(1974), *J. Mol. Biol. 85,* 103.
Spradling, A., Penman, S., Campo, M., and Bishop, J. (1964), *Cell 3,* 23.
Stavnezer, J., Huang, R., and Stavnezer, E. (1974), *J. Mol. Biol. 88,* 43.
Studier, F. (1965), *J. Mol. Biol. 11,* 373.
Sullivan, N., and Roberts, W. (1973), *Biochemistry 12,* 2395.
Tonegawa, S. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 203.
Tonegawa, S., Bernardini, A., Weimann, B., and Steinberg, C. (1974a), *FEBS Lett. 40,* 92.
Tonegawa, S., Steinberg, C., Dube, S., and Bernardini, A. (1974b), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4027.

Purification and Properties of Guanosine 5′,3′-Polyphosphate Synthetase from *Bacillus brevis*†

Jose Sy* and Hugh Akers

ABSTRACT: A ribosome-independent guanosine 5′,3′-polyphosphate synthetase has been highly purified from *Bacillus brevis* (ATCC 8185). The enzyme has a molecular weight of 55 000, as measured by sucrose density gradient centrifugation. Like the ribosome-connected stringent factor of *Escherichia coli,* it catalyzes the synthesis of the guanosine 5′,3′-polyphosphates by a pyrophosphoryl transfer mechanism from adenosine triphosphate (ATP) to guanosine di- or triphosphates (GDP, GTP). It has an apparent K_m of 0.14 mM for GDP and 0.77 mM for GTP, and is specific for the guanosine ribonucleotides as pyrophosphoryl acceptors. Several ATP analogues were tested for their ability to donate the pyrophosphoryl group. Mg^{2+} was required as a counter ion for the nucleotide substrate; however, an excess of Mg^{2+} was inhibitory. The property of the *B. brevis* enzyme is compared with the ribosome-linked enzyme of *E. coli* and an extracellular enzym excreted by several types of *Streptomyces* reported upon recently.

There is increasing evidence to support the notion that ppGpp[1] exerts a variety of effects in bacterial metabolism (Cashel and Gallant, 1969). It acts as an inhibitor of the well known stringent effect by shutting off stable RNA synthesis (Cashel, 1969; Reiness et al., 1975), and, furthermore, it inhibits key enzymes in several metabolic pathways, such as the biosynthesis of phospholipids (Merlie and Pizer, 1973), nucleotides (Gallant et al., 1971), polyamines (Hölttä et al., 1974), as well as the uptake of purine and pyrimidine (Hochstadt-Ozer and Cashel, 1972). Recently, it has been suggested that ppGpp modulates the transcriptional control of the histidine operon (Stephens et al., 1975) and other catabolic pathways (Yang et al., 1974).

The in vitro biosynthesis of ppGpp and pppGpp was found first in *Escherichia coli* as a ribosome-linked reaction which required the presence of a factor released by washing of ribosomes of stringent strains and, in addition, mRNA plus uncharged tRNA (Haseltine et al., 1972; Pederson et al., 1973; Haseltine and Block, 1973). However, it was found that, in the absence of ribosomes, the stringent factor showed low transfer activity which could be markedly increased by the addition of methanol (Sy et al., 1973). The enzyme, furthermore, was found to catalyze reverse pyrophosphoryl transfer using a high concentration of AMP as acceptor (Sy, 1974), indicating the reaction to be essentially reversible: (p)ppG + pppA ⇌ (p)pp$^{5'}$G$^{3'}$pp + pA.

Recently, we discovered in *Bacillus brevis* a guanosine 5′,3′-polyphosphate synthetase that is quite independent of the ribosomal complex (Sy, 1976). It was not stimulated by either the ribosome mRNA–uncharged tRNA complex or by methanol. Another such ribosome-independent synthetase has recently been found to be released into the extracellular fluid by a number of *Streptomyces* (Nishino and Murao, 1975; Oki et al., 1975). We report here on the large scale preparation, purification, and further characterization of the *B. brevis* synthetase, including a comparison with the other two synthetases.

Materials and Methods

Materials. [α-^{32}P]GTP was obtained from ICN. [^{14}C]GDP, [γ-^{32}P]ATP, and [^{3}H]ATP were purchased from New England Nuclear Corp. All other nucleotides were obtained from P-L Biochemicals except for ppppG which was a product of Sigma Chemical Co.

Preparation of B. brevis Extracts. B. brevis (ATCC 8185) was cultured on 10 g of beef extract (Difco) and 10 g of peptone (Difco) per l. At late log phase (OD_{650} = 3.5–3.8) the culture was chilled, and the cells were collected by centrifugation and stored frozen. Thawed cells (107 g) were washed with 100 ml of buffer A (20 mM Tris-OAc, pH 8.1, 14 mM $Mg(OAc)_2$, 60 mM KOAc, 1 mM dithiothreitol) by centrifugation. The washed cells were resuspended in 100 ml of buffer A that contained 100 μg of electrophoretically pure DNase (Worth-

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[1] Abbreviations used are: ppGpp, guanosine 5′-diphosphate 3′-diphosphate; pppGpp, guanosine 5′-triphosphate 3′-diphosphate; ppppG, guanosine 5′-tetraphosphate; ATP, GTP, CTP, UTP, ITP, adenosine, guanosine, cytidine, uridine, and inosine 5′-triphosphates; ATPase, GTPase, adenosine and guanosine 5′-triphosphatase; dATP, dGTP, 2′-deoxyadenosine and 2′-deoxyguanosine 5′-triphosphates; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrito)tetraacetic acid.

TABLE I: Purification of Guanosine 5′,3′-Polyphosphate Synthetase from *Bacillus brevis*.[a]

Purification Step	Vol (ml)	Protein (mg)	Sp. Act.[b] (nm μg^{-1} hr^{-1})	Yield (%)	Fold Purification
Ammonium sulfate 0-45%	40	2740	0.013	(100)	(1)
DEAE-cellulose	5	100	0.29	74	22
Hydroxylapatite	1.1	6	1.11	17.5	85
Sephadex G-100	1.8	0.7	4.47	8.7	344

[a] Protein concentration was determined by the Lowry procedure (Lowry et al., 1951) using bovine serum albumin as standard. [b] Specific activity is defined as the nm of GTP converted to ppGpp + pppGpp per μg of enzyme protein per h at 30 °C.

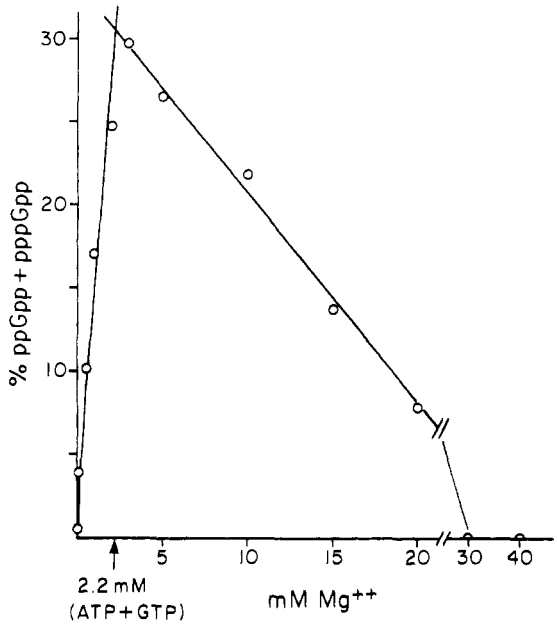

FIGURE 1: Mg^{2+} optimum for guanosine 5′,3′-polyphosphate synthetase. Guanosine 5′,3′-polyphosphates were assayed in 25 μl of reaction mixture containing 50 mM Tris-OAc, pH 8.1, 4 mM dithiothreitol, 0.2 mM [α-^{32}P]GTP (17 Ci/mol), 2 mM ATP, 1 mg/ml of bovine serum albumin, 0.4 μg of enzyme, and the indicated concentration of Mg^{2+} as the acetate salt. Incubations were performed at 30 °C for 45 min and the reaction was stopped by the addition of HCOOH. The resulting guanosine polyphosphates were then assayed as described under Materials and Methods.

ington) and passed through a French press at 18 000 psi. The homogenate was centrifuged at 14 000 rpm for 30 min; the resulting supernatant fluid was centrifuged for 30 min in a Spinco 60 Ti rotor at 30 000 rpm, and for a further 2.5 h at 50 000 rpm in the same rotor. The supernatant fraction from the final centrifugation (S-100) was used for enzyme purification.

Assay for the Synthesis of Guanosine Polyphosphate. The standard assay was carried out in 25 μl of reaction mixture containing 50 mM Tris-OAc, pH 8.1, 4 mM dithiothreitol, 2.2 mM $Mg(OAc)_2$, 2 mM ATP, 0.2 mM [α-^{32}P]GTP (10-40 Ci/mol), 1 mg/ml of bovine serum albumin, and 0.4 μg of purified enzyme fraction, and was incubated at 30 °C for 60 min; the reaction was terminated by the addition of HCOOH. The guanosine polyphosphates formed were then analyzed by thin-layer chromatography on poly(ethylenimine) cellulose as described (Sy and Lipmann, 1973).

Purification of B. brevis Guanosine 5′,3′-Polyphosphate Synthetase. Ammonium sulfate (34.6 g) was added to the S-100 fraction (125 ml) to 45% saturation. The precipitate was redissolved in 50 mM Tris-OAc, pH 8.1, 1 mM dithiothreitol, 0.1 mM EDTA, and then dialyzed against 20 mM Tris-OAc, pH 8.1, 1 mM dithiothreitol. The protein fraction (2740 mg) was adjusted to 0.1 M KCl, and applied to a column (4 × 19 cm) of DE-52 (Whatman) that was preequilibrated with 0.1 M KCl, 20 mM Tris-OAc, pH 8.1, 1 mM dithiothreitol. The enzyme was eluted from the column by a linear gradient of 0.1-0.5 M KCl in 20 mM Tris-OAc, pH 8.1, 1 mM dithiothreitol (750 × 750 ml) at a flow rate of 1.2 ml/min. Fractions of 9.5 ml were collected and 15 μl from each was assayed for guanosine polyphosphate synthesis. The active fractions were pooled and precipitated by ammonium sulfate (60% saturation). The precipitate was redissolved in 50 mM Tris-OAc, pH 8.1, 1 mM dithiothreitol, 0.1 mM EDTA, and dialyzed against 20 mM Tris-OAc, pH 8.1, 1 mM dithiothreitol.

The DE-52-purified enzyme fraction (100 mg) was adjusted to 5 mM $KHPO_4$, pH 7.5, and applied to a column (1.5 × 6 cm) of hydroxylapatite that had been preequilibrated with 5 mM $KHPO_4$, pH 7.5, 1 mM dithiothreitol, 10% glycerol. The enzyme was eluted from the column with a linear gradient from 5 to 120 mM $KHPO_4$, pH 7.5, containing 1 mM dithiothreitol and 10% glycerol (100 × 100 ml) at a flow rate of 0.32 ml/min. Fractions of 4.3 ml were collected and 1 μl of each was assayed for enzymatic activity. The active fractions were pooled and their protein was precipitated by ammonium sulfate (60% saturation). The precipitate was dissolved in 50 mM Tris-OAc, pH 8.1, 5 mM dithiothreitol, 10% glycerol, and dialyzed against 10% glycerol, 20 mM Tris-OAc, pH 8.1, 1 mM dithiothreitol, 50 mM KCl.

The dialyzed hydroxylapatite fraction (6 mg) was applied to a Sephadex G-100 column (1.5 × 73 cm), preequilibrated with 40 mM Tris-OAc, pH 8.1, 250 mM KCl, 1 mM dithiothreitol, and the column was eluted with the same buffer at a flow rate of 12 ml/h. Fractions of 2.6 ml were collected and 15 μl from each was assayed for activity. The active fractions were pooled and concentrated by pressure dialysis through an Amicon membrane. The enzyme fraction was stored in liquid nitrogen in small aliquots and was stable for many months, remaining so through several cycles of freezing and thawing.

Results

The summary of enzyme purification in Table I shows that the best preparation of the synthetase was purified ca. 340-fold compared with the initial ammonium sulfate precipitate protein fraction as baseline, since the specific activity determination in the crude S-100 fraction was unreliable due to the presence of alkaline phosphatase. The purest enzyme fraction obtained from Sephadex G-100 was not homogeneous as it contained multiple protein staining bands on gel electrophoresis. However, it was practically free of ATPase, GTPase, or adenylate kinase; only a contamination with 5′-GMP kinase was detectable. The enzyme tended to aggregate at low salt

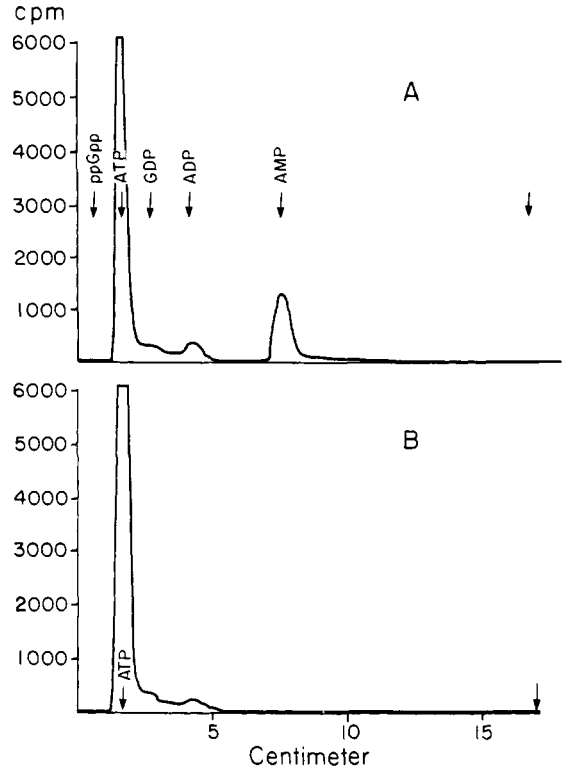

FIGURE 2: Determination of AMP as a product of guanosine 5′,3′-polyphosphate synthetase reaction. The conditions for guanosine 5′,3′-polyphosphate synthesis are described under Materials and Methods. The reaction mixture contained in 25 μl: 50 mM Tris-OAc, pH 8.1, 4 mM dithiothreitol, 1 mg/ml of bovine serum albumin, 2 mM $Mg(OAc)_2$, 1 mM [^{3}H]ATP (300 Ci/mol), 0.4 μg of enzyme, and for A 1 mM GDP, and for B no further addition. Incubations were at 30 °C for 120 min and the reaction was stopped with HCOOH. Aliquots of 20 μl were then applied to a 1.5-cm strip of poly(ethylenimine) cellulose thin-layer sheets, which were then desalted by washing with methanol and developed in 1.0 M LiCl. The chromatograms were air-dried and scanned in a Varian radioscanner.

concentration but, unlike the *E. coli* enzyme, did not precipitate.

The Mg^{2+} optimum (Figure 1) of the purified enzyme was equal to that concentration of Mg^{2+} which titrated the nucleotides, indicating Mg–ATP and Mg–GTP complexes to be substrates. Increasing the Mg^{2+} concentration above the titration point decreased the enzymatic activity, indicating inhibition by free Mg^{2+}. The Mg^{2+} optimum curve for the synthetase from *B. brevis* was similar to the *E. coli* synthetase when assayed in the presence of methanol (Sy et al., 1973), but differed from the synthetase released by *Streptomyces* where 30 mM or greater Mg^{2+} concentration was reported to be optimal (Oki et al., 1975).

To determine if the *B. brevis* guanosine polyphosphate synthetase used a similar pyrophosphoryl transfer mechanism to that found for the *E. coli* stringent factor (Sy and Lipmann, 1973), the enzyme was incubated with [^{3}H]ATP and unlabeled GTP, and the resulting products were analyzed by thin-layer chromatography. Figure 2A shows that the only radioactive nucleotide produced was [^{3}H]AMP. No AMP was formed when GTP was absent from the incubation mixture (Figure 2B). This result indicates the *B. brevis* guanosine polyphosphate synthetase is likewise a pyrophosphoryl transferring enzyme.

Table II shows that GDP is the preferred substrate for the *B. brevis* enzyme, with an apparent K_m of 0.14 mM, that for GTP is fivefold higher at 0.77 mM and the K_m for ATP was found to be 0.6–0.7 mM. Mg^{2+} is the preferred cation for the

TABLE II: Divalent Cation Specificity of Guanosine 5′,3′-Polyphosphate Synthetase.[a]

Ion Species	% GTP Converted to ppGpp + pppGpp	
	60 min	480 min
Mg^{2+}	22	61
Ca^{2+}	0	0
Mn^{2+}	7	23

[a] The assays were carried out in 25 μl of reaction mixture containing 26 mM Tris-OAc, pH 8.1, 2.6 mM dithiothreitol, 1.06 mM ATP, 0.5 mM [α-^{32}P]GTP (4 Ci/mol), 0.8 mg/ml of bovine serum albumin, 0.4 μg of enzyme, and 1.6 mM of the appropriate cations. Incubation was performed at 30 °C for the indicated times, 5-μl aliquots were withdrawn, and the reaction was stopped by applying directly to poly(ethylenimine) cellulose plates. Formation of ppGpp and pppGpp was then determined as described (Sy and Lipmann, 1973).

TABLE III: Apparent K_m for Various Nucleotide Substrates.[a]

Nucleotide	Apparent K_m (mM)
GDP	0.14
GTP	0.77
ATP (with GDP)	0.62
ATP (with GTP)	0.71

[a] K_m values were obtained from Lineweaver–Burk plots. The standard assay condition was used with the following modification: (1) K_m for GDP was determined with [^{14}C]GDP (1.1×10^5 cpm/reaction) and 4 mM ATP; (2) K_m for GTP was determined with [α-^{32}P]GTP (1.25×10^5 cpm/reaction) and 4 mM ATP; (3) K_m for ATP was determined under two conditions, with 0.3 mM [^{14}C]GDP (18 Ci/mol) and with 2.5 mM [α-^{32}P]GTP (1.3 Ci/mol). All nucleotides were added as a mono-Mg^{2+} complex. Incubations were performed at 30 °C and the ppGpp or pppGpp formed was determined as described (Sy and Lipmann, 1973). Several time points per concentration of nucleotides were determined and the resulting linear rate was used for calculation. V_m for both reactions was about 25 nm μg^{-1} h^{-1}.

reaction (Table III), Mn^{2+} can substitute but at a lower rate; Ca^{2+}, however, is inactive as a counter ion for the nucleotides. In the presence of the optimal Mg^{2+} concentration the addition of Mn^{2+} is quite inhibitory (data not shown), but addition of Ca^{2+} has no such effect.

We had shown (Sy, 1976) that the *B. brevis* enzyme is quite specific for ATP and that dATP acts only as a poor pyrophosphate donor. Continuing tests for specificity showed that adenosine 5′-(β,γ-imino)triphosphate and the fluorescent analogue 1,N^6-ethyladenosine triphosphate do not function with the enzyme (Table IV); the former, however, acts as a very strong competitive inhibitor. On the other hand, 2′-*O*-methyl-ATP and 8-bromo-ATP can serve as weak pyrophosphate donors. The guanosine nucleotides appear to be quite specific pyrophosphate acceptors for the *B. brevis* enzyme, but neither ATP, UTP, CTP, dGTP, dGDP, 2′-*O*-methyl-GDP nor 7-methyl-GDP will accept; only ITP and guanosine 5′-tetraphosphate are pyrophosphorylated, but at a very slow rate (Table V). dGTP and dGDP act only as weak competitive inhibitors, whereas 2′-*O*-methyl-GDP is not inhibitory (data not shown). These results indicate that a free 2′-OH group is essential on the acceptor but is less critical at the donor site.

The molecular weight of the *B. brevis* guanosine polyphosphate synthetase was estimated by the sucrose density gradient centrifugation method using bovine serum albumin (mol wt

TABLE IV: Pyrophosphate Donor Activity of Various ATP Analogues.[a]

Nucleotide	% GTP Converted to ppGpp + pppGpp	
	60 min	480 min
ATP	21	72
AMPPNP[b]	0	0
2′-*O*-MeATP	3	20
1,N^6-ϵATP	0	0
8-BrATP	4	11

[a] Guanosine 5′,3′-polyphosphate synthesis was assayed as described under Materials and Methods. The reaction mixture in 25 μl contained: 60 mM Tris-OAc, pH 8.1, 4 mM dithiothreitol, 0.8 mg/ml of bovine serum albumin, 2 mM $Mg(OAc)_2$, 0.75 mM [α-^{32}P]GTP (4 Ci/mol), 0.4 μg of enzyme, and 2.5 mM of the indicated ATP analogue. Incubations were done at 30 °C and, at the indicated times, 5-μl aliquots were withdrawn for guanosine polyphosphate assay. [b] Abbreviations: AMPPNP, adenosine 5′-(β,γ-imino)triphosphate; 2′-*O*-MeATP, 2′-*O*-methyl-ATP; 1,N^6-ϵATP, 1,N^6-ethyladenosine triphosphate; 8-BrATP, 8-bromo-ATP.

TABLE V: Pyrophosphate Acceptor Activity of Various Nucleotides.[a]

Nucleotide	% ATP Transferred to Nucleoside 5′,3′-Polyphosphate
ATP	0
GDP	32
GTP	33
ppppG	9
dGTP	0
dGDP	0
UTP	0
CTP	0
ITP	4
2′-*O*-Me-GDP	0
7-Me-GDP	0

[a] Nucleoside 5′,3′-polyphosphate synthesis was assayed in 25 μl of reaction mixture containing 50 mM Tris-OAc, pH 8.1, 3 mM dithiothreitol, 0.5 mM [γ-^{32}P]ATP (4.5 Ci/mol), 0.8 mg/ml of bovine serum albumin, 1.5 mM $Mg(OAc)_2$, 0.4 μg of enzyme, and 1 mM of the indicated nucleotide. Incubations were at 30 °C for 60 min and 5-μl aliquots were analyzed for nucleoside 5′,3′-polyphosphate synthesis as described (Sy and Lipmann, 1973).

69 000), creatine kinase (mol wt 82 000), myokinase (mol wt 22 000), and nucleotide diphosphate kinase (mol wt 109 000) as molecular-weight markers. Since the enzyme tends to aggregate at low salt concentration, the test was carried out in the presence of 0.25 M KCl. Under these conditions, the enzymatic activity sedimented as a single symmetrical peak with an approximate molecular weight of 55 000.

Discussion

The previous report on the ribosome-independent nature of the *B. brevis* guanosine 5′,3′-polyphosphate synthetase (Sy, 1976) is further supported by preparation of an extensively purified enzyme that synthesizes ppGpp or pppGpp in only buffer, Mg^{2+}, ATP, and GDP or GTP. The *B. brevis* enzyme, as does the *E. coli* stringent factor, catalyzes a transfer of the ATP β,γ-pyrophosphate group. Its affinity for GDP is five times higher than for GTP, and GDP is the predominant product if it is present as a significant fraction in an incubation mixture containing it and GTP.

Comparison between the Different Guanosine Polyphosphate Synthetases. In addition to the ribosome-dependent and ribosome-independent polyphosphate synthetases found in *E. coli* and *B. brevis*, a similar enzyme from *Streptomyces* has recently been characterized (Nishino and Murao, 1975; Oki et al., 1975). Of these, the *E. coli* enzyme (stringent factor) is the best studied. It has a molecular weight of 75 000 (Cochran and Byrne, 1974; Block and Haseltine, 1975; Sy, 1974) and is strongly stimulated by a ribosome mRNA-uncharged tRNA complex. The *B. brevis* enzyme is smaller at 55 000 daltons and is completely ribosome independent. The ribosome-independent *Streptomyces* enzyme has a molecular weight of 25 000 and thus is the smallest of the group. Since it is found in extracellular fluid, it is apparntly excreted by the organism or is easily washed off. However, no guanosine polyphosphate synthetase activity could be detected in the extracellular fluid of our *B. brevis* culture. It may be worth noting that with increasing complexity of the enzyme its molecular weight increases with increments corresponding to the size of the smallest of the group.

The *B. brevis* and *E. coli* enzymes are most similar in their high substrate specificities; both are specific for GDP or GTP as acceptors. The deoxyguanosine nucleotide series, as well as ATP, CTP, and UTP, are inactive; however, GMP and guanosine 5′-tetraphosphate are acceptors, although at a much reduced rate (Sy, 1975). Both enzymes are also similarly specific for ATP as the pyrophosphoryl donor. In contrast, the *Streptomyces* enzyme is reported to have a much broader substrate specificity, as it can also utilize adenine nucleotides as acceptors of pyrophosphate in the 3′ position. Furthermore, the three enzymes differ widely in their Mg^{2+} optimum. As is well documented by Block and Haseltine (1973) and Cashel (1975), the *E. coli* 5′,3′-polyphosphate synthetase is the product of a rel A gene and is the mediator of the stringent response. In contrast, a physiological function for the *B. brevis* and *Streptomyces* enzymes has not yet been found.

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References

Block, R., and Haseltine, W. A. (1973), *J. Mol. Biol. 77,* 625.
Block, R., and Haseltine, W. A. (1975), *J. Biol. Chem. 250,* 1212.
Cashel, M. (1969), *J. Biol. Chem. 244,* 3133.
Cashel, M. (1975), *Annu. Rev. Microbiol. 29,* 301.
Cashel, M., and Gallant, J. (1969), *Nature (London) 221,* 838.
Cochran, J. W., and Byrne, R. W. (1974), *J. Biol. Chem., 249,* 353.
Gallant, J., Irr, J., and Cashel, M. (1971), *J. Biol. Chem. 246,* 5812.
Haseltine, W. A., and Block, R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1554.
Haseltine, W. A., Block, R., Gilbert, W., and Weber, K. (1972), *Nature (London) 238,* 381.
Hochstadt-Ozer, J., and Cashel, M. (1972), *J. Biol. Chem. 247,* 7067.
Hölttä, E., Jänne, J., and Pispa, J. (1974), *Biochem. Biophys. Res. Commun. 59,* 1104.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R.

J. (1951), *J. Biol. Chem. 193,* 265.
Merlie, J. P., and Pizer, L. I. (1973), *J. Bacteriol. 116,* 355.
Nishino, T., and Murao, S. (1975), *Agric. Biol. Chem. 39,* 1007.
Oki, T., Yoshimoto, A., Sato, S., and Takamatsu, A. (1975), *Biochim. Biophys. Acta 410,* 262.
Pederson, F. S., Lund, E., and Kjeldgaard, N. P. (1973), *Nature (London) New Biol. 243,* 13.
Reiness, G., Yang, H. L., Zubay, G., and Cashel, M. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2881.
Stephens, J. C., Artz, S. W., and Ames, B. N. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4389.
Sy, J. (1974), *Lipmann Symp.: Energy, Regul. Biosynth. Mol. Biol., 1974,* 599.
Sy, J. (1975), *Biochemistry 14,* 970.
Sy, J. (1976), *Biochemistry 15,* 606.
Sy, J., and Lipmann, F. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2145.
Sy, J., Ogawa, Y., and Lipmann, F. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 306.
Yang, H. L., Zubay, G., Urm, E., Reiness, G., and Cashel, M. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 63.

Temperature-Sensitive DNA Polymerase Induced by a Bacteriophage T5 Mutant: Relationship between Polymerase and Exonuclease Activities†

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ABSTRACT: DNA polymerase induced by bacteriophage T5*ts*53, a mutant with temperature-sensitive polymerase, was purified to about 95% purity as judged by dodecyl sulfate gel electrophoresis. The 3′ → 5′ exonuclease associated with the polymerase had higher activity than that associated with the parent wild-type enzyme. It was more stable to heat than the polymerase, and it degraded primer-template even in the presence of 4 dNTP's at higher temperature. However, the evidence presented shows that the inhibition of DNA synthesis by higher temperature was primarily due to defects in polymerase function rather than to overactive exonuclease. The presence of primer-template DNA stabilized the polymerase to heat. Purified *ts*53 polymerase was also shown to discriminate against incorporation of BrdUMP, especially at higher temperature. This is in agreement with observations made in vivo with *ts*53-infected bacteria.

Analysis of DNA synthesized in vivo in *Escherichia coli polA*$^-$ infected with T5*ts*53, a mutant with temperature-sensitive DNA polymerase (Dewaard et al., 1965), showed residual T5 DNA synthesis at nonpermissive temperatures which was due to phage-induced protein(s) (Fujimura, 1971a). This raised the question whether this synthesis is due to the phage-induced polymerase already identified or to another polymerase. Another difference observed at that time was that the repair-type synthesis at nonpermissive temperatures incorporated hardly any BrdUMP,[1] while replication at the permissive temperature did incorporate BrdUMP (Fujimura, 1971b). So another question raised was whether discrimination against BrdUMP incorporation at higher temperature was due to the polymerase.

To answer the above questions, all the detectable polymerases in *E. coli polA*$^-$ and *polA*$^-$, *polB*$^-$ mutants infected with T5*ts*53 were analyzed. There was only one phage-induced polymerase fraction (Fujimura, 1973a,b); it had activity to both denatured DNA and nicked DNA as primer-templates. Activities to two types of primer-templates were characterized further with wild-type T5 DNA polymerase purified to homogeneity and shown to be an intrinsic property of one DNA polymerase protein (Fujimura and Roop, 1976).

In preliminary work with purified temperature-sensitive polymerases (*ts*53 and *ts*5E), we have observed enhanced exonuclease activity at higher temperatures, which degraded primer-templates even in presence of 4 dNTPs (Fujimura, 1974). The purified wild-type polymerase was shown to have exonuclease associated with it (Steuart et al., 1968a) and to be 3′ → 5′ exonuclease (Das and Fujimura, 1976). This raised a possibility that inhibition of synthesis at higher temperatures with *ts*53 or *ts*5E polymerase may be due to higher 3′ → 5′ exonuclease activity.

This report presents evidence that inhibition of DNA synthesis at higher temperatures with *ts*53 polymerase is primarily caused by a defect in polymerase function of the enzyme (not by overactive exonuclease). It further shows that the purified *ts*53 polymerase does discriminate against BrdUMP incorporation at higher temperatures. A possible reason is also given for residual synthesis at higher temperatures in vivo.

Materials and Methods

Organisms. Bacteriophage T5*ts*53 was obtained from the late F. Lanni. Wild-type T5 phage T5$^+$, which is the parent strain of the mutant, was obtained from Y. Lanni. T5*rev* is a revertant strain picked from T5*ts*53 stock. Host bacteria for these phages are *E. coli* R15 *polA*$^-$ obtained from S. Kondo (Kato and Kondo, 1970).

Enzyme Assays. Both polymerase and nuclease assays were carried out as described previously (Fujimura and Roop, 1976)

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; BrdUMP, bromodeoxyuridine monophosphate; EDTA, ethylenediaminetetraacetic acid.