
Affinity labeling of eukaryotic elongation factors using N^ε-bromoacetyl-Lys-tRNA

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ABSTRACT

eEF-T and eEF-Tu from rabbit reticulocyte and from *Artemia* were affinity labeled using N^ε-bromoacetyl-Lys-tRNA prepared with either yeast or *E. coli* tRNA. Only the eEF-Tu polypeptide was crosslinked when eEF-T was incubated with the reactive aminoacyl-tRNA analogue, which indicates that at least part of the aminoacyl-tRNA binding site is the same in both eEF-Tu and the multi-subunit eEF-T. Complex formation (eEF-Tu·aa-tRNA·GTP) was required for cross-linking, since no covalent reaction with eEF-Tu occurred in the absence of GTP. The yield of crosslinked product was greatly reduced by adding either unmodified rabbit liver aminoacyl-tRNA or unmodified *E. coli* Lys-tRNA to the incubation to compete for the aminoacyl-tRNA binding site on eEF-T or eEF-Tu, indicating that the covalent reaction occurs while the N^ε-bromoacetyl-Lys-tRNA is bound in this site. The affinity labeling of a prokaryotic and two different eukaryotic elongation factors by the same reagent suggests that there may be conservation of structure in the region of the proteins which binds the aminoacyl end of the aminoacyl-tRNA.

INTRODUCTION

Aminoacyl-tRNA (aa-tRNA) binding to polysomes is catalyzed by protein elongation factors in both prokaryotes and eukaryotes (1,2). In prokaryotes, a ternary complex containing aa-tRNA, GTP, and elongation factor Tu (EF-Tu) binds to the ribosome in response to the appropriate message (1,2). Following GTP hydrolysis, an EF-Tu·GDP complex is released from the ribosome, and a second protein, EF-Ts, catalyzes the exchange of GTP for GDP in the EF-Tu complex (1,2).

However, the situation is not as well defined in eukaryotes, either structurally or functionally. The eukaryotic elongation factor eEF-T (3) contains three polypeptide chains, and it exists in multiple forms in various tissues (1,2,8-11). Two of the chains of eEF-T (the α and γ chains) have functional activities which are analogous to those of the prokaryotic EF-Tu and EF-Ts, respectively, and are referred to as eEF-Tu and eEF-Ts (7,10-12). The function of the third polypeptide, the β chain, has not yet been established. Its existence and the isolation of high molecular weight species of

the eukaryotic factors raise several questions about the structural and functional nature of the elongation factor complex in eukaryotes.

Of particular interest is the nature of the interaction between aa-tRNA and the various forms of the eukaryotic elongation factors. One technique which can provide direct structural information about the aa-tRNA binding sites in these proteins is affinity labeling. As described in this paper, we have used this approach to investigate eEF-T and eEF-Tu from two different sources, utilizing N^ε-bromoacetyl-Lys-tRNA (εBrAcLys-tRNA) as a probe. εBrAcLys-tRNA is a chemically reactive and functionally active analogue of aa-tRNA, and has previously been found to affinity label *E. coli* EF-Tu (13), as well as *E. coli* ribosomes (14).

MATERIALS AND METHODS

Proteins. eEF-T and eEF-Tu from rabbit reticulocyte and *Artemia* were prepared as described (4,8,15). Crystalline EF-Tu·GDP was purified from *E. coli* B (16). Peptidyl-tRNA hydrolase and S-100 enzymes were purified from *E. coli* A-19 or MRE 600 (13).

Aminoacylation of tRNA. Unfractionated *E. coli* MRE 600 (Boehringer) or K-12 (Grand Island Biologicals) tRNA was aminoacylated with radioactive lysine (New England Nuclear) as detailed elsewhere (13). Unfractionated tRNA from brewer's yeast (Plenum Scientific Research) was aminoacylated by the same procedure using *E. coli* S-100 enzymes. Unfractionated rabbit liver tRNA (40 A₂₆₀ units) was aminoacylated in a 1.25 ml incubation (37°C, 15 min) containing: 0.1 M potassium phosphate (pH 7.2); 10 mM MgCl₂; 4 mM ATP; 1 mM dithioerythritol; 20 μM in 19 non-radioactive amino acids; 20 μM [¹⁴C]Leu (New England Nuclear; 670 cpm/pmol); and 5 mg of charging enzymes from rabbit reticulocytes (17). Following phenol extraction and ethanol precipitation, the tRNA was further purified by Sephadex G-25 chromatography at 4°C in H₂O, and then lyophilized. Prior to use the tRNA was resuspended in 1 mM potassium acetate (pH 5). The total concentration of aa-tRNA in the solution was estimated using a value of 7% for the tRNA^{Leu} content in rabbit liver tRNA (18) and assuming that all tRNAs were aminoacylated to the same extent.

Pure *E. coli* tRNA^{Lys} (2 A₂₆₀ units; Boehringer) was aminoacylated in a 3 ml incubation (37°C, 30 min) containing: 0.1 M HEPES (pH 8.0); 10 mM magnesium acetate; 10 mM KCl; 5 mM dithiothreitol; 10 μM [¹⁴C]Lysine; 4 mM ATP; 100 μM CTP; and 1.0 mg of *E. coli* S-100 enzymes. Following phenol extraction and ethanol precipitation, the Lys-tRNA^{Lys} (0.7 ml) was further purified by Sephadex G-25 chromatography (1.1 cm I.D. x 18 cm) at 4°C in 1 mM

potassium acetate (pH 5.0), and then reprecipitated. The tRNA pellet, again obtained by ultracentrifugation (27,000 rpm, 4°C, 90 min, SW 27 rotor), was resuspended in and dialyzed against 1 mM potassium acetate (pH 5.0) prior to storage in liquid nitrogen.

Modification of Lys-tRNA. ϵ BrAcLys-tRNA was prepared from either yeast or *E. coli* Lys-tRNA as described (14), except that only unfractionated *E. coli* ϵ BrAcLys-tRNA was treated with peptidyl-tRNA hydrolase. The preparation of *E. coli* N ^{α , ϵ} -dibromoacetyl-Lys-tRNA (DiBrAcLys-tRNA) has also been described previously (14).

Affinity Labeling. Affinity labeling incubations (typically 37°C, 2 hr, 175 μ l) contained 50 mM HEPES (pH 7.4), 5 mM magnesium acetate, 64 mM total KCl + NH₄Cl (usually 14 mM KCl), 1 mM phosphoenolpyruvate (PEP), 10 μ M GTP (where indicated), 3.1 units of pyruvate kinase (Sigma), elongation factor as indicated, and tRNA as indicated. Prior to the addition of tRNA and then factor, the solution was incubated (37°C, 10 min) to convert any contaminating GDP to GTP. Aliquots (usually 25 μ l) were assayed either for cold trichloroacetic acid-insoluble radioactivity (lysine covalently bound to protein or to tRNA) or for hot (85°C, 10 min) trichloroacetic acid-insoluble radioactivity (lysine covalently bound to protein). Standard procedures and Millipore (HAWP, 0.45 μ) filters were used in the assays. Because the stock solutions of *Artemia* factors contained 25% (v/v) glycerol, the *Artemia* affinity labeling incubations contained as much as 11% (v/v) glycerol.

Electrophoresis. Samples of crosslinked reticulocyte eEF-T were prepared for electrophoretic analysis by scaling up the standard incubations 6 to 10-fold. These incubations were terminated after 2 hours at 37°C by the addition of 2-mercaptoethanol (to 0.3 M) and further incubation (20 min, 37°C). This eliminated unreacted bromoacetyl moieties and prevented non-specific crosslinking during the subsequent procedures. The samples were dried under nitrogen, resuspended in 50 μ l of buffer containing 1% sodium dodecyl sulfate (19) and dansylated reticulocyte eEF-T (8), boiled for 2 min, and electrophoresed on 12.5% acrylamide gels according to Laemmli (19). Following electrophoresis, the position of the dansylated α and γ chains in the gels were determined using ultraviolet light. Gel slices (1.5 mm) were then processed for counting as described elsewhere (8).

Isoelectric Focusing. Samples of crosslinked reticulocyte eEF-T were prepared as described in the preceding section, then resuspended and incubated in 60 μ l 9.5 M urea, 2% NP-40, 2% ampholines (pharmalyte, pH3-10, Pharmacia Fine Chemicals) for 1 hour at 37°C. One-half of the material was focused on

a 4.5% acrylamide gel containing 8 M urea and 3.3% ampholytes prepared as described by Breithaupt *et al.* (20), except that the concentration of amino acids in the gel was twice that reported and NP-40 was omitted. The sample was electrophoresed for 16 hours at room temperature exactly as described (20). At the end of the run, the pH gradient was measured with a microelectrode and the gel was cut transversely into 1 mm slices. Two consecutive slices were added to 0.5 ml of 30% H₂O₂ and incubated for 16 hours at 60° in tightly stoppered scintillation vials. The dissolved slices were counted in 5 ml of ACS scintillation cocktail (Amersham).

RESULTS

Covalent Reaction between eEF-T and εBrAcLys-tRNA. When rabbit reticulocyte eEF-T was incubated with *E. coli* εBrAcLys-tRNA in the presence of GTP, a covalent crosslink was formed between the protein and the aa-tRNA analogue. The time dependence of the crosslinking is shown in Fig. 1 by the appearance of hot trichloroacetic acid-insoluble radioactivity. The radioactive lysine which precipitated must have been covalently attached to protein, because aa-tRNA is hydrolyzed in hot trichloroacetic acid and free amino acid is not

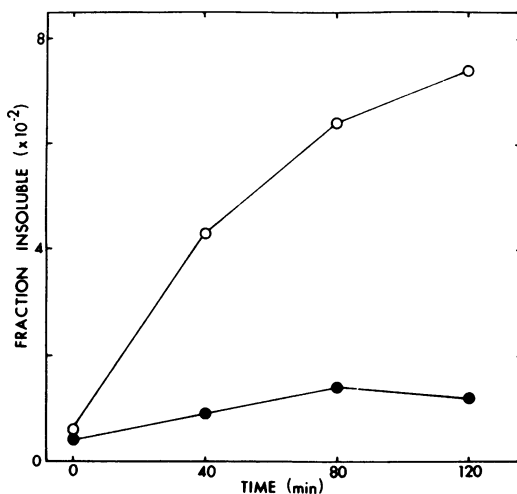


Fig. 1. Incorporation of [¹⁴C]Lys from εBrAcLys-tRNA into hot trichloroacetic acid-insoluble material. Incubation (280 μl; as in Materials and Methods) contained 128 μg of rabbit reticulocyte eEF-T and 0.70 A₂₆₀ units of *E. coli* εBrAcLys-tRNA (prep A; described in Table 5) with (○) or without (●) 10 μM GTP. A background value of 0.028, obtained from an incubation containing GTP but no eEF-T, was subtracted from the data.

retained by the filters. The amount of crosslinking was not increased significantly by extending the incubation time to three hours.

Control experiments demonstrated that an intact bromoacetyl group was required for crosslinking. Since sulfhydryl-containing reagents react efficiently with primary alkyl bromides under the appropriate conditions, we exposed ϵ BrAcLys-tRNA to 0.6 M 2-mercaptoethanol (0°C, pH 7.4, 8 min) prior to incubation with eEF-T and GTP. This eliminated any increase in hot trichloroacetic acid-insoluble radioactivity (either + or - GTP) during the incubation. Therefore, the crosslinking reaction involves replacement of the bromine by an eEF-T nucleophile.

GTP Dependence of Crosslinking. Since ternary complex (aa-tRNA·eEF-T·GTP) formation requires GTP, any crosslinking which results from normal complex formation should be GTP-dependent. Therefore, the amount of crosslinking which constitutes true affinity labeling is given by the difference between the + GTP and - GTP hot trichloroacetic acid-insoluble cpm in Fig. 1. This is confirmed below, where it is shown that the - GTP crosslinking is non-specific.

Affinity labeling of eEF-T by ϵ BrAcLys-tRNA was also observed when the non-hydrolyzable GTP analog, guanylylimidodiphosphate (GMPPNP), at 200 μ M was substituted for GTP in the incubation (data not shown). The extent of covalent reaction was appropriate for the amount of complex formation, as measured by aminoacyl bond protection (discussed below).

Peptidyl-tRNA does not form a ternary complex (2,21). Hence, one would expect DiBrAcLys-tRNA, a reactive analogue of peptidyl-tRNA, to exhibit little or no GTP-dependent crosslinking to elongation factors. The data in Table 1 shows that the GTP-dependent crosslinking is much less with DiBrAcLys-tRNA than with ϵ BrAcLys-tRNA (cf. Table 2). The small amount of covalent reaction which was GTP-dependent in Table 1 probably resulted from the crosslinking of eEF-T to the ϵ BrAcLys-tRNA present in the DiBrAcLys-tRNA preparation (legend to Table 1).

Inhibition of Affinity Labeling by *E. coli* Lys-tRNA. If the covalent reaction with eEF-T occurs while ϵ BrAcLys-tRNA is bound to the protein's aa-tRNA binding site, the addition of unreactive aa-tRNA to the incubation should decrease the amount of crosslinking by competing with ϵ BrAcLys-tRNA for these binding sites. As shown in Table 2, the presence of unmodified *E. coli* Lys-tRNA in the solution greatly reduces the amount of GTP-dependent crosslinking. At high Lys-tRNA concentrations, the affinity labeling is eliminated. This indicates that the covalent reaction is binding site-specific and therefore

Table 1. GTP dependence of covalent reaction between elongation factors and reactive peptidyl-tRNA.

Factor	³ H Lys precipitated in hot trichloroacetic acid, pmol		
	+GTP	-GTP	ΔGTP
<i>Artemia</i> eEF-Tu	1.03	.91	.12
Rabbit reticulocyte eEF-T	1.53	1.12	.41

Incubations (175 μl) contained 0.50 A₂₆₀ units of *E. coli* DiBr-[³H]Lys-tRNA (3230 cpm/pmol Lys) and either eEF-Tu (32 μg) or eEF-T (80 μg). The DiBrAc-Lys-tRNA solution added to each incubation contained 26.9 pmol of lysine esterified to tRNA (86.5% α, ε-dilabeled; 8% ε-labeled; 0.5% unmodified; 94% of the DiBrAcLys contained reactive bromine). Further details are given in Materials and Methods. Data shown is the average of two (rabbit) or three (*Artemia*) separate experiments.

represents true affinity labeling.

The crosslinking reaction was also inhibited by unacylated tRNA (Table 2). This indicates that the eEF-T-GTP complex associates with unacylated tRNA, though apparently much less strongly than with aa-tRNA since 80 times as many unacylated tRNA molecules as Lys-tRNA molecules did not provide the same amount of inhibition of affinity labeling. Prokaryotic EF-Tu has also been found to interact with unacylated tRNA (13,22).

The amount of hot trichloroacetic acid-insoluble radioactivity in the incubations lacking GTP was not eliminated by the presence of Lys-tRNA or

Table 2. Effect of unmodified *E. coli* tRNA on the affinity labeling of rabbit reticulocyte eEF-T.

Incubation	¹⁴ C Lys precipitated in hot trichloroacetic acid, pmol			Affinity labeling, % of control
	+GTP	-GTP	ΔGTP	
A. control	1.79	.44	1.35	100
B. +tRNA	1.31	.39	.92	68
C. +Lys-tRNA (x7)	.86	.55	.31	23
D. +tRNA	.80	.36	.44	33
E. +Lys-tRNA (x25)	.40	.40	0	0

Each incubation (175 μl; Materials and Methods) contained 80 μg of rabbit reticulocyte eEF-T and 18.6 pmol of *E. coli* εBrAc-[¹⁴C]Lys-tRNA (0.48 A₂₆₀ units; prep B; described in Table 5). Unacylated tRNA or Lys-tRNA was added just prior to addition of εBrAcLys-tRNA and eEF-T. Total unfractionated *E. coli* tRNA in incubation was 0.5 A₂₆₀ units; in B and C, 2.5; in D and E, 7.4. Total unmodified *E. coli* Lys-tRNA added to incubation C was 134 pmol, and to E, 470 pmol. Data shown is the average of three separate experiments.

unacylated tRNA (Table 2). This indicates that the crosslinking in the absence of GTP was a non-specific reaction and did not require complex formation between ϵ BrAcLys-tRNA and factor. Furthermore, a significant amount of hot trichloroacetic acid-insoluble radioactivity was observed in some factor-free incubations (Fig. 1), so it is possible that the tRNA solution contains some crosslinked material. This background was subtracted only from the data in Fig. 1.

Inhibition of Affinity Labeling by Rabbit Aminoacyl-tRNA. Several investigators have demonstrated that the reticulocyte cell-free protein synthesizing system does not discriminate against heterologous tRNA (e.g., 23,24), and we have shown that the reticulocyte lysate will incorporate N^{ϵ} -acetyl-lysine from *E. coli* N^{ϵ} -acetyl-Lys-tRNA into tetrameric hemoglobin (25). This indicates that the non-mammalian tRNA interacts with the components of the reticulocyte system in the same manner as the reticulocyte tRNA. However, it is conceivable that the *E. coli* ϵ BrAcLys-tRNA reacts covalently with eEF-T and/or eEF-Tu from a specific binding site other than that normally occupied by the mammalian aa-tRNA. For this reason the sensitivity of the crosslinking to the presence of rabbit aa-tRNA was investigated.

The data in Table 3 demonstrate that GTP-dependent crosslinking to either eEF-T or eEF-Tu from rabbit reticulocytes was decreased by the addi-

Table 3. Inhibition of affinity labeling of rabbit reticulocyte eEF-T and eEF-Tu by rabbit aminoacyl-tRNA.

<u>Incubation</u>	<u>Factor</u>	<u>GTP-dependent Affinity Labeling, % of control</u>
A. Control	eEF-T	100
B. A + aa-tRNA (x 0.8)	eEF-T	87
C. A + aa-tRNA (x 1.6)	eEF-T	59
D. Control	eEF-Tu	100
E. D + aa-tRNA (x 1.6)	eEF-Tu	61

Incubations A, B, and C (175 μ l; Materials and Methods) contained 80 μ g of rabbit reticulocyte eEF-T and 0.48 A_{260} units of *E. coli* ϵ BrAc-[14 C]Lys-tRNA (prep B; described in Table 5). Incubations D and E were the same, except that each incubation contained 40 μ g of rabbit reticulocyte eEF-Tu and 0.03 A_{260} units of purified *E. coli* ϵ BrAc-[14 C]Lys-tRNA^{Lys} (described in Table 5). Aminoacylated rabbit tRNA was added just prior to the addition of ϵ BrAcLys-tRNA and eEF-T or eEF-Tu. Incubation B received 14.3 pmoles of rabbit aa-tRNA (0.01 A_{260} units; 1.0 pmol of [14 C]Leu-tRNA; Materials and Methods), while incubations C and E received twice as much rabbit aa-tRNA. No hot trichloroacetic acid-precipitable [14 C]Leu was found in incubations which lacked only ϵ BrAcLys-tRNA. GTP-dependent affinity labeling (100%) in A totaled 0.95 pmol, and in D, 0.49 pmol.

tion of rabbit aa-tRNA to the affinity labeling incubation. Unacylated rabbit tRNA also inhibited the crosslinking, but much less than did the aminoacylated tRNA (data not shown). The sensitivity of the affinity labeling to the presence of rabbit aa-tRNA demonstrates that this aa-tRNA and the *E. coli* εBrAcLys-tRNA compete for binding to eEF-T or eEF-Tu. This indicates that the covalent reaction occurs while the εBrAcLys-tRNA is bound to the functional aa-tRNA binding site on eEF-T and eEF-Tu.

Ternary Complex Formation. When an aa-tRNA associates with EF-Tu and GTP to form a ternary complex, the aminoacyl ester bond is protected from hydrolysis (26). Therefore, a reduction in the rate of hydrolysis is indicative of complex formation. The extent of aminoacyl bond protection has been used to determine binding constants for aa-tRNA association with EF-Tu·GTP (27). To demonstrate ternary complex formation with eEF-Tu, GTP, and εBrAcLys-tRNA, the hydrolysis of this aa-tRNA was measured under conditions which either prevented (+GDP) or promoted (+GTP, converted from GDP by pyruvate kinase and phosphoenolpyruvate) association with elongation factors. As shown in Table 4, the rate of hydrolysis of εBrAcLys from tRNA was lower in the incubations which contained GTP. Although the differences in the hydrolysis rates with and without GTP was small in the case of eEF-Tu, it was reproducible. Thus, *E. coli* εBrAcLys-tRNA forms a ternary complex with both the eukaryotic and prokaryotic factors.

It is also clear from Table 4 that εBrAcLys-tRNA associates much more strongly with EF-Tu·GTP than with eEF-Tu·GTP. The affinity of εBrAcLys-tRNA for eEF-Tu·GTP was not reduced because of the modification of the Lys-tRNA, because the extent of hydrolysis of *E. coli* Phe-tRNA was the same after 30

Table 4. The interaction of *E. coli* εBrAcLys-tRNA with *E. coli* EF-Tu and *Artemia* eEF-Tu.

Factor	Fraction of aminoacyl bonds hydrolyzed			¹⁴ C]Lys precipitated in hot trichloroacetic acid, pmol		
	+PEP	-PEP	ΔPEP	+PEP	-PEP	ΔPEP
<i>E. coli</i> EF-Tu	.18	.64	.46	8.62	.11	8.51
<i>Artemia</i> eEF-Tu	.65	.72	.07	.99	.37	.62

These incubations differed from the standard 175 μl incubations (Materials and Methods) only in that each contained 10 μM GDP; 1 mM phosphoenolpyruvate (PEP) was included as indicated. Each incubation also contained 0.48 A₂₆₀ units of εBrAc-[¹⁴C]Lys-tRNA (prep B; described in Table 5), and either 20 μg of eEF-Tu or 15 μg of EF-Tu.

min in solutions containing either 1.4×10^{-5} M rabbit eEF-Tu·GTP or 6.5×10^{-7} M *E. coli* EF-Tu·GTP. Thus, a higher concentration of eEF-Tu·GTP than of EF-Tu·GTP was required to obtain the same degree of protection of the aminoacyl bond of an unmodified aa-tRNA. Nor was the ternary complex with the eukaryotic factor weaker because heterologous tRNA was being used. Even with a 7-fold greater concentration of rabbit eEF-Tu than of *E. coli* EF-Tu in parallel incubations, the homologous ternary complex formed with rabbit eEF-Tu·GTP and rabbit aa-tRNA provides less aminoacyl bond protection than does the heterologous complex formed with *E. coli* EF-Tu·GTP. This is demonstrated by a semi-log plot of the hydrolysis data (Fig. 2) which shows that the rate of deacylation was much faster in the eEF-Tu-containing sample. Although we cannot rule out the possibility that eEF-Tu does not protect the aminoacyl bond of a complexed aa-tRNA as well as does EF-Tu, our results are consistent with those of others which show that the eukaryotic elongation factor forms

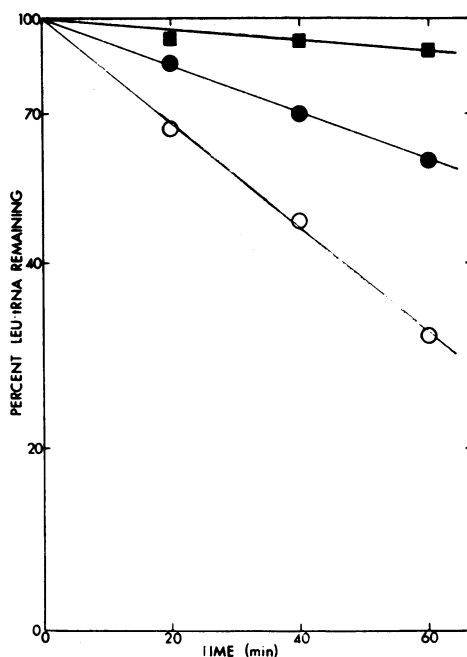


Fig. 2. Elongation factor-dependence of aminoacyl bond hydrolysis. Standard 175 μ l incubations at 37°C contained 71 pmol of rabbit aa-tRNA (5 pmol of [14 C]Leu-tRNA; Materials and Methods), and either 40 μ g of rabbit reticulocyte eEF-Tu (●) or 5 μ g of *E. coli* EF-Tu (■) or no factor (○). Aliquots (20 μ l) were precipitated in cold trichloroacetic acid with 1.1 A₂₆₀ units of ribosomal RNA as carrier. Data shown is the average of two separate experiments.

a much weaker ternary complex than does the prokaryotic factor (2).

Extent of Covalent Reaction. The extent of crosslinking of ϵ BrAcLys-tRNA to eEF-Tu is much less than it is to EF-Tu (Table 4). It is probable that this results primarily from the large difference in the strength of ternary complex formation (Table 4, Fig. 2), and hence from the large difference in the time the ϵ BrAcLys-tRNA and the factor are associated and in a position to react covalently. But the efficiency of covalent reaction also depends upon the particular protein nucleophile involved in the crosslinking and its accessibility to the bromoacetyl group. The degree to which the latter contributes to the difference in the crosslinking of EF-Tu and eEF-Tu has not yet been established.

In an effort to improve the aminoacyl bond protection and the efficiency of crosslinking to the eukaryotic factors, we surveyed a range of incubation conditions which included those found optimal by various groups (e.g., 6,12, 28). But the protection and crosslinking were either reduced or not significantly changed when the following changes were made in the standard protocol: $[Mg^{2+}]$ at 1, 3, or 10 mM; $([NH_4^+] + [K^+])$ at 15, 43, 93, 100 or 143 mM; pH at 7.6, 7.8, or 8.0; [GTP] at 0.1 mM; addition of glycerol to 25%; or addition of bovine serum albumin to 0.5 mg/ml. The extent of GTP-dependent affinity labeling was increased by increasing either the factor or the ϵ BrAcLys-tRNA concentration, but these increases were not directly proportional at concentration levels above those used in the standard incubations.

The extent of crosslinking observed in supposedly equivalent incubations varied somewhat (e.g., compare Tables 2 and 5). This has also been observed in other systems (13,14). The variations probably result from differences in the particular macromolecule preparations utilized, either in terms of the fraction of intact bromoacetyl groups in the ϵ BrAcLys-tRNA or in the fraction of functional molecules in the eEF-Tu or eEF-T.

Specificity of Affinity Labeling. The affinity labeling is not much affected by the source of the tRNA, since the extent of GTP-dependent crosslinking was about the same for both yeast and *E. coli* ϵ BrAcLys-tRNA (Table 5). As expected, small variations in the amount of reactive peptidyl-tRNA (DiBrAcLys-tRNA) in the incubations did not affect the association of ϵ BrAcLys-tRNA with, and its crosslinking to, eEF-T. This is shown by the similar data obtained using two different preparations of *E. coli* ϵ BrAcLys-tRNA (Table 5). The small difference in crosslinking obtained with these two ϵ BrAcLys-tRNA solutions probably results primarily from a difference in the percentage of intact bromoacetyl groups in the samples. The cause of the large amount of

Table 5. Affinity labeling of rabbit reticulocyte eEF-T with various ϵ BrAcLys-tRNAs.

ϵ BrAcLys-tRNA	^{14}C Lys precipitated in hot trichloroacetic acid, pmol		
	+GTP	-GTP	Δ GTP
<i>E. coli</i> , prep A	1.25	.27	.98
<i>E. coli</i> , prep B	1.47	.33	1.14
purified <i>E. coli</i>	2.15	.62	1.53
yeast	2.70	1.66	1.04

Each incubation (175 μ l; Materials and Methods) contained 80 μ g of rabbit reticulocyte eEF-T. The ϵ BrAc- ^{14}C Lys-tRNA (prep A) added to the indicated incubations contained 17.7 pmol of lysine esterified to tRNA (90% ϵ -labeled; 3% α , ϵ -dilabeled; 3% α -labeled; 4% unmodified; 79% of ϵ -bromoacetyl groups intact; 665 cpm/pmol Lys), and totaled 0.44 A_{260} units. The corresponding values for ϵ BrAc- ^{14}C Lys-tRNA (prep B) were 24.1, 77%, 9%, 3%, 11%, 87%, 620, and 0.48 A_{260} units. For purified ϵ BrAc- ^{14}C Lys-tRNA^{lys}, 22.9, 85%, 10%, 0.5%, 4.5%, 88%, 565, and 0.03. For yeast ϵ BrAc- ^{14}C Lys-tRNA, 24.5, 69%, 17%, 3%, 11%, 80%, 555, and 0.44. Data shown is the average from two (purified *E. coli*), three, or four (yeast) separate experiments.

crosslinking of yeast ϵ BrAcLys-tRNA to rabbit eEF-T in the absence of GTP has not yet been determined. It may result from binary complex formation between the tRNA and the factor. Such a complex has been detected between yeast Phe-tRNA and *Artemia* eEF-Tu (28).

Both eEF-T and eEF-Tu from *Artemia* (brine shrimp) are affinity labeled by *E. coli* ϵ BrAcLys-tRNA, as are both eEF-T and eEF-Tu from rabbit reticulocytes. Table 6 shows the extent of crosslinking observed with each of these factors. The differences in crosslinking efficiency may reflect intrinsic differences in the factors, or - more likely - differences in the activity of these particular preparations of the factors.

Subunit Specificity of Affinity Labeling. The similarity in the extent of alkylation of eEF-T and eEF-Tu (Table 6) suggests that ϵ BrAcLys-tRNA reacts covalently only with the eEF-Tu polypeptide in the multisubunit eEF-T. This interpretation was confirmed as follows. Rabbit reticulocyte eEF-T was incubated as usual with ϵ BrAcLys-tRNA, both in the absence and the presence of GTP. The eEF-T subunits were dissociated, and then separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The radioactivity found in the gels was distributed as shown in Fig. 3.

Radioactivity co-electrophoresed with large molecular weight polymers

Table 6. Affinity labeling of various eukaryotic elongation factors.

Factor	$[^{14}\text{C}]\text{Lys}$ precipitated in hot trichloroacetic acid, pmol		
	+GTP	-GTP	ΔGTP
Rabbit reticulocyte eEF-T	1.20	.22	.98
Rabbit reticulocyte eEF-Tu	1.55	.47	1.08
<i>Artemia</i> eEF-T	1.02	.26	.76
<i>Artemia</i> eEF-Tu	1.36	.29	1.07

The incubations (175 μl ; Materials and Methods) contained 80 μg of rabbit reticulocyte eEF-T, 40 μg of rabbit eEF-Tu, 80 μg of *Artemia* eEF-T, or 32 μg of *Artemia* eEF-Tu. Assuming that eEF-Tu constitutes 40% of the eEF-T preparations (8), the *Artemia* incubations contained equivalent amounts of eEF-Tu. The rabbit eEF-Tu incubation contained 0.48 A_{260} units of *E. coli* $\epsilon\text{BrAc}-[^{14}\text{C}]\text{Lys-tRNA}$ (prep B; see Table 5), and the others contained 0.44 A_{260} units of *E. coli* $\epsilon\text{BrAc}-[^{14}\text{C}]\text{Lys-tRNA}$ (prep A; see Table 5).

only in the incubation which contained GTP, and the position of the radioactive peak coincided with the location of the eEF-Tu polypeptide in the gel. In neither incubation (+ or - GTP) did the eEF-Ts (MW = 30,000; ref. 4) react

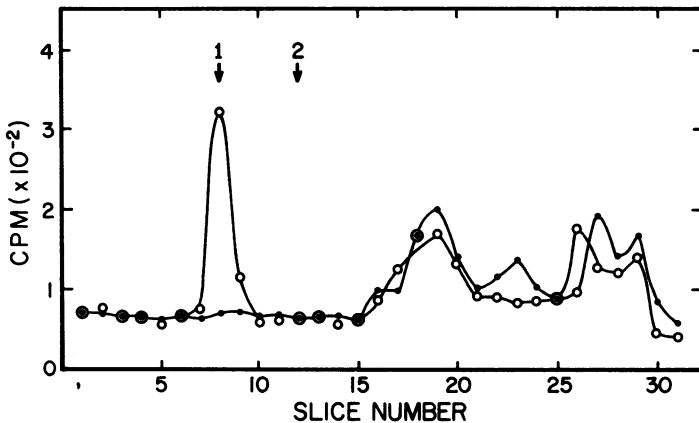


Fig. 3. Analysis of crosslinked products by SDS-gel electrophoresis. The +GTP (o) incubation (1.05 ml; Materials and Methods) contained 2.88 A_{260} units of *E. coli* $\epsilon\text{BrAc}-[^{14}\text{C}]\text{Lys-tRNA}$ (prep B; Table 5) and 480 μg of rabbit reticulocyte eEF-T. The -GTP (o) incubation (1.75 ml; Materials and Methods) contained 4.8 A_{260} units of the same tRNA and 800 μg of eEF-T. Portions of each sample (210 μg of +GTP; 350 μg of -GTP) were electrophoresed using procedures detailed in Materials and Methods. The eEF-Tu marker was located at position 1 in the gel, and the eEF-Ts marker at position 2.

covalently with ϵ BrAcLys-tRNA. Since eEF-Tu and the β chain have similar molecular weights (53,000 and 51,000, respectively; ref. 8), it was conceivable that the radioactivity associated with the high molecular weight species was covalently attached to the β polypeptide, even though the presence of the β chain in an affinity labeling incubation did not increase the extent of crosslinking (Table 6). This possibility was examined using a different technique. Purified rabbit reticulocyte eEF-T is resolved into three component polypeptides after isoelectric focusing: an eEF-Tu (α) chain with an isoelectric point of 8.5; a β chain with an isoelectric point of 6.5; and an eEF-Ts (γ) chain with an isoelectric point of 5.5 (15). When a sample of crosslinked rabbit eEF-T was examined by isoelectric focusing, no radioactivity was found associated with the β polypeptide (Fig. 4). Thus, only eEF-Tu is affinity labeled by ϵ BrAcLys-tRNA. The absence of covalent reaction with eEF-Ts and the β chain is additional evidence that the crosslinking is a specific reaction.

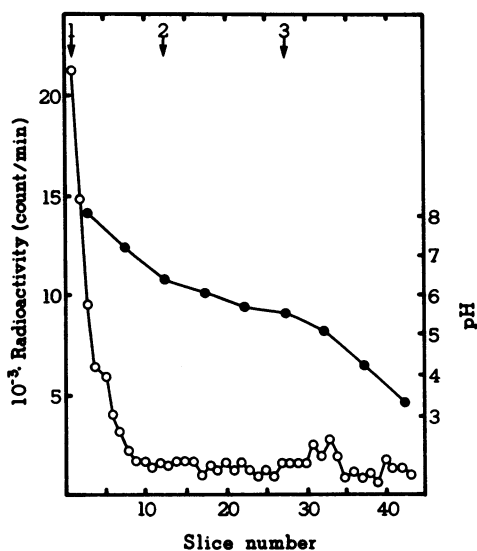


Fig. 4. Analysis of crosslinked products by isoelectric focusing. The incubation (525 μ l; Materials and Methods) contained: 1.84 A_{260} units of *E. coli* ϵ BrAc-[³H]Lys-tRNA (166 pmol of lysine esterified to tRNA; 75% ϵ -labeled; 11% α , ϵ -dilabeled; 6% α -labeled; 8% unmodified; 47% of ϵ -bromoacetyl groups intact; 9880 dpm/pmol Lys; not treated with peptidyl-tRNA hydrolase); 10 μ M GTP; and 250 μ g of reticulocyte eEF-T. Approximately 110 μ g of eEF-T was analyzed using the procedures described in Materials and Methods. Radioactivity is shown by the open circles (o), and pH by the closed circles (\bullet). The numbers 1, 2, and 3 refer to the focused positions of eEF-Tu, β chain, and eEF-Ts, respectively.

The data in Fig. 3 also shows that eEF-Tu was crosslinked to *E. coli* ϵ BrAcLys-tRNA only when GTP was present in the incubation. This indicates that the hot trichloroacetic acid-insoluble radioactivity observed in incubations lacking GTP resulted from ϵ BrAcLys-tRNA crosslinking to small molecular weight peptides, presumably a fraction of the small molecular weight material shown in Fig. 3. This covalent reaction is not specific and does not result from a functional association between eEF-Tu and ϵ BrAcLys-tRNA, since the reaction is GTP-independent. This result is not surprising in view of the fact that the - GTP crosslinking was not affected by the presence of tRNA molecules which could compete for a legitimate aa-tRNA binding site (Table 2). The identity and origin of the small peptides is not known.

Similar results were obtained when affinity labeling incubations containing DiBrAcLys-tRNA instead of ϵ BrAcLys-tRNA were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (data not shown). Only crosslinking to an eEF-Tu-sized polypeptide was GTP-dependent, and this probably resulted primarily from reaction with the ϵ BrAcLys-tRNA which was present as a contaminant in the DiBrAcLys-tRNA preparation (see legend to Table 1).

DISCUSSION

The chemical crosslinking described in this paper constitutes an affinity labeling reaction because (i) complex formation between ϵ BrAcLys-tRNA, eEF-Tu, and GTP was required for crosslinking (Fig. 1,3), and (ii) crosslinking occurred from the normal binding site for aa-tRNA on the protein (Tables 2,3). Furthermore, a reactive tRNA which does not normally associate with eEF-Tu exhibited little, if any, GTP-dependent crosslinking to the protein (Table 1).

The GTP-dependent affinity labeling by ϵ BrAcLys-tRNA was specific for eEF-Tu, even in the presence of eEF-Ts and the β chain (Fig. 3,4). The eEF-Tu residue(s) which reacts with ϵ BrAcLys-tRNA must be on the surface of the protein, accessible, and located less than 9 Å (the fully extended length of the ϵ BrAcLys side-chain) along the surface from the position of the lysine α -carbon in the ternary complex. We do not yet know with certainty whether eEF-Tu dissociates from the β and γ chains of eEF-T upon ternary complex formation. If the complex remains undissociated, then two conclusions follow. First, because the other subunits of eEF-T are not affinity labeled and do not prevent the affinity labeling of eEF-Tu (Fig. 3,4; Table 5), the aminoacyl end of the aa-tRNA binding site on eEF-Tu does not appear to be close

to the other subunits. Second, because the affinity labeling results are the same with both eEF-T and eEF-Tu, it appears that the aminoacyl part of the aa-tRNA binding site is the same in both eEF-Tu and the multisubunit eEF-T.

The successful affinity labeling of eEF-Tu will provide direct structural information about the protein-nucleic acid complex. In particular, the identification of the reactive eEF-Tu residue(s) will provide a reference point(s) for establishing the orientation of the macromolecules in the complex, once the crystal structure of eEF-Tu has been determined. It is also worth noting that the covalent complex we have described may facilitate crystallization of the protein-nucleic acid complex, since it is non-dissociable. However, the low efficiency of covalent reaction in this case may be an almost insurmountable barrier. Thus, such studies, as well as investigations of the functional activity of the crosslinked eukaryotic complex (the crosslinked prokaryotic complex is functionally active (13)), must await the establishment of efficient preparation and purification methodology.

Our data suggest that the aa-tRNA binding sites of the prokaryotic and eukaryotic factors differ considerably, since the association constant between aa-tRNA and factor-GTP is much higher with EF-Tu than with eEF-Tu (Table 4, Fig. 2). It is possible that this difference in the ability to form ternary complexes may contribute to the observed difference in the rate of protein chain elongation in prokaryotes and eukaryotes (29,30). On the other hand, the affinity labeling of bacterial, shrimp, and rabbit elongation factors by the same reagent is striking, and suggests that the factors may have significant structural similarities at the aminoacyl end of the aa-tRNA binding sites. It will be interesting to compare the affinity-labeled residues and peptides of EF-Tu and eEF-Tu.

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