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# SYNTHESIS OF SUGAR-MODIFIED NUCLEOSIDE 5′-TRIPHOSPHATES WITH PARTIALLY PURIFIED NUCLEOTIDE KINASES FROM CALF THYMUS

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## Summary

The partial purification of some nucleoside monophosphate kinases (ATP:nucleosidemonophosphate phosphotransferases, EC 2.7.4.4) from calf thymus by chromatography on Blue Sepharose to remove interfering phosphatase activity is described. Their specificities towards nucleoside monophosphates modified in the sugar are investigated. Pyrimidine nucleoside monophosphate kinase is not very much affected by such modifications, whereas GMP kinase does not tolerate such alteration. The effect on AMP kinase is intermediate.

## Introduction

Nucleoside 5′-triphosphates are substrates and/or effectors for a large number of enzymes. It is, therefore, not surprising that many reactions exist for the conversion of nucleoside 5′-monophosphates to the corresponding 5′-triphosphates, and both chemical and enzymic methods have been developed for this purpose [1—7]. When working on a small scale and with labelled compounds enzymic phosphorylation is generally preferred. However, the enzymes used often exhibit specificities which prevent their application for the phosphorylation of modified nucleotides. We are particularly interested in sugar-modified nucleosides [8—10] and therefore require enzymes which can tolerate such structural alterations of the substrate. During a recent investigation [10] it

Abbreviations: NMP kinase, nucleoside monophosphate kinase; NDP kinase, nucleoside diphosphate kinase.

was observed that nucleoside monophosphate kinases (ATP:nucleoside monophosphate phosphotransferases, EC 2.7.4.4) from calf thymus are less specific with regard to the sugar moiety than those from *Escherichia coli*. Although the separation of nucleotide kinases from calf thymus has been reported [11—13], the procedures described are rather involved and we felt a simpler isolation procedure particularly to remove interfering phosphatase activity should be developed. This report describes the partial purification of some of the nucleoside monophosphate kinases from calf thymus on Blue Sepharose and their specificities with regard to the sugar moiety.

## Materials and Methods

Blue Sepharose CL-6B was purchased from Pharmacia. Phospho*enol*pyruvate (potassium salt), NADH (disodium salt), lactate dehydrogenase from pig muscle, pyruvate kinase from rabbit muscle were purchased from Boehringer (Mannheim, F.R.G.).

The four 2′-*O*-methylnucleoside 5′-phosphates and 5-methyl UMP were purchased from P.L. Biochemicals (Milwaukee, U.S.A.). 5-Methyl dCMP, araCMP, TDP, araCTP and dCTP were purchased from Sigma Chemical (St. Louis, U.S.A.). AraAMP was purchased from Serva Feinbiochemica (Heidelberg, F.R.G.). Other natural nucleotides were obtained from Pharma Waldhof (Mannheim, F.R.G.). 2′-Deoxy-2′-azido- and 2′-deoxy-2′-chloronucleoside 5′-phosphates, 3′-deoxy-3′-azido and 2′,3′-dideoxy-3′-azidonucleoside 5′-phosphates were prepared from the corresponding nucleosides by a slight modification of the method of Yoshikawa et al. [14]. AraUMP was prepared from 2,2′-cyclo UMP by hydrolysis. 3′-Deoxy-3′-azido dATP, 2′-deoxy-2′-azido CTP and -UTP, were prepared from the corresponding 5′-phosphates by the methods of Hoard and Ott [3]. Aminonucleotides were prepared from the corresponding azidonucleotides by reaction with triphenylphosphine [15].

The coupled pyruvate kinase-lactate dehydrogenase assay system [16] was used to assay the enzymes throughout the purification and also to estimate initial velocities of phosphorylation of the various nucleoside 5′-phosphates. The assays were performed at pH 8.9 [11—13]. The reaction mixture contained in 1 ml: 100 mM diethanolamine/HCl (pH 8.9), 2 mM ATP, 10 mM $MgCl_2$, 1 mM potassium phospho*enol*pyruvate, 40 mM KCl, 0.14 mM NADH, 1.7 μg pyruvate kinase, 5 μg lactate dehydrogenase, 1 mM deoxynucleoside 5′-phosphate or thymidine 5′-diphosphate (for the assay of NDP kinase) and an appropriate amount of kinase mixture. In the case of the pyrimidine nucleoside monophosphate kinase assay, 0.25 mM dithiothreitol was also added, since the enzyme shows full activity only in the presence of thiols [11]. For the phosphorylation of 2′-azido CMP or -UMP, 4.8 mM 2-mercaptoethanol was used instead of dithiothreitol since it has been reported that dithiothreitol reacts with alkyl azides [17]. Enzyme activity was measured by following the decrease in absorbance at 340 nm. A small rate obtained with the enzyme sample in the absence of substrate was subtracted. All reactions were carried out at 25° C. For the alkaline phosphatase assay, phospho*enol*pyruvate, NADH, pyruvate kinase, lactate dehydrogenase and 5′-nucleotide were omitted and 5 mM sodium *p*-nitrophenylphosphate was added. The increase in absorbance

at 405 nm was then measured. Protein concentration was determined by the method of Warburg and Christian [18].

The reaction mixture for the preparation of nucleoside triphosphates with the partially purified kinase mixture contained in a volume of 125 µl, 200 mM diethanolamine/HCl (pH 8.9), 1 mM ATP, 12 mM $MgCl_2$, 0.25 mM dithiothreitol for pyrimidine nucleoside monophosphates (4.8 mM mercaptoethanol for the reaction with 2′-azido CMP and -UMP), 32 mM potassium phospho*enol*pyruvate, 1.7 µg pyruvate kinase, 4 mM (0.5 µmol) nucleoside 5′-phosphate and 5—25 µl kinase mixture. The reaction was carried out at 37° C and the reaction course followed by high-performance liquid chromatography (HPLC) on a Packard-Becker 8200 chromatograph equipped with a Packard 1170 UV detector operating at 254 nm. The strong anion exchanger Nucleosil 10 SB from Machery and Nagel was packed into a stainless steel column (40 cm × 2 mm). Elution was effected with buffer A (0.05 M $KH_2PO_4$/0.25 M KCl, pH 4.5) or buffer B (0.05 M $KH_2PO_4$/0.45 M KCl; flow rate, 1 ml/min). Nucleoside 5′-triphosphates were identified by comparison of retention times with authentic samples. The yields were calculated from the peak area.

*Purification of kinases*

*Streptomycin-precipitated extract from calf thymus.* The preparation of a streptomycin-precipitated extract from calf thymus was performed essentially following the method of Thelander (private communication). 90 g frozen calf thymus was homogenized in a mixerblender with 270 ml 0.05 M Tris-HCl (pH 7.8). The homogenate was centrifuged at 25 000 × *g* for 30 min and the

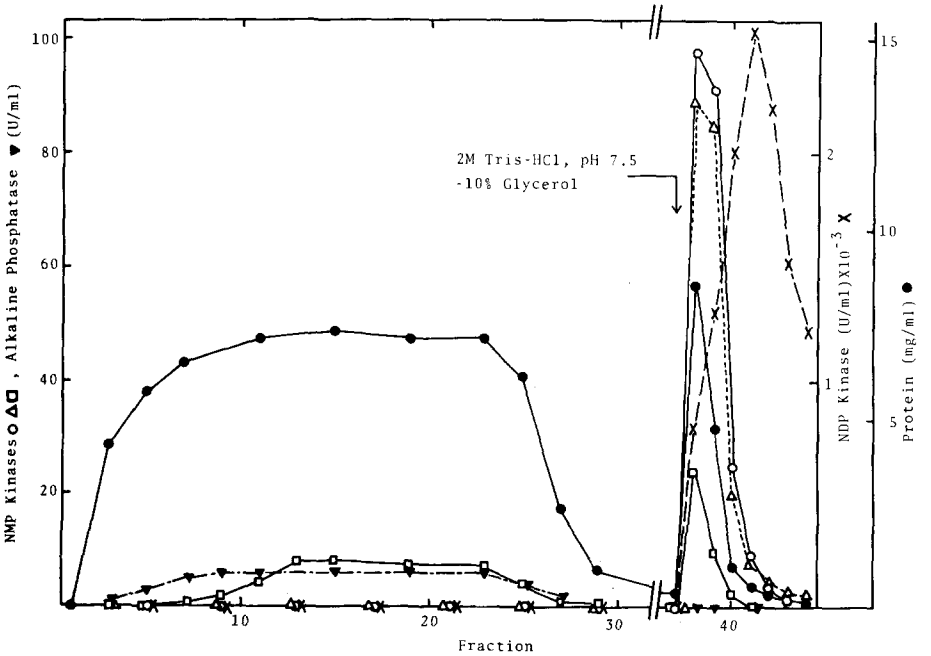


**Fig. 1. Chromatography of the streptomycin-precipitated and dialyzed extract on Blue Sepharose CL-6B.** o———o, **dCMP kinase;** Δ------Δ, **dAMP kinase;** □———□, **dGMP kinase;** X------X, **NDP kinase;** ▼------▼, **alkaline phosphatase;** ●———●, **protein.**

resulting supernatant collected. To this fluid was added 18 ml 7.5% streptomycin sulfate with stirring. The mixture was centrifuged at 25 000 × *g* for 30 min. The supernatant was collected and dialyzed overnight against 0.01 M Tris-HCl (pH 7.5), 1 mM dithiothreitol. The slight turbidity was removed by centrifugation at 25 000 × *g* for 30 min and the supernatant was used for further purification.

*Chromatography on Blue Sepharose.* The streptomycin-precipitated and dialyzed extract from calf thymus (30 g) was applied to a column of Blue Sepharose CL-6B (5 g, 1.6 × 10 cm) in a cold room. The column was washed with 40 ml 0.01 M Tris-HCl (pH 7.5), 1 mM dithiothreitol. 75% of the dGMP kinase activity was eluted in the void volume which also contained the majority of the protein. Activity due to other NMP kinases and NDP kinases was not detected in the column effluent. All of the required kinases were eluted with 2.0 M Tris-HCl (pH 7.5), 1 mM dithiothreitol and 10% glycerol. The first 7.3 ml of the eluate (fractions 38 and 39), containing most of the NMP kinase activity (Fig. 1 and Table I), was collected and dialyzed against 0.01 M Tris-HCl (pH 7.5), 15% glycerol (v/v), 1 mM dihiothreitol. This fraction also contained NDP kinase [19] which was sufficient to phosphorylate the products of the NMP kinase reaction to the corresponding triphosphates.

## Results and Discussion

Four nucleoside monophosphate kinases (TMP kinase, AMP kinase, GMP kinase and CMP-UMP kinase) are reported to be present in mammalian tissue [20,21], the main contaminant in crude extract being a phosphatase. The presence of this enzyme makes it impossible to phosphorylate poor substrates with the crude kinase mixture since both the phosphate donor and phosphate acceptor are destroyed during the prolonged incubation times required. In order to remove this interfering enzyme activity, chromatography on Cibacron Blue with its affinity for ATP-requiring enzymes seems to be an appropriate method [22]. Recently, nucleotide kinases were isolated by this method from human

TABLE I

ACTIVITIES OF NMP KINASES AND NDP KINASE BEFORE AND AFTER BLUE SEPHAROSE TREATMENT

Units are μmol NTP produced/h.

| | Before blue Sepharose treatment | | After blue Sepharose treatment | |
|---|---|---|---|---|
| | Total activity (units) | Specific activity (units/mg protein) | Total activity (units) | Specific activity (units/mg protein) |
| dAMP kinase | 970 | 1.3 | 690 | 13.3 |
| dCMP kinase * | 1 050 | 1.4 | 760 | 14.7 |
| dGMP kinase | 720 | 1.0 | 130 | 2.5 |
| NDP kinase | 73 300 | 100 | 8120 | 157 |
| Alkaline phosphatase ** | 690 | 0.94 | 0.2 | 0.004 |

* Pyrimidine nucleoside monophosphate kinase as assayed with dCMP.

** Units are μmol *p*-nitrophenol produced/h.

TABLE II

NUCLEOSIDE 5′-PHOSPHATES AS PHOSPHATE ACCEPTORS

Relative initial velocity taking the values of dCMP, dGMP, dAMP as 100 for pyrimidine nucleotides, guanine nucleotides and adenine nucleotides, respectively.

| Substrate | $V_0$ | Substrate | $V_0$ | Substrate | $V_0$ | Substrate | $V_0$ |
|---|---|---|---|---|---|---|---|
| CMP | 56 | UMP | 136 | GMP | 115 | AMP | 240 |
| dCMP | 100 | dUMP | 41 | dGMP | 100 | dAMP | 100 |
| 2′-Deoxy-2′-amino CMP | 129 | 2′-Deoxy-2′-amino UMP | 81 | 2′-Deoxy-2′-amino GMP | 2 | 2′-Deoxy-2′-amino AMP | 51 |
| 2′-*O*-methyl CMP | 91 | 2′-*O*-methyl UMP | 18 | 2′-*O*-methyl GMP | 0 | 2′-*O*-methyl AMP | 2 |
| 2′-Deoxy-2′-azido CMP | 155 | 2′-Deoxy-2′-azido UMP | 45 | 2′-Deoxy-2′-azido GMP | 0 | 2′-Deoxy-2′-azido AMP | 13 |
| 2′-Deoxy-2′-chloro CMP | 164 | 2′-Deoxy-2′-chloro UMP | 55 | 2′,3′-Dideoxy-3′-azido dGMP | 0 | 2′,3′-Dideoxy-3′azido dAMP | 0.1 |
| araCMP | 104 | araUMP | 144 | | | araAMP | 12 |
| 5-Methyl dCMP | 0 | 5-Methyl UMP | 2 | 2′,3′-Dideoxy-3′-amino dGMP | 0 | 2′,3′-Dideoxy-3′amino dAMP | 8 |
| | | TMP | 0 | | | 3′-Deoxy-3′-amino AMP | 72 |
| | | | | | | 3′-Deoxy-3′-azido AMP | 1.2 |
| | | | | | | 3′-Deoxy AMP | 53 |

erythrocytes and acute myelocytic leucemia cells [23]. As can be seen from Table I a purification of the various kinase activities from calf thymus could be achieved by passage of the crude mixture over a Cibacron Blue column. TMP kinase activity could not be detected presumably because of instability in the absence of substrate [24]. Most importantly, phosphatase activity was no longer present in the kinase fraction.

The rates of phosphorylation of various nucleotides with the NMP kinases isolated by this procedure and ATP are described in Table II. Although there are a few reports [20] concerning substrate specificity of nucleoside monophosphate kinases using base-modified nucleoside 5′-monophosphates, this is the first systematic investigation with respect to the sugar moiety. Two 5-substituted pyrimidine nucleotides were included in this survey since modifications at this position are of potential interest for affinity labeling [25].

Neither the 2′- nor the 3′-hydroxyl group of natural nucleotides are essential for pyrimidine nucleoside monophosphate kinase. However, they are important for AMP kinase and almost indispensable for GMP kinase. Methylation of the hydroxyl groups ore replacement by an azido or amino group in AMP lowers considerably the $V_0$ value. The same modifications in GMP abolish the substrate properties. In spite of the nonspecificity with regard to the sugar residue, pyrimidine nucleoside monophosphate kinase does not tolerate a methyl group at position 5 of the pyrimidine base.

Several nucleoside 5′-phosphates were phosphorylated to triphosphates with the partially purified kinase mixture on a 0.5 μmol scale and the reaction mixtures were analyzed by HPLC. The yields and retention times are described in Table III. It can clearly by seen that this kinase system can be applied widely for the synthesis of triphosphates of sugar-modified nucleosides apart from most of the guanosine derivatives. The yields were generally quantitative. Even very poor substrates such as 3′-deoxy-3′-azido dAMP, 3′-deoxy-3′-amino dAMP and 2′-deoxy-2′-amino GMP can be phosphorylated in good yields using longer incubation times. On the other hand, the yield was not more than 10% when 3′-azido dAMP was phosphorylated with the crude streptomycin-precipitated extract which indicates the interference of the phosphatase. This enzyme

TABLE III

PREPARATION OF NUCLEOSIDE TRIPHOSPHATES

Detailed experimental conditions as described under Materials and Methods. Retention time of ATP: with buffer A, 15.3 min; with buffer B, 7.9 min.

| Substrate (0.5 μmol) | Enzyme (unit) | Incubation time (h) | Yield | Buffer for elution | Retention time (min) | |
|---|---|---|---|---|---|---|
| | | | | | Starting material | Product |
| araCMP | 0.5 | 0.5 | quant. | A | 0.5 | 2.6 |
| 2′-Deoxy-2′-azido CMP | 0.5 | 0.5 | quant. | A | 1.7 | 6.7 |
| dCMP | 0.5 | 0.5 | quant. | A | 0.8 | 3.4 |
| 2′-Deoxy-2′-azido UMP | 0.5 | 1 | quant. | A | 2.1 | 6.5 |
| 2′,3′-Dideoxy-3′-amino dAMP | 0.5 | 6 | quant. | A | 0.7 | 4.2 |
| 2′,3′-Dideoxy-3′-azido dAMP | 2.4 | 72 | 58% | B | 4.5 | 11.7 |
| 2′-Deoxy-2′-amino GMP | 0.5 | 48 | 65% | A | 0.5 | 2.4 |

preparation should, thus, be very useful for the preparation of sugar-modified nucleoside triphosphates.

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