Inactivation of Escherichia coli Elongation Factor T₃ by the Arginine-specific Reagent Butanedione* 

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Elongation factor T₃ (EF-T₃) is inactivated by the arginine-specific reagent 2,3-butanedione. Excess elongation factor T₃ (EF-T₃), the protein substrate for EF-T₃, protects EF-T₃ from this inactivation. Borate ion affects the inactivation kinetics in a manner consistent with the formation of an arginine residue-butanedione-borate complex. The butanedione inactivation is rapidly reversible in the absence of borate ion by simple dilution, Sephadex G-25 gel filtration, or dialysis, but is irreversible in the presence of millimolar borate concentrations. Butanedione-containing solutions of EF-T₃, rapidly establish an equilibrium between free, active EF-T₃, and inactive EF-T₃-butanedione complex that is described by a dissociation constant of 3.3 ± 0.7 mM at pH 8.0. The addition of a borate molecule to the butanedione-inactivated protein showed a dissociation constant of 120 ± 50 μM. A reaction order for butanedione uptake by active EF-T₃, of 0.8 ± 0.1 mol of reagent/mol of EF-T₃, was derived under pseudo-first order conditions from inactivation rate constants. The pH profile of these rate constants showed that the protein active site becomes increasingly available for attack at more alkaline pH values irrespective of the presence of borate. Amino acid analysis of partially inactivated EF-T₃, showed the loss of 2.3 ± 0.2 arginine residues/molecule of EF-T₃, when extrapolated to 100% loss of enzymatic activity. On the other hand, inactivation of EF-T₃, by butanedione does not affect the reactivity toward N-ethylmaleimide of the cysteine of EF-T₃, which is the only other residue known to be essential to its interaction with EF-T₃. We conclude that EF-T₃, contains 2 reactive arginine residues, one of which is essential and protected by its interaction with EF-T₃.

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Escherichia coli elongation factor T₃ functions in protein synthesis to promote the recycling of EF-T₃¹ in aminoacyl-tRNA binding to the ribosome. EF-T₃, bound to GDP is released from the ribosome. EF-T₃, catalyzes the exchange of this nucleotide with free GTP in a reaction which requires the intermediate formation of a binary EF-T₃-GTP complex. This catalytic exchange precedes the formation of the EF-T₃-GTP·aminoacyl-tRNA complex which mediates aminoacyl-tRNA binding to the ribosome (1). Little is known about which features of EF-T₃, are required for the reaction except that the protein contains one essential sulphydryl group which is unreactive in the EF-T₃-GTP complex (2, 3). We have recently observed that both EF-G (4) and EF-T₃ (5) contain an essential arginine residue as judged by their inactivation by butanedione in borate buffer. These observations led us to investigate the role of arginine residues in the function of EF-T₃, and we report here on the results of these experiments.

During the past few years, arginine-specific reagents of the dicarbonyl type have been used to show the importance of arginine in the protein binding sites of many anionic substrates and effectors. Some examples are the carbamyl phosphate and ATP and CTP binding sites of aspartate transcarbamylase (6), the ATP binding site of creatine kinase (7), the NADP binding site of glutamate dehydrogenase (8), and the NADH binding sites of alcohol dehydrogenases (9). Most of the work has emphasized the importance of arginine residues in small molecule binding sites on proteins and only a few cases of an arginine requirement in protein-protein interactions have been elucidated by using arginine-modifying reagents. Some of these are carboxypeptidase A (10, 11), carboxypeptidase B (12, 13), pepsin (14), and ovine pituitary lutropin binding to cell surface receptors (15). Thus, this investigation of an essential arginine in EF-T₃, not only expands our knowledge of the active site of the protein but also adds to the significance of arginine residues in protein-protein interactions.

EXPERIMENTAL PROCEDURES

Materials—Midlog E. coli B cell paste was purchased from Grain Processing Corp. [8-³H]GDP (11.3 Ci/mmol) and N-ethyl-2-³H]ethylmaleimide (150 Ci/mmol) were from New England Nuclear. 2,3-Butanedione from Sigma Chemical Co. was freshly redistilled before use. All other chemicals were of the highest purity commercially available.

EF-T₃ and EF-T₃ Preparation—EF-T₃ with a specific activity of 1.7 × 10⁶ units/mg was prepared from E. coli B by the dissociation of the EF-T₃·ITP complex as described by Arai et al. (16). The molarity of EF-T₃ was calculated assuming 1 pmol = 56 units. EF-T₃·GDP with a specific activity of 20,000 units/mg was prepared either by the method of Arai et al. (16) or by the method of Miller and Weissbach (17). The molarity of EF-T₃ was calculated assuming 1 pmol = 1 unit. Protein concentrations were determined by the method of Lowry et al. (18). Nucleotide-free EF-T₃ was prepared by a modification of the procedure of Wittinghofer and Leberman (19). EF-T₃·GDP was incubated with [8-³H]GDP at 37°C for 10 min and dialyzed overnight at 0°C against 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, and 1 mM dithiothreitol to remove unbound GDP and magnesium ion. ITP (final concentration, 20 mM) was added to the dialyzed sample and this solution was applied to a 1-ml DEAE-Sephadex A-50 column equilibrated with diethylamine buffer containing 4 mM ITP. The column was washed with this starting buffer until no more 'H eluted from the column. The EF-T₃·ITP was eluted from the column with starting buffer containing 0.3 M KCl. The pooled EF-T₃·ITP was then passed over a Sephadex G-50 desalting column equilibrated with diethylamine buffer. The resultant EF-T₃ was used immediately after preparation and was free of ITP and contained less than 0.1 mol GDP/mol of EF-T₃.

EF-T₃ Activity Assay—The standard assay for EF-T₃ catalysis of nucleotide exchange with EF-T₃·GDP was performed by the method of Miller and Weissbach (20) except that incubations were for 1
Butanedione Inactivation of EF-T<sub>s</sub>

instead of 5 min at 0°C. One unit of EF-T<sub>s</sub>, catalyzed the exchange of 1 pmol of [8-<sup>3</sup>H]GDP into the EF-T<sub>s</sub>-GDP complex under these conditions. Assay reaction mixtures (50 μl) contained less than 0.02 μM EF-T<sub>s</sub>, plus 50 mM Tris-HCl (pH 7.4), 10 mM MgCl<sub>2</sub>, 50 mM NH<sub>4</sub>Cl, 10 mM β-mercaptoethanol, 3.4 μM EF-T<sub>s</sub>-GDP, 2.8 mM arginine, and 3.1 mM [8-<sup>3</sup>H]GDP. After 1 min of incubation at 0°C, the reaction was termin-ated by adding 9 volumes of incubation solution without EF-T<sub>s</sub> or butanedione while taking aliquots for assay of activity as described above.

RESULTS

Inactivation of EF-T<sub>s</sub>, by Butanedione in the Presence of Borate—Incubation of EF-T<sub>s</sub>, with several different concentrations of butanedione in the presence of borate resulted in the pseudo-first order inactivation of the protein as shown in Fig. 1. Pseudo-first order rate constants were derived from the slope of these semilog plots. These rate constants were in turn plotted versus the butanedione concentration in log-log plots and curve fitted to Equation 2 below. The reaction order and apparent second order inactivation rate constant were obtained from this plot as the slope and intercept, respectively. This method of determining constants follows the procedure of Levy et al. (31). The pseudo-first order rate constant (K′) is assumed to be defined by the equation:

\[ K' = K''(\text{butanedione})^n \]  

where K′′ = apparent second order inactivation rate constant and n = butanedione reaction order. Taking the logarithm of Equation 1 gives:

\[ \log K' = \log K'' + n \log(\text{butanedione}) \]  

The above curve fit yielded a reaction order of 0.8 ± 0.1 mol of butanedione/mol of EF-T<sub>s</sub>, and an apparent second order inactivation rate constant of 3.2 ± 0.4 M<sup>−1</sup> min<sup>−1</sup> at pH 8.0 and 30°C.

Riordan (10) has reported that the modification of arginine by butanedione/borate followed by acid hydrolysis causes the loss of arginine upon subsequent amino acid analysis. Consequently, loss of arginine residues from such analyses is an accepted method of quantitating arginine residue modification by butanedione/borate. The amino acid composition of un-modified EF-T<sub>s</sub>, was reasonably consistent with the two sets of published data for the protein (29, 33). Based on the molecular weight average of 32,000 (22, 23) we found EF-T<sub>s</sub>, to contain 9.4 mol of arginine residues/mol of protein. The data in Fig. 2 correlate the inactivation of EF-T<sub>s</sub>, by butanedione/borate with the concomitant loss of arginine residues. At activity levels greater than 20%, an approximately linear relationship was observed. Extrapolation to 100% inactivation yielded a loss of 2.3 ± 0.2 arginine residues/mol of EF-T<sub>s</sub>,. Upon prolonged incubation, the arginine residue further reduces activity.

Reversal of Inactivation—Simple dilution reversal was described in the section covering dissociation constant determinations. In the Sephadex G-25 gel filtration reversal, EF-T<sub>s</sub>, (6.4 μM) was incubated for 37 mm with 3.42 mM butanedione and 50 mM borate at pH 8.0 and 30°C. Carrier bovine serum albumin (50 μg) and β-mercaptoethanol (50 μM final concentration) to terminate the N-ethylmaleimide reaction, were then added. This mixture was brought to 10% (w/v) trichloroacetic acid to precipitate protein and filtered onto a Millipore filter for "H analysis of the precipitate. The filters were air-dried and tritium was determined by liquid scintillation counting.

**FIG. 1.** Butanedione concentration dependence of the inactivation of EF-T<sub>s</sub>,. The reactions were conducted in the usual way with butanedione millimolar concentrations as indicated in the figure. Incubation of EF-T<sub>s</sub>, (0.11 μM) was at 30°C in 50 mM borate buffer (pH 6.0). Time zero was defined by the addition of butanedione to the EF-T<sub>s</sub>, solution.
Except for the loss of tyrosine, no other amino acid showed significant change as a result of this modification. Yankeelov et al. (24) have observed that tyrosine residues are lost during the acid hydrolysis of butanedione/borate-modified proteins. This loss was not observed when phenol was included during hydrolysis. Accordingly, we hydrolyzed inactivated EF-T, in the presence of 1% phenol and under these conditions arginine was the only residue the recovery of which changed as the result of incubation with butanedione and borate. This result indicates that the tyrosine modification of butanedione-inactivated EF-T, occurred during acid hydrolysis and not during the inactivation reaction.

EF-T, the protein substrate for EF-T, almost completely protected EF-T, from butanedione/borate inactivation under the experimental conditions shown in Fig. 3. EF-T, was rapidly inactivated under these conditions in the absence of EF-T,. Since EF-T, is also inactivated by butanedione/borate, but more slowly at pH 9.5, the molar ratio of EF-T, to EF-T, was set at 16 to retain sufficient active EF-T, for protection. The protection of EF-T, by EF-T, is unlikely to have resulted from nonspecific effects because an equal concentration of bovine serum albumin did not affect the inactivation rate of EF-T, (data not shown).

EF-T, contains 2 cysteine residues, 1 of which in the native protein is reactive toward sulfhydryl reagents (2, 3). This reactive cysteine is apparently at or near the protein active site because its modification destroys activity and it is not reactive in the EF-T,. EF-T, complex (2, 3). Our preparations of EF-T, incorporated 0.85 mol of N-ethylmaleimide/mol of protein under the conditions described under "Experimental Procedures." The reaction was complete in 5 min. In order to determine if modification of the essential arginine residue affected the reactivity of this cysteine toward N-ethylmaleimide, we examined the uptake of N-[ethyl-2-3H]ethylmaleimide by EF-T, inactivated by butanedione. EF-T, inactivated >98% by butanedione/borate incorporated N-[ethyl-2-3H]ethylmaleimide at the same rate and to the same extent as unmodified EF-T,.

EF-T, was inactivated under pseudo-first order conditions by butanedione with or without borate buffer with a pH dependence as shown in Fig. 4. Between pH 6 and 11, EF-T, incubated as described but without butanedione, was negligibly altered in activity during the time intervals needed to determine the plotted rate constants. The apparent second order inactivation rate constants were calculated from Equation 1, assuming a reaction order of 1. Below pH 8.5, the inactivation rate was slow and unaffected by the presence or absence of borate. Above pH 8.5, the inactivation rate increased with increasing pH but to a lesser extent in the presence of borate. Presumably, borate caused this rate decrease at alkaline pH by lowering the effective butanedione concentration due to a direct borate interaction with free butanedione. We have observed that butanedione incubated in 50 mm borate at alkaline pH values slowly loses its characteristic 282 nm UV absorbance. Other buffers such as Hepes or bicarbonate did not produce a spectral change. We assume that this spectral change resulted from a chemical interaction between borate ion and free butanedione. Riordan has also reported this spectral observation (10). EF-T, inactivation rate measurements above pH 11 were unobtainable due to the lability of both butanedione and enzyme activity.

EF-T, inactivated by butanedione in borate buffer could be reactivated by the concomitant removal of the butanedione and borate. Removal of only butanedione did not result in reactivation. EF-T, was reacted with butanedione (3.4 mm)
and borate (50 mM) in the usual way until only 8% of the original activity remained. The sample was then divided in half and desalted on two Sephadex G-25 columns which were identical except one was eluted with buffer containing borate (50 mM). The sample desalted in the presence of borate remained 8% active for 8 h at 24°C. The sample that was desalted in the absence of borate under the same conditions increased in activity in a linear fashion reaching a value of 50% in 7 h. Upon prolonged incubation, 65% of the original specific activity was regained. Similar results were obtained by dialysis at 0°C, but reactivation required several days and did not exceed 65%.

**The Mechanism of Butanedione Inactivation of EF-T, in Borate Buffer**—As originally proposed by Riordan (10), the inactivation of a protein by butanedione in borate buffer appears to proceed in two steps. In the first step, butanedione adds to the guanidino group of an arginine side chain, forming a ring structure with vicinal hydroxyls. In the second step, a borate ion joins covalently with the vicinal hydroxyls generated in the first step. Mixtures of borate-buffered protein and butanedione, therefore, should contain arginine residues in three states: unreacted, reacted with butanedione, and reacted with butanedione/borate. Both reactions should be reversible and exhibit an equilibrium under appropriate conditions. EF-T, mixed with butanedione in the absence of borate, initially loses catalytic activity with pseudo-first order kinetics and then levels off to a constant per cent activity the magnitude of which is dependent on the reagent concentration (Fig. 5). The most direct interpretation of this result is that butanedione rapidly modified an EF-T, active site arginine residue, reversibly forming an inactive adduct. Using this model and the usual method used to correlate inactivation and butanedione incorporation is hydrolysis of modified protein and subsequent amino acid analysis. Acid hydrolysis converts the normally reversible butanedione-arginine complex into an unknown stable structure (10). The degree of modification is then determined by quantification of unmodified arginine by amino acid analysis. Subjecting partially modified EF-T, samples to this procedure resulted in a linear relationship between activity loss and arginine loss, which extrapolated to 2.3 ± 0.2 residues modified with complete inactivation. Thus, only 2 out of a possible 10 arginine residues in the native protein reacted with butanedione under these conditions. The known active site cysteine residue was shown not to be responsible for EF-T, inactivation by butanedione. N-Ethylmaleimide incorporation into this residue was not altered either in rate or extent by extensive butanedione/borate modification. Thus, the modification of arginine residues, as described here, does not obstruct the active site cysteine directly or indirectly in a way that alters its susceptibility to N-ethylmaleimide attack.

**DISCUSSION**

When EF-T, was incubated with butanedione in borate buffer, its activity was rapidly lost with pseudo-first order kinetics. Several lines of evidence point to an active site arginine as the single altered feature of EF-T, causing this inactivation. These are amino acid analysis, substrate protection by EF-T, and inactivation chemistry consistent with the Riordan mechanism (10) for arginine modification by butanedione in borate buffer.

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**TABLE I**

<table>
<thead>
<tr>
<th>Borate (mM)</th>
<th>Per cent EF-T, activity</th>
<th>Association constant K1 (μM)</th>
<th>Reaction order</th>
<th>Dissociation constant K2 (nM)</th>
</tr>
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The borate addition reaction was also examined. As already shown in Fig. 1, butanedione in the presence of 50 mM borate ion inactivated EF-T, with pseudo-first order kinetics to negligible activity. This is in contrast to the leveling off of activity in the absence of borate (Fig. 5). Apparently, this high borate concentration rapidly drives the reaction in the direction of the EF-T,-butanedione-borate adduct at a rate which is defined by the rate of butanedione addition. Lowering the borate concentration allowed us to observe a borate concentration-dependent equilibrium inactivation of EF-T,. Table I summarizes experiments where borate addition to mixtures of EF-T, and butanedione in equilibrium lowered the EF-T, activity plateau, dependent on the borate concentration. On the basis of the above assumptions, an average dissociation constant of 120 ± 50 μM was calculated for borate from the EF-T,-butanedione-borate adduct.
Indirect evidence for the arginine specificity of the inactivation is that its chemical characteristics follow Riordan’s mechanism (10) of sequential addition of 1 molecule of butanedione and borate ion to the arginine residue being modified. These additions both appear reversible and display dissociation constants of 3.3 mM and 120 μM, respectively, under our conditions at pH 8. Secondly, we determined the apparent second order rate constant for butanedione addition to the EF-Tᵢ active site arginine to be 3.2 and 5.2 M⁻¹ min⁻¹ in the presence and absence of borate, respectively. The decreased rate constant observed when borate is present is presumably due to the previously discussed reduction of the effective butanedione concentration caused by the interaction of free butanedione and borate.

The apparent second order rate constants for EF-Tᵢ inactivation by butanedione both in the presence and absence of borate increase with pH between 6 and 11. This could indicate that susceptibility to the reagent depends on the ionization of an arginine residue or another group in its vicinity. However, we were unable to define a pK value, leaving open other explanations for this dependence.

Although the amino acid analysis showed 2 arginines modified, incorporation of 1 molecule of butanedione/EF-Tᵢ molecule appeared to result in complete inactivation. The incorporation involved in the active site can be determined kinetically by the reaction order (12). By this method, it was determined that the incorporation of 0.8 molecule of butanedione resulted in complete inactivation. The simplest interpretation of this and the amino acid analysis result is that EF-Tᵢ possesses 2 arginine residues with comparable reactivity toward butanedione, only 1 of which is essential to its interaction with EF-Tᵢ⁺.

The results presented here taken together with earlier work with sulfhydryl reagents (2) indicate that EF-Tᵢ⁺ contains 1 cysteine and 1 arginine residue, both of which are essential to its role in the binding to and recycling of EF-Tᵢ₋.

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