Biochemical Pharmacology, Vol. 53, pp. 913–920, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00831-3

3′-Azido-3′-deoxythymidine Uptake Into Isolated Rat Liver Mitochondria and Impairment of ADP/ATP Translocator

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ABSTRACT. To gain some insight into the mechanism by which 3′-azido-3′-deoxythymidine (AZT) impairs mitochondrial metabolism, [^{14}C]AZT uptake by rat liver mitochondria (RLM) *in vitro* was investigated. AZT accumulated in mitochondria in a time-dependent manner and entered the mitochondrial matrix. The rate of AZT uptake into mitochondria showed a hyperbolic dependence on the drug concentration and was inhibited by mersalyl, a thiol reagent that cannot enter mitochondria, thus showing that a membrane protein is involved in AZT transport. Investigation into the capability of AZT to affect certain mitochondrial carriers demonstrated that AZT was able to impair the ADP/ATP translocator, but had no effect on Pi, dicarboxylate, tricarboxylate, or oxodicarboxylate carriers. AZT inhibited ADP/ATP antiport in either mitochondria or mitoplasts in a competitive manner with different sensitivity (K_i values were 18.3 ± 2.9 and 70.2 ± 5.8 μM, respectively). Consistent with this were isotopic measurements showing that AZT accumulates in the intermembrane space. AZT does not use ADP/ATP carrier to enter mitochondria, as shown by the failure of both carboxyatractyloside (CAT) to inhibit AZT transport into mitochondria and AZT to induce ATP efflux from ATP-loaded mitochondria. ADP/ATP translocator impairment by AZT as one of the biochemical processes responsible for the ATP deficiency syndrome is discussed. BIOCHEM PHARMACOL 53;7:913–920, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. 3′-azido-3′-deoxythymidine; rat liver mitochondria; AZT transport; anion carriers; ADP/ATP carrier

3′-Azido-3′-deoxythymidine (AZT[6])* is the most widely used drug in acquired immunodeficiency syndrome (AIDS) therapy, as it is a powerful and selective inhibitor in human immunodeficiency virus replication [1]. Although the AZT's high toxicity limits the clinical efficacy of long-term AZT therapy [2], the mechanism by which AZT toxicity occurs remains to be established. Mitochondria have been found to be a cellular target for AZT: AZT treatment in humans resulted in myopathy, with ragged-red fibers in skeletal muscle and depletion of muscle phosphocreatine, ATP, and mtDNA levels [3–5].

As a result of AZT administration in rats, abnormal skeletal and cardiac muscle mitochondria were generated with deleterious effects in the electron transport system and concomitant high lactic acid levels [6, 7].

The distortion of the cristae and other mitochondrial abnormalities were observed in AZT-grown muscle cells in tissue culture [6]; in AZT-grown human and murine cell lines, the number of mitochondrial DNA molecules per mitochondrion was reduced, and selective mitochondrial toxicity has been proposed to be responsible for delayed AZT toxicity in patients [8, 9]. On the other hand, early AZT effects on oxidative phosphorylation were also observed with a decrease in ATP synthesis [10].

AZT causes both a dose-dependent inhibition of NADH-linked respiration and NADH-cytochrome c reductase activity when added to isolated rat skeletal muscle, brain, and liver mitochondria [11]. Inhibition of the adenylate kinase in rat liver mitochondria (RLM) was also found [12]. Moreover, externally added AZT can inhibit mitochondrial DNA synthesis [13], most likely via the powerful inhibition of DNA polymerase γ by the triphosphate AZT derivative [14, 15].

Although the above-reported findings suggest the existence of AZT/AZT derivative transport into mitochondria, to date such a transport has not been investigated in detail. This article work aims to ascertain whether and how AZT can enter isolated RLM as well as to establish whether AZT can impair metabolite traffic across the mitochondrial

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* *Abbreviations:* AIDS, acquired immunodeficiency syndrome; AP_5A, P_1,P_5-di (adenosine-5′) pentaphosphate; ATP D.S., ATP detecting system; AZT, 3′-azido-3′-deoxythymidine; CAT, carboxyatractyloside; G6P-DH, glucose-6-phosphate dehydrogenase; HK, hexokinase; NEM, *N*-ethyl-maleimide; RLM, rat liver mitochondria.

Received 22 February 1996; accepted 8 October 1996.

membrane. We demonstrate the active entry of AZT into RLM. Moreover, AZT was found to inhibit the ADP/ATP carrier.

MATERIALS AND METHODS

Chemicals

$[^3H]_2O$, [U-^{14}C] sucrose and [6,6'(n)-^{3}H] sucrose were from Amersham (Buckinghamshire, England, UK). [^{14}C]AZT and all other chemicals were from Sigma (St. Louis, MO, USA). Mitochondrial substrates were used as Tris salts at pH 7–7.3.

Mitochondria and Submitochondrial Fraction Preparation and Protein Assay

RLM were isolated from male Wistar rats (150–200 g) as described [12], with mitochondrial protein determined according to [16]. Mitochondria integrity was verified during the course of each experiment, essentially by measuring the respiratory control of the mitochondrial preparations by means of a Gilson 5/6 oxygraph (Gilson Italia, MI, Italy) using a Clark Electrode (Gilson Italia, MI, Italy). Mitochondrial preparations with a respiratory control index lower than 3 were discarded. Mitoplasts and submitochondrial fractions were obtained as described in [17], with purity tested by measuring the activities of adenylate kinase (EC 2.7.4.3) and glutamate dehydrogenase (EC 1.4.1.3) as in [18, 19].

Measurements of [^{14}C]AZT Uptake

Uptake experiments were carried out as follows: RLM (3–4 mg protein) were incubated at 25°C for 1 min, in 1 mL of a standard medium consisting of 0.25 M sucrose, 20 mM Tris-HCl pH 7.25, 1 mM EDTA-Tris, in the presence of $[^3H]_2O$ (0.1 μCi). The uptake reaction was started by the addition of [^{14}C]AZT and stopped when appropriate by rapidly centrifuging (15000 rpm, 4 min) in a refrigerated Ole Dich microcentrifuge (Ole Dich Instrumentmakers ApS, Hvidovre, Denmark) equipped with an Eppendorf rotor (Ole Dich Instrumentmakers ApS, Hvidovre, Denmark). Perchloric extracts from mitochondrial pellet were obtained as in [20] and dissolved in 4 mL of Ready Solv EP Beckman scintillation cocktail for radioisotopic counting performed with a Beckman LS 7800 scintillation counter (Beckman Instrument, Fullerton, CA, USA). In parallel experiments, use was made of [^{14}C]sucrose in order to measure intramitochondrial volume as previously described [20–22]. The amount of [^{14}C]AZT actually taken up, the fold accumulation, i.e. the ratio of intra- to extramitochondrial drug concentration, and the space or volume of distribution of labelled compounds in the mitochondrial pellets were calculated as previously described [21–23].

Measurements of AZT Uptake by HPLC

To measure the amount of AZT taken up, RLM (10–12 mg protein) were incubated for 1 min at 25°C in 1 mL of a medium consisting of 0.2 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES-Tris pH 7.2, in the presence of $[^3H]_2O$ (0.2 μCi) and, in parallel experiments, in the presence of [^{14}C]sucrose (0.1 μCi) as described above and in [20–22]. AZT uptake was started by adding 15 μM AZT and was stopped 3 min later by rapid centrifuging (see above). Aliquots (20 μL, 1.4 mg protein) of perchloric extracts, obtained as in [20], were analysed using a Kontron Instruments HPLC system (Kontron Instrument, MI, Italy), including a mod. 420 pump and mod. 425 gradient former equipped with a 430 UV/VIS detector (set at 265 nm wavelength) and a 450 MT2 data system integrator). Separation was achieved on a Spherisorb 5 μ ODS2 Kontron Analytical reverse phase column (25 cm × 4.6 mm) (Kontron Instrument, MI, Italy) eluted at a flow rate of 1 mL/min. An isocratic separation with 25 mM ammonium phosphate buffer pH 5.7 was used; 15 min later the same buffer was added with 10% CH_3CN. Under these conditions, properly developed to assay both nucleoside monophosphate and AZT, the AZT peak was found to appear after approximately 25 min. AZT was calibrated in a 0 to 50 μM concentration range by using standard solutions diluted in the perchloric extraction solutions.

Fluorimetric and Photometric Mitochondrial Carrier Activity Assays

Pi uptake was measured as essentially in [24], with RLM (1–2 mg protein) incubated at 25°C in 2 mL of a medium consisting of 250 mM sucrose, 2 mM Tris-succinate, 4 mM $MgCl_2$, 5 mM Tris-acetate pH 6.5 in the presence of 2 μg rotenone, and swollen by the addition of 160 μM $CaCl_2$. On phosphate addition an increase in absorbance at 546 nm was observed, with the rate of optical change determined as tangent to the initial part of the progress curve.

Succinate/malate exchange (via dicarboxylate carrier), *cis*-aconitate transport (via tricarboxylate carrier) and oxaloacetate uptake (via oxodicarboxylate carrier) were followed as described elsewhere [25], with RLM (1 mg protein) incubated at 25°C in 2 mL of a standard medium consisting of 0.2 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes-Tris pH 7.2.

The spectrophotometric measurements of ADP/ATP exchange were carried out essentially as previously reported [26]. Briefly, RLM (1.4 mg protein) were incubated at 25°C in 2 mL of the standard medium in the presence of the ATP detecting system (ATP D.S.), consisting of glucose (2.5 mM), hexokinase (HK) (EC 2.7.1.1) (0.5 E.U.), glucose-6-phosphate dehydrogenase (G6P-DH) (EC 1.1.1.49) (0.2 E.U.) and $NADP^+$ (0.2 mM). NADPH formation in the extramitochondrial phase, which reveals ATP appearance due to externally added ADP, was followed spectrophotometrically at 340 nm. The exchange reaction was started by adding ADP. The rate of absorbance increase, expressed as nmol $NADP^+$ reduced/min · mg protein, was obtained as a tangent to the initial part of the curve.

Loading Procedure of RLM with ATP and Fluorimetric Measurements of Intramitochondrial ATP Efflux Induced by ADP

Loading measurements of RLM with 1 mM ATP were performed essentially as previously reported [26]. This procedure gave an intramitochondrial ATP concentration equal to 3.5 mM, as enzymatically assayed in mitochondrial extracts according to [27].

ATP efflux, induced by externally adding ADP (50 μM) to loaded mitochondria, was continuously monitored using 334 nm and 456 nm as excitation and emission wavelengths, by means of the ATP D.S. described above, in the presence of rotenone (2 μg), antimycin (2 μg), oligomycin (5 μg), and 10 μM P_1,P_5-di (adenosine-5′)pentaphosphate (AP_5A), added to prevent any mitochondrial ADP phosphorylation.

RESULTS

Evidence of [^{14}C]AZT Uptake in Isolated RLM

In order to determine whether and how isolated RLM can take up externally added AZT, [^{14}C]AZT was added to mitochondria, with the amount of the drug actually taken up into the organelle measured as described in Materials and Methods.

In Fig. 1 the [^{14}C]AZT space/[^{3}H]$_2$O space ratio, the [^{14}C]AZT space/[^{14}C]sucrose space ratio, and fold accumulation, i.e. the ratio between intra/extramitochondrial AZT concentration, are reported as studied at two different AZT incubation times. After 45 sec incubation of mitochondria with 10.8 μM AZT, ratios of 1.1 and 1.3 were calculated for the [^{14}C]AZT space/[^{3}H]$_2$O space and [^{14}C]AZT space/[^{14}C]sucrose space ratios respectively, with fold accumulation higher than 2. Following 120 sec incubation, ratios were 1.3, 1.5, and 3.9. In a parallel experiment (not shown), the time course of [^{14}C]AZT uptake was followed. AZT uptake into the organelle occurred linearly with time up to 20 sec and reached completion after 3 min. These results clearly show that AZT accumulates into isolated mitochondria and that this process is time dependent, thus excluding the possibility of the drug's binding to the external surface of mitochondria.

To clarify the mechanism of drug uptake into the organelles, the dependence of [^{14}C]AZT uptake on extramitochondrial AZT concentration was investigated. In Fig. 2A, the fold accumulation of AZT into RLM was plotted as a function of extramitochondrial AZT concentration. After 20 sec incubation of RLM with 5–10 μM AZT, intra/extramitochondrial AZT concentration ratios were close to 3. Varying along with AZT concentration, fold accumulation decreased, reaching a value of 1 for an AZT concentration higher than 40 μM. The inverse relationship between the accumulation gradient and drug concentration clearly suggests saturation characteristics for AZT uptake [22, 23, 28].

To confirm such a conclusion, the dependence of the rate of AZT uptake on increasing AZT concentration was

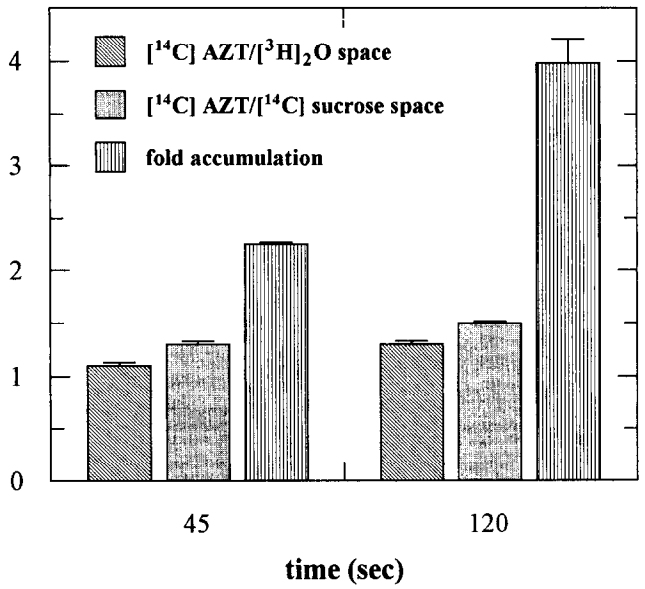

FIG. 1. Dependence of [^{14}C]AZT uptake in RLM on incubation time. Mitochondria (3.5 mg protein) were incubated at 25°C under the experimental conditions described in Materials and Methods in the presence of [^{3}H]$_2$O (0.1 μCi). The uptake reaction was started by the addition of 10.8 μM [^{14}C]AZT and, when indicated, the reaction was stopped and the radioactivity measured in mitochondrial extracts as described in Materials and Methods. In parallel experiments, [^{14}C]sucrose (0.2 μCi) instead of [^{14}C]AZT was added to mitochondrial suspension. AZT uptake was expressed as both a [^{14}C]AZT space/[^{14}C]sucrose space ratio and a [^{14}C]AZT space/[^{3}H]$_2$O space ratio. Calculation made of intramitochondrial volumes allows for measurements of [^{14}C]AZT fold accumulation, i.e. the ratio of intra- to extramitochondrial drug concentration. The standard deviations of the data were determined from the mean of three experiments.

studied (Fig. 2B). Saturation kinetics were found, with K_m and V_{max} values equal to 10 ± 4 μM and 180 ± 27 pmol/min · mg protein, respectively, as obtained by using Grafit software (Erithacus Software Ltd., Staines, UK).

To gain further insight into the mechanism by which [^{14}C]AZT uptake takes place, an inhibition study was performed by testing the sensitivity of AZT uptake by RLM to certain thiol reagents. Both mersalyl and *N*-ethylmaleimide (NEM), which differ in their capability of entering mitochondria [29], were found to inhibit AZT (15 μM) uptake. The nonpenetrant SH reagent, mersalyl (0.5 mM), gave ca. 60% inhibition, whereas NEM (1 mM), which can cross the mitochondrial membrane, was much less effective. As expected, unlabelled AZT added at a concentration 30 times higher than [^{14}C]AZT inhibited nucleoside translocation by approximately 90%.

Effect of AZT on the Activity of Certain Mitochondrial Carriers: Inhibition of ADP/ATP Antiport

The inhibition of the AZT uptake rate by mersalyl strongly indicates that AZT transport into mitochondria involves a carrier.

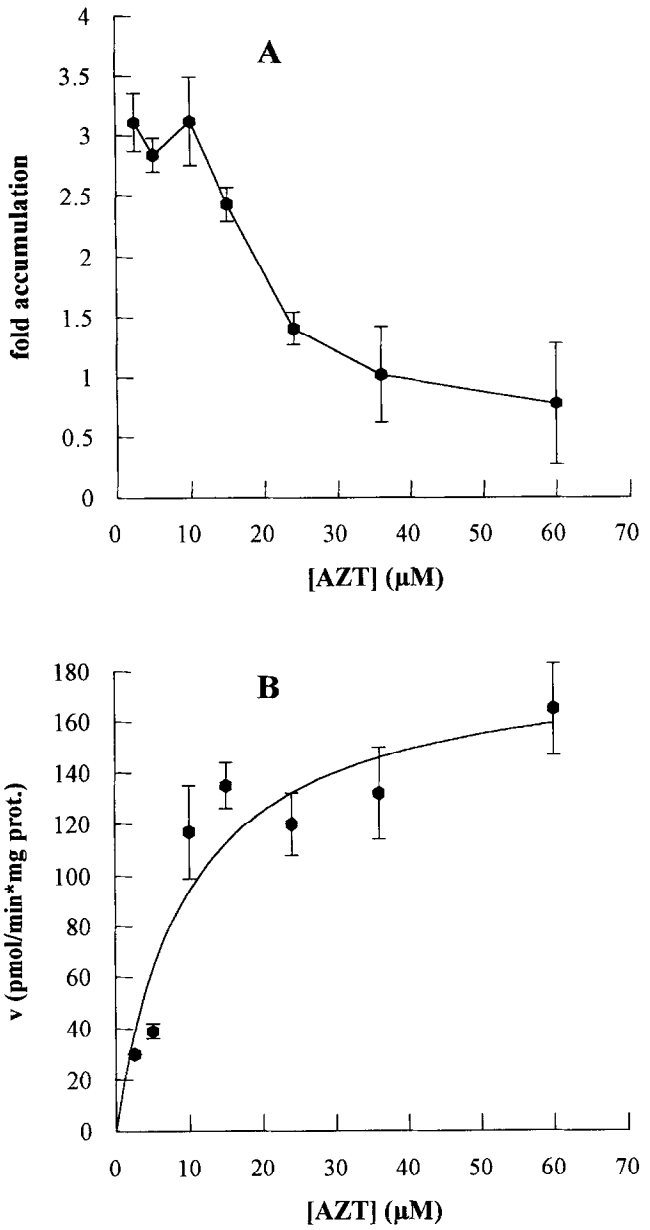

FIG. 2. Dependence of [^{14}C]AZT uptake on extramitochondrial AZT concentration. Mitochondria (3.3 mg protein) were incubated under the same experimental conditions described in Fig. 1. The uptake reaction was started by the addition of increasing concentrations of [^{14}C]AZT and stopped 20 sec later, with the amount of [^{14}C]AZT taken up by mitochondria measured as described in Materials and Methods. In (A), the Y axis represents the fold accumulation of [^{14}C]AZT in the organelles. In (B), the AZT uptake rate (V) is expressed as pmol AZT taken up/min · mg mitochondrial protein. The standard deviations of the data were determined from the mean of three experiments.

To gain a degree of insight into the translocator involved in the drug transport, the effect of AZT on the activity of certain mitochondrial carriers previously found to be sensitive to mersalyl was investigated [30]; thus, the capability of 10 μM AZT to inhibit the Pi, dicarboxylate, oxodicarboxylate, or tricarboxylate carriers was tested as in [24, 25]. In each case, the concentration of the substrate used was close to the K_m value of each substrate for mitochondrial translocator, as determined under the same experimental conditions.

In a number of experiments (Table 1), AZT failed to inhibit the tested anion transport rate. In the same experiments, the addition of mersalyl, butylmalonate, 1,2,3, benzenetricarboxylate, and phenylsuccinate, which are powerful inhibitors of phosphate, dicarboxylate, tricarboxylate, and oxodicarboxylate carriers [24, 25, 30] and which were used as a control, completely inhibited anion translocation.

The AZT effect on ADP/ATP carrier activity was then studied, making use of an experimental procedure described in [26] to continuously monitor ADP/ATP exchange in RLM where oxidative phosphorylation can occur (Fig. 3A). ATP concentration outside mitochondria was negligible, because no change in the absorbance of externally added $NADP^+$ (0.2 mM) was found in the presence of ATP D.S. consisting of glucose (1 mM), HK (0.2 E.U.), and G6P-DH (0.1 E.U.). The addition of 5 μM ADP to RLM caused a rapid increase in absorbance, resulting in ATP appearing in the extramitochondrial phase. The rate of absorbance increase was shown to reflect the rate of ADP/ATP exchange, as demonstrated in a control experiment carried out by using carboxyatractyloside (CAT) as a nonpenetrant inhibitor of the transport (see [26, 30]). A possible contribution of adenylate kinase to ATP formation is excluded, owing to the presence of AP_5A (10 μM), a powerful inhibitor of adenylate kinase [31]. Consistent with this finding was the inhibition of the rate of ADP/ATP exchange determined by CAT (0.5 μM). Interestingly, AZT (20 μM) significantly inhibited the rate of ADP/ATP exchange (37% inhibition). AZT inhibition of the ADP/ATP exchange rate is better demonstrated in Fig. 3B as a Dixon plot [32], where the dependence of the rate of ADP/ATP exchange on AZT concentration is compared at 5 and 50 μM ADP concentrations. Competitive inhibition was found in this experiment, with a value of K_i equal to 18 μM. In three different experiments carried out with different mitochondrial preparations, a mean K_i value of 18.3 ± 2.9 μM was calculated.

In another series of experiments, use was made of mitoplasts, which lack the outer mitochondrial membrane. When ADP/ATP exchange was measured in mitoplasts under the same experimental conditions described for mitochondria, using ADP as substrate, a K_m value of 4.5 ± 1.7 μM and a V_{max} value of 7.5 ± 0.9 nmol/min · mg protein were measured using different preparations. In Fig. 4, a typical experiment is shown in which the dependence of the rate of ADP/ATP exchange on AZT concentration was measured and data were plotted according to a Dixon analysis. A competitive inhibition by AZT on the ATP efflux rate was found; interestingly, the K_i value was equal to 65 μM in this experiment (70.2 ± 5.8 in three different experiments), i.e. almost four times higher with respect to the value measured with mitochondria.

TABLE 1. The effect of AZT on the activity of certain mitochondrial carriers

Mitochondrial carrier	Substrate	Addition	V_o
			(ΔA_{546}/min·mg prot.)
		none	0.5 ± 0.1
Phosphate	Pi	AZT	0.5 ± 0.1
		Mersalyl	0.1 ± 0.1
			(nmol/min·mg prot.)
		none	5.2 ± 0.2
Dicarboxylate	Succinate	AZT	5.1 ± 0.3
		Butylmalonate	0.9 ± 0.1
			($\Delta F_{334/456}$/min·mg prot.)
		none	83 ± 3
Tricarboxylate	*cis*-Aconitate	AZT	80 ± 2
		1,2,3 Benzenetricarboxylate	10 ± 2
			($\Delta F_{334/456}$/min·mg prot.)
		none	350 ± 9
Oxodicarboxylate	Oxaloacetate	AZT	352 ± 7
		Phenylsuccinate	25 ± 4

Carrier activity was measured as reported in Materials and Methods by using 0.1 mM Pi, 0.15 mM succinate, 0.1 mM *cis*-aconitate and 0.1 mM oxaloacetate as specific substrates. When present, the other compounds were used at the following concentrations: 10 μM AZT, mersalyl (20 nmol/mg prot), 1 mM butylmalonate, 1 mM 1,2,3, benzentricarboxylate, 1 mM phenylsuccinate. The initial rates of the uptake were measured as previously reported [24, 25]. The standard deviations of data were determined from the mean of three experiments.

This finding raises the question as to whether AZT can accumulate in the intermembrane space, thereby accounting for the apparent discrepancy between mitochondria and mitoplasts towards AZT sensitivity of the ADP/ATP exchange.

To ascertain in which mitochondrial compartment(s) AZT is sequestered, experiments were performed to determine the subcellular localisation of the AZT taken up. Thus, RLM were incubated for 5 min with 10.8 μM [^{14}C]AZT in the presence of [^{3}H]sucrose, which does not permeate the inner mitochondrial membrane, and measurements were taken of the [^{14}C]AZT space/[^{3}H]sucrose space ratio (Fig. 5). As expected, this ratio in the mitochondrial pellet was found to be 1.4. In mitoplasts, obtained from loaded mitochondria as described, the ratio was found to be 2.5. More than 90% of [^{14}C]AZT radioactivity present in mitoplasts was found in the matrix, where a [^{14}C]AZT space/[^{3}H]sucrose space ratio of 2.9 was determined. This result further confirms that AZT is able to permeate the inner mitochondrial membrane and shows that AZT, after crossing this membrane, can accumulate in the matrix space. Surprisingly, the [^{14}C]AZT space/[^{3}H]sucrose space ratio was found to be higher than one in the intermembrane space fraction, thus showing that AZT accumulation of the drug can occur in this compartment. No significant radioactivity was found to be associated with either membrane fraction.

Failure of ADP/ATP Translocator in Transporting AZT Into Mitochondria

The inhibition by AZT on the ADP/ATP exchange rate suggests that this translocator is involved in AZT transport into mitochondria. To find out whether AZT can enter mitochondria in antiport with intramitochondrial ATP via the ADP/ATP translocator, mitochondria were loaded with ATP and either AZT or ADP (used as a control), added to the mitochondria. AZT up to a concentration of 0.5 mM failed to cause ATP efflux, as enzymatically revealed outside mitochondria. In the same experiment, externally added ADP, as expected, caused ATP efflux (3.5 nmol/mg protein in 10 min exchange), which occured in a manner sensitive to the inhibitor of ADP/ATP translocator CAT.

To definitively ascertain whether AZT can enter mitochondria via the ADP/ATP translocator, the sensitivity of AZT uptake to CAT, as measured by HPLC, was tested (Fig. 6). AZT content was measured in perchloric extracts from mitochondria to which no addition was made (A), or to which 15 μM AZT (B), 15 μM AZT plus mersalyl (40 nmol/mg protein) (C), or 15 μM AZT plus 5 μM CAT (D) were added, respectively. Comparison of HPLC peak areas shows that AZT entered mitochondria and that its uptake was completely insensitive to CAT, but inhibited by mersalyl.

Consistent with [^{14}C]AZT uptake measurements, the amount of AZT actually taken up by mitochondria, as calculated as a mean of three HPLC experiments, was equal to 70 ± 0.7, 12 ± 0.7, and 70 ± 0.7 pmol/mg protein for B, C, and D, respectively.

DISCUSSION

This article clearly shows that AZT per se can enter mitochondria, as shown by both isotopic and HPLC measurements. Moreover, AZT transport is shown to occur in a carrier-mediated manner.

Such a conclusion derives from experimental findings showing that AZT can localise in the mitochondrial matrix

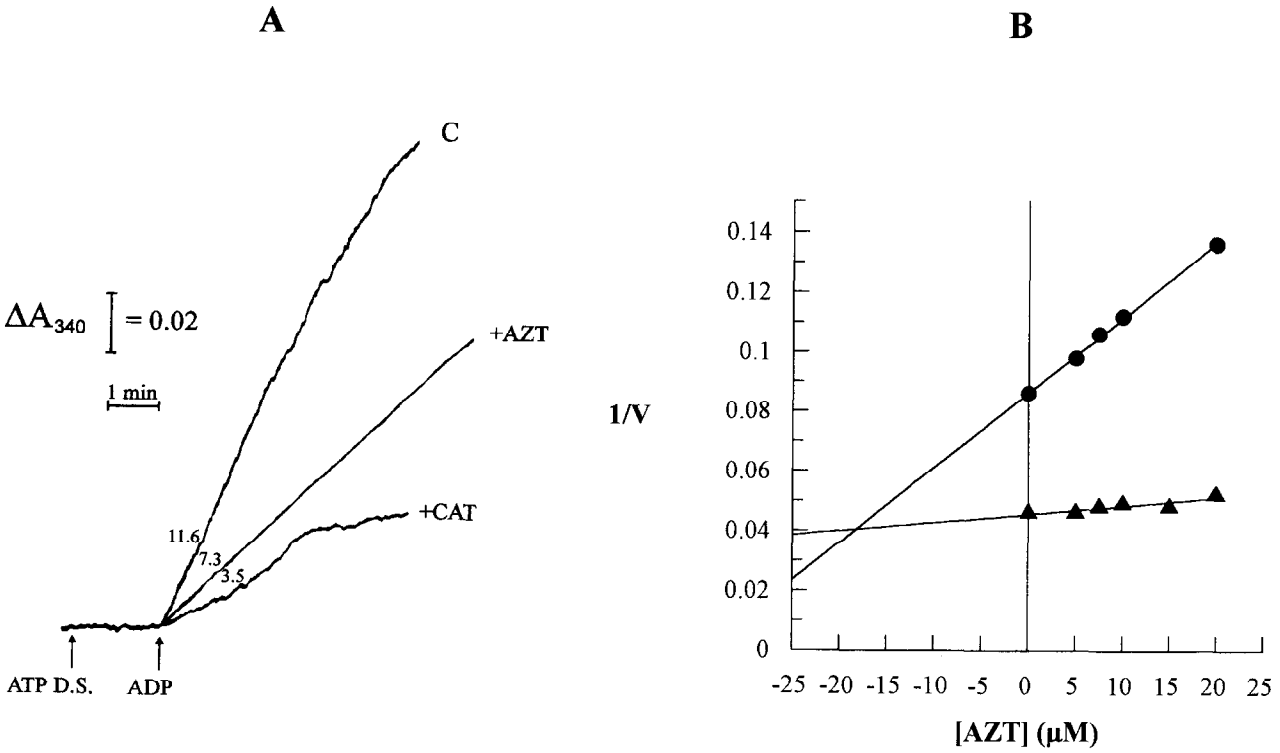

FIG. 3. Inhibition by AZT of the rate of ADP/ATP exchange in RLM. (A) RLM (1.4 mg protein) were incubated at 25°C, as described in Materials and Methods, in the presence of 10 μM AP_5A. At the arrows, additions were as follows: ATP D.S. [1 mM glucose, 0.2 mM $NADP^+$, HK (0.2 E.U.) G6PDH (0.1 U.E.)] and 5 μM ADP. When present, either 20 μM AZT or 0.5 μM CAT were added 1 min before ADP. The trace shows the increase in A_{340} and the numbers alongside give the rate of $NADP^+$ reduction in nmol/min × mg mitochondrial protein, measured as the tangent at the initial part of the progress curve. (B) Dixon plot of the inhibition by AZT of the ADP/ATP exchange rate. The efflux of ATP was measured by using 5 (●) and 50 (▲) μM ADP, either in the absence or presence of AZT at the indicated concentrations. The rate V is expressed as nmol of $NADP^+$ reduced/min · mg mitochondrial protein.

and that transport is consistent with certain criteria commonly used to demonstrate the occurrence of a carrier-mediated translocation, i.e. the inverse relationship between uptake and drug concentration, the hyperbolic dependence of the rate of the uptake on the substrate concentration, and the uptake inhibition caused by non-penetrant compounds.

Unfortunately, the identification of the carrier(s) that can translocate externally added AZT remains to be established; however, the experiments reported in this article rule out the possibility that the Pi, dicarboxylate, tricarboxylate, and oxodicarboxylate translocators are involved in AZT transport. In fact, AZT fails to inhibit their activity when used at a concentration roughly close to that obtained *in vivo* in AIDS therapy.

Externally added AZT was found to inhibit the appearance of ATP outside both mitochondria and mitoplasts, which occurs as a result of externally added ADP. Given that AZT can enter the mitochondrial matrix, its possible inhibition of ATP synthase should be considered. Indeed, application made of the control strength criterion [26] to the Dixon plots reported in Figs. 3 and 4 demonstrates that AZT inhibits the rate of the ADP/ATP exchange that was found to limit the rate of NADPH formation under our experimental conditions. However, even if AZT is a powerful inhibitor of ADP/ATP exchange in RLM and mitoplasts, it does not use the ADP/ATP translocator to enter the organelles. This conclusion is based both on the failure of AZT to cause efflux of intramitochondrial ATP, and more importantly, on the insensitivity of AZT uptake to CAT, a powerful inhibitor of the ADP/ATP carrier.

The fate of taken up AZT merits further discussion: indeed, in studying the AZT–mitochondria interaction, particular attention should to be paid to if and how a sufficient level of AZTTP can be formed inside the mitochondria as well as to the origin of this nucleotide, which has been found to inhibit mitochondrial DNA synthesis [9, 10]. In this regard, in addition to a putative AZTTP synthesis in cytosol and subsequent uptake into mitochondria, we might suppose that neointernalised AZT could be phosphorylated in the organelles via a reaction catalysed by certain mitochondrial enzyme(s), as for instance thymidine kinase [33–35]. However, AZTTP localisation in mitochondria requires further investigation.

On the other hand, a comparison between the time course of AZT uptake into mitochondria, which is complete in 3 min, and the observed inhibition of the ADP/ATP carrier, which does not require AZT uptake and is found immediately after AZT addition to mitochondria, raises an important question: under physiological condi-

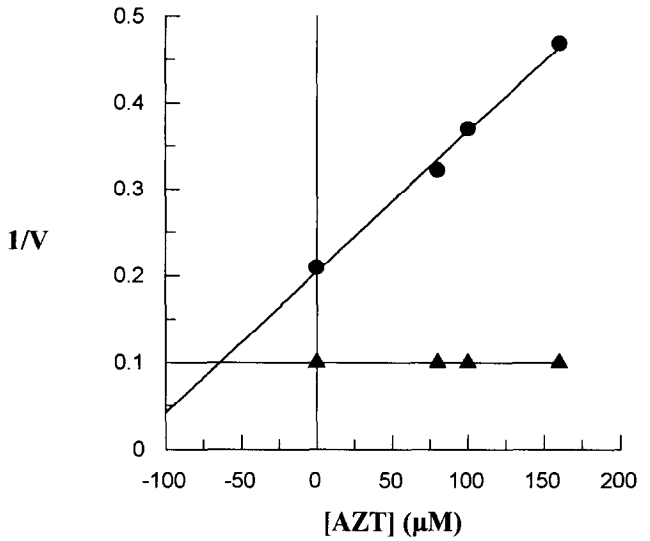

FIG. 4. Dixon plot of the inhibition by AZT of the ADP/ATP exchange rate in mitoplasts. The efflux of ATP was measured by using 10 (●) and 50 (▲) μM ADP, either in the absence or presence of AZT at the indicated concentrations. The rate V is expressed as nmol of $NADP^+$ reduced/min · mg mitochondrial protein.

tions, could the short-term effect of AZT on mitochondrial metabolism be involved with the delayed effect due to the inhibition of the mitochondrial γ-DNA polymerase (which requires ca. 20 hr [10, 12], with both effects causing a cellular ATP deficiency syndrome. At present, this possibility is still a matter of debate. Nonetheless, our previous work

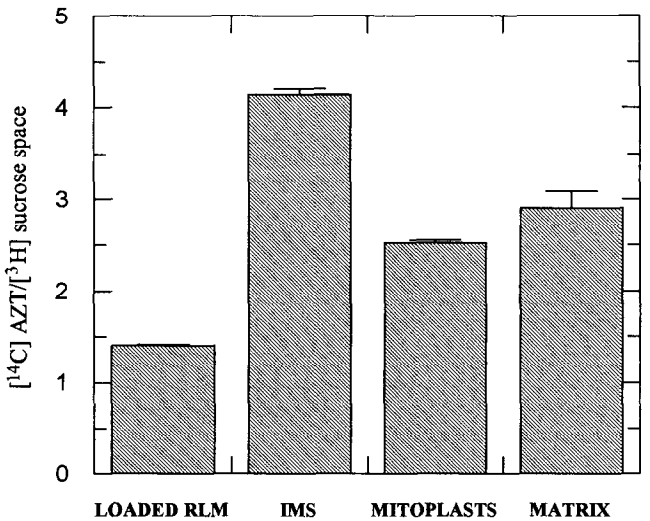

FIG. 5. Subfractionation of [^{14}C]AZT-loaded RLM. RLM (150 mg protein) were loaded with [^{14}C]AZT under the experimental conditions described in Materials and Methods in the presence of [^{3}H]sucrose (0.2 μCi). Both [^{14}C]AZT and [^{3}H]sucrose radioactivity were measured in loaded mitochondria, in the intermembrane space (IMS), in mitoplasts and in the mitochondrial matrix obtained from loaded mitochondria as described in Materials and Methods. The [^{14}C]AZT space/[^{3}H]sucrose space ratio in each fraction was calculated as described in Materials and Methods.

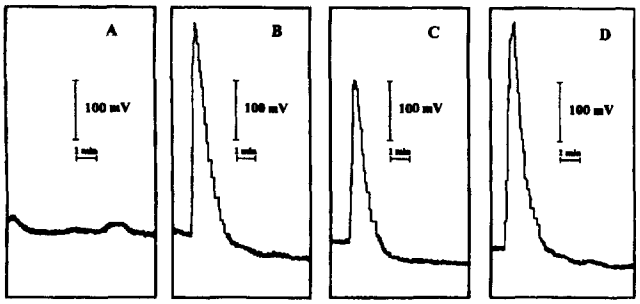

FIG. 6. HPLC measurements of AZT uptake in RLM. The amount of AZT in perchloric extracts of RLM (1.4 mg protein) was measured as described in Materials and Methods. (A) No addition, (B) RLM incubated with 15 μM AZT. (C) RLM incubated with 15 μM AZT in the presence of mersalyl (40 nmol/mg protein). (D) RLM incubated with 15 μM AZT in the presence of 5 μM CAT.

[12] and the present article show that AZT per se can impair mitochondrial energy metabolism, because the activity of both adenylate kinase and ADP/ATP translocator are impaired. In particular, we suggest that in spite of the putative low AZT concentration in cytosol, the capability of mitochondria to accumulate AZT in the intermembrane space could account for a physiological inhibition of ADP/ATP carrier. This is consistent with the significant difference found in the AZT K_i for ADP/ATP antiport inhibition between mitochondria and mitoplasts (18.3 ± 2.9 and 70.2 ± 5.8 μM, respectively). This apparent discrepancy could be explained in terms of the reported drug accumulation in the intermembrane space of mitochondria, with a consequent apparent increase in the inhibitory effect. Such an accumulation cannot occur in mitoplasts.

In conclusion, we propose that AZT causes a significant reduction in the ATP availability in the cytosol owing to AZT inhibition of the ADP/ATP transport, which takes place even though AZT does not use ADP/ATP carrier to enter mitochondria. Thus, the impairment of ADP/ATP translocator, together with the previously shown inhibition of adenylate kinase [12], could well be the biochemical process that exerts an early direct effect on oxidative phosphorylation, which in turn, could be responsible for the ATP deficiency syndrome induced in cells treated with AZT and which is proposed to be a severe AZT side effect in AIDS therapy [8, 9].

If AZT uptake in mitochondria and AZT inhibition of ADP/ATP transport play a significant role in ATP deficiency syndrome, then the discovery of a proper inhibitor of AZT transport in mitochondria, as well as a proper AZT chemical modification that could prevent ADP/ATP carrier impairment and/or uptake into mitochondria could be valuable goals for further research.

The authors thank Dr. Steve Reshkin for his critical revision of the manuscript. The skillful technical assistance of Mr. Vito Giannoccaro is gratefully acknowledged. This work was partially financed by C.N.R. (95.02118.CT04) and M.U.R.S.T. (40% funding).

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