100.
Playtis, A. J., and Leonard, N. J. (1971), *Biochem. Biophys. Res. Commun. 45*, 1.
Robins, M. J., Hall, R. H., and Thedford, R. (1967), *Biochemistry 6*, 1877.
Skoog, F., and Armstrong, D. J. (1970), *Annu. Rev. Plant Physiol. 21*, 359.
Swaminathan, S., Bock, R. M., and Skoog, F. (1977), *Plant Physiol.* (in press).
Thimmappaya, B., and Cherayil, J. D. (1974), *Biochem. Biophys. Res. Commun. 60*, 665.
Vreman, H. J. (1973), Ph.D. Thesis, University of Wisconsin.
Vreman, H. J., Schmitz, R. Y., Skoog, F., Playtis, A. J., Frihart, C. R., and Leonard, N. J. (1974), *Phytochemistry 13*, 31.
Vreman, H. J., Skoog, F., Frihart, C. R., and Leonard, N. H. (1972), *Plant Physiol. 49*, 848.

Selective Chemical Modification of *Escherichia coli* Elongation Factor G: Butanedione Modification of an Arginine Essential for Nucleotide Binding†

Michael S. Rohrbach‡ and James W. Bodley*

ABSTRACT: Treatment of *Escherichia coli* elongation factor G with the arginine reagent, 2,3-butanedione, leads to the inactivation of the enzyme when performed in sodium borate buffers. The inhibition follows pseudo-first-order kinetics until 95% of the activity has been lost and further incubation results in complete inhibition. Removal of the borate by exhaustive dialysis results in the restoration of approximately 85% of the original activity. The pH dependence of the reaction suggests that the ionization of a group in the protein with a pK_a of approximately 8.8 facilitates the reaction with butanedione. A reaction order of 1.01 ± 0.13 was calculated for the inhibition reaction, indicating that the incorporation of one butanedione per elongation factor G results in the inactivation of the enzyme. The kinetics of inhibition in the presence of GTP indicate that the elongation factor G·GTP complex is refractory to butanedione inhibition. Elongation factor G which has been partially inactivated by butanedione has the same apparent K_m for GTP as does the native enzyme. These results indicate that elongation factor G contains only one essential arginine residue which is reactive with butanedione and that this residue is located at its nucleotide binding site.

In spite of its central role in protein synthesis, the nature of the structural features of elongation factor G (EF-G)[1] which are important for its function remains largely unknown. Although EF-G has long been known to catalyze the hydrolysis of GTP in the presence of the ribosome (Nishizuka and Lipmann, 1966), it was not known until recently whether the nucleotide binding site was on EF-G, the ribosome, or was generated by the interaction of these two molecules. Several lines of evidence now indicate that the nucleotide binding site exists on EF-G. These include equilibrium binding measurements (Arai et al., 1975; Baca et al., 1976), steady-state kinetic analysis (Rohrbach and Bodley, 1976), and the protection of EF-G by guanine nucleotides against sulfhydryl reagents (Marsh et al., 1975; Baca et al., 1976).

The existence of the binding site on EF-G for the negatively charged nucleotide suggested that basic amino acid residues in EF-G play an important part in the binding of nucleotide. Indeed with the development by Riordan (1973) of 2,3-butanedione as a highly selective arginine modifying reagent, a number of enzymes which act upon phosphate-containing substrates have been shown to contain arginyl residues at their active sites. Some notable examples of these enzymes are alcohol dehydrogenase (Lange et al., 1974), mitochrondrial and supernatant malate dehydrogenase (Foster and Harrison, 1974; Bleile et al., 1975), alkaline phosphatase (Daemen and Riordan, 1974), fructose-1,6-bisphosphate aldolase (Lobb et al., 1975), and mitochondrial ATPase (Marcus et al., 1976).

With this background, it seemed likely that arginyl residues could be important in the binding of guanine nucleotides to EF-G. In this report we describe the results of our study on the interaction of butanedione with EF-G.

Experimental Section

Materials. Elongation factor G was purified to homogeneity from *Escherichia coli* B (obtained from Grain Processing Corp.) by the method of Rohrbach et al. (1974). 2,3-Butanedione was purchased from Sigma Chemical Co. [^{3}H]GTP and [α-^{32}P]GTP were obtained from New England Nuclear Inc. Fusidic acid was a generous gift of Dr. W. O. Godtfredsen of Leo Pharmaceutical Products. All other chemicals were of the highest purity commercially available.

pH Dependence of Butanedione Inhibition. Reaction solutions (103 μL) containing 1.8 μM EF-G, 4.06 mM butanedione in either 50 mM sodium citrate–50 mM sodium borate–5 mM β-mercaptoethanol (pH 5.74 to 8.98) or 50 mM sodium bo-

† From the Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received October 8, 1976.* This investigation was supported by grants from the National Institutes of Health (GM-17101 and GM-21359). This paper is No. 24 in the series: Studies on Translocation. The preceding paper in this series is Rohrbach and Bodley, submitted for publication.

‡ Present address: Thoracic Disease Research Unit, Mayo Clinic/Foundation, Rochester, Minn. 55901.

[1] Abbreviations used: EF-G, elongation factor G; Tris, tris(hydroxymethyl)aminomethane.

rate–5 mM β-mercaptoethanol (pH 7.56 to 10.97) were incubated at 30 °C for 15 min. Aliquots (3 μL) were then withdrawn and the activity of EF-G was measured by the formation of the EF-G·[^{3}H]GDP·ribosome·fusidic acid complex. This complex was quantitated by the standard Millipore assay of Highland et al. (1971) with one modification. 50 mM Tris-Cl was used in place of 10 mM Tris-Cl in the assay solution in order to assure the correct pH. Controls containing no butanedione were run at each pH and remained 100% active over the entire range examined.

Determination of Reaction Order. Reaction solutions (103 μL) containing 50 mM sodium borate (pH 9.5), 5 mM β-mercaptoethanol, 1.9 μM EF-G, and variable concentrations of butanedione were incubated at 30 °C. Aliquots (3 μL) were removed at timed intervals and the EF-G activity was determined as described above. All reactions were allowed to proceed until a minimum of 60% of the original activity had been lost. The pseudo-first-order rate constants at each butanedione concentration were determined from a plot of log activity vs. time. The calculation of the reaction order and second-order rate constant for inhibition from the pseudo-first-order rate constants is described in the text.

Protection by GTP against Inhibition. Reaction solutions (107 μL) containing 50 mM sodium borate, 5 mM β-mercaptoethanol, 1.9 μM EF-G, 5.33 mM butanedione, and variable [^{3}H]GTP concentrations were incubated at 30 °C. Aliquots (3 μL) were removed at timed intervals and the activity was determined as described above. Pseudo-first-order rate constants were determined from a plot of log activity vs. time.

Kinetics of GTP Hydrolysis. EF-G was inactivated as described above until 40% of the original activity remained. It was then diluted tenfold into 10 mM Tris-Cl (pH 7.4), 20 mM magnesium acetate, 10 mM NH_4Cl, and 5 mM β-mercaptoethanol. Aliquots of this solution were then used in the hydrolysis reaction. The hydrolysis solution (100 μL) contained 50 mM Tris-Cl (pH 8.0), 20 mM magnesium acetate, 80 mM NH_4Cl, 5 mM β-mercaptoethanol, 7 nM EF-G, 210 nM ribosomes, and [α-^{32}P]GTP from 110 μM to 33 μM. The hydrolyses were conducted and analyzed as previously described (Rohrbach and Bodley, 1976).

Results

Inhibition of EF-G by Butanedione. When EF-G was incubated with butanedione in 50 mM sodium borate buffer, it was rapidly inactivated. The inactivation followed pseudo-first-order kinetics until over 95% of the activity had been lost, and further incubation resulted in complete inactivation. The rate of inactivation under these conditions exhibited a pronounced pH dependence (Figure 1). As shown in this figure, the second-order rate constant for inactivation increased over tenfold as the pH was raised from 7.5 to 10.0. This observed pH dependence suggests that the ionization of a group in the protein with a pK_a of approximately 8.8 facilitated the reaction with butanedione. In order to utilize conditions which maximized the rate of butanedione inhibition and minimized the pH denaturation of EF-G, all of the subsequent studies were performed at pH 9.5.

In his original investigation of the use of butanedione as an arginine specific reagent, Riordan (1973) observed that the inhibition was stabilized by the presence of borate ion and reversible in its absence. This behavior has been observed for the butanedione modification of a number of other enzymes (Daemen and Riordan, 1974; Foster and Harrison, 1974; Lange et al., 1974; Marcus et al., 1976) and was also observed

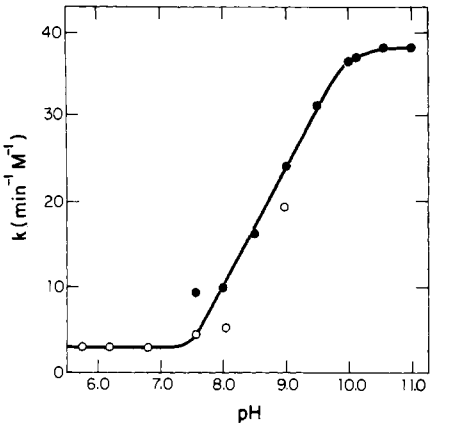

FIGURE 1: pH dependence of the second-order rate constant for butanedione inhibition of EF-G. Second-order rate constants for inhibition were obtained by dividing the pseudo-first-order rate constant at each pH by the butanedione concentration. The inhibition reactions were performed as described in Materials and Methods in either 50 mM sodium borate–50 mM sodium citrate–5 mM β-mercaptoethanol buffer (○) or 50 mM sodium borate–5 mM β-mercaptoethanol buffer (●).

TABLE I: Relationship between the Apparent Pseudo-First-Order Inhibition Constant and Butanedione Concentration.[a]

Butanedione (mM)	$k' \pm$ SD (min^{-1})
10.96	0.409 ± 0.011
5.33	0.192 ± 0.004
2.66	0.099 ± 0.003
1.60	0.058 ± 0.001

[a] The pseudo-first-order rate constants were determined from a least-squares analysis of all the points from plots of log activity vs. time for each of the butanedione concentrations listed.

for the inhibition of EF-G. This was demonstrated by inhibiting EF-G until 27% of the original activity remained, and then exhaustively dialyzing the modified enzyme against either 50 mM sodium borate (pH 9.5), 5 mM β-mercaptoethanol or 10 mM Tris-Cl (pH 7.4), 20 mM magnesium acetate, 10 mM NH_4Cl, 5 mM β-mercaptoethanol. The sample dialyzed against borate buffer remained at the same level of inhibition (23%), while the sample dialyzed against Tris buffer regained 85% of its original activity upon removal of excess butanedione and borate.

The reaction order with respect to butanedione for the inhibition of EF-G in 50 mM borate buffer (pH 9.5) was determined from the relationship between the pseudo-first-order rate constants for inhibition and the corresponding butanedione concentrations employed (Table I). For pseudo-first-order reactions:

$$k' = k[\mathrm{I}]^n \qquad (1)$$

where k' is the pseudo-first-order rate constant, k is the second-order rate constant, and n is the number of molecules of the inhibitor (I) reacting with each enzyme active site to produce the inactive enzyme·inhibitor complex. Taking the logarithm of both sides of eq 1:

$$\log k' = \log k + n \log[\mathrm{I}] \qquad (2)$$

Thus a replot of log k' vs. log[I] yields both the reaction order and the second-order rate constant. This method for determination of reaction order for enzyme inhibition reactions has

TABLE II: Dependence of the Pseudo-First-Order Rate Constant for Butanedione Inhibition on GTP Concentration.[a]

GTP (μM)	$k' \pm$ SD (min^{-1})
0	0.161 ± 0.003
110	0.096 ± 0.001
194	0.068 ± 0.001
267	0.058 ± 0.001
534	0.034 ± 0.001

[a] All inhibition reactions were carried out at a fixed butanedione concentration (5.33 mM) and the variable concentrations of GTP shown in the table. Pseudo-first-order rate constants were determined from a least-squares analysis for all the points from plots of log activity vs. time for each GTP concentration.

been successfully applied by a number of investigators (Levy et al., 1963; Scrutton and Utter, 1965; Keech and Farrant, 1968; Hollenberg et al., 1971; Marcus et al., 1976). When the data shown in Table I were plotted according to eq 2, a reaction order of 1.01 ± 0.13 and a second-order rate constant of 31.3 ± 3.0 M^{-1} min^{-1} were obtained. This suggested that the reaction of one arginyl residue per active site produced the observed inhibition.

Since the activity measurements were based upon the ability of EF-G to form the quaternary EF-G·[^{3}H]GDP·ribosome·fusidic acid complex, modification which would prevent the binding of GTP, the ribosome, or fusidic acid to EF-G would result in the observed inhibition. In order to determine if the modification was occurring at the nucleotide binding site, the inhibition was carried out at constant butanedione concentrations in the presence of variable concentrations of GTP (Table II). Scrutton and Utter (1965) have derived the followed equation for the reaction of an inhibitor with an enzyme in the presence and absence of the enzyme's substrate:

$$\frac{k'}{k'_0} = \frac{k_2}{k_1} + K_d\left[\frac{1 - (k'/k'_0)}{[S]}\right] \quad (3)$$

where k'_0 and k' are the pseudo-first-order rate constants in the absence and presence of substrate [S], k_1 and k_2 are the second-order rate constants in the absence and presence of substrate, and K_d is the dissociation constant for the enzyme–substrate complex. When k'/k'_0 is plotted against $[1 - (k'/k'_0)]/[S]$, the intercept is a measure of the maximum protection by substrate against inhibition with complete protection producing a value of 0, and the slope is equal to K_d. When the inhibition data in Table II were plotted according to eq 3, an intercept of −0.01 ± 0.11 was obtained, indicating that the reactive arginyl residue is completely protected when the nucleotide binding site is saturated with GTP. From the slope of this plot, a value of 173 ± 42 μM was calculated for the dissociation constant of the EF-G·GTP complex under these conditions.

Clearly the ability of EF-G to participate in quaternary complex formation with the ribosome, GDP, and fusidic acid is destroyed by the incorporation of one molecule of butanedione per EF-G selectively at the nucleotide binding site. We therefore examined the effect of this modification on the ability of EF-G to participate with the ribosome in the catalytic hydrolysis of GTP. EF-G was reacted until 40% of its binding activity remained and then was diluted tenfold into 10 mM Tris-Cl (pH 7.4)–20 mM magnesium acetate–10 mM NH_4Cl–5 mM β-mercaptoethanol. Upon dilution, binding activity was partially regenerated over the first 6 min and reached a stable value of 72% activity. The apparent V_{max} for GTP hydrolysis decreased following modification from 0.104 to 0.074 nmol/min, while the apparent K_m was the same for both the partially modified and control EF-G. Furthermore the ratio of the V_{max} for the partially inhibited and control EF-G was 0.71, indicating that the binding activity as measured by the formation of the quaternary complex and the ability to hydrolyze GTP are affected to the same extent by modification.

Discussion

When EF-G was incubated with butanedione in sodium borate buffer, activity was rapidly lost via pseudo-first-order kinetics. The pH dependence of this reaction (Figure 1) indicated that the ionization of a group with a pK_a of 8.8 facilitated the reaction. This pH dependence differed from that reported by Riordan (1973) for the butanedione modification of carboxypeptidase A which was maximal at pHs 7.5 and above. Since free arginine (pK_a > 12) was easily modified at pH 7.5, Riordan attributed the pH dependence to the ionization of a group other than arginine in the active site of the protein which dominated the local environment and affected the reactivity of the arginine. The same explanation would appear to be most reasonable for the pH dependence of the butanedione modification of EF-G. Alternatively this pH profile may be the reflection of borate ionization.

Although butanedione caused the complete inactivation of EF-G and the inactivation followed pseudo-first-order kinetics, the correlation between inactivation and incorporation of butanedione could not be performed by the usual method. This correlation has generally been obtained by removing aliquots at time intervals during the reaction and subjecting them to hydrolysis in vacuo in 6 N HCl. This converts the normally reversible butanedione·arginine complex into an irreversible covalent adduct of unknown structure (Riordan, 1973). The degree of modification is then determined by quantitation of arginine by amino acid analysis. Unfortunately, EF-G is a single polypeptide chain which contains 34 arginyl residues per mole (Rohrbach et al., 1975) and the loss of one arginine out of 34 is beyond the uncertainty of the amino acid analysis. Fortunately, the reaction order, i.e., the number of butanedione molecules incorporated per active site of the enzyme, can be kinetically determined. This method has been applied by a number of investigators faced with a similar dilemma (Levy et al., 1963; Scrutton and Utter, 1965; Keech and Farrant, 1968; Hollenberg et al., 1971; Marcus et al., 1976). From the kinetic analysis, it was determined that the incorporation of 1.01 molecules of butanedione would result in complete inhibition. As a check on this method, the incorporation of butanedione was determined from the kinetic data presented by Foster and Harrison (1974) on the butanedione inhibition of malate dehydrogenase. A value of 1.03 butanedione per active site was calculated kinetically as compared with a value of 1.2 as reported by the authors based upon amino acid analysis. While this method does not present direct evidence for the modification of arginyl residues, the known specificity of butanedione for arginine, the selectivity of the inactivation of EF-G, and the partial reactivation following removal of borate are consistent with the modification of an arginine.

The kinetics for GTP hydrolysis with partially modified EF-G demonstrated that the K_m was unaffected by modification. Thus the loss of activity in partially modified EF-G can be attributed to the unmodified enzyme still present rather than to a modified enzyme with different properties. In addition, the extent of inhibition when measured by hydrolysis was

identical with that measured by the stoichiometric formation of the EF-G·[^{3}H]GDP·ribosome·fusidic acid complex. This result is consistent with the observation that the modification occurred at the nucleotide binding site. Our recent examination of the mechanism of GTP hydrolysis catalyzed by EF-G (Rohrbach and Bodley, 1976; Baca et al., 1976) demonstrated that the reaction was ordered with GTP being the first substrate to bind to EF-G. Thus any modification which prevents GTP binding should result in identical loss of activity when measured either by hydrolysis or quaternary complex formation. Since GTP completely protects against this inactivation, it may be concluded that there are no other reactive arginines in EF-G which are essential to its role in GTP hydrolysis on the ribosome.

The existence of an essential arginyl residue in the nucleotide binding site of EF-G would be in agreement with the proposal of Lange et al. (1974) that arginyl residues may be essential for the binding of phosphate-containing substrates. This postulate has been reinforced by the recent reports on the role of arginyl residues in ATP requiring enzymes. Creatine kinase (Borders and Riordan, 1975), glutamine synthetase and carbamoyl phosphate synthetase (Powers and Riordan, 1975), and mitochondrial ATPase (Marcus et al., 1976) have all been shown to contain arginyl residues at the ATP binding site. In light of our present identification of an essential arginyl residue at the guanine nucleotide binding site of EF-G, it would be of interest to examine the other GTP requiring protein synthesis factors to determine if this is a general characteristic of the nucleotide binding site in these proteins.

Acknowledgments

We thank Mr. Ardin MarSchel for his helpful discussions throughout this work.

References

Arai, N., Arai, K., and Kaziro, Y. (1975), *J. Biochem. (Tokyo) 78,* 243–246.

Baca, O. G., Rohrbach, M. S., and Bodley, J. W. (1976), *Biochemistry 15,* 4570–4574.

Bleile, D. M., Foster, M., Brady, J. W., and Harrison, J. H. (1975), *J. Biol. Chem. 250,* 6222–6227.

Borders, C. L., and Riordan, J. F. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 647.

Daemen, F. J. M., and Riordan, J. F. (1974), *Biochemistry 13,* 2865–2871.

Foster, M., and Harrison, J. H. (1974), *Biochem. Biophys. Res. Commun. 58,* 263–267.

Highland, J. H., Lin, L., and Bodley, J. W. (1971), *Biochemistry 10,* 4404–4409.

Hollenberg, P. F., Flashner, M., and Coon, M. J. (1971), *J. Biol. Chem. 246,* 946–953.

Keech, D. B., and Farrant, R. K. (1968), *Biochim. Biophys. Acta 151,* 493–503.

Lange, L. G., Riordan, J. F., and Vallee, B. L. (1974), *Biochemistry 13,* 4361–4370.

Levy, H. M., Leber, P. D., and Ryan, E. M. (1963), *J. Biol. Chem. 238,* 3654–3659.

Lobb, R. R., Stokes, A. M., Hill, H. A. D., and Riordan, J. F. (1975), *FEBS Lett. 54,* 70–72.

Marcus, F., Schuster, S. M., and Lardy, H. A. (1976), *J. Biol. Chem. 251,* 1775–1780.

Marsh, R. C., Chinali, G., and Parmeggiani, A. (1975), *J. Biol. Chem. 250,* 8344–8352.

Nishizuka, Y., and Lipmann, F. (1966), *Arch. Biochem. Biophys. 116,* 344–351.

Powers, S. G., and Riordan, J. F. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2616–2620.

Riordan, J. F. (1973), *Biochemistry 12,* 3915–3923.

Rohrbach, M. S., and Bodley, J. W. (1976), *Biochemistry 15,* 4565–4569.

Rohrbach, M. S., Bodley, J. W., and Mann, K. G. (1975), *J. Biol. Chem. 250,* 6831–6836.

Rohrbach, M. S., Dempsey, M. E., and Bodley, J. W. (1974), *J. Biol. Chem. 249,* 5094–5101.

Scrutton, M. C., and Utter, M. F. (1965), *J. Biol. Chem. 240,* 3714–3723.