Cardiac Mitochondrial DNA Polymerase-γ Is Inhibited Competitively and Noncompetitively by Phosphorylated Zidovudine

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Abstract Zidovudine (azidothymidine [AZT]) inhibits human immunodeficiency virus replication and reduces the severity of acquired immunodeficiency syndrome. A limiting side effect of AZT is a mitochondrial cardiac and skeletal myopathy in which the pharmacologically active derivative of AZT (AZT triphosphate) plays a critical role. The present study determined biochemical mechanisms of AZT-induced mitochondrial toxicity and identified AZT triphosphate as an inhibitor of DNA polymerase-γ in vitro. Inhibition kinetics were defined using purified bovine cardiac mitochondrial DNA polymerase-γ and AZT triphosphate in vitro. The Kᵣ for deoxythymidine triphosphate was 0.8±0.3 μmol/L. AZT triphosphate incubation with DNA polymerase-γ in vitro resulted in mixed kinetics with a competitive Kᵣ of 1.8±0.2 μmol/L and a noncompetitive Kᵣ' of 6.8±1.7 μmol/L. These Kᵣ and Kᵣ' values were strikingly higher than values for retroviral reverse transcriptase but lower than values for other cellular DNA polymerases. These data support previous molecular and morphological findings in clinical AZT mitochondrial myopathy and in models of AZT myopathy in vivo. Biochemical findings suggest that inhibition of mitochondrial DNA polymerase-γ may be integral to the pathogenesis of AZT-induced myopathy.

Zidovudine (azidothymidine [AZT]), 2',3'-dideoxythymidine, and other dideoxynucleosides are useful in the treatment of acquired immunodeficiency syndrome (AIDS) because they interfere with action of reverse transcriptase (RT) of human immunodeficiency virus-1 (HIV) and inhibit retroviral replication. Pharmacologically, AZT is phosphorylated intracellularly to AZT triphosphate (AZTTP) in three successive steps. AZTTP interferes with the action of HIV RT either by competing with deoxythymidine triphosphate (dTTP) for the active site of HIV RT or by chain termination of DNA, but both mechanisms may be operative.

Limiting side effects of AZT therapy in AIDS patients include bone marrow toxicity and skeletal myopathy. Dalakas et al reported a zidovudine-induced mitochondrial myopathy. Molecular studies revealed decreased skeletal muscle mitochondrial DNA in DNA extracts of muscle biopsies from AZT-treated AIDS patients. Clinical evidence suggests that AZT myopathy develops slowly and cumulatively with respect to AZT treatment.

A postulated mechanism for the AZT-induced mitochondrial myopathy suggested that AZTTP interfered with mitochondrial DNA polymerase-γ (DNA pol-γ), the enzyme for mitochondrial DNA replication. Studies here offered biochemical evidence in vitro to define subcellular events in the pathogenesis of AZT toxicity and related this to altered mitochondrial DNA replication found in vivo.

We explored enzymologic events in the interaction of purified DNA pol-γ with AZTTP. Toxic mechanisms in AZT mitochondrial myopathy may be analogous in some ways to pharmacologic mechanisms in the antiretroviral action of AZT. The present study showed that AZTTP inhibited DNA pol-γ with mixed inhibition kinetics. AZTTP acted both as an alternate substrate for dTTP with DNA pol-γ and competed with dTTP for DNA pol-γ nucleotide binding (as seen with AZTTP and some cellular polymerases).

Materials and Methods

Materials
Reagents were analytic grade I. AZTTP was from Moravek Biochemicals, Brea, Calif. [3H]dTTP was from Amersham. All enzyme inhibitor assays were replicated from five to eight times, and each assay point was performed in triplicate within each run. Calculated arithmetic means from the triplicate assays were used to plot kinetic data.

Mitochondrial Isolation Procedures
For mitochondrial isolation, all procedures took place either on ice or at 4°C and resembled those used by us and by others in the past. Fresh bovine hearts were obtained from the slaughterhouse (courtesy of Kluener Packing Co, Cincinnati, Ohio) approximately 20 minutes before beginning the preparation. Tissue was minced into 1-cm cubes and homogenized in buffer M that consisted of (mmol/L) sucrose, 250; Tris-HCl, 50 (pH 8.0); 2-mercaptoethanol (2-ME), 5; EDTA, 1; MgCl₂, 5; and KCl, 25. Buffer volume was increased 10-fold, and the mixture was homogenized with a hand-held Potter-Elvehjem homogenizer (Fisher Scientific, Pittsburgh, Pa). Debris was pelleted at 50g. The mitochondria were pelleted from the supernatant solution by centrifugation at 8500g for 10 minutes. The resultant pellet was washed in buffer M, and the proce-
dure was repeated twice. The final pellet was distributed into 50-mL tubes, frozen in liquid N2, and stored at −80°C for use.

**Mitochondrial Extraction of DNA Pol-γ**

For DNA pol-γ extraction from isolated mitochondria, methods were modified from previous work. 7,9 Frozen pellets of isolated mitochondria were disrupted by grinding with levigated alumina (2:1 w/w) at −20°C with a mortar and pestle. The mitochondrial paste was placed on ice and suspended in cold extraction buffer (buffer E, consisting of 50 mmol/L Tris-HCl (pH 8.0), 5 mmol/L 2-ME, 1 mmol/L EDTA, 20 mmol/L MgCl2, and 1 mmol/L NaCl). Alumina was removed by centrifugation at 500 g for 10 minutes. The extract was then centrifuged for 1 hour at 165,000g in a 50-Ti rotor (Beckman, Irvine, Calif). The resulting supernatant solution was brought to 43% saturation with saturated (NH4)2SO4 (pH 7.5). This precipitate was collected by centrifugation at 12,000 g for 15 minutes in an SS-34 rotor (Du Pont, Sorvall). The precipitate was then dissolved in a small volume of buffer (TME buffer, consisting of [mmol/L] Tris-HCl, 25 [pH 8.0]; 2-ME, 5; and EDTA, 1) and was dialyzed for 16 hours against three changes of buffer (at least 50-fold volume of dialysate). Dialyzed fractions underwent centrifugation at 12,000 g for 15 minutes.

The resulting supernatant solution was retained and was subjected to column chromatography on DEAE-Sephadex (Pharmacia-LKB, Bromma, Sweden). The column was equilibrated first by washing with 20 bed volumes of TME buffer. Fraction collection began on application of the sample. Elution occurred by using a step gradient while washing the column sequentially with 3 bed volumes each of TME that contained increasing salt concentrations. The following gradient was used: TME, TME plus 80 mmol/L NaCl, TME plus 150 mmol/L NaCl, and TME plus 300 mmol/L NaCl. All fractions were assayed for DNA pol-γ activity as described below. A peak that contained DNA pol-γ enzyme activity eluted between 80 and 150 mmol/L NaCl. Fractions were pooled with the peak if they exhibited DNA pol-γ activity in excess of 25% of the peak value. Pooled fractions were dialyzed in buffer P (mmol/L: Tris, 25; NaCl, 150 [pH 7.5]; 2-ME, 5; and EDTA, 1) for 8 hours with three changes of dialysate. The resultant dialyzed fraction was submitted to column chromatography with phosphocellulose. The column was equilibrated and washed with 20 bed volumes of buffer P. Enzyme was eluted with a continuous gradient of 150 to 600 mmol/L NaCl in buffer P. Bovine serum albumin (final concentration, 1 mg/mL; Sigma Chemical Co, St Louis, Mo) was added to the peak fractions. Fractions were frozen in liquid N2 and stored at −80°C.

**Preparation of the Poly(A) · Oligo(dT)12-18 Template/Primer**

For template preparation, poly(A) was mixed with oligo(dT)12-18 in buffer containing 0.01 mol/L Tris-HCl and 1.0 mmol/L EDTA at a nucleotide ratio of 5:1. The mixture was heated to 65°C for 5 minutes and cooled to room temperature. A nucleotide ratio of 5:1 was optimal and was used in all experiments. Activated DNA was prepared according to Loeb. 10

**DNA Pol-γ Assays**

DNA pol-γ assays were performed in 50-μL volumes in buffer that consisted of 25 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 5 mmol/L 2-ME, 1 mmol/L MnCl2, 50 μmol/L (final concentration) of poly(A) · oligo(dT)12-18 as template/primer, 10 μCi/mL [3H]dTTP (final concentration of 2 μmol/L unless noted), and 0.01 to 0.04 U per assay. Reactions were carried out for 30 minutes at 30°C and were stopped by placing the mixture on ice. Carrier DNA (150 μg per tube) was added, and the product was precipitated with ice-cold 10% trichloroacetic acid containing 20 mmol/L sodium pyrophosphate. The precipitate was collected on GF filters and washed twice in 1 mol/L HCl containing 20 mmol/L sodium pyrophosphate and once in 95% ethanol. Radioactivity present in the dried filters was counted in a liquid scintillation spectrometer (Beckman, Irvine, Calif) in vials containing 10 mL scintillation cocktail. Alternatively, the product was collected directly on DEAE-cellulose filters without carrier DNA. 7,9 When calf thymus DNA was used as template/primer, its final concentration was 50 μg/mL. In those experiments, 10 mmol/L MgCl2, and the three unlabeled substrates (dATP, dCTP, and dGTP at final concentrations of 15 μmol/L each) were used.

**Results**

**Purification of DNA Pol-γ**

Bovine cardiac DNA pol-γ was purified to near homogeneity. Bovine cardiac DNA pol-γ activity resembled the activity of DNA pol-γ extracted previously from porcine and rodent liver 7,9 and recently from bovine liver (R.R. Meyer and W. Lewis, unpublished data).

**Effect of Poly(A) · Oligo(dT)12-18 Template/Primer**

The effect of poly(A) · oligo(dT)12-18 template/primer concentration was determined on DNA pol-γ activity. Nucleotide incorporation reached a maximum at approximately 6 pmol in the assay (Fig 1). The Kₘ for the (poly(A) · oligo(dT)12-18 template was 5.1 μmol/L (nucleotide) as determined by a Lineweaver-Burk plot.

**Kinetics of dTTP Incorporation by Bovine Cardiac DNA Pol-γ**

By use of dTTP as substrate for bovine cardiac DNA pol-γ, incorporation reached a maximum at 5 pmol. On
a representative Lineweaver-Burk plot (Fig 2), the $K_m$ for dTTP with bovine cardiac DNA pol-γ was determined to be 0.7 μmol/L dTTP (0.8±0.3 [mean±SD]) μmol/L dTTP, n=8).

Inhibition of DNA Pol-γ by AZTTP

AZTTP was an effective inhibitor of cardiac DNA pol-γ in the in vitro assays. A family of lines was generated using a Lineweaver-Burk plot (Fig 3). When dTTP was used at several different concentrations, the concentration of the inhibitor (AZTTP) was titrated from 0 to 3.5 μmol/L AZTTP. As shown in Fig 3, lines intersected above the x axis and to the left of the y axis. This indicated that the inhibition was mixed and that both noncompetitive and competitive mechanisms of inhibition were operative.11

By use of a derivative of the Lineweaver-Burk plot in which the slope and the vertical intercept were plotted as a function of AZTTP concentration, two lines were generated (Fig 4), yielding two distinct $K_s$.11 By using the absolute value of the respective x intercepts, the competitive $K_i$ was determined to be 1.8 μmol/L AZTTP (1.8±0.2, n=5), and the noncompetitive $K'_i$ was 5.7 μmol/L AZTTP (6.8±1.7, n=5) on this representative graph.

Comparison of Kinetic Constants of dTTP and AZTTP: Relation to Other Cellular Polymerases

Effects of dTTP and AZTTP on DNA pol-γ were compared with their corresponding reported effects on other cellular DNA polymerases. The kinetic constants we derived for dTTP ($K_m$) and AZTTP ($K_i$ and $K'_i$) with DNA pol-γ were compared with those determined by investigators who examined the interaction of AZTTP with the cellular DNA polymerases1,11 and with HIV RT.14,15 Of the known cellular DNA polymerases, DNA pol-γ exhibited the lowest $K_m$ for dTTP (Table). This $K_m$ was approximately threefold to sixfold lower than that determined with DNA pol-β or DNA pol-δ and between one and two orders of magnitude lower than that determined with DNA pol-α or DNA pol-ε. Similarly, the $K_i$ of AZTTP with DNA pol-γ was the lowest reported among the cellular polymerases. It was exceeded by that for DNA pol-α, DNA pol-β, DNA pol-δ, or DNA pol-ε by over two orders of magnitude. The $K_i$ for AZTTP with DNA pol-α or with DNA pol-β each exceeded the $K'_i$ with DNA pol-γ by over two orders of magnitude (Table).
Coupled with pathological evidence that pinpoints the target organelles as mitochondria, the present in vitro data biochemically support the hypothesis that inhibition of DNA pol-γ is a pathogenetic mechanism for the development of AZT-induced myopathy. In other cellular DNA polymerase systems, AZTTP competed with dTTP for binding. Similarities may be inferred between AZTTP inhibition of DNA pol-γ and AZTTP inhibition of HIV RT. Proposed mechanisms of inhibition of both DNA pol-γ and HIV RT involve DNA chain termination and competitive inhibition.

Previous experimental findings in vivo and in vitro are supported by and corroborate the present enzymologic data. In our work, we determined that oral AZT in vivo caused decreased abundance of rat cardiac and skeletal muscle mitochondrial DNA and RNA, decreased polypeptide synthesis in isolated mitochondria, and damaged mitochondrial cristae ultrastructure but did not change DNA pol-γ activity from muscle extracts. As may have been expected, mitochondrial DNA pol-γ activity was not affected in that system under the conditions used. First, assays performed in those studies used relatively crude extracts of muscle DNA pol-γ. Second, DNA pol-γ is encoded by nuclear genes, not mitochondrial genes.

Nuclear DNA replication has not been shown to be an important target for AZT toxicity. However, in vitro studies supported the role of AZT as a mitochondrial toxin. Mitochondrial DNA was decreased in human lymphoblastoid cells by AZT in vitro, and selective loss of mitochondrial DNA occurred with MOLT-4F lymphoblasts exposed to 2',3'-dideoxycytidine, AZT, and other dideoxynucleosides in vitro. Use of DNA pol-γ derived from calf thymus, AZTTP inhibited DNA pol-γ activity approximately 30% at 4 μmol/L. This is consistent with findings in our study. AZT (25 μmol/L) inhibited [3H]thymidine incorporation in isolated mitochondria by 90% in vitro. Of note, the compartmentalization of AZT in mitochondria, the size of the intramitochondrial nucleotide pool, and the intramitochondrial phosphorylation of AZT were not addressed here but may play important roles in AZT myopathy in vivo.

Beyond the explicit results that demonstrated inhibition of DNA pol-γ by AZTTP, the present study offers some insights into the role of DNA pol-γ function in altered mtDNA replication in some heritable mitochondrial myopathies. It is possible that processivity of DNA pol-γ, coupled with either a relative or absolute increase in abundance of small mtDNA fragments or perhaps a relative or absolute decrease of intact mtDNA genomes in mitochondria, may play a role in some forms of genetic mitochondrial illnesses.

There exists a genetic illness that closely resembles AZT myopathy in which there is a ragged red-fiber myocardopathy and decreased abundance of mitochondrial DNA. Because of the similarity between some heritable myopathies and the AZT-induced myopathy, it may be possible to use the AZT myocardy model in vivo and in vitro as an effective tool to dissect molecular aspects of the pathogenesis and natural history of some mitochondrial myopathies.

In summary, the $K_m$ of dTTP was 0.8±0.3 μmol/L with DNA pol-γ. AZTTP inhibited DNA pol-γ effect.
tively with both competitive and noncompetitive mechanisms. The mixed inhibition of DNA pol-γ by AZTTP contrasted with the competitive inhibition mechanisms of AZTTP reported with other cellular DNA polymerases in vitro. Further studies may prove conclusively that the toxic mechanism of AZTTP in vivo relates to the mixed inhibition of DNA pol-γ in vitro and may define the relative contribution of these inhibition mechanisms to the pathogenesis of AZT myopathy in vivo.

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References


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