Inhibition of nucleoside diphosphate kinase in rat liver mitochondria by added 3′-azido-3′-deoxythymidine

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Abstract The effect of 3′-azido-3′-deoxythymidine on nucleoside diphosphate kinase of isolated rat liver mitochondria has been studied. This is done by monitoring the increase in the rate of oxygen uptake by nucleoside diphosphate (TDP, UDP, CDP or GDP) addition to mitochondria in state 4. It is shown that 3′-azido-3′-deoxythymidine inhibits the mitochondrial nucleoside diphosphate kinase in a competitive manner, with a K_i value of about 10 μM as measured for each tested nucleoside diphosphate. It is also shown that high concentrations of GDP prevent 3′-azido-3′-deoxythymidine inhibition of the nucleoside diphosphate kinase.

Key words: Mitochondrion; 3-Azido-3′-deoxythymidine; Nucleoside diphosphate kinase

1. Introduction

A large body of evidence shows that mitochondria are selective cell targets of 3′-azido-3′-deoxythymidine (AZT), with many dramatic structural and functional changes described, induced by both long-term and short-term AZT treatment (for a review see [1]). Although it is commonly accepted that mitochondria-AZT interaction is the principal cause of AZT cytotoxicity, the elucidation of the processes leading to the impairment of the energy metabolism, i.e. the ATP deficiency syndrome [2], remains far from being exhaustively clarified. Thus, the identification of the AZT mitochondrial molecular targets is still a suitable goal to pursue. In this regard, we have recently found that adenylate kinase [3] and the ADP/ATP carrier [4] are strongly inhibited by AZT in rat liver mitochondria (RLM) isolated in vitro.

This work deals with the effect of AZT on the mitochondrial nucleoside diphosphate kinase (NDPK) (EC 2.7.4.6), the enzyme which plays a crucial role in the regulation and the channelling of intracellular energy phosphate metabolites [5], being an important link between the cellular pool of ATP, maintained by catabolic metabolism, and the NTP utilised for various intracellular anabolic reactions.

In RLM, NDPK is mainly localised in the intermembrane space [6], where it catalyses the reversible transfer of the terminal phosphoryl group of ATP or an other NTP to NDP acceptors. Thus, it seems worthwhile to find out whether AZT interaction with the mitochondrial enzyme can occur. In order to achieve this, experiments were made aimed at ascertaining whether and how externally added AZT can affect NDPK as measured in coupled RLM in vitro by using separately TDP, UDP, CDP and GDP as phosphate acceptor for newly synthesised ATP via oxidative phosphorylation.

We show that AZT is a potent competitive inhibitor of the mitochondrial NDPK in isolated RLM and that externally added GDP can prevent AZT inhibition.

2. Materials and methods

All chemicals were from Sigma Chemical Co. (St. Louis, MO, USA). Mitochondrial substrates were used as Tris salts at pH 7–7.3.

The RLM were isolated as described [4] from male Wistar rats, (200–250 g) fed ad libitum and suspended in the standard medium containing 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.25. Mitochondrial preparations showing a respiratory control index (RCI) lower than three were discarded. Mitochondrial protein was determined according to Waddel and Hill [7].

Oxygen uptake measurements were carried out at 25°C by means of a Gilson 5/6 oxygraph using a Clark electrode. RLM (2 mg protein) were added to 2 ml of the respiration medium consisting of 210 mM mannitol, 70 mM sucrose, 0.1 mM EGTA, 20 mM Tris-HCl, 5 mM potassium phosphate (pH 7.4), 3 mM $MgCl_2$ in the presence of 2 μg rotenone. 5 mM succinate was used as a respiratory substrate and 160 μM ADP was used to induce state 3 respiration.

The NDPK reaction was assayed by measuring the stimulated respiration rate (SRR) due to TDP, UDP, CDP or GDP addition (up to 150 μM) to RLM at the completion of state 3/state 4 transition [6]. The SRR was calculated as the difference between the NDP_{rate} and the state 4 post ADP_{rate}, where the NDP_{rate} refers to the initial rate of respiration obtained when each single NDP was added and state 4 post ADP_{rate} represents the rate of respiration following the state 3 to state 4 transition. Measurements made in triplicate with the same mitochondrial preparation gave a S.D. lower than 3%.

3. Results

In order to investigate the effect of AZT on NDP-dependent SRR in coupled mitochondria, in a typical experiment (Fig. 1A) RLM were added with succinate (5 mM) with the oxygen uptake continuously measured. ADP addition (160 μM) to RLM was found to stimulate the rate of oxygen uptake (RCI higher than 4). At the completion of the state 3/state 4 transition, mitochondria were added with TDP (a), UDP (b), CDP (c) or GDP (d) (50 μM each) either in the absence or in the presence of AZT (15 μM). All the tested NDPs were found to stimulate the respiratory rate: thus the SRRs were 23, 26, 10 and 24 ngat. O/min/mg protein for TDP, UDP, CDP and GDP, respectively. Externally added AZT (15 μM) was found to decrease the NDP-dependent

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Abbreviations: AZT, 3′-azido-3′-deoxythymidine; CAT, carboxyatractyloside; NDP, nucleoside diphosphate; NDPK, nucleoside diphosphate kinase; NTP, nucleoside triphosphate; RCI, respiratory control index; RLM, rat liver mitochondria; SRR, stimulated respiration rate

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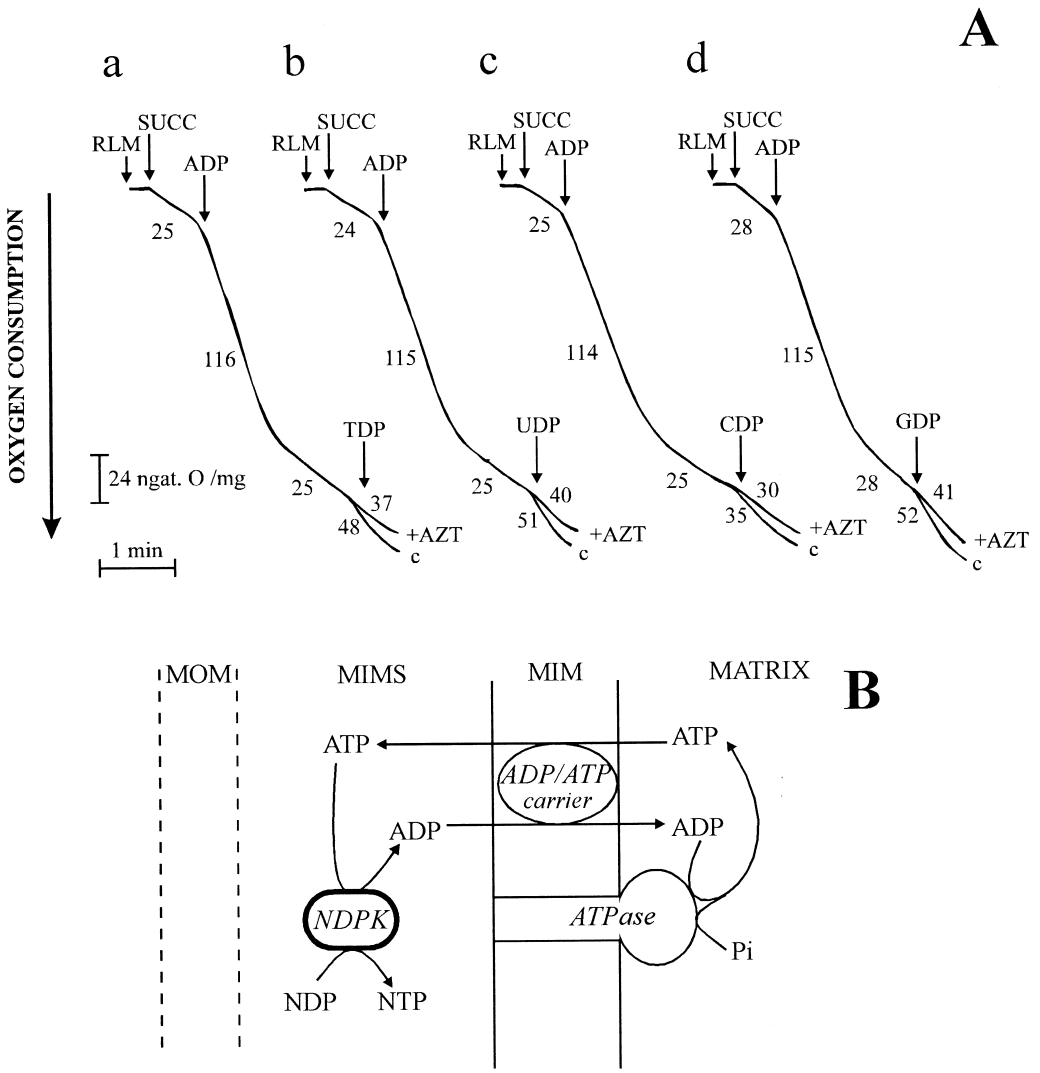

Fig. 1. A: The effect of AZT on the NDP-dependent SRR in isolated coupled RLM. RLM (2 mg of protein) were incubated at 25°C in 2 ml of the respiration medium in the presence of 2 μg of rotenone. At the arrows, the following additions at the indicated concentrations were made: succinate (SUCC) (5 mM), ADP (160 μM) and TDP (a), UDP (b), CDP (c) or GDP (d) (50 μM each). When present, AZT (15 μM) was added to the mitochondrial suspension immediately before the NDP addition. Numbers along the traces indicate the rate of oxygen uptake expressed as ngat. O/min/mg protein. B: Mechanism of stimulation of respiration rate induced by NDP addition to RLM. The scheme reports the three different steps involved in the NDP-dependent SRR.

SRR by 48, 42, 50 and 46% for TDP, UDP, CDP and GDP, respectively. In agreement with [6], in a control experiment, GDP (600 μM) proved to prevent SRR completely (data not shown).

As previously proposed [6], the explanation for the observed NDP-dependent SRR is that the externally added NDP is phosphorylated in the intermembrane space to the corresponding NTP by the effluxed ATP. The newly synthesised ADP enters the matrix, via an ADP/ATP carrier, where it is phosphorylated to ATP, via ATP synthase, with the consequent stimulation of the respiration (Fig. 1B).

Since SRR inhibition due to externally added AZT could be a result of the impairment of the reaction catalysed by NDPK, the ADP/ATP carrier or ATP synthase, the control strength criterion [8] was applied to identify the rate-limiting step of the NDP-dependent SRR under our experimental conditions. Thus, two inhibitors were used: carboxyatractyloside (CAT), the inhibitor of the ADP/ATP carrier and oligomycin, the inhibitor of the ATP synthase. Unfortunately, no inhibitor of the mitochondrial NDPK [9] can be used at present. In a typical experiment (Fig. 2), TDP (150 or 300 μM) was used as a NDPK substrate in the presence of increasing concentrations of CAT, oligomycin or AZT. At 150 μM TDP, no SRR inhibition was found when either CAT (0–0.015 μM) (Fig. 2A, ♦), or oligomycin (0–0.05 μg/mg protein) (Fig. 2B, ▲) was added to RLM, showing that neither the ADP/ATP translocator, nor the ATP synthase can limit the measured SRR, which reflects the reaction catalysed by NDPK. Under these conditions, SRR was inhibited by AZT (Fig. 2C, ●). Obviously, the inhibition observed at increasing CAT or oligomycin concentration is explained by the rate of the ADP/ATP antiport or ATP synthesis, respectively, becoming the rate-limiting step of the measured process. On the other hand, SRR induced by 300 μM TDP was inhibited by CAT (Fig. 2A, ◇), but was insensitive to both oligomycin (0–0.05 μg/mg protein) (Fig. 2B, △) and AZT (Fig. 2C, ○), showing that the activity of the ADP/ATP carrier limits the rate of the TDP-dependent SRR. Similar results were ob-

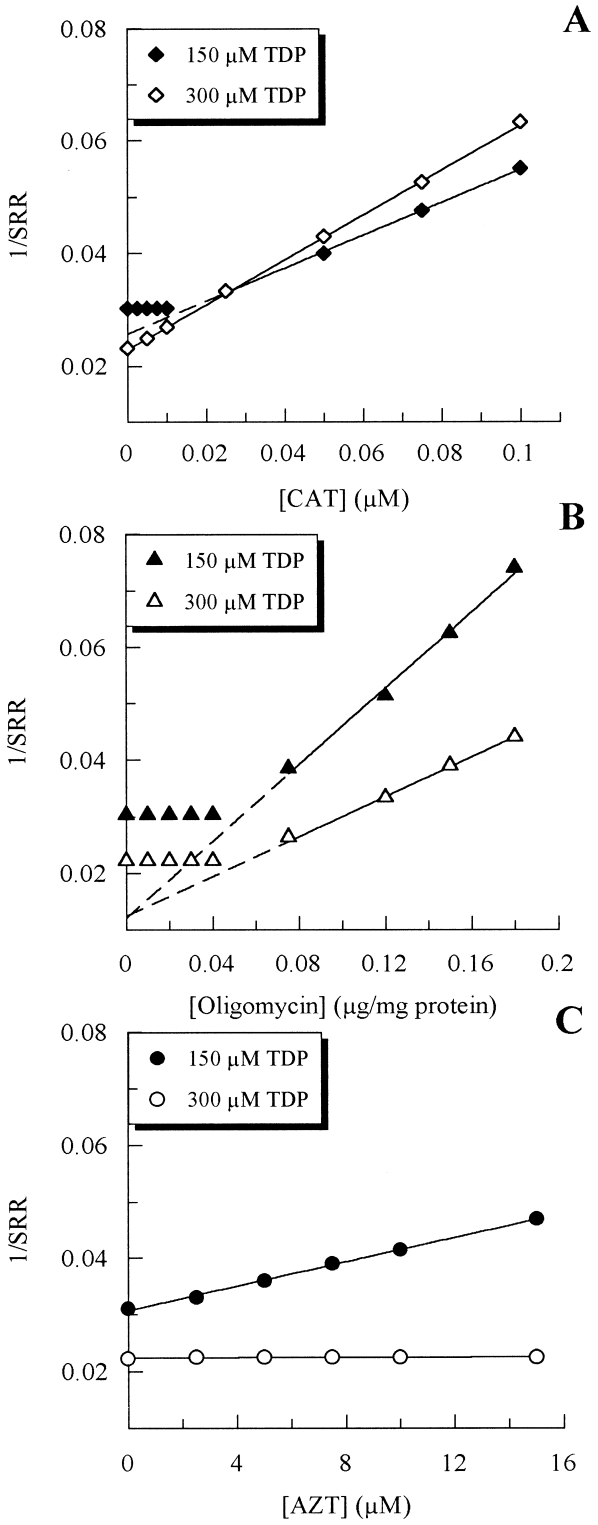

Fig. 2. A Dixon plot of the inhibition by CAT, oligomycin and AZT of the TDP-dependent SRR. RLM (2 mg protein) were incubated under conditions described in the legend to Fig. 1. Either 150 μM or 300 μM TDP were added to RLM in the absence or in the presence of increasing concentrations of CAT (A), oligomycin (B) or AZT (C). TDP-dependent SRR values, measured as described in Section 2 are expressed as ngat. O/min/mg protein.

tained using UDP and CDP (either 150 μM or 300 μM) and GDP as NDPK substrates, as 300 μM GDP per se inhibited the SRR [6], this substrate was used up to 150 μM (data not shown). These results clearly show that AZT decreases the NDP-dependent SRR by inhibiting the NDPK reaction, which can be assayed by using NDP concentrations up to 150 μM. Thus, the dependence of NDP-induced SRR on substrate concentrations was studied by using NDP concentrations up to 150 μM (data not shown). Hyperbolic kinetics were found: mean K_m values of 51 ± 6, 57 ± 6, 103 ± 11 and 36 ± 4 μM for TDP, UDP, CDP and GDP, respectively, were obtained, as calculated in three experiments carried out with different mitochondrial preparations. These values are in fairly good agreement with literature values [6].

The kinetic analysis of AZT inhibition of the mitochondrial NDPK was carried out according to Dixon [10] by measuring the dependence of SRR caused by single addition of TDP, UDP, CDP, or GDP on AZT concentration (0–15 μM) (Fig. 3). AZT was found to inhibit NDPK reaction in a competitive manner, with K_i values of 9.5 (Fig. 3A), 10.0 (Fig. 3B), 9.4 (Fig. 3C) and 9.1 μM (Fig. 3D), in this experiment. A statistical analysis, carried out by comparing results from three different experiments, showed that the K_i values are not statistically different ($P > 0.2$).

In order to distinguish between pure and partial competitive inhibition, the same data were plotted as the reciprocal of the fractional inhibition (*i*) versus the reciprocal of the AZT concentration (see insets in Fig. 3). Pure competitive inhibition was found to occur up to 100 μM NDP, as judged by the ordinate intercept which is equal to 1, while, a partial com-

Table 1
The effect of GDP on the AZT inhibition of NDPK reaction

Substrate	Addition	SRR (ng at. O/min × mg protein)	% $I_{measured}$	% $I_{theoretical}$
TDP (50 μM)	None	28	–	–
TDP (50 μM)	AZT	16	43	43
TDP (100 μM)	None	37	–	–
TDP (100 μM)	AZT	27	27	25
TDP (50 μM)	**GDP**	**41**	**–**	**–**
TDP (50 μM)	**GDP+AZT**	**37**	**10**	**28**
UDP (50 μM)	None	26	–	–
UDP (50 μM)	AZT	14	46	46
UDP (100 μM)	None	35	-	–
UDP (100 μM)	AZT	27	23	23
UDP (50 μM)	**GDP**	**40**	**–**	**–**
UDP (50 μM)	**GDP+AZT**	**33**	**18**	**26**
CDP (50 μM)	None	18	–	–
CDP (50 μM)	AZT	9	50	50
CDP (100 μM)	None	28	–	–
CDP (100 μM)	AZT	17	39	40
CDP (50 μM)	**GDP**	**34**	**–**	**–**
CDP (50 μM)	**GDP+AZT**	**28**	**18**	**24**
GDP (50 μM)	None	29	–	–
GDP (50 μM)	AZT	18	38	38
GDP (100 μM)	**None**	**36**	**–**	**–**
GDP (100 μM)	**AZT**	**26**	**28**	**28**

TDP, UDP, CDP or GDP at the indicated concentrations were added to RLM (2 mg of protein) either in the absence or in the presence of GDP (50 μM). When indicated, AZT (15 μM) was also present. The SRR values, calculated for each single NDP (either in the absence or in the presence of GDP) in the absence of AZT, were taken as 100% of control. $I_{theoretical}$ (expressed in percentage) is calculated according to the equation: $\% I_{theoretical} = (1 - V_i/V_o) \times 100$, where: $V_i = V_{max}$ [NDP]/(K_{mNDP} (1+[AZT]/K_i)+[NDP]) and $V_o = V_{max}$ [NDP]/(K_{mNDP}+[NDP]). The K_m values used in the reported equations were 51, 57, 103 and 36 μM for TDP, UDP, CDP and GDP, respectively and the K_i value used was 10 μM. When GDP was present, the K_m used to calculate $I_{theoretical}$ was taken equal to 36 μM. For further details see the text.

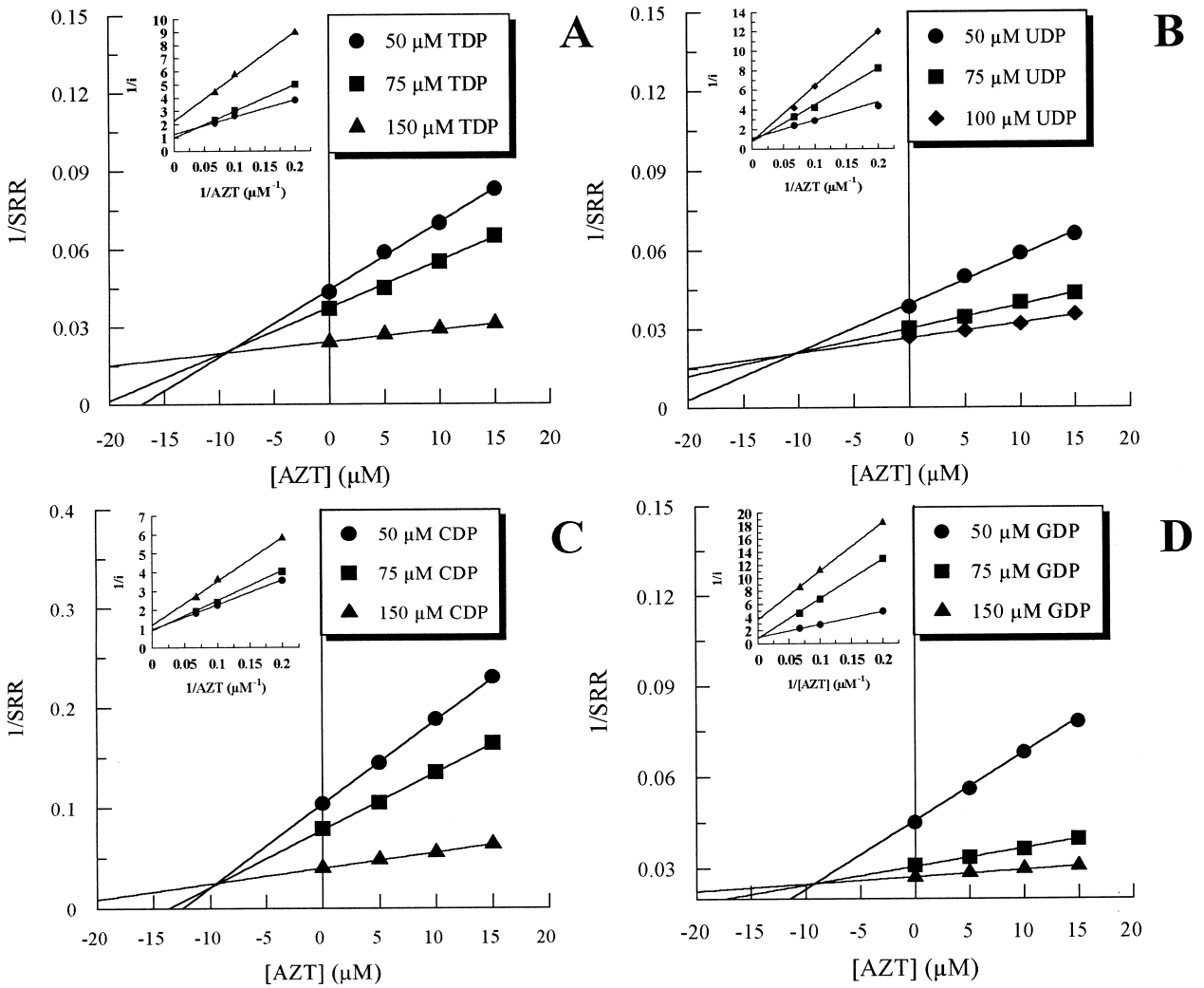

Fig. 3. A Dixon plot of the inhibition by AZT of the rate of NDPK reaction. RLM (2 mg protein) were incubated under the conditions described in the legend to Fig. 1. TDP (A), UDP (B), CDP (C) or GDP (D) was added to RLM in the absence or in the presence of increasing concentrations of AZT. The insets of each panel report the plots of $1/i$ against 1/AZT: where $1 = 1 - SRR_i/SRR_o$, SRR_i and SRR_o being the rates measured in the presence and absence of the inhibitor, respectively.

petitive inhibition was found at the higher NDP concentration used, as demonstrated by the ordinate intercept value which is greater than 1 [11].

In order to identify a possible candidate to prevent AZT inhibition of NDPK, SRRs were measured by using either 50 or 100 μM of each single NDP or 50 μM NDP plus 50 μM GDP. In each case, NDP was added in the absence and in the presence of 15 μM AZT (Table 1). We calculated the theoretical inhibition due to AZT, assuming competitive inhibition, with the above reported K_m and AZT K_i values. When NDP plus GDP were added together as substrates, the lowest K_m value (36 μM) was used, thus accounting for the least inhibition expected in the case of competitive inhibition. The comparison made between the experimental and the theoretical inhibition shows that GDP can somehow prevent AZT inhibition.

In order to further confirm the capability of GDP to prevent AZT inhibition and in the light of the reported capability of GDP to act both as a substrate (up to 150 μM) and as an inhibitor (above 150 μM) of NDPK [6], AZT (15 μM) was added to RLM and SRR was measured as a function of the GDP concentration (Fig. 4). In the 50–150 μM GDP concentration range, competitive inhibition of SRR by AZT was found (see inset in Fig. 4), as in Fig. 3D. On the other hand, in the 150–250 μM GDP concentration range, no inhibition by AZT was observed, thus confirming the capability of GDP to prevent AZT inhibition.

4. Discussion

This paper shows that AZT can inhibit the rate of NDPK reaction in isolated coupled RLM, whatever the NDP used.

The experimental conditions used to assay the NDPK reaction merit a specific discussion. In fact, since NDPK reaction, ADP/ATP transport via the carrier and ATP synthesis via ATP synthase are involved in the observed process, it is necessary to verify that SRR actually reflects NDPK activity. This was made by applying the control strength criterion [8] to the Dixon plots reported in Fig. 2. Our results demonstrated that up to concentrations of 150 μM NDP, SRR was a measure of NDPK reaction. It should be noted that this was not previously considered [6].

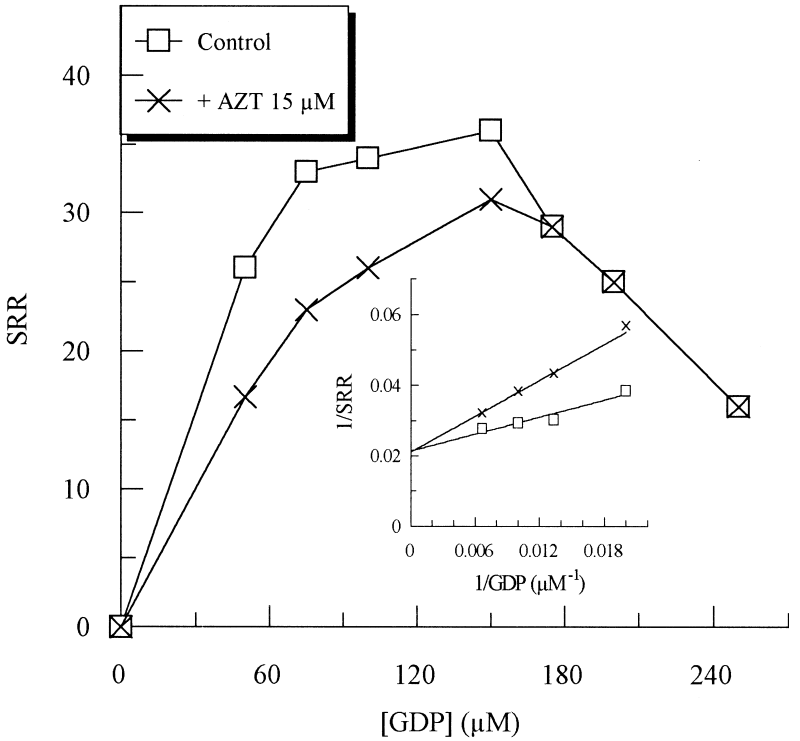

Fig. 4. The dependence of NDPK reaction rate on GDP concentration: effect of AZT. RLM (2 mg protein) were incubated under the conditions described in the legend to Fig. 1. Increasing concentrations of GDP were added in the absence or in the presence of AZT (15 μM). The inset reports the plot of 1/SRR against 1/GDP as measured either in the absence (□) or in the presence (×) of AZT.

The kinetic analysis of the nature of the inhibition by AZT gives some insight into the AZT-NDPK interaction. We show that AZT is a competitive inhibitor of NDPK, with a K_i of about 10 μM, as measured for each NDP used as a substrate. In the light of both the K_i value and the NDP K_m values measured here and, moreover, in the light of the reported AZT accumulation in the intermembrane space [4], we assume that such an inhibition could occur also in vivo. Taking in consideration the physiological role of the mitochondrial NDPK in the NTP production, coupled to oxidative phosphorylation, and in their channelling in various anabolic processes [5], the in vivo AZT inhibition might reduce the cellular availability of the NTP.

Interestingly, the nature of AZT inhibition on NDPK was found to change with the concentration of the NDP used as substrates of NDPK. In fact, up to 75–100 μM NDP, the AZT inhibition of NDPK was pure competitive, making it likely that AZT competes with NDP for the same binding site. In the presence of 150 μM NDP, AZT behaves as a partial competitive inhibitor, thus showing that AZT can bind to another saturable site. Among the tested nucleotides, high concentrations of GDP were found to prevent the AZT inhibition. Such a prevention is consistent with the binding of GDP as a NDPK inhibitor, perhaps to the enzyme site responsible for the partial competitive inhibition by AZT. Thus, we suggest that in the presence of high NDP/NTP concentrations, the NDPK conformation changes, thus allowing GDP regulation in vivo and perhaps AZT inhibition. Although the mechanism of GDP-AZT NDPK inhibition remains to be elucidated, the usefulness of GDP in ATP deficiency syndrome prevention merits further research.

The impairment of the mitochondrial NDPK together with the inhibition of adenylate kinase [3] and the ADP/ATP carrier [4], could be the biochemical process that causes an early damage to the energy metabolism, responsible for the ATP deficiency syndrome induced in cells treated with AZT [2].

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