Isolation and Properties of a Ribosome-bound Factor Required for ppGpp and pppGpp Synthesis in *Escherichia coli*

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SUMMARY

A scheme for isolating the stringent factor, a factor which stimulates the pyrophosphorylation of GTP and GDP, from high salt extracts of ribosomes derived from stringent strains of *Escherichia coli* is reported. The stringent factor was purified 400-fold from the high salt-ribosome eluates. The final preparation was judged to be 85% homogeneous by sedimentation and electrophoretic criteria. The results of sodium dodecyl sulfate-gel ionophoresis and sedimentation analysis indicated that the factor is a protein consisting of a single polypeptide chain with a molecular weight of 77,000.

In addition to the stringent factor, the conversion of GTP to the guanosine polyphosphates, ppGpp and pppGpp, was shown to require ATP, Mg²⁺, and ribosomes. The *in vitro* reaction was further characterized with respect to kinetic parameters, substrate specificity, and its sensitivity to known inhibitors of the *in vivo* synthesis of ppGpp.

From measurements of stringent factor activity in fractions obtained by differential centrifugation of crude cell extracts it was concluded that essentially all of the factor in the extracts was bound to ribosomes.

Extracts prepared from several different relaxed mutants were shown to be devoid of stringent factor activity. Evidence is presented that it is the stringent factor rather than the ribosomes which is impaired in these mutants. The implication of these results in relation to the function of the *rel* gene and the control mechanism underlying the stringent response is discussed.

Many auxotrophic strains of *Escherichia coli* respond almost immediately to the loss of an essential amino acid by restricting the synthesis of a variety of critical metabolic products such as stable species of RNA (1), purine nucleoside triphosphates (2), and lipids (3). This response, termed the "stringent" response, is under the control of the *rel* gene (4) and is characteristic of *rel*⁺ or stringent strains of *E. coli*. *rel*⁻ or relaxed mutants on the other hand fail to curb their metabolism directly upon depletion of a required amino acid (5). This is not the only trait, however, that distinguishes the stringent from the relaxed phenotype. Cashel and Gallant (6) discovered that during amino acid starvation stringent strains rapidly accumulate the two guanosine polyphosphates (7, 8), ppGpp and pppGpp, whereas, relaxed strains do not. These pleiotropic effects of mutations in the *rel* gene suggest that (9) (a) the ppGpp and pppGpp nucleotides may be responsible for the restricted metabolic activity peculiar to the stringent response; and (b) the function of the *rel* gene is related directly or indirectly to the synthesis of these compounds.

Quite recently Haseltine et al. (10) have defined conditions for forming ppGpp and pppGpp in vitro. The synthetic reaction involves the transfer of a pyrophosphoryl group from ATP to GDP or GTP (7, 8) in the presence of ammonium chloride-washed ribosomes and the stringent factor, a putative protein(s) released from *rel*⁺ ribosomes by washing them with concentrated ammonium chloride solutions. Since no such stimulatory factor was found in the ammonium chloride-ribosome washes prepared from several relaxed mutants, these authors concluded that the *rel*⁺ stringent factor was inactivated as a consequence of mutation in the *rel* locus. This implicit connection between the stringent factor and the gene determining stringency indicated that a clearer notion of the nature and properties of this factor might further our understanding of the control mechanism underlying the stringent response.

In this communication, procedures for obtaining highly purified stringent factor and a partial characterization of the purified factor are presented.

EXPERIMENTAL PROCEDURE

Materials

Cells of the stringent *E. coli* strain, K-12 (ATCC 10798), were obtained from Miles Laboratories. H₃P₀₄ and [8-³H]guanosine 5'-triphosphate (5.28 Ci per mmole) were purchased from New England Nuclear. All unlabeled nucleotides except GDP, a *rel* whose phenotype is designated "relaxed"; ppGpp, 5'-diphosphosphate-3'-diphosphate guanosine; pppGpp, 5'-triphosphate-5'-triphosphate guanosine; GDP, G-factor, the elongation factor required for the translocation step in protein synthesis; A-site, the ribosomal acceptor site for tRNA.

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product of P-L Biochemicals, t-chloramphenicol, streptomycin sulphate, and dithiothreitol were supplied by Sigma. Phosphocellulose (Whatman P11) was obtained from Reeve-Angel Co.; hydroxyethylpase (Hyapatite C) from the Clarkson Chemical Corporation; ammonium sulphate and sucrose (both "special catyogenic grade") from Mann; polyethyleneimine thin layer plates (Cel 300 PEI/UV 254) from Brinkmann Instruments; and puromycin dihydrochloride from Nutritional Biochemicals. Oxytetracycline dihydrate and sodium fusidate were gifts from Dr. Paul Lietmann of Johns Hopkins University. Purified factor G was generously provided by Dr. Nathan Brot of the Roche Institute of Molecular Biology.

Methods

Assay

The standard assay employed was a modification of that described by Haseltine et al. (10). The reaction mixture contained in 0.05 ml, 50 mM Tris-Cl, pH 8.0; 18 mM MgCl2; 10 mM KCl; 2 mM dithiothreitol; 1.25 mM EDTA; 1 mM GTP; 1 mM ATP; 1 x 10⁶ cpm of [γ-32P]ATP or 8 x 10⁴ cpm of [3H]GTP; 2.7 μM NH4Cl-washed 70S ribosomes; and stringent factor. Incubations were for 10 min at 35°C. Reactions were terminated by adding 10 μl of 6 M formic acid, mixing, and chilling on ice. To those terminated reactions containing [3H]GTP instead of [γ-32P]ATP, 4 nmoles each of unlabeled ppGpp and pppGpp in 5 μl were added as chromatographic markers. The reaction contents were transferred to microfuge tubes and spun in a Spinco microfuge for 1 min. Twenty microliters of the supernatants were spotted on polyethyleneimine thin layer plates and dried. The plates were then soaked in methanol, dried, and developed in 1.5 M KH₂PO₄, pH 3.4, as described by Cashel et al. (11). After chromatography the plates were dried and then autoradiographed, using Kodak "Blue Brand" medical x-ray film, to detect 32P labeled product or exposed to ultraviolet light provided by a Mineralight UVS-11 lamp to localize the guanosine polyphosphate markers added to the [3H]GTP-containing reactions. The spots on the chromatograms corresponding to ppGpp and pppGpp were cut out and placed in scintillation vials for counting. Aliquots of [γ-32P]ATP or [3H]GTP were spotted on developed chromatograms and counted to determine the specific radioactivities of these compounds.

Control reactions from which stringent factor was omitted were included in each set of experiments. The stringent factor activity was calculated by summing the amount of radioactivity incorporated into the ppGpp and pppGpp spots, subtracting the background incorporation measured in the control experiments, and dividing the difference by the product of the specific activity of ATP or GTP and the time of incubation.

A unit of stringent factor activity is defined as the amount of factor forming 1 nmole of product (ppGpp plus pppGpp) per min at 35°C.

Preparation of [γ-32P]ATP

The method used was that of Weiss et al. (12). The initial specific radioactivity of the purified [γ-32P]ATP was 6.8 x 10⁶ cpm per μmole.

Preparation of ppGpp, pppGpp, and [32P]pppGpp

ppGpp and pppGpp were synthesized under standard assay conditions in separate 10-ml reactions. The ppGpp reaction was catalyzed by 130 units of the ammonium sulphate-fraction stringent factor, and the pppGpp reaction, by 130 units of the phosphocellulose-fraction stringent factor. [32P]pppGpp was prepared in a 0.5-ml reaction containing 1 x 10⁶ cpm of [γ-32P]-ATP and 6.5 units of the phosphocellulose-fraction stringent factor. Incubations were for 1 hour. The guanosine tetra- and pentaphosphates were isolated and concentrated according to the procedures of Cashel and Kalbacher (7). The yields of purified, unlabeled ppGpp and pppGpp were 2.5 and 1.8 amoles, respectively. The initial specific radioactivity of the purified [32P]pppGpp was 5.6 x 10⁶ cpm per μmole.

G-Factor Hydrolysis of pppGpp

The reaction mixture contained in 0.05 ml, 50 mM Tris-Cl, pH 8.0; 18 mM MgCl₂; 10 mM KCl; 2 mM dithiothreitol; 1.25 mM EDTA; 0.52 mM [3H]pppGpp (5.6 x 10⁶ cpm per μmole); 2.7 μM NH₄Cl-washed 70S ribosomes; and purified G-factor. Incubations were for 10 min at 35°C. Reactions were terminated and assayed for ppGpp and pppGpp as described under "Assay."

Sodium Dodecyl Sulfate-Gel Electrophoresis

The method adopted was essentially that of Weber and Osborn (13). One-tenth-milliliter samples in sealed tubes were boiled for 90 s in the presence of 2% sodium dodecyl sulfate and 5% β-mercaptoethanol and then dialyzed for 1 hour against 250 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate, 0.1% β-mercaptoethanol, and 20% glycerol. The gels contained 7.5% acrylamide, 0.15% cross-linker (methylen bisacrylamide), 0.05 M sodium phosphate buffer, pH 7.0, and 0.1% sodium dodecyl sulfate. Electrophoresis was conducted at a constant current of 8 ma per gel until the tracking dye was approximately 1 cm from the bottom of the tube. The gels were then stained in freshly prepared solutions of 0.25% Coomassie blue and destained in 7% acetic acid.

Sucrose Gradient Centrifugation

Linear gradients of sucrose from 5 to 20% were made up according to Martin and Ames (14) in 0.05 M Tris-Cl buffer, pH 7.6, 0.001 M MgCl₂, 0.2 M (NH₄)₂SO₄, and 0.002 M dithiothreitol. One-tenth-milliliter samples were layered onto the gradients (4.6 ml) and centrifuged in an SW 39L swinging bucket rotor for 24 hours at 38,000 rpm and 3°C. At the end of the run 8-drop fractions were collected by piercing the bottom of the tube and assayed.

Protein Measurement

Protein concentration was estimated by the method of Lowry et al. (15).

Preparation of High Salt-washed Ribosomes

The ribosomes pelleted in the ammonium chloride extraction step of the stringent factor purification described below were re-suspended in standard buffer containing 0.5 M NH₄Cl at a concentration of 8 mg per ml. The solution was stirred for 30 min and then 24-ml portions of it were layered onto 6 ml of standard buffer containing 0.5 M NH₄Cl and 20% glycerol in 30-ml screw-capped centrifuge tubes. The tubes were spun in a Beckman Ti 60 rotor for 3 hours at 50,000 rpm. The transparent pellets were washed an additional time by repeating the cycle of resuspension in standard buffer containing 0.5 M NH₄Cl and centrifugation through a 20% glycerol cushion at 50,000 rpm. The ribosomes were finally dissolved in standard buffer containing 10% glycerol at a concentration of 65 mg per ml and stored at -20°C.
**Purification of Stringent Factor**

The following steps were carried out at temperatures ranging from 0°-4°.

**Preparation of Crude Extracts and Isolation of Ribosomes—** Five-hundred grams, wet weight, of frozen cells were thawed and suspended in 500 ml of standard buffer (0.01 M Tris-Cl buffer, pH 7.6, 0.01 M MgCl₂, 0.01 M NH₄Cl, and 0.001 M dithiothreitol). The cells were disrupted by three passages through a Manton-Gaulin laboratory homogenizer. The cell debris was removed by two centrifugations of 25 min each at 27,000 × g in a Sorvall RC2-B centrifuge. The supernatants were transferred to 30-ml screw-capped centrifuge tubes fitted with IEC metal caps and spun in Ti 60 rotors at 50,000 rpm for 2.5 hours in Beckman model L2-65B preparative ultracentrifuges. The supernatants were aspirated off and pooled. The pelleted ribosomes were resuspended in 190 ml of standard buffer containing 10% glycerol and stored frozen overnight at -20°.

**Ammonium Chloride Extraction of Ribosomes and Ammonium Sulfate Precipitation—** The frozen suspension of ribosomes was thawed and 190 ml of standard buffer containing 1.0 M NH₄Cl were added with stirring. The solution was stirred for an additional 90 min, and then 24-ml portions of it were layered onto 6 ml of standard buffer containing 0.5 M NH₄Cl and 20% glycerol in 30-ml screw-capped centrifuge tubes. The tubes were centrifuged for 3 hours at 50,000 rpm in Ti 60 rotors. The clear supernatants were aspirated off and pooled. The pelleted ribosomes were saved and used in the preparation of high salt washed ribosomes as described under “Experimental Procedure.” To the stirred supernatant fluid finely ground ammonium sulfate was added to 45% of saturation. After a further 10 min of stirring the precipitate was collected by centrifugation for 15 min at 27,000 × g. The pellets were dissolved in 40 ml of Buffer A (0.02 M Tris-Cl buffer, pH 7.4, 0.001 M EDTA, 0.002 M dithiothreitol, and 10% glycerol) containing 0.1 M NH₄Cl and dialyzed for 2 hours against 2 liters of the same buffer. Any precipitate that appeared during dialysis was removed by centrifugation.

**Phosphocellulose Chromatography—** The dialyzed preparation was diluted with sufficient Buffer A to reduce its conductivity to 6 mhos as measured by a YSI model 31 conductivity bridge and then applied at a flow rate of 0.3 ml per min to a P11-cellulose column (2 × 42 cm) which had been equilibrated with Buffer A containing 0.1 M NH₄Cl. The column was washed with the equilibrium buffer until the absorbance of the effluent at 280 nm was less than 0.2 units. Protein was eluted with a linear gradient consisting of 500 ml of Buffer A containing 0.1 M NH₄Cl in the mixing chamber and 500 ml of Buffer B containing 0.6 M NH₄Cl in the reservoir at a flow rate of 0.6 ml per min. Eight-milliliter fractions were collected and assayed for stringent factor activity and absorbance at 280 nm. The resultant chromatogram is shown in Fig. 1. Fractions, 31 through 34, were pooled and dialyzed for 2 hours against 1 liter of Buffer B (0.01 M Tris-Cl buffer, pH 7.2, 0.002 M MgCl₂, 0.002 M dithiothreitol, and 10% glycerol) containing 0.1 M (NH₄)₂SO₄.

**Hydroxyapatite Chromatography—** After adjustment of its conductivity to 7.6 mhos, the dialyzed phosphocellulose fraction was applied at a flow rate of 0.23 ml per min to a column (0.9 × 25 cm) of Hypatite C previously equilibrated with Buffer B containing 0.1 M (NH₄)₂SO₄. The column was then washed with 50 ml of equilibration buffer. Chromatography was carried out with a linear gradient of ammonium sulfate (150 ml of 0.1 M (NH₄)₂SO₄ in Buffer B and 150 ml of 0.5 M (NH₄)₂SO₄ in the same buffer). Fractions (3.4 ml) were collected at a flow rate of 0.32 ml per min and assayed. Fig. 2 shows the elution profile from the hydroxylapatite column. Fractions, 39 through 43, were pooled and concentrated by adding solid ammonium sulfate to 70% of saturation. After collection by centrifugation the ammonium sulfate precipitate was dissolved in 0.5 ml of Buffer C (0.01 M Tris-Cl buffer, pH 7.6, 0.001 M MgCl₂, 0.002 M dithiothreitol, and 0.2 M (NH₄)₂SO₄) and dialyzed for 1 hour against 100 ml of the same buffer.

**Glycerol Gradient Centrifugation—** The dialyzed hydroxylapatite fraction was layered on a 12.6-ml linear glycerol gradient (10 to 30% (v/v) of glycerol in Buffer C) and centrifuged at 40,000 rpm in an SW 40 Ti rotor for 48 hours at 4°. The bottom of the tube was pierced, 0.5-ml fractions collected, and both the absorbance at 280 nm and stringent factor activity in each were assayed. The distribution of stringent factor activity and A₂₆₀ in the gradient are depicted in Fig. 3. The pooled fractions (14 through 16) were divided into 0.2-ml aliquots and stored frozen in microtube tubes at -90° in a Revco ultralow temperature freezer. Storage of the purified factor under these conditions resulted in the loss of only 10% of the original activity after 5 months.

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*Fig. 1. Phosphocellulose chromatography. A column (2 × 42 cm) of phosphocellulose P11 was loaded with 160 mg of protein from Fraction 2, Table I. After washing with Buffer A containing 0.1 M NH₄Cl to remove unadsorbed protein, the column was eluted with a linear gradient of 0.1 M to 0.6 M NH₄Cl in Buffer A. Eight-milliliter fractions were collected at a flow rate of 0.6 ml per min and assayed for absorbance at 280 nm and stringent factor activity as described under ‘Methods.’*
Fig. 2. Hydroxylapatite chromatography. Fractions of high specific stringent factor activity from the phosphocellulose column were pooled, dialyzed against Buffer B containing 0.1 M ammonium sulfate, and passed onto a column (0.9 × 25 cm) of Hypap- tite C. After rinsing the column with 3 column volumes of di-
alysis buffer, chromatography was carried out using a linear gradient of ammonium sulfate in Buffer B extending from 0.1 m
 to 0.5 M. Fractions of 3.4 ml were collected at 10.5-min interval
s and assayed for stringent factor activity as described under “Ex-
perimental Procedure.”

Fig. 3. Glycerol gradient centrifugation. Fractions from the hydroxylapatite column containing stringent factor at high spec-
cific activity were combined, precipitated with solid ammonium sulfate, and dialyzed against Buffer C for 1 hour. Five-tenths of a milliliter of the concentrated, dialyzed sample was layered on top of a 12.6-ml linear glycerol gradient (10 to 30% (v/v) of gly-
cerol in Buffer C) and centrifuged at 40,000 rpm in an SW 40Ti rotor for 48 hours. Fractions of 0.5 ml were collected by punc-
turing the bottom of the tube and assayed for stringent factor activity as described under “Methods.”

A summary of the purification procedure is presented in Table I. Assays performed on steps preceding ammonium chloride extraction yielded low values for total activity indicating that the reaction was inhibited in crude extracts. Consequently, the results of these assays are not reported in the table.

**RESULTS**

**Criteria of Purity**—In the glycerol gradient sedimentation profile (Fig. 3) the peak of stringent factor activity is coincident with an absorbance peak. The specific activities of the very active fractions (tubes 14, 15, and 16) are essentially constant. However, the activity peak is bracketed by two absorbance peaks. To determine the degree of purity of the stringent factor present in the final step of purification with greater accuracy, aliquots from the glycerol gradient fraction were subjected to electrophoresis in sodium dodecyl sulfate gels. The electrophoretic pattern obtained is shown in Fig. 4. One major band and several minor ones are evident. Microdensitometry of sev-
eral sodium dodecyl sulfate gel ionograms prepared from samples of the glycerol gradient fraction indicated that the minor bands accounted for approximately 15% of the protein in the gel.

TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total Activity (X 10^3 units)</th>
<th>Specific Activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ammonium chloride extract</td>
<td>435</td>
<td>1.34</td>
<td>66.0</td>
<td>11</td>
<td>100</td>
</tr>
<tr>
<td>2. Ammonium sulfate</td>
<td>40</td>
<td>4.05</td>
<td>60.8</td>
<td>37</td>
<td>92</td>
</tr>
<tr>
<td>3. Phosphocellulose</td>
<td>32</td>
<td>0.10</td>
<td>42.2</td>
<td>825</td>
<td>64</td>
</tr>
<tr>
<td>4. Hydroxylapatite</td>
<td>17</td>
<td>0.04</td>
<td>13.0</td>
<td>1920</td>
<td>20</td>
</tr>
<tr>
<td>5. Glycerol gradient</td>
<td>2</td>
<td>0.05</td>
<td>4.4</td>
<td>4380</td>
<td>7</td>
</tr>
</tbody>
</table>
FIG. 4. Sodium dodecyl sulfate-gel electrophoretic pattern for purified stringent factor from the glycerol gradient fraction. Two hundred microliters of the glycerol gradient fraction (Fraction 5 in Table I), containing 10 ng of stringent factor, were treated with sodium dodecyl sulfate and subjected to electrophoresis as described under "Methods." The direction of electrophoresis was from bottom to top.

However, it has not been demonstrated that the major band of denatured protein in the sodium dodecyl sulfate gels possesses stringent factor activity. Attempts to subject samples to electrophoresis from the glycerol gradient fraction in acrylamide gels under conditions which would permit the assay of both protein and stringent factor activity after electrophoresis have been unsuccessful owing to the tendency of the factor to aggregate in the low ionic strength buffers required for electrophoresis. Nevertheless, the close agreement between the molecular weights obtained for the major protein band in sodium dodecyl sulfate gels and for active stringent factor in sucrose gradients (see "Molecular Weight Determination" below) supports the view that the major protein band of sodium dodecyl sulfate gels is derived from the stringent factor. On the basis of the above results it is tentatively concluded that the stringent factor was 85% pure after glycerol gradient centrifugation.

Molecular Weight Determination—An estimate of the molecular weight of the stringent factor was obtained from measurements of its sedimentation velocity in sucrose gradients using the procedures of Martin and Ames (14). Employing human hemoglobin as a reference protein (mol wt of 64,500 (16)) a molecular weight of 74,500 was calculated for the stringent factor.

A subunit molecular weight for the factor was approximated by comparing its electrophoretic mobility with those of known molecular weight markers in sodium dodecyl sulfate gels under the conditions developed by Weber and Osborn (13). The protein standards used were glyceraldehyde phosphate dehydrogenase, ovalbumin, pyruvate kinase, bovine serum albumin, and phosphorylase A. The results of this analysis indicated a molecular weight of 77,000 for the sodium dodecyl sulfate-denatured stringent factor. Since this value for the subunit molecular weight is very close to that of the undenatured factor, a monomeric quaternary structure is proposed for the stringent factor.

Requirements of Stringent Factor-stimulated Reaction—The stringent factor-catalyzed formation of ppGpp and pppGpp from GTP or GDP is almost absolutely dependent upon ATP, Mg2+, and ribosomes. Omission of any one of these essential ingredients from a reaction reduces the rate of pyrophosphorylation to less than 3% of that measured in a fully supplemented incubation mixture.

In all of the following studies requiring stringent factor only the highly purified factor obtained in the glycerol gradient fraction was employed. Unless otherwise specified 5 μl of this fraction corresponding to 0.2 ng of stringent factor were added to each reaction.

pH Activity Profile—The effect of pH on the rate of conversion of GTP to ppGpp and pppGpp is illustrated in Fig. 5. The rate of product formation increases with increasing pH from pH 6.5 to 9.0 and then declines sharply at pH values above 9.0. Whether it is the association of ribosomes and stringent factor to form the catalytic unit or the actual catalysis or both which is sensitive to high pH remains to be elucidated.

Magnesium Dependence—Magnesium ions stimulated the reaction. A broad concentration optimum ranging from 12 to 30 mM was observed (Fig. 6). A similar magnesium requirement curve but displaced toward lower concentrations of magnesium has been obtained by Pedersen et al. (17) using a partially purified preparation of stringent factor. This displacement between the two curves can be attributed to the fact that these workers expressed their results in terms of the effective magnesium concentration obtained by correcting the apparent magnesium concentration for the binding of the cation to ATP and GTP.

Specificity for Pyrophosphate Acceptors—A variety of nucleotides were surveyed for their relative capacity to accept pyrophosphate groups in the transfer reaction catalyzed by stringent factor. Of the 10 ribo- and deoxyribonucleotides tested only the guanosine derivatives, GDP and GTP, and their analogs, GDP-C6P and ITp, were found to be active as pyrophosphatase acceptors (Table II). Apparently the structural requirements for nucleotides which can serve as pyrophosphatase acceptors under the standard assay conditions is quite restricted as indicated by
FIG. 6. Influence of Mg\textsuperscript{2+} concentration on the rate of ppGpp plus pppGpp formation. Assays were performed as described under "Methods" with the concentration of MgCl\textsubscript{2} as indicated. The designated Mg\textsuperscript{2+} concentrations were not corrected for the binding of the cation to ATP and GTP.

**TABLE II**

*Pyrophosphate acceptor activity of nucleotides*

Each substrate at an initial concentration of 1 mM was incubated with and without stringent factor under the standard assay conditions (cf. "Methods"). Following chromatography of the reaction mixtures the pyrophosphorylated products were identified from radioautograms as the labeled compounds whose syntheses were stringent factor-dependent and whose \( R_p \) values were less than those of the homologous substrates. In Experiment 1 \([\gamma^3P]ATP (2 \times 10^6 \text{ cpm per } \mu\text{mole})\) was the labeled substrate. In Experiment 2 each of the indicated substrates was labeled with \(^{14}\text{C}\) at a specific activity of 1 \( \times 10^6 \text{ cpm per } \mu\text{mole. ATP was unlabeled. The amount of product formed from each substrate was calculated from the difference in the amounts of } [\gamma^3P]\text{phosphosphate or } [\gamma^4C]\text{nucleotide incorporated into product in the control and enzyme-catalyzed reactions (cf. "Methods").}"

<table>
<thead>
<tr>
<th>Nucleotide substrate</th>
<th>Amount of product formed</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>4.0</td>
<td>100</td>
</tr>
<tr>
<td>GDP</td>
<td>3.7</td>
<td>92</td>
</tr>
<tr>
<td>GDPCP</td>
<td>1.3</td>
<td>32</td>
</tr>
<tr>
<td>ITP</td>
<td>0.8</td>
<td>20</td>
</tr>
<tr>
<td>dGTP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dGDP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ATP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GMP</td>
<td>&lt;0.1</td>
<td>&lt;3</td>
</tr>
<tr>
<td>UTP</td>
<td>N.P.</td>
<td></td>
</tr>
<tr>
<td>CTP</td>
<td>N.P.</td>
<td></td>
</tr>
</tbody>
</table>

*No pyrophosphorylated derivative produced.*

the reduced relative acceptor activities of GDPCP and ITP which are closely related structurally to GTP.

**Specificity for Pyrophosphate Donors**—Eight potential donors of pyrophosphoryl groups were assayed under standard conditions for their ability to stimulate ppGpp and pppGpp synthesis. Significant pyrophosphorylation occurred only in the presence of ATP or dATP (Table III). The dATP substrate was shown by chromatography to be free from appreciable ATP contamination. Moreover, it is unlikely that trace amounts of ATP in the dATP would account for the high relative donor activity of dATP (33%).

**Effect of Antibiotic Inhibitors of Protein Synthesis**—Several drugs known to inhibit the formation of ppGpp and pppGpp in the intact bacterial cell (18) were tested for their effect upon the \( \text{in vitro} \) synthesis of these compounds. All of the drugs examined share the common property of interfering with various ribosome-mediated steps in protein biosynthesis (19). They were added to reaction mixtures at concentrations comparable to those which are effective \( \text{in vivo} \). In agreement with results reported previ-

**TABLE III**

*Pyrophosphate donor activity of nucleoside triphosphates*

Each incubation mixture contained at a concentration of 1 mM \([H]GTP (1.6 \times 10^6 \text{ cpm per } \mu\text{mole})\) and in place of ATP one of the several, designated nucleoside triphosphates. The amount of ppGpp and pppGpp formed was measured as described under "Assay."

<table>
<thead>
<tr>
<th>Nucleotide triphosphate</th>
<th>Amount of ppGpp + pppGpp formed</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>4.0</td>
<td>100</td>
</tr>
<tr>
<td>GTP\textsuperscript{a}</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UTP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CTP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dATP</td>
<td>1.5</td>
<td>33</td>
</tr>
<tr>
<td>dGTP</td>
<td>&lt;0.1</td>
<td>&lt;3</td>
</tr>
<tr>
<td>dCTP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dTTP</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*The concentration and specific radioactivity of GTP in this control reaction were the same as in the other reactions.*
discovered that these losses in activity were attributable chiefly to a strong tendency of the high salt-extracted stringent factor activity present in the ammonium chloride extract was suffered during purification. Only 7% of the total stringent factor were isolated. This low yield is due, in part, to losses over-whelming production in vivo relative to pppGpp. In vitro crude preparations of stringent factor preferentially catalyze the formation of ppGpp regardless of whether GTP or GDP is the substrate. However, with purified stringent factor GDP is converted to ppGpp regardless of whether GTP or GDP is the substrate. In the presence of purified stringent factor, but rather, the small amount of factor originally extracted from the ribosomes. Assuming the factor to be 85% pure in the glyceral gradient fraction and correcting for fractionation losses it was calculated that 0.7 mg of active stringent factor was released from each gram of ribosomes by treatment with ammonium chloride. No stringent factor was detected on the washed ribosomes or in the nonribosomal fraction of the K-12 cell extracts (see below). Unquestionably, our efforts to purify the stringent factor were seriously handicapped by this scarcity of active factor in the crude K-12 lysates. Despite a nearly 400-fold purification of the stringent factor from the high salt ribosomal eluates, the factor was still inhomogeneous as judged by sodium dodecyl sulfate-gel electrophoresis.

The distribution of stringent factor in cell extracts was determined from measurements of its activity in the membrane fraction obtained by low speed centrifugation of cell lysates and in the supernatant (S-100) and particulate fractions resolved by high speed centrifugation of membrane-free extracts. Virtually no activity was found in the membrane fraction. No activity could be detected in the S-100 fraction even after phosphocellulose chromatography. Essentially all of the stringent factor activity was confined to the particulate fraction. Several cycles of suspension and centrifugation in standard buffer failed to release significant amounts of stringent factor activity from this fraction. From these studies it was inferred that all of the factor present in the cell was bound to the ribosomes.

To obtain a satisfactory reaction velocity under the standard assay conditions required a 50:1 mole ratio of ribosomes to stringent factor. The need for such an excess of ribosomes over factor might arise from a low affinity of high salt-washed ribosomes for factor or from the presence of a significant proportion of catalytically defective particles in the ribosome preparation. Saturation kinetic studies in which ribosomes were titrated against stringent factor and vice versa indicated that only a small fraction of the ribosomes stimulate ppGpp and pppGpp synthesis. Assuming that only 1 molecule of stringent factor is to aggregate in solutions of low ion strength. By maintaining the ion strength above a level corresponding to a conductance of 6 mhos throughout the purification aggregation was minimized but not completely obviated. However, it was not the depletion of stringent factor activity over the course of purification that was mainly responsible for the low yield of purified factor, but rather, the small amount of factor originally extracted from the ribosomes. Hence, the functioning of such a degradative pathway would explain the selective synthesis of ppGpp in vivo as well as the shift to predominantly pppGpp production in reactions stimulated by purified stringent factor preparations from which G-factor has been removed.

**DISCUSSION**

From 500 g of K-12 cells only 100 µg of highly purified stringent factor were isolated. This low yield is due, in part, to losses suffered during purification. Only 7% of the total stringent factor activity present in the ammonium chloride extract was recovered in the glycerol gradient fraction (cf. Table I). It was discovered that these losses in activity were attributable chiefly to a strong tendency of the high salt-extracted stringent factor to aggregate in solutions of low ion strength. By maintaining the ion strength above a level corresponding to a conductance of 6 mhos throughout the purification aggregation was minimized but not completely obviated. However, it was not the depletion of stringent factor activity over the course of purification that was mainly responsible for the low yield of purified factor, but rather, the small amount of factor originally extracted from the ribosomes. Assuming the factor to be 85% pure in the glyceral gradient fraction and correcting for fractionation losses it was calculated that 0.7 mg of active stringent factor was released from each gram of ribosomes by treatment with ammonium chloride. No stringent factor was detected on the washed ribosomes or in the nonribosomal fraction of the K-12 cell extracts (see below). Unquestionably, our efforts to purify the stringent factor were seriously handicapped by this scarcity of active factor in the crude K-12 lysates. Despite a nearly 400-fold purification of the stringent factor from the high salt ribosomal eluates, the factor was still inhomogeneous as judged by sodium dodecyl sulfate-gel electrophoresis.

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bound per ribosome it was calculated that 3% of the ribosomes formed active complexes in the presence of saturating amounts of factor. This finding is consistent with the recent discovery (17, 21) that the ribosome must assume a specific configurational state in order to participate in the reaction. ppGpp and pppGpp synthesis in vitro is contingent upon stringent factor associating with ribosomes that are complexed to mRNA and which possess codon-selected, uncharged tRNA species in their acceptor sites (A-sites). The small fraction of ammonium chloride-treated ribosomes that stimulated ppGpp and pppGpp synthesis may represent a residue of configurationally acceptable particles which survived the preparative procedures used in their isolation. Furthermore, the requirement that competent ribosomes contain uncharged tRNA molecules in their A-sites may account for the inhibition of ppGpp and pppGpp production by oxytetracycline and fusidic acid. Both drugs may interfere with the binding of uncharged tRNA to the A-site and thus prevent the ribosome from assuming its catalytically active state. Oxytetracycline is believed to block the association of aminocycloheptane tRNA with the ribosome’s A-site (22). It may exclude uncharged as well as charged tRNA from this site. Fusidic acid by inhibiting the translocation step in protein synthesis (23) may prevent the A-site from being “cleared” of peptidyl-tRNA rendering the site unavailable to a deacylated tRNA molecule.

Unlike stringent strains, rel- mutants do not accumulate ppGpp and pppGpp under the stimulus of amino acid deprivation (6). This may be due to a direct effect of mutation in the rel gene upon the stringent factor-ribosome complex responsible for their production. Preparations of both stringent factor and ribosomes derived from rel- strains were checked for their ability to catalyze ppGpp and pppGpp synthesis in the standard assay reaction. In agreement with the studies of Haseltine et al. (10) no stringent factor activity was detected in extracts prepared from several different rel- mutants. Moreover, fractionation of these extracts according to the procedures described for purifying stringent factor did not result in the recovery of any factor activity. The ribosomes isolated from rel- strains by the ammonium chloride procedure were unimpaired by mutation since on a molar basis they were equally effective as rel+ ribosomes in stimulating guanosine polyphosphate synthesis in the presence of purified stringent factor. These results strongly imply that the stringent factor component of the catalytic complex is inactivated either directly or indirectly by mutation in rel- strains. However, this demonstrable lack of stringent factor activity in rel- extracts is at variance with the well documented ability of rel- strains to form ppGpp and pppGpp under special physiological conditions (24–26). This apparent contradiction can be explained in several ways. An inhibitor of the stringent factor-stimulated reaction may be present in rel- extracts. Attempts by us to demonstrate the presence of an inhibitor in rel- extracts by adding aliquots of their membrane, S-100, and crude particular fractions to reactions containing purified stringent factor and rel+ ribosomes were unsuccessful. Possibly the stringent factor-catalyzed pathway for ppGpp and pppGpp biosynthesis is lost in rel- strains and an alternative mechanism for their synthesis which is not detected by the standard assay system exists (10). Finally, the stringent factor, although functional in the growing rel- mutants examined by us, could be rendered highly labile by mutation so that it does not survive cell disruption. At present there is insufficient evidence available to permit any conclusions as to the specific cause of stringent factor inactivation in rel- extracts. Nevertheless, the strict correlation between the absence of stringent factor activity in vitro and the rel- genotype suggests that the rel locus either contains the structural gene for the stringent factor or acts epigenetically to determine the functional integrity of the factor. Furthermore, these conclusions are consistent with the proposal that ppGpp and pppGpp play a central role in regulating the expression of the stringent response.

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Isolation and Properties of a Ribosome-bound Factor Required for ppGpp and pppGpp Synthesis in *Escherichia coli*
Joanne W. Cochran and Raymond W. Byrne


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