Limited Trypsinolysis of Native *Escherichia coli* Elongation Factor G†

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ABSTRACT: Native elongation factor G possesses three peptide bonds or limited regions of the molecule which are especially sensitive to trypsinolysis. Cleavage at these sites occurs in sequence. Initially, the protein, a single peptide chain of 74,000 daltons, is rapidly split into a fragment of 71,000 daltons and one or more small peptides totaling 3000 daltons. This initial scission does not alter the gross three-dimensional structure of the protein, as the native and trypsin-cleaved protein have the same K_d on Sephadex gel filtration. Although cleaved, the modified elongation factor G retains full activity as measured by its ability to form complexes with the ribosome and guanine nucleotides. A single peptide bond (or region) in the 71,000-dalton fragment is then completely cleaved, yielding fragments of 47,000 and 29,000 daltons. These fragments do not remain associated under native conditions, as they are clearly resolved on gel filtration. Loss of activity is concomitant with the scission of the 71,000-dalton fragment. The third cleavage is incomplete under the conditions employed here and occurs in the 47,000-dalton peptide generating a fragment of 45,000 daltons and one or more small peptides totaling 2000 daltons. Prolonged treatment with higher levels of trypsin ultimately reduces the protein to small peptides but without generating further discrete fragments visible on sodium dodecyl sulfate gel electrophoresis.

Limited proteolysis under nondenaturing conditions has been a useful tool in the analysis of the structural and functional properties of a number of enzymes which are composed of large single peptide chains. This approach can provide information about the overall organization of the peptide chain and is of particular benefit when applied to enzymes which catalyze complex reactions. Notable examples of the application of this approach are found in the study of DNA polymerase (Setlow et al., 1972; Setlow and Kornberg, 1972) and amino acyl-tRNA synthetase (Piszkiewicz and Goitein, 1974; Koch et al., 1974; Lee, 1974).

Elongation factor G (EF-G)[1] is composed of a single peptide chain of 74,000 daltons (Rohrbach et al., 1975). The protein interacts with the ribosome and GTP in bringing about the seemingly complicated process of translocation. Little is known about the overall structure of EF-G or of the features of the molecule which are involved in its various interactions. As part of an ongoing investigation of the structure of the protein and its function in protein synthesis we report here on the generation of specific peptide fragments when native EF-G is subjected to trypsinolysis.

Experimental Section

Material. Elongation factor G was purified to homogeneity from *Escherichia coli* B (obtained from Grain Processing Corp.) by the method of Rohrbach et al. (1974). Bovine pancreatic trypsin and soybean trypsin inhibitor were purchased from Sigma Chemical Co.[^{3}H]H_2O, [^{3}H]GTP, [α-^{32}P]GTP, and [^{3}H]GDP were obtained from New England Nuclear Inc. GMPPCP was purchased from Miles Laboratories and tritiated by catalytic exchange by New England Nuclear Corp. The labeled nucleotide was subsequently purified by linear gradient elution (0.1–0.5 *M* LiCl) from a column (0.45 × 45 cm) of DEAE-Sephadex A-25. [^{3}H]EF-G was prepared as described by L. Lin and J. W. Bodley (manuscript in preparation). Fusidic acid was a generous gift from Dr. W. O. Godfredsen of Leo Pharmaceutical Products. All other chemicals were of the highest purity available.

Assays of Binding Activity. The activity of EF-G was measured by the formation of the EF-G·ribosome·[^{3}H]GDP complex which was quantitated by the standard Millipore assay of Highland et al. (1971). The ability of trypsin treated EF-G to form this complex with other guanine nucleotides was determined by the substitution of either [^{3}H]GDP or [^{3}H]GMPPCP for [^{3}H]GTP in the assay.

Tryptic Hydrolysis of EF-G. EF-G (285 μg/ml in 0.1 *M* Tris-Cl (pH 8.1) containing 5 m*M* β-mercaptoethanol) was incubated with trypsin (5 μg/ml) at 37°. At timed intervals, aliquots (60 μl) were removed, and 0.8 μg of soybean trypsin inhibitor was added to terminate the reaction. Control experiments indicated that trypsinolysis was arrested under these conditions and that trypsin and trypsin inhibitor did not interfere with the assay of EF-G. Samples (2 μl) from these quenched enzyme solutions were assayed for binding activity as described above. The remainder of each solution was denatured with sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis (see below).

Electrophoresis. Sodium dodecyl sulfate gel electrophoresis of EF-G and its digestion products was conducted in two ways. In both cases protein was visualized by staining with Coomassie Blue followed by diffusion destaining in 7.5% acetic acid. For the purposes of comparative qualitative visualization, as shown in the photographs here, samples were analyzed on the slab gel apparatus as described by Hawley et al. (1973) and modified by Rohrbach et al. (1974). For the quantitative analysis of the amounts and

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[1] The abbreviation GMPPCP is used for guanosine (β,γ-methylene)triphosphate and EF-G designates elongation factor G in accordance with the convention published in *Science 176,* 195–197 (1972).

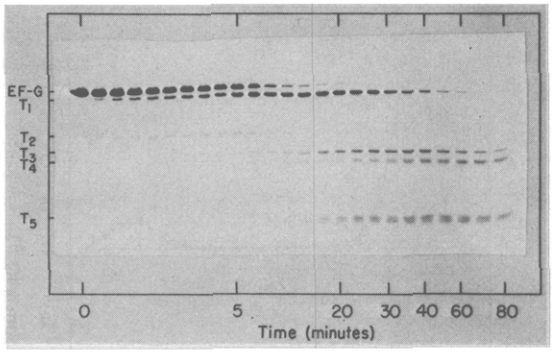

FIGURE 1: Time course for the generation of tryptic fragments. Aliquots were removed from the tryptic digestion mixture at timed intervals and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis as described in the Experimental Section. Each well contained 3 μg of protein.

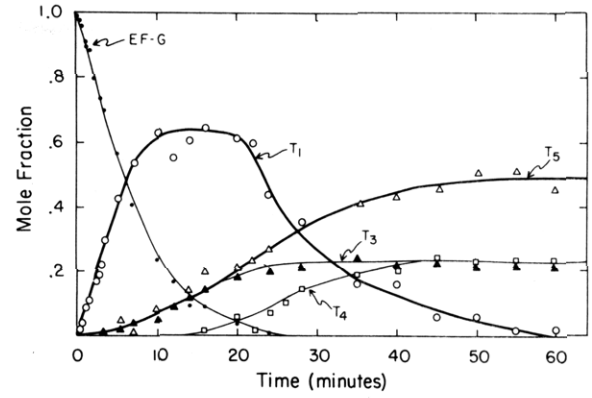

FIGURE 2: Kinetics of the generation of the tryptic peptides. The mole fractions of the peptides at each time point were calculated from the densitometer tracing of the sodium dodecyl sulfate polyacrylamide gel electrophoresis as described in the Experimental Section. The peptides shown in the figure are EF-G (●), T1 (○), T3 (▲), T4 (□), and T5 (△).

molecular weights of the various digestion products, samples were analyzed on 0.5 × 7.5 cm cylindrical gels by the method of Weber and Osborn (1969) as modified by Mann et al. (1971). Molecular weights were determined by comparison to the following standards: EF-G (74,000), bovine serum albumin (69,000), catalase (57,000), ovalbumin (43,000), carbonic anhydrase (30,000), chymotrypsinogen (23,600). The gels were scanned at 500 nm on a Gilford Model 221A spectrophotometer to determine the quantity of the digestion products. The mole fraction of each fragment was calculated from its corresponding area and molecular weight using the following equation:

$$\text{mole fraction} = \frac{\text{area/mol wt}}{\Sigma(\text{area/mol wt})}$$

Implicit in this calculation are the assumptions that the area of any fragment is proportional to its mass and that this proportionality is constant for all fragments.

Gel Filtration. Chromatography of native and trypsin modified EF-G was performed on columns (0.5 × 50 cm) of Sephadex G-100. The proteins were eluted with 50 m*M* Tris-Cl (pH 7.4) containing 5 m*M* β-mercaptoethanol. [^{3}H]EF-G and [^{3}H]H_2O were added to each sample prior to chromatography and were used to normalize the elution profiles of the individual chromatographies. The protein elution profile was determined from the 235-nm adsorbance of each fraction. The internal markers, [^{3}H]EF-G and [^{3}H]H_2O, were located by counting 20-μl aliquots from each fraction. The elution position of binding activity was assayed as described above using [α-^{32}P]GTP in place of [^{3}H]GTP. For those chromatographies in which the tryptic fragments were subsequently examined by electrophoresis, aliquots from each fraction were denatured in sodium dodecyl sulfate and subjected to electrophoresis as described above.

Results

When native EF-G is incubated with trypsin, only a limited number of the potentially sensitive peptide bonds are cleaved. A time course of tryptic digestion analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis is shown in Figure 1. A total of five large tryptic peptides,[2] ranging in size from 71,000 to 29,000 daltons (see below), were generated during the course of hydrolysis. The three smaller fragments appear as terminal cleavage products under these conditions while the larger two are transitory intermediates in the degradation sequence.

Table I: Molecular Weights, R_f, and Maximum Mole Fractions of Tryptic Peptides.

Fragment	R_f	Molecular Weight	Maximum Mole Fraction
EF-G	0.205	74,000	1.00
T1	0.235	71,000	0.64
T2	0.390	53,000	0.02
T3	0.450	47,000	0.25
T4	0.480	45,000	0.24
T5	0.685	29,000	0.51

The complexity of the fragment pattern shown here prevented the determination of the degradation pathway by simple inspection. However, the degradation sequence can be elucidated from the relationship among the mole fractions of each fragment as a function of time. Calculation of the fragment mole fractions required the prior determination of the molecular weights of these fragments which were estimated from their electrophoretic mobilities on sodium dodecyl sulfate polyacrylamide gels. The mobilities of six molecular weight standards when plotted as the log of their molecular weight vs. their R_f yielded a linear relationship (data not shown). The molecular weights of the tryptic peptides were determined by reference to these standards and the resulting values are summarized in Table I. Using these molecular weights, the mole fractions were calculated as described in the Experimental Section. The resulting plot of the mole fraction for intact EF-G and each fragment as a function of time of trypsinolysis is shown in Figure 2.

This figure suggests the following sequence of events during tryptic hydrolysis of native EF-G. Upon addition of trypsin, a single peptide bond is rapidly cleaved. This scission generates fragment T1 (71,000 molecular weight) and one or more small peptides too small to be detected under these analytical electrophoretic conditions. The molecular weight of this small peptide(s) is approximately 3000 as calculated from the difference in molecular weights of EF-G and T1. Only after the initial cleavage which generates T1 does a second peptide bond become accessible to trypsin. The hydrolysis of this bond splits T1 into fragments

[2] The apparent doublet corresponding to T5 is an electrophoretic artifact of this particular electrophoresis.

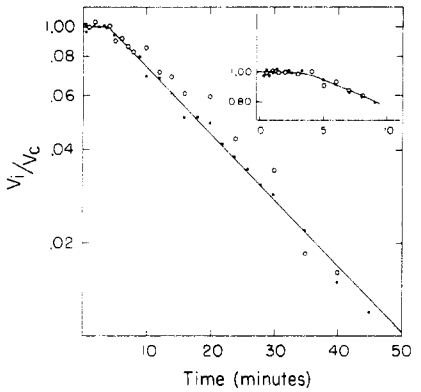

FIGURE 3: Kinetics of the loss of activity. Aliquots of the digestion mixture were removed at the indicated times and the trypsinolysis was terminated by the addition of soybean trypsin inhibitor. The activity (●) was measured by Millipore filtration as described in the Experimental Section. Also shown is the sum of the mole fractions of EF-G plus T1 (○) at each time as determined in Figure 2. The insert to the figure represents an expansion of the first 10 min of digestion with additional data points.

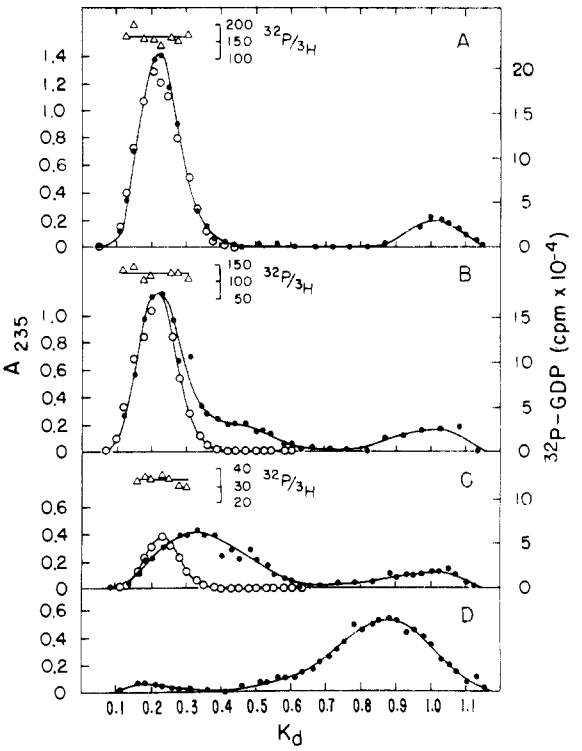

FIGURE 4: Sephadex G-100 chromatographies of digestion solutions. Internal markers of $[^3H]H_2O$ and [^{3}H]EF-G were included in each chromatography to normalize elution positions. The absorbance at 235 nm (●) and binding activity (○) were measured as described in the Experimental Section. The ratio of [^{32}P]GDP binding activity of the digested protein to internal [^{3}H]EF-G marker (△) is shown in the inserts of the figure. Panel A is undigested EF-G. Panels B and C are digests terminated after 18 and 36 min, respectively. Panel D is a total digestion of EF-G.

T3 (molecular weight 47,000) and T5 (molecular weight 29,000). While the sum of the molecular weights of T3 and T5 is greater than that of T1, the discrepancy is within the 10% error limits intrinsic to the gel electrophoresis method of molecular weight determination (Weber and Osborn, 1969). The generation of T3 exposes a previously unavailable peptide bond in T3 which is partially cleaved by trypsin yielding fragment T4 (molecular weight 45,000) and one or more undetectable small fragments totaling approximately 2000 daltons. The supposition that T4 is generated by cleavage of T3 is supported by the observation that the sum of the mole fractions of T3 and T4 is equal to the mole fraction of T5 throughout the course of hydrolysis. Since these are the only fragments visible after prolonged digestion, their mole fractions (Figure 2 and Table I) sum to unity.

Two features of the kinetics of fragment appearance (Figures 1 and 2) suggest that the three specific cleavages observed here must occur in sequence. First, it is clear that fragment T4 appears only after a substantial time lag. In fact, the kinetics of its appearance indicate that the cleavage which generates it can occur in T3 but not in T1. Close inspection of these data revealed a similar but shorter lag in the appearance of T3 and T5 while T1 appeared without lag. In addition, if the cleavage which generates T3 and T5 could occur in both EF-G and T1 then either T3 or T5 should be accompanied, at least transiently, by a second band with an apparent molecular weight 3000 higher. A similar argument applies to the cleavage which generates T4 and no such doublets were detected.

The maximum yield (expressed as mole fraction) of each fragment during the course of hydrolysis is listed in Table I. The mole fraction of T2 did not exceed 0.02. Its appearance only in the first 15 min of digestion and the lack of any detectable fragments which would correspond to its breakdown suggested that T2 arose through a minor cleavage. It was therefore not included in Figure 2.

The mole fraction values for T3, T4, and T5 did not change appreciably from their values after 60 min of digestion even when the digestion was carried out for 24 hr. This may have resulted from the autodigestion of trypsin. However, a second addition of a larger amount of trypsin, followed by overnight incubation, resulted in complete conversion to peptides too small to be detected on electrophoresis. This additional cleavage occurred without the intermediate generation of detectable discrete fragments and was therefore random cleavage by this criterion (data not shown).

We have not examined the terminal sequences of the fragments generated during this digestion. As a consequence we were unable to distinguish between cleavage at unique peptide bonds as opposed to cleavage at one of several bonds in limited regions of the molecule. The only observation which bears on this point is the apparent homogeneity of the fragments on sodium dodecyl sulfate gel electrophoresis. When scanned, the individual bands on these gels produced sharp symmetrical profiles (data not shown). While this result does not exclude multiple scission points, at the least it indicates that they must occur within closely defined areas of the molecule.

Somewhat surprisingly, even though EF-G was immediately and rapidly cleaved by trypsin, binding activity was unchanged during the first several minutes of hydrolysis. A plot of binding activity as a function of time of hydrolysis is shown in Figure 3 (closed circles). The insert in the figure represents an expansion of the first 10 min of digestion with additional data points, which more clearly illustrates the initial lag phase. Following this initial lag phase, there was a rapid, pseudo-first-order inactivation of the protein which was essentially complete (activity reduced to less than 10%) in 50 min. Similar results were obtained when either GDP or GMPPCP was used in place of GTP in the assay (data not shown).

The retention of full binding activity during a time period

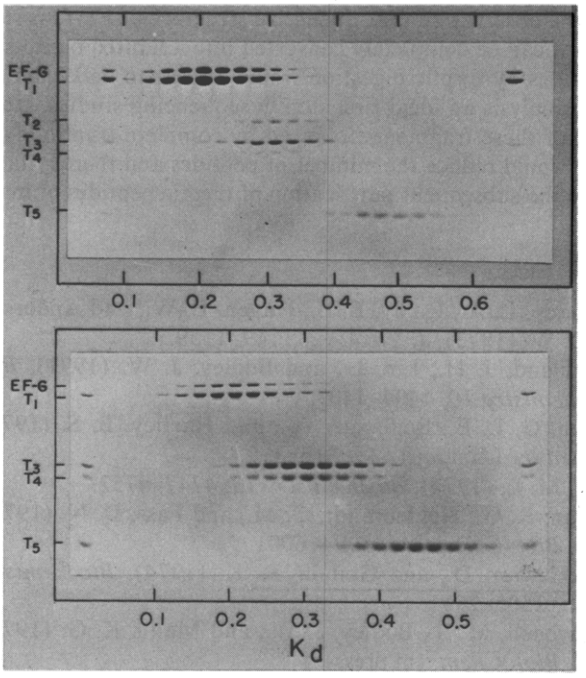

FIGURE 5: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the gel chromatography fractions. The top panel corresponds to the chromatogram in 4B, while the bottom panel corresponds to 4C. In both electrophoretograms, the first and last well contained the unchromatographed digests.

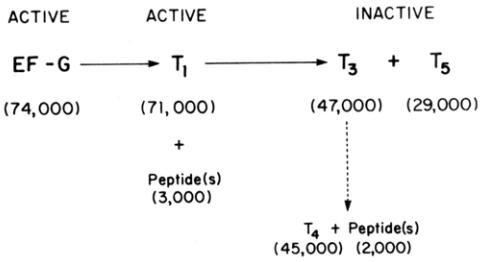

FIGURE 6: Tryptic degradation scheme of native elongation factor G. The molecular weights of the tryptic fragments are shown in parentheses under each fragment. Solid arrows indicate a complete cleavage while the dashed arrow represents a partial cleavage.

when up to 40% of the EF-G had been cleaved by trypsin suggested that the initial scission did not affect the ability of the protein to form nucleotide-containing complexes. This was confirmed by the demonstration that the curve expressing the sum of the mole fractions of EF-G and T1 as a function of time is identical with that for the loss of activity (Figure 3). This observation also indicates that the loss of activity accompanies the degradation of T1.

The trypsin fragments of EF-G were also subjected to gel filtration chromatography under nondenaturing conditions in order to determine the extent to which they remained in association. These experiments might also be expected to reveal low level activity associated with the fragments derived from T1 which would not have significantly altered the relationship shown in Figure 3. In order to maximize the reproducibility of these columns internal markers of [^{3}H]H_2O and [^{3}H]EF-G were included. [^{3}H]EF-G, which was present at levels too low to be detected by assay, also provided a sensitive reference with which to detect changes in molecular weight which might have resulted from the release of the small peptide(s) produced by the first cleavage.

Two digests were subjected to this analysis. The first (Figure 4B) was selected to maximize T1 and minimize EF-G and the second to maximize T3, T4, and T5 (Figure 4C). For comparative purposes chromatograms are also shown for undigested (Figure 4A)[3] and completely digested EF-G (Figure 4D). The A_{235} elution profile of both digests revealed significant reduction in the apparent molecular weight of a portion of the protein. In both cases, however, the remaining EF-G activity was coincident with the internal [^{3}H]EF-G marker. This coincidence is particularly apparent in the ratio of activity ([^{32}P]GDP binding) to [^{3}H]EF-G marker shown in the inserts of the figure.[4]

It seems likely on the basis of this result that the small peptide(s) generated during the first cleavage remains associated with T1. However, since we have not looked directly for this peptide(s), the possibility remains that its dissociation is not accompanied by a corresponding decrease in the hydrodynamic volume of the remaining protein.

In these analyses, the digested protein of lower molecular weight was not resolved into discrete components as judged by simple absorbance measurements of the column effluent. Therefore, the effluent was subjected to sodium dodecyl sulfate gel electrophoresis analysis in order to establish the position of the individual fragments. It is clear from this analysis (Figure 5) that T3 and T5 are almost entirely resolved from each other. Moreover, chromatography on these columns of the standard proteins employed in the calibration of the sodium dodecyl sulfate gels indicated that T3 and T5 eluted in positions predicted on the basis of their molecular weight (data not shown). On this basis these fragments do not appear to interact with each other.

As already noted, no activity was detected in the portions of the Sephadex chromatogram which corresponded to the elution positions of any fragments but T1. Fractions containing the various peptides were assayed for activity. As shown in Table II, these peptides either individually or in combination with one another were inactive in complex formation.

Discussion

When native EF-G is subjected to limited tryptic digestion, only three peptide bonds appear to be available for cleavage. These bonds are cleaved in sequential order. The proposed tryptic degradation scheme of native EF-G is shown in Figure 6.

In its native conformation, only one potentially sensitive bond in EF-G is accessible to trypsin. While this peptide bond is rapidly cleaved, the scission neither detectably alters the gross three-dimensional structure of the protein nor affects its ability to form a complex with the ribosome, GDP, and fusidic acid. The initial cleavage does, however, expose a previously inaccessible peptide bond. Cleavage at this site destroys activity and the two resulting peptides dissociate under native conditions. The final cleavage site is made accessible only after fragment T3 has been generated. In contrast to the first two scissions, this final cleavage is not complete under the experimental conditions employed

[3] The small peak of 235-nm absorbing material occurring in the column volume of the chromatogram of undigested EF-G resulted from a slight excess of β-mercaptoethanol in the reaction mixture over the column elution buffer.

[4] In control experiments with this column, bovine serum albumin (69,000) was clearly separated from EF-G. The A_{235} albumin/[3H]EF-G ratio varied by a factor of 4 across the peak.

Table II: Nucleotide Binding Activity of Tryptic Peptides.[a]

Peptides	Cpm
EF-G and T1	16,000
T3 and T4	100
T5	0
Small peptides	102
T3 and T4 + T5	0
T5 + small peptides	200
T3 and T4 + T5 + small peptides	150

[a] The ability of the tryptic peptides to form nucleotide-containing complexes was determined by Millipore filtration as described in the Experimental Section. Shown are the cpm of [α-^{32}P]GDP retained on the filters.

here.

A crude description of the three-dimensional structure of EF-G emerges from these results. It would appear that its single polypeptide chain is folded in such a way that the molecule is formed into two unequal sections with only minimal noncovalent interactions between them. A lysine or arginine residue located 25 to 30 amino acids from one terminus of the chain is readily accessible to the solvent and is the site of the initial rapid cleavage by trypsin. This cleavage does not alter the gross three-dimensional structure of the protein, but does cause a local conformational change which exposes a second trypsin-sensitive bond. This bond is probably located in a section of the polypeptide chain which joins the two large sections together. Its cleavage destroys activity and allows the two sections to separate.

The observation that the native structure of EF-G is such that it can be completely converted into a limited number of peptides by tryptic digestion would appear to make limited trypsinolysis an ideal first step in sequencing studies. Isolation of these fragments followed by complete tryptic digestion would reduce the number of peptides and thereby facilitate the subsequent purification of tryptic peptides of interest.

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