DONOR ACTIVITY OF 5′-O-PHOSPHONYLMETHYL ANALOGUES OF ATP AND GTP IN THE PHOSPHORYLATION OF URIDINE CATALYZED BY URIDINE KINASE FROM MOUSE LEUKEMIC CELLS

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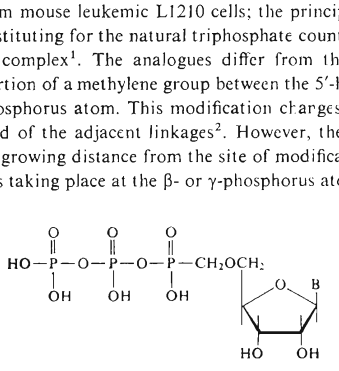
The phosphonate analogues of ATP and GTP can function as phosphate donors in uridine kinase reaction. The K_m constants for ATP and its analogue ATP_c (*I*) are identical, the V_{max} value for ATP is five times higher than that for ATP_c. Also the V_{max} constants with respect to uridine follow the same pattern (250 nmol for ATP, 35·7 nmol for ATP_c). The optimum Mg^{2+} concentration for the phosphonate is 3 times higher compared with ATP.

Recently we have demonstrated that the 5′-O-phosphonylmethyl analogues of UTP and CTP (*III*, *IV*) inhibit the phosphorylation of uridine catalyzed by uridine kinase partially purified from mouse leukemic L1210 cells; the principle of this effect consists probably in substituting for the natural triphosphate counterparts in the formation of the ternary complex[1]. The analogues differ from the natural nucleotide derivatives by an insertion of a methylene group between the 5′-hydroxyl group of the nucleoside and α-phosphorus atom. This modification changes the character of the phosphorus atom and of the adjacent linkages[2]. However, these effects are bound to diminish with the growing distance from the site of modification. For this reason the enzymic reactions taking place at the β- or γ-phosphorus atoms of the nucleotide



I, B = adenin-9-yl
II, B = guanin-9-yl
III, B = uracil-1-yl
IV, B = cytosin-1-yl

phosphonyl analogues might be feasible to at least a limited extent. Consequently, t was of interest to investigate the phosphate transfer reactions mediated enzymatically by substituting the triphosphate analogues *I* and *II* for natural triphosphates which usually function as phosphate donors. Uridine kinase from mouse leukemic cells which requires ATP or GTP as phosphate donors was chosen as a model enzyme or this purpose. The analogues of ATP (ATP_c, *I*) and GTP (GTP_c, *II*) were investigated in two respects: (*a*) in their ability to serve as phosphate donors in the absence of natural triphosphates, and (*b*) in their ability to specifically inhibit the utilization of the "natural" phosphate donors.

MATERIALS AND METHODS

Chemicals. [2-^{14}C]Uridine (2 000 MBq/mmol) was delivered by the Institute for Research Production, Application and Use of Radioisotopes, Prague. Adenosine 5'-triphosphate, cytidine 5'-triphosphate, guanosine 5'-triphosphate and uridine were purchased from Calbiochem, Luzern. The synthesis of compounds *I*, *II* and *IV* was described previously[3]. Uridine kinase fraction from L1210 mouse leukemic cells was prepared and purified as described earlier[1].

Assay of uridine kinase activity. The reaction mixture contained Tris-HCl buffer (pH 7·4) $5 . 10^{-2}$ mol l^{-1}; adenosine (guanosine) 5'-triphosphate or an appropriate analogue, $1 . 10^{-3}$ mol l^{-1}; 2-mercaptoethanol, $5 . 10^{-3}$ mol l^{-1}; Mg^{2+} ions, $1 . 10^{-3}$ mol l^{-1}; [2-^{14}C]uridine, $5 . 10^{-5}$ mol l^{-1} and 100 μg of the enzyme protein in a total volume of 0·3 ml. The mixture was incubated for 20 min at 37°C in a Dubnoff incubator and analyzed as described previously. The formation of the reaction product was linear over the time period studied. Further procedures were described in the preceding paper[1].

HPLC *analysis* was performed on a glass column of Separon SI C18 (5 μm, 3·3 × 150 mm); elution (0·4 ml/min) by 0·1 mol l^{-1} triethylammonium hydrogen carbonate (pH 7·5) containing 5% (v/v) methanol. Sensitivity, 0·16 absorbancy unit per full scale at 254 nm. The identification of the components of reaction mixtures was performed by the internal standard method.

RESULTS AND DISCUSSION

Uridine kinase from mouse leukemic cells catalyzes the phosphorylation of uridine in the presence of the analogues ATP_c (*I*) and GTP_c (*II*) as the only phosphate donors. The time course of this process is demonstrated by Fig. 1. It is evident that the donor activities of both compounds are comparable, although considerably lower than those of ATP or GTP. The formation of UMP has been confirmed using either labelled uridine, or direct HPLC analysis of the incubation mixture. The low overall yield of the reaction with ATP_c does not enable us to ascertain whether the transfer of the phosphate group to the acceptor results in the formation of AMP_c or ADP_c. However, since ATP_c (I) is stable in the presence of the enzyme (omitting the acceptor in the incubation mixture) and also UMP does not undergo any changes in the presence of ATP or ATP_c and the enzyme, we can conclude that UMP is the primary product of the reaction which is due to the transfer of γ-phosphate rele-

ased from the analogue. Although the enzyme preparation contains small amounts of nucleolytic activities splitting to a similar extent ATP to ADP and AMP as well as UMP to Urd this does not substantially affect our results.

All known phosphate displacement reactions require cooperative binding of divalent metal cations[4]. In the uridine kinase reaction, both ATP and ATP_c (I) markedly differ in their response to the divalent metal catalysis. At a constant donor (ATP, ATP_c) concentration (1 mmol l^{-1}), the optimum Mg^{2+} concentration for ATP amounts to 1 mmol l^{-1}, whereas with ATP_c, the optimum concentration is 3 mmol . . l^{-1} Mg^{2+}. Mn^{2+} ions are unable to stimulate the phosphorylation of uridine in the presence of ATP_c (Fig. 2). This effect can be interpreted in terms of an altered structure of the donor molecule: the Mg^{2+}–ATP complexes possess a rather rigid geometry whose formation is determined by the chelation of the cation involving the adenine ring, as well as β- and α-phosphate residues. In case of ATP_c, the involvement of the modified α-phosphate is affected by the electron-positive CH_2 group attached to the phosphorus atom. An increased Mg^{2+} concentration will be required to reinforce the transition state complex with the participation of ATP analogue. However, the loss of chelating forces is irreparable by increasing the Mn^{2+} ion concentration.

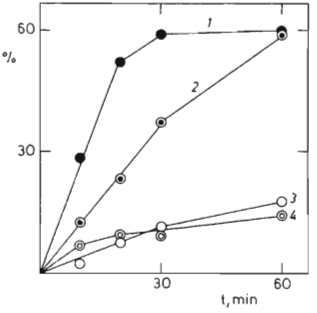


FIG. 1

Time course of uridine kinase activity in the presence of ATP (*1*), GTP (*2*), ATP_c (*3*) or GTP_c (*4*) at $1 . 10^{-3}$ mol l^{-1}. Uridine concentration was set at $5 . 10^{-5}$ mol l^{-1}. The enzyme activity is given in % of newly formed uridine 5'-monophosphate

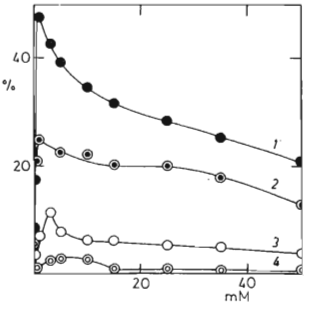


FIG. 2

Uridine kinase activity in the presence of ATP-Mg^{2+} (*1*), ATP-Mn^{2+} (*2*), ATP_c-Mg^{2+} (*3*) or ATP_c-Mn^{2+} (*4*). mM, concentration of Mg^{2+}- or Mn^{2+}-ions; %, enzyme activity

A still more detailed analysis of the situation can be based on the kinetics of the ATP_c-mediated phosphorylation of uridine. Although the donor activity of this compound is inferior to that of ATP (Fig. 1), the K_m constants for both compounds are identical. The values of V_{max}, however, are widely different (Fig. 3, Table I). These findings suggest an identical affinity of both donors for the rest of the ES-complex components and, at the same time, a marked rate difference of the catalyzed reaction. The affinity of the acceptor (uridine) for the systems containing different donors is, as anticipated, nearly identical (Table I). The mutual compatibility of ATP and its analogue, ATP_c (*I*) towards the enzyme is also supported by the inhibitory activity of ATP_c upon the ATP-mediated reaction: this inhibition ($K_i = 7{\cdot}6 \cdot 10^{-4}$ . . mol l^{-1}) has a strict competitive character (Table II).

In the previous communication, we have shown that CTP_c (*IV*) causes a feedback inhibition of uridine kinase[1]. Therefore we have investigated the behaviour of this compound in the system utilizing ATP_c as the donor molecule. The results (Table II)

TABLE I

Michaelis constants of uridine kinase for ATP, ATP_c and Urd

Compound	Second substrate	$K_m \cdot 10^{-4}$ mol l^{-1}	V_{max}, nmol
ATP	Urd	2·56	66·7
ATP_c	Urd	2·56	12·9
Urd	ATP	1·56	250·0
Urd	ATP_c	1·10	35·7

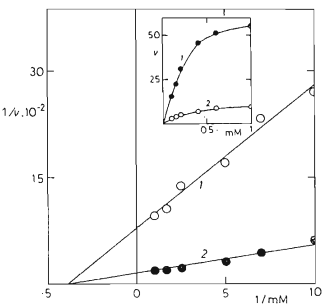


FIG. 3

The Lineweaver–Burk plot with ATP (*1*) or ATP_c (*2*) as phosphate donors. *v*, nmol uridine 5′-monophosphate mg^{-1} of enzyme protein 20 min^{-1}; mM, different concentrations of ATP or ATP_c

TABLE II

K_i constants of uridine kinase for CTP, CTP_c and ATP_c

Inhibitor	Substrate	K_i, mol l^{-1}	Inhibition
CTP	ATP_c	$1{\cdot}80 \cdot 10^{-6}$	competitive
CTP_c	ATP_c	$6{\cdot}40 \cdot 10^{-5}$	competitive
CTP	Urd	$3{\cdot}80 \cdot 10^{-5}$	non-competitive
CTP_c	Urd	$2{\cdot}50 \cdot 10^{-5}$	non-competitive
ATP_c	ATP	$7{\cdot}60 \cdot 10^{-4}$	competitive

demonstrate a competitive character of this inhibition, *i.e.* identical with its effect upon ATP-mediated reaction. The K_i value of this inhibition is thirty times lower compared with the value for the inhibition caused by CTP. On the contrary, the K_i values for CTP and CTP_c (*IV*) with respect to uridine (at a constant ATP_c concentration) are within the same order of magnitude, the process being non-competitive in both cases (Table II); this situation is fully in agreement with our previous findings using natural phosphate donors[1].

Thus, it can be concluded that the α-modified nucleoside 5'-triphosphates of the type *I* and *II* are capable of the phosphate release following the activation by uridine kinase from leukemic cells. The lower yield of the transfer reaction to compare with the natural donor might be due to the altered ability of the analogue to form the proper transition state complex probably by affecting the metal chelating forces involved. This failure, however, merely results in kinetic changes in distinction to other enzymes, namely DNA dependent RNA polymerase which is capable of specific binding of triphosphate analogues *I*–*IV* (ref.[5]). Further study of phosphonyl analogues of different nucleoside 5'-triphosphates would be rewarding in extending our knowledge of various other enzymes catalyzing the transfer of nucleotidyl residues and phosphate groups.

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