PROPERTIES OF SOLUBLE AND METAL ION PRECIPITATED URIDINE KINASES FROM CALF BRAIN

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Uridine kinase precipitated by divalent metal ions from partially purified calf brain extract differs from the soluble enzyme preparation. In the precipitated form of uridine kinase the highest activity is obtained using Pb^{2+}- or Zn^{2+}-ions. The apparent K'_m constants of Pb- and Zn-precipitated uridine kinases are quite similar, but lower than those of the soluble enzyme. Thermal stability of Pb- and Zn-precipitated uridine kinases suspended in an aqueous medium is lower than that of the native enzyme. However, the freeze-dried metal-containing enzyme fractions are highly stable. The insoluble form of uridine kinase is more sensitive to feedback inhibition with CTP, this effect being especially pronounced at lower ATP concentrations. The biological importance of precipitated uridine kinase is mentioned briefly.

Uridine kinase as a representative enzyme of the salvage pathway of pyrimidine synthesis[1-3] plays a key role in tissues in which RNA synthesis is dependent on the supply of preformed pyrimidine bases and nucleosides. Depending on their stage of development and organogenesis various tissues of higher organisms make effective use of the salvage pathway[4-6]. The activity of uridine kinase is generally considered to reflect the efficiency of the system to utilize the salvage pathway. As early as 1954 Rutman and coworkers[7] reported that uracil was utilized for nucleic acid synthesis by a chemically induced liver tumour to a greater extent than by a normal liver. Consequently, uridine kinase was expected to play an important role in the synthesis of nucleic acids in neoplastic tissues. The assumed importance of uridine kinase was further strengthened by the finding that the emergence of resistance of various tumour cells towards 6-azauridine, 5-fluorouridine, or 5-azacytidine was paralleled by the deletion of this enzyme in mutant cell lines[8].

In this paper a simple method of obtaining an insoluble form of uridine kinase coupled to metal ions is described. Calf brains were used as the source of the enzyme. Simultaneously, properties of uridine kinase precipitated by means of Pb^{2+}- and Zn^{2+}-ions were compared with those of the soluble enzyme preparation. Preliminary results of this work were presented elsewhere[9].

EXPERIMENTAL

Reagents. Adenosine 5′-triphosphate and cytidine 5′-triphosphate were obtained from Calbiochem, Luzern. 6-Azauridine and 6-azauridine 5′-phosphate were prepared in this Institute.

6-Azauridine-[4,5-^{14}C] (80 mCi/mmol) was delivered by the Institute for Research, Production and Uses of Radioisotopes, Prague. Lead acetate and zinc chloride were of analytical grade purity, ammonium sulfate was a 3times recrystallized product of Lachema, Brno.

Purification of uridine kinase. In all experiments calf brains obtained from an abattoir within 45 min of decapitation and kept at −30°C were used. Uridine kinase was partially purified by a modified method of Canellakis[10] and Orengo[11]. Brains were homogenized with 3 volumes of cold 25 mM-Tris-HCl buffer (pH 7·4) containing 25 mM-KCl and 5 mM-$MgCl_2$. The homogenate was centrifuged (10000 *g*, 30 min, 2°C), sediment discarded and the clear supernatant fraction brought to 20% saturation by powdered ammonium sulfate. The precipitate was removed by centrifugation and the supernatant, containing uridine kinase, saturated with ammonium sulfate to 35% concentration. The solution was left 1 h at 3°C and precipitated proteins were dissolved in 50 mM-Tris-HCl buffer, pH 7·4, and the solution (fraction III) was used a source of soluble uridine kinase. The enzyme was purified about 10times.

Precipitation of uridine kinase by metal ions. The insoluble form of uridine kinase was prepared from the enzyme fraction III by adding 0·5M lead acetate or zinc chloride under mild stirring to a final concentration of 3 mM. Precipitated proteins were centrifuged, washed twice with distilled water and the suspension was freeze-dried. Elementary analysis and measurement of the content of metals in the precipitated and lyophilized protein fractions was carried out by Dr J. Horáček of the Analytical Department of this Institute.

Assay of uridine kinase activity. The activity of uridine kinase was assayed with 6-azauridine-[4,5-^{14}C] as substrate [12,13]. Radioactive 0·05 mM 6-azauridine was incubated at 37°C with 3 mM-ATP and equimolar Mg^{2+}-ions in the presence of soluble or precipitated uridine kinase preparations in a total volume 0·3 ml of 50 mM-Tris-HCl buffer (pH 7·4). Proteins were determined according to the method of Lowry and coworkers[14]. Chromatographic analysis of the incubation mixture was carried out on Whatman paper No 1 in a solvent system composed of isobutyric acid–ammonium hydroxide–water (44 : 1 : 22). The radioactive zones were cut out and their radioactivity was measured with a Packard liquid-scintillation spectrometer.

RESULTS

An attempt to purify uridine kinase from calf brain using Pb^{2+}-ions[9] to precipitate differentially individual proteins in the cell-free extract resulted in the finding of an insoluble enzyme fraction with high uridine kinase activity. The precipitated proteins formed a stable complex making release of the enzyme in soluble form impossible[15]. In the present study the effect of several divalent cations on the activity and precipitation of partially purified calf-brain uridine kinase was measured. The results (Table I) indicate differential precipitation of the enzyme by individual metal ions. While Mn^{2+}, Co^{2+} and Ni^{2+}-ions resulted in about 25% precipitation, Cu^{2+}, Zn^{2+} and especially Pb^{2+}-ions precipitated the enzyme almost completely. Using 5 mM concentration of these ions no activity of uridine kinase in the soluble fraction was detected. Fe^{2+}-ions had no effect on uridine kinase precipitation even at 10 mM concentration.

In the preparation of partially purified uridine kinase we proceeded as described previously with the Pb-precipitated enzyme[9]. Comparison of the effect of Pb^{2+}-

TABLE I

Precipitation of Calf Brain Uridine Kinase by Metal Ions

Samples of fraction III (2 ml, specific activity 11·5 nmol/mg proteins, 1·02 mg proteins per 0·1 ml) in 50 mM Tris-HCl buffer (pH 7·4) were mixed in ice-water bath with 0·5M salt solutions (acetates and chlorides) to give a final concentration of cations 2 and 5 mM, respectively. After 10 min of mixing the precipitates were removed by centrifugation (5000 *g*, 3 min, 3°C) and the activity of uridine kinase (in 0·1 ml of supernatants and sediments suspended in 2 ml of the buffer) was measured during a 10-min incubation period.

Metal ions	Uridine kinase, nmol		total %	uridine kinase, nmol		total %
	supernatant	precipitate		supernatant	precipitate	
	2 mM ions			5 mM ions		
Pb^{2+}	4·65	5·85	91·4	0·75	7·10	68·2
Cu^{2+}	0	6·05	52·6	0	4·50	38·1
Zn^{2+}	0·85	5·85	58·3	0	6·80	59·1
Mn^{2+}	7·90	2·15	87·2	7·75	2·55	90·0
Co^{2+}	7·15	2·45	83·5	7·10	2·58	83·9
Ni^{2+}	6·50	2·60	80·7	4·72	2·85	65·7

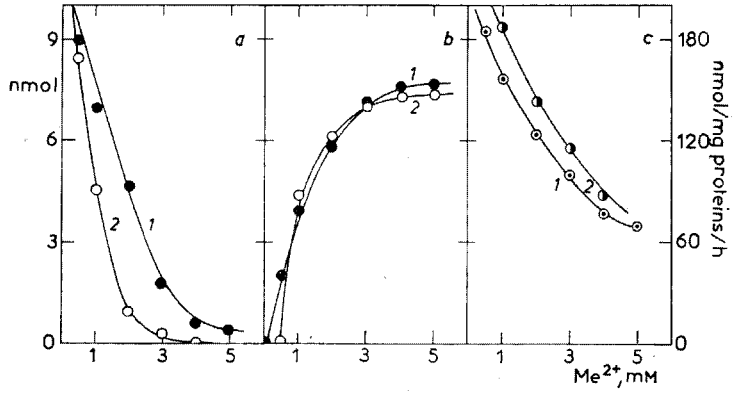

FIG. 1

Precipitation of Uridine Kinase in the Presence of Pb^{2+}- and Zn^{2+}-Ions

5 ml samples of fraction III (1·0 mg proteins per 0·1 ml) were kept in ice-water bath and mixed with 0·5M lead acetate *1* or 0·5M-$ZnCl_2$ 2. After 10 min of mixing the precipitates were centrifuged (5000 *g*, 5 min, 2°C) and uridine kinase activity as well as protein content in 0·1 ml of individual supernatant fractions and sediments, suspended in 5 ml of 25 mM-Tris-HCl buffer, were measured. *a* The activity of uridine kinase expressed as nmol of phosphorylated 6-azauridine in the supernatant; *b* in the precipitate; *c* specific activity of uridine kinase (nmol/mg protein per h) in the precipitate.

and Zn^{2+}-ions on both the activity and the extent of enzyme precipitation is depicted in Fig. 1. Fig. 1*a* shows the activity of enzyme remaining in the supernatant fluid. However, the results obtained so far do not permit us to distinguish between enzyme inactivation and inhibition. The activity of uridine kinase in precipitated protein fractions was the same (Fig. 1*b*), and also the specific activities of both enzyme preparations were similar (Fig. 1*c*). However, isolation of Pb- and Zn-precipitated enzyme fractions on a preparative scale often revealed slight differences between the specific activities of both enzyme preparations. Elementary analysis of the lyophilized enzyme fractions coupled to Pb^{2+}- and Zn^{2+}-ions indicate that on molar bases the concentration of both metals in the two enzyme precipitates is the same.

The aim of the present study was to get more insight into the kinetic properties of the soluble and precipitated forms of uridine kinase. The time course of phosphorylation when 6-azauridine is used as substrate of uridine kinase is shown in Fig. 2. The reaction was linear up to 10–15 min of incubation using given amounts of individual enzyme fractions. The maximum amount of reacted substrate (about 60% using 0·05 mM 6-azauridine) was the same no matter if precipitated or soluble uridine kinases were used. The kinetic constants of soluble and precipitated uridine kinases with respect to 6-azauridine and ATP as substrates, calculated from the Lineweaver–Burk plots (Fig. 3), are presented in Table II. The apparent Michaelis

TABLE II

Kinetic Constants of Soluble and Precipitated Uridine Kinases with 6-Azauridine and ATP as Substrates

Uridine kinase was assayed in a reaction mixture (0·5 ml) containing 0·05 mM 6-azauridine and varying concentrations of ATP with equimolar Mg^{2+}-ions, and 2 mM ATP with equimolar Mg^{2+}-ions and varying concentrations of 6-azauridine, respectively. Incubation was carried out for 10 min at 37°C with soluble (1·10 mg proteins), Pb-precipitated (0·50 mg proteins) and Zn-precipitated (0·50 mg proteins) uridine kinases. The activity of enzymes was expressed in mol/l of 6-azauridine phosphorylated during 10 min of incubation in the presence of 1 mg protein in used enzyme preparations.

Uridine kinase	Substrate	Apparent K'_m mM	Apparent V'_{max} mM
Soluble	ATP	2·0	0·23
	6-azauridine	0·1	0·30
Pb-precipitated	ATP	1·0	0·50
	6-azauridine	0·05	0·38
Zn-precipitated	ATP	1·0	0·21
	6-azauridine	0·05	0·26

constants K'_m of the Pb- and Zn-precipitated enzymes were identical, but lower than those of soluble kinase. Also, the apparent maximum velocities of soluble and Zn-precipitated fractions were practically the same; however, V'_{max} of the Pb-precipitated enzyme was higher. Compared with the soluble enzyme fraction, uridine kinases precipitated by metal ions are more sensitive in solution to thermal inactivation (Fig. 4). Similar changes in enzyme inactivation were observed with enzyme preparations subjected in aqueous solutions to preincubation at 50°C using both different and equal initial activities and protein contents of the enzyme preparations.

Uridine kinase is susceptible to feedback inhibition by pyrimidine nucleoside 5'-triphosphates[2,11,17]. Effective inhibition *in vitro* was obtained using UTP and especially CTP. The inhibitory mechanism is dependent on, and modulated by the level of ATP in the reaction mixture[11,18]. A marked difference between the inhibitory effect of CTP on the soluble and Pb-precipitated uridine kinase was reported earlier[9]. Inhibition by CTP was more marked in the precipitated enzyme than in the soluble one. In the present study we obtained similar results using also Zn-precipitated uridine kinase (Fig. 5). There was no apparent difference in CTP-induced feedback inhibition between the Pb- and Zn-precipitated uridine kinases. The inhibitory effect of CTP on different uridine kinase preparations in relation to ATP concentration is shown in Fig. 6. It is evident that ATP at higher levels prevented feedback inhibition, this effect being different using either soluble or precipitated form of uridine kinase.

DISCUSSION

The occurrence of uridine kinase catalyzing the phosphorylation of uridine and cytidine was first described in mammalian liver[10] and Ehrlich ascites tumour[19], and later in extracts from a variety of tissues[20]. The enzyme was partially purified from different tissues and organisms and its molecular properties were described[11,21–24].

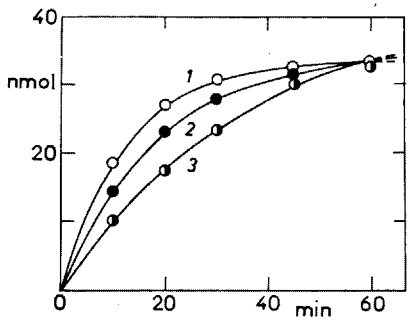

FIG. 2

Time Course of 6-Azauridine Phosphorylation Catalyzed by Soluble and Precipitated Uridine Kinases

The activity of uridine kinase was assayed at 37°C with soluble Fraction III *1*, Pb-precipitated *2* and Zn-precipitated *3* enzyme preparations.

Divalent metal ions were found to be necessary for its activity, Mg^{2+}-ions being the most effective[21,22].

Precipitation of uridine kinase by metal ions results in the formation of a protein complex irreversibly coupled to these ions. Preliminary results indicate that the enzyme cannot be released from this complex without loss of activity (see also[15]). There is evidence indicating that uridine kinase of dry corn seeds survives for years partly as a stable complex[25]. Again, attempts to dissociate the enzyme from its putative complex resulted in inactivation of uridine kinase activity.

Both Pb- and Zn-precipitated uridine kinases suspended in aqueous media are more sensitive to thermal denaturation (Fig. 4). However, the precipitated enzyme preparations were found to be highly stable in dry state[16], even at 100°C. Thus, the greater thermal lability of the metal-containing fractions of uridine kinase in solution may be due to coagulation of these preparations at higher temperatures. It is also

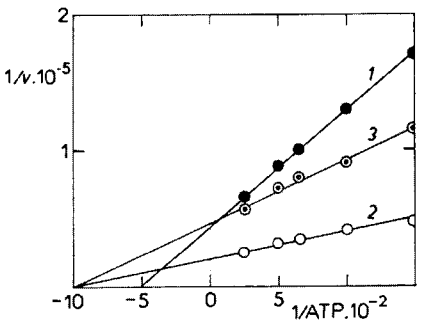

FIG. 3

Lineweaver–Burk Plot for Soluble and Precipitated Uridine Kinases with Respect to Concentration of ATP

The activity of uridine kinase was assayed in a reaction mixture (0·5 ml) containing varying concentrations of ATP with equimolar Mg^{2+}-ions, 0·05 mM 6-azauridine-[4,5-^{14}C] and soluble (1.10 mg proteins; *1* Pb-precipitated (0·50 mg proteins; *2* and Zn-precipitated (0·56 mg proteins; *3* uridine kinases. Incubation was 10 min at 37°C. The amount of 6-azauridine 5′-phosphate formed in the presence of 1 mg protein as well as the concentration of ATP are expressed in mol per litre.

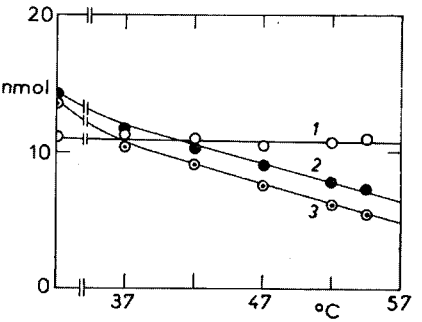

FIG. 4

Thermal Stability of Soluble and Precipitated Uridine Kinases

Soluble (1·04 mg proteins; *1* Pb-precipitated (0·61 mg proteins; *2* and Zn-precipitated (0·73 mg proteins; *3* uridine kinases in 50 mM-Tris-HCl buffer (pH 7·4) were preincubated for 20 min at different temperatures (°C). Thereafter to a total volume 0·3 ml of an incubation mixture 6-azauridine-[4,5-^{14}C] (0·05 mM) and ATP with equimolar Mg^{2+}-ions (3 mM) were added and uridine kinase activity (nmol) was assayed at 37°C during a 10-min incubation period.

conceivable that interaction of the enzyme with metal ions can lead to changes in subunit-subunit interaction or relation of the two isozyme forms of uridine kinase[26,27] which are reflected in alteration of thermal stability.

Changes in thermal stability of uridine kinase have been observed previously under a variety of experimental conditions. A marked difference was found using uridine kinases isolated from mouse leukemic cells either sensitive or resistant to 5-azacytidine[23], or from Ehrlich ascites tumour cells sensitive and resistant to 5-fluorouracil[28]. Changed thermal stability was observed also with uridine kinases present in the liver of control and 5-azacytidine-treated rats[29] as well as with two molecular forms of the enzyme isolated from Novikoff ascites hepatoma[26] and Ehrlich ascites tumour cells[27]. It seems that alterations in the secondary or tertiary structure of uridine kinase are responsible for the observed changes in the thermal stability of the enzyme.

Since the fraction of uridine kinase used in this study for the precipitation by meta

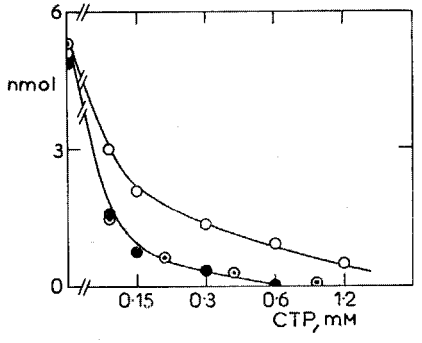

FIG. 5

Feedback Inhibition of Soluble and Precipitated Uridine Kinases with CTP

Soluble (0·65 mg proteins; ○) Pb-precipitated (0·34 mg proteins; ●) and Zn-precipitated (0·41 mg proteins; ⊙) uridine kinases in 50 mM-Tris-HCl buffer (pH 7·4) were mixed with 0·04 mM 6-azauridine-[4,5-^{14}C], 2 mM ATP with equimolar Mg^{2+}-ions and varying concentrations of CTP in a total volume 0·4 ml. Uridine kinase activity (nmol) was assayed at 37°C during a 10-min incubation period.

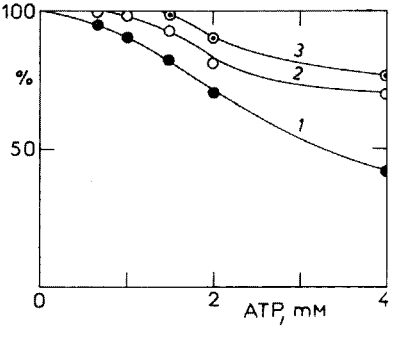

FIG. 6

Effect of ATP on Feedback Inhibition of Soluble and Precipitated Uridine Kinases

Soluble (1·10 mg proteins; *1* Pb-precipitated (0·50 mg proteins; *2* and Zn-precipitated (0·62 mg proteins; *3* uridine kinases in 60 mM-Tris-HCl buffer (pH 7·4) were mixed with 0·05 mM 6-azauridine-[4,5-^{14}C], 0·2 mM CTP or buffer and varying ATP with equimolar $MgCl_2$ in a total volume 0·5 ml. Uridine kinase was assayed at 37°C during a 10-min incubation period. Inhibition with 0·2 mM CTP is expressed as per cent of the corresponding control incubation without CTP.

ions is of low purity, it is difficult to decide whether the modification by divalent cations represents a separation of isozymes, coprecipitation and/or interaction of uridine kinase with inert proteins. Changed structural organization of the soluble and precipitated uridine kinase molecules might be responsible for the changed feedback inhibition in either form of the enzyme by CTP (Figs 5 and 6). Higher sensitivity of precipitated enzymes to the action of CTP presumably reflects differences in the accessibility of ATP and the feedback inhibitor to the active and regulatory sites of the flexible and alternatively fixed polypeptide chains of the soluble and precipitated forms of uridine kinase.

The ability of the insoluble form of uridine kinase to phosphorylate cytostatic nucleoside analogues could be of practical importance. Utilization of the salvage pathway by rapidly proliferating tissues including different tumours often serves to supply pyrimidine precursors for nucleic acid synthesis[3]. Moreover, the level of uridine kinase is considered a limiting factor in the transformation of cytostatic uridine and cytidine analogues to biologically active 5'-nucleotides. This becomes even more evident in cells resistant to these drugs with deleted uridine kinase[8]. In such a case the administration of uridine kinase coupled to Zn^{2+}-ions might promote phosphorylation of the drugs used.

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