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# MECHANISMS OF TOXICITY OF 3′-AZIDO-3′-DEOXYTHYMIDINE

## ITS INTERACTION WITH ADENYLATE KINASE

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**Abstract**—Recent experiments from our laboratory have indicated that the inhibitory effect of 3′-azido-3′-deoxythymidine (AZT) on oxidative phosphorylation may occur directly, in addition to being brought about by its inhibition of mtDNA replication. We report here studies on the effect of AZT on adenylate kinase, an enzyme crucial to oxidative phosphorylation. AZT decreased the aromatic residues fluorescence of rabbit muscle adenylate kinase, indicating binding of AZT to the enzyme. Of three other enzymes studied as controls, AZT bound only to those that possessed ATP/ADP binding sites. Up to concentrations of 15 $\mu$M, AZT was a more potent effector of fluorescence quenching than were ATP, ADP, AMP, and the AZT control, deoxythymidine. AZT strongly inhibited adenylate kinase in the direction of ATP synthesis ($K_i$, 8 $\mu$M), the inhibition being of the partial competitive type, whereas deoxythymidine inhibition, also partially competitive, was much weaker ($K_i$, 90 $\mu$M). When measured in the direction of ADP synthesis, AZT failed to demonstrate any inhibition at concentrations up to 10 $\mu$M. Experiments on isolated intact rat liver mitochondria with the enzyme activity measured in both directions confirmed the isolated enzyme results. Respiratory control by these mitochondria was not affected by AZT. The finding of AZT affinity for ATP/ADP binding sites may open new avenues of approach to the study of AZT toxicity.

*Key words:* 3′-azido-3′-deoxythymidine toxicity; AZT toxicity; adenylate kinase toxicity; mitochondria, AZT and adenylate kinase; AZT binding

The major toxic side-effects of the anti-HIV-1 ddN¶ analogs used in AIDS therapy are bone marrow suppression, skeletal myopathy and cardiomyopathy, all induced by AZT, and peripheral neuropathy induced by ddC, ddI (and d4T). Strong evidence has accumulated that a cellular target of this class of drugs is the mitochondrion. The mitochondrial enzyme, DNA polymerase $\gamma$, is susceptible to many of these drugs because they impair its ability to replicate DNA; many ddNTP analogs are excellent substrates for the enzyme and upon incorporation into DNA cause chain termination, whereas others may act as competitive inhibitors [1]. An impairment of mtDNA replication would result in severe consequences to oxidative phosphorylation and probably to other mitochondrial processes and, in turn, to cellular function.

Evidence for the view that the mitochondrion is a target of the anti-HIV-1 ddN analogs comes from a wide spectrum of systems ranging from isolated enzymes to AIDS patients. *In vitro* evidence includes: the inhibition of DNA replication by isolated DNA polymerase $\gamma$ by the ddNTP analogs of naturally occurring deoxynucleoside triphosphates [2, 3]; the exertion of such inhibition by many non-naturally occurring ddNTPs, which possess strong anti-HIV-1 activity [4]; the inhibitory effect on DNA replication of ddNs added to isolated intact rat liver mitochondria [5]; the inhibition of DNA replication in mitochondria isolated from the Friend murine erythroleukemic cell (a bone marrow model) grown in AZT [4, 6] or the PC12 cell (a peripheral neuronal model) grown in ddC [7]; the depletion of mtDNA as well as an increased rate of glycolysis in Molt-F cells grown in ddC, the first demonstration indicating a dideoxynucleoside-induced (delayed) impairment of oxidative phosphorylation) [8, 9]; the reduction in the number of mtDNA molecules per mitochondrion in AZT-grown Friend cells** [6]; the distortion of or lack of cristae in the mitochondria of ddC-treated Molt-F cells [9]; the distortion of the crystae and other mitochondrial abnormalities in AZT-grown muscle cells in tissue culture [10]; and

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¶ Abbreviations: ddN, dideoxynucleoside; ddNTP, dideoxynucleoside triphosphate; AZT, 3′-azido-3′-dideoxythymidine; ADK, adenylate kinase; $P_i$, inorganic orthophosphate; and $AP_5A$, P1,P5-di (adenosine-5′) pentaphosphate.

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the rescue of the PC12 cell from ddC by means known to rescue cells completely depleted of their mtDNA [7]. The evidence obtained from *in vivo* studies on animals includes: onset of cardiomyopathy in rats fed AZT, accompanied by disorganization and disappearance of mitochondrial cristae [10, 11] and depression of transcription of the mitochondrial Cyb gene [11], and skeletal muscle showing similar structural effects [10] as well as deleterious effects at various points in the terminal electron transport system leading to uncoupling of oxidative phosphorylation with concomitant high lactic acid levels [10]. Finally, studies on humans reveal that AIDS patients treated with AZT show myopathy, ragged-red fibers in skeletal muscle (an indication of mitochondrial damage), and severely reduced levels of mtDNA [12, 13].

Recently, during the course of a study of the effect of AZT on the growth and metabolism of Friend cells, we noticed that even a short exposure of the cells to AZT would result in metabolic changes. Thus, after 3 or 4 hr of cell growth in the presence of this drug, the cells showed evidence of impaired oxidative phosphorylation, increased glycolysis, an increase in the number of mitochondria per cell, and a sharp decrease followed by an increase in cellular ATP. Such an exposure period would be too short a fraction of the 18–20 hr doubling time of the cell to bring about such changes were they the result of an inhibition of mtDNA replication; the consequences of such an inhibition should affect only daughter mitochondria and the time of exposure to AZT is only a small fraction of the cell doubling time. We therefore asked whether, in addition to the delayed effect of AZT caused by such a mechanism, AZT might also be exerting some direct effect, perhaps on oxidative phosphorylation itself or on some reaction closely associated with it.

To answer this question, we have made use of three well-studied and well-defined mitochondrial systems, each of which yields different information. One of these permits measurement of the rate of ATP synthesis by isolated rat liver mitochondria and the extent to which this rate is affected by AZT addition. The second permits measurement of how AZT addition affects respiratory control in isolated rat liver mitochondria. Results on these two systems will be published elsewhere.* The third system permits determination of the effect of AZT on isolated purified adenylate kinase. This mitochondrial enzyme catalyzes the reversible reaction, ATP + AMP $\longleftrightarrow$ 2 ADP which, in the forward direction, is critical to oxidative phosphorylation, serving to replenish the supply of ADP, the sole $P_i$ acceptor in oxidative phosphorylation. In this report, we describe our studies on the adenylate kinase system, specifically the propensity of AZT to both bind to the enzyme and to inhibit its activity solely in the direction of ATP synthesis.

## MATERIALS AND METHODS

All chemicals and enzymes were from Sigma (St. Louis, MO). Mitochondrial substrates were used as Tris salts at pH 7.0 to 7.4. The Sigma ADK (EC 2.7.4.3) was from rabbit muscle.

The AZT–ADK interaction was monitored by measuring changes in the aromatic residues fluorescence due to externally added AZT essentially as described in Ref. 14, by means of an LS50 Perkin–Elmer spectrofluorimeter, in a 1-cm cuvette, using 280 nm as excitation wavelength. ADK (5 $\mu$g, 1.5 E.U.) was suspended at 20° in 2 mL of medium consisting of 10 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes–Tris, pH 7.2. Because of the relatively high concentrations (with respect to the extent of UV absorbance) of nucleotides used in these experiments, corrections for inner filter effects were made. Correction factors were obtained for all data points by measuring the fluoresence quenching in the presence of tryptophan, which does not chemically interfere with adenine nucleotides.

The ADK-catalyzed enzymatic reaction was spectrophotometrically monitored essentially as described [15, 16] by following in a coupled reaction the pyridine nucleotide redox state changes at 340 nm at 25°. The reaction in the direction of ATP production was followed using a reaction mixture consisting of 10 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes–Tris, pH 7.2, 1 mM glucose, hexokinase (0.2 E.U.) glucose-6-phosphate dehydrogenase (0.1 E.U.), 0.2 mM $NADP^+$ and ADP at the concentrations indicated in the legends, in a final volume of 2 mL. The reaction was started by the addition of ADK. The reaction in the direction of ADP production from AMP and ATP was followed under the experimental conditions described in Ref. 16. The rate of change in absorbance was measured as tangent to the initial part of the progress curve. The $\varepsilon_{340}$ value measured for both NADPH and NADH was found to be 6.2 $mM^{-1}$ $cm^{-1}$.

Mitochondria were isolated from 200–250 g male Wistar rats as previously described [17]. The final mitochondrial pellet was suspended in an isolation medium consisting of 0.25 M sucrose, 20 mM Tris–HCl, pH 7.2, 1 mM EDTA–Tris to give a protein concentration, determined according to Ref. 18, of 50–60 mg/mL. Mitochondrial preparations showing a respiratory control index lower than 3 were discarded.

The ADK reaction in rat liver mitochondria was monitored using an ATP or ADP detection system and following the pyridine nucleotide redox state fluorimetrically with excitation wavelength set at 334 nm and emission at 456 nm. The reaction mixture used to measure the ADK-catalyzed synthesis of ATP from ADP, previously described in detail [19], consisted of 1 mM glucose, hexokinase (2.6 E.U.), glucose-6-phosphate dehydrogenase (1.3 E.U.), 0.2 mM $NADP^+$, 5 $\mu$M carboxyatractyloside, and 5 $\mu$g oligomycin. For the reverse reaction, ADP appearance following ATP + AMP addition to mitochondria, the reaction mixture consisted of phosphoenolpyruvate (0.45 mM), pyruvate kinase (2.6 E.U.), lactate dehydrogenase (1.8 E.U.) NADH (30 $\mu$M), rotenone (2 $\mu$g), antimycin (2 $\mu$g), oligomycin (5 $\mu$g) and carboxyatractyloside (5 $\mu$M), in a final volume of 2 mL. The rate of fluorescence change resulting from the addition of the adenine

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nucleotide(s) to the reaction mixture was calculated as tangent to the initial part of the progress curve and expressed as nanomoles of nucleotide synthesized per milligram of mitochondrial protein. Controls were done to make certain that the rate of fluorescence change was independent of the concentration of substrates and enzymes of the ATP/ADP detecting systems. To obtain quantitative measurements of the rates of appearance of both the ATP and ADP in the extramitochondrial phase, the fluorimetric response was calibrated according to Ref. 20.

Oxygen uptake measurements were carried out at 25° in 2 mL of a medium consisting of mitochondria (3.4 mg protein), 0.2 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes–Tris, pH 7.2, and 2 $\mu$g rotenone, by means of a Gilson 5/6 oxygraph using a Clark Electrode. The rate of oxygen uptake resulting from the addition of 10 mM succinate was measured and expressed as natoms O per minute per milligram of protein.

## RESULTS

*Binding of AZT to adenylate kinase.* Changes in aromatic residues fluorescence are often used to measure ligand binding to protein, and this technique has been used successfully in studies on the binding to ADK of its adenine nucleotide substrates. We have used this method to determine whether the thymidine derivative, AZT, is capable of binding to ADK. In a typical experiment, ADK fluorescence emission spectra were measured in the absence and in the presence of AZT, which *per se* was found not to fluoresce under the same experimental conditions. As a result of the drug addition, a marked change in the spectrum was observed (Fig. 1A); its area was found to decrease about 27%. (No significant shift in the peak wavelength was observed.) To address the question of the specificity of the binding of AZT to ADK and, more specifically, whether the enzyme needs to possess an ATP, ADP, or AMP binding site, control experiments were done in which the interaction of AZT with three other enzymes, all of which bind adenine nucleotides, was studied. Two of these (hexokinase and fructose-6-phosphokinase) use ATP as one of their substrates, converting the ATP to ADP. Upon addition of AZT, both enzymes showed fluorescent changes similar to those observed for ADK (data not shown). However, the third enzyme, glucose-6-phosphate dehydrogenase, which binds a nucleotide derivative ($NADP^+$) but which possesses no ATP/ADP binding site, showed no fluorescent effect upon AZT addition (Fig. 1B). Thus, these preliminary results are consistent with the hypothesis that AZT is binding to an ATP/ADP site, but further studies are required to support this view.

The dependence of the fluorescence quenching on drug concentration is shown in Fig. 2A. The extent of quenching ($\Delta F$) was approximately linear up to about 15 $\mu$M AZT when it plateaued sharply. Up to this point, it appeared that AZT was a more powerful effector than ATP, ADP, or AMP (Fig. 2, panels B, C and D). However, these curves did not plateau at higher concentrations. Since AZT is a derivative

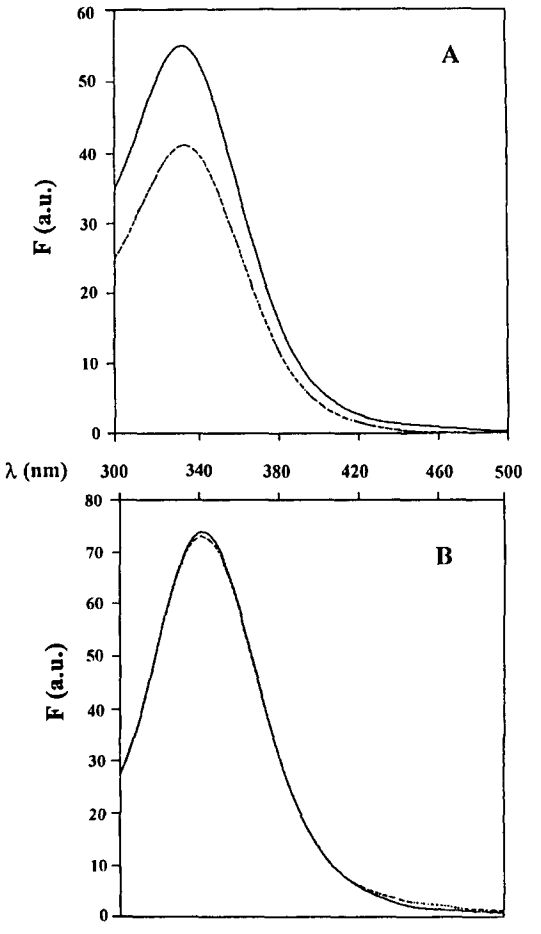


Fig. 1. Effect of AZT on the fluorescence emission spectrum of adenylate kinase. See legend to Fig. 2 for assessment of the standard deviations. Key: F (a.u.), fluorescence intensity in arbitrary units; absence (——) or presence (----) of 10 $\mu$M AZT. (A) Adenylate kinase. (B) Glucose-6-phosphate dehydrogenase.

of thymidine, this nucleoside was also studied. It was less potent at 15 $\mu$M than AZT, but it did not plateau at this point and it was the strongest effector at 50 $\mu$M (panel E).

*Effect of AZT on enzyme activity of isolated ADK.* The previously described results suggested that AZT may bind to the ATP/ADP binding site of adenylate kinase, thus raising the question of whether it can also inhibit the enzyme. This possibility was tested using ADP as the substrate and assaying for ATP formation. Substantial inhibition by AZT was found, and the kinetics of the inhibition were studied using ADP concentrations between 37.5 and 300 $\mu$M. The results, shown in a Dixon plot (Fig. 3A), demonstrated inhibition that was apparently of the competitive type with a $K_i$ value of about 8 $\mu$M, as calculated from the abscissa value of the intersection point of the curves [21]. However, Dixon plots are fairly insensitive in distinguishing between inhibition that is competitive and that which is partially

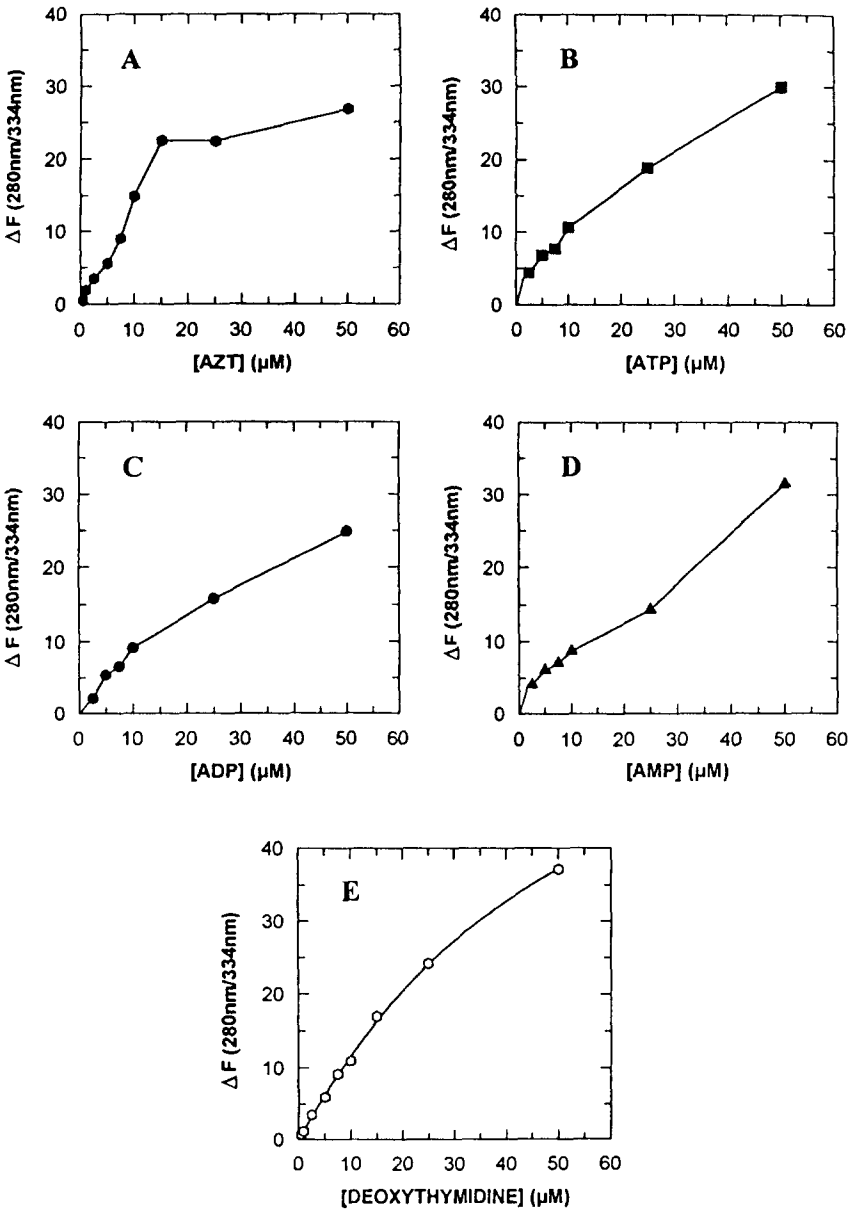


Fig. 2. Comparison of the binding to adenylate kinase of AZT, adenine nucleotides and deoxythymidine. The binding was measured fluorimetrically before and after addition of the compounds to ADK, the difference being reported on the $y$ axis in arbitrary units (a.u.). Excitation was set at 280 nm and emission was measured at 334 nm. The standard deviations of the data point values of $\Delta F$, as determined from the mean of 4 experiments, fell within the diameter of the curve symbols with the following exceptions: curve A, 10 $\mu$M and 50 $\mu$M points, $\pm 2.5$; curve B, 50 $\mu$M point, $\pm 2.7$; curve C, 50 $\mu$M point, $\pm 2.7$; curve D, 10 $\mu$M point, $\pm 1.9$; 25 $\mu$M point, $\pm 2.3$; 50 $\mu$M point, $\pm 2.7$; curve E, 7.5 $\mu$M point, $\pm 1.7$; 10 and 25 $\mu$M points, $\pm 2.0$; 15 and 50 $\mu$M points, $\pm 2.2$. Panels: (A) AZT; (B) ATP; (C) ADP; (D) AMP; and (E) deoxythymidine.

competitive [21]. The data, therefore, were plotted as the reciprocal of the fractional inhibition versus the reciprocal of the AZT concentration (Fig. 3B). The inhibition appeared to be of the partial competitive type as judged by the ordinate intercept, which was greater than 1. The $K_m$ for ADP was found to be about 75 $\mu$M, in fairly good agreement with that reported previously [22]. As a control, deoxythymidine, the parent compound of AZT, was similarly studied and partially competitive inhibition was also found (Fig. 4B). In contrast to AZT, however, its inhibitory effectiveness was an order of magnitude less than that of AZT, its $K_i$ being 90 $\mu$M (Fig. 4A).

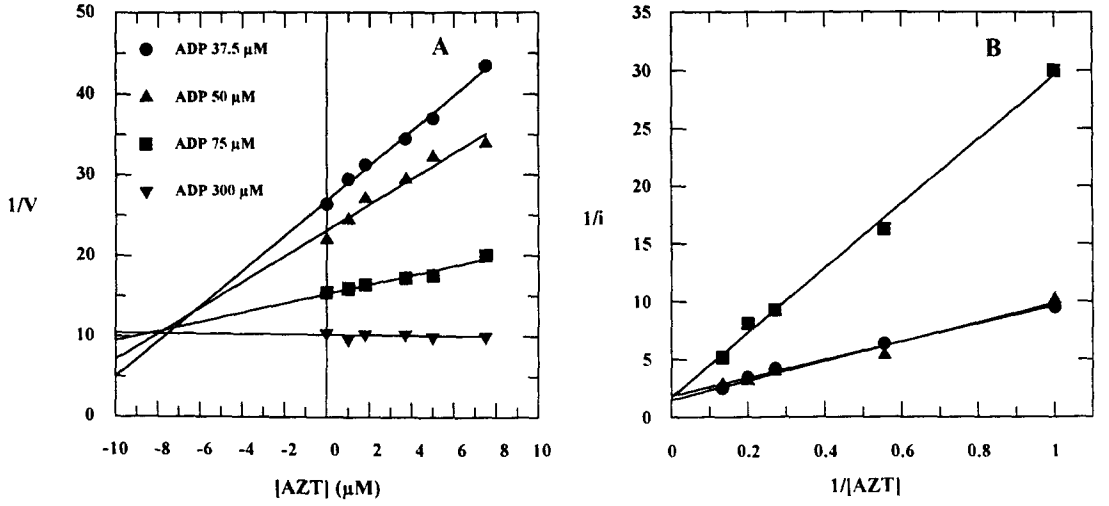


Fig. 3. Kinetics of the inhibition by AZT of the ADK reaction in the direction of ATP/AMP formation. Reaction and assay conditions are described in Materials and Methods. Panels: (A) Dixon plot [21]; (B) plot of 1/i against 1/[AZT] where $i = 1 - (V_1/V_0)$, $V_1$ and $V_0$ being the rate in the presence and absence of the inhibitor, respectively. Curve symbols of the three curves in panel B correspond to those in panel A.

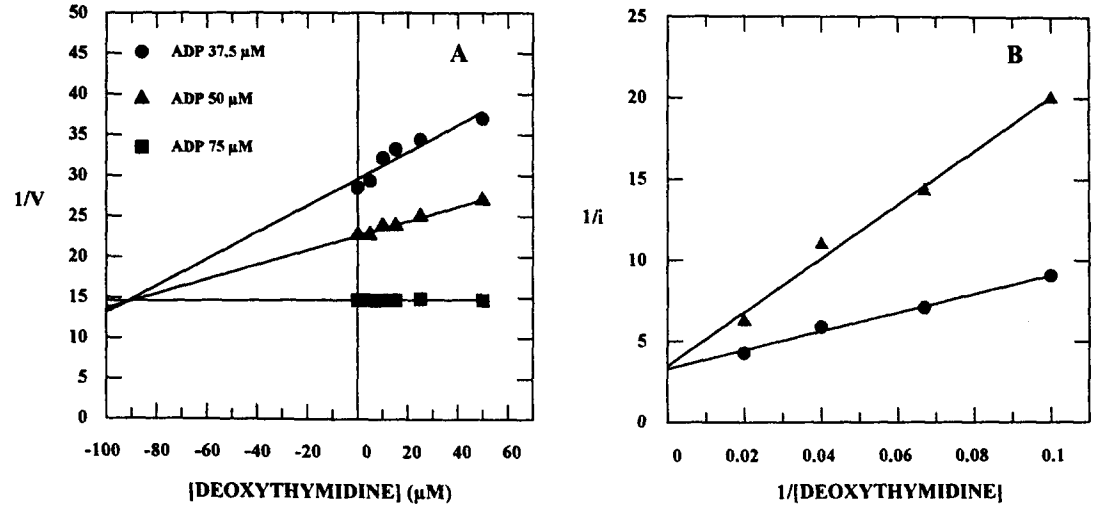


Fig. 4. Kinetics of the deoxythymidine inhibition of the ADK reaction in the direction of ATP/AMP formation. Reaction and assay conditions are described in Materials and Methods. Curve symbols of the two curves in panel B correspond to those in panel A.

The sole $P_i$ acceptor in oxidative phosphorylation is ADP; AMP does not so function. Any AMP produced in the cell, e.g. that from the pyrophosphate cleavage reactions of ATP, must first be converted to ADP before it can be converted to ATP by oxidative phosphorylation and this is one of the physiological roles of mitochondrial adenylate kinase. It was therefore crucial to determine whether AZT inhibits the enzymatic reaction in the direction of ADP production from ATP + AMP. The series of experiments was carried out by studying the effect of AZT at various concentrations of AMP (0–1 mM) at 1 mM ATP and at various concentrations of ATP (0–1 mM) at 1 mM AMP; in another experiment, AMP and ATP each at 10 mM were used. The results showed that at AZT concentrations ranging from 0 to 10 μM, no inhibition whatsoever could be observed and, therefore, no inhibition kinetics were shown. The failure of AZT to inhibit the reaction in this direction suggests that if the immediate target of AZT is some crucial step in the overall process of oxidative phosphorylation, the ADK-catalyzed conversion of ATP + AMP to ADP is not that step.

Any conclusion of pharmacological import which

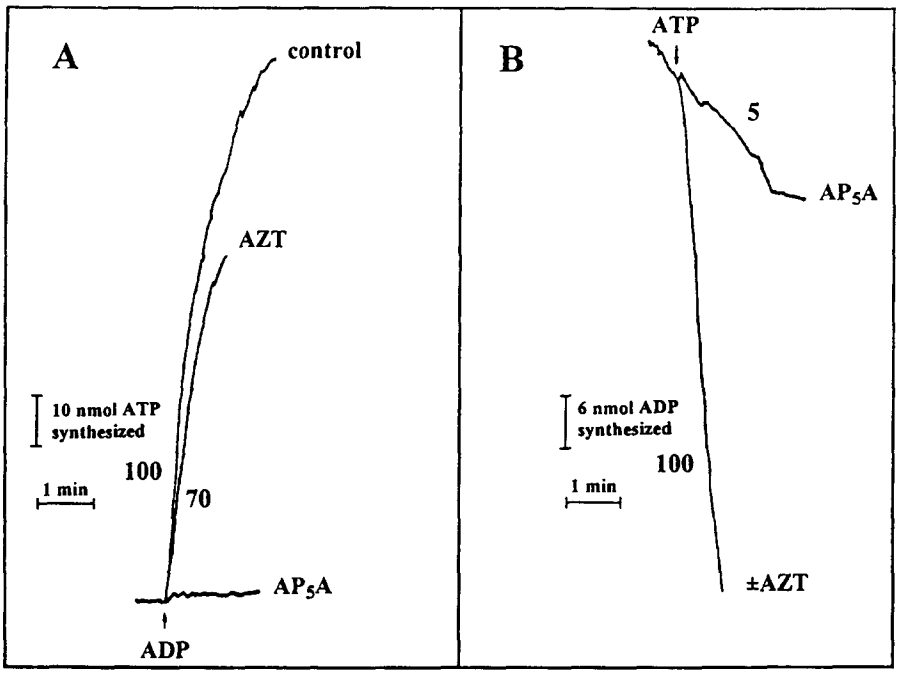


Fig. 5. Effect of AZT addition to mitochondria on the intramitochondrial adenylate kinase reaction. Conditions of the reactions and fluorimetric assays are described in Materials and Methods. When used, 10 $\mu$M $Ap_5A$ or 10 $\mu$M AZT was added 1 min before adenine nucleotide addition. Panel A, substrate was 10 $\mu$M ADP. Panel B, substrates were 10 $\mu$M ATP and 10 $\mu$M AMP; the AMP was present in the basic reaction mixture and the reaction was started by addition of the ATP. The bars represent the scales on the $x$ and $y$ axes: horizontal bars, time ($x$ axis); vertical bars, amount of nucleotide synthesized ($y$ axis).

might be drawn from these studies on an isolated mitochondrial enzyme would be fortified if confirmation could be obtained in intact mitochondria. To this purpose, the effect of AZT on ADK was studied in isolated mitochondria. In the first experiment, mitochondria were incubated with added ADP and assayed for ATP formation. Oligomycin was present to block ATP synthesis via oxidative phosphorylation. Carboxyatractyloside was used to inhibit any ATP that might be so synthesized from being exported and therefore assayed by the external ATP assay system. The results showed (Fig. 5A) a 30% inhibition of ATP formation in the presence of 10 $\mu$M AZT, a value in reasonable accord with the previously observed (Fig. 3) $K_i$ value of 8 $\mu$M for AZT.

When assayed in the direction of ADP synthesis, however, additions to the reaction mixture now being ATP, AMP, and again 10 $\mu$M AZT, oligomycin and carboxyatractyloside, it can be seen that AZT exerted no inhibition whatsoever (Fig. 5B). That the enzyme is indeed susceptible to strong inhibition *in situ* was shown by the effect of added $AP_5A$, a powerful inhibitor of ADK in both directions, which was found to give a fast 100% and a time-dependent 95% inhibition in Fig. 5, panels A and B, respectively. Thus, the results of these mitochondrial experiments confirm the view that AZT inhibits ADK in the direction of the formation of ATP but not of ADP.

The possibility remained that since AZT does inhibit the ADK reaction in one direction, even if that direction is not that of ADP formation, it could somehow interfere with the mechanism of respiratory control. This was tested by the classical technique of measuring the effect of ADP addition on mitochondrial oxygen uptake. In this case, the respiratory substrate was succinate and the reaction was carried out in the presence of rotenone to prevent oxidation of NADH to $NAD^+$, the latter being required for the oxidation of malate to oxaloacetate, an inhibitor of succinate oxidation. Two control experiments (Fig. 6) demonstrated that respiratory control was operating properly: added ADP (curve A) caused a strong stimulation of the rate of oxygen uptake, from 36 to 104 natoms/min, yielding an acceptable respiratory control index (about 3). Substitution of added ATP and AMP for ADP (curve B) also resulted in stimulation of oxygen uptake, somewhat less than that in the ADP experiment probably because the rate of formation of ADP is limiting. However, when AZT was added prior to the addition of the ATP and AMP (curve C), no AZT effect was observed, confirming the previous observations that ADK reaction in the direction of ATP is not inhibited by AZT and that AZT does not affect respiratory control. An additional control experiment showed that when a strong inhibitor of the ADK reaction in both

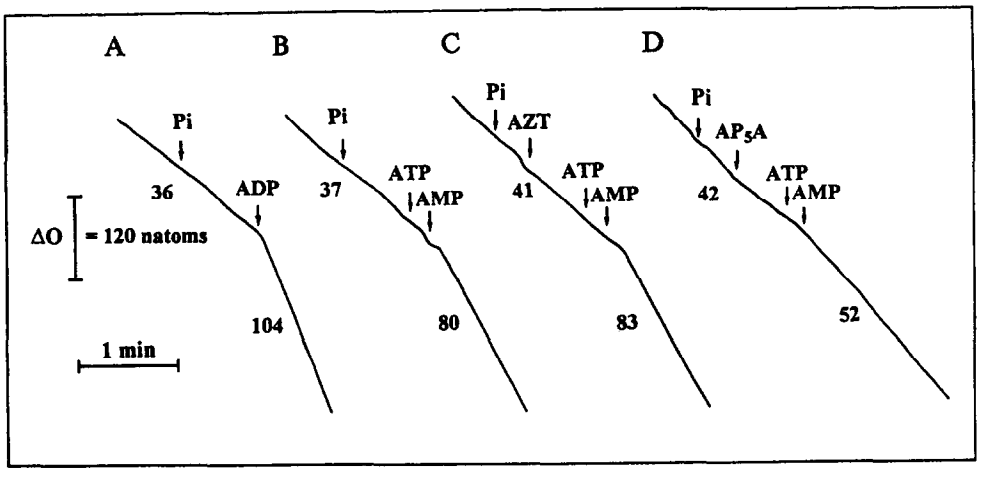


Fig. 6. Effect of AZT on respiratory control, i.e. on the stimulation of oxygen uptake induced by ATP + AMP addition to mitochondria. The rate of oxygen uptake caused by 10 mM succinate addition was measured and expressed as natoms O/min/mg protein. Additions at the arrows were $P_i$ (1 mM), ADP (200 $\mu$M), ATP (50 $\mu$M), AMP (50 $\mu$M), AZT (100 $\mu$M), $AP_5A$ (10 $\mu$M). The bars represent the scales on the $x$ and $y$ axes: horizontal bar, time ($x$ axis); vertical bar, increase in oxygen uptake over that with no additions ($y$ axis). The numerical values adjacent to the curves express rates of oxygen uptake, $\Delta O$/min. Curve A: Control, ADP added; Curve B: Control, ATP and AMP added; Curve C: AZT added prior to ATP and AMP; Curve D: Control, $AP_5A$ added in place of AZT.

directions was added prior to ATP + AMP (curve D), ADP could not be formed and respiratory control was virtually eliminated.

## DISCUSSION

Previous results from our laboratory on the Friend murine erythroleukemic cell have suggested that, in addition to the indirect inhibitory effect of AZT on oxidative phosphorylation, mediated by its inhibition of mtDNA replication, AZT may also have a direct effect on the phosphorylation process. (The conclusions reached in a recent study [23] are consistent with this view. However, the results are difficult to interpret since the AZT concentrations used were from two to three orders of magnitude higher than pharmacological plasma levels or than those that inhibit cell growth in culture.) In any case, the question of a possible direct effect has led us to an investigation designed to identify the specific step in oxidative phosphorylation that might be the target of AZT.

The results of the experiments reported here do not provide evidence that the AZT-associated impairment of oxidative phosphorylation in the intact Friend cell could result from an inhibition of the ADK reaction. Although this nucleotide analogue does inhibit ADK, it does so effectively only in the direction of ATP formation from ADP. That the ADK-catalyzed formation of ADP, a substance absolutely required as the phosphate acceptor in the last step of oxidative phosphorylation, is not affected by AZT was shown directly in the isolated enzyme system and in isolated mitochondria. In addition, the ADK reaction in mitochondria permits respiratory control by ATP + AMP addition and such control remained unaffected by the presence of AZT. It remains possible that in the intact cell the ADK reaction in the direction of ATP synthesis plays some important but as yet unknown metabolic role, which could be interrupted by ATP.

The interaction of AZT with adenylate kinase raises an interesting question concerning a possible mechanism of toxicity of AZT. The binding of AZT to ADK accompanied by its inhibition of the enzyme suggests that AZT binds at or near the ATP/ADP binding site. This view is strengthened by results showing that AZT binds to two additional enzymes that have ATP/ADP binding sites but does not bind to an enzyme that does not possess such a paired site. While such binding and inhibitor studies need to be extended to a spectrum of other enzymes before such a hypothesis becomes more than speculative, it is tempting to propose that AZT could be exerting its toxic effects by targeting one or more of a variety of nucleotide-utilizing enzymes.

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