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Steady State Kinetic Analysis of the Mechanism of Guanosine Triphosphate Hydrolysis Catalyzed by *Escherichia coli* Elongation Factor G and the Ribosome†

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ABSTRACT: The mechanism of guanosine triphosphate (GTP) hydrolysis catalyzed by elongation factor G and the ribosome in the absence of other participants in protein synthesis was examined by steady-state kinetic analysis. Optimal hydrolytic conditions were determined to be approximately pH 8.0, 20 mM Mg^{2+}, and 80 mM NH_4^+. Kinetic analyses were performed under these conditions at constant elongation factor G concentrations and variable ribosome and GTP concentrations. The resulting double-reciprocal plots in conjunction with the inhibition patterns obtained with GDP indicated that the reaction occurs by an ordered mechanism in which GTP is the leading obligatory substrate. Dissociation constants for GTP and guanosine diphosphate (GDP), as well as limiting Michaelis constants for GTP and ribosomes, were calculated from the double-reciprocal plots. These values are: K_s^{GTP} = 37.0 μM, K_s^{GDP} = 16.5 μM, K_M^{GTP} = 8.0 μM, K_M^R = 0.22 μM. Inhibition was also observed at high ribosomal concentrations and suggests that inhibition was due both to the decreased breakdown of the tertiary elongation factor G·GDP·ribosome posthydrolytic complex and to the formation of a nonproductive elongation factor G·ribosome complex. A sequential mechanism with a dead-end elongation factor G·ribosome complex has been constructed to describe the hydrolysis of GTP catalyzed by elongation factor G and the ribosome.

Since its discovery (Nishizuka and Lipmann, 1966), the uncoupled GTPase[1] reaction catalyzed by elongation factor G (EF-G) and the ribosome has served as a convenient model system for the examination of the mechanism of EF-G action. The failure to detect any binary complexes or the postulated tertiary EF-G·ribosome·GTP Michaelis complex has confined the majority of the investigations of this mechanism to events occurring after the formation of the tertiary complex. These studies have shown that a relatively stable posthydrolytic EF-G·ribosome·GDP complex is formed, indicating that release of P_i precedes the release of GDP (Brot et al., 1969; Parmeggiani and Gottschalk, 1969). It has also been shown that the hydrolytic step in the mechanism is irreversible and that cleavage occurs between the γ-phosphorus atom and the oxygen bridging the β- and γ-phosphorus atoms (Rohrbach et al., 1974).

Early kinetic analyses of the reaction employed a single fixed

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[1] Abbreviations used are: EF-G, elongation factor G; GTPase, guanosine triphosphatase; GTP, GDP, guanosine tri- and diphosphates; P_i, inorganic phosphate; in denoting equilibrium constants, the superscript R refers to ribosome.

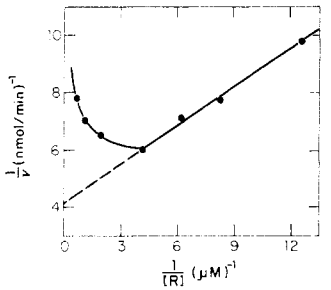

FIGURE 1: Inhibition of hydrolysis at high ribosomal concentration. The reaction solutions contained 22 nM EF-G, 13 μM GTP, and ribosomal concentrations from 1.36 to 0.08 μM. Initial velocities were determined as described under Methods after 5 min of incubation.

concentration of both EF-G and ribosomes with GTP as the only variable (Nishizuka and Lipmann, 1966; Kaziro et al., 1969). More recent kinetic studies (Chinali and Parmeggiani, 1973; Yamamoto et al., 1972) have demonstrated that the observed initial velocities are a complex function of the concentrations of EF-G and ribosomes, as well as GTP.

The recent observation in our laboratory of an EF-G·ribosome binary complex (Lin and Bodley, 1976) has prompted the present kinetic analysis of the uncoupled GTPase reaction. In this communication, we report our finding that the addition of GTP and ribosomes to EF-G is ordered with GTP being the first bound. The previously observed EF-G·ribosome complex appears to be a dead-end complex which is not involved in the hydrolytic pathway. A mechanistic model based on these findings is proposed, and the resulting equilibrium constants derived from the model are calculated.

Experimental Procedure

Midlog cells of *Escherichia coli* B were purchased from Grain Processing Corp. and were the source of both ribosomes (Bodley, 1969) and EF-G which was purified to homogeneity as previously described (Rohrbach et al., 1974). [α-^{32}P]GTP (initial sp act. 100 Ci/mmol) was purchased from New England Nuclear Corp. GTP and GDP were obtained from Sigma Chemical Co. Polygram Cel 300 PEI thin-layer chromatography plates were purchased from Brinkman Instruments, Inc. All other chemicals were of the highest grade commercially available.

Measurement of Initial Velocities. All kinetic analyses were performed at 37 °C, and, unless otherwise stated, the reaction mixtures contained 50 mM Tris-Cl, 20 mM magnesium acetate, 80 mM NH_4Cl, 5 mM β-mercaptoethanol, and varying concentrations of EF-G, ribosomes, and nucleotide. The final pH of these solutions was 8.0. Reaction solutions (100 μl) containing all components except nucleotide were preincubated for 5 min. Hydrolysis was initiated by the addition of nucleotide and terminated after periods ranging from 3 to 15 min by the addition (30 μl) of 30% formic acid. Controls containing no EF-G were run under the same conditions.

After acidification, initial velocities were determined by quantitation of the conversion of [α-^{32}P]GTP to [α-^{32}P]GDP. Triplicate samples (10 μl) from each acidified reaction solution were chromatographed on polyethyleneimine-impregnated cellulose thin-layer plates. The chromatograms were developed with 0.75 M potassium phosphate, pH 3.4. After chromatography, the individual spots containing GTP and GDP were located by radioautography, excised, placed in scintillator fluid, and quantitated in a Beckman LS-100C liquid scintillation counter. Hydrolysis was a linear function of time up to 20% conversion to GDP, and all experiments were performed in this range. Hydrolysis due to EF-G alone was insignificant (Rohrbach et al., 1974). Hydrolysis due to ribosomes alone extrapolated to 2.5 mol min^{-1} mol of ribosomes^{-1} at infinite [GTP]. Blank values for hydrolysis in the absence of EF-G were subtracted from each determination.

All stated ribosomal concentrations represent the concentration of active ribosomes as determined from their ability to form the EF-G·ribosome·GDP·fusidic acid complex (Highland et al., 1971). As the formation of this complex from GTP requires a single round of hydrolysis (Bodley et al., 1970), this measurement was judged to be a reflection of their ability to participate in GTP hydrolysis. Ribosomal preparations used in this study varied from 59 to 62% active, and the appropriate correction of spectrophotometrically determined concentrations was made.

Calculation of Rate Equations. Rate equations for various mechanistic models were calculated by the diagrammatic method of King and Altman (1956). Among the mechanisms for which rate equations were derived were the four ordered on, ordered off; the two ordered on, random off; the two random on, ordered off; and the random on, random off mechanisms.

Results

Determination of pH, Mg^{2+}, and NH_4^+ Optima. The effect of pH on initial velocity was measured over the pH range 7.5–9.2. The resulting pH profile followed a bell-shaped curve with maximum hydrolysis at pH 8.0. While this pH optimum is lower than that reported either by Kaziro et al. (1969) or Yamamoto et al. (1972), the observation that the pH of the complete hydrolysis solution was 0.6–1.0 pH unit lower than the hydrolysis buffer alone may account for this difference.

Magnesium ion is an absolute requirement for hydrolysis and, at concentrations below 5 mM, no hydrolysis was detected. The Mg^{2+} optimum was effected by the presence of NH_4^+. In its absence, the Mg^{2+} optimum was 10 mM, while the presence of 160 mM NH_4^+ shifted the Mg^{2+} optimum to 20 mM. In addition, NH_4^+ increased the rate of hydrolysis observed at the Mg^{2+} optimum. The NH_4^+ optimum, in contrast to the Mg^{2+} optimum, was rather broad with maximal hydrolysis occurring at 80 mM NH_4^+. In the experiments which follow, the hydrolyses were performed at pH and ionic conditions chosen to yield approximately maximum initial velocities, i.e., pH 8.0, 20 mM Mg^{2+}, and 80 mM NH_4^+.

Inhibition of Hydrolysis at High Ribosomal Concentrations. The uncoupled hydrolysis of GTP was examined at constant concentrations of EF-G and variable concentrations of ribosomes and GTP. Since ribosomes are not consumed in this reaction, it was possible that, at high ribosomal concentrations, inhibition would occur in a manner analogous to simple product inhibition. As shown in Figure 1, inhibition does occur at ribosomal concentrations greater than 0.25 μM. In order to avoid this inhibition and the resulting nonlinear double-reciprocal plots, the ribosomal concentrations used in the following experiments were kept below 0.25 μM. As a result, the range of ribosomal concentrations which could be employed was limited.

Dependence of Initial Velocity on Ribosomal and GTP Concentrations. The dependence of the initial velocity (v) on the concentration of GTP (437–32.8 μM) was determined at fixed variable concentrations of ribosomes. Four ribosomal concentrations, 254, 173, 127, and 104 nM, were employed and the double-reciprocal plot of v^{-1} vs. [GTP]$^{-1}$ was linear for each. The intercept values for each of the ribosomal concen-

trations employed were 6.5, 7.0, 9.0, and 10.6 min/nmol, respectively, and the slopes were 0.190, 0.218, 0.288, and 0.345 min/μl, respectively. As indicated by these data, both the apparent V_{max} and K_M are dependent upon ribosomal concentration. This result rules out three possible mechanisms. A ping-pong mechanism is eliminated, since K_M is a function of ribosomal concentration. Mechanisms in which ribosomes either are the first bound and last released component or must bind GTP before binding to EF-G are also eliminated. For both of these mechanisms V_{max} is independent of ribosomal concentration. When the observed slopes and ordinate intercepts were replotted as a function of $[R]^{-1}$, linear relationships were obtained.[2] The replot of slope vs. $[R]^{-1}$ had an ordinate intercept of 0.028 min/μl and a slope of 3.29×10^{-8} μmol min/$(\mu l)^2$, while the replot of intercept vs. $[R]^{-1}$ had an ordinate intercept of 3.05 min/nmol and a slope of 7.64×10^{-4} min/μl. From these results, the dependence of the initial velocity (v) on the concentration of GTP and ribosomes in the absence of GDP and at low ribosomal concentrations is given by:

$$\frac{1}{v} = \phi_0 + \frac{\phi_1}{[GTP]} + \frac{\phi_2}{[R]} + \frac{\phi_{12}}{[GTP][R]} \quad (1)$$

From the replots of slope and intercept vs. $[R]^{-1}$, the four kinetic constants in eq 1 were calculated. These values, in turn, were used to determine the limiting Michaelis constants and dissociation constants from the following relationships:

$$K_s^{GTP} = \frac{\phi_{12}}{\phi_2}; K_M^{GTP} = \frac{\phi_1}{\phi_0}; K_M^{R} = \frac{\phi_2}{\phi_0}$$

The values for these constants are summarized in Table I and represent the average values from two independent determinations performed at two different EF-G concentrations. In addition, the turnover number calculated from these data was 350 mol min^{-1} mol^{-1}.

While the low ribosomal concentrations employed in the experiment described above resulted in linear replots, the use of higher ribosomal concentrations (>0.25 μM) led to nonlinearity in the replots of both slope and intercept vs. $[R]^{-1}$ (data not shown). Thus, at higher ribosomal concentrations, two additional terms are required in the rate equation to describe the observed inhibition, and the rate equation becomes:

$$\frac{1}{v} = \phi_0 + \frac{\phi_1}{[GTP]} + \frac{\phi_2}{[R]} + \frac{\phi_{12}}{[GTP][R]} + \phi_3[R] + \phi_4\frac{[R]}{[GTP]} \quad (2)$$

While the double-reciprocal plots of v^{-1} vs. $[GTP]^{-1}$ in the absence of GDP eliminate a number of possible mechanisms, they cannot be used to distinguish between a mechanism which is ordered with GTP as the leading substrate and ones which have either random addition of substrates or release of products. However, the product inhibition patterns obtained with GDP can be used to distinguish among these mechanisms. The dependence of the initial velocity on the concentration of GTP (109–32.8 μM) was determined at fixed GDP (39 μM) and fixed variable ribosomal concentrations. Four ribosomal concentrations, 208, 173, 127, and 104 nM, were employed and the double-reciprocal plots of v^{-1} vs. $[GTP]^{-1}$ were linear for each. The intercept values for each of the ribosomal concentrations were 6.6, 7.5, 9.9, and 10.7 min/nmol, respectively, and the slopes were 0.850, 0.915, 1.05, and 1.28 min/μl, respectively.

[2] In all cases, greater than 97% of the ribosomes were free and it was assumed that the concentration of free ribosomes equaled that of total ribosomes.

TABLE I: Kinetic Constants for the Interactions among EF-G, the Ribosome, GTP, and GDP.

Kinetic Constant	(μM)
K_M^{GTP}	8.0 ± 1.0
K_M^{R}	0.22 ± 0.02
K_s^{GTP}	37.0 ± 6.0
K_s^{GDP}	16.5
K_s^{R}	0.15

Replots of the ordinate intercepts and the slopes vs. $[R]^{-1}$ were linear. The replot of the intercept vs. $[R]^{-1}$ had an ordinate intercept of 3.0 min/nmol and a slope of 7.94×10^{-4} min/μl. A comparison of these values with those obtained from the intercept vs. $[R]^{-1}$ replot in the absence of GDP (see above) indicated that GDP had no effect, within experimental error, on either the intercept or slope of the replot. The replot of the slope vs. $[R]^{-1}$ had an ordinate intercept of 0.45 min/μl and a slope of 8.38×10^{-8} μmol min/$(\mu l)^2$. Comparison of these values with those obtained in the absence of GDP (see above) indicated that GDP had a pronounced effect on both the intercept and slope of the secondary plot of slope vs. $[R]^{-1}$. Thus, the rate equation in the presence of GDP at low concentrations of ribosomes is:

$$\frac{1}{v} = \phi_0 + \frac{\phi_1}{[GTP]} + \frac{\phi_2}{[R]} + \frac{\phi_{12}}{[GTP][R]} + \frac{\phi_5[GDP]}{[GTP]} + \frac{\phi_6[GDP]}{[GTP][R]} \quad (3)$$

Values of ϕ_5 and ϕ_6 were calculated from the secondary plot of slope vs. $[R]^{-1}$ in the presence of GDP. These values were used, in turn, to determine the dissociation constant for GDP and the dissociation constant (K_s^R) for the release of ribosomes from the EF-G·R·GDP complex from the following equations:

$$K_s^{GDP} = \frac{\phi_{12}}{\phi_6}; K_s^{R} = \frac{\phi_6}{\phi_5 - \frac{\phi_1}{K_s^{GDP}}}$$

The values for these two dissociation constants are listed in Table I.

This pattern of GDP inhibition is consistent with an ordered mechanism in which GTP is the leading substrate. In mechanisms which involve either random addition of substrates or release of products, both the ordinate intercepts and the slopes should be dependent on GDP.

Dependence of Initial Velocity on GTP at a Fixed High Concentration of Ribosomes. While all of the above observations support an ordered mechanism, it was possible that the mechanism was random and only appeared ordered due to the high levels of GTP and low levels of ribosomes which were of necessity employed. In order to investigate this possibility, the dependence of initial velocity on GTP at a single fixed high concentration of ribosomes was examined. The ribosomal concentration used was 21 times the level at which inhibition was observed and GTP concentrations up to 5 times K_s^{GTP} were employed. These conditions should allow random addition if it were possible.

If the mechanism were random, a concave upward nonlinearity should be exhibited under these conditions. The plot of v^{-1} vs. $[GTP]^{-1}$ was linear over all GTP concentrations em-

TABLE II: Predicted Effects of Ribosomes and GDP on the Double-Reciprocal Plots for Various Reaction Mechanisms.[a]

Mechanism[b]	1	2	3	4	5	6	7	8	9	10	Obsd
V^{-1} vs. $[GTP]^{-1}$											
Intercept dependent on R	−	+	+	+	+	+	+	+	+	+	+
Slope dependent on R	+	+	+	+	+	+	+	+	+	+	+
Intercept dependent on GDP	−	+	−	−	+	+	+	+	+	−	−
Slope dependent on GDP	+	+	+	+	+	+	+	+	+	+	+
Nonlinearity at high R	−	−	−	−	−	−	+	+	+	−	−
Replot intercept vs. $[R]^{-1}$											
Nonlinearity at high R	−	+	−	+	−	+	+	+	+	+	+
Intercept dependent on GDP	−	+	−	−	+	+	+	+	+	−	−
Slope dependent on GDP	−	+	−	−	+	+	−	+	+	−	−
Replot slope vs. $[R]^{-1}$											
Nonlinearity at high R	−	−	+	−	+	+	+	+	+	+	+
Intercept dependent on GDP	+	+	+	+	+	+	+	+	+	+	+
Slope dependent on GDP	−	+	−	+	+	+	+	+	+	+	+

[a] A rate equation was derived for each mechanism by the method of King and Altman (1956), and used to determine the predicted effects listed in the table. A plus (+) indicates that the described parameter will be affected; a minus (−) indicates no effect. [b] All mechanisms refer to the order of GTP and ribosome binding to EF-G and the order of release of GDP and ribosome from the EF-G·GDP·ribosome complex. The mechanisms are: (1) R first bound, R last released; (2) R first bound, GDP last released; (3) GTP first bound, R last released; (4) GTP first bound, GDP last released; (5) R first bound, random release; (6) GTP first bound, random release; (7) random binding, R last released; (8) random binding, GDP last released; (9) random binding, random release; (10) GTP first bound, GDP last released, formation of dead-end EF-G·R complex.

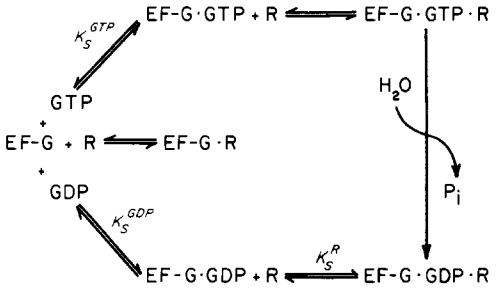

FIGURE 2: Proposed mechanism for hydrolysis of GTP by EF-G and the ribosome.

ployed (data not shown). Thus, even at high ribosomal concentrations the sequence of addition of GTP and ribosomes is ordered.

Discussion

The kinetic analysis of the mechanism of GTP hydrolysis catalyzed by EF-G and the ribosome in the absence of all other components required for protein synthesis presents an unusual problem. Most multicomponent systems contain one macromolecule which is clearly the enzyme. However, in this reaction, two of the three components which form the Michaelis complex are macromolecules, and the identity of the enzymatic species is less clear. Three observations led us to initially assume that the "active enzyme" is the EF-G·ribosome complex. First, the number of moles of GTP hydrolyzed greatly exceeds the number of moles of EF-G and ribosomes present, indicating that both EF-G an ribosomes function catalytically in the reaction. Secondly, neither EF-G nor ribosomes alone possess detectable hydrolytic activity (Rohrbach et al., 1974). Finally, an EF-G·ribosome binary complex has been observed (Lin and Bodley, 1976). From these observations, it appears that the generation of the active catalytic site requires the presence of both EF-G and the ribosome.

Based on the assumption that the "active enzyme" was the EF-G·ribosome complex, we expected the results of the kinetic analysis to resemble those for a metal-activated enzyme, i.e., only K_M and not V_{max} would be influenced by variations in ribosomal concentrations. Somewhat surprisingly, the intersecting pattern obtained in the kinetic analysis indicated that both K_M and V_{max} were dependent upon ribosomal concentration. This result eliminated both a ping-pong mechanism and an ordered mechanism in which the ribosome was the first bound and last released component.

Two important points emerge with the elimination of these two mechanisms and the observation that ribosomes, to our knowledge, are not covalently altered during the course of the reaction. First, high concentrations of ribosomes should cause inhibition. Secondly, the patterns of inhibition due to GDP and high ribosomal concentrations allow not only the elucidation of the reaction mechanism, but also the order of substrate binding in a sequential mechanism. Rate equations were derived by the method of King and Altman (1956) for ordered, partially random, and totally random mechanisms. The effects of GDP and high ribosomal concentrations on the kinetic plots, as predicted from the rate equation for each mechanism, are summarized in Table II.

Experimentally it was observed that high ribosomal concentrations cause nonlinearity in the secondary plots of both intercept vs. $[R]^{-1}$ and slope vs. $[R]^{-1}$. GDP had no effect on the secondary plot of intercept vs. $[R]^{-1}$ but did affect both the slope and intercept of the secondary plot of slope vs. $[R]^{-1}$. These data are consistent with one mechanism listed in Table II. A model for this kinetic mechanism is shown in Figure 2.

In this model, the tertiary EF-G·GTP·ribosome Michaelis complex is formed by an ordered pathway in which GTP is the first component bound. The dissociation of the posthydrolytic EF-G·GDP·ribosome complex is also ordered with GDP being the last component released. The inclusion of a binary EF-G·ribosome complex in this mechanism was required to explain the observed nonlinearity of the secondary plot of slope vs. $[R]^{-1}$ at high ribosomal concentrations. Although this binary complex has been previously observed and the requirements for its formation have been shown to be similar to those for hydrolysis (Lin and Bodley, 1976), in the mechanism it is a dead-end complex which does not enter into the hydrolytic

sequence. All of the steps in the mechanism are reversible except for the hydrolytic step (Rohrbach et al., 1974) whch results in the overall reaction being irreversible.

One rather unexpected result of these studies is the predicted existence of binary complexes involving EF-G and guanine nucleotides. It would appear that the binding site for guanine nucleotides resides on EF-G. This is in sharp contrast to the requirement of both ribosomes and EF-G for the generation of an active catalytic site. In the following paper (Baca et al., 1976), a number of different physical techniques have been used to demonstrate the formation of complexes involving EF-G and guanine nucleotides. In addition, during the preparation of this manuscript, Marsh et al. (1975) reported the existence of a nucleotide binding site on EF-G based on the protection against inhibition of EF-G by sulfhydryl reagents afforded by the presence of guanine nucleotides. These results lend strong support to the validity of the model proposed here. It should be noted that Yamamoto et al. (1972) have proposed a sequential model for this reaction in which ribosomes first bind GTP and then EF-G. This model does not agree with either the kinetic data presented here or the binary complexes which we have observed (Lin and Bodley, 1976; Baca et al., 1976).

Finally, two notes of caution should be sounded in the interpretation of the model presented here. First, the kinetic constants reported are apparent constants and are valid only for the concentrations of Mg^{2+} and NH_4^+ used in this investigation. As we have shown, the initial velocity is very sensitive to changes in the concentrations of these ions. As the original intent of this investigation was to describe the mechanism by which GTP hydrolysis occurs under maximal hydrolytic conditions, the elucidation of the effects of variable Mg^{2+} and NH_4^+ concentrations on the individual kinetic constants is beyond the scope of this work. Second, the mechanism proposed here is valid only for the hydrolysis catalyzed by EF-G and the ribosome alone. Further experimentation will be required to extend this model to the process of translocation per se. The presence of mRNA, deacylated tRNA, and peptidyl-tRNA on the ribosome will probably affect at least some of the reactions described here.

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