Identification of a Histidine Residue near the Aminoacyl Transfer Ribonucleic Acid Binding Site of Elongation Factor Tu†

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ABSTRACT: The complex of elongation factor Tu with GTP (EF-Tu·GTP) reacts with N^{ϵ}-bromoacetyl-Lys-tRNA (ϵBrAcLys-tRNA) to form a functional covalently linked complex (XLTC). The site of cross-linking must be near the site on EF-Tu·GTP that binds the aminoacyl moiety of aminoacyl transfer ribonucleic acid (AA-tRNA). For identification of this site, a nanomole of purified XLTC prepared from ϵBrAc[^{14}C]Lys-tRNA was digested first with RNase A and then with trypsin, and the peptides were resolved by high-performance liquid chromatography using a C_8 reverse-phase column. A single peptide contained 80% of the label. The amino acid composition of this peptide was identical with that of residues 59–74 in EF-Tu. The NH_2-terminal sequence of the peptide was determined to be Gly-Ile-Thr-Ile, which are residues 59–62 in EF-Tu. The modified amino acid was identified as π-(carboxymethyl)histidine, which establishes that His-66 is at or near the AA-tRNA binding site on EF-Tu·GTP.

Elongation factor Tu promotes the binding of aminoacyl-tRNA to ribosomes during protein biosynthesis. When EF-Tu[1] binds GTP, it can form a strong ternary complex with AA-tRNA. EF-Tu·GDP, in contrast, shows no affinity for AA-tRNA. The role of GTP may be described as that of an allosteric effector which alters the protein's tertiary structure to expose an AA-tRNA binding site. The nature of the AA-tRNA binding site and how it is affected by GTP and GDP are at present unknown.

The tertiary structure of EF-Tu·GDP is being studied by X-ray crystallographic methods (Gast et al., 1977; Morikawa et al., 1978; Jurnak et al., 1980). It has not been possible to obtain suitable crystals of EF-Tu containing its other ligands; however, it should be possible to delineate the binding sites for these substances by chemical methods if suitable cross-linked derivatives could be obtained. We have previously reported that EF-Tu·GTP reacts with ϵBrAcLys-tRNA to form a functional cross-linked ternary complex (XLTC) (Johnson et al., 1978). XLTC reacts as readily with poly(A)-programmed ribosomes as the natural complex. GTP in the complex is hydrolyzed, but EF-Tu·GDP remains bound to ribosomes via the covalent bond to Lys-tRNA (Johnson et al., 1978). Thus, it is likely that the structures of XLTC and the natural ternary complex are similar. In this paper, we describe the identification of His-66 as the site of cross-linking.

Experimental Procedures

Preparation of XLTC. EF-Tu·GTP was prepared from *Escherichia coli* B as previously described (Miller & Weissbach, 1974). ϵBrAc[^{14}C]Lys-tRNA (specific activity 600 dpm/pmol) was prepared according to a previously described procedure (Johnson & Cantor, 1980). Radioactive *N*-bromoacetoxysuccinimide was synthesized by using bromo-[1-^{14}C]acetic acid (Amersham) and a 40% molar excess of both *N*-hydroxysuccinimide and dicyclohexylcarbodiimide and was used without recrystallization for the preparation of ϵBr[^{14}C]AcLys-tRNA as described (Johnson & Cantor, 1980). The conditions for the cross-linking reaction were similar to those previously reported (Johnson et al., 1978). The reaction mixtures (2.0 mL) contained 10 nmol of ϵBrAcLys-tRNA, 50 nmol of EF-Tu·GTP, and 75 μg of pyruvate kinase in a buffer composed of 0.25 mM GTP, 2 mM PEP, 10 mM $MgCl_2$, 50 mM Hepes (pH 7.6), and 50 mM KCl. EF-Tu·GDP was incubated with pyruvate kinase and PEP for 10 min at 37 °C prior to adding ϵBrAcLys-tRNA. The incubation was continued for 80 min, at which time 55% of the BrAcLys-tRNA had reacted with EF-Tu·GTP. The extent of reaction was assayed by precipitation with hot trichloroacetic acid.

The XLTC preparation was freed of nonbound tRNA and pyruvate kinase by gel filtration on Ultrogel AcA-44 (a product of LKB) in a buffer composed of 20 mM Tris-HCl, pH 7.4, 0.1 mM $MgCl_2$, 100 mM NH_4Cl, and 1 mM DTT. The $MgCl_2$ concentration of the pooled XLTC-containing fractions were raised to 10 mM. Most of the excess EF-Tu was also removed by this procedure; the remaining free EF-Tu was removed by filtration through a stack of cellulose nitrate filters (Type BA-85, Schleicher & Schuell). The purified complex was concentrated by pressure dialysis in an Amicon stirred cell using a UM-10 membrane.

Isolation of Labeled Peptide. Intact XLTC resists tryptic cleavage; therefore, the tRNA moiety was removed first by RNase A digestion. The course of the reaction was followed by a cellulose nitrate filter assay (Miller & Weissbach, 1974), wherein intact complexes pass through the filter but EF-Tu free of AA-tRNA binds to the filter. XLTC (2.5 nmol) was incubated at 37 °C for 1 h with 10 μg of RNase, at which time 80% of the tRNA was removed. The free protein was then digested with trypsin (5 μg) at 0 °C. After 30 min, the mixture was warmed to room temperature, and the digestion was allowed to continue for 7 h. The tryptic peptides were resolved by HPLC. The initial separation was performed by using a 15-cm Altex reverse-phase octyl column eluted with a linear gradient of 0–20% 1-propanol in 0.5 M formic acid–pyridine

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[1] Abbreviations used: EF-Tu, peptide chain elongation factor Tu; tRNA, transfer ribonucleic acid; AA-tRNA, aminoacyl-tRNA; ϵBrAcLys-tRNA, N^{ϵ}-bromoacetyl-Lys-tRNA; XLTC, cross-linked ternary complex, GTP·EF-Tu–AcLys-tRNA; PEP, phosphoenolpyruvate; HPLC, high-performance liquid chromatography; Ptc, phenylthiocarbonyl; π-(carboxymethyl)histidine, π designates substitution at the imidazole nitrogen nearer the side chain; τ-(carboxymethyl)histidine, τ designates substitution at the more distant nitrogen (IUPAC–IUB Commission on Biochemical Nomenclature, 1975); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

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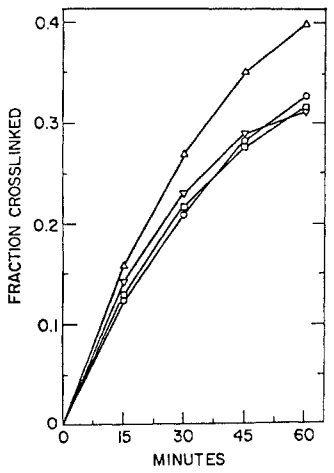

FIGURE 1: Dependence of the rate of cross-linking upon pH. The reaction mixtures (500 μL) contained 50 pmol of ϵBrAc[^{14}C]Lys-tRNA (644 dpm/pmol), 350 pmol of EF-Tu·GTP, 23 μg of pyruvate kinase, 10 μM GTP, 2 mM PEP, 10 mM $MgCl_2$, 50 mM KCl, and 50 mM Hepes, adjusted to the indicated pH values, and were measured at 37 °C. At intervals, 25-μL aliquots were assayed for radioactivity precipitable in hot trichloroacetic acid, as previously described (Johnson et al., 1978). pH values are 7.0 (○), 7.4 (□), 7.6 (△), and 7.9 (▽).

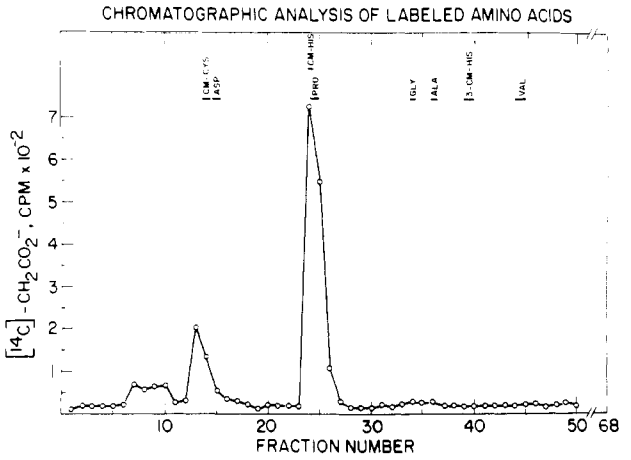

FIGURE 2: Chromatographic analysis of carboxymethylated amino acids. XLTC labeled with ^{14}C in the carboxymethyl group was digested for 20 h at 100 °C in 6 N HCl, and the amino acids were chromatographed on the Jeol amino acid analyzer. Fractions were collected directly, bypassing the detection system. The radioactivity in these fractions was measured, and aliquots of several fractions were rechromatographed on the analyzer to determine which amino acid they contained. The points of elution of these amino acids and of the authentic carboxymethyl amino acids are denoted at the top of the figure.

buffer (pH 4.0) (Lewis et al., 1979). Ten percent of the effluent was diverted through a fluorescamine detection system (Bohlen et al., 1975). The fraction containing radioactivity was rechromatographed on a 15-cm RP-18 column using the previously described elution conditions.

Amino Acid Composition. Amino acid analyses of the peptide were performed with the microcolumn analyzer equipped with a fluorescamine detection system (Stein et al., 1973). Carboxymethyl amino acids were identified by comparison to authentic samples. τ-(Carboxymethyl)histidine was kindly provided by Dr. Stanford Moore, and π-(carboxymethyl)histidine and *S*-(carboxymethyl)cysteine were obtained from Calbiochem. N^{ϵ}-(Carboxymethyl)lysine was synthesized according to a published procedure (Gundlach et al., 1959).

Peptide Sequence Determination. A sequential Edman degradation of the peptide was performed manually according to the procedure described by Tarr (1977). By use of the method of Lai (1977), the Ptc amino acids extracted at each step were hydrolyzed and identified by amino acid analysis.

Results

pH Dependence of the Modification Reaction. The previously reported study characterized the reaction of ϵBrAcLys-tRNA with EF-Tu·GTP to form a functional covalently bound complex (Johnson et al., 1978). The time course of the reaction is not strongly influenced by pH in the range 7.0–7.9 (Figure 1). The increased rate of deacylation of ϵBrAcLys-tRNA at higher pH values (data not shown) probably contributes greatly to the apparent decrease in the rate of cross-linking at pH 7.9. Nonetheless, it appears that cross-linking occurs through an amino acid residue with a side-chain group that is substantially deprotonated above pH 7. This residue is likely to be histidine or an unusually acidic cysteine. The amino terminus is not a possibility because this residue is blocked in EF-Tu (Miller & Weissbach, 1977; Laursen et al., 1977). Aspartate and glutamate cannot be regarded as likely reactive residues because the carboxymethylated residue is stable to acid hydrolysis at 100 °C.

Identification of the Reactive Residue. XLTC formed from N^{ϵ}-Br[^{14}C]AcLys-tRNA was digested in 6 N HCl, and the labeled residues were resolved on an amino analyzer (Figure 2). The elution times of the labeled amino acids were determined by rechromatographing the labeled fractions on the analyzer, the large quantity of unlabeled amino acid in each fraction being readily detectable by ninhydrin. It was found that the major labeled residue emerged with proline, where an authentic preparation of π-(carboxymethyl)histidine also elutes. A smaller peak of radioactivity comprising about 20% of the total label emerged slightly ahead of aspartic acid, where authentic *S*-(carboxymethyl)cysteine appears. No π-(carboxymethyl)histidine or N^{ϵ}-(carboxymethyl)lysine was observed. The elution times of our carboxymethyl amino acid standards agree with those published earlier by Crestfield et al. (1953).

Isolation of the Labeled Peptide. Because only a limited amount of cross-linked ternary complex (XLTC) could be conveniently prepared, it was necessary to isolate and analyze nanomole quantities of peptides. The HPLC system originally developed by Lewis and co-workers for the isolation of brain peptides (Lewis et al., 1979) was well-suited for this purpose. The initial chromatography of the tryptic digest revealed only one major labeled peptide (Figure 3A), which contained 75% of the radioactivity applied to the column. A minor amount of radioactivity that failed to bind to the column probably consisted of free amino acids. Upon rechromatography on an RP-18 column, the labeled peptide was resolved from an unlabeled contaminant (Figure 3B). About 85% of the radioactivity applied to the column was recovered in a single fraction.

One-fifth of the purified peptide (250 pmol) was hydrolyzed in 6 N HCl and subjected to amino acid analysis using the microcolumn analyzer with a fluorescamine detector. The analysis (Table I) revealed very little leucine or alanine, which indicates that the peptide was free of substantial contaminants. A new peak in the chromatogram appeared where proline was expected (proline does not give a fluorescent derivative with fluorescamine). By comparison to known standards, this peak was assigned to π-(carboxymethyl)histidine.

The composition of the peptide agrees well with that expected from the tryptic peptide containing EF-Tu residues 59–74 (Arai et al., 1980; Jones et al., 1980): Gly^{59}-Ile-Thre-Ile-Asn-Thr-Ser-His*-Val-Glu-Tyr-Asp-Thr-Pro-Thr-Arg^{74}. The cross-linking reaction converted the histidine to π-(carboxymethyl)histidine and added the lysyl residue to the labeled peptide.

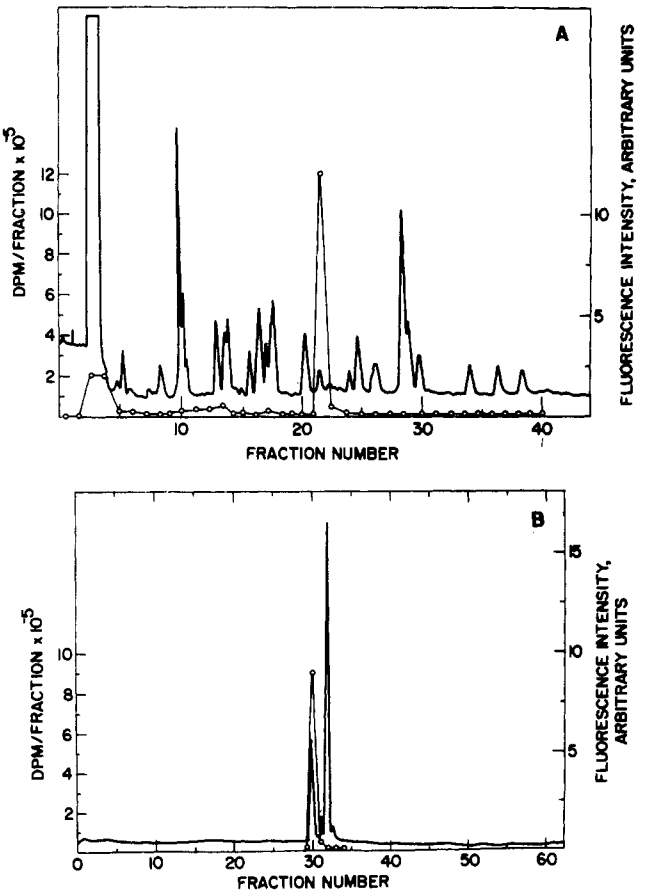

FIGURE 3: Chromatography of tryptic peptides. (A) The tryptic digest of 2.5 nmol of XLTC was applied to a 15-cm Altex reverse-phase octyl column and was eluted with a linear gradient of 0–20% 1-propanol in 0.5 M formic acid–pyridine buffer (pH 4.0) at a flow rate of 36 mL/h. (B) The fraction containing the labeled peptide was applied to a 15-cm Altex reverse-phase octadecyl column and was eluted with a linear gradient of 12–15% 1-propanol in 0.5 M formic acid–pyridine buffer (pH 4.0) at a flow rate of 22 mL/h. In both separations, 1-mL fractions were collected, peptides were detected by reaction with fluorescamine (solid lines), and radioactivity was measured in an aliquot of each fraction (open circles).

Table I: Amino Acid Composition of Labeled Peptide

amino acid	labeled peptide (pmol)	ratio	EF-Tu-residues 59–74
Asp	503	2.0	2
Thr	937	3.7	4
Ser	246	1.0	1
Glu	266	1.1	1
π-CM-His	300	1.2	0
Gly	287	1.2	1
Ala	24	0.1	0
Val	254	1.0	1
Ile	481	1.9	2
Leu	16	0.05	0
Tyr	247	1.0	1
His	17	0.05	1
Lys	253	1.0	0
Arg	255	1.0	1
Pro	ND[a]		1

[a] ND, not determined.

Amino-Terminal Sequence of Labeled Peptide. The remainder of the purified peptide was subjected to manual Edman degradation (Table II). Four degradation cycles result in the following sequence: Gly-Ile-Thr-Ile-. This tetrapeptide corresponds to residues 59–62 in the EF-Tu sequence (Arai et al., 1980; Jones et al., 1980). The amino acid composition

Table II: Manual Sequence Analysis of NH_2-Terminal Sequence of the Modified Peptide

Edman Cycle			
	residue	pmol	% yield[b]
0[a]		810	100
1	Gly	762	94
2	Ile	420	55
3	Thr	250	59
4	Ile	172	69

Composition of Peptide after 4 Cycles of Edman Degradation

residue	pmol[c]	residue	pmol[c]
Asx	543 (2)	Tyr	202 (1)
Thr	673 (3)	Arg	267 (1)
Ser	273 (1)	Pro[d]	(1)
Glx	376 (1)	Lys	71 (1)
Val	297 (1)	His	77 (1)
Ile	140 (0)		

[a] Initial picomoles based on amino acid composition. [b] Yield based on previous cycle. [c] Analysis was corrected for buffer blank; numbers in parentheses indicate number of residues in peptide containing residues 63–74. [d] Proline not determined.

of the peptide remaining after four cycles agreed closely with that expected for residues 63–74. The low yields of His and Lys are not readily explained. It is possible that the cross-linked derivative they form undergoes an alternative reaction during the Edman degradation.

Discussion

A general approach to determining how proteins interact with nucleic acids is to generate specific cross-links between the molecules at their sites of interaction in sufficient yield to permit analysis. That few of these interaction sites have been identified attests to the difficulty in satisfying the requirements of yield and specificity.

In an earlier report, we showed that a specific cross-linked reaction occurs between EF-Tu·GTP and εBrAcLys-tRNA (Johnson et al., 1978). The specificity of the reaction is further supported by the finding of only one labeled peptide in the tryptic digest and one major carboxymethylated amino acid after acid hydrolysis. The small amount of *S*-(carboxymethyl)cysteine found in the digests may be formed by reaction with the nearby Cys-81.

Although the yield of this cross-linking reaction often exceeds 50%, limitations in the availability of the reactants required purification and analytical techniques applicable to subnanomole quantities. The HPLC system gave a good recovery of the purified peptide, and the fluorescamine-based detection system provided the sensitivity necessary for the amino acid analysis.

The labeled peptide was identified as residues 59–73 of EF-Tu by both its composition and amino-terminal sequence. The modified amino acid found in both the labeled peptide and total protein hydrolysates was determined to be π-(carboxymethyl)histidine; therefore, the site of covalent attachment must be His-66, the only His residue in the peptide. The small effect of pH upon the cross-linking rate indicates that the nucleophile is deprotonated above pH 7, which is also consistent with the residue being histidine.

Other evidence suggests that a histidine residue lies near the tRNA binding site. Nakano et al. (1980) found that AA-tRNA binding to EF-Tu·GTP from *Thermus thermophilus* is inhibited by photooxidation. The His residue protected by AA-tRNA binding could be associated with a single NMR peak. It is unknown whether this residue corresponds to His-66 in *E. coli* EF-Tu. Jonak & Rychlik (1980) have reported that the AA-tRNA binding activity of *E. coli* EF-

Tu·GTP is also inactivated by photooxidation. Again it is not known whether the His residue destroyed is His-66. The amino-terminal region of EF-Tu is rich in histidine. Seven of the eleven His residues are located in the first 85 residues; four of the nineteen residues 66–84 are histidine. Modification of any of these residues might be expected to interfere with AA-tRNA binding.

Modification of EF-Tu at either of two nearby sites has been shown to inhibit AA-tRNA binding. Alkylating Cys-81 with NEM (Miller et al., 1971; Wade et al., 1975; Arai et al., 1976) reduces the affinity of EF-Tu·GTP for AA-tRNA by several orders of magnitude, as does tryptic cleavage at Arg-44 and Arg-58 (F. Jurnak and D. L. Miller, unpublished results; Arai et al., 1976; Wittinghofer et al., 1980).

Whether His-66 is directly involved in AA-tRNA binding remains uncertain. The N^{ϵ}-(bromoacetyl)lysyl side chain could extend 0–7 Å from the β carbon of the lysyl residue. Thus, His-66 might be involved in binding either the aminoacyl moiety or the 3′-terminal adenosine.

The identification of His-66 as the site of the cross-link will locate the AA-tRNA binding site in the tertiary structure models now under construction in other laboratories. In a tentative model, His-66 lies in a cleft separating the two major domains of the molecule (J. Nyborg and T. LaCour, personal communication). It may be significant that the GDP binding site lies only about 12 Å away from His-66. This residue may be part of the allosteric mechanism that opens the AA-tRNA binding site when EF-Tu·GDP is converted to EF-Tu·GTP.

Our previous study (Johnson et al., 1978) showed that the cross-linked ternary complex reacted with poly(A)-programmed ribosomes as rapidly and extensively as Lys-tRNA·EF-Tu·GTP and that the GTP in the complex was hydrolyzed in the process. We were not, however, able to observe peptidyl transfer from AcLys-tRNA bound in the peptidyl site. This lack of reactivity may be explained by the cross-linked lysyl residue lying in the cleft between the two domains, where it would be sterically hindered from reacting at the peptidyl transferase center. Thus, it is necessary for EF-Tu to release the aminoacyl residue before peptide bond formation can occur.

Elongation factor Tu resembles EF-G in some of its functions; however, EF-G does not bind AA-tRNA. A striking sequence homology between EF-Tu and EF-G has been discovered in the region 58–86, where 15 of the residues are identical (Laursen & Duffy, 1978). This identity was recognized by considering that a 10-residue α-helical insertion occurred in EF-G between residues 67–68 (EF-Tu numbering). It is provocative to speculate that EF-G lost its AA-tRNA binding capacity because of this insertion at the AA-tRNA binding site.

A major purpose of our cross-linking experiments is to construct a model of the ternary complex in the absence of crystallographic data on this unstable entity. Identifying one more contact site between AA-tRNA and EF-Tu·GTP should permit a reliable orientation of the structural models of tRNA and the protein.

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