Registry No. Lysozyme, 9001-63-2; Trp, 73-22-3; gadolinium, 7440-54-2.

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Limited Cleavage of Eucaryotic Elongation Factor Tu by Trypsin: Alignment of the Tryptic Fragments and Effect of Nucleic Acids on the Enzymatic Reaction†

Lawrence I. Slobin,* Ralph V. Clark, and Mark O. J. Olson

ABSTRACT: Treatment of eucaryotic elongation factor Tu (eEF-Tu; M_r 53 000) with trypsin in the presence of 25% (v/v) glycerol results in cleavage of the factor at two sites and generates a single polypeptide of 43 000 daltons termed eEF-Tu^t and low molecular weight peptide fragments [Slobin, L. I., Clark, R. V., & Olson, M. O. J. (1981) *Biochemistry 20*, 5761–5767]. Digestion of eEF-Tu with carboxypeptidase A for varying lengths of time indicated that the carboxyl-terminal sequence of the factor is Ala-Ser-COOH. The presence of serine at the carboxyl-terminal position was confirmed by hydrozinolysis. eEF-Tu^t was found to possess the same carboxyl-terminal sequence, proving that the low molecular weight peptide fragments released by trypsin, designated T1 and T2, originate from the amino-terminal end of the factor. Peptide T2 contains a blocked amino-terminal residue, as does the starting eEF-Tu, and has a M_r of ca. 4000 based on gel electrophoresis and amino acid composition. Peptide T1 has a M_r of 6000 based on gel electrophoresis and amino acid composition. Taken together, T1, T2 and eEF-Tu^t account for essentially all of the mass of eEF-Tu. Amino-terminal sequence analysis of T1 reveals as striking sequence homology with the amino-terminal sequence of EF-Tu. In particular, the sequence Glu-Lys-Phe-Glu occupies positions 3–6 in T2 and 3–6 in EF-Tu. Other sequence homologies are evident in the first 14 residues of T1 and EF-Tu. Treatment of eEF-Tu in the presence of 28S rRNA markedly accelerates the rate of trypsin cleavage of the factor, whereas treatment of eEF-Tu in the presence of 28S rRNA and in the absence of glycerol stabilizes eEF-Tu^t against trypsin cleavage. By contrast, cleavage of eEF-Tu by trypsin is strongly inhibited by the presence of aminoacyl-tRNA and GTP in reaction mixtures. These results, when combined with additional structural information, suggest that the aminoacyl-tRNA binding site of eEF-Tu is located at the amino-terminal end of the factor.

Recent work in this laboratory has revealed some structural homology between bacterial elongation factor Tu and its functional homologue in eucaryotic cells, eEF-Tu (Slobin et al., 1981).[1] It was found that treatment of eEF-Tu with trypsin resulted in cleavage of the factor at at least two sites, producing a single polypeptide of 43 000 daltons, eEF-Tu^t, and two or more unidentified peptides of M_r ca. 5000. Amino-terminal sequence analysis of eEF-Tu^t showed that the first

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[1] Abbreviations: EF-Tu, procaryotic elongation factor Tu; eEF-Tu, eucaryotic elongation factor Tu; aa-tRNA, aminoacyl-tRNA. eEF-Tu has been referred to by other investigators as EF-I_α or EF-1_L.

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four residues, Gly-Ile-Thr-Ile, are identical with the first four residues of a 37 000-dalton tryptic fragment of *Escherichia coli* EF-Tu. Other sequence homologies were evident in the first 12 amino-terminal residues of the corresponding tryptic fragments. Evidence was presented that at least one of the tryptic peptides derived from eEF-Tu originated from the amino-terminal end of the factor.

In this report we show that both low molecular weight tryptic peptides originate from the amino-terminal end of eEF-Tu. A partial sequence of one of these peptides indicates that it is structurally homologous to the amino-terminal end of EF-Tu from *E. coli*. Finally, it has been shown elsewhere that eEF-Tu contains two distinct nucleic acid binding sites (Slobin, 1983). Using sensitivity to trypsin cleavage as a structural probe, we present evidence that the aminoacyl-tRNA binding site of eEF-Tu is located at the amino-terminal end of the factor, whereas the other nucleic acid binding domain is located within eEF-Tu^t.

Experimental Procedures

Materials. Purified eEF-Tu from rabbit reticulocytes was prepared as described previously (Slobin et al., 1981). Trypsin treated with N^{α}-tosyl-L-phenylalanine chloromethyl ketone was obtained from Worthington. Soybean trypsin inhibitor, carboxypeptidase A treated with diisopropyl fluorophosphate, molecular weight marker proteins, nucleotides, and synthetic polynucleotides were purchased from Sigma. All other chemicals were reagent grade or better. 28S rRNA from rabbit reticulocyte polysomes was purified as described (Slobin, 1983). Rabbit liver transfer RNA was charged with [^{3}H]-lysine by using crude rabbit reticulocyte aa-tRNA synthetases (Slobin, 1983). The charged tRNA contained 62 pmol of lysine/A_{260} unit of nucleic acid.

Trypsin Treatment of eEF-Tu. Tryptic hydrolysis of eEF-Tu was performed exactly as described previously (Slobin et al., 1981). The buffer used for trypsin treatment (buffer A) contained 50 mM Tris-HCl, pH 7.5, 5 mM magnesium acetate, 1 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.5 mM ethylenediaminetetraacetic acid, and 25% (v/v) glycerol. The reaction was stopped by the addition of a 2-fold excess (w/w) of soybean trypsin inhibitor.

Amino Acid and Sequence Analysis. Proteins and peptides were hydrolyzed in 5.7 M HCl at 110 °C for 22 h in tubes flushed with nitrogen and sealed in vacuo. Amino acid compositions were determined on a Beckman 119 CL amino acid analyzer.

Amino acid sequence analyses were performed on a Beckman 890-C sequenator equipped with a cold trap attachment and a Sequemat P-6 auto converter. A program similar to that of Brauer et al. (1975) employing 0.1 M Quadrol was used. The PTH-amino acids at each cycle of Edman degradation were identified by high-performance liquid chromatography on a Varian 5000 liquid chromatograph equipped with a Beckman-Altex dedicated PTH-amino acid reverse-phase Ultraphere-ODS column. Separation was achieved by use of a linear gradient with an initial solvent of 4.2 mM sodium acetate (pH 4.95) containing 5% tetrahydrofuran up to 40% of the second solvent (which consisted of 10% tetrahydrofuran in acetonitrile [program supplied by Beckman Instruments]). Confirmations of PTH-amino acids in some steps were made by isocratic runs using various proportions of the two solvents.

Intact eEF-Tu was subjected to hydrazinolysis essentially according to the method of Schroeder (1972) and the hydrazides were extracted with benzaldehyde (Akabori et al., 1952). The free carboxyl-terminal amino acids were quantitated on the amino acid analyzer.

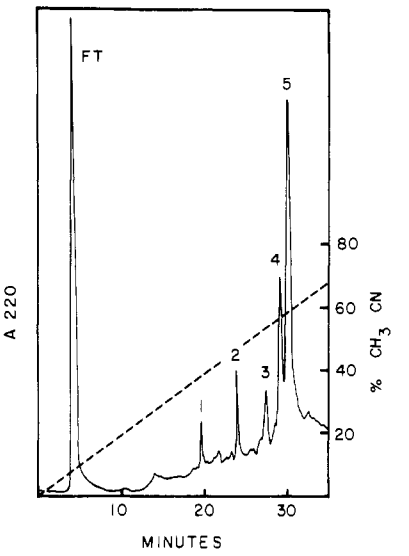

FIGURE 1: HPLC of trypsin digest of eEF-Tu. Native eEF-Tu (1 mg/mL) was digested with trypsin at a protease concentration of 2% (w/w) for 30 min at 25 °C. After addition of a 2-fold excess (w/w) of soybean trypsin inhibitor over trypsin, a sample of the digest containing 50 μg of eEF-Tu was applied to the HPLC column. The column was developed as described under Experimental Procedures. The peaks of optical density in the chromatogram were identified at 1 (tryptic peptide 1), 2 (tryptic peptide 2), 3 (soybean trypsin inhibitor), 4 (eEF-Tu^t), and 5 (eEF-Tu). FT signifies the solvent flow through. See the text for details.

High-Performance Liquid Chromatography. Tryptic digests were applied directly to a column (250 × 4.1 mm) of Synchropak RP-P (Synchrom, Inc., Linden, IN) previously equilibrated with 0.1% trifluoroacetic acid. Liquid chromatography was performed on a Perkin-Elmer 3B liquid chromatograph using a linear gradient of 0.1% trifluoroacetic acid in acetonitrile according to Mahoney & Hermodson (1980). Peptides were detected by absorbance at 220 nm.

Results and Discussion

Separation and Characterization of the Peptides Produced by Limited Tryptic Digestion of eEF-Tu. Previous work in this laboratory has shown that when eEF-Tu (M_r 53 000) is treated with trypsin in buffers containing 25% (v/v) glycerol, a 43K trypsin resistant fragment is produced via a 48K intermediate (Slobin et al., 1981). These results suggested that at least two low molecular weight peptides were cleaved from eEF-Tu by trypsin. However, no low molecular weight peptide fragments were observed when digests of the factor were analyzed on acrylamide gels by straining with Coomassie blue.

In an attempt to identify these peptides, tryptic digests of eEF-Tu were analyzed by HPLC. As shown in Figure 1, five absorbancy peaks were found. Subsequent analysis of each peak by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate produced the following results: peak 5 was found to contain intact eEF-Tu, peak 4 was found to contain eEF-Tu^t, and the peptide in peak 3 migrated to the position of soybean trypsin inhibitor. Material from peaks 1 and 2 did not give detectable bands when stained with Coomassie blue R, suggesting that they correspond to the low molecular weight peptides released from eEF-Tu by trypsin. However, as shown in Figure 2, these peptides could be visualized by different staining procedures. Peptide T1 could be stained with silver nitrate and migrated on an acrylamide gel to the same position as a 6000-dalton cyanogen bromide fragment of myoglobin (Figure 2B). Although T2 did not appear to silver stain, it could be detected by reacting it with

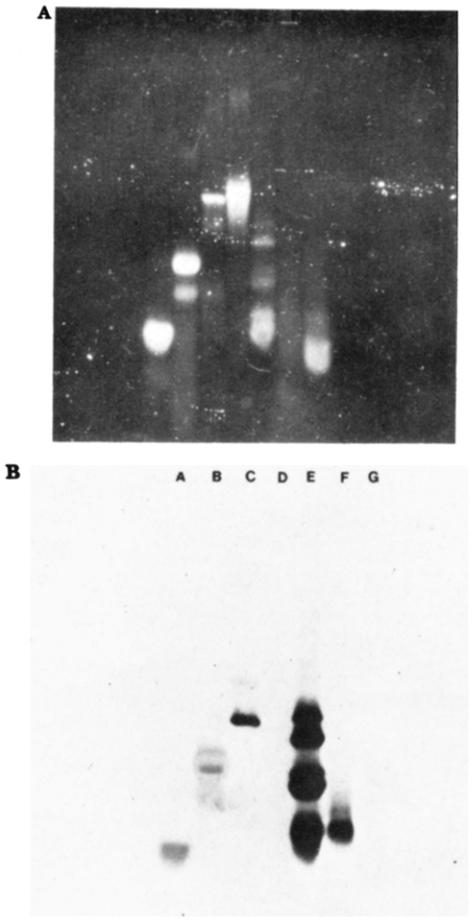

FIGURE 2: Polyacrylamide gel electrophoresis of tryptic peptides T1 and T2. Exponential 15–30% acrylamide gels were prepared according to Laemmli (1970) and run at a current of 25 mA/gel until the bromophenol blue dye marker reached the bottom of the gel. T1 and T2, as well as marker peptides, were reacted with fluorescamine (Eng & Parkes, 1975) prior to electrophoresis. The gel was photographed under UV light (Figure 2A) and subsequently strained with silver nitrate (Figure 2B) by the procedure of Oakley et al. (1980). (Lane A) Insulin B chain (M_r 3400); (lane B) cytochrome *c* (M_r 12 500); (lane C) apomyoglobin (M_r 17 800); (lane D) β-lactoglobulin (M_r 18 400); (lane E) cyanogen bromide digest of apomyoglobin prepared according to Gross & Witkop (1967) (M_r of peptides are 17 800, 14 400, 10 680, 8138, and 6289); (lane F) T1 (6 μg of peptide); (lane G) T2 (4 μg of peptide). From (A) it may be seen that T1 apparently did not reaction with fluorescamine. (B) shows that neither β-lactoglobulin nor T2 reacted with the silver strain.

fluorescamine prior to electrophoresis (Figure 2A). The molecular weight of T2 was calculated to be approximately 4000.

The amino acid compositions of T1 and T2 are given in Table I. From the composition data minimum molecular weights of 5.9×10^3 and 3.9×10^3 were calculated for T1 and T2, respectively. These values correspond closely to the molecular weight estimates for these peptides obtained from acrylamide gels.

We next attempted to locate peptides T1 and T2 with the structure of eEF-Tu. As reported previously, eEF-Tu contains a blocked amino-terminal residue, whereas eEF-Tu^t contains an amino-terminal glycine residue (Slobin et al., 1981). Consequently, we expected that either T1 or T2 would be blocked at the amino-terminal end. Analysis of both peptides in the sequenator indicated that T2 contained a blocked amino-terminal residue and, therefore, originates from the amino terminus of eEF-Tu.

To resolve the question as to whether T1 is adjacent to T2 in the eEF-Tu sequence or originates from the carboxyl-terminal end of the factor, the carboxyl-terminal sequences of eEF-Tu and eEF-Tu^t were analyzed by carboxypeptidase A digestion. A time course for the carboxypeptidase A digestion of eEF-Tu is shown in Figure 3. Only four amino acids, serine, alanine, lysine and glycine, were released after treatment with carboxypeptidase A for 2 h. Both alanine and serine were released in near stoichiometric amounts and at comparable rates; lysine and glycine were released at slower rates and in substoichiometric amounts. The data suggest that

Table I: Amino Acid Analysis of Tryptic Peptides T1 and T2[a]

	T1		T2	
	ratios of amino acids	integer residues	ratios of amino acids	integer residues
Asp	5.4	5	4.0	4
Thr	4.1	4	1.1	1
Ser	3.9	4	2.9	3
Glu	6.2	6	2.2	2
Pro	2.0	2	2.7	3
Gly	6.0	6	4.1	4
Ala	3.7	4	3.7	4
Cys	0	0	0	0
Val	4.2	4	2.9	3
Met	1.1	1	0.1	0
Ile	3.8	4	1.2	1
Leu	3.1	3	3.1	3
Tyr	1.0	1	1.4	1
Phe	1.1	1	1.7	2
His	3.4	3	1.5	2
Lys	6.4	6	3.4	3
Arg	0.8	1	0.8	1
no. of residues		55		37
M_r	5.9×10^3		3.9×10^6	

[a] Amino acid analysis was performed as described under Experimental Procedures. The amide content and tryptophan were not determined. Values represent the average of duplicate determinations which differed by less than 10%.

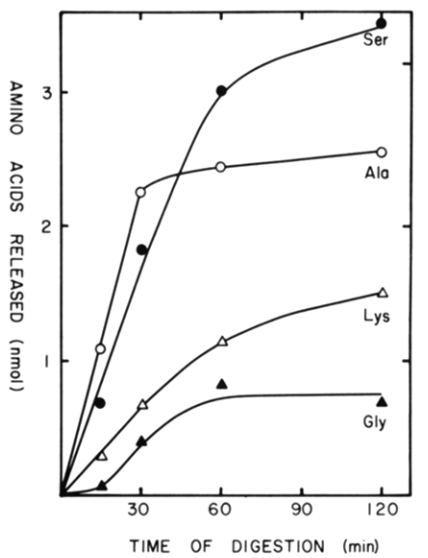

FIGURE 3: Carboxypeptide A digestion of eEF-Tu. A stock solution of eEF-Tu [1 mg in 125 μL of a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM 2-mercaptoethanol, and 25% (v/v) glycerol] was diluted with 8 volumes of *N*-ethylmorpholine acetate buffer, pH 8.5, and 20 μg of carboxypeptidase A was added. After incubation at 37 °C for the indicated times aliquots (200 μL of the reaction mixture) were removed, and the digestion was stopped by the addition of an equal volume of HPLC-grade 1 M acetic acid. Any precipitated protein was removed by centrifugation and the clear supernatant was lyophilized. The dried residue was dissolved in 0.2 M sodium citrate buffer, pH 2.2, and analyzed for amino acids on a Beckman 119 CL amino acid analyzer. A zero digestion time value was subtracted from each data point.

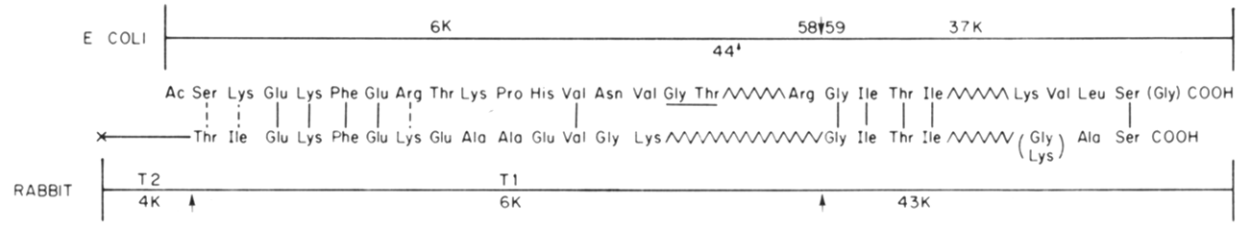

FIGURE 4: Structural homology between eEF-Tu and EF-Tu from *E. coli*. The amino terminal of T1 from eEF-Tu is aligned with the amino-terminal end of EF-Tu (Arai et al., 1980). The solid arrows indicate the sites of trypsin cleavage of the native factors. The X at the amino-terminal end of eEF-Tu indicates a blocked α-amino group (Slobin et al., 1981). The dashed lines connecting the two sequences indicate that an amino acid residue in one sequence can be converted to the residue shown in the other sequence by a single base substitution in the genetic code. The solid lines in the figure starting at residue 59 in the *E. coli* sequence indicate part of the homology previously established between the amino-terminal end of eEF-Tu^t and *E. coli* EF-Tu (Slobin et al., 1981). The uncertainty in the order of Gly and Lys residues at the carboxyl-terminal end of eEF-Tu is indicated in parentheses. See the text for a discussion. Both Gly and Ser are found at the carboxy-terminal end of EF-Tu (Arai et al., 1980). The zigzag lines indicate polypeptide chains which, for the most part in the case of eEF-Tu, are of as yet undetermined sequence.

Table II: NH_2-Terminal Sequence Analysis of Peptide T1

cycle	amino acid residue	yield[a] (nmol)
1	Thr	0.42
2	Ile	0.60
3	Glu	1.25
4	Lys	1.42
5	Phe	0.42
6	Glu	0.76
7	Lys	1.10
8	Glu	0.72
9	Ala	0.56
10	Ala	0.58
11	Glu	0.45
12	Val/Met[b]	0.16
13	Gly	0.20
14	Lys	0.66

[a] The PTH-amino acids were identified by HPLC as described under Experimental Procedures. Approximately 2.5 nmol of peptide was applied to the sequencer. [b] The HPLC system did not separate PTH-valine from PTH-methionine when the gradient system was used. Sufficient quantities of this residue were not obtained for a run on a second system which separates these two PTH derivatives.

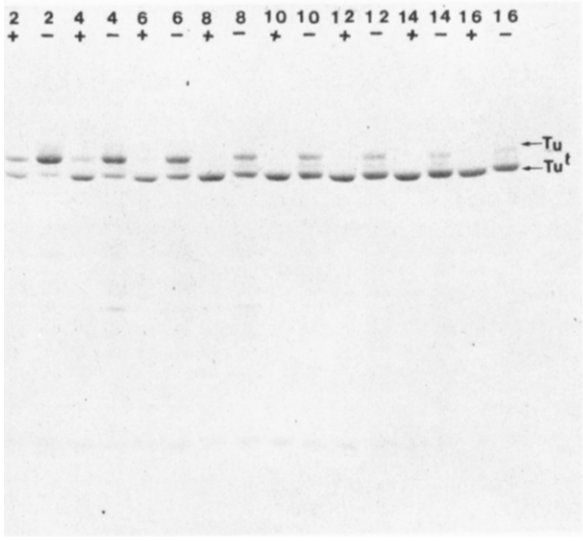

FIGURE 5: Effect in the presence of 25% (v/v) glycerol of 28S rRNA on the cleavage of eEF-Tu by trypsin. eEF-Tu (1 mg/mL in buffer A) was incubated at 25 °C for 10 min in the presence (+) or absence (−) of 11.8 A_{260} units/mL rabbit reticulocyte 28S rRNA. The molar ratio of eEF-Tu/28S rRNA was 54. Trypsin was added to both reaction mixtures to a final concentration of 20 μg/mL, and at 0, 2, 4, 6, 8, 10, 12, 14, and 16 min 20-μL aliquots of the digestion mixture were removed and hydrolysis was stopped by the addition of 0.8 μg of soybean trypsin inhibitor. After the last aliquot was withdrawn samples were treated with 1% sodium dodecyl sulfate and electrophoresed on a 10% acrylamide gel in the presence of sodium dodecyl sulfate and stained with Coomassie blue R as described previously (Slobin et al., 1981). The numbers above the lanes indicate the time of trypsin treatment in minutes. The arrows adjacent to Tu and Tu^t indicate the positions of migration of eEF-Tu and eEF-Tu^t, respectively.

serine, which was released in the largest quantity and which is usually released slowly by carboxypeptidase A (Ambler, 1972), is the carboxyl-terminal residue of eEF-Tu, followed by alanine in the pentultimate position. This conclusion was confirmed by subjecting a sample of eEF-Tu to hydrazinolysis; only serine was found to be released.

The order of lysine and glycine in the polypeptide chain is less certain. While lysine was released at faster rates and in greater amounts, glycine is released very slowly by carboxypeptidase A (Ambler, 1972) and the protein blank (zero time) values for glycine were high.

A sample of eEF-Tu was digested with trypsin for 2 h, at which time greater than 90% of the starting eEF-Tu was converted to eEF-Tu^t (Slobin et al., 1981). eEF-Tu^t was further purified by HPLC and digested for 60 min with carboxypeptidase A as described for EF-Tu (see the legend to Figure 3). The amino acid serine was the major digestion product (0.81 mol of serine/mol of eEF-Tu^t); alanine, lysine, and glycine were detected in lesser amounts. We conclude that eEF-Tu^t has the same carboxyl-terminal sequence as a eEF-Tu and that T1 must originate from a position adjacent to T2 at the amino-terminal end of eEF-Tu. It remains possible that T2 and T1 are separated by a small peptide which has escaped our detection.

Amino-Terminal Sequence of T1 and Sequence Homology between eEF-Tu and EF-Tu. Peptide T1 was purified by HPLC and taken for amino acid sequence analysis by using a Beckman sequenator. One predominant PTH-amino acid was released at each cycle (Table II). To our surprise the amino-terminal sequence of T1 showed a clear homology to the amino-terminal end of EF-Tu from *E. coli* (Figure 4). The sequence Glu-Lys-Phe-Glu (positions 3–6 in T1) is identical with the sequence at positions 3–6 at the amino-terminal end of EF-Tu. Of the remaining three residues in the first seven positions in T1, all are related to corresponding residues in EF-Tu by a single base substitution in the genetic code. If a gap of 2 residues is placed between positions six and seven in T1, then six or possibly seven identities in sequence exist among the first 14 residues in T1 and first 16 residues at the amino-terminal end of EF-Tu.

Figure 4 compares the primary structure of EF-Tu from *E. coli* with the structure of rabbit reticulocyte eEF-Tu. Although only about 7% of the primary structure of eucaryotic factor has been determined, it seems certain that eEF-Tu and EF-Tu share a common evolutionary origin. The two factors, however,

differ by about 10000 daltons in mass, and we previously suggested that a large percentage of this mass difference is due to the insertion during evolution of additional sequences at the amino-terminal end of the factor (Slobin et al., 1981). The data presented in this paper suggest that the sequence of peptide T2 represents an N-terminal extension of the eucaryotic factor for which there is no structural counterpart in EF-Tu. This hypothesis is based not only upon the sequence homology between T1 and the amino-terminal end of EF-Tu but also upon the nearly exact size correspondence between T1 (~6000 daltons) and the peptide released from EF-Tu by trypsin cleavage at Arg-58 (Wittinghofer et al., 1980).

A number of features of the structural relationship between EF-Tu and eEF-Tu are remarkable. Both regions of strong homology between the two factors originate at a trypsin-sensitive site. The conservation of sequences at these sites suggests that they represent functionally important parts of the factor molecule (see below). The accessibility of these sites to trypsin in the case of eEF-Tu (the same is true of the trypsin-sensitive sites of EF-Tu) and the apparent unavailability of the numerous other potential trypsin-sensitive bonds, both in the intact factor and in T1 and T2, suggest that both T1 and T2 represent distinct domains of the factor that are linked to each other and to eEF-Tu^t through particularly exposed structures.[2]

Both eEF-Tu and tufB gene products of *E. coli* contain a carboxyl-terminal serine residue (An & Friesen, 1980). While our data for the carboxyl-terminal sequence of eEF-Tu is not unequivocal, it does suggest some homology with the carboxyl-terminal sequence of EF-Tu. Should such homology exist, then the additional 60 or so residues which comprise the difference in mass between eEF-Tu^t and residues 59–393 of *E. coli* EF-Tu must exist as an insertion into the bacterial sequence rather than as an extension of the carboxyl-terminal end of the molecule.

Effect of Nucleic Acids on the Cleavage of eEF-Tu by Trypsin. The sensitivity of EF-Tu to trypsin has been used successfully as a probe to investigate structure–function relationships of the factor (Blumenthal et al., 1977; Douglass & Blumenthal, 1979). In the case of the eucaryotic factor we have shown that both GDP and eEF-Ts appear to markedly retard the rate of trypsin cleavage of eEF-Tu (Slobin et al., 1981). Work in a number of laboratories has shown that eEF-Tu binds to a variety of different nucleic acids (Domogatsky et al., 1978; Kolb et al., 1978). Recently it has been shown that eEF-Tu contains two distinct nucleic acid binding sites; one for ribosomal or other high molecular weight RNAs and another for aa-tRNA and that the factor can bind both types of nucleic acids simultaneously (Slobin, 1983). The existence of these two distinct binding sites suggested that the action of trypsin on eEF-Tu in the presence of the aforementioned nucleic acids might help reveal their binding loci within the factor.

To study the effect of rRNA on the trypsin sensitivity of eEF-Tu, complexes of the factor and 28S rRNA were formed and treated with trypsin in the presence of glycerol for varying times. As seen in Figure 5, the presence of 28S rRNA in

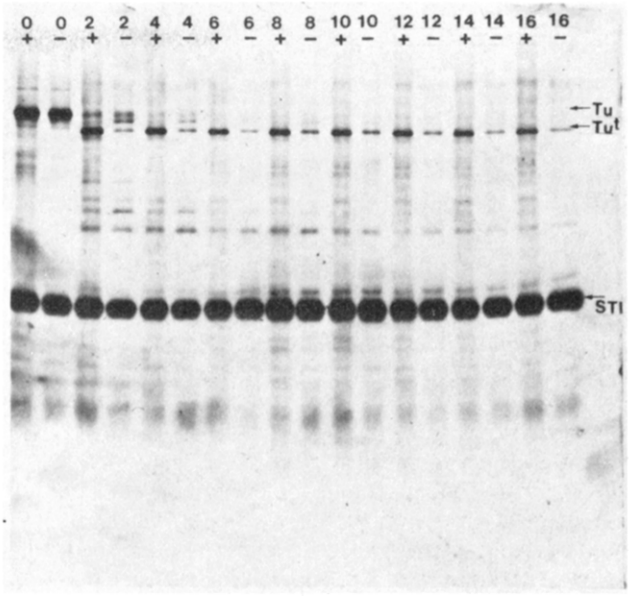

FIGURE 6: Effect of 28S rRNA on the cleavage of eEF-Tu by trypsin in the absence of glycerol. eEF-Tu was incubated with (+) or without (−) 28S rRNA for 10 min at 25 °C and then treated with trypsin as described in the legend to Figure 5, except that glycerol was omitted from buffer A. At the times indicated, digest samples containing 0.5 μg of eEF-Tu were taken for electrophoresis and the protein bands were stained with silver nitrate (Oakley et al., 1980). STI signifies soybean trypsin inhibitor. See the legend to Figure 5 for additional details.

digestion mixtures greatly enhances the rate of trypsin cleavage. When the hydrolysis of eEF-Tu by trypsin was quantitated by scanning stained gels of the digest and treated as a first-order reactions, we found that the $t_{1/2}$ for the disappearance of eEF-Tu decreased from 11.5 to 4 min in the presence of rRNA. These results indicate that the amino-terminal end of eEF-Tu has become more accessible to trypsin. Other RNAs which bind strongly to eEF-Tu, such as poly(G) (Slobin, 1983), also accelerate the rate of trypsin hydrolysis of eEF-Tu and stabilize eEF-Tu^t against further degradation.

It is well established that eEF-Tu activity is extremely unstable in the absence of high concentration of glycerol (Nagata et al., 1976; Slobin & Moller, 1976). This functional instability is reflected in the fact that trypsin rapidly degrades eEF-Tu to small peptides in the absence of glycerol (Slobin et al., 1981). When eEF-Tu was digested with trypsin in the absence of glycerol, the presence of 28S rRNA in the reaction mixture led to a significantly enhanced stability of eEF-Tu^t (Figure 6). We interpret these results, together with the results shown in Figure 5, to signify that the binding site for 28S rRNA on eEF-Tu resides in eEF-Tu^t. It should be noted, however, that we have failed to detect binding between eEF-Tu^t, purified as described previously (Slobin et al., 1981) and 28S rRNA using a nitrocellulose filter to detect protein–nucleic acid complexes (unpublished observations). It is possible that the rRNA binding site is rather labile and does not survive the steps required for eEF-Tu^t purification. An additional possibility that could account for the failure of eEF-Tu^t to bind RNA is that the 43K fragment needs to be associated with peptide T1 in order to form an effective RNA binding site (see below).

When eEF-Tu was incubated with GTP and aa-tRNA instead of 28S rRNA, prior to trypsin treatment and in the absence of glycerol, the stability of the factor was greatly enhanced (Figure 7). Although some cleavage of the factor does occur during the first 14 min of trypsin treatment, it is possible that some, if not all of it, can be ascribed to hydrolysis

[2] We have investigated the kinetics of formation of T1 and T2 from eEF-Tu by trypsin using HPLC. As expected from our previously published data (Slobin et al., 1981), peptide T2 is released at a somewhat faster rate than peptide T2. However, both peptides appear to stabile once released, at least during the first hour of digestion. Only after 2 h of digestion did we notice a significant decline in the relative yield of these two peptides. Several smaller peaks of peptide material besides T1 and T2 appear in the chromatogram and have not been further charactgerized. They may represent some additional cleavage of T1 and T2 or of the starting eEF-Tu.

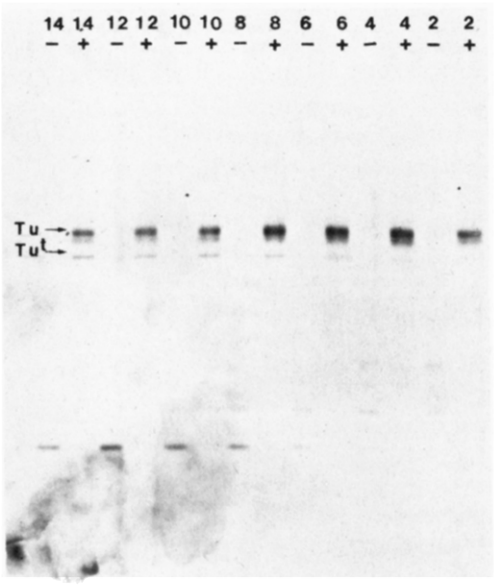

FIGURE 7: Effect of aminoacyl-tRNA on the cleavage of eEF-Tu by trypsin in the absence of glycerol. eEF-Tu was incubated with (+) or without (−) 25 μM rabbit liver Lys-tRNA (molar ratio of Lys-tRNA to eEF-Tu equals 1.3) for 10 min at 25 °C and then treated with trypsin as described in the legend to Figure 4, except that glycerol was omitted from buffer A. At the time indicated digest samples containing 0.5 μg of eEF-Tu were taken for electrophoresis and the protein bands stained with silver nitrate. See the legends to Figures 5 and 6 for additional details.

of the aminoacyl bond in aa-tRNA.[3] The increased resistance of eEF-Tu to the action of trypsin in the presence of aa-tRNA is unlikely to be due directly to the binding of GTP to the factor, since the nucleotide by itself has been shown to have little effect on the digestion reaction (Slobin et al., 1981). Thus, aa-tRNA and 28S rRNA affect the trypsin sensitivity of eEF-Tu in opposite ways.

It has been reported by several laboratories (Arai et al., 1976; Jurnak et al., 1977) that trypsinized EF-Tu cannot form a ternary complex with aa-tRNA and GTP. Others, however, have found that the trypsinized factor is active in ternary complex formation (Jacobson & Rosenbusch, 1976; Wittinghofer et al., 1980). Nonetheless, there seems to be agreement that the interaction of EF-Tu with aa-tRNA is considerably reduced (Wittinghofer et al., 1980) or eliminated (Jurnak et al., 1977) when the small peptide which spans the region from alanine-45 to arginine-58 is excised from the polypeptide chain. Recently it has been reported that His-66 in EF-Tu reacts covalently with the affinity label N^{ϵ}-bromoacetyl-Lys-tRNA (Guerrier-Takada et al., 1981).

We have reported previously that the loss of factor activity during trypsin digestion (as measured by its ability to bind aa-tRNA to 80S ribosomes) is much slower than the rate of eEF-Tu' formation (Slobin et al., 1981). However, no ribosome-dependent activity was detectable after purification of eEF-Tu' by ion-exchange chromatography followed by gel filtration. This apparent discrepancy may be accounted for by the observation that peptide T1 remains associated with eEF-Tu' when trypsin digests of the factor are subjected to gel filtration (unpublished observations). The association of T1 with eEF-Tu resembles the association of residues 1–44 with residues 59–393 subsequent to the tryptic cleavage of EF-Tu (Wittinghofer et al., 1980).

[3] Uncharged tRNA has a low affinity for eEF-Tu (Slobin, 1983) and has little effect on the rate of trypsin cleavage of eEF-Tu (unpublished observations).

A number of findings reported here implicate peptide as T1 having an important role in binding of aa-tRNA to eEF-Tu. The protection against tryptic hydrolysis by aa-tRNA suggests that part or all of the sequence of T1 is associated with aa-tRNA. It remains possible that the effect of aa-tRNA is indirect, i.e., binding of aa-tRNA to loci in eEF-Tu' produces a conformational alteration at the amino-terminal end of the factor. However, the binding site of 28S rRNA appears to be located within eEF-Tu' (Figure 6), as does the binding site for guanine nucleotides (Slobin et al., 1981). Furthermore, as mentioned previously, eEF-Tu can bind aa-tRNA and 28S rRNA simultaneously.

Perhaps the most telling agrument in favor of T1 as the locus of aa-tRNA binding is the remarkable conservation of structure at the amino-terminal end of eEF-Tu (Figure 4). The sequence homologies between EF-Tu and eEF-Tu in that region imply functional significance and, as we have already noted, evidence in the case of both factors suggests direct involvement of the amino-terminal end in aa-tRNA binding.

Despite the homologies between EF-Tu and eEF-Tu illustrated in Figure 4, notable structural and functional differences exist between the two factors (Slobin et al., 1981; Slobin, 1981). We expect that peptide T2 has no direct counterpart in EF-Tu and that the sequences responsible for rRNA binding in eEF-Tu do not exist in the bacterial factor. The functional role of these eucaryotic-specific structures remains to be established.

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Registry No. Trypsin, 9002-07-7; glycerol, 56-81-5; GTP, 86-01-1.

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Nuclear Magnetic Resonance Studies of Amino Acids and Proteins. Side-Chain Mobility of Methionine in the Crystalline Amino Acid and in Crystalline Sperm Whale (*Physeter catodon*) Myoglobin†

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ABSTRACT: We have obtained deuterium (^2H) nuclear magnetic resonance (NMR) spectra and spin–lattice relaxation times (T_1) of L-[ϵ-$^2H_3$]methionine, L-[ϵ-$^2H_3$]methionine in a D,L lattice, and [*S-methyl*-$^2H_3$]methionine in the crystalline solid state, as a function of temperature, in addition to obtaining ^2H T_1 and line-width results as a function of temperature on [ϵ-$^2H_3$]methionine-labeled sperm whale (*Physeter catodon*) myoglobins by using the method of magnetic ordering [Rothgeb, T. M., & Oldfield, E. (1981) *J. Biol. Chem. 256*, 1432–1446]. The results indicate that in the L-amino acid, methyl rotation having an activation energy (ΔE^*) of 8.3 ± 1 kJ dominates T_1 at low temperatures ($\lesssim$−10 °C), while at higher temperatures an additional large-amplitude side-chain motion occurs which causes changes in the ^2H NMR line shape and T_1. This motion is inhibited in the D,L lattice, indicating that lattice effects may have a strong effect on the mobility of anhydrous amino acids in the solid state. Further substitution at S^δ to form the sulfonium salt [*S-methyl*-$^2H_3$]-methionine causes a large increase in ΔE^*, to 15.9 ± 2 kJ, a value comparable to the 14–16 kJ found in valine and leucine, which contain the structurally similar isopropyl moiety. These results suggest that the very low barriers to methyl rotation in the methionine side chain are due to long C–S bond lengths and the presence of only two substituents on sulfur, while the anomalous high-temperature behavior is due to a lattice-packing effect. ^2H T_1 results with methionine-labeled myoglobin are complex, reflecting the presence of fast large-amplitude side-chain motions, in addition to rapid methyl rotation. Our data indicate that Met-55 and Met-131 are motionally inequivalent in crystalline cyanoferrimyoglobin, in contrast to solution NMR results. We have also recorded ^{13}C cross-polarization "magic-angle" sample-spinning NMR spectra of [ϵ-^{13}C]methionine-labeled crystalline cyanoferrimyoglobin (at 37.7 MHz, corresponding to a magnetic field strength of 3.52 T) and of the same protein in aqueous solution. Cross-polarization transfer rates and proton rotating-frame relaxation time results again indicate that Met-55 and Met-131 are motionally inequivalent in the solid state, and the T_{CH} data indicate that Met-55 is more solidlike. However, we find that ^{13}C chemical shifts in solution and those in the crystalline solid state are in very close agreement, suggesting that the average solution and crystal conformations are the same, in the area of Met-55 and Met-131.

There is currently considerable interest in investigating the dynamic structures of proteins in solution, in membranes, and in the crystalline solid state (Williams, 1978; Frauenfelder et al., 1979; Frauenfelder & Petsko, 1980; Artymiuk et al., 1979; Gurd & Rothgeb, 1979; Karplus & McCammon, 1981; Keniry et al., 1982; Schramm et al., 1981; Schramm & Oldfield, 1982). Nuclear magnetic resonance (NMR)[1] spectroscopy, because of its sensitivity to the wide range of time scales over which the motions occur, is one of the best techniques capable of yielding such information (Oldfield et al., 1981; Schramm et al., 1981).

In this paper, we discuss motions of the amino acid methionine, using ^2H and ^{13}C NMR spectroscopy. In another paper (Keniry et al., 1982), we have discussed the motions of methyl groups in alanine, valine, threonine, and leucine, in a variety of systems. We showed that, in general, the motions observed were rather simple, consisting solely of fast methyl group rotation. However, with the larger leucine side chain, additional motions occurred in some instances since the observed ^2H NMR spectra had nonzero asymmetry parameters (η). Similar effects have been observed by Batchelder et al.

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[1] Abbreviations: NMR, nuclear magnetic resonance; CP/MAS, cross-polarization "magic-angle" spinning; Tempamine, 4-amino-2,2,6,6-tetramethylpiperidinyloxy; TSP, sodium 3-(trimethylsilyl)[2,3-$^2H_4$]propionate; Me_4Si, tetramethylsilane; Mb, myoglobin; CoMb, cobalt derivative of myoglobin, "coboglobin"; MbH_2O, aquoferrimyoglobin; MbCN, cyanoferrimyoglobin; MbF, fluoroferrimyoglobin; MbCO, carboxyferromyoglobin; $\Delta\nu_Q$, quadrupole splitting; T_1, spin–lattice relaxation time.

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