PROPERTIES OF Pb-PRECIPITATED URIDINE KINASE ENABLING ITS USE IN PREPARATIVE-SCALE SYNTHESIS OF RADIOACTIVE 6-AZAURIDINE 5'-PHOSPHATE

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The addition of lead acetate to partly purified calf brain uridine kinase leads to the precipitation of the proteins present without a marked drop of the activity of the enzyme. The complex of the precipitated proteins with lead ions is considerably stable; attempts to release uridine kinase in soluble form from the complex were unsuccessful. The lyophilized precipitate of uridine kinase does not loose its enzymatic activity when stored at low temperature. It was employed for preparative synthesis of radioactive 6-azauridine 5'-monophosphate from 6-azauridine-4,5-[^{14}C]. The optimum conditions of incubation, which permit a 70–80% yield to be obtained, are described.

During the past few years considerable attention has been devoted to the preparation of immobilized enzymes attached to a solid support[1,2]. These modified enzymes show numerous advantages compared to soluble enzyme preparations, such as, *e.g.* a higher thermal stability, the possibility of repeated use, *etc.* This paper reports on the preparation of the insoluble form of uridine kinase; in this process, the support is substituted to a certain degree by precipitated proteins. We have found recently[3,4] that the addition of certain heavy metal ions brings about the precipitation of uridine kinase without any substantial drop of the activity of the enzyme. A complex precipitate is formed in which the protein moiety is coupled to metal ions. This paper describes certain characteristics of the complex and the use of Pb-precipitated calf brain uridine kinase in preparative-scale synthesis of 6-azauridine 5'-phosphate. A procedure suitable for the preparation of labeled uridine and thymidine nucleotides has been described by us elsewhere[5,6].

EXPERIMENTAL

Reagents and Animals

6-Azauridine-4,5-[^{14}C] (80 mCi/mmol) was synthetized in the Institute for Research, Production and Use of Radioisotopes, Prague. Adenosine 5'-triphosphate was purchased from Calbiochem, Luzern. Ammonium sulfate was an analytical grade, 3-times recrystallized product of Lachema,

Brno. Calf brains were obtained from an abattoir within 45 min after decapitation and kept at −30°C before use.

Purification and Precipitation of Uridine Kinase

The brains were homogenized in a glass homogenizer with a tigh-fitting Teflon pestle in 3 volumes of cold 25 mM Tris-HCl buffer, pH 7·4, containing 25 mM KCl and 5 mM $MgCl_2$ (Fraction I, see Table I). Uridine kinase was further purified partially by a modified method of Orengo[7]. The homogenate was centrifuged (10000 *g*, 20 min, 2°C) and the supernatant fraction (Fraction II) brought to 20% saturation with ammonium sulfate. The precipitate was discarded and the supernatant fraction saturated with ammonium sulfate to 35% saturation. The solution was left overnight at 5°C and the proteins precipitated were removed by centrifugation and dissolved in 50 mM Tris-HCl, pH 7·4 (Fraction III). To the cold solution 0·5M lead acetate was added to a final concentration of 3 mM; the precipitated proteins were spun down and repeatedly washed with distilled water. The sediment suspended in water (Fraction IV) was freeze-dried, and yielded relatively stable Pb-precipitated uridine kinase (Fraction V). The protein content was determined according to Lowry and coworkers[8].

Measurement of Uridine Kinase Activity

The uridine kinase activity was determined is a standard reaction mixture (usually 0·3 ml) containing 60 mM Tris-HCl buffer, pH 7·4, 3 mM adenosine 5′-triphosphate, 1·5 mM $MgCl_2$, 0·05 mM 6-azauridine-4,5-[^{14}C] as substrate[9], and soluble or metal-ion precipitated uridine kinase pre-

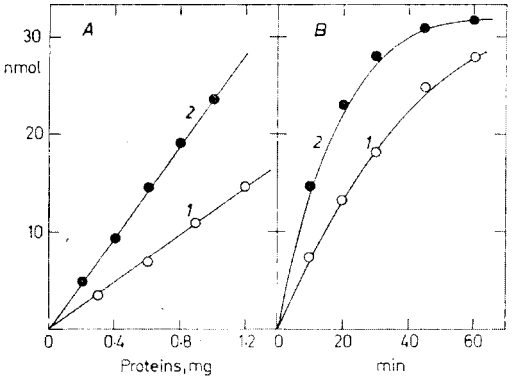

FIG. 1

Standard Assay of Uridine Kinase Activity Catalyzed by Soluble and Pb-Precipitated Form of Enzyme

A: 10 min incubation of 0·05 mM 6-azauridine-4,5-^{14}C at 37°C in the presence of 4 mM adenosine 5′-triphosphate with 1·5 mM $MgCl_2$ in a total volume of 0·3 ml. Fraction III *1* and Fraction V *2*. *B*: Time course of the reaction in the presence of *1* soluble (Fraction III, 0·69 mg of proteins) and *2* Pb-precipitated enzyme (Fraction V, 0·57 mg of proteins). The activity of the enzyme is expressed in nmol of phosphorylated 6-azauridine.

parations. The incubation was carried out with shaking at 37°C and aliquots of the reaction mixture were analyzed by descending chromatography on a Whatman No 1 paper in n-butanol–acetic acid–water (10 : 1 : 3) or in a solvent system composed of isobutyric acid–ammonium hydroxide–water (66 : 1·5 : 33). The radioactive zones of 6-azauridine and of its 5′-phosphate were cut out after comparison with standards and their radioactivity was assayed in a Packard Tri Carb liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Uridine kinase, which catalyzes the phosphorylation of uridine and cytidine by adenosine 5′-triphosphate, plays an important role in the synthesis of nucleic acids in rapidly proliferating tissues[10]. The enzyme is not absolutely specific and reacts with uridine and cytidine analogs, *e.g.* 5-fluorouridine[11], 5-fluorocytidine[11], 6-azauridine[12], 6-azacytidine[13], 2-thiouridine[14], 5-hydroxyuridine[15], 5-azacytidine[16,17], *etc.* Since the level of uridine kinase reflects the efficiency of the system to utilize preformed pyrimidine precursors by the salvage pathway, the finding[18] that brain utilizes preformed pyrimidines to a much greater extent than the *de novo* pathway led us to use

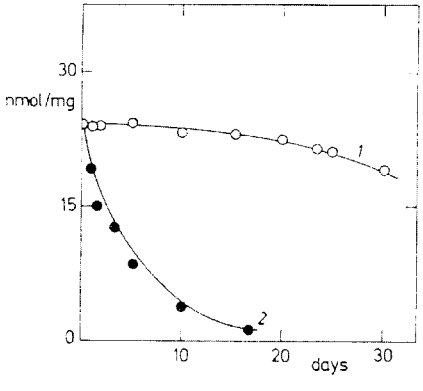

FIG. 2

Stability of Freeze-Dried Pb-Precipitated Uridine Kinase

Fraction V was stored at 5°C *1* or 37°C *2* in flasks with glass stoppers and at various time intervals (days) the activity of uridine kinase was assayed during a 10 min incubation period.

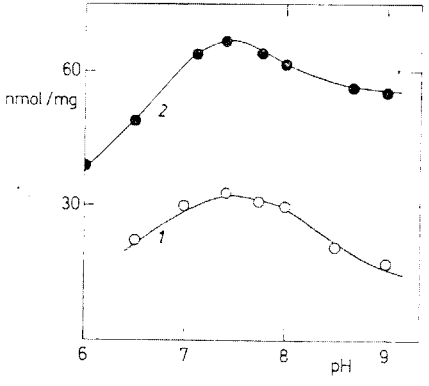

FIG. 3

Activity of Soluble and Pb-Precipitated Uridine Kinase at Different pH

Soluble uridine kinase *1*, (Fraction III, 0·91 mg of proteins) and precipitated enzyme *2* (Fraction V, 0·80 mg of proteins) were incubated 15 min at 37°C in 50 mM Tris-HCl buffer at different pH values with 0·1 mM 6-azauridine-4,5-^{14}C, *4* mM adenosine 5′-triphosphate and 2 mM $MgCl_2$. The enzyme activity is expressed in nmol of reacted 6-azauridine per mg protein.

calf brain as a source of the enzyme. Uridine kinase has already been isolated and purified from various sources, especially from Ehrlich ascites tumor cells[11,19], mouse leukemic cells[20], and recently from rat liver[21] and calf thymus[17].

The procedure of isolation of uridine kinase and its salting out by ammonium sulfate, resulting in a mild increase of the specific activity of the enzyme, are shown in Table I. The precipitation of the proteins, giving rise to an insoluble form of uridine kinase, was carried out as in our earlier experiments[4] where the addition of lead acetate did not cause a more significant loss of the enzyme activity during the precipitation. The measurement of the activity of uridine kinase in the process of purification and precipitation was effected by a method using 6-azauridine as substrate[9,22]. The conditions of the assay and the linear dependence of the course of the reaction on the length of the incubation period and the quantity of the enzyme preparation added are shown in Fig. 1.

The precipitate of proteins obtained after the addition of lead acetate to a solution of partly purified uridine kinase was repeatedly washed with water to remove adsorbed lead ions and lyophilized. No substantial loss of enzymatic activity of uridine kinase was observed during storage of the precipitate in solid state at a low temperature. The changes which take place during storage of the lyophilisate of Pb-precipitated uridine kinase at $+5°$ and 37°C are obvious from the data shown in Fig. 2.

As can be seen, the precipitated enzyme preparation is sufficiently stable even at 37°C. By contrast, a lower thermal stability of the Pb-precipitated form of the enzyme compared to the soluble form of uridine kinase was observed[4] if preincubation had been carried at higher temperature in a buffered medium (Fraction III). No difference was observed when the phosphorylation of 6-azauridine was carried out with soluble or Pb-precipitated uridine kinase at various pH-values (Fig. 3). Neutral pH (7·0 – 7·5) was found to be most suitable for the reaction.

The Pb^{2+}-ions are obviously linked to the free SH-bonds of the individual proteins present in the precipitate of partly purified uridine kinase. The complex is stable

TABLE I

Preparation of Partially Purified Calf Brain Uridine Kinase Precipitated by Pb^{2+}-Ions

Fraction No	Total activity		Specific activity	
	μmol per fraction, %		nmol/mg proteins increase	
I	8·86	100	1·29	1·0
II	4·16	47	2·07	1·6
III	4·08	46	12·32	9·6
IV	2·13	24	24·28	18·8
V	2·02	23	26·18	20·3

TABLE II

Effect of Various Reagents on Activity of Uridine Kinase Coupled to Pb^{2+}-Ions

To Pb-precipitated uridine kinase suspended in 1 ml of water (0·23 mg of proteins) were added compounds reacting with Pb^{2+}-ions. After 10 min of mixing in an ice water bath, the precipitate was centrifuged (5000 *g*, 5°C, 3 min) and the activity of uridine kinase in the supernatant and the sediment (suspended in 1 ml of water) was determined. Activity of control = = 100%.

Compound	Concentration mM	Uridine kinase, nmol		Activity %
		precipitated	soluble	
—	—	6·1	—	100
NaS	2	3·4	0	55·8
NaS	3	2·3	0	37·7
EDTA	2	4·8	0	78·7
Oxalic acid	2	0·8	0	13·0
Anthranylic acid	2	6·3	0	103·0

TABLE III

Conditions of Synthesis of 6-Azauridine 5′-Phosphate from 6-Azauridine Using Pb-Precipitated Uridine Kinase

1 mM 6-Azauridine-4,5-^{14}C was incubated in Dubnoff shaking incubator at 37°C, in 50 mM Tris-HCl, pH 7·4, with 40 mM adenosine 5′-triphosphate and 20 mM $MgCl_2$ in a total volume of 4 ml.

Pb-precipitated uridine kinase mg	6-Azauridine 5′-phosphate, nmol			
	15 min	30 min	60 min	120 min
8	320	450	520	480
16	400	560	630	720
24	480	620	740	810

and the presence of lead ions can be detected by certain reagents; we have not been able, however, to release uridine kinase in soluble form (Table II). Lead sulfide, EDTA, and oxalic acid, reacting to a different degree with the Pb^{2+}-ions coupled to the proteins precipitated, inhibit rather significantly the activity of uridine kinase. When the Pb-precipitate was suspended in the incubation medium, the presence of free lead ions was not detected in the solution; hence the possibility of contamination of the end product of the reaction is very little probable.

In view of the fact that Pb-precipitated uridine kinase can easily be prepared and the lyophilized preparation is stable when stored at a low temperature, we examined the possibility of practical use of this modified enzyme fraction for preparative-scale synthesis of radioactive 6-azauridine 5'-monophosphate. This nucleotide is of major biological importance since it is the actual substance with inhibitory activity which is formed in the cell after the application of 6-azauridine[12,23]. At a sufficiently high level of the precipitated enzyme, an up to 80% conversion of 6-azauridine into the corresponding 5'-monophosphate takes place in the incubation mixture (Table III). A reversal cleavage of the newly formed nucleotide to 6-azauridine was not observed during the incubation. If necessary, the precipitated uridine kinase preparation can be isolated from the mixture by centrifugation and used directly for an additional incubation. The reaction product can then be isolated by common procedures from the incubation medium which has been freed of proteins under these mild conditions.

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