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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 (5-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 regulation by UTP and CTP which exhibit competitive inhibition with respect to the phosphate donor (Anderson and Brockman, 1964; Liacouras *et al.*, 1975) and regulation by changes in quaternary structure caused by orthophosphate, ATP, and CTP (Payne and Traut, 1982a).

A number of investigators have reported multiple forms of uridine kinase from normal and neoplastic tissues when crude or partially purified preparations of the enzyme are subjected to gel filtration (Krystal and Webb, 1971; Krystal and Scholefield, 1973; Keefer *et al.*, 1975; Greenberg *et al.*, 1977; Otal-Brun and Webb, 1979), to ion exchange chromatography (Sköld, 1963; Fulchignoni-Lataud *et al.*, 1976; Dubinina *et al.*, 1982), to native isoelectric focusing (Ahmed and Welch, 1979; Ullman *et al.*, 1979; Absil *et al.*, 1980; Ahmed and Baker, 1980; Ahmed, 1982), and to affinity chromatography (Veselý and Smrt, 1977). Except for Krystal and Scholefield (1973) who suggested that the two molecular weight forms of uridine kinase observed in preparations from Ehrlich ascites cells could be a monomer and tetramer of the same species, the other investigators have invariably suggested that the multiple forms of uridine kinase constitute isoenzymes. Using normal and neoplastic tissues, gel filtration studies have shown multiple uridine kinase peaks, leading to the interpretation that one or more isoenzymes of uridine kinase are preferentially associated with the neoplastic state (Krystal and Webb, 1971; Greenberg *et al.*, 1977). Preparations of uridine kinase from fetal and postnatal rat liver also show multiple molecular weight forms when chromatographed on Sepharose 6B, leading to the interpretation that differential expression of uridine kinase isoenzymes is taking place during development (Krystal and Webb, 1971). In addition, the development of resistance to pyrimidine nucleoside analogs has been attributed to the preferential expression of one of the uridine kinase isoenzymes (Sköld, 1963; Keefer *et al.*, 1974; Keefer *et al.*, 1975; Greenberg *et al.*, 1977).

Previous experiments in our laboratory have suggested an additional or alternative interpretation: uridine kinase can exist in a variety of different aggregation states that can be interconverted by appropriate effectors such as substrates and inhibitors (Payne and Traut, 1982a). Even highly purified uridine kinase will exhibit several different polymer forms that only slowly equilibrate with each other; the enzyme will therefore exhibit multiple peaks on a gel filtration column.

The preparation of homogeneous uridine kinase has never been previously achieved with any eukaryotic source; most studies have used enzyme preparations that were purified less than 10-fold. In the present study, Ehrlich ascites cells were used because no other cell or tissue has higher uridine kinase activity, and since it was previously reported that Ehrlich ascites cells may contain isozymes of uridine kinase (Krystal and Scholefield, 1973).

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EXPERIMENTAL PROCEDURES AND RESULTS[1]

DISCUSSION

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 M 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.

Routing the P-11 gradient eluant through two hollow fiber dialysis units facilitated the rapid equilibration of the phosphocellulose column eluant to conditions necessary for uridine kinase binding to the ATP-agarose resin. This procedure reduced the phosphate concentration from 80 mM to about 5 mM and simultaneously added to the enzyme preparation 5 mM Mg^{2+} necessary for adsorption to ATP-agarose. Phosphate concentrations greater than 20 mM inhibited uridine kinase adsorption to the ATP 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) or with native isoelectric focusing (Ahmed and Welch, 1979; Ahmed and Baker, 1980; Ahmed, 1982; Ullman *et al.*, 1979; Absil *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.

Acknowledgment—We are grateful to W. Evans Kemp, Jr. for technical assistance.

[1] Portions of this paper (including "Experimental Procedures," "Results," Figs. 1–6, and Tables I and II) are presented in miniprint at the end of this paper. The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; BSA, bovine serum albumin; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; PK/LDH, pyruvate kinase/lactate dehydrogenase. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-580, cite the authors, and include a check or money order for $7.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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URIDINE KINASE FROM EHRLICH ASCITES CARCINOMA
PURIFICATION AND PROPERTIES OF HOMOGENEOUS ENZYME

Robert C. Payne, Nancy Cheng, and Thomas W. Traut

EXPERIMENTAL PROCEDURES

Materials

[5,6-^{3}H] uridine was obtained from ICN Chemicals. All pyrimidine and purine compounds, alpha-1 antitrypsin, aprotinin, adenosine 5'-triphosphate-agarose (linked via ribose hydroxyls), streptomycin sulfate, enzyme grade ammonium sulfate and protein molecular weight standards were obtained from Sigma Chemical Company. DE-52 cellulose, P-11 phosphocellulose and 2.5 cm DE-81 filter discs were purchased from Whatman. Hollow fiber bundles with molecular weight cutoff of 5000 (176 fibers per bundle, 2.0 ml nominal volume) were obtained from Spectrum Medical Industries. Reagents for gel electrophoresis were obtained from Bio-Rad or BRL (Bethesda Research Laboratories) with the exception of Servalyte 3/10 40% ampholytes which were obtained from Serva Fine Biochemicals. Chlorohexidine gluconate (20% Solution) was a gift from Lonza Inc., Fairlawn, N.J. Ehrlich ascites cells were propagated and harvested from CF1 mice as previously described (Payne and Traut, 1982b).

Enzyme assays

With crude enzyme preparations (all steps before the phosphocellulose column), uridine kinase activity was measured with a radiometric assay at 37°C in a volume of 50 or 200 µl. Reaction mixtures contained 50 mM Tris-HCl (pH 7.4 at 37°C), 10 mM ATP (stock ATP solution's pH adjusted to 7.4 with 2 N KOH prior to addition), 11 mM $MgCl_2$ (Mg^{2+} concentration determined by EDTA titration as described by Skoog and West, 1976), and 1 mM [5,6-^{3}H] uridine (25 Ci/mole). Reactions were initiated by the addition of enzyme. The product UMP was measured either by thin layer chromatography on polyethyleneimine cellulose plates (Brinkman) as previously described (Payne and Traut, 1982b), or by binding to Whatman DE-81 filter discs (Ahmed, 1981). When the DE-81 binding assay was used, aliquots of the reaction mixture were quenched into 5 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 enzymes pyruvate kinase and lactate dehydrogenase; the oxidation of NADH is then dependent on ADP, a product of uridine kinase. Assays were performed on a Beckman Model 25 recording spectrophometer at 22°C. For the standard assay, the decrease in absorbance at 340 nm was monitored in a 1 ml total reaction volume containing 50 mM HEPES (pH 8.0 @ 22°C), 50 mM KCl, 10 mM ATP (pH of stock ATP solution adjusted to 8.0 with 2.0 N KOH), 12 mM $MgCl_2$ (stock Mg^{2+} concentration determined by EDTA titration as described by Skoog and West, 1976), 6 mM phosphoenolpyruvate tri(cyclohexylammonium) salt, 2 mM NADH, 70 units pyruvate kinase, and 100 units lactate dehydrogenase (Sigma Product # 40-7). Centrifuge columns containing Sephadex G-25 were employed to remove ammonium sulfate in the PK/LDH mix (Christopherson, et al. 1979). After the addition of enzyme, the change in absorbance was followed until endogenous ADP had been consumed. Reactions were then initiated with the addition of uridine (1 mM). For kinetic studies where the concentration of ATP was varied the $MgCl_2$ concentration was adjusted to maintain the free Mg^{2+} concentration constant at 1 mM. Any contribution to the ATP and/or Mg^{2+} concentration by the enzyme sample was included in this calculation as well as the Mg^{2+} chelation by phosphoenolpyruvate, ATP, and if present, pyrimidine nucleotides (O'Sullivan and Smithers, 1979).

The use of the different buffers described above was based on the following considerations. Phosphate interacts 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, the ability of Tris to bind both Mg^{2+} and ATP (O'Sullivan and Smithers, 1979) makes it less desirable, and HEPES buffer was used; pH 8 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 Cleland (1970).

Buffer Composition

Buffers designated as KPGM contained 10 mM potassium phosphate, 10% glycerol (v/v), and 10 mM magnesium chloride at a pH of 7.5. The composition of TCE buffer was 50 mM Tris-Base and 0.01 mM EDTA which had been titrated to a pH of 6.6 with 1 M citric acid. KPME buffer contains 5 mM potasium phosphate, 5 mM magnesium chloride and 0.005 mM EDTA at a pH of 7.5. KPGM buffers were sterilized by filtration through a or 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 3 times with 5 volumes of 2 M NaCl followed by 3 washes of 5 volumes of deionized H_2O before proceeding with the 0.5 N HCl and 0.5 N KOH treatment. Washed DE-52 cellulose was stored at 4°C in 5 mM EDTA and 0.006% (v/v) chlorohexidine gluconate. DE-52 was equilibrated by a batch procedure in 10 mM potassium phosphate, pH 7.5, before packing of the column. Equilibration was considered complete when the pH and conductivity were identical to 10 mM potassium phosphate buffer, pH 7.5. DE-52 cellulose was recycled using the same procedures.

Before use, P-11 phosphocellulose was washed according to the following schedule. The resin was initially washed 3 times in 5 volumes of 2M NaCl and then suspended in 5 volumes of 0.25 N KOH and stirred gently for 10 minutes, followed by extensive washes with deionized H_2O until the pH was below 8. The resin was then twice resuspended in 5 volumes of 0.25 N HCl and stirred gently for 5 minutes followed by extensive rinsing with deionized H_2O until the pH was above 5.

The resin was then transferred to a large column and washed with 50 volumes of 1 mM EDTA. This is an essential step that removes an unidentified, UV absorbing compound whose presence results in significant, if 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 mM EDTA at 4°C or used immediately. P-11 phosphocellulose was initially equilibrated in 5 volumes of 10 X TCE buffer, making adjustments to pH 6.6 with either 0.5 M Tris-Base or 1 M Citric acid. The material was then resuspended in TCE buffer several times until both pH and conductivity were identical to TCE buffer. After use, the P-11 phosphocellulose was recycled by repeating the washes with KOH 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 5 x 10 volumes of deionized H_2O. The resin was then suspended in 5 volumes of 70% ethanol and stirred gently for 10 minutes before a final H_2O 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 Electrophoresis

Slab gels (0.75 mm x 11 cm x 16 cm) containing 12% acrylamide and 0.32% N,N'-methylenebisacrylamide were cast and run at 12°C in a Model SE-600 Vertical Slab Gel Unit (Hoefer Scientific Instruments) essentially according to the procedure of Laemmli (1970). A 1.5 cm stacking gel consisted of 4% acrylamide and 0.1% N,N'-methylenebisacrylamide. Samples containing 100-800 ng protein were applied to sample wells. Electrophoresis was intitiated at 10 mA per gel until samples had completely entered the stacking gel; the current was then increased to 30 mA per gel.

Two-Dimensional Electrophoresis

Two dimensional electrophoresis was carried out according to O'Farrell (1975), using lysis buffer containing 2% Bio-Rad ampholytes (pH 3/10); 2% SDS, and 4 mM DTT. The sample overlay solution had 1% ampholytes pH 3/10. The tube gels contained: 3.74% acrylamide and 0.21% bis-acrylamide, 2% ampholytes 3/10, and 0.034% ammonium persulfate. The ends of the tubes were not covered with dialysis tubing. The tube gels were prefocused (at constant voltage) at 400 V before loading, and the gels were run 16 hrs at 450 V. The tube gels were placed on ice and loosened with a 1 µl Hamilton syringe prior to ejection from the tubes. The SDS sample buffer used for gel equiliberation 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 consisted of 12% acrylamide, 0.32% bis-acrylamide and 0.1% TEMED.

Silver Staining Procedure for Visualizing Proteins in Polyacrylamide Gels

The silver staining method of Oakley et. al. (1980) was employed with the following modification. Gels were fixed in 50% methanol - 10% acetic acid overnight and washed 1 hour with several changes of deionized H_2O prior to proceeding with the glutaraldehyde step.

Gel Scanning

Gels were scanned at 400 nm on a Gilford Model 2600 Microprocessor spectrophotometer equipped with a gel scanning apparatus and a Hewlett-Packard Model 7225B XY plotter. Peak integration was performed by an internal subroutine procedure.

Protein Assays

A dye-binding assay using Coomassie Blue G as described by Read and Northcote (1981) was used to determine protein concentration in all samples except the final fraction from the ATP-agarose column. Dye reagent containing 0.01% Serva Blue G in 1.6 M phosphoric acid and 0.8 M ethanol was used. Bovine serum albumin ($E^{1\%}_{280nm}$ = 6.6) was used as a standard.

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 16 cm x 14 cm) was prepared as described above. As shown in Fig. 1, plots were constructed of peak area versus ng BSA applied per lane, and µl of sample applied per lane. Slopes of both plots were calculated and uridine kinase protein concentration was determined by taking the slope ratio of the two plots as indicated by the equation in Figure 1. This method is extremely sensitive and is capable of measuring protein concentration in samples containing less than 0.25 µg per 1 ml.

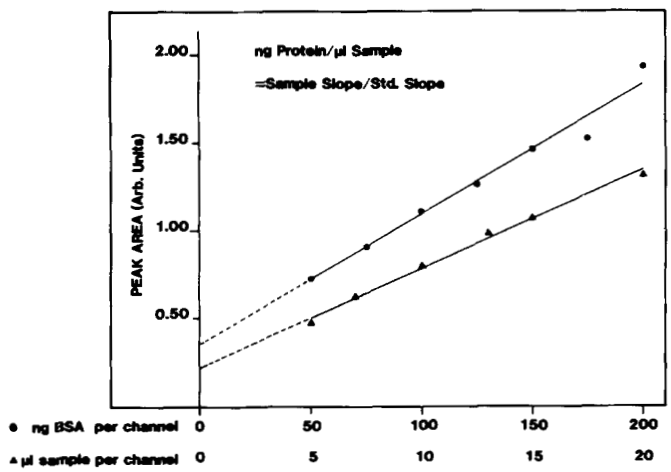

Figure 1. Determination of protein concentration in ATP-agarose column fractions. An SDS-polyacrylamide gel was prepared as described under Methods. 50 µl samples were applied to each of 15 sample wells containing 50 to 200 ng of BSA or 5 to 20 µl of uridine kinase. Gels were stained with silver and scanned at 400 nm to measure the intensity of staining for protein bands. The integrated areas under the peaks were used as a measure of protein in each sample. The protein concentration of uridine kinase in the sample is then obtained by dividing the change in peak area per µl sample (slope of sample curve) by the change in peak area per ng protein standard (slope of standard curve).

Chromatographic Analysis of Purified Enzyme

Gel filtration was done on an ultrogel AcA34 column (1.0 x 50 cm) equilibrated with 50 mM Tris HCl (pH 7.5), 50 mM NaCl, and 3 mM MgATP.

Ion exchange chromatography was done on DEAE-silica (SAX-300, 4.6 mm x 25 cm) using high pressure liquid chromatography. The column was equilibrated with 0.5 M NaAc, 20 mM TrisAc (pH 8.0). The purified uridine kinase sample was eluted with a gradient of phosphate (pH 6.0) from 0 to 1 M.

RESULTS

Purification of Uridine Kinase

Results of the purification are summarized in Table 1. All procedures were performed at 0-4°C. With about 300-500 grams of cells, this purification takes 7 consecutive days. 300 grams (wet packed weight) of Ehrlich ascites cells were suspended in 480 ml of cold 10 mM potassium phosphate buffer (pH 7.5) containing 25 mg/l α-1-antitrypsin and 2.5 ml/l aprotinin (10-20 trypsin inhibitor units per ml),10 µM leupeptin, and 10 µM pepstatin. The cells were homogenized for 5 minutes with a Tekmar Tissumizer at maximum speed. Homogenization was continued in a Dounce homogenizer with 4 strokes of a tight fitting pestle. The homogenate was centrifuged for 20 min at 20,000 x g to remove cellular debris. The supernatant was decanted and the cellular debris was extracted a second time with 480 ml of the same buffer. Resuspension was achieved with a 1 min burst of the Tekmar homogenizer. The resuspended material was centrifuged for 20 min at 20,000 x g. The two supernatant fractions were combined and designated as S-20 supernatant in Table I. Fifty microliters of a 10% streptomycin sulfate solution was added per ml of S-20 supernatant and the mixture was stirred on ice for 30 min. Precipitated nucleic acids and subcellular aggregates were removed by centrifugation at 150,000 x g for 2 hours. The resulting S-150 supernatant was dialyzed overnight against two changes of 12 l of 10 mM potassium phosphate buffer, (pH 7.5). Precipitated protein in the S-150 dialysate was removed by centrifugation at 20,000 x g for 30 min. The clarified S-150 dialysate was applied, at a flow rate of 250 ml/hr, to a DE-52 column (4.0 cm x 45 cm with a bed volume of 550 ml) that was equilibrated with 10 mM potassium phosphate buffer, (pH 7.5). The column was further washed with 2 bed volumes of 10 mM potassium phosphate buffer, (pH 7.5). Uridine kinase was eluted with 100 mM potassium phosphate buffer, (pH 7.5).

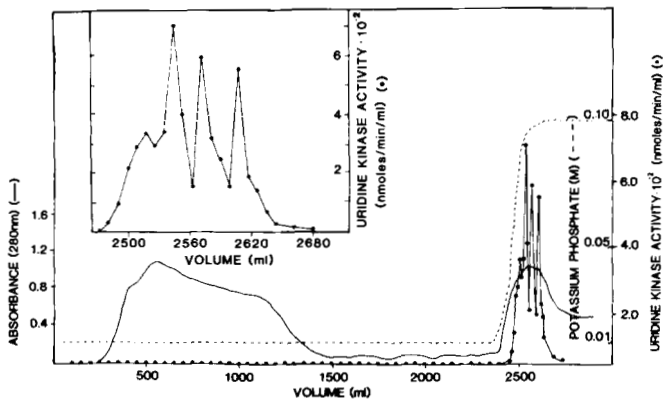

Figure 2. DE-52 cellulose chromatography. 960 ml of S-150 clarified dialysate were loaded on a DE-52 cellulose column 10.5 ml fractions were collected. (●) uridine kinase activity; (——)absorbance at 280 nm; (---) potassium phosphate concentration. Inset: the uridine kinase elution expanded to show multiple peaks of enzyme activity.

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 38% of saturation (at 0°C) 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 53% saturation, stirred for 45 min at 0°C. The solution was centrifuged as before and the protein pellet dissolved in a minimal volume of 10 mM potassium phosphate buffer, (pH 7.5). The dissolved 38-53% ammonium sulfate fraction may be stored at -20°C for later processing but uridine kinase activity decreases according to a first order decay with a $t_{1/2}$ of 7 weeks. For optimal recovery it is therefore advantageous to proceed directly to the P-11 phosphocellulose column.

The 38-53% ammonium sulfate fraction was diluted with 40 volumes of TCE buffer and applied to the P-11 column (2.5 cm x 43 cm, bed volume = 200 ml) equilibrated in TCE buffer. The P-11 column was then washed with approximately 1 liter of TCE buffer. Uridine kinase activity was eluted from the column with a linear gradient from 0 to 200 mM potassium phosphate in TCE buffer. After completion of the gradient, elution was continued with 300 ml of 200 mM potassium phosphate in TCE buffer, (pH 6.6). 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 sample was lowered to approximately 5 mM by use of a hollow fiber unit immersed in 2 l of KPGM buffer. The dialysis buffer was changed twice, and the enzyme sample was then adjusted to 10 mM $MgCl_2$.

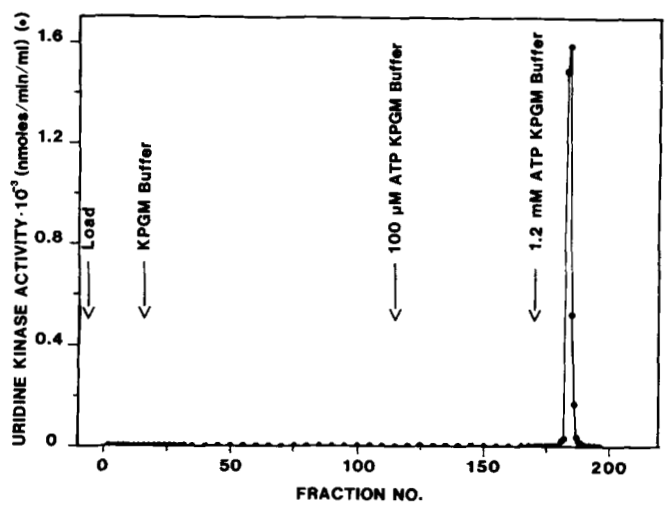

Figure 3. Affinity chromatography on ATP-Agarose. The pooled P-11 cellulose fraction was applied to the ATP-Agarose column. Homogeneous uridine kinase was eluted with 1.2 mM ATP in KPGM buffer. Fractions contained 5.4 ml.

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 30 cm with a bed volume of 60 ml) equilibrated in KPGM buffer. After sample addition was complete, the column was washed with 10 column volumes of KPGM buffer. This was followed by 5 column volumes of 100 µM ATP in KPGM buffer. Homogeneous uridine kinase was step-eluted with 2 column volumes of 1.2 mM ATP in KPGM (after addition of ATP to the buffer, the pH was readjusted to pH 7.5 with 2.0 N KOH). Enzyme eluted with the leading edge of the 1.2 mM ATP solvent front (Figure 3). Active fractions were individually sterilized by filtration through a Millex-GS (Millipore Corporation) 0.22 m filter and stored unfrozen at 0°C in sterile cryotubes. Alternatively, fractions can be adjusted to a final concentration of 50% glycerol and stored at -20°C. 80% of the uridine kinase activity remained in samples stored sterilized at 0°C (unfrozen) for 2 months, while no loss in activity was observed in samples stored in 50% glycerol at -20°C.

The major loss in enzyme activity occurred at the step using DEAE cellulose chromatography, and may represent proteolysis since it took 24 h from addition of enzyme to the column to its final elution. Thereafter, recovery of uridine kinase activity was excellent for subsequent steps. Recoveries after the ammonium sulfate step were always greater than 100%; this most likely reflects inhibition by ammonium sulfate during the assay of this fraction.

Table I. 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)						
183	5.5[a]	0.076	259 500	20 000	17.7 } 39.8	55 900
184	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.

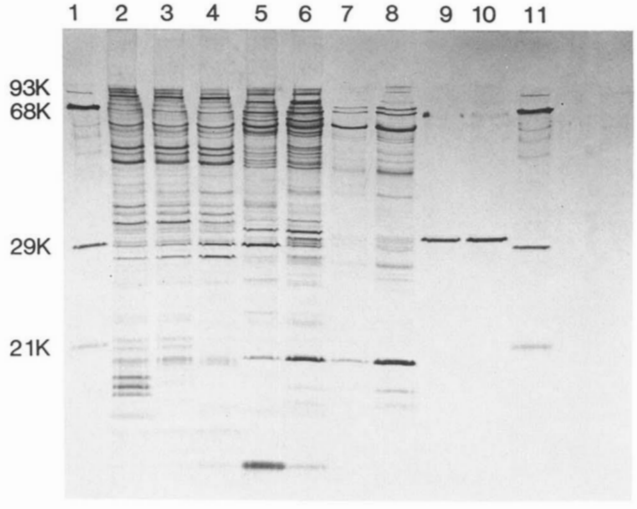

Figure 4. SDS-polyacrylamide electrophoresis of uridine kinase at different stages of purification. Samples were treated with SDS and mercaptoethanol before analysis on Laemmli gels. Molecular weight markers (Lanes 1 and 11) contained 70 ng each of phosphorylase b (93,000), bovine serum albumin (68,000), carbonic anhydrase (29,000), and soybean trypsin inhibitor (21,000). Samples containing 800 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 µl of pooled P-11 column fractions, respectively. Lanes 9 and 10 contained 70 ng 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. This value is in agreement with a monomer molecular weight of 34,000 determined previously by gel filtration (Payne and Traut, 1982a). 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.

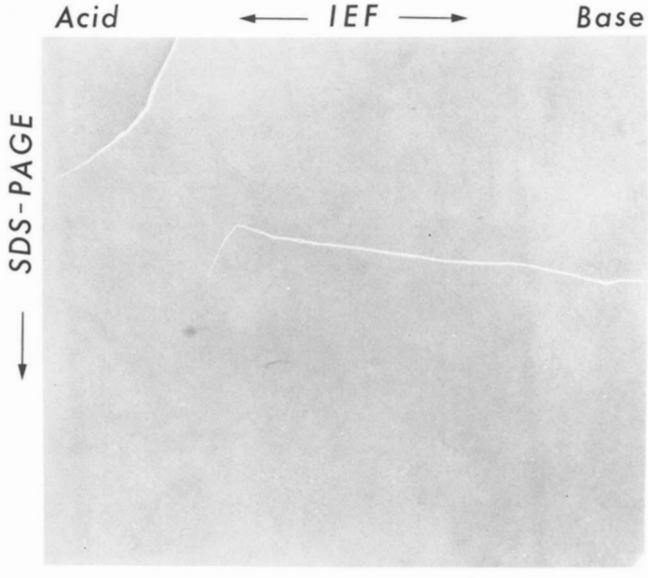

Figure 5. Two-dimensional electrophoresis of homogeneous uridine kinase. The sample consisted of 500 ng of uridine kinase protein in 30 µl of solubilizing buffer.

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.

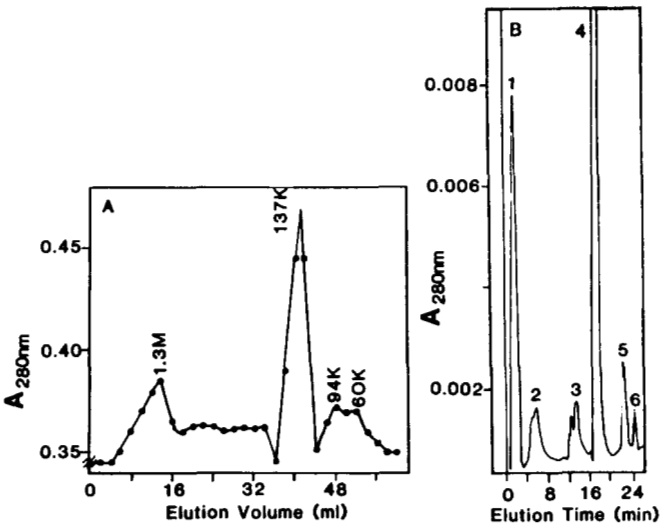

Fig. 6. Chromatography of homogeneous uridine kinase. The purified enzyme was eluted from (A) an AcA34 ultrogel column or (B) a DEAE-silica column.

Both the gel filtration column and the ion-exchange column were able to resolve native uridine kinase into several peaks (Fig. 6). The multiple molecular weight species seen with the ultrogel column resembled the results obtained with crude uridine kinase (Payne and Traut, 1982a). The ion exchange column resolved at least 6 peaks (Fig. 6B); peak 4 represents ATP (present in the enzyme sample) as determined by thin layer chromatography, the absorption spectrum of the peak, and by comparison to the elution of ATP. The other peaks all had active uridine kinase activity (data not shown).

Kinetic Studies

The purified enzyme had a specific activity of 283 μmol/min/mg enzyme at 22°C. This leads to a turnover number of about 150 s^{-1}. Initial velocity patterns with homogeneous uridine kinase using uridine and ATP as substrates were consistent with a sequential mechanism as opposed to a ping-pong mechanism (data not shown). This is consistent with the findings of Liacouras and Anderson (1975) who used a 250-fold purified uridine kinase preparation from murine mast tumor P815. Both CTP and UTP showed competitive inhibition with respect to ATP. Ki's for CTP and UTP inhibition were 10 and 61 μM, respectively. The Km'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 Km's for 5-fluorouridine, 3-deazauridine, and 6-azauridine were 69, 200, and 340 μM, respectively.

Table II. Kinetic Parameters for Substrates and Inhibitors of Uridine Kinase[a]

Substrates	Km(μM)	Ki(μM)
Uridine	40	
Cytidine	57	
5-Fluorouridine	69	
3-Deazauridine	200	
6-Azauridine	340	
Adenosine 5'-Triphosphate	450	160
Cytidine 5'-Triphosphate		10
Uridine 5'-Triphosphate		61

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