INHIBITION BY CTP AND UTP ANALOGUES OF URIDINE KINASE FROM MOUSE LEUKEMIC CELLS

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The new phosphonate analogues of CTP and UTP (CTP_c and UTP_c) inhibit the phosphorylation of uridine catalysed by uridine kinase in the presence of ATP and Mg^{2+}-ions. The inhibition is competititve with respect to phosphate donor, and non-competitive with respect to phosphate acceptor. With respect to uridine the K_i constants for CTP_c and UTP_c are $7{\cdot}5 \cdot 10^{-5}$ mol l^{-1} and $1{\cdot}0 \cdot 10^{-4}$ mol l^{-1}, respectively. With respect to ATP the K_i value for CTP_c ($3{\cdot}6 \cdot 10^{-6}$ mol . l^{-1}) is 3× lower than that for CTP. The novel analogues could be useful for further study of uridine kinase.

In mammalian tissues uridine kinase (ATP : uridine 5′-phosphotransferase, E 2.7.1.48) is essential for the activation of uridine and cytidine[1] and of their analogues[2] which are phosphorylated in the presence of a phosphate donor. The enzyme catalyses the phosphorylation of uridine or cytidine in the presence of ATP and Mg^{2+}-ions to UMP or CMP, and is a part of the salvage pathway of pyrimidine nucleosides. This anabolic transformation is apparently the initial and rate-limiting step in the biosynthesis of the nucleoside 5′-triphosphates from the preformed pyrimidine ribonucleosides[3].

Eukaryotic cells with a long G_1 cell cycle period appear to have a minimal level of uridine kinase acivity, whereas rapidly dividing cells use the enzyme to a significant extent[4]. Since the cells with a high rate of mitosis, *e.g.* different kinds of malignant cells, rely extensively on the salvage route, various uridine and cytidine analogues have been devised in an effort to arrest the malignant cell proliferation by producing metabolic blocks at different points. Consequently, uridine kinase is also of pharmacological interest[2].

It is well known that a number of enzymes are subject to feed-back modulation of their activity by terminal products of the metabolic sequence involved[5]. Thus CTP and UTP have been shown to be inhibitors of uridine-cytidine kinase activity[3]. In this report we describe the effect of new analogues of the natural 5′-triphosphates, 5′-O-diphosphorylphosphonylmethyluridine (UTP_c) (*I*) and/or -cytidine (CTP_c) (*II*) (ref.[6]), on the modulation of the activity of uridine kinase partially purified from L1210 mouse leukemic cells.

EXPERIMENTAL

Chemicals. [2-^{14}C]Uridine (2 000 MBq/mmol) was delivered by the Institute for Research, Production and Uses of Radioisotopes, Prague, adenosine 5'-triphosphate, uridine 5'-triphosphate, cytidine 5'-triphosphate and uridine came from Calbiochem. Luzern. The synthesis of CTP_e and UTP_e was described previously[6]. All compounds were homogeneous with paper and HPLC chromatography and with paper electrophoresis in 0·1M triethylammonium hydrogen carbonate (pH 7·5).

Uridine kinase fraction: Cell-free extract was prepared from L 1210 mouse leukemic cells, washed twice with 10^{-2} mol l^{-1} Tris-HCl buffer (pH 7·4), $1{\cdot}5 \cdot 10^{-1}$ mol l^{-1} KCl, and $5 \cdot 10^{-3}$ mol l^{-1} 2-mercaptoethanol (TKM buffer). The cell suspension was repeatedly subjected to freezing at the temperature of dry ice followed by subsequent thawing. After centrifugation (105 000*g*, 4°C, 60 min) the supernatant (20 mg of protein per ml) was adjusted to 29—38% saturation with ammonium sulfate. The final sediment was dissolved in the TKM buffer, dialysed overnight against 20 volumes of TKM buffer at 4°C under mixing, and the preparation was kept at −70°C. The protein content was about 10 mg of protein per ml. The enzyme activity was stable for at least two months.

Assay of uridine kinase. The reaction mixture was incubated for 20 min at 37°C usually in a total volume of 0·4 ml in a Dubnoff shaker bath, and contained Tris-HCl buffer (pH 7·4), $3{\cdot}75 \cdot 10^{-2}$ mol l^{-1}; adenosine 5'-triphosphate, $7{\cdot}5 \cdot 10^{-3}$ mol l^{-1}; 2-mercaptoethanol and Mg^{2+}-ions, $2{\cdot}75 \cdot 10^{-3}$ mol l^{-1}; [2-^{14}C]uridine, $7{\cdot}5 \cdot 10^{-5}$ mol l^{-1}; and 50 μg of enzyme protein. The reaction was terminated by heating in a boiling water bath for 1 min. Aliquots of the reaction mixture (0·1 ml) were chromatographed on a Whatman paper No 1 in a solvent system composed of ethanol–tert-butanol–formic acid–water (60 : 20 : 50 : 150, *v/v*) with appropriate standards. Chromatographic spots were cut out, and their radioactivity was counted in a liquid scintillation counter (Isocap/300, Searle, Nuclear Chicago Div.). Unless otherwise stated, uridine kinase activity is expressed as nmol of uridine 5'-monophosphate formed in a 20 min period per mg of protein.

HPLC *analysis* was performed on a Spectraphysics liquid chromatograph; column 3·3 × × 150 mm Separon SI C18 (5 μ) (Laboratorní přístroje. Prague), mobile phase 0·1 mol l^{-1} triethylammonium hydrogen carbonate (pH 7·5) containing 2·5% (*v/v*) methanol. Flow rate, 0·4 ml/min, detection at 254 nm (0·016 AUFS). Under the above assay conditions, CTP_e was found unchanged.

RESULTS

The time course of uridine kinase activity and its relation to the amount of the enzyme protein is given in Fig. 1. Only negligible quantities of uracil due to phosphorolysis and of 5'-di- and triphosphates due to contaminating phosphokinases were formed in the presence of the 29—38% saturation fraction. (With crude extracts these compounds arise in considerable amounts[7].)

The inhibitory effect of CTP_e (*II*) on uridine kinase is indicated by the Dixon plot of 1/initial velocity against CTP_e at two fixed concentrations of the phosphate acceptor[8] (Fig. 2). The inhibitor was varied over a 6-fold range, and the concentration of uridine was 100—150-fold less than that of ATP. The plot shows the intercept

on the base line which is indicative of a non-competitive inhibition with respect to uridine, and gives the K_i value of $7{\cdot}5 \cdot 10^{-5}$ mol l^{-1}. The K_i constant for CTP determined simultaneously was $6{\cdot}5 \cdot 10^{-5}$ mol l^{-1} (Table I).

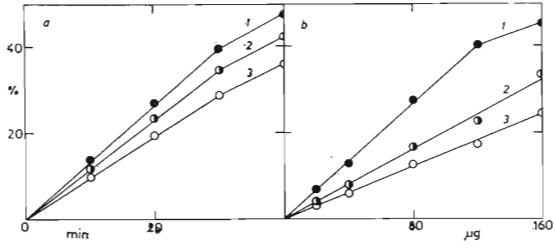

FIG. 1

Time course of uridine kinase activity and its relation to the amount of enzyme protein. *a* 0·1 m of reaction mixture (0·8 ml) was chromatographed directly at given time intervals. *b* reaction mixtures were incubated for 20 min. *1* control, *2* CTP_c $5 \cdot 10^{-5}$ mol l^{-1}, *3* CTP $5 \cdot 10^{-5}$ mol l^{-1}. The enzymic activities are given in %

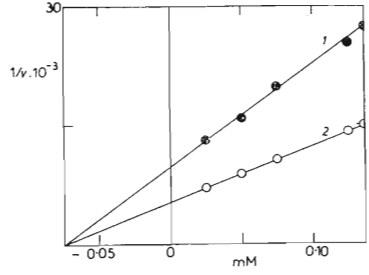

FIG. 2

Inhibition by CTP_c of uridine kinase with respect to uridine. *1* uridine 20 nmol, *2* uridine 30 nmol

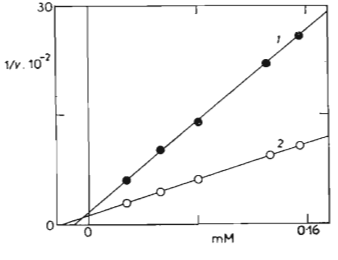

FIG. 3

Inhibition by CTP_c of uridine kinase with respect to ATP. *1* ATP 0·2 μmol, *2* ATP 0·4 μmol

The second compound under study, UTP_c (*I*), was varied over a 3·3-fold range and tested in the same system as CTP_c. In this case, the Dixon plot indicated a non-competitive inhibition with respect to uridine, and the K_i constant of 10^{-4} mol l^{-1}, while the same value for UTP determined simultaneously was $5{\cdot}5 \cdot 10^{-5}$ mol l^{-1} (Table I). The character of inhibition for UTP was also non-competitive.

However, the inhibition of uridine kinase by CTP_c at different concentrations of ATP was a competitive process with the K_i constant of $3{\cdot}6 \cdot 10^{-6}$ mol l^{-1} (Fig. 3) whereas the corresponding value for CTP amounted to $1{\cdot}1 \cdot 10^{-5}$ mol l^{-1} (Table I). The competitive inhibition of ATP by CTP_c suggested that the latter compound might function as a phosphate donor; this, however, was clearly excluded in an experiment in which different concentrations of CTP_c instead of ATP were added to the usual reaction mixture; under these conditions no formation of UMP was observed. At the same time, HPLC analysis of the incubation mixture confirmed that no dephosphorylation of CTP_c takes place in the presence of the enzyme with or without uridine.

Table I indicates the K_i constants for CTP, UTP, CTP_c and UTP_c with respect to uridine and ATP as well as the K_m constants for both substrates. The K_m constants were determined by different authors for the enzyme from P815 mouse tumour cells[9], Novikoff rat hepatoma[4], AKR leukemic cells[7], Ehrlich ascites cells[10,11,12], calf thymus[13] and a ciliate protozoa *Tetrahymena pyriformis*[14]; for ATP, the values were within the range of $3{\cdot}6 \cdot 10^{-3}$ mol l^{-1} in P815 neoplasma and $5 \cdot 10^{-5}$ mol l^{-1} in Ehrlich ascites cells. Our value (Table I) is close to the value of $1{\cdot}5-3{\cdot}1 \cdot 10^{-4}$ mol $\cdot l^{-1}$ found in the Novikoff rat tumour. The K_i constants previously measured for CTP with respect to phosphate acceptor were estimated in AKR mouse leukemic cells[7] ($7{\cdot}9 \cdot 10^{-5}$ mol l^{-1}) and in *Tetrahymena*[14] ($6{\cdot}3 \cdot 10^{-5}$ mol l^{-1}); the value for CTP_c obtained with L1210 mouse leukemic cells (Table I) is in agreement with these data. It is also evident that UTP_c is the least efficient inhibitor with respect to uridine ($K_i = 10^{-4}$ mol l^{-1}).

TABLE I

Michaelis constants and inhibition constants of uridine kinase for CTP, TUP and their analogues

Substrate		Uridine	ATP
K_m, mol l^{-1}		$1{\cdot}56 \cdot 10^{-4}$	$2{\cdot}56 \cdot 10^{-4}$
K_i, mol l^{-1}	CTP	$6{\cdot}50 \cdot 10^{-5}$	$1{\cdot}10 \cdot 10^{-5}$
	UTP	$5{\cdot}50 \cdot 10^{-5}$	—
	CTP_c	$7{\cdot}50 \cdot 10^{-5}$	$3{\cdot}60 \cdot 10^{-6}$
	UTP_c	$1{\cdot}00 \cdot 10^{-4}$	—

The inhibitory constants fo CTP and CTP_c with respect to phosphate donor (Table I) are very low, especially that for CTP_c, although the K_m value for ATP is $2{\cdot}5 \cdot 10^{-4}$ mol l^{-1}.

DISCUSSION

Uridine kinase catalyses the bisubstrate single displacement reaction in which both substrates add to the enzyme to form a ternary complex. Single displacement reaction is characterized by the pattern of intersecting lines when $1/v$ is plotted against $1/s$ at different substrate concentrations[9,15]. The enzyme is subject to allosteric inhibition by CTP and UTP, and has 3 binding sites: one for the phosphate donor, one for the phosphate acceptor, and the third binding site for the allosteric inhibitor[4]. CTP and UTP function as competitive inhibitors with respect to ATP and non-competitive ones with respect to uridine. In contrast to other 5'-triphosphates, CTP and UTP are known to be poor phosphate donors[10,13,14].

It seemed therefore of interest to investigate the modulation of uridine kinase activity by the recently synthesized analogs *I*, *II* which differ from the natural 5'-triphosphates of uridine and cytidine by an insertion of CH_2 group between the sugar hydroxyl group of the nucleoside molecule and the α-phosphate atom. These compounds cannot undergo degradation of the α-P−C−O linkage, but on ground of the adaptability of this grouping, might adopt approximately the conformation of natural allosteric inhibitor.

It is evident (Table I) that with respect to uridine the K_i constant for CTP_c and UTP_c are increased by 15−82% to compare with the corresponding values for the natural 5'-triphosphates and the inhibition pattern is non-competitive (Fig. 2). With respect to phosphate donor, inhibitions by CTP_c and by CTP are competitive; nevertheless, the K_i value for CTP_c ($3{\cdot}6 \cdot 10^{-6}$ mol l^{-1}) is about 3× lower than that of CTP (Table I). This suggests that the binding of the CTP analogue in the ternary complex is tighter than the binding of CTP itself.

It should be noted that uridine kinase has not yet been purified to homogeneity; consequently, the kinetic data obtained with partially purified preparations are of preliminary character. However, our results show that the phosphonate analogues of CTP and UTP (*I*, *II*) could be of interest as suitable means for a further study of this enzyme.

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