Biochimica et Biophysica Acta, 410 (1975) 262—272

BBA 67650

PURINE NUCLEOTIDE PYROPHOSPHOTRANSFERASE FROM *STREPTOMYCES MOROOKAENSIS*, CAPABLE OF SYNTHESIZING pppApp AND pppGpp

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(Received April 4th, 1975)
(Revised manuscript received August 12th, 1975)

Summary

Purine nucleotide pyrophosphotransferase was purified to apparent homogeneity from a culture filtrate of *Streptomyces morookaensis*. It is a monomeric protein with a molecular weight of 24 000—25 000, and its isoelectric point is 6.9.

The enzyme synthesizes purine nucleoside 5′-phosphate (mono, di, or tri) 3′-diphosphates such as pppApp, ppApp, pApp, pppGpp, ppGpp and pppIpp by transferring a pyrophosphoryl group from the 5′-position of ATP, dATP and pppApp to the 3′-position of purine nucleotides. The purified enzyme catalysed the formation of 435 μmol of pppApp and 620 μmol of pppGpp from ATP and GTP per min per mg protein under the standard conditions. The enzyme requires absolutely a divalent cation for activity, and optimum pH for the enzyme activity lay above 10 for Mg^{2+} and Mn^{2+}, for Co^{2+} and Zn^{2+} from 9 to 9.5, and for Fe^{2+} from 7.5 to 8. The following Michaelis constants were determined: AMP, 2.78 mM; ADP, 3.23 mM; GMP, 0.89 mM; GDP, 0.46 mM and GTP, 1.54 mM, in the case of ATP donor. The enzyme is inhibited by guanine, guanosine, dGDP, dGTP, *N*-bromosuccinimide, iodacetate, sodium borate and mercuric acetate.

Introduction

There has been increasing interest in the properties and physiological functions of a factor present in the ribosomal wash of stringent *Escherichia coli* [1,2] and the unusual nucleotides, pppGpp, ppGpp, ppApp and pppApp,

Abbreviations: $p(CH_2)ppG$, β,γ-methylene guanosine 5′-triphosphate; $p(CH_2)ppA$, β,γ-methylene adenosine 5′-triphosphate; $pp(CH_2)pA$, α,β-methylene adenosine 5′-triphosphate; $p(CH_2)pA$, α,β-methylene adenosine 5′-diphosphate.

which are known to be ubiquitously distributed in nature [3—16].

Recently, Murao and Nishino found and purified a novel ATP : nucleotide pyrophosphotransferase in *Streptomyces adephospholyticus* [11—14]. This enzyme was characterized to transfer the β-γ pyrophosphate of ATP to adenine, guanine and hypoxanthine nucleotides, and to synthesize nucleoside 5′-phosphate (mono, di or tri) 3′-diphosphates [15—16].

We have investigated the occurrence and physiological significance of pppApp-synthesizing enzyme in microorganisms, and found that the pppApp-synthesizing activity occurred widely in actinomycetes [17]. The present paper describes the purification procedure of a nucleotide pyrophosphotransferase from *Streptomyces morookaensis* ATCC19166, and some of its molecular and catalytic properties.

Experimental procedure

Materials

Chemicals. Common nucleotides and nucleosides were purchased from either Sigma Chem. Co. or Boehringer Mannheim GmbH. Nucleotide analogues, $p(CH_2)ppG$, $p(CH_2)ppA$, $pp(CH_2)pA$ and $p(CH_2)pA$ were purchased from Miles Lab., Inc. Crystalline bovine serum albumin was obtained from General Biochem; human γ-globulin and bovine ribonuclease Grade I from Miles Servac; egg white lysozyme, 6 times crystallized, from Seikagaku Kogyo Co.; chymotrypsin, 3 times crystallized, and crystalline ovalbumin from Sigma Chem. Co. [8-^{3}H] AMP diammonium salt (14 Ci/mmol), [8-^{3}H] ADP trilithium salt (17 Ci/mmol), [8-^{3}H] ATP trilithium salt (17 Ci/mmol), [8-^{3}H] GMP diammonium salt (10 Ci/mmol), [8-^{3}H] GDP trilithium salt(10 Ci/mmol) and [8-^{3}H] GTP tetrasodium salt (9.35 Ci/mmol) were purchased from Schwarz/Mann.

Methods

Microorganism and cultural conditions. Streptomyces morookaensis ATCC19166 was grown in a medium composed of 2% glycerin, 4% polypeptone, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 2ppm Mn^{2+} and Fe^{2+}, pH 7.0. Cultivation was carried out in a 20-liter jar fermentor containing 10 liters of the medium at 28°C for 48 h under aeration (5 liters per min) and agitation (250 rev./min).

Enzyme assay. Procedure A: The standard assay system consisted of 62.5 mM glycerine/NaOH buffer, pH 10.0, 5 mM $MgCl_2$, 10 mM [8-^{3}H] ATP ($1.66 \cdot 10^6$ dpm, 2 Ci/mol) and 0.1 to 0.2 units enzyme in a final volume of 0.2 ml. After incubation at 37°C for 10 min, the reaction was terminated by addition of 20 μl of 1 M acetic acid, mixing and chilling on ice. To 20 μl of the reaction mixture 5 μg of authentic unlabeled pppApp were added as chromatographic marker and this mixture was spotted on a polyethyleneimine cellulose F thin-layer sheet (DC-Alufolien PEI-cellulose F, E. Merck AG), dried, and developed in 1.15 M or 0.75 M KH_2PO_4, pH 3.4. After chromatography the sheets were dried and exposed to ultraviolet light provided by a Manasul UV lamp to localize the pppApp formed. The area on the chromatograms corresponding to pppApp was cut out into small pieces, and transferred into a scintillation vial containing 0.5 ml of 0.1 M HCl. After boiling the contents for 15 min, radio-

activity was measured in a Aloka scintillation spectrophotometer Model LSC-653. Enzyme activity was calculated by the amount of radioactivity incorporated into pppApp spot, subtracting the background incorporation measured in the control experiments. A unit of enzyme activity is defined as the amount of enzyme forming 1 μmol of pppApp per minute at 37°C. Specific activity of enzyme was expressed as units per mg protein.

Procedure B. As reported by Murao and Nishino [13], the terminal phosphate at the 3'-position of pppApp is very labile in acid, especially in sulfuric acid, therefore this nucleotide could be estimated as inorganic phosphate. The standard reaction mixture contained 100 mM glycine/NaOH buffer, pH 10, 5 mM ATP, 5 mM $MgCl_2$ and enzyme solution (0.2 to 1 unit) in a final volume of 0.5 ml. After incubation at 37°C for 10 min, the reaction was terminated by addition of 0.5 ml of 0.2 M acetic acid, and chilled on ice. Inorganic phosphate was determined by the method of Fiske and SubbaRow [18].

It was elucidated that the formation of ppp[8-^{3}H]App was proportional to the amount of inorganic phosphate in the reaction mixture; 1.04 μmol of P_i was determined in 1 μmol of pppApp formed under the above assay conditions. Therefore, a unit of enzyme activity is defined as the amount of enzyme forming 1.04 μmol of P_i per min.

Isolation and identification of the reaction products. New nucleotides in the reaction mixture were isolated by the methods of Hamagishi et al. [16] and Cashel [19]. Identification of pApp, ppApp, pppApp, pGpp, ppGpp and pppGpp was carried out by co-chromatography on PEI-cellulose sheet with authentic samples kindly provided by Dr S. Murao and Dr M. Cashel, by resistance to periodate oxidation, and on the basis of molar ratio of P_i to nucleotide.

Polyacrylamide gel electrophoresis. Analytical electrophoresis on polyacrylamide gel (7.5%) was performed at pH 9.4 at a current of 2 or 5 mA per tube at 4°C according to the method of Davis [20]. Electrophoresis on polyacrylamide gel (7.5%) containing 0.1% sodium dodecyl sulfate was performed according to the method of Weber and Osborn [21].

Isoelectric focusing on polyacrylamide gels was carried out by the method of Gronow and Griffiths [22]. The enzyme was extracted with 1 ml of 0.1 M KCl solution from the unfixed gels (2 mm slice), and enzyme activity and pH were determined.

Glycerol gradient centrifugation. Linear 15 to 55% (v/v) glycerol gradients (0.01 M Tris · HCl, pH 7.4, and 0.2 M NaCl) were prepared as described by Britten and Roberts [23]. The centrifugation was carried out at 7°C and 39 000 rev./min in a RPS40 rotor of a Hitachi centrifuge Model 65P for 48 h.

Isoelectric focusing. The method described by Vesterberg and Svensson [24] was used. The isoelectric focusing was performed in an LKB8100 electrofocusing column using carrier ampholite (LKB Instruments, Inc.) at pH 3 to 10 or 5 to 8. The column was run for 48 h at 700 V with cooling by water circulated at 10°C. After the run, 2 ml-fractions were assayed for the enzyme activity, pH and absorbance at 280 nm.

Chemical assay. Protein was determined by the method of Lowry et al. [25] with crystalline bovine serum albumin as a standard; with most column fractions, protein concentrations were monitored by absorbance at 280 nm.

Inorganic phosphate was determined by the method of Fiske and SubbaRow [18], and the total phosphate was determined as inorganic phosphate after ashing by the method of Allen [26].

Results

Purification of the enzyme

All operations were carried out at 0 to 4°C, unless otherwise specified.

Step 1: Ammonium sulfate fractionation. To the culture filtrate obtained as described under Methods, ammonium sulfate was added to 60% saturation. The precipitate was dissolved in 5 liters of tap water and dialyzed for 34 h against running water.

Step 2: DEAE-Cellulose column chromatography. The dialyzed preparation was applied to a DEAE-cellulose column (75 × 10 cm) equilibrated with 0.01 M acetate buffer (pH 5.5) containing 0.001 M $MgCl_2$ (Buffer A), and the enzyme passed through the column without retention to DEAE-cellulose. 10 560 ml of the eluates were pooled.

Step 3: CM-Sephadex C-25 column chromatography. The enzyme solution from Step 2 was applied to a CM-Sephadex C-25 column (50 × 5.5 cm) equilibrated with Buffer A. After the column was washed with the same buffer, impure protein was eluted with 1500 ml of the buffer supplemented with 0.1 M NaCl, and then the enzyme was eluted with 0.01 M Tris · HCl, pH 8.7, containing 0.01 M $MgCl_2$ (Buffer B) and 0.2 M NaCl. The active fractions were pooled (620 ml) and concentrated by ammonium sulfate precipitation (60% saturation). The enzyme was dissolved in Buffer B, followed by dialysis for 24 h against 200 volumes of the same buffer.

Step 4: DEAE-Sephadex A-25 column chromatography. The enzyme preparation from Step 3 was applied on a DEAE-Sephadex A-25 column (50 × 3 cm) which had been equilibrated with Buffer B. After the column was washed with the same buffer supplemented with 0.02 M NaCl, the enzyme was eluted with

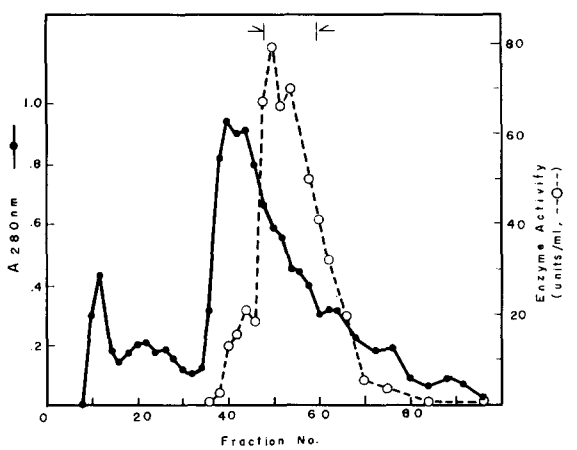

Fig. 1. DEAE-Sephadex A-25 column chromatography of the enzyme. After washing with Buffer B containing 0.02 M NaCl, the column was eluted with 0.05 M NaCl in Buffer B. Fractions of 7 ml were collected at a flow rate of 0.8 ml per min, and those between the arrows were used for the next step.

Buffer B supplemented with 0.05 M sodium chloride. Fig. 1 shows a typical elution profile. Active fractions were combined (105 ml) and concentrated to 10 ml with the aid of a collodion bag.

Step 5: Gel filtration on a Sephadex G-75 column. The concentrated enzyme solution from Step 4 was applied to a Sephadex G-75 column (77 × 3 cm) equilibrated with 0.05 M glycine/NaOH buffer, pH 9.0, containing 0.01 M $MgCl_2$. Elution was carried out with the same buffer and 3-ml fractions were collected at a flow rate of 8 ml per h. Enzyme fractions were eluted as a single and symmetrical peak with high specific activity and recovery (87%), and were combined and stored at 4°C for 2 months without loss of activity. The enzyme has been stored in the same buffer containing 20% glycerol at −20°C.

The overall purification achieved was about 4800-fold with a yield of about 3%. The purified enzyme catalyzed the formation of 435 μmol of pppApp from ATP and 620 μmol of pppGpp from ATP and GTP per min per mg protein under the standard conditions. Typical results of the purification are presented in Table I.

Molecular properties

Molecular weight and isoelectric point. When run on a calibrated column of Sephadex G-75 in 0.05 M glycine/NaOH buffer, pH 9.0, containing 0.1 M KCl and 0.01 M $MgCl_2$, the enzyme gave an apparent molecular weight of 25 000 comparing with the reference proteins. The mobility of the enzyme on a sodium dodecyl sulfate (0.1%) polyacrylamide gel (7.5%) was compared with that of bovine serum albumin, γ-globulin and egg white lysozyme. The enzyme migrated as a single band and its molecular weight was calculated to be 24 000. Sedimentation coefficient of the enzyme upon glycerol gradient centrifugation was estimated to be 2.60 S using bovine serum albumin, γ-globulin, and RNAase as standard proteins. These results suggest that the purified enzyme preparation is homogenous and consists of a monomeric protein with a relatively small molecular weight. Isoelectric point of the enzyme was at pH 6.90 on electrofocusing column and 6.97 in polyacrylamide gel, as only a single active band.

Catalytic properties

Identification of 3'-pyrophosphorylated products synthesized from 5'-

TABLE I

PURIFICATION OF NUCLEOTIDE PYROPHOSPHOTRANSFERASE FROM *STREPTOMYCES MOROOKAENSIS*

Purification step	Volume (ml)	Protein (mg)	Total units	Specific activity (units/mg protein)	Recovery (%)
Culture filtrate	20000	1201000	103200	0.09	100
Ammonium sulfate	8590	245100	61500	0.25	59.6
DEAE-Cellulose	10560	58740	54650	0.95	53.0
CM-Sephadex C-25	235	980	18530	18.9	18.0
DEAE-Sephadex A-25	120	65	6860	105.6	6.7
Sephadex G-75	9	6.8	2960	435.3	2.9

purine nucleotides. Pyrophosphorylated products of 5′-purine nucleotides were isolated and purified by the method of Hamagishi et al. [16] using DEAE-Sephadex A-25 column chromatography, and were characterized by the co-chromatography with authentic samples, resistance to periodate oxidation and molar ratio of phosphate, as shown in Table II.

The reaction products co-migrated with the authentic nucleoside 5′-phosphate 3′-diphosphate on PEI-cellulose sheet in 1.15 M KH_2PO_4. R_F values of reaction products are shown in Table II; pApp and pGpp which were synthesized from ATP and AMP or GMP differed from those of ATP and GTP. On the other hand, the assay results of the reaction products for total phosphate indicated that two phosphates were added to the acceptor by the enzyme. In order to establish the phosphorylated position, reaction products were treated with sodium metaperiodate and chromatographed on PEI-cellulose sheet. Only ATP and GTP mobilities on thin-layer were affected and they were completely degraded; the R_F values of ATP and GTP changed from 0.35 and 0.40 to 0.1 and 0.06, respectively. Therefore, the resistance of reaction products to periodate oxidation indicates that the ribose residue is substituted at positions 2′, or 3′.

Effects of pH and divalent metal ions. The enzyme has an optimum reactivity in the pH range of 7.5 to 11 depending upon divalent cations (Fig. 2). In the presence of $5 \cdot 10^{-3}$ M metal ions, optimum pH lay above 10 for magnesium and manganese ions, while for cobalt and zinc ions in 9 to 9.5, and for ferrous ions are shifted down from 7.5 to 8, respectively. Mg^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+} satisfied the requirement to various extents as shown in Fig. 3, but Ni^{2+}, Sr^{2+}, Cd^{2+}, Hg^{2+}, Cu^{2+}, Ca^{2+} and Pb^{2+} did not. Magnesium ions most actively stimulated the reaction with a broad concentration optimum ranging from 30 to 100 mM. Cobalt ions stimulated the reaction at an optimum con-

TABLE II

CHARACTERISTICS OF NUCLEOSIDE 5′-PHOSPHATE (MONO, DI, OR TRI) 3′-DIPHOSPHATE

Donor	Acceptor	Pyrophosphorylated product	R_F on PEI-cellulose sheet in KH_2PO_4, pH 3.4		Nucleotide tested* (μmol)	Total phosphate (μmol)	Molar ratio of P_i to nucleoside	Periodate** oxidation
			1.15 M	0.75 M				
ATP	AMP	pApp	0.54	0.37	0.26	0.77	2.96	resistance
	ADP	ppApp	0.44	0.18	0.31	1.24	4.01	resistance
	ATP	pppApp	0.35	0.08	0.14	0.66	4.91	resistance
	Authentic	ATP	0.52	0.33	0.87	2.72	3.12	degraded
	GMP	pGpp	0.44	0.22	0.23	0.71	3.08	resistance
	GDP	ppGpp	0.23	0.07	0.36	1.37	3.81	resistance
	GTP	pppGpp	0.12	0.01	0.26	1.25	4.89	resistance
	Authentic	GTP	0.40	0.16	—	—	—	degraded

* Calculated assuming an extinction coefficient (ϵ) of 15 000 at 260 nm for ATP and 11 800 at 260 nm, for GTP, pH 7.0.

** Nucleotide preparations (0.02 ml, 50 mM) were treated with 0.025 ml of 0.1 M sodium metaperiodate for 10 min at 30°C, then with 0.025 ml of 1 M lysine for 40 min at 45°C. After adding 0.05 ml of 4 M formic acid, 0.01 ml samples were spotted on PEI-cellulose sheet and chromatographed in 1.15 M KH_2PO_4, pH 3.4.

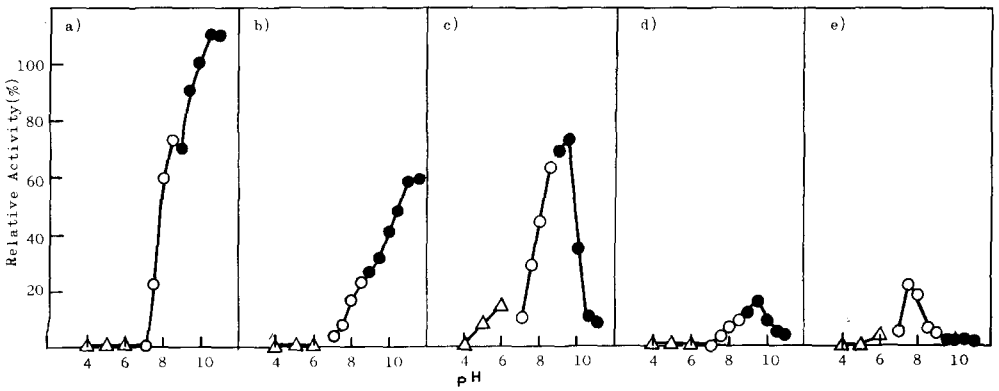

Fig. 2. Effect of pH on the pppApp formation in the presence of various metal ions. The reaction mixture contained 2.5 μmol of Mg^{2+} (a), Mn^{2+} (b), Co^{2+} (c), Zn^{2+} (d) or Fe^{2+} (e), 2.5 μmol of ATP, 2 units of enzyme, and 50 μmol of the following buffer in a final volume of 0.5 ml. Incubation was carried out at 37°C for 10 min. △, acetate buffer; ○, Tris · HCl buffer; ●, glycine/NaOH buffer, adjusted to the pH indicated.

centration of 60 mM with 65% range against relative activity at 50 mM Mg^{2+}, and manganese ions have a 50% range at the broad concentration ranging from 10 to 100 mM. The stimulations by ferrous and zinc ions were relatively low as shown by the relative activity of 18% at the optimum concentration of 10 to 20 mM for zinc, and 15% at 50 to 60 mM for ferrous ions.

Stability of the enzyme. At the concentration of over 1 mg protein per ml, the purified enzyme was stable without loss of activity for at least several months on storage at 4°C in 0.05 M glycine/NaOH buffer, pH 9.0, containing 0.01 M $MgCl_2$, and in 60% saturated ammonium sulfate. Handling the purified enzyme at low protein concentrations (below 10 μg per ml) caused a considerable loss in enzyme activity at 4°C (up to 80%); bovine serum albumin (10 μg

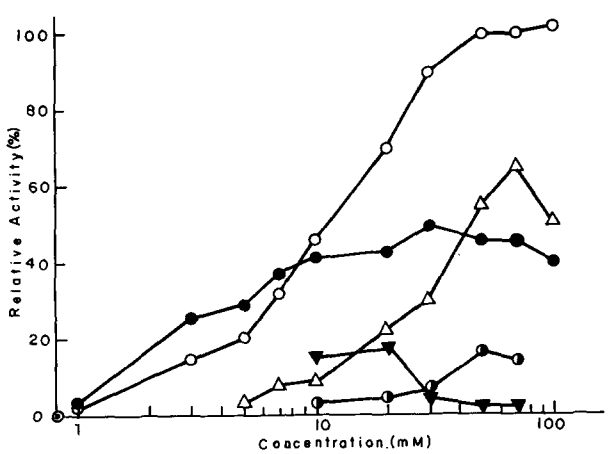

Fig. 3. Influence of various concentrations of divalent cations on the pppApp formation. Enzyme reaction and assay were performed according to Procedure B as described under Methods with the concentration of divalent cations as indicated. ○, Mg^{2+}; ●, Mn^{2+}; △, Co^{2+}; ◑, Fe^{2+}; ▼, Zn^{2+}.

per ml) and non-ionic surface active agents such as Tween 40 (0.01 to 0.001%) were not only protective for thermal and pH inactivation, but stimulated the enzyme reaction. Stimulation of the enzyme reaction by these substances will be reported elsewhere. Upon heat treatment of the enzyme at 40°C, 50% of original enzyme activity was lost during 10 min at pH 10, but this inactivation was prevented by the addition of substrate, non-ionic surface active agents or protein. When the enzyme solution containing over 50 μg protein per ml was heated at 100°C in 0.05 M glycine/NaOH buffer containing 0.01 M $MgCl_2$, no significant loss of activity was observed for at least 15 min between pH 9 to 11. Over 90% of original activity was retained between pH 7 to 11 at 37°C for 2 h, but the enzyme was quite unstable at acidic pH.

Inhibitors. The effect of various inhibitors upon the pyrophosphorylation of [8-^{3}H]ATP to ppp[8-^{3}H]App was shown in Table III. Guanine, guanosine, dGDP and dGTP produced marked inhibition, while other bases, nucleosides and nucleotides were not inhibitory. *N*-Bromosuccinimide which is a brominating agent for tryptophan residue characteristically inhibited the enzyme reaction at the concentration of 0.1 mM. Mercuric acetate, sodium borate and iodoacetate were also inhibitory. The details of the kinetic analyses of the inhibition will be described in a following paper.

Specificity for pyrophosphate donors. Pyrophosphorylation of GTP was assayed with the use of an equimolar mixture of nucleotide and [8-^{3}H]GTP under the standard conditions of Procedure A for their ability to stimulate pppGpp synthesis. ATP, dATP and pppApp were the only potential donors as shown in Table IV, but GTP, GDP, GMP, ADP, ITP, IDP, CTP and UTP were not. The enzyme was also inactive against *p*-nitrophenyl phosphate, glucose

TABLE III

EFFECT OF INHIBITORS ON NUCLEOTIDE PYROPHOSPHOTRANSFERASE

The enzyme was first incubated with inhibitors at 37°C for 10 min. Pyrophosphorylation of ATP was then initiated by addition of [8-^{3}H]ATP, and determined by Procedure A.

Compound	Final concentration (mM)	Relative activity (%)
None		100
Guanine	10	35.2
Guanosine	10	50.9
dGDP	10	13.1
dGTP	10	34.1
p-Chloromercuribenzoate	1	91.0
Mercuric acetate	5	51.8
	1	101.3
Mono iodoacetate	5	17.0
	1	96.2
Sodium arsenate	1	94.5
N-Bromosuccinimide	0.1	0
N-Ethylmaleimide	5	100
NaF	10	96.2
KCN	10	102.7
NaN_3	10	95.5
Thioglycolate	1	97.8
Ethylmercaptan	1	99.4
Urea	10	108.9
Sodium borate	10	55.9

TABLE IV

PYROPHOSPHATE DONOR ACTIVITY OF NUCLEOSIDE TRIPHOSPHATES

ppp [8-^{3}H]Gpp formed by pyrophosphorylation of [8-^{3}H]GTP was assayed under the standard conditions of Procedure A.

Donor	Amount of pppGpp formed (nmol/10 min)	Relative activity (%)
ATP	621	100
GTP	0	0
UTP	0	0
CTP	0	0
dATP	250	40.2
dGTP	0	0
pppApp	200	32.2
p(CH_2)ppA	0	0
p(CH_2)ppG	0	0

1-phosphate, 3-phosphoglycerate and inorganic pyrophosphate.

Specificity for pyrophosphate acceptors. The tritiated products pyrophosphorylated at the 3'-position were detected by co-chromatography on PEI-cellulose sheet with authentic samples. Among 32 purine and pyrimidine bases, nucleosides and nucleotides tested, only adenosine, guanosine and inosine 5'-phosphate (mono, di or tri) served as pyrophosphate acceptors to synthesize nucleoside 5'-phosphate 3'-diphosphates. Synthetic ATP and GTP analogues, p(CH_2)ppA and p(CH_2)ppG, were also pyrophosphorylated. In order to determine pyrophosphate acceptor activity of 5'-purine nucleotides, 2000 nmol of [8-^{3}H]nucleotide as acceptor were incubated with equimolar ATP under the standard assay conditions. As is shown in Table V, the preference of pyrophosphate acceptor was seen in order of descending activity by guanine, adenine and hypoxanthine nucleotides and by diphosphate, monophosphate and triphosphate of nucleoside, independently of the pyrophosphate donor.

TABLE V

PYROPHOSPHATE ACCEPTOR ACTIVITY OF PURINE NUCLEOTIDES

Each of the adenine and guanine nucleotides labeled with tritium at the 8-position of the purine base was used for determining the pyrophosphorylation activity according to Procedure A.

Acceptor	Donor	Amount of product formed (nmol/10 min)		
		ATP	dATP	pppApp
[^{3}H]AMP		462	146	273
[^{3}H]ADP		969	480	762
[^{3}H]ATP		1279	146	104
p(CH_2)ppA		120	+	+
[^{3}H]GMP		1368	275	651
[^{3}H]GDP		1450	460	1619
[^{3}H]GTP		720	248	200
p(CH_2)ppG		915	+	+
IMP		+	+	+
IDP		+	+	+
ITP		+	+	+

+, Pyrophosphate acceptor activity was determined by PEI-cellulose thin-layer chromatography.

TABLE VI

APPARENT MICHAELIS CONSTANTS FOR VARIOUS NUCLEOTIDES

K_m and V values were obtained from Lineweaver-Burk plots. The reaction mixture consisted of various concentrations of [^{3}H]nucleotide ($1.53 \cdot 10^6$ cpm/μmol), 12.5 μmol of glycine/NaOH buffer, pH 10.0, 1 μmol of $MgCl_2$, 2 μmol of unlabeled ATP, and $1 \cdot 10^{-3}$ units of the enzyme in a final volume of 0.2 ml. Incubation was carried out at 37°C for 5 min. Radioactive 3′-pyrophosphorylated products were identified by the localization of ultraviolet absorption and co-chromatography with a non-labeled sample of authentic nucleotide. The velocity was expressed as μmol of 3′-pyrophosphorylated product per min per unit.

Donor	Acceptor	K_m (mM)	V (μmol/min/unit)
ATP	[^{3}H] AMP	2.78	0.48
	[^{3}H] ADP	3.23	1.01
	[^{3}H] GMP	0.89	1.18
	[^{3}H] GDP	0.46	1.11
	[^{3}H] GTP	1.54	1.59
dATP	[^{3}H] ADP	1.67	4.75
	[^{3}H] GDP	0.25	0.49
	[^{3}H] GTP	0.47	0.35
pppApp	[^{3}H] ADP	1.61	0.34
	[^{3}H] GDP	0.20	1.41
	[^{3}H] GTP	1.67	2.43

Kinetics. The Michaelis constants and maximum velocities for various nucleotides in the pyrophosphorylation catalyzed by the enzyme were estimated by the method of Lineweaver and Burk [27]. These kinetic data are summarized in Table VI.

Discussion

pppApp-synthesizing activity was first found in the culture filtrate of a new species, *St. adephospholyticus* by Murao and Nishino [12,14], and a novel ATP : nucleotide pyrophosphotransferase was purified and characterized [12—16].

We found the activity of synthesizing pppApp and ppApp from ATP in *St. morookaensis* ATCC19166, *St. aspergilloides* ATCC 14804, *St. hachijoensis* IF012782, *Actinomyces violascens* IF012920, and *Streptoverticillium spectatum* ATCC27464 among 825 authorized strains of bacteria, fungi, yeasts, fungi imperfecti and actinomycetes(19). From the culture filtrate of *St. morookaensis* ATCC19166, purine nucleotide pyrophosphotransferase was purified to near homogeneity and it was a monomeric protein with a molecular weight of 24 000. A 4800-fold purification was required for obtaining 6.8 mg of a homogeneous enzyme preparation starting from 20 liters of the culture broth with an overall yield of about 3%. This low yield is due to losses suffered in the rapid inactivation at a low concentration of protein and to aggregation in solution of low ionic strength during purification.

This enzyme synthesized purine nucleoside 5′-phosphate 3′-diphosphate by transferring a pyrophosphoryl group from the 5′-position of ATP, dATP and pppApp to the 3′-position of purine nucleotides; 5′-mono, di and triphosphates of adenosine, guanosine and inosine, and purine nucleotide analogues, $p(CH_2)$ppA and $p(CH_2)$ppG, in the presence of divalent cations and in alkaline.

This enzyme is very similar to that of *St. adephospholyticus* in the molecular weight and substrate specificity [14,15]. However, there seem to be some differences in inhibition with nucleotides and its related compounds, stimulation of the enzyme reaction with non-ionic surface active agents and proteins, pH optima, and metal ion requirements between this enzyme and that of *St. adephospholyticus* [15]. In this enzyme the pyrophosphorylation of ATP to pppApp was remarkably inhibited with guanine, guanosine, dGDP, dGTP, and *N*-bromosuccinimide, which is a brominating agent for tryptophan residue. In addition, kinetic studies revealed that guanine nucleotides, especially GDP, were better acceptor than other nucleotides. The apparent K_m for GTP was 1.54 mM which is about three times higher comparing with the K_m of 0.55 mM in purified stringent factor [3].

The extracellular excretion of purine nucleotide pyrophosphotransferase and the possible regulatory role for nucleoside polyphosphates during the growth of actinomycetes are of great interest. The relationship between the enzyme in *Streptomyces* and the stringent factor in bacteria may be physiologically significant, since Cochran and Byrne [3] and Block and Haseltine [4] have reported that the stringent factor of *E. coli* is an enzyme.

Acknowledgements

The authors are grateful to Drs. S. Murao and T. Nishino, College of Agriculture, University of Osaka Prefecture, and Dr. M. Cashel, Lab. Molecular Genetics, National Institute of Child Health and Human Development N.I.H., U.S.A., for their helpful advice and a supply of authentic pppApp, ppApp, pApp, pGpp, pppIpp and pppGpp.

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