Different Effect of dGTP on 2′-Deoxyadenosine Metabolism in Mitochondria and Cytosol

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Two enzymes participating in 2′-deoxyadenosine (dAdo) metabolism: dAdo kinase (dAdoK EC 2.7.1.76) and adenosine deaminase (ADA, EC 3.5.4.4) were partially purified from rat liver mitochondria and cytosol and influence of nucleosides and nucleotides on the activity of these enzymes were investigated. Mitochondrial and cytosol dAdoK are separate proteins, while ADA from both subcellular fractions possesses similar physical properties. dGTP, a competitive inhibitor of mitochondrial dAdoK, inhibits cytosol ADA in a mixed way but activates mitochondrial ADA and cytosol dAdoK. A possible effect of dGTP on dAdo metabolism in mitochondria and cytosol is discussed.

Introduction

Two enzymes are directly responsible for dAdo concentration in the cell: dAdoK and ADA. Inherited deficiency of ADA activity in acute lymphoblastic leukemia [1] is associated with high levels of deoxyribonucleoside phosphorylating activity, and low levels of deoxyribonucleotide dephosphorylating activity, as well as with toxic concentration of dATP and immunological disfunction [2, 3]. On the contrary, ADA inhibited normal human peripheral blood lymphocytes excreted dAdo and the peak of dAdo excretion is concomitant with the maximal incorporation of dThd into DNA [4]. Although the mechanism of dAdo toxicity is still unresolved, the inhibition of ribonucleotide reductase by dATP and S-adenosyl-homocysteine hydrolase by dAdo have been proposed as its important effects [5]. Basing on these data we have undertaken a study of nucleoside and nucleotide influence on partially purified dAdoK and ADA obtained from mitochondria and cytosol.

Abbreviations: dAdoK, deoxyadenosine kinase; ADA, adenosine deaminase; dThd, 2′-deoxythymidine; dAdo, 2′-deoxyadenosine; dGuo, 2′-deoxyguanosine; dIno, 2′-deoxyinosine.

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Materials and Methods

Chemicals

U¹⁴C Ado and dAdo were obtained from Amersham Corp. (England). Unlabelled ribo- and 2′-deoxyribonucleosides and their 5′-mono-, di- and triphosphates were purchased from Sigma Chemical Company (U.S.A.) and from Calbiochem-Behring Corp. (U.S.A.). Other reagents used were highest quality commercial products of Pharmacia Fine Chemicals, Whatman, Fluka AG Loba Chemie (Wien), Koch-Light Lab and POCh (Poland).

Enzyme assays

Enzyme activity was measured under optimal conditions at linear kinetics. dAdoK assay was performed as described earlier [6]. For ADA activity assay, the incubation mixture of 0.5 ml final volume contained (in final concentration) 80 mM Tris[(hydroxymethyl)aminomethane]-HCl buffer (pH 8.0), 1 mM Ado/dAdo (0.05 µCi per sample) and purified enzyme (30−40 µg per sample). After incubation at 37 °C for 40 min, the reaction was stopped by immersion of the tubes for 2 min in boiling water and 100 µl of the supernatant ob-

tained after removal of denatured protein was spotted on Whatman DE 81 chromatography paper. Ado or dAdo were separated from Ino or dIno in 2 mM ammonium formate (with R_f for Ado, dAdo, Ino and dIno of 0.58, 0.27, 0.24 and 0.08, respectively), and spots corresponding to standards were counted in an LKB liquid scintillation counter. K_m values were calculated from eight substrate concentrations using equations described in [6]. K_i values were calculated according the same method after the kind of inhibition had been determined (each value of K_m and K_i: the mean ± S.D. for four experiments).

Enzyme preparation

dAdoK purification was performed as described previously [7]. Partially purified ADA was obtained according to Tsukada and Yoshino method [8] with our own modifications as follows: mitochondria from livers of 15 female Wistar rats, isolated as described in [7] were suspended for 8 h in 25 mM Tris-HCl buffer (pH 7.4) containing 25 mM KCl and 5 mM $MgCl_2$ (buffer A) and sonicated (MSE ultrasonic disintegrator). Disrupted mitochondria were centrifuged (105000 × g for 60 min) and the supernatant was heated at 65° for 8 min. The precipitate was discarded (15000 × g for 40 min) and $CaCl_2$ (to the final concentration of 3 mM) and $(NH_4)_2SO_4$ (to give 65% saturation) were added to the supernatant. The precipitate was removed by centrifugation (15000 × g for 40 min), dissolved in 50 mM borate buffer (pH 7.6), containing 2 mM $CaCl_2$ (buffer B) and dialyzed overnight against buffer B. Dialyzed solution was applied to DEAE cellulose column (0.9 × 30 cm) and the protein was eluted from the column with linear gradient of NaCl (from 0.12 M to 0.4 M) in buffer B. The fractions with ADA activity, concentrated by dialysis against Ficoll, were then transferred to Sephadex G-150 column (2.5 × 40 cm). The protein was eluted by buffer B containing 0.15 M NaCl and fractions with ADA activity were concentrated as above. The cytosol ADA obtained from 10% (v/w) liver homogenate in buffer A, as a result of postmitochondrial supernatant centrifugation (105000 × g for 60 min) was prepared under the same conditions. Polyacrylamide gel electrophoresis, gel isoelectric focusing and molecular weight determination by gel filtration in Sephadex G-150 were carried out as described previously [7]. Protein concentrations were determined as in [9], with bovine serum albumin as standard.

Results and Discussion

dAdoK from mitochondria and cytosol was purified 932-fold and 290-fold, respectively; ADA from the mentioned subcellular fractions was purified 927-fold and 1022-fold. The apparent molecular weight of dAdoK was estimated to be 40 kDa for mitochondrial enzyme and 78 kDa for the cytosol enzyme. ADA from both mitochondria and cytosol have mol. wt. of 34 kDa. pI values were found to be 6.7 ± 0.2 and 7.7 ± 0.3 for mitochondrial and cytosol dAdoK, respectively, and 5.2 ± 0.2 for mitochondrial and cytosol ADA. dAdoK and ADA showed Michaelis-Menten kinetics. K_m of mitochondrial and cytosol dAdoK were obtained at 8.5 ± 1.8 μM and 750 ± 167 μM, respectively, and at 30.8 ± 6.0 μM and 28.7 ± 5.8 μM for, correspondingly, mitochondrial and cytosol ADA (using dAdo as substrate).

Influence of nucleosides and nucleotides on both mitochondrial and cytosol enzymes and character of changes of dAMP synthesis and dAdo deamination in presence of dGTP, are presented in Table I and Fig. 1. Using the compounds indicated, only dGTP modified dAdo metabolism in mitochondria and cytosol, however the influence of dGTP on dAdo phosphorylation and dAdo deamination is quite different. In mitochondria dGTP strongly competitively inhibits dAMP synthesis (K_{mi}/K_m = 502, reduction of dAdo phosphorylation by 97.5%, Fig. 1a, a_1, Table I), and activates dAdo deamination (activation of dAdo deaminase activity by 44% (Fig. 1c, Table I). In cytosol, on the contrary, dGTP activates dAdo phosphorylation by 57% (Fig. 1b, Table I) and inhibits dAdo deamination by mixed inhibition (reduction of dAdo deamination by 64%, Fig. 1d, Table I). Activating effect of dGTP on dAdo deamination in mitochondria is probably connected with changes of conformation of the catalytic centre of ADA, without a similar effect of dAMP synthesis in cytosol (different K_m value in presence of dGTP for mitochondrial dAdo deaminase, Fig. 1c; lack of changes of K_m value with dGTP for cytosol dAdo kinase, Fig. 1b).

The cells either utilize the anabolic way of phosphorylating dAdo or reduce the concentration of
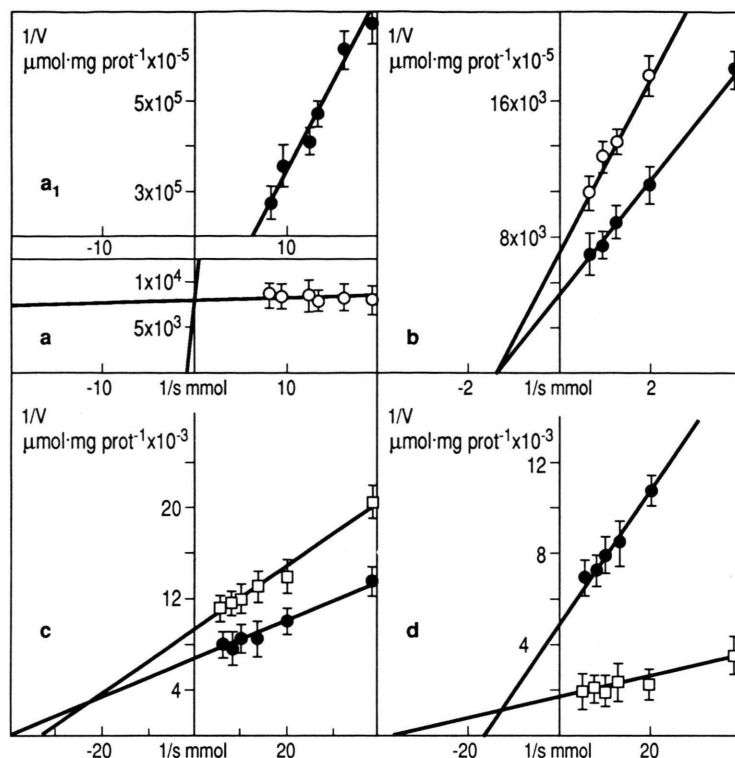
Fig. 1. Lineweaver-Burk plots of dAdo kinase ($-\bigcirc-\bigcirc-\bigcirc-$) and dAdo deaminase (ADA, $-\square-\square-\square-$) activities in mitochondria and cytosol in presence dGTP ($-\bullet-\bullet-\bullet-$), 932- and 290-fold purified mitochondrial and cytosol dAdo kinase (spec. activity 13 μmol × mg prot.$^{-1}$ × 10^{-5} and 14 μmol × mg prot.$^{-1}$ × 10^{-5}, respectively) and 927- and 1022-fold purified mitochondrial and cytosol dAdo deaminase (spec. activity 107 μmol × mg prot.$^{-1}$ × 10^{-3} and 586 μmol × mg prot.$^{-1}$ × 10^{-3}, respectively) were used in experiments. Each line was calculated for six substrate concentrations at constant dGTP concentration (0.1 mmol). Four of five substrate concentrations were marked in some parts of the figure due to the used scale. Each point of the line − the mean ± S.D. for four experiments.

a Inhibition of mitochondrial dAdo kinase activity by dGTP. Line of the enzyme activity without inhibitor, crosses X-axis at value −118 (which points to $K_m = 8.47 \times 10^{-3}$ mmol); line in presence of dGTP crosses X-axis at value −0.23 (which points to $K_{mi} = 4.25$ mmol). Both straight lines cross Y-axis at value 7690 (which points to V_{max} value 13 μmol × mg prot.$^{-1}$ × 10^{-5}). Course of the line indicates a competitive type of inhibition.

a_1 Rest of straight line of enzyme activity in presence of dGTP. The scale of Y-axis 20 times smaller then for the Y-axis in a, caused a different slope of the line.

b Activation of cytosol dAdo kinase activity by dGTP. The increase of the enzyme activity has no effect on K_m value but almost twice increases the V_{max} value.

c Activation of mitochondrial dAdo deaminase activity by dGTP. The increased enzyme activity is accompanied by a decrease of K_m value and rise of V_{max} value.

d Inhibition of cytosol dAdo deaminase activity in presence of dGTP. Inhibition of the enzyme activity proceeds with twice increased K_m value and three times diminished V_{max} value, which suggests a mixed type of inhibitione.
Regression equations of the enzyme activity lines without and in presence of dGTP are: a, a_1: y = 19.5 × + 8298 and y = 33286 × + 105; b: y = 4700 × + 8680 and y = 3439 × + 4590; c: y = 0.32 × + 8.8 and y = 0.11 × + 7.6; d: y = 0.027 × + 2.03 and y = 0.24 × + 5.03.

Table I. Effect of nucleosides and nucleotides on mitochondrial and cytosol dAdo kinase and dAdo deaminase activity. The compounds (0.1 mM) were tested using dAdo as substrate (0.1 mM and 1.5 mM for mitochondrial and cytosol dAdo kinase, respectively), and 0.1 mM for both mitochondrial and cytosol dAdo deaminase.

| | dAdo kinase [μmol × mg protein^{-1} × 10^{-5}] | | dAdo deaminase [μmol × mg protein^{-1} × 10^{-3}] | |
	Mitochondria	Cytosol	Mitochondria	Cytosol
Cytosol	13.0 ± 1.1	14.2 ± 1.2	107 ± 7.6	586 ± 37
dGuo	0.14 ± 0.0013 $K_i = 0.1$ (comp.), p = 0.001	0.5 ± 0.04 $K_i = 2$ (comp.), p = 0.001	104 ± 7.0 (NS)	510 ± 30 (NS)
dGMP	4.6 ± 0.6 $K_i = 4.8$ (comp.), p = 0.001	15.2 ± 1.4	123 ± 8.8 (NS)	588 ± 37
dGTP	0.33 ± 0.03 $K_i = 0.2$ (comp.), p = 0.001	22.3 ± 1.8 (act. 37%), p = 0.005	154 ± 9.2 (act. 44%), p = 0.005	212 ± 15 $K_i = 87$ (m. inh.), p = 0.001
GTP	11.2 ± 0.7 (NS)	4.7 ± 0.3 $K_i = 33$ (comp.), p = 0.001	109 ± 7.6 (NS)	570 ± 3.1 (NS)
dATP	5.3 ± 0.4 $K_i = 79$ (m. inh.), p = 0.001	5.2 ± 0.4 $K_i = 122$ (m. inh.), p = 0.001	112 ± 8.0 (NS)	515 ± 28 (NS)

Each value: The mean ± SEM for four experiments. The values of p were calculated using Student's test. NS-mean non-significant (*i.e.* p > 0.05). K_i represents μmol. act., activation; comp. or m. inh. indicates competitive or mixed inhibition.
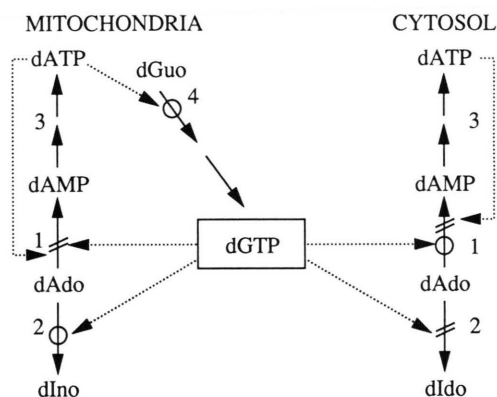

Fig. 2. Relations between dGTP and dAdo kinase/deaminase activity in mitochondria and cytosol.
1-deoxyadenosine kinase, 2-deoxyadenosine deaminase (ADA), 3-dAMP/dADP kinases, 4-deoxyguanosine kinase.
⟶ metabolic pathway, ·····▶ ⊖ activation, ·····▶ ≠ inhibition.

dAdo in a catabolic process in which ADA is the main enzyme [10, 11] to protect themselves against the accumulation of toxic dAdo [12]. Beside dAMP synthesis following dAdo phosphorylation, dAMP formed during dATP catabolism is dephos-

phorylated to dAdo which, in turn, is deaminated to dIno by ADA [13]. It helps to understand a different effect of dGTP upon deoxyadenosine phosphate synthesis in mitochondria and cytosol. In mitochondria dGTP prefers catabolism of dAdo by simultaneously activating ADA and inhibiting dAdo kinase. In cytosol, on the other hand, dGTP privileges dAdo anabolism by activating dAMP synthesis and decreasing dAdo deamination (Fig. 1 b, d, Table I). In case of dGTP deficiency in mitochondria, the synthesis of dATP is elevated, which by activating dGuo kinase [14] and increasing dGTP concentration restores the relation showed in Fig. 2. It is a possible protective mechanism of mitochondria against the effects of toxic dATP accumulation. dGTP deficiency in cytosol changes both − activation of dAMP synthesis and inhibition of dAdo deamination to a comparable extent (Fig. 2, Table I). It prompts suggestions that dIno synthesis privilege is one of the mechanisms by which the changes of dGTP concentrations in cytosol are used in the regulation of toxic dATP concentration. The analysis of mechanisms, controlling the accumulation of toxic dAdo and dATP in mitochondria and cytosol and connected with changes of dGTP concentrations, should also

comprise the fact that dAMP synthesis is suppressed by dATP by a feedback inhibition (Fig. 2, Table I). Quantitatively the effect is similar both in mitochondria and cytosol (K_{mi}mit/K_{mi}cyt = 0.64, Table I). However, it concerns mainly cytosol, where dGTP activates dAMP synthesis and not mitochondria, where dGTP increases dAdo deamination (Fig. 1 b, c).

It is striking that mitochondrial and cytosol ADA, having the same physical properties, behave completely differently in the presence of dGTP.

The fact of stoichiometric quantity of ADA basic and acidic amino acids in ADA [15] capable of producing electrostatic bindings between their side chains suggests that different sensitivity of mitochondrial and cytosol ADA on dGTP is the consequence of a different tertiary structure of the enzyme. Although this argumentation may seem too speculative, further studies will confirm the distinctions between mitochondrial and cytosol ADA.

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