RELEASE OF ACTIVE URIDINE KINASE FROM Zn-PRECIPITATED PROTEIN FRACTIONS BY TRITON X-100

A. ČIHÁK

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received June 22nd, 1976

Precipitation of partially purified uridine kinase from various tissues of rats by means of Zn^{2+}-ions results in the formation of stable complex containing the enzyme and contaminating proteins. Uridine kinase can be solubilized from the complex by Triton X-100 and 0·5 mM-EDTA facilitates the release of the enzyme into the soluble form. The solubilization of uridine kinase depends on the tissue from which the complex has been prepared and on the aging of precipitated proteins. Uridine kinase precipitated by Zn^{2+}-ions from the kidney and small intestine of rats was released by 2% Triton X-100 to about 40% while no enzyme activity in the soluble fraction was observed using the highly active Zn-precipitated enzyme complex from the brain. The solubilized uridine kinase is rather stable and can be separated on a DEAE-cellulose column as a "native" soluble enzyme.

The precipitation of partially purified uridine kinase with contaminating proteins by means of heavy metal ions resulting in the formation of stable and active complexes[1,2] might be useful for the better understanding of uridine kinase complexes existing in living cells. The enzyme has not been obtained in a sufficient purity so far and differences in the size of uridine kinase isolated from various sources[3–7] may reflect its association with other macromolecules. Thus, uridine kinase purified from the microsomal fraction of mouse salivary glands was characterized by a molecular weight of more than two million[6]. Similarly, the enzyme isolated from ungerminated corn seeds of *Zea mays* appeared to have a molecular weight of several million[7]. Attempts to dissociate the enzyme from a high-molecular-weight fraction resulted in inactivation of the enzyme activity.

The insoluble complexes of uridine kinase prepared in our laboratory from various tissues of higher organisms[2] represent an active form of the enzyme immobilized by means of metal ions. The kinetic properties of the soluble and Zn-precipitated forms of uridine kinase are rather similar[8], but there are differences between the stability of two forms of the enzyme towards thermal inactivation and digestion with proteinases[9]. The first attempt to solubilize uridine kinase from Zn- or Pb-complexes was unsuccessful and resulted in inactivation of the enzyme activity[9]. In this report evidence is presented that using a nonionic detergent Triton X-100 it is pos-

sible to solubilize uridine kinase from its complexes without a substantial loss of activity.

EXPERIMENTAL

Material. 6-Azauridine-[4,5-^{14}C] (80 mCi/mmol) and uridine-[2-^{14}C] (48 mCi/mmol) were delivered by the Institute of Research, Production and Uses of Radioisotopes, Prague. Radioactive 5-fluorouridine was prepared from 5-fluorouracil-[2-^{14}C] (24 mCi/mmol) and α-D-ribose-1-phosphate (Calbiochem) by the described method[10] using a cell-free extract of *Escherichia coli*. 6-Azauridine was product of Spofa, Prague, and adenosine 5'-triphosphate was obtained from Calbiochem, Luzern. Zinc chloride was from Merck, EDTA, disodium salt, and ammonium sulfate, 3times recrystallized product, were from Lachema, Brno and DEAE-cellulose was from Pharmacia, Uppsala. Triton X-100 was obtained from Dr P. Riches, London.

Preparation of uridine kinase. Female rats (180—200 g) kept under standard conditions were used for the experiments. The animals were killed by cervical dislocation, bled, the tissues under investigation were removed, weighed and homogenized at 3°C in 3 vol (w/v) of cold 25 mM Tris-HCl buffer (pH 7·4) containing 25 mM-KCl and 5 mM-$MgCl_2$ using glass homogenizer with a tightly fitting Teflon pestle. The small intestine, throughly washed free of debris, was homogenized in a frozen state. The tissue homogenates were centrifuged (10000*g*, 20 min, 2°C) and uridine kinase present in postmitochondrial supernatant fractions was precipitated by 20—35% saturation by powdered ammonium sulfate[3,11]. The precipitated proteins were centrifuged, dissolved in water and the solution was used as a source of the soluble uridine kinase. The insoluble form of the enzyme was prepared[1] by adding 0·5M-$ZnCl_2$ to the cooled solution of the soluble enzyme to a final concentration of 5 mM. Coagulated proteins were centrifuged (10000*g*, 20 min, 2°C), washed twice with cold water and freeze-dried.

Uridine kinase activity was measured at 37°C in 0·3 ml of the incubation mixture containing 60 mM-Tris-HCl buffer (pH 7·4), 3 mM-ATP, 1·5 mM-$MgCl_2$ and 0·05 mM 6-azauridine-[4,5-^{14}C] as substrate[12]. Zn-precipitated and lyophilized samples of uridine kinase were kept at 3°C, the soluble fraction of the enzyme at −30°C. The analysis of the incubation mixture was done chromatographically on Whatman No 1 paper in a solvent system composed of isobutyric acid–ammonium hydroxide–water (44 : 1 : 22). The radioactive zones of unreacted substrate and of the newly formed 5'-phosphate were cut out with respect to the position of standards and their radioactivity was measured in a Packard liquid-scintillation spectrometer. The activity of uridine kinase is expressed as nmol of phosphorylated substrate during the course of 10 min incubation period at 37°C usually per 1 mg of protein.

Solubilization of uridine kinase was carried out by adding the solution of Triton X-100 in water to Zn-precipitated and freeze-dried fractions (usually 0·3 ml to 1 mg samples) and after 5 min of mixing at 2°C followed by a short-term centrifugation. Chromatography of solubilized uridine kinase on DEAE-cellulose was carried out according to Liacouras and Anderson[13] using 0·1M potassium phosphate (pH 7·5) for the enzyme elution. Protein content in fractions of uridine kinase containing Triton X-100 was measured by a modified procedure of Lowry and coworkers[14]. The presence of Triton X-100 resulted in the formation of a yellow precipitate, probably the complex of the detergent and phosphomolybdate[15]. The precipitate formed after the addition of Lowry reagent was centrifuged (2000*g*, 2 min) just prior to reading the absorbance which was proportional to protein concentration.

RESULTS

There are only slight differences in kinetic properties of the soluble and Zn-precipitated preparations of uridine kinase[8]. Both forms of the enzyme utilize uridine, cytidine as well as synthetic pyrimidine analogues as substrate (Fig. 1). Using 6-azauridine for the measurement of uridine kinase precipitated by Zn^{2+}-ions from various tissues of rats it was shown that phosphorylation of the analogue displays a linear course over a wide range of protein concentration in a reaction mixture (Fig. 2). From the data presented it is apparent that the highest activity in Zn-precipitated form is obtained using partially purified uridine kinase from the kidney while a similar preparation from the liver shows the lowest uridine kinase activity.

To solubilize Zn-complexed form of uridine kinase different compounds were used. Although enzymatically active, the metal ion precipitated protein fractions are completely insoluble in aqueous solutions. Of the compounds tested (Table 1) only the addition of deoxycholate or Triton X-100 resulted in a partial solubilization of metalloprotein complexes. While deoxycholate led at higher concentrations

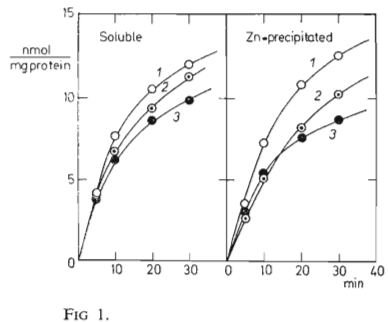

FIG 1.

Time Course of the Activity of Soluble and Zn-Precipitated Uridine Kinases from Rat Kidney using Uridine *1*, 5-Fluorouridine *2* and 6-Azauridine *3* as Substrates

0·05 mM [^{14}C-] labelled substrates were incubated at 37°C with 3 mM-ATP and 1·5 mM-$MgCl_2$ in the presence of 1 mg protein in the soluble or Zn-precipitated fractions of the enzyme. min, Length of incubation; nmol/mg protein, level of phosphorylation.

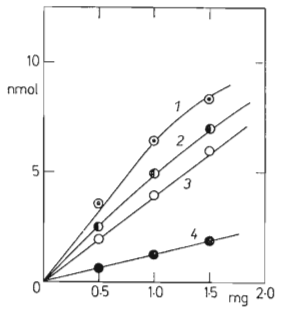

FIG. 2

Uridine Kinase Activity of Zn-Precipitated Protein Fractions from Various Tissues of Rats

Incubation was carried out at 37°C with 0·05 mM 6-azauridine-[4,5-^{14}C], 3 mM-ATP and 1·5 mM-$MgCl_2$. *1* Kidney; *2* spleen; *3* brain and *4* liver. nmol, Activity of the enzyme; mg, the amount of Zn-precipitated uridine kinase added into the reaction mixture.

to a pronounced inhibition of the enzyme activity, uridine kinase solubilized by Triton X-100 (0·5 – 4·0% solution in water) was highly active.

The fraction of uridine kinase released by Triton X-100 into the solution represents a high-molecular-weight complex of the enzyme with coprecipitating proteins. The specific activities of uridine kinase in supernatant fractions (using 2% Triton X-100 ± ± 0·5 mM-EDTA for solubilization) in relation to the speed of centrifugation of the released enzyme are shown in Fig. 3. The results indicate that uridine kinase solubilized by Triton X-100 should be considered as a high-molecular-weight complex easily sedimenting at higher centrifugation forces. The combination of EDTA with the detergent facilitates the release of uridine kinase into the solution beside causing a slight enhancement of the enzyme activity[2].

The course of uridine kinase solubilization from Zn-complexed fraction of the enzyme prepared from rat kidney in relation to the concentration of Triton X-100 is shown in Fig. 4. Under optimal conditions about 40% of the total activity of uridine kinase was found in the soluble supernatant fraction. The presence of 0·5 mM-EDTA resulted in the enhancement of solubilized portion of the enzyme; at higher concentrations the agent caused the inhibition of enzyme activity and had no effect on further release of uridine kinase.

TABLE I

Effect of Different Compounds on the Solubility of Zn-Complexed Uridine Kinase from Rat Kidney

To 1 mg of 7 days old Zn-precipitated and lyophilized uridine kinase preparations suspended in water were added different compounds to a total volume 0·3 ml and after 5 min of mixing at 2°C the samples were centrifuged (2500*g*, 1 min, 2°C). The activity of uridine kinase in sediments suspended in 0·1 ml of water and in aliquots of supernatants was assayed.

Compounds (conc.)	Uridine kinase, nmol		Inhibition %
	insoluble	soluble	
Control	6·0	0	—
Tween 80 (4%)	6·2	0	0
Digitonin (2 mM)	6·1	0	0
Deoxycholate (2 mM)	6·0	0·1	0
Deoxycholate (5 mM)	0	0·42	93·0
Dodecyl sulfate Na^+ (1 mM)	1·6	0	73·3
Triton X-100 (2%)	4·1	2·0	0
EDTA (0·5 mM)	7·0	0	0
EDTA (5 mM)	0·4	0	93·3
Na_2S (2 mM)	3·3	0	45·0
Mercaptoethanol (7 mM)	5·0	0	16·7

Similar procedure was applied to solubilize uridine kinase from Zn-complexed protein fractions prepared from the brain, liver, small intestine and spleen of the rats. In these instances marked differences of the release of uridine kinase have been observed. While Zn-complexed uridine kinase from the small intestine was solubilized similarly as the enzyme from the kidney (Fig. 5), only traces of enzyme activity using Zn-precipitates from the spleen and, especially, from the liver and brain were observed in the soluble fraction. In this case EDTA had only slight effect on the solubilization of the enzyme.

The solubilizing effect of Triton X-100 on uridine kinase from Zn-precipitated fractions depends on the duration of storage of the complexed enzymes. The highest release of the enzyme into the soluble fraction was observed using the newly prepared

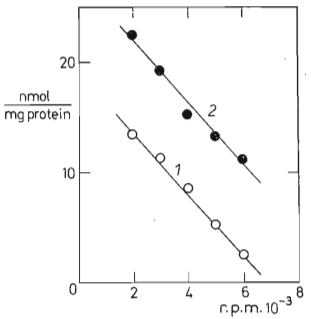

FIG. 3

Specific Activity of Uridine Kinase in Supernatant Fractions of 2% Triton X-100 ± 0·5 mM-EDTA in Relation to the Speed of Centrifugation

2 mg samples of freeze-dried Zn-precipitates of uridine kinase 8 days after preparation were mixed for 5 min at 2°C with 0·6 ml of Triton X-100 without *1* or with 2 0·5 mM-EDTA before 2 min centrifugation [rpm × 10^{-3}] at 2°C. The activity of uridine kinase (nmol/mg protein) was measured at 37°C during a 10-min incubation period.

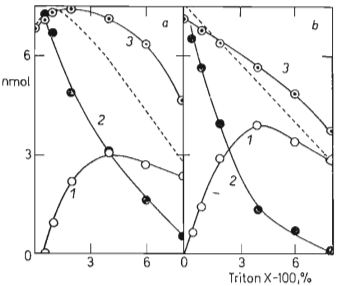

FIG. 4

Release of Uridine Kinase from Rat Kidney Zn-Precipitates in Relation to Concentration of Triton X-100 without *a* and with *b* 0·5 mM EDTA

1 mg samples of freeze-dried precipitates 6 days after preparation were mixed for 5 min at 2°C with 0·3 ml of Triton X-100 dissolved in water and centrigufed (2 500*g*, 1 min, 2°C). *1* The activity of uridine kinase (nmol) in supernatant fractions; 2 the activity remaining in the sediment suspended in water and *3* the activity of samples treated with Triton X-100 without centrifugation. Dotted, the sum of activities ad *1* and 2.

Zn-precipitates (Fig. 6). After few days of storage of freeze-dried samples at low temperature the portion of the solubilized enzyme was decreased and higher concentrations of Triton X-100 were necessary for the solubilization of the enzyme. The released uridine kinase is relatively stable at 37°C. However, during heating in Triton X-100 at 50°C the enzyme underwent rapid inactivation (Fig. 7).

The solubilized uridine kinase can be separated on a DEAE-cellulose column similarly as the enzyme that has not been precipitated by metal ions. Uridine kinase solubilizedfrom the rat kidney complex was eluted with the yield amounting to 42% and with a 6·5 times higher specific activity than the starting material. However, the enzyme was highly unstable and its complete inactivation was observed few hours after elution. An attempt to isolate different molecular forms of uridine kinase[5,16,17] from the solubilized fraction of the enzyme from rat kidney using the affinity chromatography on Sepharose 6B coupled to N^4-(5-aminopentyl)cytidine revealing three different forms of the enzyme in cytosolic fraction of rat kidney[18]

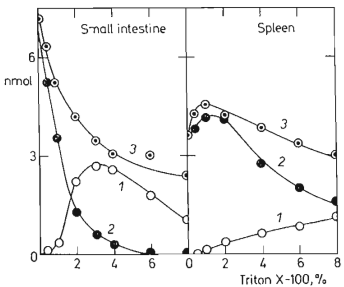

FIG. 5

Differences in Solubilization of Uridine Kinase by Triton X-100 from Zn-Precipitates Prepared from Spleen and Small Intestine of Rats

The treatment of 5 days old Zn-precipitates with Triton X-100 was the same as in Fig. 4. *1* The activity of uridine kinase (nmol) in supernatants; *2* the activity remaining in sediments and *3* the activity of 1 mg samples treated with Triton X-100 without centrifugation.

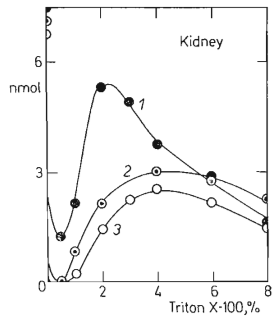

FIG. 6

Release of Uridine Kinase by Triton X-100 from Rat Kidney Zn-Complexed Fractions in Relation to Aging of Complexes at 2°C

The treatment with Triton X-100 was the same as in Fig. 4. *1* The activity of uridine kinase (nmol) released from the complex 24 h after preparation; *2* 6 days and *3* 12 days later.

was not successful probably due to the contamination of the column with Triton X-100.

DISCUSSION

Uridine kinase, an enzyme of the salvage pathway of pyrimidine synthesis, has a key role in tissues dependent on the supply of preformed pyrimidine bases and nucleosides necessary for the synthesis of nucleic acids[19–24]. The activity of uridine kinase is considered to reflect the efficiency of the tissue to utilize the salvage pathway and the enzyme is especially important in tissues with a high growth rate. The assumed importance of the enzyme in neoplasias is strengthened by the observation that the development of resistance of tumor cells towards cytostatics belonging to the group of uridine and cytidine analogues is paralleled by the deletion of uridine kinase in mutant cell lines[25–27].

The finding that uridine kinase can be precipitated by means of heavy metal ions[1,2] and the freeze-dried enzyme preparations are highly stable[9] offers the opportunity to prepare the enzyme for various purposes. In this study we attempted to release uridine kinase from its insoluble complexes. Triton X-100 was found as the best agent (Table I) leading to the solubilization of uridine kinase without a substantial loss of its activity. Only at higher concentrations the detergent caused the decrease of uridine kinase activity (Fig. 4).

Triton X-100 is a nonionic detergent with an average molecular weight of the monomer between 602–640. Above the critical concentration it forms in aqueous solutions micelles with a 100times higher molecular weight. Only the monomeric form of the detergent interacts with proteins[28] and the agent has been used for the solubilization of various proteins without causing

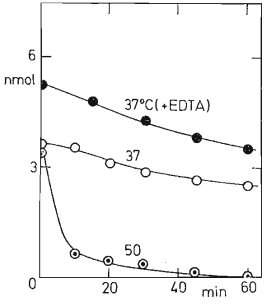

FIG. 7

Thermal Stability of Uridine Kinase Released by Triton X-100 from Rat Kidney Zn-Complexed Enzyme Fractions

10 mg samples of freeze-dried Zn-precipitates 12 days after preparation were mixed at 2°C with 1 ml of 2% Triton X-100 ± 0·5 mM-EDTA. After 5 min the suspensions were centrifuged (2500*g*, 2 min, 2°C) and the clear supernatant fractions preincubated (min) at 37 or 50°C before the estimation of uridine kinase activity.

denaturation[29,30]. Nevertheless, Triton WR-1339 administered to experimental animals is known to produce striking changes followed by the appearence of secondary lysosomes in various tissues filled with the detergent[31–33].

The presence of EDTA in Triton X-100 used for the solubilization of Zn-precipitated proteins results in the enhanced activity of the portion of uridine kinase in the soluble fraction (Fig. 4) and prevents its reprecipitation as the detergent is diluted. In the absence of the chelating agent the precipitation of solubilized uridine kinase by a simple dilution of Triton X-100 was easily demonstrated. Uridine kinase released into the soluble fraction was relatively stable (Fig. 7) as has also been shown by its separation on a DEAE-cellulose column. However, the eluted enzyme was extremely unstable. A similar lability of uridine kinase was observed after the separation of three forms of the enzyme from rat kidney cytosole fraction[18]. The low stability of uridine kinase can be explained by the loss of some factor(s) during purification necessary for the enzyme stability or by the fact that the enzyme is stable only in the association with other macromolecules[7]. The experiments are in progress to characterize proteins coprecipitating with uridine kinase and/or necessary for the enzyme stability.

The author wishes to express appreciation to Dr J. Veselý for his advice during preparation of the manuscript.

REFERENCES

1. Čihák A.: FEBS (Fed. Eur. Biochem. Soc.) Lett. *51*, 133 (1975).
2. Čihák A., Havránek M.: Int. J. Biochem. *7*, 187 (1976).
3. Orengo A.: J. Biol. Chem. *244*, 2204 (1969).
4. Plunkett T. W., Moner J. G.: Biochim. Biophys. Acta *250*, 92 (1971).
5. Krystal G., Scholefield P. G.: Can J. Biochem. *51*, 379 (1973).
6. Durham J. P., Galanti N.: J. Biol. Chem. *249*, 1806 (1974).
7. Deng Q. I., Ives D. H.: Biochim. Biophys. Acta *377*, 84 (1975).
8. Čihák A.: This Journal *41*, 1096 (1976).
9. Čihák A.: Hoppe-Seyler's Z. Physiol. Chem. *357*, 345 (1976).
10. Čihák A., Šorm F.: Biochem. Pharmacol. *21*, 607 (1972).
11. Canellakis E. S.: J. Biol. Chem. *227*, 329 (1957).
12. Čihák A., Seifertová M., Veselý J.: Arch. Biochem. Biophys. *148*, 400 (1972).
13. Liacouras A. S., Anderson E. P.: Arch. Biochem. Biophys. *168*, 66 (1975).
14. Lowry O. H., Rosebrough M. J., Farr A. L., Randall R. J.: J. Biol. Chem. *193*, 265 (1951).
15. Eibl H., Lands W. M.: Anal. Biochem. *30*, 51 (1969).
16. Keefer R. C., Morris H. P., Webb T. E.: Cancer Res. *34*, 2260 (1974).
17. Keefer R. C., McNamara D. J., Webb T. E.: Cancer Biochem. Biophys. *1*, 107 (1975).
18. Veselý J., Smrt J.: Neoplasma, in press.
19. Sköld O.: Biochim. Biophys. Acta *44*, 1 (1960).
20. Weichsel M. E., Hoogenraad N. K., Levine R. L., Kretchmer N.: Pediatr. Res. *6*, 682 (1972).
21. Čihák A., Veselý J.: J. Biol. Chem. *248*, 1307 (1973).

22. Rutman N. R., Cantarow A., Patschkis, F. S.: Cancer Res. *14*, 119 (1954).
23. Kára J., Šorm F., Winkler A.: Neoplasma *10*, 3 (1963).
24. Plagemann P. G. W., Ward G. A., Mahy B. W. J., Korbecki M.: J. Cell. Physiol. *73*, 233 (1969).
25. Pasternak C. A., Fischer G. A., Handschumacher R. E.: Cancer Res. *21*, 110 (1961).
26. Reichard P., Sköld O., Klein G., Révész G., Magnusson P. H.: Cancer Res. *22*, 235 (1962).
27. Veselý J., Čihák A., Šorm F.: Cancer Res. *30*, 2180 (1970).
28. Robinson N. C., Tanford C.: Biochemistry *14*, 369 (1975).
29. Helenius A., Simon K.: J. Biol. Chem. *247*, 3656 (1972).
30. Frasch C. E., Gotschlich E. C.: J. Exptl. Med. *140*, 87 (1974).
31. Wattiaux R., Wattiaux de Coninck S.: Nature *216*, 1132 (1967).
32. Horvat A., Baxandall J., Touster O.: J. Cell Biol. *42*, 469 (1969).
33. Huter S., Phillips M. J., Wherrett J. R.: Lab. Invest. *33*, 305 (1975).