Purification and Properties of ATP:GTP 3'-Pyrophosphotransferase (Guanosine Pentaphosphate Synthetase) from *Streptomyces antibioticus*

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Two forms of ATP:GTP 3'-pyrophosphotransferase (guanosine pentaphosphate synthetase) have been purified from *Streptomyces antibioticus*. The larger form has an M_r of 88,000, while the M_r of a smaller form is 47,000. Both synthetase forms are active in the formation of guanosine 5'-triphosphate, 3'-diphosphate in reaction mixtures containing methanol. Unlike the RelA protein from *Escherichia coli*, the synthetases from *S. antibioticus* do not use GDP efficiently as a substrate. Experiments using crude extracts of *S. antibioticus* mycelium and the 88,000- M_r form of guanosine pentaphosphate synthetase strongly suggest that the 47,000- M_r species is produced by proteolysis of the larger species. This conclusion is supported by the observation that antibody to either protein reacts with the other protein. Thus, the 88,000- M_r species may be the catalytically relevant protein in vivo. Unlike the RelA protein, the 88,000- M_r protein is not activated by ribosomes. Modest levels of guanosine pentaphosphate synthesis were observed in mycelial extracts derived from nine other actinomycetes.

When growing cells of *Escherichia coli* are starved for amino acids, a number of physiological and biochemical changes occur which are referred to collectively as the stringent response (reviewed in reference 4). Perhaps the most striking feature of the stringent response to amino acid starvation is a rapid and dramatic decrease in the rate of stable RNA (rRNA and tRNA) synthesis. There is strong evidence for the involvement of a highly phosphorylated guanine nucleotide, guanosine 3',5'-bispyrophosphate (also referred to as guanosine tetraphosphate, ppGpp, magic spot I, and MSI) as an effector of the stringent response (2, 4). One pathway for the production of ppGpp in *E. coli* involves the product of the *relA* locus, an enzyme [ATP:GTP(GDP) 3'-pyrophosphotransferase] which catalyzes pyrophosphoryl transfer from ATP to GTP or GDP (4, 22). Mutations in the *relA* gene which abolish its expression lead to a relaxed response to amino acid starvation in which stable RNA synthesis continues (4). In vivo, the initial product of the *relA*-dependent pathway for ppGpp synthesis is guanosine pentaphosphate (also referred to as pppGpp, magic spot II, and MSII). The synthesis of pppGpp occurs on idling ribosomes in vivo in the presence of mRNA and uncharged tRNA (9, 23). Guanosine pentaphosphate is converted to ppGpp by the action of a phosphohydrolase (7). While many cellular activities are affected in the stringent response, the requirements for (p)ppGpp synthesis in vivo are consistent with the hypothesis that one function of the stringent response is to regulate the balance between stable RNA levels and the cellular demand for protein synthesis.

Evidence exists for the function of ppGpp as a positive and negative regulator of RNA polymerase (14, 25, 27). ppGpp can also be synthesized by a *relA*-independent pathway. Evidence has been presented which implicates the *spoT* gene product as a bifunctional enzyme capable of both synthesis and degradation of (p)ppGpp (10, 35).

The stringent response in the actinomycin-producing streptomycete *Streptomyces antibioticus* has recently been studied.

Ochi isolated a mutant of *S. antibioticus* IMRU3720, the *relC49* mutant, which showed relaxed kinetics for RNA synthesis under conditions of amino acid starvation and which also contained levels of ppGpp lower than those in the parental strain (20). The *relC49* mutant was subsequently shown to possess ribosomes with an altered ribosomal protein (21). This observation confirmed the identity of the relaxed mutant as a *relC* mutant. Most interestingly, the *relC* mutation completely abolished the ability of the organism to produce actinomycin and severely decreased the level of one of the key enzymes involved in actinomycin biosynthesis in *S. antibioticus*, phenoxazinone synthase (20). There was no effect of the *relC* mutation on kynurenine formamidase, which is not directly connected to the actinomycin pathway (20). In more recent experiments, evidence was presented for an effect of the *relC* mutation at the level of transcription of the structural gene for phenoxazinone synthase. Thus, the *relC* mutant contained decreased levels of *phs* mRNA (15). The *relC49* mutant was also found to lack detectable levels of actinomycin synthetase I, another enzyme involved in actinomycin biosynthesis (15). In the parental strain of *S. antibioticus*, there was a significant increase in ppGpp levels just prior to the onset of actinomycin production (15).

The data just cited suggest the strong possibility that ppGpp plays a role in initiating actinomycin biosynthesis in *S. antibioticus*. To further examine this possibility, it is desirable to obtain mutants in which the production of ppGpp is reduced to negligible levels or is completely eliminated. Such is not the case in the *relC49* mutant (15, 20). An additional drawback of *relC* mutants is that the effect of the mutation on ppGpp production in those constructs is indirect. The levels of ppGpp are presumably reduced in *E. coli relC* mutants because the mutant ribosomes are unable to interact productively with the RelA enzyme, not because of changes in the enzyme itself. Furthermore, since the *relC49* mutant grows more slowly than the wild type (15), it is possible that the effects of the mutation on actinomycin biosynthesis are related to the change in growth rate rather than to the decrease in ppGpp levels. These considerations argue further for the isolation of mutants with

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specific defects in the genes which encode the enzyme(s) responsible for ppGpp synthesis.

Preliminary attempts to isolate the streptomycete analog of *E. coli relA* by using the cloned *relA* gene have proven unsuccessful (33). As an alternative strategy, the purification and characterization of enzymes involved in (p)ppGpp production in *S. antibioticus* have been undertaken. This report describes the purification and properties of two forms of ppGpp synthetase (guanosine pentaphosphate synthetase [GPS]) from *S. antibioticus*. Evidence is presented to support the proposition that the smaller of the two forms is produced from the larger form by proteolysis. To my knowledge, this report marks the first characterization of enzymes involved in ppGpp metabolism in *Streptomyces* spp.

MATERIALS AND METHODS

Growth of organisms. *S. antibioticus* IMRU3720 and other streptomycete strains were grown on NZ-amine medium and galactose-glutamic acid (GGA) medium, and mycelium was harvested from GGA medium 6 to 72 h postinoculation and was washed as described previously (5). *E. coli* CF3120, containing the cloned *relA* gene, was grown in L broth for 4 h as described by Schreiber et al. (29). Expression of *relA* was induced by the addition of isopropyl- β -D-thiogalactoside to 1 mM. Cells were harvested after a total of 9 h of growth at 37°C. CF3120 was generously supplied by M. Cashel.

GPS assays. Reaction mixtures were prepared essentially as described by Pedersen and Kjeldgaard (22). Those conditions were found to be optimal for the methanol-dependent assay of GPS. Mixtures contained the following in a total volume of 25 μ l: Tris-HCl (pH 8.0), 80 mM; MgCl₂, 40 mM; ATP, 4.4 mM; GTP, 1.3 mM; bovine serum albumin, 400 μ g/ml; [α -³²P]GTP (Dupont NEN; 3,000 Ci/mmol), 40 μ Ci/ml; methanol, 20% (vol/vol); and enzyme. Reaction mixtures were incubated at 25°C for 30 min, and reactions were quenched by the addition of 1 μ l of 88% formic acid. After 2 to 5 min at room temperature, reaction tubes were centrifuged in an Eppendorf centrifuge to remove precipitated protein, and 2.5 to 3 μ l of the resulting supernatants was spotted onto polyethyleneimine (PEI) cellulose thin-layer plates. Plates were developed as described by Cashel et al. (3), and reaction products were identified by autoradiography. The migration position of ppGpp was confirmed by comparison with the authentic compound (generously supplied by K. Ochi). In some experiments, bands corresponding to ppGpp were cut from the plates and subjected to liquid scintillation counting. In other experiments, GDP (1.3 mM) was substituted for GTP and [γ -³²P]ATP (Dupont NEN; 3,000 Ci/mmol) at 40 μ Ci/ml was substituted for [³²P]GTP. In some assays either *S. antibioticus* ribosomes or poly(U) and/or uncharged tRNA replaced methanol.

Preparation of crude extracts, ribosomes, and tRNA. *S. antibioticus* (and other streptomycetes) and *E. coli* CF3120 were grown as described above. Crude extracts were prepared by suspension of 1 to 3 g of CF3120 cells or 72-h mycelium in 5 volumes of buffer I (see below) and disruption by three passages through a French pressure cell at 12,000 lb/in². Homogenates were centrifuged for 10 min at 12,000 $\times$ g, and the resulting supernatants were used as crude extracts in GPS assays (see Fig. 1 and Table 1).

Ribosomes were prepared from crude extracts obtained from 3 g of *S. antibioticus* or *E. coli* and washed twice in 0.5 M NH₄Cl essentially as described previously (11). Total tRNA was prepared from 24-h *S. antibioticus* mycelium as described previously (11). Other streptomycetes were harvested after 24 h of growth in GGA medium and were disrupted by sonication

in buffer I (see below). Sonicates were centrifuged for 5 min in an Eppendorf centrifuge. The resulting supernatants were used in GPS assays.

Purification of GPSI and GPSII. The starting material for the purification of the GPSs was the PEI (Polymyxin P; BDH Chemicals) precipitate obtained in the purification of actinomycin synthetase I and 3-hydroxyanthranilic acid 4-methyltransferase from *S. antibioticus* (12). PEI pellets were collected from multiple purifications of those enzymes and were stored at -20°C until used in the GPS purification. All subsequent steps were performed at 4°C.

Step 1. Fifteen grams of frozen PEI pellet from 72-h *S. antibioticus* mycelium was suspended in 200 ml of buffer I (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 0.5 mM Na₂EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM dithioerythritol, 0.1 μ M pepstatin, 5% glycerol) containing 0.4 M NaCl and homogenized in a motor driven Teflon-glass homogenizer (five strokes of the pestle). The suspension was centrifuged for 10 min at 12,000 $\times$ g, and the supernatant was discarded. The resulting pellet was homogenized in 200 ml of buffer I containing 1 M NaCl (eight strokes), and the suspension was centrifuged as described above.

Step 2. The 1 M NaCl supernatant was brought to 30% saturation by the addition of solid ammonium sulfate, and the resulting suspension was stirred in the cold for 30 min. The supernatant obtained following centrifugation for 15 min at 12,000 $\times$ g was brought to 55% saturation with ammonium sulfate, the suspension was stirred for 30 min, and the precipitate was collected by centrifugation for 30 min at 20,000 $\times$ g. The supernatant was removed by suction filtration, and the pellet was redissolved in buffer I to a total volume of 12 ml.

Step 3. The enzyme solution was diluted to 200 ml with buffer I, and 100 ml was applied to a column (2 by 20 cm) of DEAE-cellulose equilibrated with buffer I. Nonadhering proteins were eluted from the column with buffer I, and the column was connected to a 250-ml linear gradient of 0.05 to 0.25 M NaCl in buffer I. Proteins were eluted at 60 ml/h, and 2.2-ml fractions were collected. Enzyme-containing fractions (fractions 49 to 65) were pooled, and the enzyme was collected by the addition of solid ammonium sulfate to 60% saturation. The resulting suspension was allowed to stand for 1 to 12 h and then centrifuged at 12,000 $\times$ g for 15 min.

Step 4. The resulting pellet was dissolved in a minimal volume of buffer I and applied to a column (1.2 by 45 cm) of Sephacryl S-400-HR equilibrated with buffer I. Fractions of 0.8 ml were collected at 5.3 ml/h. (A typical column profile is shown in Fig. 2.) Fractions 43 to 59 (peak I) and 60 to 75 (peak II) were pooled from the column.

Step 5. Peak I from the Sephacryl column was applied to a column (1.2 by 10 cm) of quaternary aminoethyl (QAE)-Sephacryl (Q-Sepharose Fast Flow; Pharmacia). The column was connected immediately to a 150-ml linear gradient of 0.15 to 0.40 M NaCl in buffer I and eluted at 60 ml/h with collection of 1.5-ml fractions. Fractions 53 to 65 were pooled. Sephacryl peak II was applied to an identical QAE-Sepharose column, and the enzyme was eluted with a 200-ml linear gradient of 0.05 to 0.40 M NaCl in buffer I. Fractions of 1.6 ml were collected at 60 ml/h, and fractions 63 to 75 were pooled. Enzyme-containing fractions were concentrated by dialysis against ammonium sulfate without stirring (30).

[³⁵S]GPS was purified from PEI pellets obtained from labeled mycelium as described above. In these experiments, each of six 800-ml *S. antibioticus* cultures was incubated for 72 h with 800 μ Ci of [³⁵S]methionine (Dupont NEN; 3,000 Ci/mmol).

Incubation of GPS enzymes with unfractionated mycelial

FIG. 1. Synthesis of phosphorylated guanine nucleotides by crude extracts of *S. antibioticus* and *E. coli*. Reaction conditions were as specified in Materials and Methods. Lane 1, no enzyme added; lane 2, HCOOH added at time zero; lane 3, *S. antibioticus* crude extract (100 µg of protein), 30-min incubation; lane 4, same as lane 3 but 60-min incubation; lane 5, same as lane 3 but 90-min incubation; lanes 6 to 8, *E. coli* crude extract (75 µg of protein), 30-, 60-, and 90-min incubations, respectively. The migration positions of pppGpp, ppGpp, and GTP are indicated.

extracts. Unfractionated extracts of 6-h *S. antibioticus* mycelium were prepared as described above with the following modifications. One gram of mycelium was disrupted in 5 ml of buffer II (identical to buffer I except that Na₂EDTA, PMSF, and pepstatin were omitted). The extracts obtained were used without centrifugation.

Generally, 12,000 cpm of [³⁵S]GPSI was incubated with 70 µg of extract protein in a total volume of 65 µl of buffer II. Mixtures were incubated for 1 to 24 h at 37°C. At the end of the incubation, mixtures were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of antibody to GPS and analysis of immunoprecipitates. Antibody to the GPSs was raised in rabbits and purified as described previously (5). Reaction mixtures for immunoprecipitation contained 30,000 to 50,000 cpm of [³⁵S]GPSI or [³⁵S]GPSII in 100 µl of phosphate-buffered saline–100 µl of purified antibody–1 mM PMSF. Mixtures were incubated for 24 h at 4°C, and immunoprecipitates were collected by centrifugation and were washed with four 1-ml portions of phosphate-buffered saline containing 1 mM PMSF and 0.05% Nonidet P-40 (Shell Chemicals). Immunoprecipitates were dissolved in sample buffer for SDS-PAGE.

Miscellaneous methods. Protein was measured as described by Bradford (1), and SDS-PAGE was performed essentially as described by Laemmli (16). Antibodies to the RelA and SpoT proteins were generously provided by Dan Gentry.

RESULTS

Properties of the GPS assay. Figure 1 shows an autoradiogram depicting the results of an assay of GPS activity in crude extracts of *S. antibioticus* IMRU3720 and *E. coli* CF3120. The crude extracts used were those obtained in the ribosome preparation (see Materials and Methods). It is apparent that both extracts contain GPS activity. Products with the mobility of authentic ppGpp and with the predicted mobility of pppGpp are observed in reaction mixtures containing enzyme extracts. It can be seen that both ppGpp and pppGpp are produced in reactions containing those extracts. The original autoradiograms and replicate experiments demonstrate clearly that the

TABLE 1. Relative amounts of ppGpp synthesized in crude extracts of *S. antibioticus* and *E. coli*^a

Incubation time (min)	% ppGpp	
	<i>S. antibioticus</i>	<i>E. coli</i>
30	9.5	42
60	15.5	46
90	18.0	48

^a GPS reactions were performed as described in Materials and Methods and the legend to Fig. 1. Regions corresponding to pppGpp and ppGpp were cut from the thin-layer plate and analyzed by liquid scintillation counting. Counts were corrected by subtracting the counts per minute from the pppGpp and ppGpp regions following chromatography of a reaction mixture containing only the assay mix. Data represent the percentage of the total amount of guanosine polyphosphate present as ppGpp. The data presented were taken from an experiment different from that whose results are shown in Fig. 1; however, the synthetase preparations were obtained by the same procedures in both experiments (see Materials and Methods).

amount of ppGpp in the reaction products increases with time of incubation of reaction mixtures. Table 1 shows the increase in the relative proportion of ppGpp observed in typical experiments with crude synthetase preparations from *E. coli* and *S. antibioticus*. These results are consistent with the observation that pppGpp is the precursor of ppGpp in *E. coli* (7). The data confirm the presence of an enzyme activity similar to the RelA protein in *S. antibioticus*.

Purification of GPSI and GPSII. In pilot experiments, it was demonstrated that GPS activity could be precipitated from crude mycelial extracts with PEI and extracted from PEI pellets with NaCl. Two forms of GPS were separated by gel filtration of partially purified enzyme preparations (Fig. 2). The RelA protein from *E. coli* was observed to precipitate in buffers of low ionic strength (22), and this property was used in the purification of that protein. While the GPSs from *S. antibioticus* did not precipitate at low ionic strength, they did aggregate under those conditions. Thus, it was possible to obtain significant purification of the synthetases by gel filtration using a matrix with a nominal exclusion limit of 8,000,000 (Fig. 2 and Table 2). The two enzyme forms were subsequently purified to near homogeneity by ion-exchange chromatography on QAE-Sepharose.

GPSI and GPSII were purified about 90- and 30-fold,

FIG. 2. Sephacryl S-400-HR chromatography of the GPS preparation from the DEAE-cellulose column. Chromatographic conditions were as specified in Materials and Methods.

TABLE 2. Purification of GPSI and GPSII from *S. antibioticus*

Step	Vol (ml)	Protein (mg)	Activity ^a	Sp act ^b	Purification (fold)	% Yield
1 M NaCl extract	200	420	14.7	0.035	1.0	100
55% ammonium sulfate	12	289	40.0	0.14	4.0	272
DEAE-cellulose	2.0	41.2	20.1	0.49	13.9	137
ammonium sulfate						
Sephacryl S-400-HR						
Peak I	11.5	4.8	2.90	0.60	17.1	20
Peak II	10.8	8.4	2.51	0.30	8.5	17
QAE-Sepharose						
Peak I	8.4	1.3	4.37	3.31	94.2	30
Peak II	8.7	2.6	2.98	1.14	32.4	20

^a Micromoles of pppGpp formed per minute.^b Micromoles per minute per milligram of protein.

respectively, relative to the specific activities of the NaCl extract of the PEI pellet (Table 2). Although the PEI pellets were used as starting materials in the experiments described here, it is possible to estimate purification levels and yields by using data obtained from studies with crude extracts such as those used for Fig. 1. Thus, it can be estimated that GPSI is typically purified about 2,000-fold relative to the crude mycelial extracts and that GPSII is purified about 750-fold. Further, such analyses suggest that the amounts of GPSI and GPSII recovered in typical purifications represent 0.45 and 0.89%, respectively, of the total soluble protein in crude extracts of 72-h *S. antibioticus* mycelium.

Both GPSI and GPSII are composed of single polypeptide chains, with molecular weights of 88,000 and 47,000, respectively, as estimated by denaturing PAGE (Fig. 3). The purification procedure yielded nearly homogeneous preparations of both enzymes as judged by PAGE (Fig. 3).

Properties of the reactions catalyzed by GPSI and GPSII. The data of Fig. 4 demonstrate the dependencies of the reactions catalyzed by GPSI and GPSII on methanol (lanes 2 and 7) and ATP (lanes 3 and 8). While some activity was

observed in the absence of methanol, significant stimulation was observed in its presence (see below). The data of Fig. 4 also demonstrate that labeled PP_i from [γ -³²P]ATP is incorporated into pppGpp by GPSI and GPSII (lanes 4 and 8). Interestingly, GDP functions poorly as a substrate for GPSI or GPSII (Fig. 4, lanes 5 and 10), unlike the situation with the RelA protein (9). Thus, it is appropriate to refer to GPSI and GPSII as ATP:GTP 3'-pyrophosphotransferases or GPSs. The radioactive material observed in lanes 5 and 10 of Fig. 4 that has the apparent mobility of pppGpp may be due to the presence of some contaminating GTP in the GDP preparations. What is noteworthy is that no product with the mobility of ppGpp was observed in lane 5 or 10 of Fig. 4.

The synthesis of ppGpp by the RelA protein occurs on idling ribosomes in the presence of mRNA and uncharged tRNA (8, 9, 23). In vitro, the RelA protein is activated by ribosomes, mRNA, and tRNA (8, 23). It was of interest to determine whether the synthetases from *S. antibioticus* showed the same

FIG. 3. PAGE of fractions from the enzyme purifications. Lane 1, size standards (molecular weights, from top: phosphorylase *b*, 97,400; bovine serum albumin, 66,200; ovalbumin, 42,699; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400); lane 2, 1 M NaCl extract (53 μ g); lane 3, Sephacryl peak I (10.5 μ g); lane 4, GPSI from QAE-Sepharose (7.8 μ g); lane 5, Sephacryl peak II (19.5 μ g); lane 6, GPSII from QAE-Sepharose (10.5 μ g).

FIG. 4. Substrate utilization by purified GPSs. Reaction mixtures for the assay of methanol-dependent pppGpp formation were prepared as described in Materials and Methods and contained the substrates indicated below. GPSI (1.6 μ g) was used for the assays in lanes 1 to 5, and GPSII (3 μ g) was used for the assays in lanes 6 to 10. Lanes 1 and 6, complete system; lanes 2 and 7, without methanol; lanes 3 and 8, without ATP; lanes 4 and 9, without [α -³²P]GTP and with [γ -³²P]ATP; lanes 5 and 10, without GTP and with GDP and [γ -³²P]ATP. Lane 11, without [α -³²P]GTP and with [γ -³²P]ATP, no enzyme. Note that the specific activity of the [γ -³²P]ATP in the reaction mixtures was 3.4 times lower than that of the [α -³²P]GTP in corresponding reaction mixtures.

FIG. 5. Incubation of GPSI with unfractionated extracts of *S. antibioticus* mycelium. Incubation conditions were as described in Materials and Methods. Mixtures were fractionated on a 10% separating gel. The migration positions of GPSI and GPSII are shown. Bands A and B have apparent M_r s of 82,000 and 69,000, respectively.

dependencies. In several experiments, performed under different conditions (e.g., 10 to 40 mM Mg^{2+}) and in the presence of poly(U) and uncharged tRNA, no stimulation of GPSI or GPSII was observed with ribosomes from *S. antibioticus* or *E. coli* (data not shown). Under the same conditions, the ribosome-dependent activity of the RelA protein was easily demonstrable (data not shown). Interestingly, the activities of GPSI and, to a lesser extent, GPSII were stimulated by poly(U) and by uncharged and charged tRNA. A thorough analysis of the stimulation of GPSI by RNAs is presented in the accompanying paper (13).

Structural relationship between GPSI and GPSII. Since it was not possible to distinguish between GPSI and GPSII in terms of their kinetic properties, other approaches were used to examine their possible structural relationship. In one approach, [^{35}S]GPSI was incubated with unfractionated extracts of *S. antibioticus* mycelium (see Materials and Methods). The rationale for this approach was the possibility that GPSII might be produced by proteolysis of GPSI and that the relevant protease(s) might be detectable in extracts of *S. antibioticus*. As can be seen in Fig. 5, incubation of GPSI at 37°C with unfractionated mycelial extracts produced a series of discrete intermediates. Two of these, A and B, have M_r s of 82,000 and 69,000, respectively. Further information on these species is provided in the accompanying paper (13). Of particular interest are the products of incubation for 4 to 12 h. A protein species with the precise mobility of purified GPSI is observed among the products of these incubations (Fig. 5). These data strongly suggest that GPSII is produced in *S. antibioticus* by proteolytic degradation of GPSI.

If the foregoing conclusion is correct, then GPSI and GPSII should have antigenic similarities. To test this hypothesis, [^{35}S]GPSI and [^{35}S]GPSII were treated with polyclonal antibody to GPSI and GPSII. The results of the electrophoretic analysis of the immunoprecipitates produced are shown in Fig. 6. It is apparent that, as expected, each antibody reacts with the corresponding antigen (lanes 2 and 5). Figure 6 also shows that antibody to either GPSI or GPSII reacts with either GPSI or GPSII (lanes 3 and 6). Taken together, therefore, the data of Fig. 5 and 6 support the identity of GPSI as the catalytically

FIG. 6. Immunoprecipitation of [^{35}S]GPSI and [^{35}S]GPSII. Incubations were carried out as described in Materials and Methods, and immunoprecipitates were fractionated by SDS-PAGE. Lanes 1 and 4, [^{35}S]GPSI and [^{35}S]GPSII, respectively; lane 2, [^{35}S]GPSI plus antibody to GPSI; lane 3, [^{35}S]GPSII plus antibody to GPSI; lane 5, [^{35}S]GPSII plus antibody to GPSII; lane 6, [^{35}S]GPSI plus antibody to GPSII.

relevant form of the enzyme and the conclusion that GPSII is a breakdown product of GPSI. Further evidence in support of these conclusions is presented in the accompanying paper (13).

GPS activity in other actinomycetes. Mycelial extracts were prepared from 10 actinomycetes, including *S. antibioticus*, as described in Materials and Methods and were tested for GPS activity. As shown in Fig. 7, pppGpp was synthesized in the methanol-dependent assay by each extract, but the amounts obtained were much greater with extract from *S. antibioticus* than with any of the other extracts tested. The level of synthesis by *Saccharopolyspora erythraea* was also somewhat higher than that by the streptomycetes other than *S. antibioticus*. Significant quantities of GDP (topmost spot in the lanes of Fig. 7) were observed in the experiments of Fig. 7 but not in similar assays whose results are shown in other figures (e.g., Fig. 1). This is probably due to the procedure used to prepare crude extracts for the experiments of Fig. 7, i.e., sonication, which is

FIG. 7. Activity of mycelial extracts of various streptomycetes in the GPS assay. Reaction mixtures contained between 50 and 100 μ g of mycelial crude extract. Other conditions were as described in Materials and Methods. Lane 1, *Streptomyces albus* ATCC 21828, lane 2, *Streptomyces ambifaciens* ATCC 15155, lane 3, *S. antibioticus* IMRU3720, lane 4, *Streptomyces chrysomallus* ATCC 11523, lane 5, *S. coelicolor* A3(2), lane 6, *Saccharopolyspora erythraea*, lane 7, *Streptomyces fradiae* UC8306, lane 8, *Streptomyces glaucescens* ETH22794, lane 9, *Streptomyces lividans* TK24, lane 10, *Streptomyces parvulus* ATCC 12434.

known to release hydrolytic enzymes from cellular membranes (24).

The synthesis of pppGpp by the extracts of the nine organisms other than *S. antibioticus* has not yet been examined under other reaction conditions.

DISCUSSION

The isolation of GPS from *S. antibioticus* apparently represents the first characterization of enzymes involved in ppGpp metabolism in *Streptomyces* spp. and extends the range of organisms in which the synthesis of (p)ppGpp has been examined. The RelA and SpoT proteins of *E. coli* have been studied in some detail, and (p)ppGpp synthetases in two *Bacillus* species have also been characterized (6, 32). GPSI from *S. antibioticus*, with an M_r of 88,000, is similar in size to the RelA (M_r , 84,000) and SpoT (M_r , 79,000) proteins (17, 28) and to the ribosome-dependent (p)ppGpp synthetase from *Bacillus stearothermophilus* (M_r , 86,000 [6]). GPSII (M_r , 47,000) has a size which is similar to that of a ribosome-independent (p)ppGpp synthetase from *Bacillus brevis* (M_r , 55,000 [32]); however, the data of Fig. 5 and 6 (see above) argue strongly for the identity of GPSII as a breakdown product of GPSI. In addition, the specific activity of GPSII observed in several experiments was variable and was always lower (by 70 to 90%) than that of GPSI. Further, although GPSII migrated as a single band on polyacrylamide gels (see, e.g., Fig. 3), N-terminal sequence analysis of several purified preparations produced two sequences in approximately equal amounts. Thus, the N-terminal end of the protein may be frayed. It is also important to note that when incubations similar to those described for Fig. 5 were extended for 24 h, essentially all of the [35 S]GPSI was completely degraded (data not shown). These observations support the conclusion that GPSII is a steady-state intermediate in the proteolysis of GPSI and may not be a catalytically relevant GPS species in vivo. For these reasons, the remainder of this discussion will focus on the properties of GPSI.

Like that of RelA, the activity of GPSI from *S. antibioticus* is stimulated by methanol. The stimulation in both cases is probably the result of a change in conformation in the presence of the organic solvent which enhances the catalytic activity of the protein. Also like RelA, GPSI appears to aggregate in buffers of low ionic strength (Fig. 2). The specific activity of GPSI (3.3 $\mu\text{mol}/\text{min}/\text{mg}$ of protein) (Table 2) is similar to that of the RelA protein (4 $\mu\text{mol}/\text{min}/\text{mg}$) (22) in the methanol-dependent assay. Unlike for RelA, GDP is a poor substrate for GPSI (Fig. 4). This observation makes it likely that at least one pathway for the synthesis of ppGpp in *S. antibioticus* involves the formation of pppGpp as the initial reaction product.

There are recent reports of the synthesis of highly phosphorylated nucleotides by a nucleotide 3'-pyrophosphokinase in streptomycetes (18, 19). This enzyme transfers the 5'- β , γ -pyrophosphoryl group from ATP or dATP to purine or pyrimidine nucleotides. Among the compounds produced by such reactions are ppGpp and ppApp (18, 19). The ability of GPSI to synthesize ppApp was tested under the conditions used for Fig. 4 in the presence or absence of methanol. No ppApp was formed in these assays (data not shown). As Fig. 4 demonstrates, GPSI does not utilize GDP efficiently as a substrate.

It was surprising to find that neither GPSI nor GPSII was stimulated by ribosomes. While that observation may relate to an inappropriate choice of reaction conditions, it must be

noted that ribosomes were obtained from mycelia of several ages (18, 24, and 72 h of growth in GGA medium), that those ribosomes were active in poly(U)-directed polyphenylalanine synthesis (data not shown), that several different Mg^{2+} concentrations were used in the GPS assays, and that the ribosome-dependent activity of the RelA protein could be demonstrated easily under the assay conditions used here. Nevertheless, the activation of GPSI by ribosomes under appropriate conditions remains a possibility.

It is also possible that the GPS system in *S. antibioticus* does not represent the analog of the *relA* (or *spoT*) system in *E. coli*. In relation to this possibility, it is perhaps significant that neither GPSI nor GPSII reacts with antibody to RelA or SpoT (data not shown). Arguing against this possibility are the observations that only minimal amounts of GPS activity remain in the soluble fraction following treatment of mycelial extracts with PEI and that essentially no additional GPS activity can be obtained from PEI pellets following extraction of GPSI and GPSII.

A second, extremely interesting possibility is that (p)ppGpp synthesis in *S. antibioticus* is not ribosome dependent. Consistent with this suggestion is the observation that the activity of GPSI is stimulated by a synthetic mRNA [poly(U)] and by charged and uncharged tRNA. Details of this stimulation are presented in the accompanying paper (13). Since *S. antibioticus* GPS is precipitated from mycelial extracts by PEI, it is possible that the enzyme is associated with ribosomes in vivo. This hypothesis is consistent with the observation that SpoT is ribosome associated even though its activity is presumably ribosome independent (26). It must be emphasized, however, that the results presented here do not eliminate the possibility of a second pathway to ppGpp production in *S. antibioticus* that is similar to the *relA* pathway in *E. coli*. The fact that *relC* mutations can be isolated in *S. antibioticus* is consistent with this possibility.

Figure 7 demonstrates modest levels of GPS activity in nine actinomycetes other than *S. antibioticus*. With the exception of that of *Saccharopolyspora erythraea*, the levels of pppGpp production observed were quite low compared with those observed in the methanol-dependent assay with *S. antibioticus* extracts. No ppGpp was observed in the reactions in which the other mycelial extracts were used. It was surprising not to detect high levels of pppGpp synthesis in *Streptomyces coelicolor*, since that organism has been shown to exhibit a stringent response to amino acid starvation and to increase intracellular levels of ppGpp on nutritional shiftdown (31, 34). A correlation between the onset of antibiotic production and the level of ppGpp has also been observed in *S. coelicolor* (31, 34). The specific role of ppGpp in initiating antibiotic synthesis has not been determined. It is possible that the necessary conditions for the efficient synthesis of (p)ppGpp were not employed in the present study and that *S. coelicolor* and the other actinomycetes are able to synthesize pppGpp efficiently in vitro. These possibilities will be examined in subsequent experiments.

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