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Azidothymidine Triphosphate Is an Inhibitor of Both Human Immunodeficiency Virus Type 1 Reverse Transcriptase and DNA Polymerase Gamma

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The reverse transcriptase from human immunodeficiency virus type 1 was purified from the virus to near homogeneity. The enzyme was shown to possess both RNA-dependent and DNA-dependent DNA-synthesizing activity. Activated DNA as a heteropolymeric substrate was used as efficiently as was the homopolymeric substrate poly(rA)-oligo(dT). The Michaelis-Menten constants were determined for each of the four nucleotides needed to elongate a natural template primer. Azidothymidine triphosphate, a well-known inhibitor of the enzyme, inhibited the enzyme competitively with respect to dTTP and noncompetitively with respect to the other nucleotides. Azidothymidine triphosphate acted as an efficient inhibitor of cellular DNA polymerase gamma, whereas other enzymes of eucaryotic DNA metabolism, namely, DNA polymerase alpha-primase and DNA polymerase beta, were not inhibited. This finding may explain why some acquired immunodeficiency syndrome patients suffer side effects during azidothymidine therapy.

It is now well accepted that human immunodeficiency virus (HIV), formerly called lymphadenopathy-associated virus or human T-cell lymphotropic virus type III (2, 3, 22), is the causative agent of acquired immunodeficiency syndrome. Being a retrovirus, HIV requires reverse transcriptase (RT) to transcribe viral RNA into proviral DNA (28). Therefore, this enzyme represents an ideal target for combatting the virus. During the past 2 years this enzyme has been cloned into expression vectors (5, 9, 11, 15, 16, 20) to (i) obtain sufficient amounts of enzyme for the screening of potential antiviral drugs and (ii) meet safety standards by handling plasmid-containing bacteria instead of hazardous virus. It has also been characterized with respect to its DNA-synthesizing capacity on the artificial homopolymeric substrates poly(rA)-oligo(dT) and poly(rC)-oligo(dG) (9, 29). Although these investigations have already led to the detection of several compounds, e.g., azidothymidine (7, 26, 29) and phosphonoformate (30), which inhibit RT, it appears likely that a test system based upon a heteropolymeric substrate would be of advantage in the search for new and, hopefully, more effective and specific drugs, because such a system would detect not only agents acting as competitors of dTTP and dGTP but also inhibitors competing with the other nucleotides that are incorporated into natural DNA.

In this communication, we describe the development of such an assay, which provides a screening system for the detection of as-yet-unknown inhibitors of RT. Substances found to inhibit RT in this test system can subsequently be evaluated for their inhibitory potential against cellular enzymes of DNA metabolism, namely, DNA polymerases alpha, beta, and gamma and DNA primase. These enzymes are involved in the replication of the cellular genome, repair-type DNA synthesis, replication of mitochondrial DNA, and synthesis of primers to be elongated by DNA polymerase alpha, respectively (6). This assay was also used to determine kinetic parameters for RT to better characterize the enzyme with respect to the action of inhibitors. As a representative inhibitor we used azidothymidine triphosphate (AzTTP), the phosphorylated form of the well-known anti-HIV drug azidothymidine. The results were compared with those obtained with DNA polymerase gamma, an essential cellular enzyme that is also inhibited by AzTTP and other nucleotide analogs.

MATERIALS AND METHODS

Isotopes. All radioactive nucleotides were purchased from Amersham/Buchler (Braunschweig, Federal Republic of Germany).

Nucleotides and substrates. Unlabeled nucleotides were obtained from Boehringer GmbH (Mannheim, Federal Republic of Germany), poly(rA)-oligo(dT) and other homopolymeric substrates were obtained from Pharmacia (Freiburg, Federal Republic of Germany), high-molecular-weight calf thymus DNA was obtained from Sigma (Munich, Federal Republic of Germany), and AzTTP was a kind gift from B. Öberg, Karolinska Institutet, Stockholm, Sweden. Activated DNA was prepared enzymatically from high-molecular-weight calf thymus DNA by the procedure described by Aposhian and Kornberg (1).

Chemicals. All chemicals were obtained in the highest quality available from E. Merck AG (Darmstadt, Federal Republic of Germany).

Columns and chromatographic equipment. A Pharmacia fast liquid protein chromatography system was used for the purification of RT and DNA polymerases. Phenyl-Sepharose, heparin-Sepharose, Mono S, Mono Q, Superose 6, and Superose 12 were obtained from Pharmacia. DEAE-cellulose DE 52 and phosphocellulose P 11 were obtained from Whatman (Maidstone, Great Britain). Hydroxylapatite was prepared as described previously (10).

Enzymes. The protocol for purification of RT was as follows. DNA polymerase alpha and primase were prepared as an enzyme complex from calf thymus essentially as described previously (24). DNA polymerases beta and gamma were purified from calf thymus by the procedure of Matsukage et al. (17) with minor modifications. By using

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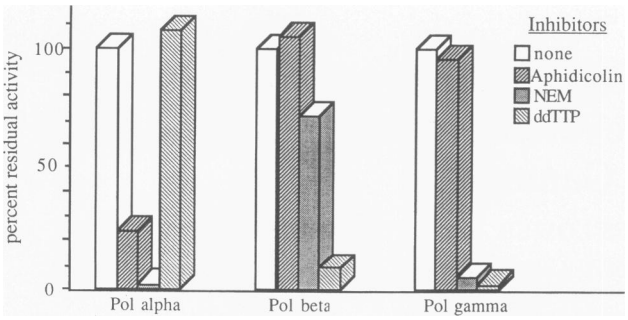

FIG. 1. Discrimination of the eucaryotic DNA polymerases. Purified enzymes (10 mU each) were incubated in the presence or absence of the inhibitors *N*-ethylmaleimide (NEM) (5 mM), ddTTP (50 μM), and aphidicolin (50 μg/ml) under DNA synthesis conditions. These inhibitors at their respective concentrations have been shown to allow discrimination of the different DNA polymerases (6): DNA polymerase alpha is sensitive to aphidicolin and NEM but relatively resistant to ddTTP, DNA polymerase beta is sensitive to ddTTP (although less so than DNA polymerase gamma) and relatively resistant to NEM and aphidicolin, and DNA polymerase gamma is very sensitive to both ddTTP and NEM but insensitive to aphidicolin.

well known inhibitors of the respective DNA polymerases, we were able to demonstrate that each DNA polymerase is well separated from the other enzymes (Fig. 1). DNase I (used to activate DNA) was purchased from Boehringer.

Purification of HIV-1 RT. For the purification of HIV type 1 (HIV-1) RT, 10 mg of density gradient-purified HIV-1 propagated in Molt 4/8 cells was suspended in 20 ml of ice-cold TADE buffer (20 mM Tris-acetate [pH 7.6], 1 mM dithiothreitol, 0.1 mM EDTA). All of the following steps were carried out at 2 to 4°C. Lysis of the virus was achieved by adding Nonidet P-40 (NP-40) and CHAPS {3-[(3-cholamidopropyl)dimethyl-ammonio]1-propanesulfonate} to 0.5 and 0.2% final concentrations, respectively, and adjusting the suspension to 1 M KCl by adding a 2.5 M KCl solution. After 15 min on ice with occasional mixing, the suspension was centrifuged for 1 h at 100,000 × *g*. The supernatant was saved, and the pellet was suspended in 10 ml of TADE buffer with the aid of a Dounce homogenizer and reextracted as described above.

The combined supernatants were filtered through a 0.2-μm-pore-diameter filter and loaded onto a 20-ml hydroxylapatite column previously equilibrated with TADE buffer containing 1 M KCl. After the column was washed with TADE buffer, proteins were eluted by applying a 200-ml gradient of 0 to 300 mM potassium phosphate (pH 7.6) in TADE buffer. Fractions containing RT with at least 15% of the activity of the peak fraction were combined and adjusted to 1 M potassium phosphate (pH 7.6). This solution was loaded directly onto a phenyl-Sepharose column (10 ml) previously equilibrated with TADE buffer containing 1 M potassium phosphate. The column was washed with TADE buffer, and RT was eluted with 1% NP-40 in TADE buffer (pH 8). RT-active fractions were pooled and passed through a 1-ml Mono S column previously equilibrated with TADE buffer (pH 8). The flowthrough containing RT was adjusted to pH 8.8 by adding 2 M Tris and applied to a 1-ml Mono Q column equilibrated with this buffer. After the column was washed with TADE buffer (pH 8.8), the enzyme was eluted with a 30-ml gradient of 0 to 500 mM potassium acetate in TADE buffer (pH 8.8) containing 10% glycerol. Peak fractions were combined, frozen in small aliquots in liquid nitrogen, and finally stored at −70°C. The enzyme thus prepared and stored is stable for at least 1 year without a significant loss in enzyme activity.

Enzyme assays. Optimal assay conditions for HIV-1 RT with the homopolymer poly(rA)-oligo(dT) were found to differ from those described for HIV-1 by other investigators (8) in that a pH optimum of 7.6 was reproducibly observed by us. In brief, enzyme activity was determined at 37°C in a total volume of 100 μl with final concentrations as follows: 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 7.6), 1 mM dithiothreitol, 0.1% NP-40, 5 mM magnesium acetate, 150 mM potassium acetate, 100 μg of bovine serum albumin per ml, 5 μM [^{3}H]dTTP (7,000 cpm/pmol); 10 μg of template primer per ml, and enzyme (the amount of which varied depending upon the experiment [see legends to figures]).

DNA polymerase alpha-primase was measured in a coupled reaction with single-stranded circular phage DNA as the template as described previously (13). DNA polymerase beta and DNA polymerase gamma were measured with activated DNA as the template as described previously (19, 34).

Unless stated otherwise, incubation periods were 30 min, after which the reactions were stopped by the addition of 3 ml of 10% trichloroacetic acid containing 20 mM sodium PP_i. After 10 min on ice, precipitates were collected on Whatman GF/C filters. The filters were washed seven times each with 3 ml of 10% trichloroacetic acid–20 mM sodium PP_i and two times each with 5 ml of 70% ethanol and then dried. Incorporated radioactivity was measured with a toluene-based scintillation fluid.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (14). Gels were stained with either Coomassie brilliant blue or silver by the procedure of Sammons et al. (25).

RESULTS

Because published procedures for the purification of RT turned out to be relatively ineffective in our experiments for obtaining a homogeneous enzyme preparation, we developed the purification scheme described in Materials and Methods. Starting with 10 mg of virus, our final yield was 50 μg of RT with a specific activity of 7,800 U/mg (one unit is defined as the amount of enzyme necessary to synthesize 1 nmol of DNA as nucleotides in 1 h). The enzyme preparation was essentially homogeneous in a silver-stained gel (Fig. 2).

To improve already existing screening systems for drugs able to inhibit retroviral RTs, especially the HIV-1 enzyme (7, 26, 33), we concentrated on finding conditions under which RT efficiently uses an inexpensive and readily available heteropolymeric substrate, such as activated DNA.

Optimal reaction conditions for DNA synthesis by RT. Starting from the assay conditions found to be optimal with poly(rA)-oligo(dT) as the template primer in our preliminary work (described in Materials and Methods), we introduced the modifications detailed below to optimize DNA synthesis with activated DNA as the template primer. To allow comparison with the results obtained with poly(rA)-oligo(dT) as the template primer, we kept concentrations of the labeled nucleotide (dTTP) and the template primer constant, whereas we adjusted the concentrations of the unlabeled nucleotides in the reactions with heteropolymeric substrate to 100 μM. Our first attempts with DNA activated by sonification, as is commonly performed to prepare template primers, yielded results similar to those reported by others, namely, very low enzyme activity in comparison with that obtained with poly(rA)-oligo(dT) as the template primer (26).

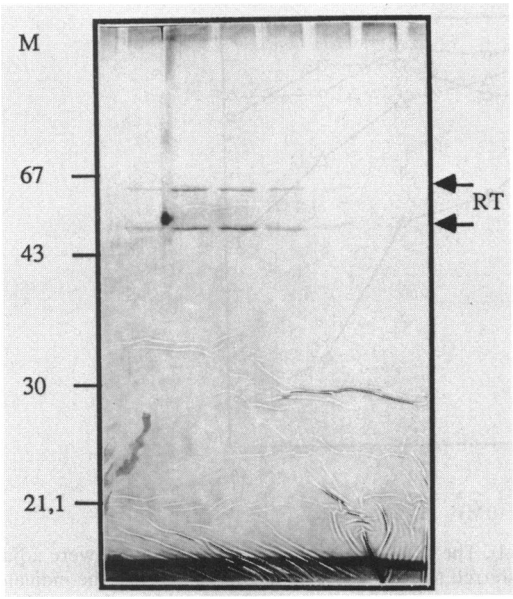

FIG. 2. Gel electrophoretic analysis of purified RT. Five percent of each fraction active in RT assays after Mono Q chromatography were prepared for gel electrophoresis by the method of Laemmli (14) with one exception, namely, mercaptoethanol was omitted to avoid artifacts in the molecular mass range of 50 to 70 kilodaltons during the staining procedures (27). The proteins were run through a 10% gel and subsequently stained with silver (25). In a parallel slot proteins with known molecular masses were coelectrophoresed to serve as markers (M). Numbers at left are in kilodaltons.

Therefore, the DNA was subsequently activated enzymatically by the procedure described by Aposhian and Kornberg (1), which resulted in a template primer that was readily utilized by RT. Gel electrophoretic analysis of this activated DNA showed the average length of the template primer to be 1,000 to 4,000 base pairs. After reexamining all parameters of the assay conditions, we found that raising the salt concentration from 150 to 225 mM potassium acetate resulted in an apparent twofold stimulation of enzyme activity. This activity could be further enhanced twofold by exchanging Tris (pH 7.6) for HEPES (pH 7.6) and simultaneously raising the buffer concentration from 50 to 140 mM. In addition, reducing the magnesium concentration from 5 to 2 mM resulted in an approximately 50% stimulation of enzyme activity. Changing the other components had no significant effect upon the enzyme activity. Figure 3 illustrates a comparison of the enzyme activities with poly(rA)-oligo(dT) and activated DNA under conditions optimal for DNA synthesis with the respective substrates. Under the assay conditions described above, RT replicated the heteropolymeric substrate slightly better than the homopolymeric one. In summary, assay conditions that resulted in an approximately 100-fold stimulation of enzyme activity with activated DNA as the template primer were developed.

Similarity of DNA synthesis from activated DNA to that from the natural template. To demonstrate a correlation between our artificial substrate and the authentic substrate of RT, viral RNA, we performed the experiments shown in Fig. 4. The inhibitory action of AzTTP was compared in endogenous and exogenous systems. The endogenous system used permeabilized HIV-1 as the source of both RT and template (viral RNA). The exogenous system used purified RT and an activated DNA substrate. It should be noted that in this particular experiment the dTTP concentration was

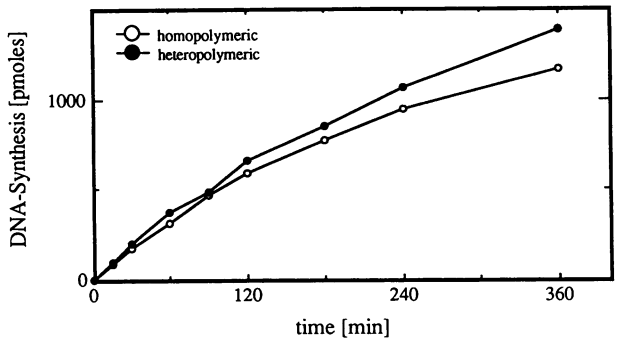

FIG. 3. Comparison of DNA synthesis by RT on homopolymeric and heteropolymeric substrates. Three U of RT was incubated for 6 h with poly(rA)-oligo(dT) and with activated DNA in a total volume of 1,000 μl under conditions found to be optimal for synthesis on the individual template primers. At the times indicated, aliquots were removed and processed for the determination of acid-insoluble radioactivity.

reduced in both reactions to 1 μM to obtain sufficient incorporation of radioactivity in the endogenous reaction. The AzTTP concentrations necessary to reduce the activity of RT by 50% were 0.06 μM in the endogenous reaction and 0.12 μM in the exogenous reaction.

Characterization of DNA synthesis catalyzed by RT. The assay conditions described above were used to characterize the enzyme with respect to Michaelis-Menten constants and sensitivity to the well-known inhibitor AzTTP. Therefore, this assay is useful not only for screening purposes but also as a tool for characterizing the enzyme. Furthermore, the results were compared with those obtained with DNA polymerase gamma and, in some cases, with DNA polymerase

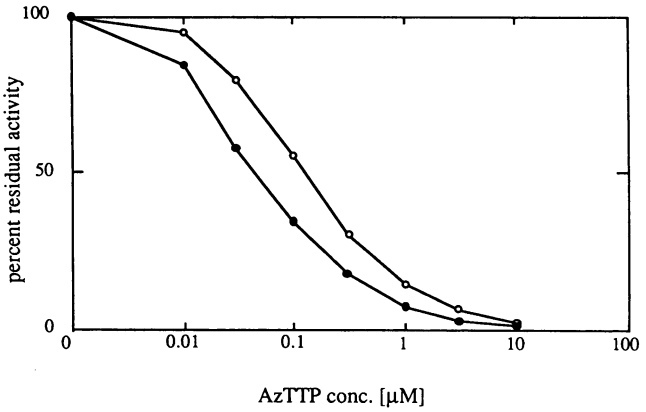

FIG. 4. Inhibition of RT activity on natural and artificial templates by AzTTP. Gradient-purified HIV-1 (10 μg) was suspended in 100 μl of TADE buffer on ice and lysed by the addition of NP-40 (final concentration, 1%). This mixture was diluted to 450 μl with 1 mM dithiothreitol and divided into nine aliquots. Concentrated stock solutions were added to yield the assay conditions found to be optimal for DNA synthesis with the heteropolymeric substrate (140 mM Tris [pH 7.6], 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 2 mM magnesium acetate, 225 mM potassium acetate, 0.1% NP-40, 100 μM each dATP, dCTP, and dGTP, 1 μM [^{3}H]dTTP). AzTTP was added to the individual mixtures to yield the indicated final concentrations (conc.). After 1 h at 37°C, samples were processed for the determination of acid-precipitable radioactivity. Similarly, instead of virus, purified RT (1 U) and activated DNA (10 μg) (○) were adjusted to the above-mentioned assay conditions and processed as described for virus.

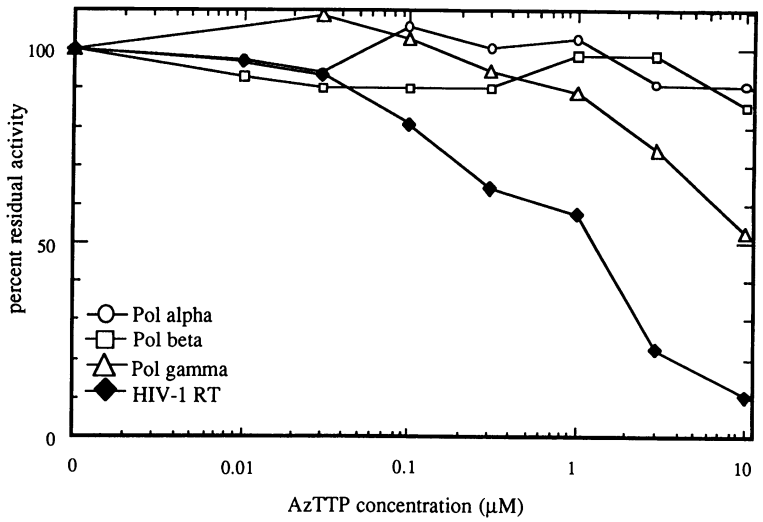

FIG. 5. Influence of AzTTP on RT and on eucaryotic DNA polymerases (pol). The respective enzymes (100 mU each) were adjusted to the individual assay conditions as described in Materials and Methods and transferred to tubes containing AzTTP to yield the indicated final concentrations of the inhibitor in a total volume of 100 μl. After 30 min at 37°C, the reactions were stopped and acid-insoluble radioactivity was determined.

alpha-primase and DNA polymerase beta. AzTTP has been shown to be a potent inhibitor of RT but does not affect DNA polymerase alpha or beta significantly (18, 26). These results were confirmed, but in addition it was observed that DNA polymerase gamma was also severely inhibited by AzTTP (Fig. 5). In this experiment a concentration of about 1 μM AzTTP was needed to inhibit RT by 50%, as compared with the 10-fold-higher concentration required to inhibit DNA polymerase gamma to the same extent.

Determination of kinetic constants for all four nucleotides. When activated DNA was used as the template primer, the influence of increasing concentrations of the four individual deoxynucleotides on the elongation of primers by RT was investigated in both the presence and the absence of AzTTP. The results obtained are shown in Fig. 6 as Lineweaver-Burk plots. In this double-reciprocal plot one can easily discriminate between competitive and noncompetitive inhibitors, because a competitive inhibitor will change the K_m, whereas a noncompetitive inhibitor will change the V_{max}. Thus, not only was it possible to determine kinetic constants for all four substrates but also it could be shown that AzTTP acts as a competitive inhibitor with respect to dTTP, as has already been shown by others with poly(rA)-oligo(dT) as the template primer (18, 26). In contrast, AzTTP acts as a noncompetitive inhibitor with respect to dATP, dCTP, and dGTP. This result was expected from a drug acting as both a nucleotide analog and a chain terminator.

DISCUSSION

In the search for drugs potentially active against HIV-1 RT and the corresponding enzymes of other retroviruses, a screening system that is both easy to handle and capable of detecting all possible inhibitors of the enzyme is needed. The first requirement has been met by a number of laboratories by developing expression vectors that allow the production and purification of large amounts of enzyme (5, 9, 11, 15, 16, 20). Our intention was to establish a screening system that would allow the detection of all inhibitors of the enzyme regardless of inhibitor structure. Such a system would be of considerable advantage because it would allow the detection of inhibitors other than thymidine derivatives, which can be tested in standard assays with poly(rA)-oligo(dT). One must keep in mind not only that chemically synthesized nucleotide analogs can act as inhibitors of RT but also that there are several examples of highly specific enzyme inhibitors derived from natural sources, so there may be highly specific and effective natural inhibitors of RT that await detection. Aphidicolin, a specific inhibitor of DNA polymerase alpha (21), is an example of an inhibitor provided by naturally occurring organisms, e.g., fungi. Such substances are not necessarily thymidine derivatives and would obviously not be detected in a screening system with poly(rA)-oligo(dT) as the template primer.

Using the assay conditions described in this communication, one can avoid the limitations mentioned above because this test system is based on the use of a heteropolymeric substrate. It has recently been reported that RT can use singly primed closed circular phage DNA as a template (32). While in that communication the phage DNA was used to determine the fidelity of RT, this template primer seems to be less suitable in a screening system for antiviral drugs because it reduces DNA synthesis to only 20% as compared with synthesis with poly(rA)-oligo(dT). Furthermore, phage DNA is not as cheap and readily available as activated DNA.

RNA is the first substrate to be transcribed into DNA during the replication of retroviruses; the resulting single-stranded DNA serves as a template for the second strand before the viral DNA can be integrated into the host cell genome (28). In both steps deoxynucleotides are incorporated into the growing DNA chain and, therefore, nucleotide analogs are expected to inhibit the enzyme by the same mechanism with both RNA and DNA as template primers. When AzTTP was used as an inhibitor of RT, a good correlation between the natural template of this enzyme and our artificial calf thymus-derived DNA template was observed, as the inhibitor concentrations needed to inhibit the enzyme by 50% differed only by a factor of two. This difference may possibly be explained by different primer concentrations in the respective assays.

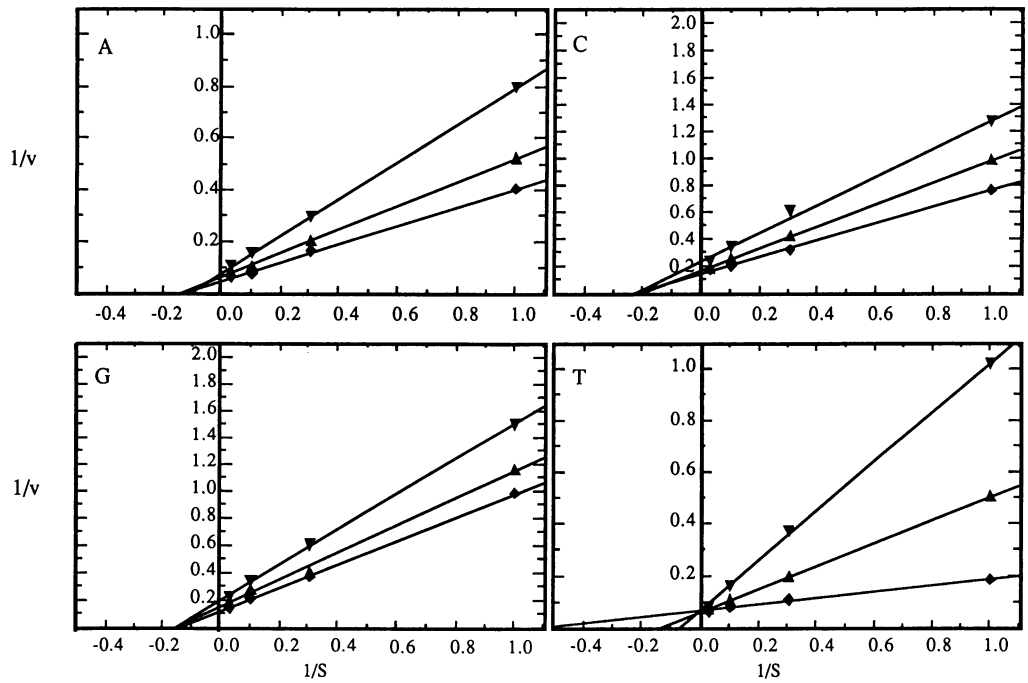

FIG. 6. Apparent K_m values and the mode of action of AzTTP. RT (10 mU) was incubated under DNA synthesis conditions as described in the text with the following differences. The concentration of the radioactive nucleotide was varied as follows: 1, 3, 10, and 33 μM dATP (A), dCTP (C), dGTP (G), and dTTP (T), respectively. The concentrations of the unlabeled nucleotides were kept constant at 100 μM. All these reactions were done in triplicate to allow the addition of different concentrations of AzTTP (0 [◆], 2.5 [▲], and 10 [▼] μM in the case of dATP, dCTP, and dGTP; 0 [◆], 0.25 [▲], and 1 [▼] μM in the case of dTTP). After 30 min at 37°C, the reactions were stopped and samples were processed for the determination of incorporation of radioactivity. The results are presented as double-reciprocal plots to allow an easy estimation of the apparent K_m values and simultaneously to allow discrimination between competitive and noncompetitive inhibition. v, Velocity; S, substrate concentration.

In previous investigations with poly(rA)-oligo(dT) and poly(rC)-oligo(dG), HIV-1 RT was partially characterized, e.g., with respect to the Michaelis-Menten constant for dTTP (26). After optimizing the assay conditions for the efficient replication of a heteropolymeric template primer, we were able not only to determine the corresponding kinetic parameters for all four nucleotides but also to analyze the mode of action of AzTTP in more detail. We found that the Michaelis-Menten constant for dTTP corresponds well with the values reported by others (26). For dATP, dCTP, and dGTP these constants were calculated from the results illustrated in Fig. 5 to be 5.8, 4.1, and 6.2 μM, respectively. Similarly, we determined the mode of inhibition by AzTTP to be competitive with respect to dTTP and noncompetitive with respect to the other three nucleotides. Obviously, these determinations could not be performed with poly(rA)-oligo(dT), because this template primer allows only the incorporation of dTMP. Thus, AzTTP acts both as a competitive inhibitor with respect to dTTP and as a chain terminator with respect to the other nucleotides.

In summary, we describe a test system which not only appears to be suitable for the screening of substances potentially active against HIV RT and other retroviruses but also provides the opportunity to characterize the mode of action of these enzymes more precisely than the commonly used test system with homopolymeric template primers. While it has already been demonstrated that AzTTP has no detrimental effect on the activity of purified DNA polymerases alpha and beta (7, 18, 26), we extended these investigations to show that DNA primase, which circumstantially uses deoxynucleotides for the synthesis of primers (12), is likewise not inhibited by AzTTP, whereas DNA polymerase gamma is readily inhibited at concentrations which are only 10-fold higher than those needed to inhibit RT. This result was not unexpected, because it is well documented in the literature that of the eucaryotic DNA polymerases, DNA polymerase gamma is most sensitive to inhibition by dideoxynucleotides (4, 6, 31; see also Fig. 1). These nucleotide analogs lack a 3′ OH group on their sugar residue, are therefore assumed to act as chain terminators, and thus are very likely to act by a mechanism similar to that of AzTTP.

It is tempting to speculate that several side effects observed in acquired immunodeficiency syndrome patients treated with azidothymidine (23) are due at least in part to the inhibition of the vital cellular DNA polymerase gamma. As mentioned above, this particular enzyme is responsible for the replication of the mitochondrial genome and, therefore, is needed to maintain a sufficient level of mitochondria in growing cells, e.g., during hematopoiesis and in epithelial cells like those in the digestive tract.

Now that the optimal assay conditions for DNA synthesis by HIV RT on a heteropolymeric substrate have been established, it is possible that additional characteristics of the DNA-synthesizing capacity of the enzyme, e.g., processivity and synthesis on a template with a secondary structure, can be determined. These questions are currently under investigation in our laboratory.

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