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NUCLEOSIDE PHOSPHOTRANSFERASE FROM YELLOW LUPIN SEEDLING COTYLEDONS *

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Summary

Nucleoside phosphotransferase (nucleotide:3′-deoxynucleoside 5′-phosphotransferase, EC 2.7.1.77) from yellow lupin seedling cotyledons was purified and the active enzyme consists of a single polypeptide chain, M_r = 72 000 ± 3000. In transphosphorylation, purine and pyrimidine nucleosides are good phosphate acceptors and 5′-nucleotides are effective phosphate donors. Among 2′- and 3′-nucleotides, only 3′-AMP and 3′-ΨMP acted as phosphate donors, and *p*-nitrophenylphosphate appeared less active in this regard. The purine and pyrimidine bases inhibit transphosphorylation. The K_m values determined for the inosine:5′-AMP pair were 400 μM for both the compounds. The enzyme showed optimum activity at pH 8.0 in 50 mM Tris-HCl buffer. Antisulfhydryl reagents and EDTA did not affect enzyme activity.

Introduction

Nucleoside phosphotransferase (nucleotide:3′-deoxynucleoside 5′-phosphotransferase, EC 2.7.1.77) catalyze the synthesis of ribo- and deoxyribonucleotides by the transfer, from suitable donors, of organically esterified phosphoric acid to a nucleoside. So far nucleoside phosphotransferases from carrot [1—3], *Escherichia coli* [4] and *Erwinia herbicola* [5] were purified and characterized. Considering the distribution of nucleoside phosphotransferases, which parallels the anabolic activity of cells and tissues [6], as well as the substrate specificity

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[4], it was suggested that these enzymes should play a role in the synthesis of nucleic acids and nucleotide coenzymes. But the metabolic role of nucleoside phosphotransferases remains unidentified. In my studies on plant enzymes metabolizing adenosine the nucleoside phosphotransferase activity was found in the cotyledons of growing yellow lupin seedlings. This paper presents the purification procedure and describes properties of the lupin enzyme, which exhibits some physical and kinetic differences from the above mentioned nucleoside phosphotransferases.

Materials and Methods

Plant materials. The cotyledons of 7-day-old *Lupinus luteus* seedlings were the source of the nucleoside phosphotransferase. The seedlings were grown on cellulose wool under natural light conditions at room temperature. Barley leaves were the source of adenosine nucleosidase which was isolated as described previously [7].

Chemicals. Chromatographically pure [U-^{14}C]adenosine was obtained as described previously [8]. [U-^{14}C]Inosine was obtained after incubation of labeled adenosine with commercial adenosine deaminase (Calbiochem, U.S.A.) and purification by thin-layer chromatography. [U-^{14}C]AMP, [U-^{14}C]guanosine, [U-^{14}C]deoxyadenosine and [U-^{14}C]cytidine were from Radiochemical Centre Amersham, U.K.; [U-^{14}C]uridine was from C.E.A., Saclay, France. All nucleobases, ribonucleosides, Aquacide and dithiothreitol were from Calbiochem, U.S.A. 5′-, 3′-, and 2′-ribonucleotides, *p*-hydroxymercuribenzoate, ribose 1-phosphate, α,β-methylene-ATP and Coomassie brilliant blue G-250 were from Sigma, U.S.A. *N*-Ethylmaleimide, 5′-dATP, NMN and proteins for markers in the determination of molecular weight were from Serva, F.R.G. Deoxyadenosine, NADP, ATP and ADP were purchased from Reanal, Hungary, *p*-nitrophenylphosphate from Carl Roth, F.R.G., 1-naphthyl-*o*-phosphate from Hopkin and Williams, U.K., DL-*O*-phosphoserine from NBCo, U.S.A., D-ribose and plastic sheets precoated with cellulose containing fluorescent indicator from Merck, F.R.G., DEAE-cellulose paper from Whatman, U.K., Sephadex G-100 from Pharmacia, Sweden, and other reagents from Polskie Odczynniki Chemiczne, Poland. Aminohexyl-Sepharose 4B was prepared as described previously [9].

Assay for nucleoside phosphotransferase. The standard incubation mixture used in the course of purification consisted of 50 mM Tris-HCl, pH 8.0, 10 mM 5′-AMP, 2 mM [U-^{14}C]inosine and 10 μl of enzyme fraction (added last) in a final volume 50 μl. The samples were incubated at 30°C for 10 min. Convenient and fast technique enabling simultaneous analysis of many samples was applied to separate the substrates and products. 5 μl of the mixture was transferred onto DEAE-cellulose sheets and the chromatograms developed in 50 mM Tris-HCl, pH 8.0, for about 40 min. After drying, the nucleotide spots (near starting points) were visualized under ultraviolet light, cut out, immersed in 5 ml of scintillation fluid and counted in Beckman LS 100 scintillation counter.

The R_F values for ribonucleosides in the system used are as follows: adenosine 0.62, guanosine 0.26, inosine 0.28, cytidine 0.78 and uridine 0.43.

In the kinetic studies, to estimate the initial velocity of the reaction, 5-μl aliquots of the incubation mixture were transferred onto DEAE-cellulose sheets after 2.5, 5, 7.5, 10, and 12.5 min. One unit of nucleoside phosphotransferase activity catalyzes the synthesis of 1 μmol of nucleotide during 1 min at 30° C.

Isomer analysis. To separate 5′-, 3′- and 2′-riboadenylates, two-dimensional thin-layer chromatography was employed. Solvent I (5% Na_2HPO_4) separates the mixture of 5′- and 2′-adenylates from 3′-isomer. In solvent II (*n*-propanol/conc. NH_3/H_2O, 11/7/2) 5′-adenylate is separated from 3′-isomer. For separation of 5′-uridylate from the mixture of 3′- and 2′-isomers the solvent II was used.

Disc electrophoresis. Analytical disc electrophoresis was carried out in 7.5% polyacrylamide gels according to the method of Davis [10]. The samples were layered directly on the sieving gel. The gels were stained with Coomassie brilliant blue G-250 according to a new staining technique described by Blakesley and Boezi [11].

Molecular weight determination. This was done either by gel filtration of the active nucleoside phosphotransferase according to the method of Andrews [12], or by SDS-polyacrylamide gel electrophoresis of the SDS-denatured enzyme in the presence of 2-mercaptoethanol as described by Weber et al. [13]. Bovine serum albumin, ovalbumin and chymotrypsinogen A were used as molecular weight standards.

Protein determination. Protein concentration was determined by the turbidimetric tannin method [14] using bovine serum albumin as standard.

Determination of K_m values. The K_m values for inosine and 5′-AMP were estimated in standard incubation conditions. The incubation mixture contained 1 μg of the Sephadex G-100 fraction of nucleoside phosphotransferase and 20 μg of bovine serum albumin. The concentration of the labelled inosine varied from 0.06 to 3.8 mM and of 5′-AMP from 0.02 to 50 mM.

Substrate specificity. In standard incubation conditions several radioactive nucleosides available were tested for their ability to accept the phosphate residue from various phosphate esters.

Other details of particular experiments are given in legends to figures.

Results and Discussion

Enzyme purification

The 7-day-old yellow lupin seedling cotyledons (fresh weight 420 g) were homogenized with two volumes 10 mM Tris-HCl buffer, pH 8.0, containing 10% glycerol in omnimixer. The slurry was passed through cheesecloth and the filtrate centrifuged (20 min, 20 000 × *g*). The nucleoside phosphotransferase was salted out between 50 and 70% of ammonium sulfate saturation. The precipitate was dissolved in 54 ml of 50 mM potassium phosphate buffer, pH 6.8, containing 10% glycerol (buffer B) dialyzed against this buffer and applied on an aminohexyl-Sepharose 4B column (5 × 16 cm) previously equilibrated with the same buffer. The enzyme activity which emerged in the break-through fraction was concentrated by ammonium sulfate precipitation (70% saturation), dissolved in 4.5 ml of buffer B and applied on the Sephadex G-100 column (2.5 × 90 cm) equilibrated with the same buffer. Active fractions which

eluted at $V_e/V_0 = 1.35$ (Fig. 1) were frozen and kept at −20° C. The Sephadex G-100 peak fraction was used for enzyme characterization. All the operations were carried out at 5° C.

The partial purification of nucleoside phosphotransferase from lupin seedling cotyledons summarized in Table I afforded over 100-fold purification of the enzyme in relation to the initial extract.

Analytical polyacrylamide gel electrophoresis of the Sephadex G-100 peak fraction revealed one distinct band comprising few percent of total protein applied, which migrated more slowly than the bulk of the other proteins (Fig. 2). After electrophoresis the gel was sliced and the enzyme activity assayed in the diffusates. It was demonstrated that both nucleoside phosphotransferase and inherent phosphatase activities were present in the slowest moving protein band (Fig. 2A and B). From the data included in Fig. 2 it can be concluded that the Sephadex G-100 fraction of lupin nucleoside phosphotransferase is free of nonspecific phosphatase or 5'-nucleotidase activities with distinct electrophoretic mobility.

The pure carrot nucleoside phosphotransferase [1,2] exhibited also a hydrolytic, phosphatase, activity when the nucleotide or some other organic phosphate esters were incubated in the absence of nucleoside, a potent phosphate acceptor.

Total activity of the enzyme markedly decreased after electrophoresis, hence, in despite of good separation of the enzyme from other proteins, this procedure cannot be recommended as an effective purification step. It can be

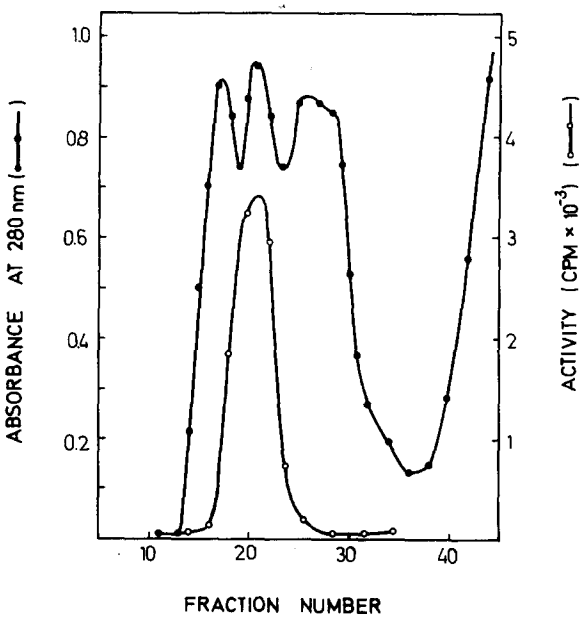

Fig. 1. Distribution of lupin nucleoside phosphotransferase activity after gel filtration. The sample (4.5 ml) containing protein after the aminohexyl-Sepharose 4B step (see text) was applied onto Sephadex G-100 column (2.5 X 90 cm). Fractions of 10 ml were collected, their absorbance at 280 nm measured and phosphotransferase activity estimated in the standard incubation mixture for 2 min at 30° C.

TABLE I

PARTIAL PURIFICATION OF NUCLEOSIDE PHOSPHOTRANSFERASE FROM YELLOW LUPIN SEEDLING COTYLEDONS

One unit (U) of nucleoside phosphotransferase activity catalyzes the synthesis of 1 μmol of nucleotide during 1 min at 30°C.

Step	Protein weight (mg)	Activity		Purification (-fold)	Yield (%)
		Total (U)	Specific (U/mg)		
Crude extract	25 600	230	0.009	1	100
50—70% $(NH_4)_2SO_4$	4 300	73	0.017	1.9	32
Sepharose-$NH(CH_2)_6NH_2$	250	36	0.144	16	15.6
Sephadex G-100 (peak fraction)	10	11	1.100	122.2	4.8

used, however, for analytical scale isolation of the enzyme for M_r and quaternary structure determination (see below).

Enzyme stability

The partially purified lupin nucleoside phosphotransferase tolerated repeated freeze-thawing. In studies on thermostability it was demonstrated that the enzyme is protected by glycerol and phosphate ions against heat inactivation (Fig. 3). One-half of the nucleoside phosphotransferase molecules was inactivated at 63°C after 3 min preincubation in 50 mM Tris-HCl buffer, pH 8.0; at 68°C in the same buffer containing 10% glycerol at at 72°C in 50 mM potassium phosphate buffer, pH 6.8, containing 10% glycerol.

Molecular weight and subunit structure

The slice diffusates obtained from eight polyacrylamide gels after electrophoresis contained sufficient amount of protein to determine molecular weight and subunit structure of the lupin nucleoside phosphotransferase. The diffusates were concentrated with Aquacide, the protein denaturated with sodium dodecyl sulfate in the presence of 2-mercaptoethanol and subjected to electrophoresis according to the procedure described by Weber et al. [13]. On sodium dodecyl sulfate-polyacrylamide gel the denatured enzyme exhibited a single band of M_r = 72 000. From these data and the data obtained from the Sephadex G-100 gel filtration it was concluded that the lupin nucleoside phosphotransferase is a single polypeptide chain with M_r = 72 000 ± 3000. As regards the molecular weight, the lupin enzyme resembles nucleoside phosphotransferase from *E. herbicola* [5]. The other plant enzyme from carrot has a molecular weight of about 44 000 and consists of two subunits [3].

Effect of anti-sulfhydryl reagents and mercaptans

The test with anti-sulfhydryl reagents, *p*-hydroxymercuribenzoate and *N*-ethylmaleimide was carried out as described previously [8]. The lupin nucleoside phosphotransferase appeared resistant to these reagents. Also 1, 10 and 50 mM 2-mercaptoethanol and dithiothreitol had no effect on the transphosphorylation.

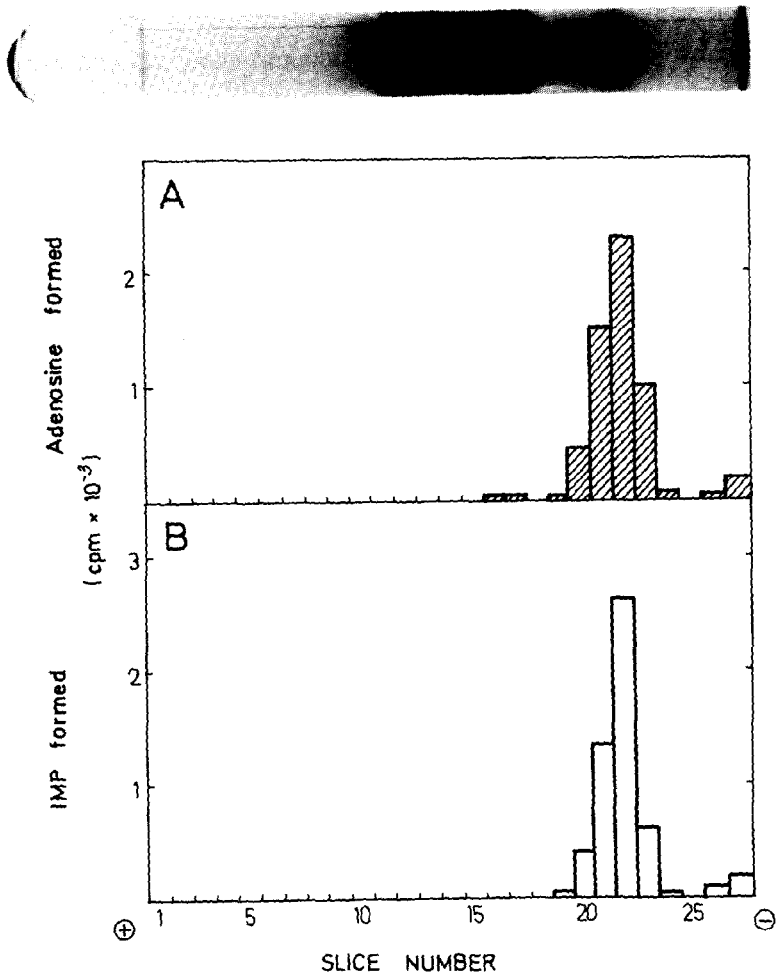

Fig. 2. Polyacrylamide gel electrophoresis of partially purified lupin nucleoside phosphotransferase. Above: distribution of protein after staining with Coomassie brilliant blue G-250. (A) Profile of phosphatase activity. (B) Profile of phosphotransferase activity. Each sample applied onto 7.5% polyacrylamide gels contained 30 μg of the Sephadex G-100 peak fraction. After electrophoresis one gel was stained and the other sliced into 2-mm pieces. Each slice was immersed in 200 μl of 50 mM Tris-HCl buffer, pH 8.0, and kept overnight at 5° C. The 10-μl portions of the diffusates were incubated at 30° C for 30 min to estimate phosphatase and phosphotransferase activities. The standard assay mixture for phosphotase contained 50 mM Tris-HCl buffer, pH 8.0, and 2 mM [U-^{14}C]AMP. The released [^{14}C]adenosine was estimated after chromatography as described in assay for nucleoside phosphotransferase.

Effect of pH

In 50 mM Tris-HCl buffer the optimum activity of the transphosphorylation was at 8.0. In this regard, the lupin enzyme differs from the carrot one [1], (pH optimum at pH 5.0), and resembles the one from *E. herbicola* [5].

Michaelis constants

Both inosine and 5'-AMP showed Michaelis-Menten type kinetics in the phosphate transfer reaction. K_m values for those substrates were 400 μM. The K_m values determined by Brunngraber and Chargaff for carrot enzyme [2] were

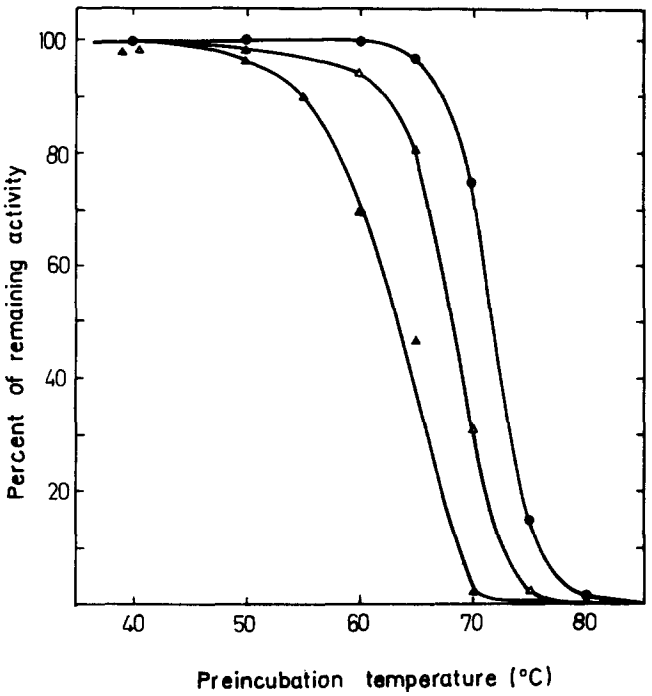

Fig. 3. Thermostability of partially purified lupin nucleoside phosphotransferase. The enzyme samples in one of the following buffers: ●, 50 mM potassium phosphate (pH 6.8), 10% glycerol; ▲, 50 mM Tris-HCl (pH 8.0); △, 50 mM Tris-HCl (pH 8.0), 10% glycerol, were preincubated for 3 min at indicated temperatures. Subsequently the samples were diluted three times with 50 mM Tris-HCl (pH 8.0) and the remaining phosphotransferase activity assayed in the standard incubation mixture.

one order of magnitude higher (3.5 mM, both for phenylphosphate used as the donor and uridine used as acceptor).

Effect of the potassium phosphate buffer ionic strength

The phosphotransferase activity was tested in the potassium phosphate buffer, pH 6.8, over the range of 10—200 mM. The reaction was slightly affected, and in 200 mM buffer was only 20% slower than in 10 mM one.

Effect of magnesium and EDTA

Magnesium chloride over range of 1—100 mM as well as 1 and 5 mM EDTA had no influence on velocity of transphosphorylation reaction.

Time course of transphosphorylation

Time course of the synthesis of UMP from [U-^{14}C]uridine and 5'-AMP catalyzed by lupin nucleoside phosphotransferase is shown in Fig. 4. Initially, when the phosphate donor is present, the transphosphorylation surpasses the inherent phosphatase activity of the nucleoside phosphotransferase. Subsequently, however, since no additional donor is supplied, all nucleotides are hydrolyzed. The time curve of transphosphorylation is modified by adenosine nucleosidase included in the incubation mixture. The rate of [^{14}C]UMP syn-

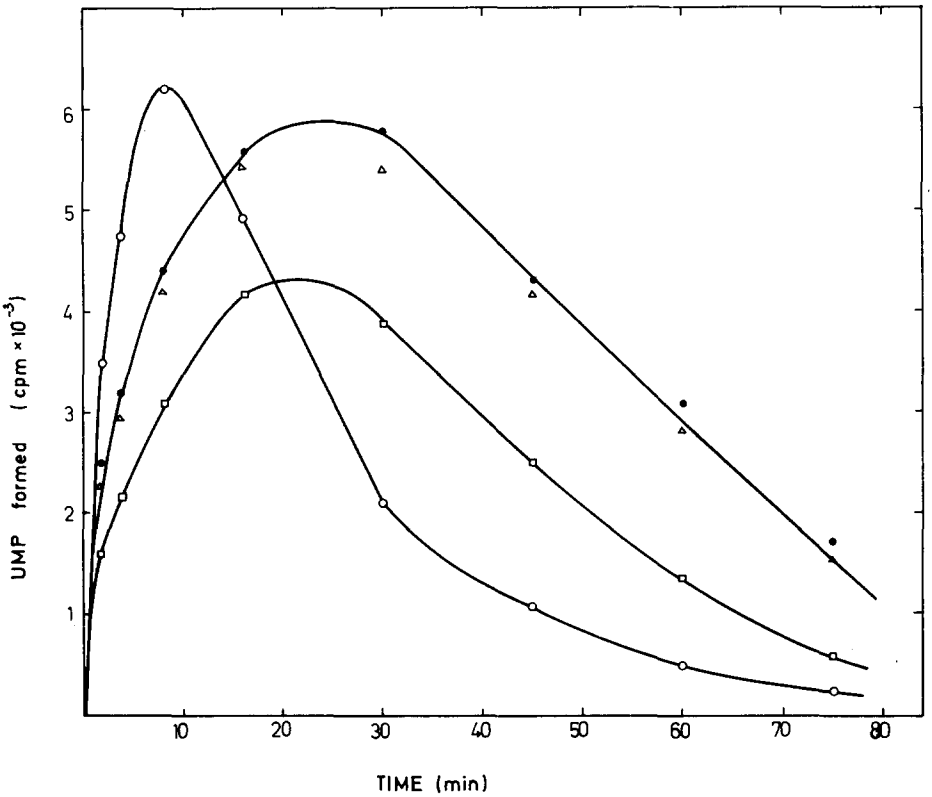

Fig. 4. Time curves of synthesis and hydrolysis of UMP by lupin nucleoside phosphotransferase; effect of adenine and ribose. Incubation mixture in total volume of 100 µl contained 50 mM Tris-HCl (pH 8.0), 2 mM [U-^{14}C]uridine, 2 mM 5′-AMP and the additives where indicated. •, mixture with no additives; ○, with about 0.2 unit of adenosine nucleosidase; □, with 2 mM adenine, or △, with 2 mM D-ribose. At intervals, 5-µl aliquots were pipetted on DEAE-cellulose sheets and developed.

thesis is first higher than in the absence of adenosine nucleosidase. It is due to the degradation of the unlabelled adenosine originating from 5′-AMP, which competes with labelled uridine for phosphate residues. At prolonged incubation, however, the labelled UMP, the only nucleotide synthesized by the enzyme, is degraded much faster than in the mixture without adenosine nucleosidase. In other parallel experiments adenine or D-ribose was added to the mixture containing labelled uridine and 5′-AMP. It was observed that while ribose did not affect the reaction, the free base, adenine, inhibited transphosphorylation competing with uridine for an acceptor (nucleoside) site. Other purine and pyrimidine bases acted also as inhibitors of the phosphotransferase activity (data not shown).

Substrate specificity

All purine and pyrimidine ribonucleosides and deoxyadenosine (the only deoxyribonucleoside tested) appeared to be good phosphate acceptors and 5′-ribonucleotides were effective phosphate donors. The initial velocities of the appropriate nucleotide synthesis from labelled ribonucleosides are summarized in Table II.

TABLE II

PHOSPHOTRANSFERASE ACTIVITY IN PRESENCE OF VARIOUS RIBONUCLEOSIDES AS ACCEPTORS USING DIFFERENT PHOSPHATE DONORS

The concentrations of all acceptors was 2 mM; of all 5′-ribonucleotides and 3′-ΨMP, 10 mM; of 3′-AMP, 5 mM, and of *p*-nitrophenylphosphate, 100 mM. The activity is expressed as μmol of nucleotide synthesized/mg protein per min under standard assay conditions.

Phosphate donor	Acceptor				
	Inosine	Guanosine	Adenosine	Cytidine	Uridine
5′-AMP	1.10	0.64	0.80	0.16	0.47
5′-GMP	1.41	0.67	0.87	0.34	1.11
5′-IMP	1.36	0.68	0.82	0.32	0.83
5′-CMP	1.16	0.74	0.58	0.64	1.48
5′-UMP	1.37	0.70	1.10	0.50	1.09
3′-AMP	0.45	0.25	0.40	0.08	0.32
3′-ΨMP	0.13	0.03	0.16	0.10	0.20
p-Nitrophenylphosphate	0.01	0.05	0.23	0.05	0.14

During preliminary experiments when the nucleoside phosphotransferase activity was assayed in crude extract of lupin cotyledons [the extract contains enzymes which readily decompose adenosine (deoxyadenosine) triphosphate to di- and monophosphate, adenosine (deoxyadenosine), adenine and ribose] it was observed that labelled inosine was converted to IMP at the same rate when either ATP, ADP or dATP was used as potential phosphate donor. These facts suggested that for lupin nucleoside phosphotransferase the deoxyribonucleaside monophosphates are probably equally effective phosphate donors as ribonucleoside monophosphates. Deoxyribonucleoside monophosphates were reported to act as good phosphate donors for nucleoside phosphotransferase from *E. herbicola* [5] and chick embryo extracts [15].

Among 2′- and 3′-ribonucleotides tested, also 3′-AMP and 3′-ψMP acted as phosphate donors. In contrast with the carrot nucleoside phosphotransferase, 100 mM *p*-nitrophenylphosphate was poorly effective as donor for the lupin enzyme.

With [^{14}C]inosine as phosphate acceptor, the following phosphate compounds used at 5 mM concentration failed to be donors of phosphate residue: 5′-NMN, NADP, ribose 1-phosphate, 1-naphthyl-*o*-phosphate, DL-*O*-phosphoserine and K_2HPO_4. The enzyme does not utilize also pyrophosphates, since ATP, ADP, dATP, α,β-methylene-ATP and $Na_4P_2O_7$ were inactive as phosphate donors. Isomer analysis of the synthesized adenylates and uridylates showed that chiefly the 5′-ribonucleotides (90%) was produced by the lupin enzyme, regardless of 5′- or 3′-nucleotides being used as phosphate donors. The remaining 10% of the synthesized adenylates was localized in 3′-isomer. Another plant nucleoside phosphotransferase from carrot favored also the formation of 5′-nucleotides [1], whereas the bacterial enzyme synthesized chiefly 3′-isomers [16].

The nucleoside phosphotransferase easily transfers phosphate residue from both purine and pyrimidine mononucleotides to nucleosides. Other phosphate esters including pyridine nucleotide, NMN, were not active in this regard. It

suggests that the enzyme controls in vivo the distribution and the balance of the purine and pyrimidine nucleoside and nucleotide pools within tissue and cell compartments. The following speculation seems to be plausible: the 5′-AMP originating from ATP and ADP degradation may, in the growing plants, be the main source of phosphate residue for nucleoside phosphotransferase, which takes part in reutilization of other ribo- and deoxyribonucleosides according to tissue and cell demand.

Acknowledgments

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