Preparation of Homogeneous Elongation Factor G and Examination of the Mechanism of Guanosine Triphosphate Hydrolysis*

MICHAEL S. ROHRBACH, MARY E. DEMPSEY, AND JAMES W. BODLEY

From the Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455

SUMMARY

A new procedure for the isolation of physically and enzymatically homogeneous Elongation Factor G based on the isolation of an Elongation Factor G-ribosome-GDP-fusidic acid complex is described. The yield from this procedure is 23 to 36% of the theoretical and results in an enzyme of specific activity 6200 to 6700 units per $A_{280}$.

The enzyme was shown to be physically homogeneous by disc gel electrophoresis under both native and denatured conditions. The enzymatic purity of the preparation with respect to enzymes utilizing guanine nucleotides was examined by quantitation of contaminating nonribosomal dependent GTase, GDPase, nucleoside diphosphate kinase, and polynucleotide phosphorylase activities. The highest contaminating activity was found to be 0.002% of the activity of Elongation Factor G.

Utilizing this homogeneous Elongation Factor G, the reaction involving the hydrolytic cleavage of GTP catalyzed by the ribosome and Elongation Factor G was examined. The position of GTP cleavage was elucidated by the technique of $\text{H}^\text{2}O$ incorporation from water. It was demonstrated that solvent oxygen is incorporated only into the inorganic phosphate derived from the $\gamma$-phosphate of GTP. The number of solvent oxygen atoms incorporated per phosphate was 0.81 ± 0.19. Neither the $\alpha$-nor $\beta$-phosphates of GDP, the other product of hydrolysis, incorporated solvent oxygen. From these observations the position of GTP cleavage was identified as occurring between the $\gamma$ phosphorus atom and the oxygen bridging the $\beta$ and $\gamma$ phosphorus atoms.

Using $^{32}\text{P}$, and [$\alpha$-$^{32}\text{P}$]GDP, it was determined that neither the exchange of $P_i$ into GTP nor the exchange of GDP into GTP are catalyzed by Elongation Factor G and the ribosome.

The purification of an enzyme which utilizes a substrate common to a number of other enzymes poses a special problem. In addition to satisfying the normal criteria for physical homogeneity, the enzymatic purity of the preparation must also be carefully ascertained. Enzymatic contamination at levels where the contaminant is physically undetectable may still result in enzymatic heterogeneity. This can occur if the contaminating enzymes have turnover numbers several orders of magnitude greater than that of the principal enzyme.

This problem is encountered during the purification of Elongation Factor G. GTP, one of the substrates for EF-G, also serves as a substrate for a wide variety of other enzymes. Some of these other enzymes, such as nucleoside diphosphate kinase (2), have turnover numbers nearly three orders of magnitude greater than that of EF-G (3). While EF-G from E. coli has been purified to physical homogeneity in a number of laboratories (3-7), the enzymatic homogeneity of these preparations has not been examined. Upon examination in our laboratory of EF-G prepared by these procedures, significant contamination by enzymes utilizing guanine nucleotides has been detected.

While this enzymatic heterogeneity had previously limited the extent of investigation of the molecular mechanism of EF-G, a great deal of information about the function of EF-G has, nevertheless, been accumulated. It has been shown to translocate the newly formed peptidyl-tRNA from the acceptor site to the donor site on the ribosome with concomitant release of the deacylated-tRNA from the donor site. This translocation process requires the hydrolysis of a molecule of GTP to GDP and $P_i$, and also results in movement of the mRNA with respect to the ribosome thus exposing the next codon for translation (3, 8). The complexity of the over-all reaction catalyzed by EF-G makes it difficult to investigate the complete reaction directly. Fortunately, a simpler usable model system exists. Ribosomes and EF-G in the absence of all other components required for protein synthesis will catalyze the hydrolysis of GTP to GDP and $P_i$ (3). While this reaction has been identified, its mechanistic basis has not been characterized.

Previous investigations of the hydrolytic mechanism have detected no long-lived phosphorylated or guanylated protein during the course of hydrolysis. EF-G and ribosomes have been reported not to catalyze the exchange of $P_i$ into GTP, and while exchange of GDP into GTP was observed, it was attributed to the action of contaminating nucleoside diphosphate kinase (3).

*This investigation was supported by National Science Foundation Grant GB-1864 and National Institutes of Health Grant GM-17101, to J. W. B. and National Science Foundation Grant GB-18664 and American Heart Association Grant 71-830 to M. E. D.

This is Paper XIV in this series and the previous paper in the series is Ref. 1.
During the course of hydrolysis of GTP by EF-G and the ribosome, a complex containing EF-G, GDP, and the ribosome has been detected (9). A burst of liberated P$_i$ equal to the moles of EF-G present was observed in the presence of fusidic acid, an antibiotic which stabilizes the EF-G:ribosome-GTP complex (10). In this paper a fast, simple procedure utilizing affinity purification is described for the preparation of physiologically and enzymatically homogeneous EF-G, which was used to answer two questions about the hydrolytic mechanism. (a) Does EF-G and the ribosome catalyze an exchange of P$_i$, or GDP into GTP? (b) Where does the cleavage occur during the hydrolysis of GTP to GDP and P$_i$? Together with existing mechanistic information, the data obtained from this investigation should allow the proposal of a rudimentary mechanism for the EF-G-catalyzed hydrolysis of GTP.

**EXPERIMENTAL PROCEDURE**

**Materials**—Mid-log cells of Escherichia coli D (unwashed and grown on minimal medium) were obtained from Grain Processing Corp. and were the source of both EF-G and ribosomes (11). [α-32P]GTP (6.7 Ci per mmole) and [32P]Pi, were purchased from New England Nuclear Corp. [α-32P]GDP was prepared by the hydrolysis of [α-32P]GTP by ribosomes and EF-G. Conversion to [α-32P]GDP was 98%. H$_2$PO$_4$ (40% atom excess) was purchased from Bio-Rad Laboratories. GTP and GDP were purchased from Sigma Chemical Co. Polygram Cel 300 PEI thin layer chromatography plates were purchased from Brinkmann Instruments, Inc. Sephadex G-150 and DEAE-A-50 were purchased from Pharmacia. Fusidic acid was a generous gift from Miss Barbara Stearns of E. R. Squibb. All other chemicals were of the highest purity obtainable.

**Assay of EF-G by Agarose Gel Chromatography**—A reaction mixture (100 μl) containing Buffer A (10 mM Tris-Cl (pH 7.4) which was 10 mM in NH$_4$Cl, 20 mM in magnesium acetate, and 5 mM in β-mercaptoethanol), 3 mM fusidic acid, 600 μg of ribosomes [α-32P]GDP (2 × 10$^6$ cpm), and the sample to be assayed (containing less than 50 pmol of EF-G) was incubated at 0°C for 3 min. It was then applied to a column (0.6 × 30 cm) of Bio-Gel A-1.5m which had been equilibrated at ambient temperature with Buffer A containing 1 mM fusidic acid. The reaction tube was rinsed twice (100 μl) with this buffer and the protein eluted with this buffer to 20 ml per hour. The first milliliter of effluent was collected and discarded. Fractions (0.1 ml) were then collected, transferred to scintillator vials, and counted as described (12).

**Assay of Enzymatic Contaminants**—All assays were initiated by the addition of 50.6 units of EF-G and were performed by incubation of the appropriate reaction mixtures (27 μl) at 37°C for 10 min. The reactions were terminated by the addition of 10 μl of 25% formic acid. Aliquots (10-μl) of the quenched reaction mixtures were chromatographed on PEI-cellulose plates with 0.75 M phosphoric acid buffer, pH 3.4. The conversion of reactants to products was determined by elution of the nucletsides from the chromatograms and counting as previously detailed (12).

GTP$^*$ → GDP$^*$ + P$_i$ (nonsynome-dependent GTPase)

The reaction mixtures contained Buffer A and GDP ranging in concentrations from 9.82 × 10$^{-4}$ M to 1.16 × 10$^{-4}$ M and [α-32P]GTP (approximately 10$^6$ cpm).

GTP + GDP$^*$ → GTP$^*$ + GDP (nucleotide diphosphate kinase) (2)

The reaction mixtures contained Buffer A, 3.85 × 10$^{-3}$ M GTP, GDP ranging in concentration from 1.31 × 10$^{-4}$ M to 8.70 × 10$^{-7}$ M, and [α-32P]GDP (approximately 10$^6$ cpm). The reaction mixtures contained Buffer A, GDP ranging in concentration from 1.4 × 10$^{-4}$ M to 8.78 × 10$^{-7}$ M and [α-32P]GDP (approximately 10$^6$ cpm).

GDP + P$_i$ → GDP$^*$ + P$_i$ (polynucleotide phosphorylase) (13)

The reaction mixtures contained Buffer A, GDP (2.63 × 10$^{-2}$ M) and P$_i$ ranging in concentration from 1.85 × 10$^{-4}$ to 10$^{-2}$ M, and 32P$^*_i$ (approximately 25,000 cpm).

**Gel Electrophoresis**—The analysis of the physical homogeneity of each step in the preparation of EF-G was performed by sodium dodecyl sulfate gel electrophoresis on a slab gel apparatus. The method of Ornstein and Davis (14) modified so that a single gel of 10% polyacrylamido was used rather than a two-phase gel.

Disc gel electrophoresis in sodium dodecyl sulfate, of the purified EF-G both reduced and nonreduced were performed by the method of Weber and Osborn (16) as modified by Mann et al. (16). Disc gel electrophoresis of the native purified EF-G were performed by the methods of Ornstein and Davis and Williams and Reisfeld (17).

**Ribosome-dependent GTP Hydrolysis**—The reaction mixture (100 μl) contained 50 mM Tris-Cl (pH 8.8), 160 mM NH$_4$Cl, 30 mM magnesium acetate, 5 mM β-mercaptoethanol, 1200 μg of ribosomes, 50 units of EF-G, 19.75 mM GTP, and 3.6 × 10$^{-4}$ M [α-32P]GTP (approximately 10$^6$ cpm). All components except GTP were mixed and incubated for 3 min at 37°C. The reaction was initiated by addition of the reaction mixture to GTP and the reaction was allowed to proceed at 37°C. Aliquots (3 μl) were removed at timed intervals and pipetted into 5% formic acid (60 μl) to stop the reaction.

The nucleotide products of the reaction were analyzed by chromatography of aliquots (10 μl) of the final reaction mixture on polyethylene-imregnated cellulose thin layer plates. The chromatograms were developed with 0.75 M potassium phosphate, pH 3.4. After chromatography, nucleotides were located by autoradiography. The individual nucleotide spots were excised, placed in scintillator fluid, and the radioactivity was quantitated in a Beckman LS-100 liquid scintillation counter.

Quantitation of the liberated P$_i$ was performed by the method of Eibl and Lands (18).

**GDP or P$_i$, Exchange into GTP**—For the analysis of GDP exchange, the reaction mixture (400 μl) contained 10 mM Tris-Cl (pH 7.4), 10 mM NH$_4$Cl, 20 mM magnesium acetate, 5 mM β-mercaptoethanol, and 1.62 mM GTP. This mixture was preincubated for 3 min and 1 μl of either 0.5 × 10$^{-3}$ M [α-32P]GDP or 0.4 × 10$^{-3}$ M [α-32P]GTP was added. The reaction was initiated by the addition of 32 units of EF-G. Aliquots (20 μl) were removed at timed intervals, and the reaction was terminated by addition of 30% formic acid (10 μl) to these aliquots. Five and one-half minutes after the initiation of the reaction, half (150 μl) of the reaction was added to 600 μg of ribosomes (20 μl), and the incubation was continued with timed samplings from both halves of the reaction mixture.

The same procedure was used for analysis of P$_i$ exchange into GTP with the following modifications. The reaction mixture contained 1.5 × 10$^{-4}$ M P$_i$; in addition to the already specified compounds, 1 μl of 32P (approximately 1.2 × 10$^6$ cpm) was added instead of [α-32P]GDP.

GTP and GDP or P$_i$, were quantitated by thin layer chromatography as described above. From the reaction containing [α-32P]GTP, the rate of GDP hydrolysis dependent upon both EF-G and the ribosome was determined. The rate of incorporation of GDP into GTP was calculated from the reaction containing [α-32P]GTP, while P$_i$, incorporation into GTP was calculated from the reaction containing P$_i$.

**Incorporation of Solvent Oxygen During Hydrolysis**—The reaction mixture (1000 μl) contained 50 mM Tris-Cl (pH 8.8), 160 mM NH$_4$Cl, 30 mM magnesium acetate, 5 mM β-mercaptoethanol, 1200 μg of ribosomes, 800 units of EF-G, 19.60 mM GTP, 1.4 × 10$^{-4}$ M [α-32P]GTP (approximately 4 × 10$^6$ cpm), and 25 μl H$_2$PO$_4$ (40% atom excess). All components except the EF-G were mixed and preincubated 3 min at 37°C. The reaction was initiated by the addition of EF-G and the incubation continued at 37°C for 90 min. The reaction was terminated by chilling to 0°C and addition of 1 ml of cold 0.6 N HCl. The separation of the hydrolyzed γ-phosphate from the GDP, hydrolysis of the α- and β-phosphates from the GDP, purification of the phosphates, and conversion of the phosphate oxygens to CO$_2$ were performed as described by Boyer and Bryan (19).
number of $^{18}O$ atoms per phosphate was corrected for both the endogenous $P_i$ content of the GTP and the exchange of phosphate $^{18}O$ with solvent $^{18}O$ during purification. The exchange correction was based on the recovery of $^{18}O$-inorganic phosphate of known enrichment purified in parallel with the sample.

RESULTS

**Purification of EF-G**

All steps in the purification and conductivity measurements were performed between 0 and 4°C unless otherwise stated. All pH measurements were obtained at ambient temperature (22°C).

**Step 1: Preparation of S-30**—Five hundred grams of cells were thawed and suspended in 500 ml Buffer A. One milliliter of DNase (4 mg per ml) was added and the cells were ruptured in a Branson sonicator. The cellular debris was removed by centrifugation in the GSA rotor of the Sorvall RC 2B centrifuge for 1 hour at 16,000 × g. After centrifugation, the supernatant was decanted and dialyzed against Buffer A. The dialyzed preparation was assayed for EF-G by the column assay.

The purification procedure is summarized in Table I. As can be seen the value for the EF-G content of the S-30 is considerably lower than would be expected. This low estimation of EF-G has been the general case and led us to postulate that the content of EF-G in such solutions must be masked by an inhibitor which interferes in some way with the assay. Since these assays appear to be unreliable, the amount of EF-G present in such solutions was estimated as follows. In *E. coli*, ribosomal RNA accounts for approximately 80% of the 260-nm absorbance (20). Also, Gordon (21) has shown that in *E. coli* the molar ratio between ribosomes and EF-G is about 1:1. Therefore, by measuring the 260-nm absorbance and calculating the number of picomoles of ribosomes present, one has an estimation, however crude, of the number of picomoles of EF-G. Since 1 unit of EF-G corresponds to 2 pmoles of EF-G (12, 22), the total number of units theoretically present can be estimated. We have used this value for the calculation of yield and purity throughout the discussion of the preparation.

**Step 2: Affinity Purification**—To the dialyzed S-30 preparation, 700,000 pmoles of ribosomes (approximately 1/3 that amount obtained from 500 g of cells) in Buffer A and 3 × 10⁶ pmoles of GTP were added and the resulting solution was made 1 mM in fusic acid. This solution was then centrifuged in the 42 rotor of the Beckman model L preparative ultracentrifuge for 8 hours at 35,000 rpm. Each rotor tube contained a 4-ml pad of 44% sucrose in Buffer A which also contained 4.3 × 10⁻⁶ M GTP and 1 mM fusic acid. After centrifugation the supernatant was decanted and saved, and the ribosomal pellets were redissolved in Buffer B (10 mM Tris-Cl (pH 7.4) containing 1 mM NH₄Cl, 20 mM magnesium acetate, and 5 mM β-mercaptoethanol). This solution was centrifuged in the 42 rotor for 8 hours at 35,000 rpm. After centrifugation the supernatant was decanted and saved. GTP (3 × 10⁶ pmoles) was added to the first supernatant and the ribosomal pellets were redissolved in this solution. After addition of sucrose pads as above, the solution was centrifuged in the 42 rotor for 8 hours at 35,000 rpm. The resulting supernatant was combined with the second supernatant and dialyzed against Buffer C (50 mM Tris-Cl (pH 7.4) containing 150 mM KCl and 5 mM β-mercaptoethanol).

The dialyzed preparation was loaded onto a column (3 × 45 cm) of DEAE-A-50 Sephadex which had been equilibrated with Buffer C. After loading, the column was washed with 3 column volumes of Buffer C. The protein was then eluted with Buffer D (50 mM Tris-Cl (pH 7.4) containing 400 mM KCl and 5 mM β-mercaptoethanol). The column was monitored at 280 nm and fractions were assayed for EF-G by the Millipore assay (12). Fractions containing EF-G were pooled and dialyzed against Buffer E (50 mM Tris-Cl (pH 7.4) containing 50% glycerol and 5 mM β-mercaptoethanol). The affinity purification resulted in a 25-fold increase in specific activity of EF-G. The yield of EF-G for this step in purification, while constant for preparations isolated from the same cell lot, has varied from one commercial cell lot to another. The values for yield have ranged from 50 to 75% of the theoretical value described above.

**Step 3: Heat Treatment**—The protein in Buffer E was heated for 7 min at 55°C, and then mixed with an equal volume of Buffer A also at 55°C. After an additional 10 min at 55°C, the solution was transferred to ice and 3 volumes of cold Buffer A were added with mixing. The resulting solution was centrifuged in the SS-34 rotor of the Sorvall RC 2B centrifuge for 15 min at 27,000 × g. The supernatant was decanted and assayed by the Millipore assay for EF-G. This solution was then dialyzed against Buffer C.

The dialyzed protein was concentrated by loading onto a 30-ml column (50 ml syringe) of Sephadex DEAE-A-50 which had been equilibrated with Buffer C. The protein was step eluted with Buffer D. The column was monitored at 280 nm and fractions were assayed for EF-G. Those fractions containing EF-G were pooled.

Conway and Lipmann (23) had previously shown that EF-G was stable for short incubations at 55°C while EF-Tu was heat-labile. While these observations have been corroborated, it was found that in order to obtain the maximum yield of EF-G, the presence of 25% glycerol during the heating was necessary. This treatment resulted in an approximate 2-fold increase in purity of EF-G, while reducing EF-Tu 10-fold.

Concentration by chromatography on a small column of DEAE-Sephadex was used throughout the preparation rather than concentration by ammonium sulfate because it was found that ammonium sulfate concentration resulted in the loss of 40 to 60% of the activity.¹

**Step 4: Sephadex G-150 Chromatography**—The dialyzed protein was applied to a column (4.1 × 107 cm) of Sephadex G-150 which had been equilibrated with Buffer C. The chromatography was performed with Buffer C at a flow rate of 15 ml per

---

¹ Unpublished observation.
hour. Fractions (10 ml) were collected and the absorbance at 280-nm was monitored. Fractions were assayed for EF-G by the Millipore assay, and those containing EF-G were pooled.

Chromatography on Sephadex G-150 separated EF-G from both higher and lower molecular weight contaminants. A 2.5-fold increase in purity was obtained on this step.

Step 5: DEAE-Sephadex A-50 Chromatography—The pooled fractions from the G-150 chromatography were applied directly to a column (1.5 × 80 cm) of DEAE-Sephadex A-50 which had been equilibrated with Buffer C. The protein was eluted at a flow rate of 20 ml per hour with Buffer C, the conductivity of which was raised to 19 mmho by the addition of solid KCl. Fractions (5 ml) were collected and the absorbance at 280 nm was monitored. Fractions were assayed for EF-G by the Millipore assay and all fractions containing EF-G were pooled.

Elution from this column resulted in coincident activity and protein peaks. The elution was performed with a buffer of constant ionic strength, 19 mmho, which is just below that required to rapidly elute EF-G from the column. This was necessary since it was found that conventional gradient elution did not completely remove minor contaminants.

The pooled fractions were diluted to a conductivity of 15 mmho by the addition of 50 mM Tris-Cl (pH 7.4), buffer containing 5 mM β-mercaptoethanol, and concentrated on a 30-ml column of DEAE-Sephadex A-50 as described in Step 3. The fractions containing EF-G were pooled and dialyzed against Buffer E and stored for use at -20°.

The over-all fold increase in purification for this preparation was 180 with 23% of the theoretically available units recovered. Repeated preparations of EF-G have resulted in the isolation of EF-G of comparable purity. However, the over-all yield has varied between 23 and 36%. The preparation described here illustrates the minimum yields which are to be expected throughout the purification. The entire purification can be completed in less than 3 weeks and should yield a minimum of 20 mg of purified EF-G for each 100 g of cells processed.

Assay of EF-G in Crude Solution—A reliable determination of the EF-G content of crude solutions is difficult to obtain. The methods which have previously been utilized are subject to high blanks. Measurement of GTPase activity due to EF-G and the ribosome can be masked by nonribosome-dependent hydrolysis of GTP. Likewise, measurement of fusidic acid enhanced, ribosomal and EF-G-dependent binding of GDP to millipore filters is obscured by the binding of GDP to the filters by EF-Tu.

An assay procedure based on the separation of the ribosome-EF-G-GDP-fusidic acid complex from other soluble protein by chromatography on an agarose column was developed for use in the assay of EF-G in crude extracts. While this assay works well with EF-G which has been partially purified, attempts to assay crude solutions by this procedure have routinely resulted in estimations 4 to 5 times lower than would be expected.

Stability of Purified EF-G—EF-G prepared by the procedure described in this paper and stored in Buffer E at -20° has shown no loss in activity upon storage for 9 months. Less pure preparations of EF-G have been stored for over 3 years under the same conditions with no loss in activity. The presence of a thiol, such as β-mercaptoethanol, is required for the maintenance of activity. For dilutions of EF-G to less than 0.2 mg per ml, the addition of nonspecific protein, such as gelatin, is required to prevent loss of activity.

Characterization of Purity of EF-G

Physical Homogeneity—The physical composition of each step in the purification is shown in Fig. 1, a photograph of a polyacrylamide sodium dodecyl sulfate slab gel electrophoresis. From left to right the samples are Step 5, Step 4, Step 3, Step 2, and Step 1 in the purification. The last two samples are purified EF-G (Step 5) and three molecular weight standards. From the top to the bottom of the gel the molecular weight standards are bovine serum albumin (mol wt 68,000, ovalbumin (mol wt 43,000), and chymotrypsinogen (mol wt 27,000). From the position of the purified EF-G relative to the molecular weight standards its molecular weight is approximately 81,000. While this is not intended as an accurate measure of molecular weight, it does confirm previously published reports that EF-G is com-

![Fig. 1](left) Sodium dodecyl sulfate slab gel electrophoresis of each step in the purification of EF-G. The samples from left to right are Step 5 (2 μg), Step 4 (2 μg), Step 3 (6 μg), Step 2 (15 μg), and Step 1 (45 μg). The last two samples are the purified EF-G (5 μg) and three molecular weight standards, bovine serum albumin, ovalbumin, and chymotrypsinogen. The conditions for electrophoresis are described in the text.

![Fig. 2](right) Disc gel electrophoresis of purified EF-G. Shown in this figure are the disc gel electrophoreses of purified EF-G under both native and denatured conditions. The conditions used for electrophoresis are described in the text. The gels from left to right are native EF-G by the method of Ornstein and Davis, native EF-G by the method of Williams and Reisfeld (17), unred EF-G in sodium dodecyl sulfate, and reduced EF-G in sodium dodecyl sulfate. All gels contained 15 μg of EF-G.
EF-G and the ribosome will hydrolyze GTP.

The enzymatic purity of the hydrolytic system is of paramount concern. To ascertain the enzymatic purity of the hydrolytic system, three properties of that system had to be demonstrated:

1. That the hydrolysis of GTP is dependent upon both ribosomes and EF-G;
2. That no exchange of products and reactants occurs due to contamination by extraneous enzymes. The last two properties are demonstrated in Table II.

**Table II**

Analysis of enzymatic contaminates in purified EF-G

<table>
<thead>
<tr>
<th>Reaction measured</th>
<th>Amount of nucleotide reacted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. GTP* → GDP* + P_i (ribosomal)</td>
<td>320 pmol/min/unit G</td>
</tr>
<tr>
<td>2. GTP* → GDP* + P_i (norrribosomal)</td>
<td>0.003</td>
</tr>
<tr>
<td>3. GDP* + GTP → GDP* + GDP</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4. GDP* + GMP → GDP* + Pi</td>
<td>0.003</td>
</tr>
<tr>
<td>5. GDP + P_i → GDP* + Pi</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

A scan of a disc gel electrophoresis performed under both native and denatured conditions is depicted in Fig. 2. From left to right the gels are native EF-G by the method of Ornstein and Davis, native EF-G by the method of Williams and Reisfeld, denatured EF-G unreduced in sodium dodecyl sulfate, and denatured EF-G reduced in sodium dodecyl sulfate. No contaminants were visible on these gels all of which contained 15 μg of EF-G.

A scan of a disc gel electrophoresis of reduced, denatured EF-G (25 μg) run in sodium dodecyl sulfate revealed that contamination by protein other than EF-G is less than 1% of the total protein applied.

**Enzymatic Homogeneity**—The contamination of the purified EF-G by enzymes which utilize GTP or GDP as substrates is depicted in Table II. The activities shown in the table, nonribosome-dependent GTPase, GDPase, nucleoside diphosphate kinase, and polynucleotide phosphorylase, were observed in significant amounts in EF-G prepared by other procedures. The quantitation of each contaminating activity has been normalized to the amount present in 1 unit of the purified EF-G. Since the turnover number for EF-G is about 320 pmoles of GTP hydrolyzed per min per unit EF-G (3, 12, 22), it can be seen that even the largest contaminant, nonribosome-dependent GTPase, will act upon GTP at only 0.002% of the rate at which EF-G and the ribosome will hydrolyze GTP.

**Investigation of Hydrolytic Reaction**

**GTP Hydrolysis**—The use of *3*O to measure the incorporation of solvent oxygen into the GTP hydrolysis products requires the generation of micromole quantities of products. Since this necessitates the use of high levels of enzyme and prolonged reaction times, the enzymatic purity of the hydrolytic system is of paramount concern. To ascertain the enzymatic purity of the hydrolytic system, three properties of that system had to be demonstrated. First, that the only products of the hydrolysis would be GDP and P_i; second, that the hydrolysis would be dependent upon both ribosomes and EF-G; third, that no exchanges of products and reactants occurs due to contamination by extraneous enzymes. The last two properties are demonstrated in Table II.

The generation of products as a function of time during the course of hydrolysis was examined by quantitation of GTP, GDP, GMP, and P_i at each time as described under “Experimental Procedure.” The result of this analysis is shown in Fig. 3. As is apparent, the hydrolysis of GTP by ribosomes and EF-G results in a quantitative conversion to GDP and P_i in 90 min. At the end of 90 min of hydrolysis, 95% of the total nucleotide is GDP. Unhydrolyzed GTP accounts for 4% of the total and GMP represents only 1% of the total nucleotide.

Also the values for GDP and P_i are in good agreement as would be expected.

The data in Fig. 3 and Table II confirm the enzymatic purity of the hydrolytic system employed for the investigation of the EF-G and ribosome-catalyzed hydrolysis of GTP.

**Incorporation of Solvent Oxygen into Products of Hydrolysis**—The incorporation of solvent oxygen into the products of the EF-G and ribosome-catalyzed hydrolysis of GTP was quantitated by use of H_2*O. The values in Table III represent the atoms of solvent *3*O oxygen incorporated per atom of phosphate. The value of 0.61 ± 0.19 for incorporation into P_i, derived from the γ-phosphate, and 0.03 ± 0.03 for incorporation into GDP, the sum of incorporation into the α- and β-phosphates, represent the average calculated from six independent determinations. These values were corrected both for the endogenous content of P_i and GDP in the GTP, and for the exchange of phosphate *3*O oxygen with solvent *3*O oxygen during subsequent purification.

The exchange correction was determined by purification and conversion to CO_2 of *3*OP_i of known enrichment under conditions identical with those employed for the purification and conversion of phosphate derived from GTP. The per cent exchange due to these procedures was 29%.

**Exchange of P_i or GDP into GTP**—The exchange of P_i or GDP into GTP was quantitated by use of either *3*P_i or [α-3*P]-GDP under conditions identical with those used for the hydrolysis of GTP. Fig. 4 illustrates the results of the exchange experiments. Enzymatic contamination is a major concern. Since the exchange should be dependent upon both EF-G and ribosomes, any exchange observed in the absence of ribosomes is probably due to contaminants such as nucleoside diphosphate...
Fig. 4. Exchange of P<sub>i</sub> or GDP into GTP catalyzed by EF-G and ribosomes. The hydrolysis of GTP and exchange of P<sub>i</sub> or GDP into GTP is depicted in both the absence (○) and presence (●) of ribosomes. The reaction mixtures described in the text were incubated at 37°. At 5.5 min, ribosomes were added to half of the reaction mixture and incubation continued. The results are presented as the percentage of reactants converted to products. Top, percentage of [γ-<sup>32</sup>P]GTP hydrolyzed to [γ-<sup>32</sup>P]GDP as a function of time. Middle, percentage of P<sub>i</sub> converted to [γ-<sup>32</sup>P]GTP. Bottom, percentage of [γ-<sup>32</sup>P]GDP converted to [γ-<sup>32</sup>P]GTP.

Discussion

Due to its central role in the process of protein synthesis, EF-G has been the focus of investigation in a number of laboratories over the past several years. Several procedures for the purification of EF-G to apparent physical homogeneity have been published (3-7). Investigations of the process of protein synthesis using these preparations of EF-G have resulted in the elucidation of the role of EF-G in this process and have thereby contributed much basic information. However, EF-G isolated by these procedures has proven to be insufficiently pure for the examination of the mechanism of EF-G on a molecular level. These preparations proved to be contaminated with enzymes utilizing GDP or GDP as a substrate, such as nucleoside diphosphate kinase, in sufficient amounts to obscure all attempts to examine the molecular mechanism of EF-G in our laboratory.

In order to obtain EF-G free of these enzymatic contaminants a new procedure for the purification of EF-G was developed which utilized an affinity technique as the initial step in purification. The purification procedure described in this paper resulted in the isolation of EF-G in 23 to 36% yield with a specific activity of 6200 to 6700 units per A<sub>280</sub>. This protein was judged to be physically homogeneous by both native and denatured disc gel electrophoresis.

Of equal importance was the enzymatic purity of the purified EF-G. Contamination by GDPase or polynucleotide phosphorylase was less than the minimum detectable limit (0.001 pmole per min per unit G). While trace activities due to nonribosomal GDPase and nucleoside diphosphate kinase were detected, the levels of contamination were acceptably low. For every picomole of GTP hydrolyzed by the nonribosomal GDPase, 64,000 pmoles would be hydrolyzed by EF-G and the ribosome; and for every picomole of GDP exchanged into GTP by nucleoside diphosphate kinase, 107,000 pmoles of GDP would be hydrolyzed by EF-G and the ribosome.

The examination of the contaminants of the purified EF-G has also answered the question, does EF-G catalyze the hydrolysis of GTP in the absence of the ribosome? EF-G prepared in our laboratory by other methods was able to hydrolyze GTP in the absence of the ribosome at about 5% the rate of hydrolysis in the presence of the ribosome. Purification of EF-G by the procedure described in this paper has shown that the previously observed rate of GTP hydrolysis by EF-G alone was not an intrinsic property of EF-G, but rather, was due to enzymatic contamination. EF-G alone does not appear to possess GDPase activity.

In order to investigate the enzymatic mechanism of EF-G, the generation of products from reactants must be due only to the action of EF-G and the ribosome. Once generated, the products should not undergo further reaction by enzymes other than EF-G and the ribosome. If these conditions are met, then the reactions catalyzed solely by EF-G and the ribosome can be characterized by the analysis of reactants and products. Enzymatic contamination at a level where the amount of either reactants or products is modified by the action of the contaminants would make the analysis of the mechanism of EF-G difficult, if not impossible. The EF-G prepared by the method described here meets the requirements of enzymatic purity necessary for investigation of the hydrolytic mechanism.

Since 1966, when the role of EF-G in protein synthesis was first established, the broad description of the reactions in which it participates have been detailed. However, a description of the mechanism on a molecular level has not been performed. The data in this paper together with the previously published observations (3, 9, 10) on the hydrolytic mechanism, represent the basis for the first rudimentary description of the mechanism on a molecular level.

The incorporation of solvent oxygen into the products, GDP and P<sub>i</sub>, resulting from the hydrolysis of GTP catalyzed by EF-G and the ribosome was measured by the uptake of H<sub>2</sub>18O. Solvent oxygen was incorporated only into P<sub>i</sub> which was derived from the γ-phosphate of GTP and liberated during the hydrolytic reaction. Solvent oxygen (0.81 ± 0.19 atom) was incorporated into the liberated P<sub>i</sub> while incorporation into GDP was...
within experimental error of zero. From these data it can be concluded that the position of GTP cleavage during the hydrolytic reaction occurs between the γ-phosphorus atom and the oxygen atom bridging the β- and γ-phosphorus atoms. However, due to the relatively large standard deviation for the incorporation of solvent oxygen into the liberated Pi, no conclusive statement can be made about the ability of the phosphate oxygen to exchange with solvent oxygen. While analysis of 18O-labeled phosphates of known enrichment resulted in precision of ±2% repeated attempts to improve the precision in the analysis of phosphate derived from GTP have failed. We are at present unable to explain this large standard deviation.

Four possible mechanisms for the hydrolysis of GTP by EF-G and the ribosome were considered. Mechanism 1 involves the direct nucleophilic attack by water on the γ-phosphate of GTP. In this mechanism, 18O from the solvent would incorporate into the phosphate derived from the γ position and no covalent intermediates or exchange reactions would occur. In Mechanism 2, the γ-phosphate of GTP undergoes nucleophilic attack by an amino acid residue either on the ribosome or EF-G. Again 18O from the solvent would incorporate into the phosphate derived from the γ position but one would predict the occurrence of a phosphorylated intermediate and possible exchange of GDP into GTP. Mechanism 3 involves the direct nucleophilic attack of water on the β-phosphate of GTP. In this mechanism, 18O from the solvent would incorporate into the phosphate derived from the β position and one would predict no occurrence of covalent intermediates or exchange reactions. Finally, in Mechanism 4, the β-phosphate of GTP undergoes nucleophilic attack by an amino acid residue either on the ribosomes or EF-G. 18O from the solvent would incorporate into the phosphate derived from the β position and the formation of a guanylated intermediate and the possible exchange of Pi into GTP would be predicted from this mechanism.

The incorporation of solvent oxygen during hydrolysis only into the phosphate derived from the γ position is consistent with either Mechanism 1 or 2. However, it rules out both Mechanisms 3 and 4.

While we have no evidence which would distinguish between Mechanisms 1 and 2, Yamamoto et al. (25) have recently reported evidence suggesting the formation of a high energy, short lifetime phosphorylation of the ribosome as an intermediate step in the hydrolytic reaction. The exchange of GDP into GTP might be observed if a phosphorylated intermediate occurs during the hydrolytic cleavage. However, this exchange was not observed. The absence of GDP-GTP exchange in this case could be attributed to the ordered release of products. If the high energy phosphorylated intermediate was hydrolyzed before the bound GDP could exchange with GDP in solution, no measurable exchange of GDP into GTP would occur.

Fig. 5 details the proposed Mechanisms 1 and 2. In both mechanisms GTP, the ribosome, and EF-G reversibly and noncovalently bind to form the active Complex I. In Mechanism 1, the γ-phosphate of GTP undergoes direct nucleophilic attack by water. This results in the cleavage of GTP, liberating Pi, and the formation of Complex II containing noncovalently bound GDP. In Mechanism 2, the γ-phosphate of GTP undergoes nucleophilic attack by an amino acid residue in the ribosome or EF-G. This results in the cleavage of GTP and formation of a phosphorylated intermediate. The phosphorylated intermediate is dephosphorylated by the addition of water which results in the liberation of Pi and the formation of Complex II. In both mechanisms, Complex II then can reversibly dissociate, yielding EF-G, the ribosome, and GDP. For both mechanisms, the rate-limiting step in the over-all reaction is the dissociation of Complex II since under appropriate conditions this complex can be isolated (9).

In the presence of fusidic acid, Complex II is in equilibrium with both free components and Complex III (10, 12, 26). The equilibrium between Complexes II and III favors Complex III and the dissociation of this complex becomes rate limiting. Under these conditions a burst of Pi corresponding to a single round of hydrolysis is observed followed by hydrolysis at a slower rate, i.e. mediated by the dissociation of Complex III.

Both mechanisms proposed here are rudimentary ones and in no way preclude additional steps. In point of fact, any given step may be the sum of a number of steps. While the data presented here do not allow one to distinguish between Mechanisms 1 and 2, the position of cleavage during hydrolysis has been firmly ascertained. Further investigation will be necessary to define the actual reaction mechanism.

Acknowledgments We would like to thank Mr. Gary Clark for performing the disc gel electrophoresis, Ms. Lillian Lin both for performing the slab gel electrophoresis and for excellent assistance throughout the work, Yeh-Mei Chow and Ruth L. Boyd for performing the 18O analysis, and Dr. Paul D. Boyer for his valuable suggestions in the preparation of this manuscript.
REFERENCES

Preparation of Homogeneous Elongation Factor G and Examination of the Mechanism of Guanosine Triphosphate Hydrolysis
Michael S. Rohrbach, Mary E. Dempsey and James W. Bodley


Access the most updated version of this article at [http://www.jbc.org/content/249/16/5094](http://www.jbc.org/content/249/16/5094)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/249/16/5094.full.html#ref-list-1](http://www.jbc.org/content/249/16/5094.full.html#ref-list-1)