Pb-PRECIPITATED PROTEIN FRACTION FROM CALF BRAIN CONTAINING HIGHLY ACTIVE URIDINE KINASE WITH DIFFERENT MOLECULAR PROPERTIES

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Received 9 December 1974

1. Introduction

The de novo pyrimidine synthesis as well as pyrimidine nucleotides synthesized from preformed bases by the salvage pathway [1] can serve as a source of pyrimidines. Hogans et al. [2] observed recently that uridine was far superior to orotic acid in labelling RNA in rat brain. Among the tissues studied the preference for uridine over orotic acid for RNA synthesis was unique to neural tissue. Even though the concept that the biogenesis of pyrimidines for RNA synthesis in the whole animal occurs primarily through the de novo synthesis is generally accepted, the results suggest that rat brain utilizes preformed pyrimidines to a much greater extent than it utilizes the de novo pathway to supply its requirements for pyrimidine nucleotides.

Uridine kinase is a representative and key enzyme of the salvage pathway [3]. The enzyme has been shown in chick embryo to be of greater importance at later stage of development and organogenesis than during early stages of development [4]. A similar observation was made during the study of a developmental pattern of uridine kinase and enzymes of the de novo pyrimidine biosynthetic pathway in developing rat cerebellum [5]. It was suggested that while the de novo synthesis provides the major source of pyrimidine nucleotides required for RNA synthesis in developing rat brain, mature brain obtains its pyrimidine nucleotides primarily from utilization of preformed pyrimidine bases and nucleotides. In this study an attempt to characterize and partially purify uridine kinase from calf brain is described. Simultaneously an effect of Pb^{2+} ions, resulting in an irreversible precipitation of the enzyme without affecting seriously its activity is presented.

2. Methods

Calf brains were obtained from an abattoir within 45 min of decapitation and kept at −30°C. Uridine kinase was purified by a modified method of Orengo [6]. Brains were homogenized (see table 1), the homogenate centrifuged and the supernatant fraction brought to 20% saturation by the slow addition of ammonium sulfate. The precipitate was discarded, supernatant saturated to 35% by ammonium sulfate and precipitated proteins were removed and dissolved in 50 mM Tris–HCl, pH 7.4 (Fraction III). To the cold solution was added [7] 0.5 M lead acetate and precipitated proteins were repeatedly washed with water and freeze-dried.

The activity of uridine kinase was assayed using 0.05 mM 6-azauridine-4,5-[^{14}C] as the substrate [8]. The analogue is not degraded by the phosphorylase present in unpurified extracts and the newly formed 6-azauridine 5′-monophosphate is not metabolized to higher 5′-phosphates [9]. Conditions of incubation and composition of the reaction mixture are apparent from the legends to figures. Aliquots of the reaction mixture were analyzed by the chromatography on Whatman paper No. 1 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 using standards and their radioactivity was assayed with a Packard liquid-

Table 1
Preparation of partially purified Pb-precipitated uridine kinase from calf brain*

Fraction	Protein	Activity	Total activity		Specific activity	
	mg/ml	nmol/ml	μmol/fraction	%	nmol/mg	Increase
Homogenate	25.0	31	7.44	100	1.24	1.00
Supernatant	19.6	36	3.36	45.1	1.84	1.48
Fraction III	8.7	96	3.04	40.4	11.03	8.90
Pb-precipitate	5.5	130	1.85	24.9	23.70	19.12

* Calf brain (40 g) was homogenized with 120 ml cold 25 mM Tris–HCl buffer, pH 7.5, containing 25 mM KCl and 5 mM $MgCl_2$. The homogenate was centrifuged (10 000 *g*, 30 min, 2°C) and the supernatant fraction brought to 20% saturation by ammonium sulfate. The precipitate was discarded and the supernatant saturated with ammonium sulfate to 35% concentration. The solution was left overnight at 5°C and precipitated proteins, removed by centrifugation were dissolved in 50 ml of 50 mM Tris–HCl, pH 7.4 (Fraction III). The cold solution was mixed with 0.5 M lead acetate to a final concentration 3 mM. Precipitated proteins were removed by centrifugation, washed twice with 20 ml of cold water, suspended in water and freeze-dried.

scintillation spectrometer. The activity of uridine kinase is expressed as nmol of reacted substrate in a given incubation period.

3. Results and discussion

The addition of ammonium sulfate to the cell-free extract prepared from calf brain resulted in a precipitation of the protein fraction containing uridine kinase. An attempt to purify the enzyme by means of Pb^{2+} ions was made. The results have shown (table 1) that although the addition of Pb^{2+} ions resulted in the precipitation of uridine kinase the fraction was enzymatically highly active. All the steps resulted in 20-fold purification only, but with a yield of about 25%. The loss of enzyme activity during freeze-drying of the sample was less than 10%, and during 14 days no appreciable decrease in the activity of the lyophilized enzyme preparation was detected.

An attempt to remove Pb^{2+} ions coupled to the protein was unsuccessful and the release of soluble form of uridine kinase from the complex was not accomplished. The course of uridine kinase precipitation and its activity in relation to the level of Pb^{2+} ions in solution is indicated in fig.1. Lower concentration of the cation resulted in the enhancement of specific activity of the precipitated enzyme to compare with control without a significant loss of the activity. However, at higher level Pb^{2+} ions precipitated the soluble enzyme almost completely, the specific activity of precipitated proteins having been similar to the soluble one, and the activity of uridine kinase was lowered by 20–40%.

Soluble and precipitated uridine kinases differ markedly in their thermal stability. From the data presented in fig.2 it is evident that the precipitated enzyme is considerably less stable on heating. The changes in thermal stability of uridine kinases were observed previously using the enzyme isolated from the liver of 5-azacytidine-treated rats [8] or from the liver of mice with lymphatic leukemia sensitive and resistant to 5-azacytidine [10]. Similar difference was observed by Sköld [11], and recently by Krystal and Webb [12] who studied two molecular forms of the enzyme in Novikoff rat hepatoma. It thus seems that alteration of the secondary or tertiary structure of the enzyme molecule is responsible for the changed sensitivity towards heating.

Uridine kinase as the rate-limiting enzyme in the anabolism of uridine and cytidine is susceptible to feedback inhibition by the pyrimidine nucleoside 5′-triphosphate end products. Effective inhibition can be exerted by uridine 5′-triphosphate and especially

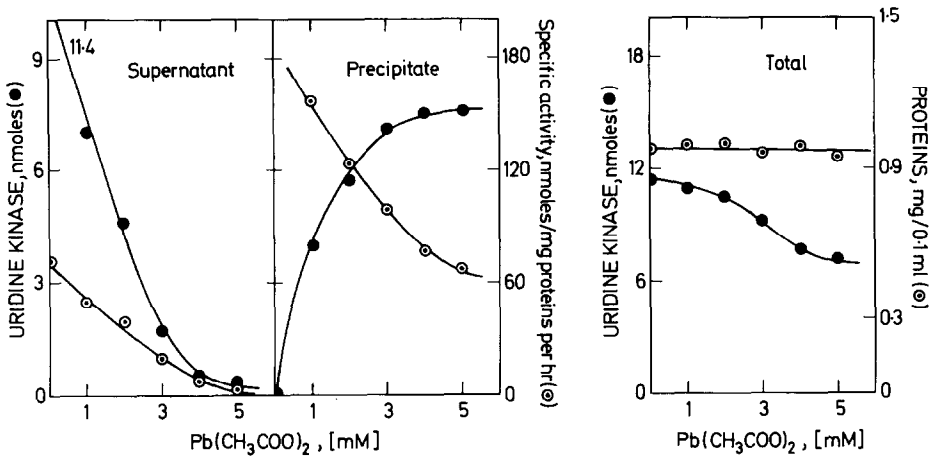

Fig.1. Precipitation of partially purified uridine kinase with Pb^{2+} ions. To 5 ml samples of Fraction III (about 50 mg proteins) kept in ice water bath 0.5 M lead acetate was added to a final concentration 1–5 mM. After 10 min of mixing the precipitate was centrifuged (5 000 *g*, 5 min, 2°C) and the activity of uridine kinase and the level of proteins in supernatants and sedimented precipitates, suspended in 5 ml of Tris–HCl buffer, were measured. The specific activity of uridine kinase is expressed as nmoles of 6-azauridine phosphorylated during 1 hr of incubation at 37°C in the presence of 1 mg of proteins.

by cytidine 5′-triphosphate [13]. Between the soluble and precipitated form of uridine kinase exist marked differences in the inhibition by CTP. The slope of curves shown in fig.3 indicates that the precipitate enzyme is much more sensitive to the inhibition by the 5′-triphosphate than the soluble one. The difference

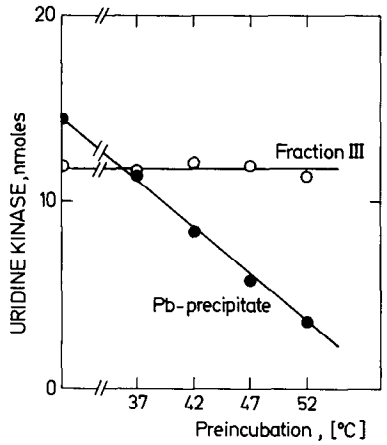

Fig.2. Different thermal stability of soluble and Pb-precipatated uridine kinase 0.1 ml of Fraction III (1.2 mg proteins) or Pb-precipitated enzyme fraction (0.6 mg proteins) were preincubated for 20 min at different temperatures (°C). Thereafter 0.1 ml of 0.05 mM 6-azauridine-4,5-[^{14}C] and 0.1 ml of 3 mM ATP with equimolar Mg^{2+} ions in 0.1 M Tris–HCl, pH 7.4, were added and the mixture was incubated for 10 min at 37°C to measure the activity of uridine kinase.

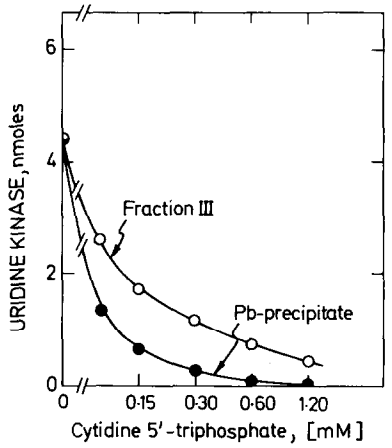

Fig.3. Feedback inhibition of soluble and Pb-precipitated uridine kinase by cytidine 5′-triphosphate. 0.1 ml of soluble (0.4 mg proteins) or Pb-precipitate enzyme fraction (0.23 mg proteins) were mixed with 0.03 mM 6-azauridine-4,5-[^{14}C], 2 mM ATP with equimolar Mg^{2+} ions and varying concentrations of CTP (mM) in a total volume 0.5 ml of 0.04 M Tris–HCl, pH 7.4. Uridine kinase activity was assayed at 37°C during a 15 min incubation period.

reflects a changed structural organization of the native and precipitated enzyme molecules and is probably based on a different accessibility of ATP and the feedback inhibitor to the active and regulatory sites of the flexible or alternatively fixed polypeptide chain of the two forms of the same enzyme.

Uridine kinase is not a metalloenzyme and subunit structure of the enzyme has not been unequivocally established at present. Precipitation of the enzyme by Pb^{2+} ions though not useful as a purification step revealed a higly active fraction with different molecular properties. The phenomenon is not specific and Zn^{2+} ions can be used to some extent for the same purpose. It should be stressed that the property of uridine kinase to phosphorylate 6-azauridine to biological active 6-azauridine 5′-phosphate [14] while irreversibly precipitated is quite unique. At present the possibility to enhance the metabolic conversion of 6-azauridine in mammalian cells in vivo by applying the enzyme coupled to metal cations is under investigation.

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