Uridine kinase from Ehrlich ascites carcinoma
PURIFICATION AND PROPERTIES OF HOMOGENEOUS ENZYME*

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Uridine kinase from Ehrlich ascites tumor cells has been purified about 60,000-fold to apparent homogeneity and with an overall recovery of about 40%. This purification was achieved using phosphocellulose and adenosine 5′-triphosphate-agarose affinity chromatography. The subunit molecular mass as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 31,000 daltons.

With two-dimensional electrophoresis, only one spot was observed, indicating the absence of isoenzymes. Multiple peaks of activity are routinely observed on ion exchange chromatography or gel filtration, for both crude preparations or homogeneous uridine kinase, in agreement with our earlier results that this enzyme exists as multiple interconvertible oligomeric forms (Payne, R. C., and Traut, T. W. (1982) J. Biol. Chem. 257, 12485–12488).

The purified enzyme has a specific activity of 283 μmol/min/mg of protein at 22 °C. Initial velocity studies using uridine and ATP are consistent with a sequential mechanism. $K_m$ values for uridine, cytidine, and ATP are 40, 57, and 450 μM, respectively. CTP and UTP are competitive inhibitors with respect to ATP, with $K_i$ values for CTP and UTP of 10 and 61 μM, respectively. The enzyme was active with several nucleoside analogs, the $K_m$ values being 69 μM (6-fluorouridine), 200 μM (3-deazauridine), and 340 μM (6-azauridine).

The pure enzyme is very sensitive to freezing, but can be maintained at 0 °C for 8 weeks with only 20% loss of activity. For long-term storage, enzyme in 50% glycerol can be maintained at −20 °C for many months with no detectable loss of activity.

Uridine kinase (ATP:uridine 5′-phosphotransferase, EC 2.7.1.48) catalyzes the phosphorylation of uridine and cytidine to their respective monophosphates. The enzyme is the rate-limiting activity of the pyrimidine salvage pathway whereby preformed pyrimidine nucleosides are recycled for nucleic acid synthesis (Anderson, 1973). It has been shown for many tissues, normal and neoplastic, that more UMP may be synthesized via the salvage route than from de novo synthesis (Weber et al., 1978; Denton et al., 1982). Two modes of regulation have been shown for the enzyme: feedback regula-

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EXPERIMENTAL PROCEDURES AND RESULTS

Uridine kinase from Ehrlich ascites cells has been purified 60,000-fold to apparent homogeneity. The essential steps in this purification involve two columns: phosphocellulose and ATP-agarose. Adsorption of enzyme to the P-11 column was inhibited by phosphate at concentrations greater than 1 mM, even though 30–80 mM potassium phosphate was necessary to elute uridine kinase from the resin. The inclusion of citrate in the TCE (50 mM Tris base and 0.01 mM EDTA titrated to pH 6.6 with 1 mM citric acid) buffer stabilized enzyme activity which otherwise is labile upon dilution in Tris buffers. P-11 chromatography increased the binding capacity of the subsequent ATP-agarose column for uridine kinase by approximately 2000-fold. This large increase is presumably affected by the selective removal on the P-11 column of ATP-binding proteins which would otherwise compete for binding sites on the ATP-agarose affinity resin.

The final chromatography on ATP-agarose had several advantages: 1) purification to homogeneity, 2) 10-fold concentration of the enzyme, and 3) the activity eluted in 1.2 mM ATP which was ideal for stabilizing enzyme activity during storage.

Multiple peaks of uridine kinase were observed on both the DE52 and the P-11 columns (Fig. 2). Similar profiles have been obtained with DEAE-cellulose chromatography (Fulchignoni-Lataud et al., 1976; Dubinina et al., 1982) and with native isoelectric focusing (Ahmed and Welch, 1979; Ahmed and Baker, 1980; Ahmed, 1982; Ullman et al., 1979; Abisil et al., 1980; Fulchignoni-Lataud and Roux, 1984). These authors have usually interpreted the multiple peaks as representing separate isozymes of uridine kinase. We have previously shown (Payne and Traut, 1982a) that uridine kinase exists as multiple aggregation states, containing different numbers of subunits, that are readily interconvertible. Since polymers of different sizes would vary in the number of exposed charged residues, they would readily separate in any method where migration is based on charge (Pharmacia, 1980; Scopes, 1982). Some interconversion is evident in our elution profiles: the four activity peaks on the DE52 column represent 49% of the initial enzyme activity, while the three peaks on the P-11 column and the one peak on the ATP column represent 43 and 40% of the initial enzyme activity, respectively. Since the recovery rate is so high, it follows that no peaks are lost in subsequent chromatography steps; rather, they are all finally converted to the same form. When this final homogeneous enzyme preparation was examined by two-dimensional electrophoresis, only one protein species was observed (Fig. 5). Multiple species were again evident when this enzyme preparation (Fig. 5) was analyzed by gel filtration or ion exchange chromatography (Fig. 6, A and B). Also contrary to the possibility of isoenzymes and separate genes for uridine kinase is the fact that uridine kinase-deficient cell lines are easily obtained (Ullman et al., 1979; Ahmed et al., 1980, Whitehouse et al., 1982). All our results, as well as those of other laboratories, are entirely consistent with our finding that different molecular weight species of uridine kinase are interconvertible (Payne and Traut, 1982a) and that the different peaks seen on ion exchange chromatography can all be converted to a single form (Figs. 2, 5, and 6).

For these reasons, it is likely that previous reports of native uridine kinase isoenzymes are due to different aggregation states of a single uridine kinase gene product.

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URIDINE KINASE FROM EHRLICH ASCITES CARCINOMA

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EXPERIMENTAL PROCEDURE

Materials

(5,6-3H) uridine was obtained from ICN Biopharmaceuticals. All pyridine and purine compounds, alpha-1 antitrypsin, aprotinin, adenosine 5′-triphosphate-agarose (linked via ribose hydroxyl), streptomycin, colistin, enzyme grade aspartic acid, and polypeptide weight standards were obtained from Sigma Chemical Company. DE-52 cellulose, P-11 phosphocelluloses and 2.5 cm DE-41 filter discs were purchased from Whatman. Hollow fiber bundles with molecular weight cut-offs of 5000 (176 fibers per 1.0 ml) and 2.0 ml nominal volume were obtained from Spectrum Medical Ltd.

Reagents for gel electrophoresis were obtained from Bio-Rad or BRL (Biochemistry Research Laboratory) with the exception of Serva dye 3/10 agarose which was obtained from Serva Fine Biochemicals. Chloroform/methanol/glacial (20 Solution) was a gift from Loma Inc., Fairview, N. J. Buffer as described were prepared and harvested from C/l acetate as previously described (Payne and Trayt, 1965).

Enzyme assays

With crude enzyme preparations (all steps before the phosphocellulose column), uridine kinase activity was measured with a radioactive assay at 37°C in a volume of 50 to 250 µl. Reaction mixtures contained 10 mM Tris-Cl (pH 7.4 at 37°C), 10 mM ATP (stock ATP solution adjusted to 7.4 with 2.5 KCl prior to addition), 11 mM MgCl2 concentration determined by EDTA titration as described by Skog and West, 1975) and 11 mM (2,5-3H) uridine (25 Ci/mM). Reactions were initiated by the addition of enzyme. The product UMP was measured either by thin layer chromatography on polyethyleneimine cellulose plates (Shimatz) as previously described (Payne and Trayt, 1965) or by binding to Whatman DE-41 filter discs (Shimatz, 1961). When the DE-81 binding assay was used, aliquots of the reaction mixture were quenched with 3 ml of non-radioactive 3 mM uridine prior to binding to DE-81 filter discs.

Enzyme activity from fractions after the ammonium sulfate step was measured with a spectrophotometric assay that used the coupling enzyme pyruvate kinase and assessed the level of pyruvate produced. NADH was then dependent on ADP, a product of uridine kinase. Assays were performed on a Beckman Model 21 recording spectrophotometer at 24°C. In the standard assay, the increase in absorbance at 340 nm was monitored at 0.1 ml total reaction volume containing 50 mM potassium phosphate buffer, pH 7.5 (on 250 mM Tris buffer solution adjusted to 0.05 with 7.5 N KOH), 12.5 mM MgCl2 (stock MgCl2 concentration determined by EDTA titration as described by Skog and West, 1975), 6 mM phosphopyruvate tri(cyanoethylammonium) salt, 2 mM NADH, 70 units pyruvate kinase, and 100 units lactate dehydrogenase (Sigma Products No. 4871). Centrifuge columns containing Sephadex G-25 were employed to remove ammonium sulfate in the P/CK/D column (Chirgwin et al, 1979). After the addition of ammonium, the change in absorbance was followed until endogenous ADP had been consumed. Reactions were terminated by the addition of 0.1 ml 0.1 N HCl. The final studies where the concentration of ATP was varied the MgCl2 concentration was adjusted to maintain constant concentration of 1 mM. Any contribution to the ATP and/or Mg2+ concentration by the enzyme sample was included in this calculation as well as the Mg2+ inhibition by phosphopyruvate, ATP, and ADP present.

The use of the different buffers described above was based on the following considerations. Phosphate interfere with uridine kinase at the catalytic site; therefore phosphate buffer is good for stabilizing the enzyme during preparation and storage. But phosphate interferes with accurate enzyme assays because phosphate is a weak competitive inhibitor, and with crude enzyme preparations that also contain nucleoside phosphorylase activity. phosphate would act as the second substrate for converting uridine to uracil. Therefore Tris buffers were used in radiometric enzyme assays. For the spectrophotometric assays, especially for kinetic studies with both Mg2+ and ATP ( binds both Mg2+ and ADP) Smithers, 1977 makes it less desirable, and HEPES buffer was used; pith is optimal for the Mg-nucleotide complexes which are the true ligands for the enzyme (O’Sullivan and Smithers, 1979). Kinetic analyses were done according to (Cletow, 1977).

Buffer Composition

Buffers designated as KPGM contained 10 mM potassium phosphate, 10 mM glycerol (v/v), and 10 mM magnesium chloride. The assay buffer was 5% Tris-Base and 0.5 mM EDTA which had been titrated to a pH of 7.4 with 1 M HCl and KPGM buffer contains 5 mM magnesium phosphate. 10 mM magnesium chloride and 0.005 nM EDTA at a pH of 7.4. KPGM buffers were sterilized by filtration through a 0.22 µm Millipore filter. Buffers were stored at -4°C and used within 1 week of preparation.

Column Resins

The DE-52 cellulose was washed initially twice in 5 volumes of 2 N NaCl followed by 1 wash of 5 volumes of 0.2 N NaCl at 4°C in a rotating test tube for 1 hour. Washed DE-52 cellulose was stored at 4°C in 5% KPGM and 0.005 nM EDTA and 0.005 nM EDTA. DE-52 was equilibrated by a batch procedure in 10 mM potassium phosphate buffer pH 7.4 for 1 hour. Equilibration was considered complete when the pH and conductivity were identical to 10 mM potassium phosphate buffer, pH 7.4. DE-52 cellulose was recycled by using the same procedures.

Before use, P-11 phosphocellulose was washed according to the following schedule. The resin was initially washed three times in 5 volumes of 2 M NaCl and then suspended in 5 volumes of 0.25 M KCl and stirred gently for 10 minutes, followed by extensive washes with distilled H2O until the pH was below 6.5. The resin was then twice resuspended in 5 volumes of 0.25 M KCl and stirred gently for 5 minutes followed by extensive rinsing with distilled H2O until the pH was above 7.

The resin was then transferred to a large column and washed with 50 volumes of 1 M EDTA. This is an essential step that removes an unidentified, UV absorbing compound whose presence results in significant, but not complete, loss of uridine kinase activity. Washing the resin on a filtration funnel is totally unsatisfactory; washing the P-11 resin on a column requires more than one week. The washed resin was stored in 5% EDTA at 4°C or used immediately. P-11 phosphocellulose was initially equilibrated in 5 volumes of 10 mM Tris buffer, making sure that the pH did not fall below 7.4. Ammonium sulfate was then resuspended in TE buffer several times until both pH and conductivity were identical to TE buffer. After use, the P-11 phosphocellulose was recycled by repeating the washes with KPGM and HCl.

Adenosine 5′-triphosphate-agarose was recycled according to the following procedure. ATP-agarose was washed 5 times with 5 volumes of 2 N NaCl to remove any bound protein. The NaCl wash was followed by 3-5 volumes of distilled H2O. The resin was then suspended in 1 volume of 0.5% Tris HCl and stirred gently for 1 hour before a final high wash and storage at -20°C in 50% glycerol. Immediately prior to use the ATP-agarose was extensively equilibrated in KPGM buffer. After chromatography, the ATP-Agarose was immediately recycled and stored in 50% glycerol at -20°C.

SDS-Polyacrylamide Gel Electrophoreses

Slab gels (0.75 mm x 11 cm x 16 cm) containing 12% acrylamide and 0.32% N,N′-methylenebisacrylamide were used and run at 12°C in a Model 6-400 Vertical Slab Gel Unit (model scientific Instruments) essentially according to the procedure of Laemmli (1970). 0.13 cm stacking gels consisted of 4% acrylamide and 0.18% N,N′-methylenebisacrylamide. Samples containing 100-800 µg protein were applied to sample wells. Each gel contained 25 µl at 30 µg/ml. Until samples had completely entered the stacking gel, the current was then increased to 50 mA per gel.

Two-Dimensional Electrophoresis

Two dimensional electrophoresis was carried out according to (O’Sullivan, 1975), using lysine buffer containing 2% Bio-rad dyes (pH 3.5/10), 2% SDS, and 4 mM DTT. The sample overlay solution had 15 imidazole pH 3.5-10. The tube gels contained: 3.14% acrylamide and 0.21% Bio-rad dyes. 2% phenol red, 1%, 0.05% mercaptamidic acid. The edge of the tube were not covered with polyethylene tubing. The tubes were run at 15 mA at 250 V. The gels were run 14 hrs at 350 V. The tube gels were placed on ice and soaked with a 1% w/v Tris buffer prior to separation from the tube. The SDS sample buffer used for gel equilibration contained 4 mM DTT, and the gels were equilibrated for 1 hr in screw cap test tubes.
The discontinuous slab gel was run using the Protein Slab Cell (Bio Rad) and prepared according to Laemmli (1970). The separating gel was 2.5 mm x 13 cm x 14 cm and contained 0.7% acrylamide and 5.7% bis-acrylamide and 0.1% TEMED.

Silver Staining Procedure for Visualizing Proteins in Polysaccharide Gels

The silver staining method of Oakley et al. (1980) was employed with the modification. Gels were fixed in 50% methanol - 10% acetic acid overnight and washed 3 times with several changes of destained Tissue prior to proceeding with the glacial acetic acid step.

Gel Staining

Gels were stained at 800 cm on a Gilford Model 2500 Microprocessor spectrophotometer using 0.25% silver nitrate and 0.1% sodium carbonate. The silver staining procedure was performed by an internal subroutine procedure.

Protein Assay

A dye-binding assay using Coomassie Blue G as described by Read and Northcote (1985) was used to determine protein concentration in all samples except the final fraction from the DE-52 column. The protein concentration was determined by taking the slope ratio of the two plots as indicated by the equation in Figure 1. This method has been found to be highly sensitive and is capable of measuring protein concentration in samples containing less than 0.25 pg per ml.

The protein concentration of the homogeneous uridine kinase preparation was determined with SDS-polyacrylamide slab gels and the silver staining procedure. A slab gel (0.75 mm x 14 cm x 14 cm) was prepared as described above. As shown in Figure 1, plates were constructed of peak area vs pg BSA applied per lane, and all of sample applied per lane. The gels were stained with silver and scanned at 510 nm on a Hewlett-Packard Model 72258 UV spectrophotometer.

Figure 2. DE-52 cellulose chromatography. 960 ml of S-150 clarified dialysate was loaded on a DE-52 column (10.5 ml fractions were collected). (a) uridine kinase activity; (b) absorbance at 280 nm; (c) potassium phosphate concentration.

The elution profile of the DE-52 column is presented in Figure 2 and shows four distinct peaks of uridine kinase activity. Fractions representing approximately 95% of the eluted activity were pooled and adjusted to 30% saturation (at pH 7.0) with ammonium sulfate. After which the solution was allowed to stir at 0°C for 45 min and centrifuged at 20,000 x g for 20 min. The pellet was discarded, and the resulting supernatant was brought to 50% saturation, stirred for 30 min at 0°C. The solution was centrifuged as before and the protein pellet dissolved in a minimal volume of 0.5 M potassium phosphate buffer (pH 7.0). The clarified 50% ammonium sulfate fraction may be stored at -20°C for later processing but uridine kinase activity decreases accordingly by a factor of 10 within 1 week for optimal recovery it is therefore advisable to proceed directly to the P-11 phosphocellulose column.

The 3.5% ammonium sulfate fraction was diluted with 40 volumes of TCE buffer and applied to the P-11 column (15.5 cm x 3.0 cm). Bed volume (100 ml) was equilibrated in TCE buffer. The P-11 column was then washed with 0.1 M ammonium sulfate after拴 TCE buffer. Uridine kinase activity was eluted from the column with a linear gradient from 0 to 200 mM ammonium sulfate phosphate in TCE buffer. After completion of the gradient elution, the column was washed with 200 mM potassium phosphate buffer. (pH 6.8). The elution profile (data not shown) contained three distinct peaks of uridine kinase activity. Recovery of activity from the P-11 column was typically between 85 and 95% of the activity recovered from the DE-52 column. The phosphate concentration of the fractions was lowered to approximately 1 mM by use of a chloroform buffer until 1 M in KPO4 buffer. The dialyzer buffer was changed twice, and the enzyme sample was then adjusted to 10 mM MgCl2.

Figure 3. Affinity chromatography on ATP-Agarose. The pooled P-11 cellulose fractions were applied to the ATP-Agarose column. Homogeneous uridine kinase was washed with 1.2 mM ATP in KPO4 buffer. Fractions contained 5.4 mM ATP.

The pooled P-11 fractions representing 95% of the recovered activity were applied at a flow rate of 10 ml/hr to the ATP-Agarose affinity column (1.6 cm x 1 cm) with a bed volume of 60 ml equilibrated in KPO4 buffer. After sample addition was complete, the column was washed with 10 volume elution buffer for 4 hours with 0.01% Triton X 1000 ml of KPO4 buffer (pH 7.5) containing 0.5% NaCl. The column was then washed with 0.01% Triton X 100 ml of KPO4 buffer. The protein concentration of the 5-150 dialysate was determined by the method of Bradford (1976) with BSA as a standard.

The major loss in enzyme activity occurred at the step using DEAE cellulose chromatography, and may represent precipitation since it took 24 h. No additional of enzyme to the column due to its low ionic strength. Therefore, recovery of uridine kinase activity was excellent for subsequent steps. However, after the ammonium sulfate step was always greater than 25%, this likely reflects inhibition by sodium dodecyl sulfate during the assay at this fraction.
Table 1. Purification of Uridine Kinase from Ehrlich Ascites Carcinoma (300 grams).

| Fraction                              | Volume (ml) | Total Protein (mg) | Specific Activity (nmoles/min/mg) | Total Activity (nmoles/min) | Recovery (%) | Relative Purification |
|---------------------------------------|-------------|--------------------|-----------------------------------|----------------------------|--------------|-----------------------|
| S-20 supernatant                      | 960         | 23 800             | 4.71                              | 113 000                    | 100          | 1                     |
| S-150 supernatant (after dialysis and clarification) | 960         | 16 000             | 6.75                              | 108 000                    | 95           | 1.5                   |
| Pooled DE-52 column fractions         | 180         | 4 500              | 10.82                             | 55 000                      | 49           | 2.3                   |
| 38-53% ammonium sulfate fraction      | 250         | 920                | 44.2                              | 38 000                      | (33)\(^b\)  | 8.9                   |
| Pooled P-11 column fractions          | 130         | 28.6               | 1714                              | 49 000                      | 43           | 370.0                 |
| ATP-Agarose column (Fraction #, see Fig. 3) | 5.5\(^a\)  | 0.076              | 259 500                           | 20 000                      | 17.7         | 55 900                |
|                                       | 5.5\(^a\)  | 0.083              | 306 600                           | 25 000                      | 22.1         | 66 100                |

\(^a\) Protein assayed by the SDS-polyacrylamide gel-silver staining procedure (Figure 1).

\(^b\) Not corrected for inhibition by ammonium sulfate.

Acid → IEF → Base

Figure 4. SDS-polyacrylamide gel electrophoresis of uridine kinase at different stages of purification. Samples were treated with SDS and mercaptoethanol before analysis on Lammeli gels. Molecular weight markers (Lanes 1 and 11) contained 70 mg each of phosphorylase b (94,000), bovine serum albumin (68,000), carbonic anhydrase (29,000), and soybean trypsin inhibitor (21,000). Samples containing 500 ng protein of the following were applied to: lane 2, whole cell homogenate; lane 3, S-20 supernatant; lane 4, S-150 supernatant after dialysis against 10 mM potassium phosphate buffer (pH 7.5), and clarification; lane 5, pooled DE-52 column fractions; lane 6, 38-53% ammonium sulfate fraction; lanes 7 and 8 contained 2 and 10 \(\mu\)l of pooled P-11 column fractions, respectively. Lanes 9 and 10 contained 70 mg each of uridine kinase protein from the two peak ATP-agarose column fractions (see Figure 3).

The SDS-polyacrylamide gel electrophoresis of the two active fractions eluted from the ATP-Agarose column are shown in Figure 4 (Lanes 9 and 10). As shown in Lane 9 the protein is apparently homogeneous with a subunit molecular weight of 31,000. The protein from Lane 10 has a subunit molecular weight of 34,000 determined previously by gel filtration (Payne and Traut, 1968). The faint bands seen at the top of Lanes 9 and 10 are artifacts of the staining procedure and not associated with any proteins, and are observed in lanes containing only sample buffer.

Electrophoresis under non-denaturing conditions has routinely produced 2 major species of uridine kinase (data not shown), in agreement with the results of Ullman et al. When homogeneous uridine kinase was subjected to two-dimensional electrophoresis (isoelectric focusing and SDS electrophoresis), only one spot was observed (Figure 5). Based on several determinations, this spot had a pI value of 6.28.
**Uridine Kinase from Ehrlich Ascites Carcinoma**

The purified enzyme had a specific activity of 283 pmol/min/mg enzyme at 22°C. This leads to a turnover number of about 150 s⁻¹. Initial velocity patterns with homogenous uridine kinase using uridine and ATP as substrates were consistent with those obtained with the enzyme from E. ascites carcinoma. This is consistent with the findings of Llompart and Andem (1975) who used a 750-fold purified uridine kinase preparation from mouse ascites tumor. Both CTP and UTP showed competitive inhibition with respect to ATP. Kᵢ's for CTP and UTP inhibition were 19 and 61 μM, respectively. The Kᵢ's for uridine and cytidine were 40 and 57 μM, respectively (Table II).

Several nucleoside analogs were also good substrates for uridine kinase. As shown in Table II, the Kᵢ's for 5-fluorouridine, 3-deazaradine, and 6-azauridine were 69, 200, and 340 μM, respectively.

### Table I. Kinetic Parameters for Substrates and Inhibitors of Uridine Kinase

| Substrates            | Kᵢ(μM) | Kᵢ(μM) |
|-----------------------|--------|--------|
| Uridine               | 40     |        |
| Cytidine              | 57     |        |
| 5-Fluorouridine       | 69     |        |
| 3-Deazaradine         | 200    |        |
| 6-Azauridine          | 340    |        |
| Adenosine 5'-Triphosphate | 450  | 160    |
| Cytidine 5'-Triphosphate | 10   |        |
| Uridine 5'-Triphosphate | 67   |        |

* Uridine kinase activity determined by the spectrophotometric method using ATP-Agarose fractions 183 or 184 (Table I).