Communication

Regulation of Uridine Kinase Quaternary Structure

DISSOCIATION BY THE INHIBITOR CTP*

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Uridine kinase from mouse Ehrlich ascites cells can exist in a variety of different aggregation states, from monomer up to aggregates that may contain 32 or more subunits. With very crude enzyme preparations, uridine kinase activity is always associated with several different coexisting molecular weight species. Changes in the aggregation state are produced in the presence of normal effectors (orthophosphate, ATP and CTP) at physiological concentrations.

With uridine kinase that has been purified 9,000-fold, enzyme activity is associated with only a single molecular weight species, but is still responsive to the same physiological effectors. In the presence of orthophosphate, uridine kinase has a molecular weight of 380,000 (appropriate for a dodecamer). In the presence of CTP, the enzyme dissociates with concomitant loss of activity. The dissociated enzyme can be reassociated to the native size. These results imply that alteration of the enzyme’s quaternary structure by normal effectors constitutes a mechanism for regulating uridine kinase activity in vivo.

URIDINE KINASE ACTIVITY—Enzyme activity was measured at 37 °C in a final volume of 50 μl containing: 50 mM Tris-HCl (pH 7.4 at 37 °C), 3 mM NaATP, 5 mM MgCl₂, 0.4 mM [5⁻³H]uridine (20 Ci/mmol), and an enzyme sample.

The product UMP was measured either by thin layer chromatography on polyethyleneimine cellulose plates (Brinkman) as previously described by us (Payne and Traut, 1982), or by binding to Whatman DE-52 filter paper disks.

Column Chromatography—Gel filtration studies were done at 4 °C using Ultrogel AcA34 (LKB). The column (2.5 X 73 cm) was pre-equilibrated with buffer plus the appropriate effectors; the enzyme sample (1 ml) was pre-equilibrated by dialysis against buffer plus effectors and eluted with the same column buffer at 25 ml/hr. To calculate apparent molecular weight values, the column was calibrated with ferritin (Mₐ = 467,000), lactate dehydrogenase (Mₐ = 142,000), hemoglobin (Mₐ = 64,500), and myoglobin (Mₐ = 17,500).

High Pressure Liquid Chromatography—Chromatography was performed on 2 Waters TSK columns joined together (a 60-cm TSK 3000 column plus a 30-cm TSK 2000 column) and a Waters pump at a flow rate of 0.8 ml/min. The column was always extensively pre-equilibrated with the appropriate buffer plus effectors, after which 100-250 μl of enzyme sample (pre-equilibrated as above) was injected.

The abbreviations used are: HPLC, high pressure liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; Pu, orthophosphate; 34K represents 34-kilodaltons, for example.

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with a variety of species, having apparent molecular weight as low as 34,000 (34K peak in Fig. 1) and as high as $1.4 \times 10^7$ million. With SDS-gel electrophoresis, uridine kinase has a molecular weight of 31K; therefore, the 34K species evident in Fig. 1 is a monomer, and the most prominent species at 130K is most likely a tetramer. When multiple molecular weights have previously been determined for uridine kinase, 2 similar sizes have been reported (Krystal and Webb, 1971; Krystal and Scholefield, 1973).

We used the same enzyme preparation for all experiments in Fig. 1, since the uridine kinase activity in this preparation was very stable, the changes in the elution pattern and in recovered activities are principally due to the different effectors used in the column buffers. In the absence of any effectors, uridine kinase activity was recovered with only a high molecular weight fraction, but with low activity (Fig. 1A). Addition of the substrate Mg-ATP produced several molecular weight species, the most prominent at 130K; Mg-ATP also aided significantly in the recovery of enzyme activity (Fig. 1B). The further addition of the inhibitor CTP at 50 $\mu$M leads to some loss of the high molecular weight species, and the appearance of a new species at 60K (Fig. 1C). Finally, CTP by itself leads to the substantial dissociation of uridine kinase, with most of the enzyme activity associated with species of 60K and 34K (Fig. 1D).

We have been able to purify uridine kinase 9000-fold with a purification scheme to be published elsewhere. This highly purified preparation contains only one significant contaminant and, therefore, is suitable for HPLC studies. With this procedure, we could directly monitor the elution of protein peaks, as measured by their absorbance at 206 nm.

In Fig. 2 is shown a series of elution profiles from HPLC experiments for each experiment, the buffer and effectors used in the HPLC column are indicated. Both Tris and CTP produce a significant background absorbance at 206 nm; when this is subtracted, the signal from the protein peaks is reduced, but the protein peaks are still readily detected. However, because of the presence of different buffers or effectors, the absolute A$_{206}$ values cannot be equated between experiments.

In the presence of CTP at 75 $\mu$M, there is a major protein peak at 133K; this peak is absent or insignificant when the enzyme is eluted down the column in Tris buffer or in phosphate buffer (Fig. 2, B and C). In the presence of CTP, uridine kinase activity is only associated with the 133K peak; with Tris or Pi, it is only associated with the larger 380K peak (data not shown).

The 200K peak is only present when we inject the 9000-fold purified enzyme, but is absent when we take a single peak from the HPLC elution and reinject it onto the column. Also, we have never seen uridine kinase activity associated with the 200K peak. Therefore, this 200K protein species is most likely the same protein contaminant that we see when our purified enzyme sample is analyzed by SDS-gel electrophoresis.

In Fig. 2D is shown the elution of the isolated 380K peak from Fig. 2C, which, when reinjected, migrates at the same position. In Fig. 2, E and F is demonstrated more clearly the reassociation of the 133K species obtained with CTP. Fig. 2F shows the UV absorbance profile of the protein in the presence of CTP; above it is shown the recovered uridine kinase activity. As indicated, 2 of the recovered fractions containing uridine kinase activity were separately reinjected on the column. Their elution (Fig. 2F) is identical to the native 380K species. For reassociation, we used Pi instead of Mg-ATP (the normal substrate, and more effective in promoting reassociation) because at mM levels, ATP totally swamps the UV signal.

**DISCUSSION**

In the experiments shown in Fig. 1, uridine kinase activity is associated with a variety of different molecular weight aggregates, and the apparent size of uridine kinase can be altered by normal physiological effectors. The enzyme is most stable in the presence of Mg-ATP (Fig. 1, B and C) or Pi (data not shown). CTP clearly leads to the dissociation of uridine kinase, as enzyme activity is now associated with dimers and monomers. As seen in Fig. 1D, when the enzyme is dissociated with CTP, the activity recovered is only 10% of that with the higher molecular weight forms produced with ATP (Fig. 1B). This suggests that the dissociated enzyme is more easily denatured, or CTP destabilizes the enzyme, and/ or that the dissociated species have less or no intrinsic cata-

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1. R. C. Payne and T. W. Traut, manuscript in preparation.
2. When the 9,000-fold purified uridine kinase preparation is analyzed with SDS-gel electrophoresis, there is one major contaminant, with a molecular weight of 51,000.
Dissociation of Uridine Kinase by CTP

FIG. 2. Changes in the quaternary state of uridine kinase measured by high pressure liquid chromatography. The TSK columns had previously been equilibrated with buffer plus effectors as indicated for each experiment. Where used, Tris buffer was 50 mM, pH 7.4; phosphate buffer (P_i) was 10 mM, pH 7.4. For experiments A, B, C, and E, uridine kinase (9000-fold purified) was injected onto the column. The peak uridine kinase activity from experiment C was reinjected onto the column for experiment D, as indicated. In similar fashion, 2 fractions from experiment E were reinjected in experiment F.

This last point needs clarification. It would seem that Fig. 1D clearly shows that monomers and dimers of uridine kinase are catalytically competent and active. However, the activity profile of Fig. 1D was obtained by incubating column samples with the substrate, Mg-ATP, at 3 mM. As seen in Fig. 1, B and C, this could reassociate monomers and dimers to higher molecular weight forms during the enzyme assay. If only the larger molecular weight species are active and some time is required for the reassociation to occur, then the reassociated species would be active for less than the full assay time, which would naturally yield lower observed product formation. Experiments are in progress to determine if the dissociated species are catalytically competent. In similar studies that we have done with UMP synthase from Ehrlich ascites cells (containing the last 2 catalytic activities of de novo UMP synthesis: orotate phosphoribosyl transferase and orotidine monophosphate decarboxylase), we showed that UMP synthase was able to exist as a monomer or as a dimer (Traut and Payne, 1980; Traut, 1982). With the highly purified enzyme preparation, it is possible to monitor the protein itself, in addition to the enzyme's activity, as shown in Fig. 2. Here again, CTP clearly leads to the dissociation of uridine kinase. Furthermore, by dialyzing a sample of the dissociated species to remove CTP, and then reinjecting it onto the HPLC column in the presence of P_i, uridine kinase was reassociated back to the native molecular weight size (Fig. 2, E and F). These last experiments clearly show that at least 2 of the different molecular weight forms of uridine kinase are interconvertible.

Molecular sieve chromatography measures the Stokes radius of a molecule, rather than the molecular weight. While the marker proteins used for calibration are reasonably globular, we have no information at this time about the shape of uridine kinase aggregates, and all molecular weight values must be considered as tentative, especially for the larger values. At this time, we can only say that the various molecular weight species seen could be assigned the following sizes:
monomer (34K), dimer (56K or 60K), tetramer (130K or 135K), and dodecamer (380K or 265-470K); species larger than 1 million could contain >32 subunits. While the molecular weight values determined for the different peaks in Fig. 1 are consistent with different oligomers of a subunit of ~34,000, it is also possible that some of the large molecular weight species represent aggregates containing uridine kinase plus one or more other protein subunits. Purified enzyme would no longer be able to form such mixed oligomers.

There are 3 major differences in the observed quaternary state properties between the purified enzyme and the crude enzyme preparation: 1) pure enzyme has activity associated with only a single molecular weight species under any specific condition; 2) activity is associated with a wider range of molecular weight species with crude enzyme; and 3) with purified enzyme, the major enzyme species has a molecular weight of 380K, while with crude enzyme, the major peak is at 130K, with a secondary peak at 365K or 470K. Some of these differences could be due to differences in the amount of enzyme protein used, since in general as enzyme protein is increased, self-association is favored. Within each set of experiments, the amount of crude or purified enzyme was not varied, but we need to do more studies on the aggregation state of uridine kinase as a function of protein concentration to resolve that. It is also possible that there are factors present in the crude preparation that directly affect the quaternary state of uridine kinase.

It is important that, with both purified enzyme and with the very crude enzyme that may be considered as being a closer approximation of conditions in vivo, CTP at physiological concentrations causes uridine kinase to dissociate. This fact, combined with the observation that uridine kinase may normally exist as several different aggregation states in equilibrium (Fig. 1) suggests that alteration of the quaternary state by CTP (and other effectors) may occur readily in vivo. For this to be a significant regulatory mechanism, it would be necessary for the smaller aggregates of uridine kinase (dimers and monomers) to have little or no catalytic activity.

When examined by chromatography on molecular sieves columns, uridine kinase was found to exist as a single molecular weight species of 60,000 when prepared from calf thymus (Lee et al., 1974), ~150,000 when prepared from mouse hepatoma P815 (Liacouras and Anderson, 1975), or ≥1 million in Novikoff ascites hepatoma (Ornberg, 1969). Multiple molecular weight species of uridine kinase have been reported for a variety of cells and tissues: 30,000 and 120,000 in rat liver (Krystal and Webb, 1971) and Ehrlich ascites cells (Krystal and Scholefield, 1973); 2 different molecular weight species of undetermined size have also been reported for various rodent or human tumors (Keef er et al., 1974; Keffer et al., 1975; Greenberg et al., 1977a,b; Otal-Brin and Webb, 1979; Ahmed and Welch, 1979; Ahmed and Baker, 1980; Ahmed, 1982).

Additional studies have reported that 2 different fractions of uridine kinase are obtained by DEAE-cellulose chromatography (Fulchignoni-Lataud et al., 1976) or by isoelectric focusing (Ahmed and Welch, 1979; Ahmed and Baker, 1980; Ahmed, 1982); 3 fractions by DEAE-cellulose (Dubimina et al., 1982) or by affinity chromatography (Vesely and Smrt, 1977); and 4 separate fractions by isoelectric focusing (Ahsel et al., 1980). In none of these studies was uridine kinase purified more than 200-300-fold.

While one of the earliest studies (Krystal and Scholefield, 1973) suggested that the 2 molecular weight species that were observed in preparations from Ehrlich ascites cells could be a tetramer and monomer of the same enzyme species, most of the subsequent reports have concluded that the various activity peaks observed represented isoforms of uridine kinase. Even separation by charge is not an unambiguous criterion for identifying isoforms; monomers and dimers of serum albumin are readily eluted as separate peaks on DEAE-columns (Pharmacia, 1980).

While we certainly have not done enough experiments to claim that uridine kinase isoforms do not exist, the data presented in Figs. 1 and 2 suggest that in many of the studies cited above the authors may have been studying different aggregation states of the same species of uridine kinase. It is becoming well documented that for a variety of bacterial and mammalian enzymes, their activity is regulated at least partly, by physiological effectors that alter the enzyme’s quaternary structure (Phillips, 1974; Klotz et al., 1975). Examples of such enzymes related to nucleotide metabolism, where changes in quaternary structure produce changes in the enzyme’s activity, include UMP synthase (Traut and Payne, 1980; Traut, 1982), phosphoribosylpyrophosphate synthetase (Becker et al., 1977; Meyer and Becker, 1977), and deoxoynucleotide kinase (Iwatsuki and Okazaki, 1967a, 1967b). With some enzymes (e.g. UMP synthase), it has been determined that effectors interact with the enzyme at a specific regulatory site, and a similar mechanism for the effect of CTP on uridine kinase has been proposed (Ornberg, 1969). Experiments are in progress to test this hypothesis.

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