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PHOSPHORYLATION OF ARABINOSYL GUANINE BY A MITOCHONDRIAL ENZYME OF BOVINE LIVER

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Abstract—Extracts of mitochondria isolated from bovine liver were shown to phosphorylate araG, forming araGMP as the sole product. When other nucleosides were used as competitors with araG as the substrate for phosphorylation, deoxycytidine, deoxythymidine and guanosine were not significantly inhibitory. However, the phosphorylation of araG was blocked by deoxyguanosine, deoxyadenosine and deoxyinosine. Deoxyguanosine was shown to be a competitive inhibitor of araG phosphorylation (apparent K_i for deoxyguanosine = 9 μM; apparent K_m for araG = 66 μM). Likewise, araG was determined to be a competitive inhibitor of mitochondrial deoxyguanosine kinase activity (apparent K_m for deoxyguanosine = 16 μM; apparent K_i for araG = 55 μM). These data suggest that the two nucleosides were phosphorylated by the same enzyme. Disc gel electrophoresis showed that the phosphorylating activity for araG migrated with deoxyguanosine kinase activity. The pH profiles of the araG and deoxyguanosine kinase activities were dissimilar. The optimum pH for deoxyguanosine kinase was 5.5; for araG kinase, it was 8.0. Collectively, these data suggest that araG is phosphorylated by mitochondrial deoxyguanosine kinase; however, separate forms of the enzyme or different reaction conditions may be involved in the optimal activities of the two catalytic events.

The purine nucleoside analog, 1-beta-D-arabinofuranosylguanine (araG†) is a drug which is being investigated currently as a potential therapeutic agent for disorders of lymphoid cells. This drug is believed to function in an analogous fashion to araC which is used to treat adult acute myelogenous leukemia [1]. In the case of araC, it is thought that the nucleoside is converted to araCTP which is incorporated subsequently into DNA. Once incorporated, the abnormal nucleoside retards further replication [2–7].

Using lymphoid cells, the administration of araG leads to the formation of araGTP. Interestingly, it was demonstrated that T cells are more sensitive than B cells to araG treatment [8–10]. It was also shown that these two cell types accumulate araGTP differently. T cells were observed to concentrate araGTP to a much greater extent than B cells. These reports suggest that the metabolism of araGTP, both its synthesis and degradation, may differ in the two cellular populations.

Shewach *et al.* [11] isolated six clones of MOLT-4 T lymphoblasts which are resistant to araG, but not to araC. Even though the metabolism of araC and araG was not defined in the study, this difference in resistance between the two strains implies that the metabolism responsible for the synthesis and/or degradation of araG is distinct from that for araC. One of those clones, 24B3, accumulated less than 10% of araGTP compared to wild type MOLT-4 cells; thus, the toxicity of araG treatment is thought to be due to the accumulation of araGTP.

The metabolic steps by which araG is converted to araGTP are not firmly established. Fridland and Verhoef [9, 10] have suggested that araG is phosphorylated by deoxycytidine kinase in both cell types, but whether or not this is the only way by which the transformation takes place is unknown. The data of Shewach *et al.* [11] and Shewach and Mitchell [12] suggest that a kinase separate from deoxycytidine kinase may be important for the phosphorylation of araG, but the details of how araGMP was transformed into araGTP were not defined.

This report documents that a mitochondrial enzyme, deoxyguanosine kinase, also has the ability to phosphorylate araG. This new understanding opens the door to further studies on araG metabolism and suggests that the cellular metabolism of araG may involve catalytic activities other than the cytosolic enzyme, deoxycytidine kinase.

EXPERIMENTAL PROCEDURES

Materials. AraG and araGTP were purchased from Calbiochem (La Jolla, CA). [^{3}H]AraG was a gift of the Burroughs-Wellcome Co. (Research Triangle Park, NC). Apyrase and all nucleosides and nucleotides were from the Sigma Chemical Co. (St Louis, MO). PEI-cellulose was supplied by Brinkmann (Westbury, NY). All other reagents were reagent grade materials.

Isolation of mitochondria. Mitochondria were isolated from beef liver by the differential centrifugation method of Chappell and Hansford [13]. The homogenization buffer contained 250 mM sucrose, 30 mM HEPES (pH 7.4), 1/2% defatted BSA, 1 mM EGTA; the isolation buffer contained 250 mM sucrose, 5 mM HEPES (pH 7.4), 0.1 mM EGTA; the final rinse buffer contained 150 mM KCl, 5 mM HEPES (pH 7.4), 0.1 mM EGTA. Each step of the

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† Abbreviations: araG, 1-β-D-arabinofuranosylguanine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; EGTA, ethyleneglycolbis(amino-ethylether)tetra-acetate; and PAGE, polyacrylamide gel electrophoresis.

Table 1. Effects of various substrate analogs on the phosphorylation of araG

Added nucleoside	Specific activity (pmole product/min/mg protein)	% Activity
None	51.2	100
dGuo	0	0
Guo	52.7	103
dThd	39.4	77
dCyd	47.1	92
dAdo	19.5	38
dIno	12.8	25

AraG kinase assays were measured using 25 μM [^{3}H]araG (0.25 μCi) and 2 mM ATP–Mg in a buffer, pH 7.0, containing 10 mM HEPES, 250 mM sucrose, and 5 mM KH_2PO_4. Reactions were assayed for 10 min. The concentration of each of the various nucleosides was 75 μM.

isolation was conducted at 4°. Tissue was minced in isolation buffer and, after being rinsed once with homogenization buffer, the tissue was ground with a Dounce homogenizer. The supernatant fraction produced by centrifugation at 700 *g* for 10 min was recentrifuged at 12,000 *g* for 10 min. The resulting pellet was resuspended in the same buffer and the process was repeated one time. This pellet was suspended in isolation buffer and sedimented for 10 min at 12,000 *g*. The mitochondria were then suspended in a final rinse buffer and centrifuged at 18,700 *g* for 10 min. This pellet was suspended in final rinse buffer. Respiratory control ratios for mitochondria isolated this way using glutamate, malate and ADP were routinely measured to be 5 to 7. The mitochondria were frozen, thawed, and sonicated, and the soluble fraction was separated from the particulate by centrifugation. The supernatant fraction was used for enzyme assay.

Assay procedure. The mitochondrial supernatant fraction was diluted so that each assay sample contained 0.2 to 0.4 mg protein. AraG kinase activity was measured by mixing the mitochondrial preparation with 2 mM ATP–Mg, 25 μM [^{3}H]araG (0.25 μCi) in buffer (pH 7.0) containing 10 mM HEPES, 250 mM sucrose, and 5 mM KH_2PO_4. The final volume was 125 μl. In some assays, [^{3}H]dGuo, [^{3}H]dAdo or [^{3}H]dCyd was substituted for [^{3}H]araG. In all cases, the radioactive substrates were purified by HPLC techniques prior to use. Reaction conditions are noted in the figures. Reactions were incubated for 10 min at 37°. After incubation, the reactions were terminated by the addition of 150 μl of cold ethanol and aliquots were spotted on PEI-cellulose. After three washes, each of 1 mM ammonium acetate followed by distilled water, the samples were dried and the remaining nucleotides were quantitated by standard liquid scintillation techniques. Reactions determined this way were linear up to 12 min and 0.5 mg protein.

Identification of product from araG phosphorylation. Nucleotide products resulting from the phosphorylation of araG in extracts of mitochondrial protein were separated and identified by chromatography on PEI-cellulose plates. The method of Schwartz and Drach [14] was used. This procedure separates mono-, di- and triphosphates in LiCl/acetic acid solvents. The migration of separated samples

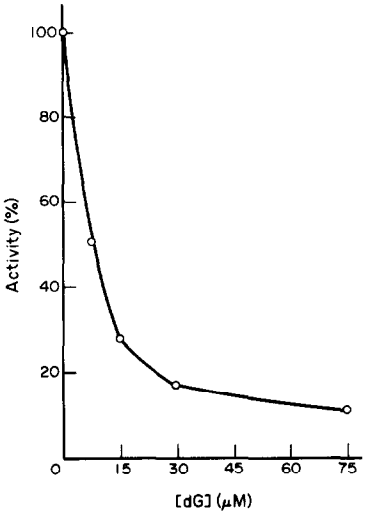

Fig. 1. Effects of deoxyguanosine as an inhibitor of araG kinase activity. The reactions were assayed as described in Experimental Procedures. dGuo was tested as an inhibitor at the indicated concentrations.

was compared to that of authentic standards of araGTP, araGDP and araGMP. The latter two standards were prepared by treatment of araGTP with apyrase.

PAGE separation of kinase activities. Standard procedures for PAGE were employed to separate the various mitochondrial kinase activities. Activities were assayed by incubating 1 mm slices of the gel in incubation media for 1 hr. Aliquots were quantified for phosphorylated products as previously described.

Separation and identification of dIno and dIMP. Products formed from the addition of [^{3}H]dAdo to the PAGE-separated proteins were fractionated on a C_{18} reverse phase column (Waters). A 40-min exponential gradient of initial buffer (0.1 M KH_2PO_4, pH 5.5) to final buffer (0.1 M KH_2PO_4, pH 5.5, with 10% acetonitrite) was run to separate the products. Reaction products were detected by radiation determination on a Radiomatic flow through scintillation counter and identified by comparison to the elution rates of authentic standards.

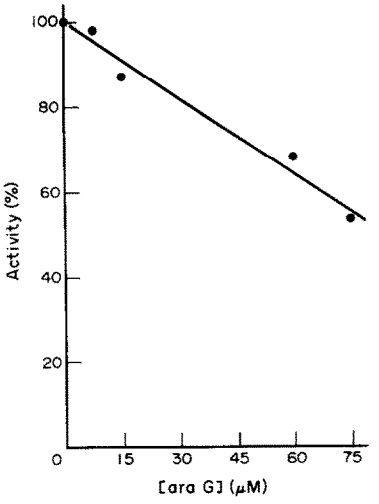

Fig. 2. Effects of araG as an inhibitor of deoxyguanosine kinase activity. Reactions were assayed as described in Experimental Procedures, except that [^{3}H]dGuo was substituted for [^{3}H]araG. Nonradioactive araG was tested as an inhibitor at the indicated concentrations.

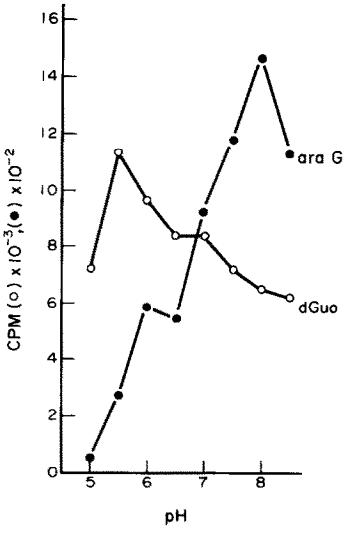

Fig. 3. pH Curve for deoxyguanosine kinase and araG kinase. Samples were assayed using either [^{3}H]dGuo or [^{3}H]araG as noted in the experimental conditions. Tris-acetate buffer (0.1 M) was substituted for HEPES.

RESULTS

When mitochondrial kinase activity was tested using [^{3}H]araG as substrate and the radioactive products were separated on PEI-cellulose, only araGMP was detected. That araGDP and araGTP were not formed was expected, since mitochondrial preparations have been shown previously to produce only dGMP when deoxyguanosine was used as a substrate [15]. Table 1 shows the effects of added nucleosides on the phosphorylation of araG. When competing nucleosides were added in a 3-fold excess of araG, deoxyguanosine completely blocked the phosphorylation of araG. Deoxyinosine and deoxyadenosine additions also caused the loss of araG kinase activity, although the influence was less than that measured for deoxyguanosine.

Since deoxyguanosine seemed to be the most potent inhibitor of araG kinase activity, the degree of its inhibition was tested by a dose–response curve. Figure 1 shows that araG kinase activity was inhibited to the 50% level at a concentration of approximately 7 μM deoxyguanosine. From a companion study of the effects of araG on the phosphorylation of deoxyguanosine, it was observed that araG was much less potent an inhibitor of deoxyguanosine kinase (Fig. 2).

These data imply that araG may be phosphorylated by mitochondrial deoxyguanosine kinase and that araG and deoxyguanosine may be corresponding substrates and competitive inhibitors for that enzyme. To test this hypothesis, Lineweaver–Burk plots of deoxyguanosine kinase activity were constructed. Since it is known that mitochondrial deoxyguanosine kinase has an acidic pH optimum [16] and Fig. 3 shows that the phosphorylation of araG was maximal at pH 8.0, the Lineweaver–Burk experiments were carried out using both acidic and basic conditions for the enzyme assays.

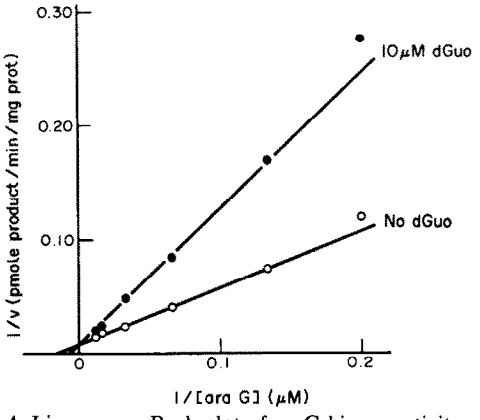

Fig. 4. Lineweaver–Burk plot of araG kinase activity and its inhibition by deoxyguanosine. The reactions were assayed as described in Experimental Procedures.

When araG kinase activity was assayed at pH 8.0 (Fig. 4) in the presence and absence of deoxyguanosine, competitive inhibition was observed. Table 2 compares kinetic constants obtained in this and a subsequent graph. An apparent K_m for araG was measured to be 66 μM and an apparent K_i for deoxyguanosine was calculated as 9 μM. When deoxyguanosine was employed as substrate and assayed at pH 8.0 (Fig. 5), araG was shown to be a competitive inhibitor; apparent K_i = 55 μM and a K_m of 16 μM for deoxyguanosine was measured. When assayed at pH 5.5, similar kinetic data were observed (Table 2).

Kinetic data support the concept that mitochondrial deoxyguanosine kinase is capable of phosphorylating araG. To further support that conclusion, mitochondrial deoxynucleoside kinases were separated by discontinuous gel electrophoresis, after which individual fractions were assayed for kinase activity. Figure 6 shows that kinase activity using

araG, deoxyadenosine and deoxyguanosine migrated at the same rate in the gel. Panel C of the figure shows that the deoxyguanosine kinase activity was inhibited by added araG. With each substrate, the kinase activity appeared as a double peak, or a combination of two isoenzymes. The slower isoenzyme appeared to favor araG as a substrate, whereas the faster isoenzyme appeared to favor deoxyguanosine. Both forms of the enzyme were inhibited by araG when deoxyguanosine was used as substrate.

When deoxyadenosine was used as substrate (panel B), analysis of the reaction revealed that only two products, deoxyinosine and dIMP, were formed. The identity of these products was determined by HPLC analysis using a C_{18} column. Since no dAMP was detected, it was concluded that no or negligible amounts of deoxyadenosine kinase were present at this position in the gel. Because of the presence of deoxyinosine in the reaction products, adenosine deaminase activity must have comigrated with deoxyguanosine kinase activity. That mitochondria possess adenosine deaminase activity has been shown [17]. Thus, deoxyadenosine appears to have been converted to deoxyinosine by the deaminase and this product was, in turn, converted to dIMP by deoxyguanosine kinase activity.

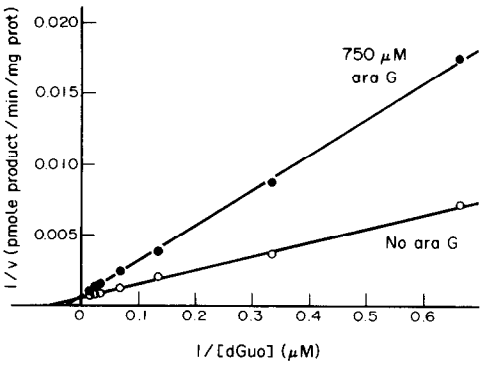

Fig. 5. Lineweaver–Burk plot of deoxyguanosine kinase activity and its inhibition by araG. The reactions were assayed as described in Experimental Procedures, except that [^{3}H]dGuo was substituted for [^{3}H]araG.

DISCUSSION

AraG has been reported to block cell growth in a manner similar to that of araC. The hypothesis is that araG is converted to araGTP and this triphosphate is then incorporated into DNA where it subsequently retards DNA synthesis [1]. The observation that araG is more inhibitory to the growth of T than B lymphoblasts suggests that this compound may have utility in the treatment of T cell lymphoproliferative disorders [12].

To date, not much is known regarding how araG is converted to araGTP, its cytotoxic form. It is important to ascertain this biosynthetic route and to identify the enzymes involved in the catalytic transformations. With this information it would be possible to pursue the development of new analogs of araG which may be even more effective than the original compound. Likewise, these data may provide answers to the question of why the drug is specifically toxic to T cells.

Fridland and Verhoef [9, 10] indicate that araGTP accumulation is greater in T than B cells and is dependent primarily on deoxycytidine kinase activity of both cell types. However, the kinase activity was four to five times higher in B than T cells. Thus, the higher araGTP levels could not be due to kinase activity. Subsequently, the 20- to 40-fold greater accumulation of araGTP in T over B cells was found to be due to the more rapid catabolism of the triphosphate in B lymphoblasts. Whereas Fridland and Verhoef suggested that the lack of catabolism of araGTP in T cells was responsible for the T cell toxic response to araGTP, Shewach and Mitchell [12] reported that no differences in the breakdown of araGTP were observed between araG-resistant and -nonresistant cells. These reports show that the overall metabolism of araG, both anabolic and catabolic, is important to the accumulation of araGTP which is thought to be the toxic form of the drug. However, there appears to be no answer as to which enzymes are the most important in maintaining cellular levels of araGTP.

In an effort to gain new information on the metabolism of araG, this compound was tested as a substrate for other enzymes known to metabolize dGuo and its analogs, namely those of mitochondrial origin. Lymphoid tissues contain few mitochondria, although they do exist in these cells and are possible sites of nucleotide metabolism. However, since it is difficult to isolate sufficient mitochondria from lymphoid cells to conduct the type of enzymatic studies reported here, liver mitochondria were isolated and these organelles provided the source of nucleoside-metabolizing enzymes.

Data reported herein support the conclusion that mitochondria contain an enzyme which phosphorylates araG, forming araGMP. Kinetic, nucleoside competition and PAGE studies suggest that mitochondrial deoxyguanosine kinase is the catalyst which forms araGMP from araG. Since the only product formed via araG phosphorylation by the mitochondrial protein preparation was araGMP, it is not known if mitochondria have the ability to further phosphorylate the compound to produce araGDP and araGTP. Other studies have shown that protein preparations of the type used here produce only dGMP and not dGDP or dGTP when deoxyguanosine is used as a substrate [15]. However, when intact mitochondria are employed, exposure to deoxyguanosine will result in the formation of all three metabolites: dGMP, dGDP and dGTP [15, 16]. It appears that the di- and triphosphorylating activities are lost when the organelle is fractured.

Others have noted that araG is phosphorylated by cytosolic deoxycytidine kinase of lymphoid cells [18]. Here we show by both nucleoside competition and PAGE analysis that mitochondrial deoxycytidine kinase does not participate in araG metabolism.

That the pH profiles for the phosphorylation of araG and dGuo were different and that two isozymes for the phosphorylation of araG and dGuo were observed may indicate that two separate enzymes

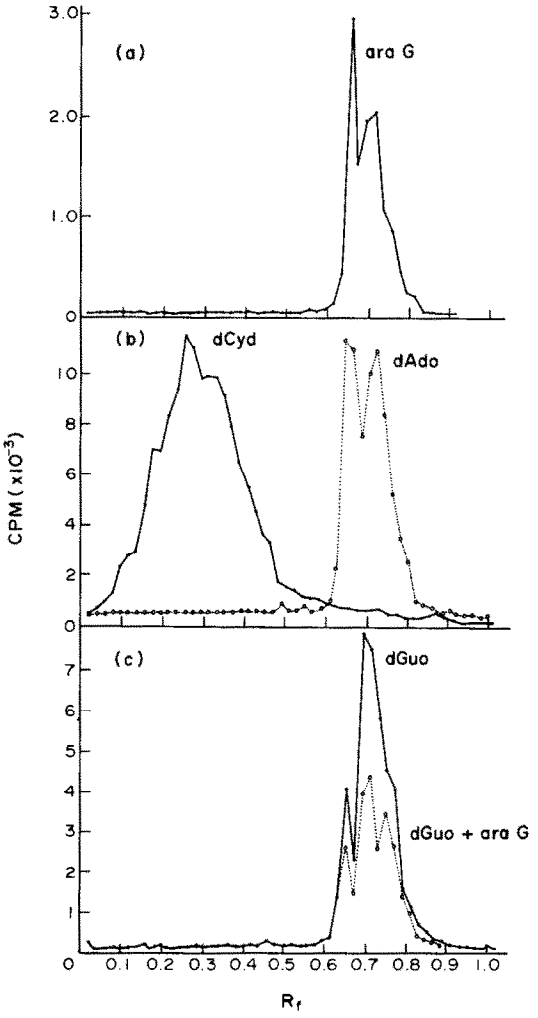

Fig. 6. Disc gel electrophoresis profiles of araG kinase activity and various deoxynucleoside kinase activities. (A) [^{3}H]araG as substrate. Assay was at pH 7.0. (B) Either [^{3}H]dCyd (—●—) or [^{3}H]dAdo (----○----) as substrate. Assay was at pH 8.0. (C) [^{3}H]dGuo (—●—) as substrate. [^{3}H]dGuo as substrate plus araG (750 μM) as inhibitor (----○----). Assay was at pH 5.5. Similar results were obtained (not shown) when the reaction was assayed at pH 8.0.

are involved in the phosphorylation of the two nucleosides. However, both isozymes were shown to metabolize the two nucleosides, but to differing degrees. It should be noted that the apparent K_m values for araG inhibition of dGuo phosphorylation were identical for both pH 5.5 and 8.0. The precise role that each of the isozymes plays in araG metabolism is not clear at this time.

Figure 6 shows that an activity which appears to phosphorylate dAdo also migrates at a rate equal to that of deoxyguanosine kinase. Since the addition of dAdo resulted in the formation of dIno and dIMP as the sole products, i.e. no dAMP was detected, it was concluded that this fraction of the gel contained both adenosine deaminase and deoxyguanosine kinase activities. This would cause dAdo → dIno → dIMP. In another report we documented that mitochondria contain a very active adenosine deaminase and that mitochondrial deoxyguanosine kinase can use dIno as a substrate [17].

Table 2. Kinetic constants derived from Lineweaver–Burk graphs

Substrate	K_m (app) (μM)	K_i (app) dGuo (μM)	K_i (app) araG (μM)	V_{max} (μmol/min/mg protein)
dGuo (pH 5.5)	17		55	2.5
dGuo (pH 8.0)	16		55	2.0
araG (pH 8.0)	66	9		0.13

Kinetic values were determined by linear regression analysis of the data points on Figs. 4 and 5.

That liver mitochondria contain adenosine deaminase and that deoxyinosine is phosphorylated by deoxyguanosine kinase have also been documented by others [19, 20]. These conclusions also explain why dAdo and dIno were inhibitory to araG phosphorylation, as shown in Table 1.

These results provide an additional explanation for the metabolism of araG by eukaryotic cells. The degree to which mitochondrial enzymes function in the pharmacology of araG is not known, but it is the focus of continued efforts.

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