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# Homogeneous Uridine Kinase from Ehrlich Ascites Tumor: Substrate Specificity and Inhibition by Bisubstrate Analogs

NANCY CHENG, ROBERT C. PAYNE,[1] W. EVANS KEMP, JR., and THOMAS W. TRAUT
*Department of Biochemistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514*
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**SUMMARY**

Uridine kinase has been purified to homogeneity from Ehlrich ascites tumor cells. For the phosphate acceptor site, the enzyme shows substrate specificity only for ribopyrimidine nucleosides and is active with various analogs that have limited structural alterations; both endocyclic and exocyclic substituents can be acceptable. Of nucleosides that have been used in the chemotherapy of cancer, 5-fluorouridine, 6-azauridine, and 3-deazauridine are good substrates, whereas arabinosylcytosine is a poor substrate. No analogs are better substrates than the physiological substrates uridine and cytidine. 5′,5‴-$P^1$,$P^4$-Bisnucleoside oligophosphate bisubstrate analogs (e.g., $Ap_4U$, $Ap_5U$) were synthesized and tested as inhibitors. The most effective compound was $Ap_4U$; with a $K_i$ of 197 μM, it bound more tightly than ATP but no better than uridine. $Ap_3A$, $Ap_4A$, and $Ap_5A$ were also tested, with the result that both $Ap_4A$ and $Ap_4U$ were most effective, suggesting that this size of bisubstrate analog most closely approaches the spacing of the catalytic site.

Uridine-cytidine kinase (EC 2.7.1.48) catalyzes the phosphorylation of uridine and cytidine, the rate-limiting step in pyrimidine salvage. It has been shown for many cells and tissues that more UMP may be synthesized via the salvage route than by *de novo* synthesis (1–4). With cultured L1210 cells, uridine at ≥12 μM in the medium supports cell growth while producing a 95% inhibition of *de novo* pyrimidine synthesis (4); thus, under optimal conditions for salvage, the *de novo* pathway may be turned off. Also, humans afflicted with orotic aciduria (resulting from a deficiency of UMP synthase in the *de novo* pyrimidine biosynthetic pathway) grow normally when uridine is included in their daily diet (5).

We have prepared homogeneous uridine kinase from mouse Ehrlich asictes cells with a specific activity of 288 μmol/min/mg (6). With rabbit antibody to the Ehrlich ascites enzyme we have shown that uridine kinase can be induced 7-fold in quiescent mouse fibroblasts stimulated with fresh serum, and about 100-fold in human lymphocytes stimulated with phytohemagglutinin.[2] These results are consistent with the observation that, in cancerous or transformed cells, uridine kinase activity becomes considerably increased (1, 2, 7–9).

Uridine kinase is considered to be important in chemotherapy in two respects. First, uridine kinase is thought to be required for the intracellular transformation of some pyrimidine nucleoside analogs to cytotoxic nucleotides. Direct administration of nucleotide analogs is ineffecitve in chemotherapy since nucleotides cannot easily diffuse across the cell membrane. Second, since enzyme activity is significantly elevated in tumor cells, the inhibition of uridine kinase itself might be important in cancer therapy.

Because minimally purified uridine kinase (specific activity 0.4–10 nmol/min/mg) was commonly used, previous studies could usually only test radioactive nucleoside analogs as substrates. Thus, it was reported that 5-fluorouridine (10), 6-azauridine (11), 5-azacytidine (12), and 3-deazauridine (13) were phosphorylated by uridine kinase from different sources. A variety of such studies is described in two reviews (14, 15). Since many reports had proposed that there might be at least two isozymes of uridine kinase (reviewed in Ref. 6), Anderson and colleagues (16–19) prepared uridine kinase from P815 mast cells that was about 1% pure (specific activity of 3 μmol/min/mg) and performed extensive kinetic studies to conclude that a single protein phosphorylated uridine, cytidine, and 5-azacytidine.

Since most compounds are not available in radioactive form, laboratories have generally tested nucleoside analogs as inhibitors of uridine kinase activity (9, 20–23). With pure enzyme from Ehrlich ascites cells we used a spectrophotometric assay to probe the catalytic site with bisubstrate inhibitors, and with a variety of nucleoside analogs tested as phosphate acceptors.

## Materials and Methods

**Chemicals.** Nucleosides, nucleotides, 1,1′-carbonyldiimidazole, alkaline phosphatase (type 1-S), venom phosphodiesterase I (type IV),

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[1] Present address: University of Virginia, Department of Chemistry, Charlottesville, VA 22901.
[2] N. Cheng and T. W. Traut, submitted for publication.

**ABBREVIATION:** Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ATP, NADH, uridine, cytidine, 5-fluorouridine, 3-deazauridine, 6-azauridine, 5-methyluridine, arabinosylcytosine, deoxyuridine, deoxycytidine, orotidine, deoxyadenosine, deoxythymidine, phospho*enol*pyruvate, pyruvate kinase-lactate dehydrogenase (No. 40-7), and Dowex 50 × 8-400 were purchased from Sigma Chemical Co. $Ap_3A$, $Ap_4A$, and $Ap_5A$ were from PL Biochemicals, and pyrazofurin was a gift from Eli Lilly. DEAE-Sephadex A-25 was obtained from Pharmacia, and polyethyleneimine cellulose thin layer chromatography plates were obtained from Brinkmann Instruments.

**Enzyme assay.** Uridine kinase was purified to homogeneity from Ehrlich ascites cells as previously described (6). Enzyme activity was measured at 22° with a spectrophotometric assay using the coupling enzymes pyruvate kinase and lactate dehydrogenase. The oxidation of NADH was dependent on ADP, one of the products of uridine kinase. The standard reaction in 1 ml total volume contained 50 mM Hepes (pH 8.0 at 22°), 50 mM KCl, 10 mM ATP, 12 mM $MgCl_2$, 6 mM phospho*enol*pyruvate, 2 mM NADH, 70 units of pyruvate kinase, and 100 units of lactate dehydrogenase. Purified enzyme (17.5 ng) was used for each assay. The assay was initiated by the addition of each nucleoside, at concentrations shown in the figure or tables. Kinetic constants were determined by the method of Cleland (24).

**Synthesis of bisubstrate analogs.** Tributylammonium salts of the nucleotides were prepared by converting their sodium salts to pyridinium salts with 40 g of Dowex 50 × 8-400 in the pyridinium form. The Dowex was first washed with 5 M HCl, water, and then pyridine. ATP (1.6 mmol) and UMP (12 mmol) were used for $Ap_4U$ synthesis. ATP (6 mmol) and UDP (0.8 mmol) were used for $Ap_5U$ synthesis. The nucleotides were mixed with the resin for 10 min and then eluted by washing the resin twice with water. Filtrates were then evaporated under reduced pressure to a volume of 10–20 ml, and a 1 molar equivalent of tributylamine per mol of phosphorus was twice added along with enough ethanol to produce a homogeneous solution. The salt was evaporated to dryness, under reduced pressure, from ethanol, and twice from chloroform for $Ap_4U$ or dry pyridine for $Ap_5U$.

$Ap_4U$ and $Ap_5U$ were synthesized according to the phosphorimidazole method of Hoard and Ott (25) with the following modifications. Dimethylformamide was made anhydrous over calcium hydride. Tributylammonium ATP for $Ap_4U$ synthesis and tributylammonium UDP for $Ap_5U$ were dissolved in a minimal amount of dry dimethylformamide. 1,1′-Carbonyldiimidazole was added to these salts (8 mmol to ATP and 4 mmol to UDP) and stirred for 1–2 hr in a stoppered flask. Methanol was then added (9.6 mmol to ATP and 4.8 mmol to UDP) and stirred for 20 min. This solution was then added to the salt of the other nucleotide (UMP for $Ap_4U$ and ATP for $Ap_5U$) in a stoppered flask, mixed until dissolved, and allowed to stand for 48 hr at room temperature. The mixture was evaporated to dryness under reduced pressure, redissolved in methanol: water (1:1) (pH adjusted to 10.5 with triethylamine), and stirred for 2 hr. The solution was evaporated to a yellow oil, redissolved in water, and chromatographed on a DEAE-Sephadex column (2.5 × 40 cm), eluting with a linear ammonium bicarbonate gradient (0–2 M). Several peaks resulted, with the peak containing $Ap_4U$ eluting at 0.36–0.43 M and the peak containing $Ap_5U$ eluting at 0.83–0.97 M. The fractions containing the peaks were pooled and desalted by repeated evaporations from water:ethanol under reduced pressure until no trace of ammonium bicarbonate remained.

The peak containing product was identified by resistance to alkaline phosphatase and by phosphodiesterase digestion products. Alkaline phosphatase was made 100 units/ml in 0.1 M glycine, 10 mM $MgCl_2$ (pH 10.5). Phosphodiesterase was made 1 mg/ml in the same buffer. The products were analyzed by polyethyleneimine cellulose thin layer chromatography run in 1.0 M LiCl to resolve the nucleotides, by cellulose thin layer chromatography run in water (pH adjusted to 10 with ammonium hydroxide) to resolve the nucleosides, and by high pressure liquid chromatography with the Whatman Partisil-10 SAX column run according to the conditions of McKeag and Brown (26). $Ap_4U$ was obtained in 3.3% and $Ap_5U$ in 4.2% yield. The products were stored in aqueous solution at −20°.

Extinction coefficients of $Ap_4U$ and $Ap_5U$ were determined by measuring the absorbance at 260 nm before and after digestion with alkaline phosphatase/phosphodiesterase, and multiplying this ratio by an extinction coefficient determined at this wavelength for an equimolar solution of ATP and UMP. The molar extinction coefficients determined were $2.05 \cdot 10^4$ for $Ap_4U$, and $2.14 \cdot 10^4$ for $Ap_5U$.

A phosphate determination was done according to the procedure of Black and Jones (27) with the following modifications. The compounds assayed were digested overnight with phosphodiesterase (0.1 mg/180 nmol of compound) and alkaline phosphatase (10 units/180 nmol of compound), before being assayed. Tripotassium phosphate was used as a standard for the assay. Results of this assay yielded 3.6 mol of phosphate per mol of $Ap_4U$, and 4.6 mol of phosphate per mol of $Ap_5U$.

## Results

Table 1 lists those nucleosides tested as phosphate acceptor for uridine kinase. The list contains nucleosides with ribose or deoxyribose for the sugar moiety, with pyrimidine or purine bases, and with various substitutions on the pyrimidine ring or ribose. Uridine was the substrate most preferred by the enzyme, but cytidine, 5-fluorouridine, 3-deazuridine, and 6-azauridine were effective substrates as well. However, uridine kinase could not use deoxypurines, and deoxypyrimidines showed slight activity as substrates.

The $K_m$ and $V_{max}$ of compounds with good activity as phosphate acceptors were determined as shown in Fig. 1. Five compounds had relatively high $V_{max}$, the lowest being for 6-azauridine and the highest for uridine, cytidine, and 5-fluorouridine. However, the $K_m$ values for these compounds were quite different. Uridine had the lowest $K_m$ (40 μM) and 6-azauridine had the highest (340 μM). Unlike these compounds, arabinosylcytosine, a threopentose, has a significantly higher $K_m$ and lower $V_{max}$.

Bisubstrate analogs have frequently proven to be effective inhibitors. 5′,5‴-$Ap_nU$ compounds are not commercially available, so comparable $Ap_nA$ compounds were tested initially as a probe for the optimal number of phosphates. As shown in Table 2, $Ap_4A$ proved most effective, with $Ap_3A$ and $Ap_5A$ having larger $K_i$ values. Accordingly, we synthesized $Ap_4U$ and $Ap_5U$. Again, $Ap_4U$ had the tightest binding, suggesting that a span of four phosphates may best approximate the spatial separation, on the enzyme, between the binding sites for the adenosine of ATP and uridine. Surprisingly, $Ap_nU$ compounds did not have much more affinity for uridine kinase than the $Ap_nA$ com-

TABLE 1

**Kinetic parameters for phosphate acceptors of uridine kinase**

| Phosphate acceptor | Percentage of activity[a] | $K_m$ | $V_{max}$ |
|---|---|---|---|
| | | μM | μmol/min/mg protein |
| Uridine | 100 | 40 | 288.2 |
| Cytidine | 62.3 | 57 | 288.2 |
| 5-Fluorouridine | 90.4 | 69 | 288.2 |
| 3-Deazauridine | 67.8 | 200 | 248.1 |
| 6-Azauridine | 56.6 | 340 | 205.7 |
| Arabinosylcytosine | 0.20 | 20,000 | 75.8 |
| 5-Methyluridine (r·thymidine) | 7.2 | ND[b] | ND |
| d·Uridine | 5.1 | ND | ND |
| d·Cytidine | 0.65 | ND | ND |
| Orotidine | 0.65 | ND | ND |
| d·Adenosine | 0 | | |
| d·Thymidine | 0 | | |
| Pyrazofurin | 0 | | |

[a] Activity determined at 10 mM ATP, and 1 mM phosphate acceptor.
[b] ND, not determined.

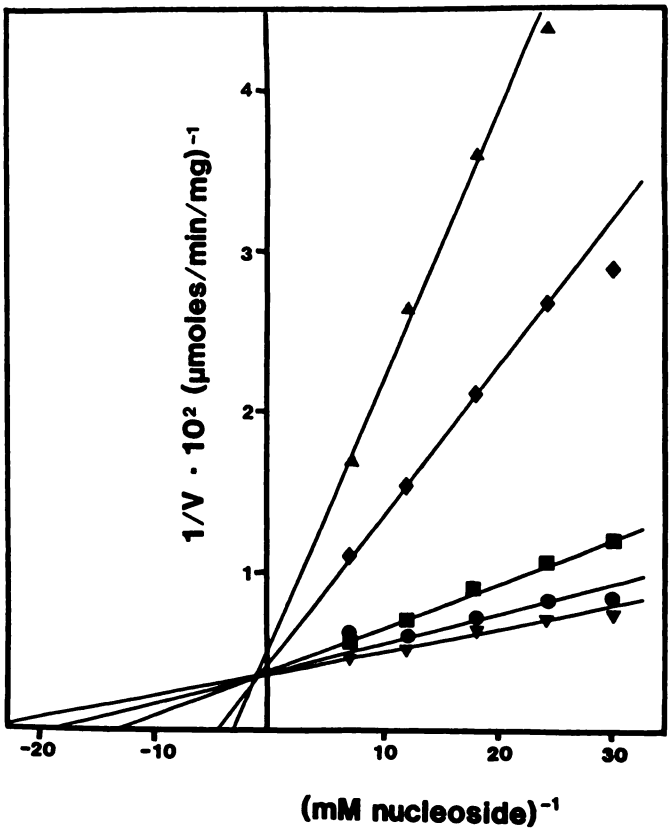



**Fig. 1.** Initial velocity of uridine kinase with various nucleoside substrates. ▼, uridine; ●, cytidine; ■, 5-fluorouridine; ♦, 3-deazauridine; ▲, 6-azauridine. ATP concentration was held constant at 10 mM.

TABLE 2
**Inhibition of uridine kinase by bisubstrate analogs**

| Compounds | $K_i$ |
|---|---|
| | mM |
| $Ap_3A$ | 2.94 |
| $Ap_4A$ | 0.305 |
| $Ap_5A$ | 0.919 |
| $Ap_4U$ | 0.197 |
| $Ap_5U$ | 1.09 |

pounds. $Ap_4U$ binds more tightly than ATP alone, but no better than uridine.

The specificity of the nucleoside acceptor site is summarized in Table 3, showing the effects of various substitutions on the pyrimidine nucleoside. The structure of pyrimidine was drawn according to the method of Saenger *et al.* (28), showing the more favorable *anti* conformation. *X* and *Y* represent endocyclic substitutions on the base. *R* represents exocyclic substituents on the base or the pentose. The categories for assessing activity were relative to uridine (where uridine is 100%): good activity (>50%), poor activity (<50%). Poor activity results from bulky substituents at positions $R_1$, $R_2$, and $R_4$. Included in this category are two potential affinity resins, with cytidine linked via spacers to a support resin. Both of these showed poor affinity for binding the enzyme. Loss of the 2′-OH group also reduces affinity, as does having the 2′-OH in a *threo* position (arabinosylcytosine).

## Discussion

The results in Table 1 give a quantitative analysis of the substrate specificity of the phosphate acceptor site on purified uridine kinase. With a partially purified preparation from Ehrlich ascites cells, Sköld (29) tested some of the same nucleosides as substrates, but no values for $K_m$ or $V_{max}$ were reported. The present results imply that uridine kinase retains its activity for those analogs derived from substitution of comparably sized and electrically neutral groups on the pyrimidine base (Table 3). However, substitution by a bulky, charged group results in poor activity. One such compound is orotidine, where the proton at the $R_2$ position is replaced by $COO^-$. This finding also agrees with the clinical trials, where treatment with allopurinol (30) or pyrazofurin (31) leads to inhibition of OMP decarboxylase and the accumulation of OMP which becomes dephosphorylated *in vivo* to orotidine; the latter is excreted instead of being salvaged. Another compound not utilized by uridine kinase is pyrazofurin; the nucleotide of this compound inhibits OMP decarboxylase (11), and activation of pyrazofurin is catalyzed by adenosine kinase (32). Various deoxy compounds were used as probes for the phosphate acceptor site on uridine kinase, and the results indicate that the 2′-hydroxyl group on the ribose is important for substrate specificity and that this group should be in the *erythro* position since arabinosylcytosine is such a poor substrate. Ribothymidine derived from tRNA is salvaged and recycled (33); the authors (33) proposed that salvage requires a pyrimidine kinase, and uridine kinase uses this nucleoside as a substrate (Table 1).

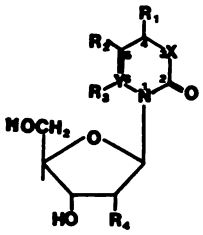
The bisubstrate analogs were synthesized as probes for the substrate-binding site. From the data obtained in Table 2, it is clear that tetraphosphate is the optimal length for a bisubstrate analog. It is expected that this analog would have a $K_i$ as small as the mathematical product of the dissociation constants for the substrates measured individually (34), as exemplified by bisubstrate analogs synthesized for adenosine kinase, AMP kinase, deoxythymidine kinase, and dTMP kinase (34). The increase in affinity is gained from the entropic advantage of saving the enzyme from searching out the substrates individually in a dilute solution. However, the $Ap_4U$ synthesized did not have nearly the affinity expected. This discrepancy might be due to steric hindrance of the bisubstrate analogs in diffusing into the active site. Kinetic studies on the reaction mechanism have shown that substrates bind in an ordered sequence, with the nucleoside binding first (34a).

Since uridine kinase activity in transformed cells is higher than in normal cells, a chemotherapeutic regimen involving the inhibition of uridine kinase itself in conjunction with inhibition of the *de novo* pathway has been suggested (8, 9, 23). Ahmed and Baker (21) have undertaken an extensive survey of compounds suitable for this purpose and Moyer *et al.* (23) have tested a large number of 5′-substituted analogs. Except for 5′-substituted pyrimidines, nucleosides that are inhibitors (i.e., versus uridine) are also themselves substrates (Table 1).

However, the functional significance of uridine kinase is still emerging. Except during fetal development, normal tissues usually have higher activity for uridine kinase than for the *de novo* pathway for UMP synthesis (1, 2, 7). Neural tissues may have a special requirement for pyrimidine salvage since the *de novo* pathway is quite low in rat brain (35) and since perfused cat brains can function only when uridine or cytidine is administered (36). It has also been shown that mice (37) and rats (38) actively salvage uridine and cytidine administered to the blood. Since plasma concentrations of uridine for many species are in the range of 4–20 μM (39–42), it is plausible that some tissues

TABLE 3

**Effect of substitutions on the ability of pyrimidine nucleosides to serve as phosphate acceptor**

| | Position | Substituents | |
|---|---|---|---|
| | | Good activity[a] | Poor activity[a] |
|  | *X* | NH, $CH_2$ | |
| | *Y* | CH, N | |
| | $R_1$ | O, $NH_2$ | 6-Aminohexyl agarose[b,c] |
| | $R_2$ | F | $CH_3$, 6-(acryloylamino)-hexanoate[b,d] |
| | $R_3$ | | $COO^-$ |
| | $R_4$ | OH[e] | H, OH[f] |

[a] Relative to activity with uridine: good (>50%); poor (<50%).
[b] Binding only, not tested for phosphorylation.
[c] $N^4$-(6-Aminohexyl)-cytidine agarose was kindly supplied by Dr. L. Frick, University of North Carolina.
[d] $C^5$[6-(Acryloylamino)-hexanoate]-cytidine was a gift from Dr. P. A. Bartlett, University of California.
[e] *erythro*.
[f] *threo*.

have become significantly or even predominantly dependent on salvage to maintain their pyrimidine nucleotide pools. Uridine concentrations of 10–40 μM can support cell growth in culture under conditions where the *de novo* pathway is substantially inhibited (3, 4). Therefore, as a means of chemotherapy the concomitant inhibition of *de novo* and salvage pyrimidine biosynthetic pathways may result in unacceptable levels of toxicity. Instead of targeting uridine kinase itself, this enzyme may be more important in chemotherapy for activating drugs that will target enzymes much closer to DNA synthesis.

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**Send reprint requests to:** Dr. Thomas W. Traut, Department of Biochemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514.