing a better insight into the mechanism of fidelity during information transfer in replication and in disease states. This information

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takes on practical importance as well, since polymerases (such as HIV-1 RT) are targets for a number of useful therapeutic agents. Moreover, identification of the parts of the dNTP molecule that specifically bind to the active center of the enzyme are useful for the design of new and more selective anti-retroviral agents.

The present study investigates the sugar part of dNTPs. In particular, the influence of the ring size of the sugar residue of a triphosphate building block on its binding capacity to the active site of RT is investigated, as well as its ability to be inserted into a DNA hybrid. Hereto, the anhydrohexitol triphosphate analogues of adenine, guanine, cytosine and thymine have been synthesised. These analogues are similar to natural dNTPs having the same base part and flexible triphosphate moiety. The sugar residue, however, consists of a six-membered anhydrohexitol ring conformationally similar to the 3′-*endo* (north) conformation found in rNTPs, in contrast to the 2′-*endo* (south) conformation mainly present in dNTPs. The anhydrohexitol triphosphate building blocks are also sterically larger as well as conformationally locked and can therefore be used to study the influence of conformation and bulkiness of the sugar moiety on the replication process. This information may be used for the design of new anti-HIV agents having a six-membered carbohydrate moiety as the glycon part.

Recently it has been reported that these anhydrohexitol triphosphates can be used as substrates for several DNA polymerases (29). Enzymes belonging to the B family seemed to be more efficient in this capacity. However, no more than two consecutive 1,5-anhydrohexitol nucleotides could be inserted into a DNA primer–template complex in a selective manner. In this study we have investigated whether RTs are more able to accept six-membered nucleoside triphosphates as substrates in DNA-templated HNA synthesis. In addition, the role of a 2′-hydroxyl group in the sugar part of a triphosphate has been investigated by studying the substrate capacity of 1,5-anhydro-2-deoxy-D-altritol nucleoside triphosphates (aNTPs) for RT. In contrast to 1,5-anhydrohexitol nucleoside triphosphates (hNTPs), aNTPs possess an adjacent hydroxyl group analogous to the hydroxyl group at the 2′-position of the sugar residue in rNTPs.

MATERIALS AND METHODS

Chemicals and DNA

The highly purified 2′-(deoxy)ribonucleoside triphosphates used in the reverse transcriptase and DNA polymerase reactions and the Mono Q 5/5 and 10/10 columns were obtained from Pharmacia. DEAE–Sephadex A-25 was purchased from Sigma.

Synthesis of hNTP and aATP

Anhydrohexitol nucleosides (hN) were prepared as previously described (30,31). Their respective monophosphates were obtained by means of the method of Yoshikawa *et al*. (32,33). The triphosphate residues (hNTP) were synthesised as described by Moffat and Khorana (34). Convertion to their sodium salts, HPLC purification and NMR identification were performed according to previously described procedures (29).

The 1,5-anhydro-2-deoxy-D-altritol nucleoside of adenine (aA) was prepared as described (35,36). The triphosphate residue was synthesised from its respective nucleoside in a two-step procedure. First, the monophosphate (aAMP) was formed according to the method of Yoshikawa *et al*. (32,33). Treatment of the nucleotide morpholidate with tributylammonium pyrophosphate yielded the triphosphate (aATP) (34). The triphosphate was than dissolved in H_2O and applied to a Sephadex column (Sephadex A-25 in the HCO_3^- form). A linear gradient of TEAB (0–0.5 M TEAB) was used to elute the product. Following collection of the major triphosphate peak, it was converted to its sodium salt by addition of a 1 M solution of NaI in acetone. The precipitate was washed and dried overnight *in vacuo* over phosphorus pentoxide at room temperature. The identity of the product was confirmed by NMR and HPLC analysis performed as described below. To remove hydrolysed triphosphate products which could be formed during storage of the material, aATP was freshly purified before use on a mono Q 10/10 column (Pharmacia) by preparative ion exchange HPLC. A linear gradient of TEAB (pH 7.5) from 0.2 to 0.65 M over 45 min was used, with a retention time of ~35 min. The overall yield for the isolated aATP was ~40%. ^{31}P NMR (aATP) δ (p.p.m.) (D_2O) –9.70 (d, γ-P), –10.0 (d, α-P), –22.15 (t, β-P). The extinction coefficient ε (λ = 260 nM) of aATP (15000 M^{-1} cm^{-1}) was used to determine the respective concentration.

HPLC

Ion exchange HPLC was performed using an L-6200 A Merck-Hitachi pump with UV monitoring. For hNTP, samples were purified and analysed as described. For aATP, analysis of the samples was achieved on a Mono Q 5/5 column using a linear gradient of TEAB (pH 7.5) from 0.1 to 0.6 M over 40 min at a flow rate of 1.0 ml/min. For preparative purposes, a semi-preparative Mono Q 10/10 column was used with a flow rate of 2 ml/min and a gradient of 0.2 to 0.65 M TEAB over 45 min.

Spectroscopy

The ^{31}P NMR spectra were recorded with a Varian Unity 500 spectrometer with 85% H_3PO_4 in H_2O as internal standard. Samples for NMR spectroscopy were prepared in a non-buffered solution of 99.96% D_2O.

Oligodeoxyribonucleotides

Oligodeoxyribonucleotides were obtained from Eurogentec. The lyophilised oligonucleotides were dissolved in DEPC-treated water and stored at –20°C. Labelling of the primer oligonucleotides at their 5′-end was achieved following standard procedures using T4 polynucleotide kinase (Life Technologies) and [γ-^{32}P]ATP (4500 Ci/mmol, 10 mCi/ml) (ICN).

Reverse transcriptase reactions

Hybridisation was performed by combining 5′-end-labelled primers with their template in a molar ratio of 1:2.5. Hybrids were denaturated by heating at 70°C for 10 min and annealed by slow cooling to room temperature over 2.5 h. Reactions were initiated by adding 3 µl of enzyme dilution to a reaction mixture of 6 µl consisting of hybrid, reaction buffer (supplied with the RTs) and triphosphate building block.

The first series of incorporation experiments were set up to investigate several commercially available RTs for their capacity to insert one, two or three hATP into the hybrids P**1**/T**1**,

P**1**/T**2** and P**1**/T**3** (Fig. 1), respectively. The enzymes considered were the RTs of avian myeloblastis virus (AMV), Moloney murine leukaemia virus [both the wild-type (M-MLV) and the RNase H$^-$ point mutant (M-MLV (H$^-$)] (all provided by Promega) and the RTs of Rous-associated virus 2 (RAV2) and HIV-1 (both from Amersham). A second series of insertion experiments focused on mutant HIV-1 RTs and their ability to incorporate three consecutive 1,5-anhydrohexitol adenine (hA) into hybrid P**1**/T**3**. Hereto, a series of reactions was performed in the presence of each of five enzymes: 4×AZT, 4×AZT/L100I, L74V, K65A and M184V. The final reaction mixtures contained 50 nM hybrid, 100 µM triphosphate building block (dATP, hATP, dGTP, dGTP + dATP or dGTP + hATP) and 0.5 U/µl commercially available RT for the first series of incorporation experiments. For the second series of insertion assays 0.005 U/µl (mutant) HIV-1 RT was used and 100 µM dATP or hATP. The reaction mixtures were incubated at 37°C and aliquots were quenched after 120 min.

Selectivity of incorporation was investigated by annealing primers P**1**, P**2**, P**3** and P**4** to template T**4** (Fig. 1) as described above. A series of 15 µl reactions was performed in the presence of M184V HIV-1 RT. The final mixture contained 50 nM primer–template complex, 10 µM each triphosphate building block (for hybrid P**1**/T**4**, dT, hT, dG, hG, dT + dG, hT + hG, dT + dG + dA, hT + hG + hA, dT + dG + dA + dC or hT + hG + hA + hC; for hybrid P**2**/T**4**, dG, hG, dA, hA, dG + dA, hG + hA, dG + dA + dC, hG + hA + hC, dG + dA + dC + dT or hG + hA + hC + hT; for hybrid P**3**/T**4**, dA, hA, dC, hC, dA + dC, hA + hC, dA + dC + dT or hA + hC + hT; for hybrid P**4**/T**4**, dC, hC, dT, hT, dC + dT or hC + hT) and 0.001 U/µl M184V. The mixture was incubated at 37°C and the reaction was quenched after 60 min.

Incorporation of multiple hN and aN nucleotides

Two series of insertion experiments were performed in the presence of either M184V or Vent (exo$^-$) DNA polymerase and a hybrid consisting of primer P**1** annealed to template T**5**. The final reaction mixture consisted of 50 nM hybrid, 10 or 1000 µM triphosphate building block (dATP, rATP, hATP or aATP) and 0.005 U/µl Vent (exo$^-$) or M184V. Reaction temperature was 55°C for Vent (exo$^-$) DNA polymerase and 37°C for M184V. Reaction times were 10 and 60 min.

Kinetic experiments

Kinetic parameters were derived from a steady-state kinetic assay (37) in the presence of dATP, hATP, dTTP and hTTP. The incorporation experiment was started by mixing M184V dilution with a mixture containing P**3**-T**4** complex (Fig. 1) for kinetic characterisation of dATP or hATP, and P**1**/T**4** complex (Fig. 1) for dTTP and hTTP. The final mixture was composed of 250 nM primer–template complex, 0.0001 U/µl M184V, reaction buffer and various concentrations of triphosphate building block. A concentration range of 0.125–25 µM was used for all four triphosphate building blocks. Reactions were incubated at 37°C. Reaction times were between 1 and 12 min.

Electrophoresis

During enzymatic incorporation assays, samples were removed and reactions were terminated by adding a double volume of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 50 mM EDTA).

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5'  CAGGAAACAGCTATGAC        3'          P1

5'  CAGGAAACAGCTATGACT       3'          P2

5'  CAGGAAACAGCTATGACTG      3'          P3

5'  CAGGAAACAGCTATGACTGA     3'          P4


3'  GTCCTTTGTCGATACTG TCCCC          5'  T1

3'  GTCCTTTGTCGATACTG TTCCC          5'  T2

3'  GTCCTTTGTCGATACTG TTTCC          5'  T3

3'  GTCCTTTGTCGATACTG ACTGAAA AA     5'  T4

3'  GTCCTTTGTCGATACTG TTTTTTT        5'  T5
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Figure 1. Sequences of primers (P) and templates (T) used in the incorporation experiments.

The products were heat denaturated at 70°C for 5 min and separated on a 0.4 mm 20% denaturating polyacrylamide gel, in the presence of 89 mM Tris–borate and 2 M EDTA buffer, pH 8.3, at 2000 V for ~2 h. Visualisation of the polymerised products was performed with a phosphorimager. The amount of radioactivity in the bands representing the respective polymerised products of enzymatic reactions was determined with Optiquant image analysis software (Packard).

RESULTS

Incorporation of hATP into a DNA hybrid and extension by RTs

In a first series of incorporation experiments, five commercially available RTs were investigated for their capacity to accept hNTPs as substrates and incorporate them into a DNA–DNA hybrid. Incorporation assays were performed by determining the insertion of one, two or three hATPs in the presence of each of five RTs and primed (P**1**) templates T**1**, T**2** and T**3**, respectively (Fig. 1). Likewise, further elongation with the natural guanosine triphosphate building block (dGTP) following insertion of the anhydrohexitol nucleotide of adenine was investigated. Table 1 gives quantitative data on the incorporation experiments in the presence of hybrid P**1**/T**1** and P**1**/T**2**. The percentages represent the amount of elongation product in comparison to the total amount of primer originally present in the sample. These results demonstrate that all RTs considered can efficiently incorporate one anhydrohexitol nucleotide with an adenine base moiety. However, the RAV2 and HIV-1 RTs are the only RTs that succeeded in incorporating two (RAV2 RT) or more (HIV-1 RT) hA opposite their natural counterparts in the template. HIV-1 RT seems to be the most error prone, since it succeeded in insertion of three and four consecutive hA, using P**1**/T**1** and P**1**/T**2** as hybrids, respectively, as a result of misincorporation opposite the natural dC building block in the template. Further elongation with a natural nucleotide after this insertion seemed possible for all the considered RTs, except M-MLV (H$^-$) RT using P**1**/T**1** as hybrid. In the presence of P**1**/T**2** further polymerisation was only possible for HIV-1 RT.

Figure 2 shows the incorporation patterns of the RTs considered in the presence of M-MLV (Fig. 2A), M-MLV (H$^-$) (Fig. 2B),

Table 1. Percentage enzymatic incorporation of hATP and further elongation in the presence of hybrids P**1**/T**1** and P**1**/T**2** by different reverse transcriptases

	Hybrid P**1**/T**1**				Hybrid P**1**/T**2**			
	hATP incorporation		hATP + dGTP		hATP incorporation		hATP + dGTP	
M-MLV RT	89%	p + 1	101.1%	p + 5	82.8%	p + 1	68.1%	p + 1
M-MLV (RNase H$^-$) RT	86.7%	p + 1	84.35	p + 1	83.0%	p + 1	79.4%	p + 1
AMV RT	94.9%	p + 1	101.0%	p + 5	94.2%	p + 1	98.3%	p + 1
RAV2 RT	97.4%	p + 1	98.0%	p + 5	94.1%	p + 2	94.4%	p + 2
HIV-1 RT	103.2%	p + 3	101.0%	p + 7	102.7%	p + 4	97.3%	p + 6

The given percentages represent the total amount of extension products in comparison to the original total amount of primer. By how many building blocks the primer was elongated is also indicated.

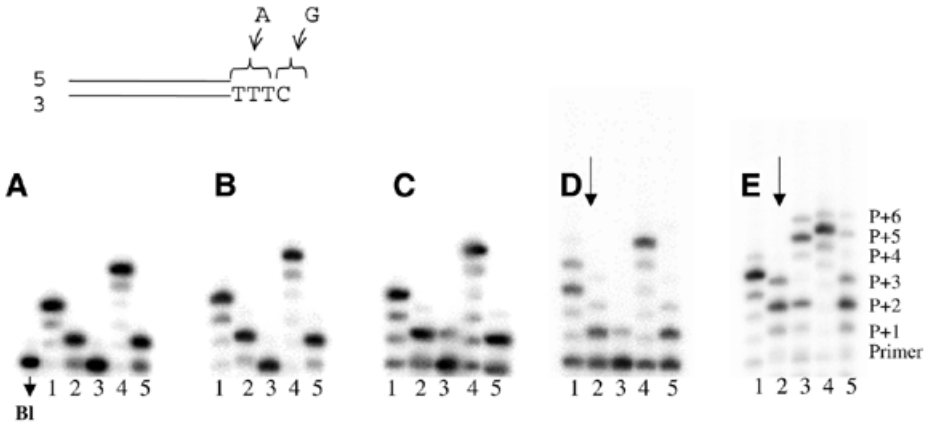

Figure 2. Phosphorimages of the enzymatic incorporation of three hA into 50 nM hybrid P**1**/T**3** in the presence of 0.5 U/µl M-MLV RT (**A**), M-MLV (H$^-$) RT (**B**), AMV RT (**C**), RAV2 RT (**D**) and HIV-1 RT (**E**) and 100 µM NTP: lane 1, dATP; lane 2, hATP; lane 3, dGTP; lane 4, dGTP + dATP; lane 5, dGTP + hATP. Bl, blank reaction in the absence of NTP. The reaction time was 120 min.

AMV (Fig. 2C), RAV2 (Fig. 2D) and HIV-1 (Fig. 2E) RTs in combination with several triphosphate building blocks, including dATP (lane 1), hATP (lane 2), dGTP (lane 3), dGTP + dATP (lane 4) or dGTP + hATP (lane 5). It is shown that M-MLV, M-MLV (H$^-$) and AMV RTs, although efficiently incorporating dA (Fig. 2A–C, lanes 1), only succeed in incorporation of one hA (Fig. 2A–C, lanes 2). The RT of M-MLV proved to have high fidelity since, under the considered reaction conditions, dG is not misincorporated opposite dT in the template (Fig. 2A and B, lanes 3). In the presence of AMV RT this misinsertion is unavoidable (Fig. 2C, lane 3). Considering RAV2 and HIV-1 RTs, an insertion of three hA is seen (Fig. 2D and E, lanes 2) (although very weak for RAV2 RT) relative to incorporation of four building blocks in the presence of dA (Fig. 2D and E, lanes 1) (the last incorporation resulting from misinsertion of dA opposite dC in the template). A relatively small amount of dG is incorporated in the presence of RAV2 RT (Fig. 2D, lane 3), in contrast to full-length extension using HIV-1 RT (Fig. 2E, lane 3). Extension with dG after inserting hA in the DNA–DNA hybrid only seemed possible in the presence of HIV-1 RT (Fig. 2E, lane 5), with a clear pause at positions p + 2 and p + 3. The promising results achieved with HIV-1 RT in the incorporation assay stimulated us to investigate whether HIV-1 RT mutants could be selected that insert three hA consecutively into a DNA hybrid. In this second series of insertion assays, five HIV-1 mutants were considered, investigating incorporation of three triphosphate building block analogues of adenine into hybrid P**1**/T**3**. In Figure 7, the different amino acids, representing the HIV-1 RT mutants, are indicated in their respective positions 67, 70, 215 and 219 (4×AZT) and 100 (4×AZT/L100I), 74 (L74V), 65 (K65A) and 184 (M184V). Results of the incorporation experiments are given in Figure 3. Wild-type HIV-1 RT (wt HIV-1 RT), under the considered reaction conditions (enzyme concentration 100 times lower than in the first series of incorporation assays), is able to insert one hA into hybrid P**1**/T**3** with an efficiency that equals that of insertion of a natural dA. RTs 4×AZT, 4×AZT/L100I and L74V succeed in incorporating a second hA, although with an efficiency that is much below that of a natural dA. RTs K65A and M184V are capable of elongating the primer with three hA. The first two building blocks are incorporated as efficiently as dA. Extension with a third hA is <50% compared to primer extension in the presence of dATP for K65A, in contrast to M184V (primer extension >50% of primer extension in the presence of dATP).

Selectivity tests

To investigate whether hNTPs could be incorporated selectively into a DNA hybrid opposite their natural counterparts, a series of insertion experiments was performed in the presence of each of four hybrids, P**1**/T**4**, P**2**/T**4**, P**3**/T**4** and P**4**/T**4**. Each hybrid was used in four series of incorporation assays in the

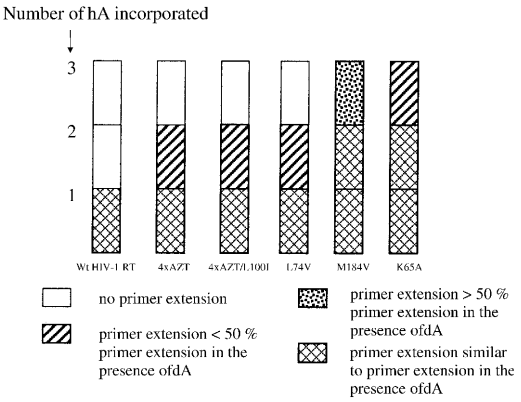

Figure 3. Representation of results of the enzymatic incorporation of three hA into 50 nM hybrid P**1**/T**3** in the presence of 0.005 U/µl wild-type HIV RT and its mutants 4×AZT, 4×AZT/L100I, L74V, M184V and K65A (indicated at the bottom of each bar) and 100 µM NTP: dATP and hATP. The reaction time was 120 min. Each bar is divided into three parts representing the insertion of one, two and three hA, respectively. The filling gives an idea of the relative amount of hA that is incorporated relative to insertion of a natural dA.

presence of different triphosphate building blocks. In this way, insertion of the hN building blocks was investigated in comparison to their natural analogues. The reaction conditions used (as described in Materials and Methods) were a compromise between an acceptable degree of incorporation and the lowest degree of misincorporation for the anhydrohexitol analogues. Figure 4 shows the resulting phosphorimages in the presence of hybrid P**1**/T**4** (Fig. 4A), hybrid P**2**/T**4** (Fig. 4B), hybrid P**3**/T**4** (Fig. 4C) and hybrid P**4**/T**4** (Fig. 4D). When taking a closer look at these pictures, it is seen that, using one triphosphate building block of the anhydrohexitol analogue as substrate, the desired incorporation pattern is seen. There is insertion of the analogue complementary to the natural base in the template (lane 3), however, no incorporation is seen using the wrong substrate (lanes 5, 13 and 15). In contrast, adding dNTP as substrate in some cases resulted in misinsertion (Fig. 4B, lane 2, and Fig. 4D, lanes 2 and 4). When using more than one triphosphate building block in the reaction mixture (lanes 6–11), the desired insertion patterns for hNTPs are observed, however, it seems that the primer could not be elongated by more than four building block analogues. Using dNTPs in the reaction mixture, full-length products could be obtained. However, more misinsertions were seen in comparison to their six-ring analogues (Fig. 4A and C, lanes 6, and Fig. 4A and B, lanes 8). From the picture it is also clear that, despite the fact that M184V is 3′→5′ exonuclease free, degradation of the primer is seen using the wrong substrate in the reaction mixture (Fig. 4A–D, lanes 4–5 and lanes 12–13).

Incorporation of multiple hN and aN nucleotides

We investigated whether elongation of a DNA hybrid with more than three anhydrohexitol triphosphate building blocks could be achieved. In addition, the substrate activity of aATP (Fig. 5) for M184V and Vent (exo⁻) was investigated. Thus, incorporation was performed in the presence of hybrid P**1**/T**5** (Fig. 6) consisting of a template with a seven base overhang of natural thymine building blocks. Figure 6A represents the incorporation pattern of the triphosphate building blocks of adenine in the presence of M184V (lanes 1–4) and Vent (exo⁻) DNA polymerase (lanes 6–9) under conditions allowing selective incorporation of the anhydrohexitol analogues. These conditions were derived in the above mentioned selectivity experiment for M184V and in previously described experiments for Vent (exo⁻) DNA polymerase (29). The picture makes clear that under selective reaction conditions (Fig. 6A), a similar insertion profile is seen for both M184V and Vent (exo⁻). However, under more drastic reaction conditions (Fig. 6B) the incorporation pattern is quite different for the two enzymes. For Vent (exo⁻), incorporation of rA (lane 7) is favoured over hA (lane 8), in contrast to M184V, which prefers hATP (lane 3) as substrate over rATP (lane 2). Neither enzyme seemed to accept the altritol analogue as a good substrate (lanes 4 and 9).

Steady-state kinetic analysis of hATP and hTTP insertion in a DNA hybrid

To gain an idea of the efficiency of incorporation by M184V of one triphosphate building block of the anhydrohexitol analogues of adenine and thymine in comparison to the respective natural nucleotides, the kinetic parameters K_m and V_{max} were determined. This experiment was performed according to the standing start assay described by Boosalis *et al.* (37). The target site was placed at the first position downstream of the 5′-end label of the primer. Quantification of radioactive spots representing polymerised products and remaining primers allowed calculation of the initial velocity of the reaction. Table 2 lists the kinetic parameters (averages of three experiments) for the four triphosphates considered. These data show a decrease in V_{max} values for both hATP and hTTP in comparison to the natural nucleotides. This decrease was more pronounced when thymine was the base. K_m values, on the other hand, increased for hATP and slightly decreased for hTTP as compared to dATP and dTTP, respectively.

DISCUSSION

Results from insertion studies investigating incorporation of hA into hybrids P**1**/T**1**, P**1**/T**2** (Table 1) and P**1**/T**3** (Figs 2 and 3) reveal that all RT considered are able to elongate a DNA primer with one anhydrohexitol building block analogue opposite its natural counterpart in the template. A comparison between the incorporation capacity of different RTs gives valuable information about the fidelity of the RT considered. Based on our results, HIV-1 RT seems to be the most error prone relative to the other RT, since it can incorporate additional hA opposite dC in templates T**1** and T**2** after insertion of one hA. AMV, M-MLV and M-MLV (H⁻) RTs, under the reaction conditions considered, cannot further elongate the DNA primer. This observation supports the generalisation that RTs of lentiviruses (EIAV, HIV-1 and HIV-2 RTs) are usually more error prone in DNA synthesis than other retroviral RTs (7,8), as was also illustrated by Morales and Kool (16). Moreover, it suggests that all RTs, although structurally related, have different characteristics that might explain their divergent capacity to accept analogues as substrates. Consequently, to support this hypothesis each polymerase must be described individually. However, the incorporation experiment in the presence of DNA primer P**1** annealed to T**2** (Table 1), investigating the ability of RTs to elongate the primer with more than one hA

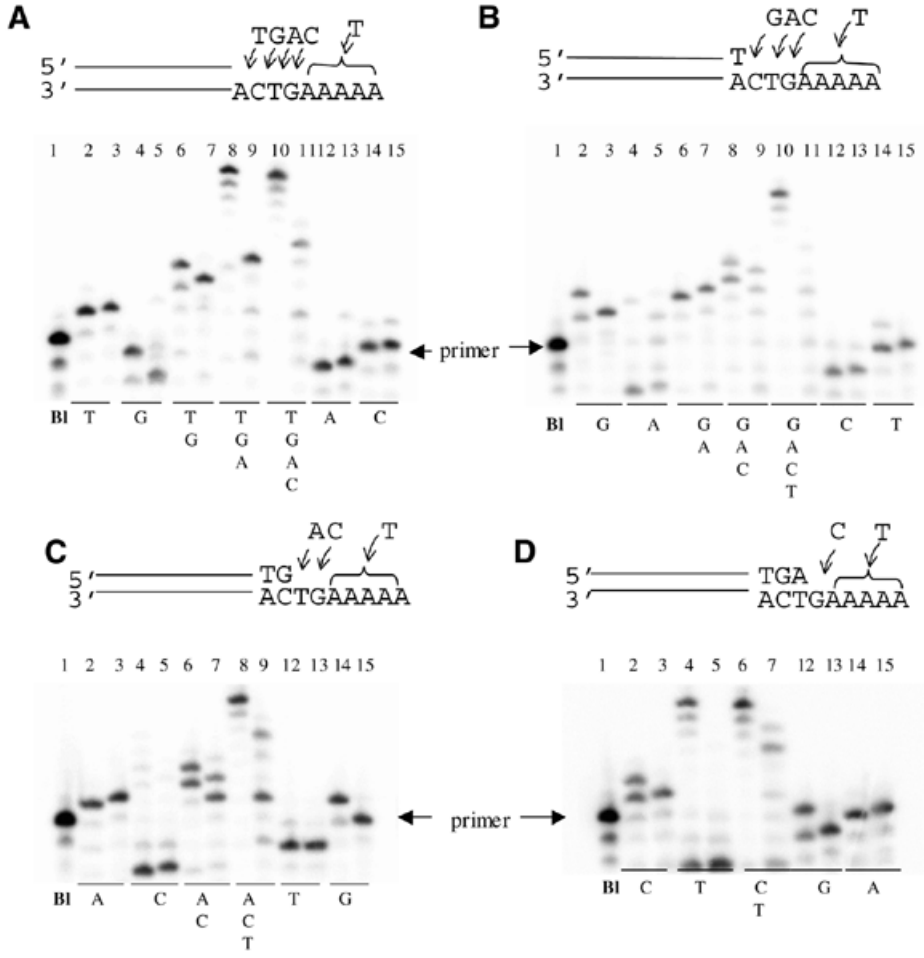

Figure 4. Phosphorimages of selectivity tests in the presence of 0.005 U/µl M184V and 50 nM hybrid P**1**/T**4** (**A**), hybrid P**2**/T**4** (**B**), hybrid P**3**/T**4** (**C**) or hybrid P**4**/T**4** (**D**). The concentration of building blocks is 10 µM. On the bottom of the resulting phosphorimages are indicated the triphosphate building blocks added as substrates. The left lane of the two columns represents the natural triphosphate building block, the right lane its anhydrohexitol analogue. (A) Lane 2, dTTP; lane 3, hTTP; lane 4, dGTP; lane 5, hGTP; lane 6, dTTP + dGTP; lane 7, hTTP + hGTP; lane 8, dTTP + dGTP + dATP; lane 9, hTTP + hGTP + hATP; lane 10, dTTP + dGTP + dATP + dCTP; lane 11, hTTP + hGTP + hATP + hCTP; lane 12, dATP; lane 13, hATP; lane 14, dCTP; lane 15, hCTP. (B) Lane 2, dGTP; lane 3, hGTP; lane 4, dATP; lane 5, hATP; lane 6, dGTP + dATP; lane 7, hGTP + hATP; lane 8, dGTP + dATP + dCTP; lane 9, hGTP + hATP + hCTP; lane 10, dGTP + dATP + dCTP + dTTP; lane 11, hGTP + hATP + hCTP + hTTP; lane 12, dCTP; lane 13, hCTP; lane 14, dTTP; lane 15, hTTP. (C) Lane 2, dATP; lane 3, hATP; lane 4, dCTP; lane 5, hCTP; lane 6, dATP + dCTP; lane 7, hATP + hCTP; lane 8, dATP + dCTP + dTTP; lane 9, hATP + hCTP + hTTP; lane 12, dGTP; lane 13, hGTP; lane 14, dATP; lane 15, hATP. (D) Lane 2, dCTP; lane 3, hCTP; lane 4, dTTP; lane 5, hTTP; lane 6, dCTP + dTTP; lane 7, hCTP + hGTP; lane 12, dGTP; lane 13, hGTP; lane 14, dATP; lane 15, hATP. Lane 1 represents the blank reaction in the absence of NTP and enzyme. The reaction time was 60 min.

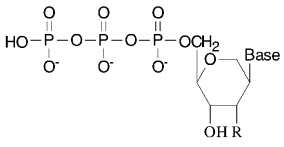

Figure 5. Structures of the anhydrohexitol nucleoside triphosphates (R = H) [base = adenine (hATP), guanine (hGTP), cytosine (hCTP) or thymine (hTTP)] and altritol nucleoside triphosphate (R = OH) of adenine [base = adenine (aATP)].

opposite their natural counterparts, allows us to divide the RTs into two distinct groups. On the one hand, the dimeric enzymes, the RTs of RAV2 and HIV-1, can insert two consecutive hA opposite dT in the template. On the other hand, the monomeric enzymes, the RTs of AMV, M-MLV and M-MLV (H^-), can extend the primer by only one building block analogue. One of the dimeric enzymes, HIV-1 RT, is the only enzyme that inserted three consecutive hA into a DNA hybrid in an efficient way (Fig. 2). Due to the discovery of HIV RT as a causative agent of AIDS in the last decade, an enormous amount of research has been performed to obtain a detailed map of the various amino acids involved in HIV-1 RT fidelity during DNA synthesis (7–15). In particular, much attention has been paid to the dNTP-binding site. To further extend our knowledge about which amino acids in HIV-1 RT are involved in sugar recognition of an incoming dNTP during DNA synthesis, five HIV-1 RT mutants were investigated for their ability to accept hA as a substrate. These investigations might be useful in the design of new nucleoside analogues active against mutated viruses. The M184V mutant of HIV-1 RT, in which Met184 is replaced by valine, resulted in a higher efficiency of incorporation of hA into a DNA hybrid (Fig. 3). Figure 7 represents the polymerase active site of HIV-1 RT complexed with a DNA template–primer duplex and an incoming dTTP (yellow) or

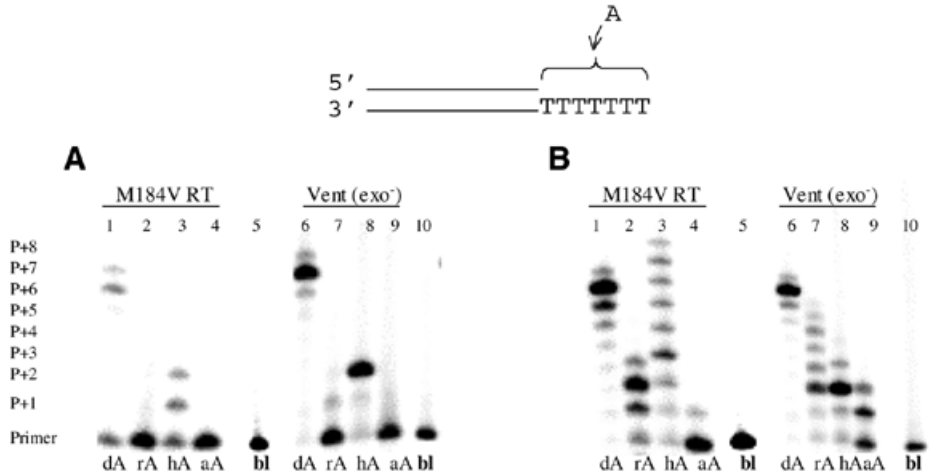

Figure 6. Phosphorimage of the enzymatic incorporation of seven A into 50 nM P**1**/T**5**. Both (A) and (B) represent incorporation patterns in the presence of 0.005 U/µl M184V (lanes 1–4) or Vent (exo⁻) DNA polymerase (lanes 6–9). To clarify the picture, the enzymes considered are indicated at the top and the triphosphate building blocks used as substrates at the bottom of the resulting phosphorimages. (**A**) A concentration of 10 µM NTP was used and the reaction time was 10 min. (**B**) Insertion in the presence of 1000 µM NTP at a reaction time of 60 min. The NTP was dATP (lanes 1 and 6), rATP (lanes 2 and 7), hATP (lanes 3 and 8) or aATP (lanes 4 and 9). Bl, blank reaction in the absence of NTP and enzyme.

Table 2. Kinetic parameters of M184V using a steady-state kinetic analysis of hATP and hTTP in a DNA hybrid

Substrate	V_{max} (% min^{-1})	K_m (µM)
dATP	2.80 ± 0.10	0.23 ± 0.04
hATP	2.17 ± 0.12	2.26 ± 0.50
dTTP	2.30 ± 0.13	0.98 ± 0.26
hTTP	1.27 ± 0.07	0.60 ± 0.16

hTTP (grey). In the figure, the different amino acids representing the HIV-1 RT mutants are indicated in the respective positions 67, 70, 215 and 219 (4×AZT) and 100 (4×AZT/L100I), 74 (L74V), 65 (K65A) and 184 (M184V). As already previously suggested (38,39), this figure further confirms that there is a direct interaction of the amino acid 184 side chain with the sugar moiety of the dNTP substrate. Met184 indeed lies in the vicinity of the sugar residue of an incoming anhydrohexitol. Amino acid 65 is also in the proximity of the sugar part. It is therefore not surprising that a different incorporation pattern of hATP is seen for M184V and K65A in comparison to wild-type HIV RT and the other mutants considered.

As indicated in our kinetic experiments in the presence of M184V, there is a difference in K_m and V_{max} values for hATP and hTTP in comparison to dATP and dTTP, respectively. The K_m values increased (adenine) or decreased slightly (thymine). The major difference between dNTPs and hNTPs is the presence of a rigid six-membered ring for hNTPs with a conformation similar to the 3′-*endo* conformation of an RNA building block. Since in nature RTs use dNTPs, having a 2′-*endo* conformation, as substrate, it is not surprising that a lower affinity of M184V for hATP is observed.

DNA polymerases are thought to catalyse DNA polymerisation by sequential conformational changes in the enzyme structure (40,41). Likewise, HIV-1 RT is considered to undergo a conformational change from the open to the closed state, positioning the nucleotide for phosphodiester bond formation (42). Since hNTPs, however, have a rigid sugar moiety, the change in conformation, necessary for elongation, is hindered, resulting in chain termination. This phenomenon is illustrated in our selectivity experiments, where no further elongation can be achieved after incorporation of four building block analogues. It confirms the observations from other researchers that a certain flexibility in conformation is indispensable for elongation (21,22). Previous selectivity experiments in the presence of Vent (exo⁻) DNA polymerase and hNTPs showed that chain termination was achieved after incorporation of only two building block analogues opposite their natural counterparts in the template under experimental conditions allowing for selective incorporation (29). This observation is in agreement with the common view that the active site of HIV-1 RT, capable of inserting two more hN under similar reaction conditions, is more flexible than those of other polymerases. That there is indeed a difference between RTs and DNA polymerases in their ability to accept triphosphate analogues for DNA replication is also demonstrated by the difference in incorporation pattern obtained in the experiment comparing insertion in a DNA hybrid of dATP, rATP, hATP and aATP in the presence of Vent (exo⁻) DNA polymerase and M184V (Fig. 6). The results for incorporation of aATP are interesting in this regard. The structure and conformation of aATP is similar to that of hATP (35,36). However, the hexitol ring in aATP has an extra hydroxyl group analogous to the OH group at the 2′ position of the sugar residue in rNTPs. No or only weak incorporation could be observed for both enzymes, in contrast to a more pronounced insertion of dATP and hATP under the same reaction conditions. These results suggest that both polymerases are hindered in their polymerisation function by the presence of the adjacent hydroxyl group. A few years ago Astatke *et al*. (43) and Gao *et al*. (44) and very recently Marquez *et al*. (24) mentioned that a single amino acid residue at the active site of the polymerases is considered to be responsible for discrimination against the 2′ residue of an incoming ribonucleotide. For DNA polymerases, this residue has been

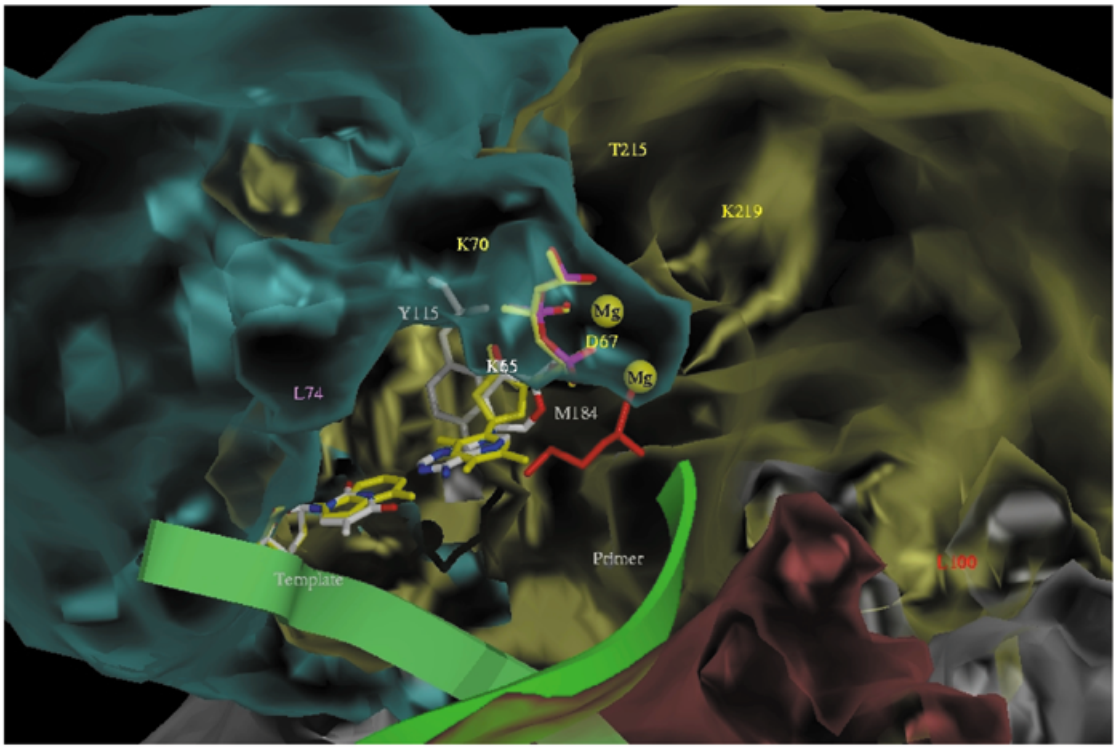

Figure 7. A model for the RT–DNA–hATP complex. The model has been created starting from the RT–DNA–dNTP structure (pdb code 1 RTD) (6). The dNTP:dA base pair (shown in yellow) was replaced by a hA:dT (CPK colours), after which the complex was energy minimised using the Amber software (45). The RT surface is rendered as blue (fingers domain), flesh (thumb domain), cream (palm domain) and white (rest). Amino acids Y115 (grey) and M184 (red), which may interact with the sugar of the entering nucleotide, are highlighted. The positions of other amino acids involved in resistance to some non-nucleoside RT inhibitors are labelled: 4×AZT (yellow), 4×AZT/L100I (red), K65A (white) and L74V (pink). The picture was generated using Bobscript and Raster3D (46–48).

identified in the Klenow fragment as Glu710 (43). For RTs, Phe155 in M-MLV RT (similar to Tyr115 in HIV-1 RT) is assumed to be responsible for this phenomenon. This is illustrated in Figure 7, showing Tyr115 lying in the vicinity of the hydroxyl group on position C_2 (dNTP) or C_3 (hNTP).

That the 2′-OH group, however, is not the only factor responsible for the decrease in efficiency is illustrated by the fact that rATP, in contrast to aATP, can be inserted by M184V (Fig. 6B, lane 2) and Vent (exo$^-$) DNA polymerase (Fig. 6B, lane 7) into the DNA hybrid under extreme reaction conditions. Since aATP and rATP have comparable conformational states it is evident that the decrease in incorporation is due to the locked form of the more bulky six-membered ring, in contrast to the flexible furanose ring in dNTP and rNTP.

CONCLUSION

It has been shown that triphosphate building blocks having an unnatural 1,5-anhydrohexitol ring can be accepted as substrates by all RTs. K_m values, however, indicate a lower affinity of the six-membered ring of the anhydrohexitol analogue for the RT active center when adenine is the base. Moreover, the presence of an adjacent hydroxyl group at the six-membered ring analogous to the hydroxyl group at the 2′ position of the sugar residue in rNTPs proves to be detrimental for substrate activity of the building block. The selectivity assay suggests that the locked form of the more bulky six-membered ring is most probably the reason for chain termination after incorporation of several hexitol nucleotides. Since previously published data in the presence of Vent (exo$^-$) DNA polymerase revealed insertion of only two anhydrohexitol triphosphates into a DNA–DNA hybrid, the hypothesis that RTs possess a ‘looser’ active site in comparison to DNA polymerases is confirmed. The observation of M184V as the most succesful HIV-1 RT mutant for consecutive incorporation of the anhydrohexitol analogues suggests a role of Met184 in HIV-1 RT in the interaction with the sugar part of an incoming triphosphate building block. The present investigations are useful for the design of new anti-HIV agents based on the anhydrohexitol scaffold.

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