Alignment of the tryptic fragments and location of sulfhydryl groups of the polypeptide chain elongation factor Tu

ARTICLE *in* BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS · SEPTEMBER 1977

Impact Factor: 2.3 · DOI: 10.1016/S0006-291X(77)80137-X · Source: PubMed

ALIGNMENT OF THE TRYPTIC FRAGMENTS AND LOCATION OF SULFHYDRYL GROUPS OF THE POLYPEPTIDE CHAIN ELONGATION FACTOR Tu

Shun Nakamura, Ken-ichi Arai, Kenji Takahashi,* and Yoshito Kaziro

Institute of Medical Science, University of Tokyo, Takanawa, Minatoku, Tokyo, and

*Primate Research Institute, Kyoto University, Inuyama, Japan

Received July 11,1977

Summary: A limited hydrolysis with trypsin of the polypeptide chain elongation factor Tu (EF-Tu) in the native state has been investigated in detail. The reaction proceeded in two stages. In the first stage, the protein was cleaved into two polypeptides, with molecular weights of 39,000 and 8,000, which were designated as Fragments A and D, respectively. In the second stage, the Fragment A was transformed to Fragments B (M.W. 23,000) and C (M.W. 12,000) which still existed as a complex molecule containing Fragment D. Analyses of N- and C-terminal sequences of the intact EF-Tu as well as these 4 fragments have revealed that Fragments D and C were derived from N- and C-termini of EF-Tu, respectively. Further, the amino acid sequence of Fragment B containing all of the three sulfhydryls of EF-Tu has been investigated. On cleavage of Fragment B with cyanogen bromide, three sulfhydryl-containing polypeptides were obtained, i.e. a 3000-dalton peptide containing the sulfhydryl essential for the binding of guanine nucleotides (SH_1), a 5000-dalton peptide containing the sulfhydryl essential for aminoacyl-tRNA binding (SH_2), and a 10,000-dalton peptide containing the non-reactive sulfhydryl (SH_3). From the partial sequence determination of these three peptides, the location of the sulfhydryls was determined as in the order of SH_2, SH_1, and SH_3 from the N- to C-termini of EF-Tu.

Previously, we have shown that a limited hydrolysis of EF-Tu·GDP by trypsin proceeded in two characteristic stages (1). In the first stage, the protein is transformed into a fragment with a molecular weight of 39,000 (Fragment A) with a concomitant loss of the ability to interact with aminoacyl-tRNA and ribosomes. Fragment A was subsequently transformed to a complex molecule containing Fragment B (M.W. 23,000) and Fragment C (M.W. 12,000), which still retained one mole of bound guanine nucleotide. Fragments A, B, and C were purified, and their amino acid compositions as well as N-terminal amino acids were determined (1). It was found that Fragment B contained all of the three sulfhydryl groups of EF-Tu and that the N-termini of Fragments A and B were identical while that of Fragment C was different (1).

We have also carried out the complete tryptic digestion of EF-Tu and isolated two sulfhydryl-containing tryptic peptides by affinity chromatography (2). The amino acid sequences of a 15-residue segment containing the sulfhydryl essential for aminoacyl-tRNA

Abbreviation used: Na-DodSO$_4$, sodium dodecylsulfate; and CPase Y, carboxypeptidase Y.

ISSN 0006-291X

binding (SH_2) and a 10-residue segment containing the non-reactive sulfhydryl (SH_3) were determined (2), thus complementing the sequence of a 42-residue segment containing the sulfhydryl essential for guanine nucleotide binding (SH_1) reported by Wade et al. (3).

In an attempt to elucidate the complete amino acid sequence of EF-Tu, we have further studied the partial digestion of EF-Tu·GDP with trypsin as well as the amino acid sequence of Fragment B. It was found that an additional small fragment (Fragment D) with a molecular weight of 8,000 was formed during the initial stage of tryptic digestion. This fragment was associated non-covalently with Fragment A, and later with Fragments B and C. As in the case of the native, undigested EF-Tu molecule (4), the N-terminal of Fragment D was blocked, indicating that this fragment presumably arose from the N-terminal of EF-Tu. The alignment of fragments in the order of D, B, and C was assigned based on the data of the N- and C-terminal sequences of these fragments and those of undigested EF-Tu. The occurrence of a small fragment with a molecular weight of 4,000-9,000 in partial tryptic digests of EF-Tu·GDP, has been reported independently by Gast et al.(5).

The sulfhydryl-containing peptides were obtained by cleavage of Fragment B with cyanogen bromide at the methionyl residues and their N-terminal sequences were determined. The location of three cysteinyl residues in Fragment B were deduced by comparing the N-terminal sequences of these peptides with the N-terminal of Fragment B, and also with the known sequences of the sulfhydryl-containing tryptic peptides (2).

During preparation of this manuscript, Laursen et al. (6) have shortly reported the location of cysteinyl and methionyl residues in EF-Tu. Their result on the location of sulfhydryl residues is in complete agreement with the result to be reported here.

Materials and Methods

Materials: Crystalline EF-Tu·GDP was prepared from E. coli MRE 600 as described elsewhere (7). N-tosyl-L-phenylalanyl chloromethylketone (TPCK)-treated trypsin and soybean trypsin inhibitor were purchased from Worthington and Sigma, respectively. Carboxypeptidase (CPase) Y was obtained from Oriental Yeast, Osaka.

Preparation of Fragment A: EF-Tu·GDP was digested with trypsin as described previously (1). To obtain Fragment A, the hydrolysis was terminated by the addition of soybean trypsin inhibitor after 8 min. Fragment A was purified by Sephadex G-150 column chromatography in the presence of 6 M guanidine-HCl (1).

Preparation of Fragments B, C, and D: 200 mg of crystalline EF-Tu·GDP (35 mg/ml) was digested with 4 mg of trypsin for 180 min, and passed through a Sephadex G-75 column (2.5 x 110 cm) equilibrated and developed at 4° with 50 mM Tris-HCl buffer (pH 7.9) and 10 mM magnesium acetate. The elution of the peptides was followed by the optical density at 230 and 280 nm. The fractions containing peptides were pooled and lyophilized, and the cysteinyl residues were carboxymethylated with iodo[^{3}H]acetic acid according to the method of Waxdal et al. (8) with a slight modification (manuscript in preparation). The carboxymethylated peptides were fractionated on a DEAE-Sephadex

column (1.8 x 55 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 7.5) containing 6 M urea, 10 mM 2-mercaptoethanol, and 0.05 M NaCl. The column was developed in a linear gradient of 0.07 to 0.4 M NaCl. The peptides were eluted in two peaks; one radioactive and the other non-radioactive. The former contained Fragment B, while the latter both Fragments C and D. Fragments C and D were further separated on a Sephadex G-150 column (2 x 85 cm) equilibrated and developed with 0.02 M Tris-HCl buffer (pH 7.8) containing 6 M urea, 0.02 M NaCl, and 10 mM ethylenediamine dihydrochloride.

Purification of sulfhydryl-containing peptides obtained from Fragment B by cleavage with cyanogen bromide: 80 mg of Fragment B labeled with iodo[^{3}H]acetic acid was cleaved with 500 mg of cyanogen bromide in 24 ml of 70% HCOOH at 25° for 26.5 hr (9). The reaction was terminated by lyophilization, and the peptides was passed through a Sephadex G-50 column (1.8 x 300 cm) equilibrated and developed with 0.1 M NH_4HCO_3. The radioactive eluate was pooled, and lyophilized. The radioactive peptides were further purified by DEAE-Sephadex A-25 column chromatography (manuscript in preparation).

Sequence determination: N-Terminal sequences-----N-Terminal sequences of peptides were determined manually by the Edman degradation with direct identification of phenylthiohydantoin-amino acids (10) and by the Na-DodSO$_4$-dansyl-Edman degradation (11). N-Terminal amino acids were also determined by the modified dansyl method of Mosesson et al. (12) and Gray (13).

C-Terminal sequences-----C-Terminal sequences of EF-Tu and the peptides were analyzed by CPase Y (14). 5 to 50 nmoles of EF-Tu or peptides were dissolved in 40 to 200 µl of 0.05 M phosphate buffer (pH 6.5) containing 1% Na-DodSO$_4$, and digested with 10 to 30 µg of CPase Y at 37°. At the times specified, aliquots were taken, and mixed with 1 ml of 10% trichloroacetic acid. The supernatant solution was separated from the precipitate by centrifugation, lyophilized, and dissolved in 1.3 ml of 0.2 M sodium citrate buffer (pH 2.2). This solution was applied to the amino acid analyzer (JEOL, model JLC-6AH). To determine the amount of the substrate in each aliquot, the precipitate was lyophilized, and hydrolyzed in 0.5 ml of 6 N HCl at 110° for 24 hr. The amount of amino acids in the precipitate was measured by amino acid analysis.

Results and Discussion

Appearance of Fragment D during limited tryptic digestion of EF-Tu—As shown in Fig. 1 (left), Fragment D (M.W. 8,000) was formed together with Fragment A at the initial stage of tryptic digestion. Fragment D was preserved throughout the second stage of digestion while Fragment A was transformed into Fragments B and C. After incubation for 180 min, the tryptic digests were chromatographed on a Sephadex G-75 column in the absence of denaturant. There was only a single peak (No. 70) of peptides and as shown in Fig. 1 (right), the peak fraction from the column was found to contain Fragments B, C, and D in an approximately equimolar ratio based on the densitometric tracing (not shown) and molecular weights. These results indicate that EF-Tu was first split into the complex of Fragments A and D, and Fragment A was further cleaved into Fragments B and C, yielding a complex containing Fragments B, C, and D.

Alignment of Fragments A, B, C, and D in the primary structure of EF-Tu—Fragments A, B, C, and D were purified as described in "Materials and Methods" to a homogeneous state as judged by polyacrylamide gel electrophoresis in the presence of Na-DodSO$_4$

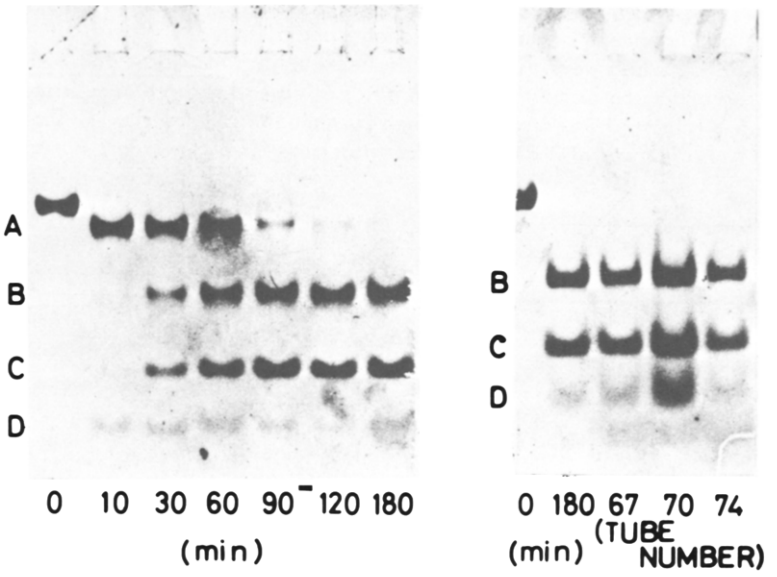


Fig. 1. Na-DodSO$_4$ polyacrylamide gel electrophoresis of trypsin-treated EF-Tu·GDP. In the left panel, 200 mg of EF-Tu·GDP was digested with 4 mg of trypsin at 0° in 8 ml of 0.1 M Tris-HCl buffer (pH 7.9) containing 10 mM magnesium acetate, 5 mM 2-mercaptoethanol, 5 mM $CaCl_2$, and 10 μM GDP. At the times specified, 2 μl aliquots were taken, and mixed with 40 μl of a solution containing 1% Na-DodSO$_4$ and 2-mercaptoethanol. Polyacrylamide gel electrophoresis in the presence of Na-DodSO$_4$ was carried out according to the method of Weber and Osborn (16) with a slab gel plate (14 x 21 x 0.12 cm). In the right panel, the hydrolysis was terminated at 180 min by the addition of 4 mg of soybean trypsin inhibitor. The hydrolyzate was immediately passed through a Sephadex G-75 column (2.5 x 110 cm) as described in "Materials and Methods", and 10-μl aliquots were withdrawn from fractions 67, 70, and 74. The electrophoretic analysis of these aliquots was performed as above.

(not shown). The molecular weight of Fragment D was estimated as 8,000 from the mobility on 15% polyacrylamide gel electrophoresis in the presence of Na-DodSO$_4$.

N-Terminal sequences of the purified fragments were investigated as described in "Materials and Methods", and were determined as: Fragment A, Gly-Ile-Thr-; Fragment B, Gly-Ile-Thr-; and Fragment C, Leu-Leu-, whereas no N-terminal amino acid was detected in Fragment D. Since the N-terminal amino acid of undigested EF-Tu is also blocked (4), the above result suggests that Fragment D was derived from the N-terminal of EF-Tu. Likewise, Fragment B presumably arose from the N-terminal portion of Fragment A. C-terminal sequences of intact EF-Tu and Fragments A, B, C, and D were analyzed with CPase Y. The results shown in Table I suggest that the tentative C-terminal sequences of intact EF-Tu as well as Fragments A and C are (Ser?, Lys, Gly, Ala_{1-2}, Val_2)-Val-Leu-Ser-COOH.

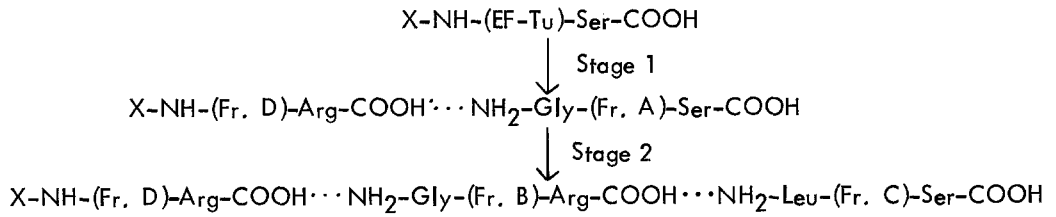
Table I. C-Terminal analyses of undigested EF-Tu, Fragment A and Fragment C. EF-Tu, Fragment A, and Fragment C were digested for 5 and 60 min at 37° with CPase Y and the amino acids released were determined as described in "Materials and Methods". The values are expressed as moles of amino acids released per mole of substrate. The amount of each substrate was also determined by amino acid analysis. A small amount of lysine and glycine was also detected after digestion for 60 min (data not shown).

Amino acid	EF-Tu		Fragment A		Fragment C	
	5 min	60 min	5 min	60 min	5 min	60 min
Ser*	0.81	1.44	0.90	0.99	0.22	0.56
Ala	0.39	1.23	0.39	1.30	0.07	0.29
Val	0.45	2.17	0.87	2.61	0.12	0.71
Leu	0.51	1.12	0.71	0.71	0.16	0.55

*) Since the buffer system of Spackman et al. (17) used for amino acid analysis did not distinguish serine , asparagine, and glutamine, the C-terminal amino acid was assigned after hydrolysis of the released amino acids in 6 N HCl at 110° for 24 hr.

On the other hand, the C-terminal amino acids of Fragments B and D were arginine (not shown). These results suggest that Fragments A and C contain the C-terminal of EF-Tu.

From the above results, the tryptic digestion of EF-Tu appears to proceed through the following stages:



It is not known whether any gap(s) exists between Fragments D and A (i.e. Fragments D and B), and Fragments B and C.

Recently, partial digestion of EF-Tu by trypsin has also been investigated by other workers (5,15). Gast et al. (5) obtained four fragments, i.e. fragments 1,2,3, and 4 having molecular weights of 39,000, 21,000, 12,000 and 4,000-9,000, respectively. Their results on the kinetics of digestion and the pattern of electrophoresis are in good agreement with ours, and we consider that their fragments 1, 2, 3, and 4 may correspond to our Fragments A, B, C, and D, respectively. Jacobson and Rosenbusch (15) obtained also a fragment lacking about 65 residues of the N-terminal end of EF-Tu by mild tryptic hydrolysis. Since the

SH_2-peptide (CNBr): Gly-Ile-Thr-Ile-Asn-Thr-Ser-His-Val-Glu-Tyr-Asp-Thr-Pro-Thr-Arg-His-Tyr-Ala-His-Val-Asp-Cys-Pro-Gly-His-Ala-Asp-Tyr-Val-Lys-
(M.W. 5,000)

SH_3-peptide (CNBr): Glu-Val-Arg-Glu-Leu-Leu-Ser-Gln-Tyr-Asp-Phe-Pro-Gly-Asp-Asp-Thr-Pro-Ile-Val-Arg-Gly-
(M.W. 10,000)

Fig. 2. Amino acid sequences of the N-terminal portion of the two sulfhydryl-containing peptides obtained from cleavage of Fragment B with cyanogen bromide.

N-terminal of the newly formed fragment was Gly-Ile-Thr-Ile-, it is probably identical with our Fragment A. According to their report, the fragment was fully active in poly (U)-dependent polyphenylalanine synthesis (15), whereas our results indicated that Fragment A·D complex could no longer interact with aminoacyl-tRNA nor ribosomes (1). The discrepancy between our results and theirs is presently unknown.

Location of three sulfhydryls in Fragment B——Three sulfhydryl-containing peptides were obtained on cleavage of Fragment B with cyanogen bromide at the methionyl residues (see "Materials and Methods"). Two sulfhydryl-containing peptides with molecular weights of about 5,000 and 10,000 were purified to a homogeneous state as judged by N-terminal group analysis, whereas the third one (M.W. 3,000) was partially purified. The N-terminal sequences of the homogeneous peptides were determined as described in "Materials and Methods" and the results are shown in Fig. 2. The N-terminal sequence of the 5,000 dalton peptide agreed with that of Fragment B, and the peptide was found to contain the sulfhydryl essential for aminoacyl-tRNA binding (SH_2) (2). On the other hand, the N-terminal sequence of a 10,000 dalton peptide (NH_2-Glu-Val-Arg-) coincided with the sequence of the C-terminus of a tryptic peptide containing the sulfhydryl essential for binding of guanine nucleotide (SH_1) previously identified as: Cys-Asp-Met-Val-Asp-Asp-Glu-Glu-Leu-Leu-Glu-Leu-Val-Glu-Met-Glu-Val-Arg (2). Furthermore, a small amount of another sulfhydryl-containing CNBr-peptide was isolated, which had an N-terminal sequence of NH_2-Val-Asp-Asp-(see above), and a molecular weight of about 10,000. From these results, the location of the three sulfhydryls in Fragment B, viz. in EF-Tu, is deduced as follows.

X-NH----------SH_2----------SH_1----------SH_3------COOH

Recently, Laursen et al. (6) have shortly reported the location of the cysteinyl and methionyl residues in EF-Tu. Their results on the location of cysteinyl residues are in complete agreement with the above results. Although these authors stated that the aminoacyl-tRNA

binding site (SH_2) may be cleaved off from EF-Tu at an early stage of tryptic digestion (6), this is unlikely since Fragment A, as we have shown previously (1), still retained SH_2. The destruction of aminoacyl-tRNA binding activity of EF-Tu might be caused, not by the direct loss of the cysteinyl residue, but by more subtle alteration of the tertiary structure of the protein due to the cleavage of the bond(s) between Fragments D and A. The work aiming at the elucidation of the complete amino acid sequences of EF-Tu is currently undrer way.

References

1. Arai, K., Nakamura, S., Arai, T., Kawakita, M., and Kaziro, Y. (1976) J. Biochem. (Tokyo) 79, 69-83
2. Nakamura, S., Arai, K., Takahashi, K., and Kaziro, Y. (1975) Biochem. Biophys. Res. Commun. 66, 1069-1077
3. Wade, M., Laursen, A., and Miller, D. L. (1975) FEBS Letters 53, 37-39
4. Blumenthal, T., Landers, T. A., and Weber, K. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1313-1317
5. Gast, W. H., Leberman, R., Schulz, G. E., and Wittinghofer, A. (1976) J. Mol. Biol., 106, 943-950
6. Laursen, R. A., Nagarkatti, S. (1977) Fed. Proc. 36, 870
7. Arai, K., Kawakita, M., and Kaziro, Y. (1972) J. Biol. Chem. 247, 7029-7037
8. Waxdal, M. J., Konigsberg, W. H., Henley, W. L., and Edelman, G. M. (1968) Biochemistry 7, 1959-1966
9. Gross, E. (1967) Methods Enzymol. II, 238-255
10. van Eerd, J. -P., and Takahashi, K. (1976) Biochemistry 15, 1171-1180
11. Weiner, A. M., Platt, T., and Weber, K. (1972) J. Biol. Chem. 247, 3242-3251
12. Mosesson, M. W., Finlayson, J. S., and Galanakis, D. K. (1973) J. Biol. Chem. 248, 7913-7929
13. Gray. W. R. (1972) Methods Enzymol., 25, 121-138
14. Hayashi, R., Moore, S., and Stein. W. H. (1973) J. Biol. Chem. 248, 2296-2302
15. Jacobson, G. R., and Rosenbusch, J. P. (1976) Biochemistry, 15, 5105-5110
16. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412
17. Spackman, D. H., Stein. W. H., and Moore, S. (1958) Anal. Chem., 30, 1190-1206