Characterization of Pyrimidine Nucleoside Monophosphokinase in Normal and Malignant Tissues

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SUMMARY

It was found that there are two kinds of pyrimidine nucleoside, monophosphokinase deoxycytidine 5'-monophosphate-deoxyuridine 5'-monophosphate (dCMP-dCMP-UMP-dUMP) kinase and cytidine 5'-monophosphate-deoxycytidine 5'-monophosphate-uridine 5'-monophosphate (CMP-dCMP-UMP-dUMP) kinase, and their molecular weights were calculated to be 46,000 and 26,000, respectively, by gel filtration.

dTMP-dUMP kinase phosphorylated dTMP with a Kₘ of 3.1 × 10⁻⁸ M and dUMP with a Kₘ of 7.7 × 10⁻⁴ M. dTMP phosphorylation catalyzed by dTMP-dUMP kinase was inhibited competitively by dUMP with a Kᵢ of 2.0 × 10⁻³ M. Similarly, phosphorylation of dUMP by this enzyme was inhibited competitively by dTMP with a Kᵢ of 2.5 × 10⁻⁴ M. CMP-dCMP-UMP-dUMP kinase of Yoshida sarcoma phosphorylated dUMP with a Kᵢ of 7.1 × 10⁻³ M and dCMP with a Kᵢ of 6.9 × 10⁻² M and 3.0 × 10⁻³ M, respectively, and phosphorylation of dCMP was inhibited competitively by dUMP with a Kᵢ of 2.2 × 10⁻³ M. Relative Vₘₐₓ activity of this enzyme was 345 nmoles/mg protein with dCMP and 127 nmoles/mg protein with dUMP.

INTRODUCTION

It has been reported in mammalian cells that dCMP kinase acts on dCMP, CMP, and UMP, but not on dTMP or dUMP, and that there is a specific enzyme for dTMP, called dTMP kinase, which also acts on dUMP. Namely, there are two nucleoside monophosphokinases involved in pyrimidine metabolism, dCMP-CMP-UMP kinase, and dTMP-dUMP kinase (7, 11). Previously, we reported a reciprocal relationship between dUMP kinase and dTMP kinase (2). However, normal rat liver cells have a low dTMP kinase activity and a high activity for phosphorylation of dUMP and do not contain the specific phosphatase. These results suggested that some enzyme other than dTMP kinase catalyzes the phosphorylation of dUMP in normal rat liver cells.

This communication reports that dCMP kinase also had a dUMP-phosphorylating activity by which low dTMP kinase and high dUMP kinase activities were shown to occur in resting cells.

MATERIALS AND METHODS

Materials. All radioactive nucleotides were purchased from the Japan Radioisotope Association, Tokyo, Japan. dUMP, dTMP, and C₅ alumina gel were obtained from Sigma Chemical Co., St. Louis, Mo.; other nucleotides were obtained from Boehringer-Mannheim Japan Co., Ltd., Tokyo, Japan. Epichlorohydrin triethanolamine cellulose and DEAE-cellulose were purchased from Seikagaku Kogyo Co., Tokyo, Japan. Hydroxylapatite was prepared by the method of Tiselius et al. (12).

Animals. Male albino Wistar-King rats, weighing 140 to 150 g, and male Donryu rats, weighing about 90 g, were maintained on laboratory chow from Oriental Yeast Co., Ltd., Tokyo, with water given ad libitum. Yoshida sarcoma cells were maintained in male Donryu rats by weekly i.p. transfer.

Enzyme Assay. For assay of dUMP kinase, a reaction mixture (0.25 ml) containing 10 μmoles of Tris-HCl (pH 8.0), 2.5 μmoles of MgCl₂, 2 μmoles of ATP, 0.25 μmole of [5-³H]dUMP (0.25 μCi), and enzyme was incubated at 37° for 30 min; it was then heated for 3 min in a boiling water bath to stop the reaction. For assay of dTMP, CMP, and dCMP kinase, 0.25 μmole of [5-³H]dTMP (0.25 μCi), 0.12 μmole of [2-¹³C]CMP, and 0.12 μmole of [2-¹³C]dCMP (0.12 μCi), respectively, were used as substrates in place of dUMP. The products were analyzed on an epichlorohydrin triethanolamine cellulose column or by paper chromatography (9, 13). dUTPase with a low Kᵢ value was assayed with the use of 0.1 mM labeled dUTP as substrate, and the activity was expressed as moles of dUMP formed after 30 min of incubation. Protein concentration was measured by the method of Lowry et al. (6) with bovine serum albumin as a standard.
RESULTS

Purification of Nucleoside Monophosphokinase for dUMP and dTMP from Yoshida Sarcoma

Preparation of Crude Extract. All the procedures described below were carried out at 4°C. Frozen Yoshida sarcoma cells (6 g) were homogenized in 10 ml of extraction buffer (5 mM potassium phosphate buffer, pH 7.5, and 5 mM 2-mercaptoethanol, 20 μM dTMP) with a Teflon homogenizer in ice. The homogenate was centrifuged at 10,000 × g for 30 min, and the supernatant was recentrifuged at 105,000 × g for 60 min. The resultant supernatant fluid was used as crude extract (Fraction 1).

Gel Filtration on Sephadex G-100. Fraction 1 (3 ml) was carefully layered on a column (2 × 50 cm) of Sephadex G-100 equilibrated with 5 mM potassium phosphate buffer, pH 7.5, containing 5 mM 2-mercaptoethanol and 20 μM dTMP. The column was eluted with the same buffer at a flow rate of about 0.4 ml/min, and 3.1-ml fractions were collected. The elution patterns of protein and enzyme activities on gel filtration are shown in Chart 1.

The elution pattern of dTMP-phosphorylating activity differed from that of dUMP phosphorylating activity, and the recoveries of the two were 90–93 and 77–19 nmoles, respectively. The dUTPase was eluted with a peak in Tube 24, separated from dUMP-phosphorylating activity. The dTMP- and dUMP-phosphorylating enzymes from Yoshida sarcoma cells were eluted in effluent volumes of 78 and 96 ml, respectively, under these conditions.

The elution volumes of the dTMP- and dUMP-phosphorylating enzymes were compared with those of several proteins of known molecular weights on a Sephadex G-100 column. From the results, the molecular weights of the dTMP- and dUMP-phosphorylating enzymes were estimated as 46,000 and 26,000, respectively.

The fractions of dTMP-phosphorylating enzyme (Tubes 24 to 27) and dUMP-phosphorylating enzyme (Tubes 29 to 33) were each combined for the next purification step.

Hydroxylapatite Column Chromatography. Ten ml of dTMP-phosphorylating enzyme fraction (Tubes 24 to 27) were applied to a hydroxylapatite column (1.5 × 3 cm) previously equilibrated with 5 mM potassium phosphate buffer, pH 7.5, containing 5 mM 2-mercaptoethanol and 20 μM dTMP and washed with the same buffer. For establishment of a linear gradient, 100 ml of the same buffer were placed in the mixing vessel, and 100 ml of 0.2 M potassium phosphate buffer, pH 7.5, containing 5 mM 2-mercaptoethanol and 20 μM dTMP were placed in the reservoir. Elution was carried out at a flow rate of 0.5 ml/min, and 3.1-ml fractions were collected. The elution profile in Chart 2A shows that the dTMP-phosphorylating enzyme was eluted in the unadsorbed fraction. This fraction contained dUMP-phosphorylating activity, and the amount of activity recovered was 180% of that applied, due to removal of the dUTPase. The adsorbed fraction contained the dUTPase but not dTMP- or dUMP-phosphorylating activity.

Next, 15 ml of dUMP-phosphorylating enzyme fraction (Tubes 29 to 33) were applied to a hydroxylapatite column under the conditions described above. The elution pattern is shown in Chart 2B. dUMP-phosphorylating activity was not detected in the unadsorbed fraction but was eluted with 30 mM potassium phosphate buffer. About 20% of the activity applied was recovered. This fraction of dUMP-phosphorylating enzyme did not phosphorylate dTMP at all.

With Sephadex G-100 column chromatography of a preparation from normal rat liver, no dTMP-phosphorylating activity was detected; but high dUMP-phosphorylating activity was eluted. This dUMP-phosphorylating enzyme activity from normal rat liver was eluted in the same position as the dUMP-phosphorylating enzyme of Yoshida sarcoma from the column.

Results on the purifications of the nucleoside monophosphokinases for dTMP and dUMP from Yoshida sarcoma are shown in Table 1.

Purification of Nucleoside Monophosphokinase for dCMP from Yoshida Sarcoma and Normal Rat Liver

For more exact characterization of the enzyme, we purified the dCMP-phosphorylating enzyme from Yoshida sar-
Separation of nucleoside monophosphokinases from Yoshida sarcoma

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<th>Yield (%)</th>
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* ND, not detectable.

Characterization of dUMP Kinase

Table 1

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* ND, not detectable.

Chromatography of the Cy Alumina Gel Eluate on DEAE-Cellulose. The sample eluted from Cy alumina gel was dialyzed and applied to a column of DEAE-cellulose (2 x 10 cm) previously equilibrated with 5 mM potassium phosphate buffer, pH 7.5, containing 5 mM 2-mercaptoethanol (Buffer 1). A linear gradient was established by placing 300 ml of Buffer 1 in the mixing vessel and 300 ml of 0.5 M KCl in Buffer 1 in the reservoir. The column was eluted at a flow rate of 1 ml/min, and 10-ml fractions were collected. The elution profile in Chart 3 shows that dUMP-phosphorylating activity was eluted in the same fractions as dCMP-phosphorylating activity. Moreover, the dUTPase activity was eluted as a wide band in Tubes 28 to 40, and the peaks of dUMP- and dCMP-phosphorylating activities were detected within this region. The dUMP/dCMP ratio was 0.08. Tubes 28 to 40 were pooled and concentrated in a collodion bag.

Chromatography on Hydroxylapatite. The DEAE-cellulose eluate was concentrated (10 ml), dialyzed against 10 mM potassium phosphate buffer (pH 7.5), and applied to a column of hydroxyapatite (2 x 3 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.5). The column was eluted with a gradient formed by placing 200 ml of the same buffer in the mixing chamber and 200 ml of 0.2 M potassium phosphate buffer (pH 7.5) in the reservoir. Elution was carried out at a flow rate of 0.5 ml/min, and 6-ml fractions were collected. The elution profile is shown in Chart 4. The phosphorylating activities for dCMP, CMP, dUMP, and UMP were found in the same fraction, but no activity for dTMP was demonstrated in these fractions. The dUTPase or dUMP kinase-masking activity was completely separated from the kinase for CMP, UMP, dCMP, and dUMP on this column.

The elution pattern of the kinase fraction (phosphorylation of UMP, CMP, dCMP, and dUMP) from normal rat liver coincided with that of the purified enzyme from Yoshida sarcoma.

Specificity for Phosphate Acceptors

The abilities of various nucleoside monophosphates to act as phosphate acceptors from ATP in the phosphoryla-
SubstrateCon
contra
tion (mm)
CMP-dCMP-UMP- dUMP kinase; Yoshida sarcoma

dCMP 0.4 100± (340) 100 (116) ND
UMP 0.4 380 400 ND
CMP 0.4 2500 2400 ND

a The relative rate of substrate phosphorylation with the use of purified CMP-dCMP-UMP-dUMP was calculated, taking dCMP-phosphorylating activity as 100.

b Numbers in parentheses, enzyme activity observed (nmoles/ml).

c The relative rate of substrate phosphorylation with the use of purified dTMP-dUMP kinase was calculated, taking dTMP-phosphorylating activity as 100.

tion of dCMP, dUMP, CMP, dTMP, and UMP are shown in Table 2. Of the 5 ribo- and deoxyribonucleoside monophosphates tested, the dCMP-phosphorylating enzyme from normal rat liver and Yoshida sarcoma acted on UMP, CMP, dCMP, and dUMP but not on dTMP, while the dTMP-phosphorylating enzyme purified from Yoshida sarcoma acted only on dTMP and dUMP. The ratios of the phosphorylations of CMP, UMP, and dUMP to that of dCMP with the crude extract and purified enzymes of normal rat liver and with the purified enzymes of Yoshida sarcoma were constant, indicating that CMP, UMP, dCMP, and dUMP kinases consisted of a single enzyme. The results showed that there are 2 kinds of pyrimidine nucleoside monophosphokinase, namely dTMP-dUMP kinase and CMP-dCMP-UMP-dUMP kinase.

Kinetics of Substrate Utilization and Inhibition

The kinetic parameters of the 2 enzymes for dCMP, dUMP, and dTMP with Mg-ATP as phosphate donor were determined from initial measurements of velocity by radioassay.

dTMP-dUMP kinase phosphorylated dTMP with a $K_M$ of $3.1 \times 10^{-5}$ M and dUMP with a $K_M$ of $7.7 \times 10^{-4}$ M (Chart 5). On the other hand, CMP-dCMP-UMP-dUMP kinase of Yoshida sarcoma phosphorylated dUMP with a $K_M$ of $3.1 \times 10^{-3}$ M and dCMP with a $K_M$ of $7.1 \times 10^{-4}$ M (Chart 6).

The inhibitory effects of pyrimidine monophosphates at concentrations of 0.5 to 5.0 mM on Yoshida sarcoma CMP-dCMP-UMP-dUMP kinase were examined (data not presented). These compounds caused different degrees of inhibition. UMP and CMP were the most potent inhibitors of the phosphorylation of dUMP and dCMP, although dUMP and dCMP also inhibited the reaction. Furthermore, dTMP, which was inactive as a phosphate acceptor, inhibited the reaction nearly 50% at a concentration of 5.0 mM.

As shown in Chart 6, dUMP phosphorylation by the enzyme was inhibited competitively by dCMP and dTMP with $K_I$'s of $6.9 \times 10^{-4}$ and $3.0 \times 10^{-3}$ M, respectively, and phosphorylation of dCMP was inhibited competitively by dUMP with a $K_I$ of $2.2 \times 10^{-3}$ M. On the other hand, dTMP phosphorylation catalyzed by dTMP-dUMP kinase was inhibited competitively by dUMP with a $K_I$ of $2.0 \times 10^{-3}$ M. Similarly, phosphorylation of dUMP by this enzyme was

Chart 5. Competitive inhibition of dTMP phosphorylation by dUMP (A) and of dUMP phosphorylation by dTMP (B). The enzyme activity was measured as described in "Materials and Methods," with the use of the unadsorbed fraction from the hydroxylapatite column in Chart 2 as dTMP-dUMP kinase.

Chart 6. Competitive inhibition of dCMP phosphorylation by dUMP (A) and of dUMP phosphorylation by dCMP (B) or by dTMP (C). The fraction on the hydroxylapatite column in Chart 4 (Tube 20) was used as the enzyme source. Enzyme activity was measured as described in "Materials and Methods" in the presence or absence of 0.8 mM dCMP or 4 mM dTMP.
inhibited competitively by dTMP with a $K_i$ of $2.5 \times 10^{-8}$ M (Chart 5). These findings indicate that there are 2 distinct enzymes catalyzing the phosphorylations of dUMP and dTMP and of CMP, UMP, dCMP, and dUMP, respectively.

**DISCUSSION**

It has been reported that dUMP kinase is identical to dTMP kinase in *Escherichia coli* (8), bacteriophage (3), chick embryonic liver (10), and mouse hepatoma (5), but that it is not identical to dCMP kinase in calf thymus (11). dCMP kinase is reported to be the same enzyme as CMP-UMP kinase (7, 11). In *E. coli* (4), DEAE-cellulose column chromatography showed that UMP kinase, but not CMP kinase, was a different enzyme from dCMP kinase. However, in contrast to the above results, we found that phosphorylation of dUMP is catalyzed not only by dTMP kinase, but also by dCMP kinase. The evidence that dCMP kinase has obvious activity for dUMP phosphorylation was obtained from the hydroxylapatite column chromatography by which the dUTP phosphatase was completely separated from dCMP kinase, thus resulting in the full appearance of dUMP phosphorylation. Therefore, the result reported by Maness et al. (7) that dCMP kinase did not phosphorylate dUMP seems to have been obtained due to the experimental condition that was less sensitive than ours for detection of dUMP phosphorylation.

The presence of the dUTP phosphatase in Yoshida sarcoma cells but not in normal rat liver explains why the dUMP/dCMP ratio for phosphorylation was not constant during its purification from normal rat liver. With removal of the phosphatase, the ratio for Yoshida sarcoma cells was the same as that for normal rat liver. The evidence that dCMP kinase is identical to dUMP kinase was also confirmed by the finding that dCMP competitively inhibited the phosphorylation of dUMP and vice versa and that the reciprocal relationship of the enzyme activity of dUMP kinase in Yoshida sarcoma and in normal rat liver is the same as that of dCMP kinase.

It is concluded that there are 2 species of enzyme molecules, the larger one with a molecular weight of 46,000 catalyzing phosphorylation of dTMP and dUMP, and the smaller one with a molecular weight of 26,000 catalyzing phosphorylation of UMP, dCMP, CMP, and dUMP, i.e., dTMP-dUMP kinase and CMP-dCMP-UMP-dUMP kinase. Resting cells contain only the latter enzyme, but in proliferating cells, dTMP-dUMP kinase is newly induced. Concomitantly, dUTP phosphatase that specifically masks dUMP kinase is also induced in rapidly growing cells, thus facilitating the supply of necessary nucleotides for DNA synthesis.

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**REFERENCES**

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