AMINO ACID SEQUENCES OF TWO SULFHYDRYL-CONTAINING TRYPTIC PEPTIDES OF THE POLYPEPTIDE CHAIN ELONGATION FACTOR Tu

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Received August 11,1975

Summary: The primary structure around the sulfhydryl groups of the polypeptide chain elongation factor Tu has been investigated. The tryptic peptides containing sulfhydryl groups were separated from other peptides by affinity chromatography using a p-chloromercuribenzoate-agarose column. Two sulfhydryl-containing polypeptides were further purified by DEAE-Sephadex column chromatography after modification with iodo[^{3}H]acetic acid. The amino acid sequences of a 15-residue segment containing the sulfhydryl essential for aminoacyl-tRNA binding (SH_2) and a 10-residue segment containing the non-reactive sulfhydryl (SH_3) have been determined using the manual method of the sequential Edman degradation. The amino acid sequences of SH_2- and SH_3-peptides were shown as His-Tyr-Ala-His-Val-Asp-Cys-Pro-Gly-His-Ala-Asp-Tyr-Val-Lys, and Ser-Thr-Cys-Thr-Gly-Val-Glu-Phe-Arg-Lys, respectively.

Polypeptide chain elongation factor Tu (EF-Tu) from *E. coli* contains three sulfhydryl groups per mole of protein (1,2), which are designated as SH_1, SH_2, and SH_3(3). SH_1 is essential for the binding of GDP or GTP, while SH_2 is essential for aminoacyl-tRNA binding (3,4), and also for interaction with ribosomes (5). The third sulfhydryl, SH_3, is non-reactive and can be titrated only after complete denaturation of the protein (3).

The conformational transition of EF-Tu near SH_2 induced by guanine nucleotides has been shown to play an important role on its interaction with aminoacyl-tRNA (3,6,7) and also with ribosomes (5). In an attempt to characterize more precisely the nature of the conformational change, and also as an initial step for determination of the complete amino acid sequence of EF-Tu, we have in-

vestigated the primary structure of sulfhydryl-containing tryptic peptides (SH-peptides) of EF-Tu.

In this communication, we report the amino acid sequences of a 15-residue segment containing SH_2 and a 10-residue segment containing SH_3. These SH-peptides were obtained by selective adsorption on and elution from the p-chloromercuribenzoate (pCMB)-agarose column followed by carboxymethylation with iodo[^{3}H]acetic acid and chromatography on a DEAE-Sephadex column. From the separate experiments in which three thiol groups of EF-Tu, i.e., SH_1, SH_2, and SH_3, were differentially labeled with iodo[^{3}H]acetic acid, and fractionated by DEAE-Sephadex column chromatography, these two SH-peptides were identified as SH_2- and SH_3-peptides, respectively. Recently, Wade et al. (8) have reported the sequence of SH_1-peptide containing 42 amino acid residues.

Materials and Methods

Crystalline EF-Tu·GDP was prepared from E. coli Q13 as described previously (9). EF-Tu·GTP and free EF-Tu were prepared from EF-Tu·GDP (10). Trypsin, and carboxypeptidases A and B were obtained from Sigma and Worthington, respectively. Iodo[2-^{3}H]acetic acid and iodo[2-^{14}C]acetic acid were purchased from Radiochemical Centre, Amersham. [^{14}C]pCMB was obtained from Commissariat à l'Énergie Atomique, France, and purified by the method of Boyer (11). pCMB-agarose was prepared according to Cuatrecasas and Anfinsen (12).

Purification of SH-peptides ------ About 100 mg of EF-Tu was denatured by acid, neutralized to pH 9.0, and digested with 2 mg of trypsin at 25° in a reaction mixture containing 25 mM Na_2SO_4 and 5 mM dithiothreitol in a total volume of 40 ml. The hydrolytic reaction was monitored with pH stat, and after the completion of digestion, the mixture was acidified to pH 4.0 and lyophilized. The residue was dissolved in 10 ml of 0.1 M acetate buffer (pH 6.0)

and treated with 0.1 M 2-mercaptoethanol to reduce the disulfides formed during tryptic digestion. The excess 2-mercaptoethanol was removed by passing through a Sephadex G-15 column, and the SH-peptides were adsorbed on a pCMB-agarose column (0.8 x 8 cm), and eluted with 0.1 M 2-mercaptoethanol. The excess 2-mercaptoethanol was again removed by passage through a Sephadex G-15 column and the SH-peptides were lyophilized. The residue was dissolved in 4 ml of 0.1 M Tris-HCl buffer (pH 8.6) containing 12.5 mM 2-mercaptoethanol, and carboxymethylated with 20 mM iodo[^{3}H]acetic acid (2 mCi/mmole). The labeled peptides were purified by DEAE-Sephadex column chromatography (0.9 x 50 cm) which was developed in a linear gradient of 0.3 to 1.5 M NH_4HCO_3.

Differential Modification of SH_1, SH_2, and SH_3 of EF-Tu ---- The selective labeling of SH_2 was performed by incubating 5 mg of EF-Tu·GTP with 10 mM iodo[^{3}H]acetic acid at 0° for 2 hours. For the selective labeling of SH_3, 2.5 mg of free EF-Tu was first incubated with 5 mM nonlabeled N-ethylmaleimide (NEM) at 0° for 1 hour, and after extensive dialysis to remove excess NEM, SH_3 was labeled with iodo[^{3}H]acetic acid in the presence of 8 M urea. For preparation of EF-Tu in which both SH_1, and SH_3 were labeled, 2.5 mg of free EF-Tu was incubated with 135 µM N-tosyl-L-phenylalanyl chloromethane (TPCK) at 0° for 1 hour to modify exclusively SH_2 (3). Excess TPCK was removed by dialysis and the modified protein was treated with iodo[^{3}H]acetic acid in the presence of 8 M urea. EF-Tu uniformly labeled at all three sulfhydryls was prepared by carboxymethylation with iodo[^{14}C]acetic acid in the presence of 8 M urea.

Analytical Methods ------- Amino acid analysis was performed according to the procedure of Spackman et al. (13). Amino acid sequence was determined by a modification (14) of the manual method

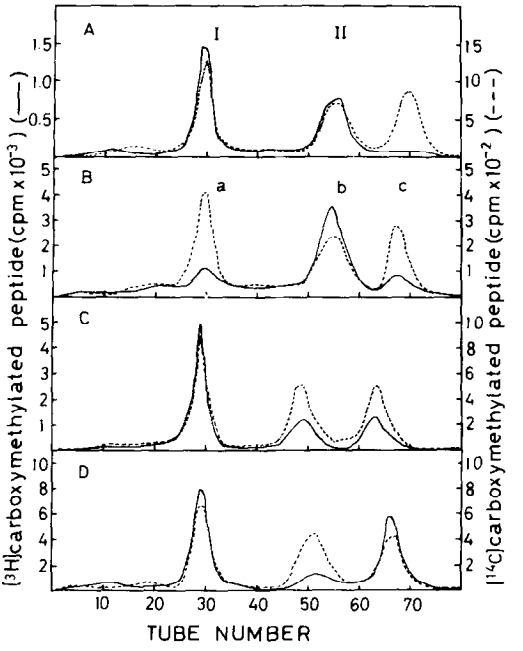

Fig. 1. DEAE-Sephadex column chromatography of labeled SH-peptides. A, SH-peptides purified by pCMB-agarose column chromatography was carboxymethylated with iodo[^{3}H]acetic acid and applied to the column together with the tryptic digests of EF-Tu uniformly labeled with iodo[^{14}C]acetic acid as an internal marker. B to D, preparations of EF-Tu differentially labeled with iodo[^{3}H]acetic acid at either SH_2(B), SH_3(C), or both SH_1 and SH_3(D), were mixed with uniformly [^{14}C]carboxymethylated EF-Tu, and were digested with trypsin. The column (0.6 x 40 cm) was developed in a linear gradient of 0.3 to 1.5 M NH_4HCO_3. Fractions (1 ml) were collected at a flow rate of 20 ml per hour. Tritium (——), and ^{14}C (---) radioactivities were measured in a toluene-based scintillation fluid mixed with Triton-X100 (2:1 v/v).

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of sequential Edman degradation (15). The phenylthiohydantoins were identified by gas-liquid chromatography on a 10 % SE-30 support (16) and by micro-polyamide thin layer chromatography on a 5 x 5-cm sheet (17). SH_2-peptide was treated with 4-sulfophenylisothiocyanate prior to Edman degradation according to Braunitzer et al. (18). The C-terminal sequences of peptides were determined by carboxypeptidases A and B according to Ambler (19).

Results and Discussion

Identification of SH-peptides ------- By the above purification procedure, two SH-peptides were obtained on DEAE-Sephadex column chromatography (Fig. 1A). They were designated as SH-peptides I and II and corresponded to radioactive Peaks a and b, respectively, of the tryptic digests (added as an internal marker) of EF-Tu which had been uniformly labeled with iodo[^{14}C]acetic acid at all three sulfhydryls. Amino acid analyses of SH-peptides I and II isolated in the absence of the marker are listed in Table I. As seen in the

Table I

Amino Acid Composition of SH-peptides I and II of EF-Tu

	SH-peptide I*		SH-peptide II*	
		Nearest integer		Nearest integer
Cys-CH_2CO_2H**	0.9	1	0.9	1
Asp	0.2	0	2.3	2
Thr	1.6	2	0.0	0
Ser	0.7	1	0.1	0
Glu	1.2	1	0.2	0
Pro	0.0	0	1.1	1
Gly	1.2	1	1.4	1
Ala	0.2	0	2.2	2
Val	1.0	1	1.8	2
Met	0.0	0	0.0	0
Ile	0.0	0	0.0	0
Leu	0.0	0	0.0	0
Tyr	0.0	0	1.4	2
Phe	1.1	1	0.0	0
Trp	0.0	0	0.0	0
His	0.0	0	2.6	3
Lys	0.8	1	1.0	1
Arg	1.0	1	0.0	0
		10		15

*The values are given in relative molar ratios taking the number of arginine and lysine residues in SH-peptides I and II, respectively, as 1.0.

**Sum of S-carboxymethylcysteine and its oxidized product.

table, these two peptides differing in amino acid composition and containing 10 and 15 amino acid residues, respectively, appeared to be sufficiently homogeneous for sequence analysis.

In order to identify these two peptides, preparations of EF-Tu selectively labeled with iodo[^{3}H]acetic acid at either SH_2, or SH_3, or both SH_1 and SH_3,[1)] were digested with trypsin and chromatographed on a DEAE-Sephadex column (Fig. 1B to D). EF-Tu uniformly labeled with iodo[^{14}C]acetic acid was mixed with each sample before tryptic digestion and treated in the same manner. As is clear from the figure, three peaks of ^{14}C-labeled tryptic peptides, Peaks a, b, and c, were found to correspond to SH_3-, SH_2-, and SH_1-peptides, respectively. From these experiments, SH-peptides I and II in Fig. 1A were identified as SH_3- and SH_2-peptides, respectively. The reason for the absence of SH_1-peptide in the eluate from the pCMB-agarose column is not clear. It may have been lost during purification, since Wade et al. (8) reported that SH_1-peptide was easily precipitated under acidic conditions.

Amino Acid Sequence of SH-peptides ------- Manual Edman degradation was performed by a modification (14) of the three-stage method of Edman (15) using 30-100 nmoles of SH-peptides. SH-peptide II was treated with 4-sulfophenylisothiocyanate prior to Edman degradation, while SH-peptide I was not. Prior to the next stage of degradation, the residual peptide in the reaction tube was placed under vacuum (less than 50 μHg) for 30 min at room temperature and for another 30 min at 50° to remove traces of reagents and volatile by-products. Identification of the major phenylthiohydantoin generated by degradation of peptides was possible up

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[1)] Since SH_1 reacts rather slowly with iodoacetic acid, it was difficult to label EF-Tu exclusively at SH_1 without labeling SH_3 at the same time. Therefore, in the experiment shown in Fig. 1D, both SH_1 and SH_3 were labeled after modifying SH_2 with TPCK.

to cycle 7 and 14, for SH-peptides I and II, respectively (Table II). The yields of the major amino acid phenylthiohydantoin identified during each cycle of degradation are given in Table II.

Table II

Manual Edman Degradation of SH-peptides I and II of EF-Tu

	SH-peptide I		SH-peptide II	
Cycle	Residue	Yield (%)*	Residue	Yield (%)*
1	Ser	258	His§	
2	Thr	102	Tyr	58
3	Cys**	27	Ala§	57
4	Thr	29	His	
5	Gly	20	Val	51
6	Val	18	Asp	58
7	Glu†	11	Cys**	38
8	(Phe)†		Pro	41
9	(Arg)†		Gly§	34
10	(Lys)†		His	
11			Ala§	56
12			Asp	
13			Tyr	30
14			Val	24
15			(Lys)†	

*Yield of the major amino acid phenylthiohydantoins determined spectrophotometrically at 269 nm (15). No correction was made for either overlap or back ground.

**Determined as phenylthiohydantoin derivative of S-carboxymethylcysteine.

§ The phenylthiohydantoin derivatives of histidine and aspartic acid were identified by thin-layer chromatography as described in Materials and Methods.

† The amino acid sequences assigned by carboxypeptidases A and B.

The C-terminal sequences of these peptides were assigned by the use of carboxypeptidases A and B (data not shown). The complete amino acid sequences of a 15-residue segment containing SH_2, and a 10-

residue segment containing SH_3 were thus determined as His-Tyr-Ala-His-Val-Asp-Cys-Pro-Gly-His-Ala-Asp-Tyr-Val-Lys, and Ser-Thr-Cys-Thr-Gly-Val-Glu-Phe-Arg-Lys, respectively.

The reactivity of EF-Tu toward aminoacyl-tRNA is qualitatively altered by the change of the guanine nucleotide ligands. Since SH_1 and SH_2 are essential for interaction with guanine nucleotides and aminoacyl-tRNA, respectively, it would be of interest to determine the location of these two sulfhydryls on the primary structure of EF-Tu. Recently we have shown that a limited hydrolysis of EF-Tu·GDP by trypsin yielded a fragment molecule of 39,000 molecular weight (Fragment A) which was subsequently transformed to a hybrid molecule containing Fragment B (M.W. 23,000) and Fragment C (M.W. 12,000) (Arai, Nakamura, Arai, Kawakita, and Kaziro, submitted for publication). Since the purified Fragment B molecule contained all three sulfhydryls of EF-Tu, it was expected that the location of these two, functionally different, thiol groups could be determined by solving the entire primary structure of the fragment molecule.

Acknowledgement —— The authors would like to thank Dr. Jean-Paul van Eerd, Department of Pharmacology, Faculty of Medicine, University of Tokyo for his valuable advice on amino acid sequence analysis.

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