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Functional covalent complex between elongation factor Tu and an analog of lysyl-tRNA

(affinity labeling/protein synthesis/N^{ϵ}-bromoacetyl-Lys-tRNA)

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ABSTRACT **Complex formation between elongation factor Tu, GTP, and N^{ϵ}-bromoacetyl-Lys-tRNA results in the crosslinking of the protein and the modified Lys-tRNA. The efficiency of affinity labeling is greater than 50%. In the presence of unmodified Lys-tRNA, the amount of crosslinking is greatly decreased. There is no covalent reaction with elongation factor Tu in the absence of complex formation. Substantial purification of the crosslinked ternary complex can be achieved by gel filtration at low Mg^{2+} concentration and passage through nitrocellulose filters. The crosslinked complex exhibits message-dependent binding to ribosomes which is accompanied by the hydrolysis of the associated GTP, as shown by both filter assays and gel filtration profiles. The crosslinked complex therefore appears to function normally except for its inability to dissociate. These experiments demonstrate that the ternary complex is the true intermediate in the binding of aminoacyl-tRNA to the ribosomes.**

The specific interactions that govern protein–nucleic acid recognition and association are not well understood, despite their importance both structurally and functionally. This results in part from the noncovalent nature of the association. Complex dissociation, for example, has stymied attempts to crystallize protein–nucleic acid complexes, except in the case of nucleosome core particles (1). Considerable effort has been directed toward obtaining a specific covalent complex of a protein and a nucleic acid. Covalent complexes between tRNAs and aminoacyl-tRNA synthetases are among those reported (2, 3). A major difficulty in this approach is demonstrating that the structure of the crosslinked complex is functionally significant and that the complex was not trapped by the crosslinking reaction in a perhaps closely related but nonfunctional conformation. In this paper we describe a crosslinked complex that is able to function *in vitro*.

In bacterial protein biosynthesis, aminoacyl-tRNA (AA-tRNA) forms a ternary complex with elongation factor Tu (EF-Tu) and GTP (4, 5). It is believed that this ternary complex then binds to ribosomes in response to the appropriate codon. After the GTP in the complex is hydrolyzed, EF-Tu·GDP and P_i are released from the ribosome, and the AA-tRNA is positioned to react with the growing peptide chain. Although this reaction sequence is consistent with the available data, it has not been established by a kinetic analysis.

Nearly all AA-tRNAs bind to EF-Tu·GTP, irrespective of their amino acid side chains, and chemical modification of the lysine side chain of Lys-tRNA does not greatly affect the binding of its ternary complex to ribosomes (6). In this report we demonstrate that ternary complex formation with the reactive AA-tRNA analog N^{ϵ}-bromoacetyl-Lys-tRNA (ϵBrAc-Lys-tRNA) results in covalent bond formation between the modified lysine and EF-Tu. This reaction represents true affinity labeling, because no covalent reaction occurs without complex formation and because competition for the EF-Tu binding site by unmodified Lys-tRNA greatly decreases the yield of crosslinked product. The crosslinking does not destroy activity: the binding of crosslinked ternary complex to ribosomes is stimulated by poly(A) and is accompanied by GTP hydrolysis.

MATERIALS AND METHODS

EF-Tu was prepared from *Escherichia coli* B (7). Partially purified peptidyl-tRNA hydrolase and S-100 enzymes were prepared from *E. coli* A-19 or MRE 600 (8). The peptidyl-tRNA hydrolase was further purified by chromatography and concentration on CM-cellulose by procedures similar to those of Kössel (9). Unfractionated *E. coli* MRE 600 (Boehringer) or K-12 (Grand Island Biologicals) tRNA was aminoacylated with radioactive lysine (New England Nuclear) as described (8), except that no sodium thiosulfate was used. Sometimes phenol extraction was used instead of benzoylated DEAE-cellulose chromatography to separate Lys-tRNA from protein. The preparation of ϵBrAcLys-tRNA is similar to that of N^{ϵ}-acetyl-Lys-tRNA (8) and will be detailed elsewhere, as will the procedures for preparing $(BrAc)_2$Lys-tRNA.

Crosslinked ternary complex was prepared in quantity by using 500-μl incubations containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) at pH 7.4, 80 mM NH_4Cl, 40 μM GTP, 2 mM phospho*enol*pyruvate, 50 μg of pyruvate kinase (Worthington), 1200 pmol of EF-Tu·GDP, and 400 pmol of ϵBrAcLys-tRNA. A preincubation (5 min, 37°) without EF-Tu·GDP and ϵBrAcLys-tRNA permitted the conversion of any contaminating GDP to GTP; after EF-Tu·GDP addition, a second preincubation (10 min, 37°) permitted the formation of EF-Tu·GTP complexes. This solution was cooled in ice before and for 5 min after ϵBrAcLys-tRNA addition to minimize the nucleophilic substitution of bromine prior to complex formation. The final incubation was typically at 37° for 60 min, although a longer time yielded more crosslinking. Because $(BrAc)_2$Lys-tRNA did not alkylate EF-Tu, the peptidyl-tRNA hydrolase incubation was eliminated from the Lys-tRNA modification procedures (8).

Crosslinked ternary complex was separated from most noncovalent ternary complex, free EF-Tu, and free Lys-tRNA by gel filtration. The low Mg^{2+} concentration causes the dissociation of the uncrosslinked ternary complex and improves the separation; however, it also causes the crosslinked ternary complex to lose GTP, which can be restored by reincubation

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Abbreviations: ϵBrAcLys-tRNA, N^{ϵ}-bromoacetyl-Lys-tRNA; Br_2Ac-Lys-tRNA, *N,N*-dibromoacetyl-Lys-tRNA; EF-Tu, elongation factor Tu; AA-tRNA, aminoacyl-tRNA; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

with GTP, Mg^{2+}, phospho*enol*pyruvate, and pyruvate kinase. Any excess EF-Tu present can be removed by filtration through type HA Millipore filters, to which the protein binds. The crosslinked ternary complex in the filtrate was concentrated in an Amicon stirred pressure dialysis cell.

The composition of a crosslinked ternary complex solution is determined by assaying the total radioactivity, the cold trichloroacetic acid-insoluble radioactivity (lysine covalently bound to protein or tRNA), the hot trichloroacetic acid-insoluble radioactivity (lysine covalently bound to protein), and the radioactivity that binds to Millipore filters (lysine covalently bound to EF-Tu in disrupted crosslinked ternary complexes). Because intact ternary complexes do not bind to filters (7), the disrupted covalent complexes either contain denatured EF-Tu or lack GTP or tRNA (see *Results*).

Ribosome binding incubations contained, in 200 μl: 7 mM magnesium acetate, 50 mM Tris·HCl (pH 7.4), 75 mM NH_4Cl, 75 mM KCl, 5 mM dithiothreitol, 0.53–0.69 A_{260} unit of poly(A) (P-L Biochemicals), 1.8 A_{260} units of *E. coli* A-19 salt-washed ribosomes (8) or MRE 600 ribosomes (10), 3.1 A_{260} units of unacylated tRNA, and crosslinked ternary complex as indicated. Ribosomes, poly(A), and salts were preincubated (25 min, 37°) before the addition of unacylated tRNA and ternary complex to complete the final incubation (30 min, 37°). The binding was assayed either by Millipore HA filter adsorption (8) or by Sepharose 6B chromatography at 4°. In the latter, a 100-μl aliquot of the binding incubation was layered on a 0.6 × 8.5 cm column equilibrated and washed with 10 mM Tris·acetate, pH 7.2/10 mM $MgCl_2$/50 mM NH_4Cl. Three-drop (0.13 ml) fractions were collected and assayed for radioactivity by using a Triton scintillator (8).

RESULTS

Ternary Complex Formation with εBrAcLys-tRNA. The kinetics of deacylation of εBrAcLys-tRNA under various conditions are depicted in Fig. 1. The rate of hydrolysis of εBrAcLys from tRNA was much less in the presence of both EF-Tu and GTP than in the absence of either (the incubation lacking phospho*enol*pyruvate was unable to convert GDP to GTP). AA-tRNA association with EF-Tu and GTP to form a ternary complex results in protection of the aminoacyl ester bond from hydrolysis (11). The data in Fig. 1 therefore demonstrate that εBrAcLys-tRNA is capable of ternary complex formation in spite of the modified lysine side chain. This result was anticipated, because earlier work showed that εBrAcLys-tRNA binding to ribosomes was EF-Tu-dependent (6).

Affinity Labeling of EF-Tu. Incubation of εBrAcLys-tRNA with EF-Tu and GTP resulted in covalent bond formation between the protein and the AA-tRNA analog. This is shown in Fig. 2 by the appearance of hot trichloroacetic acid-insoluble radioactivity in a solution that originally contained radioactive lysine only in various tRNA species. Because tRNA is hydrolyzed in hot trichloroacetic acid, only lysines covalently attached to protein will precipitate.

Ternary complexes containing Lys-tRNA are noncovalent associations, so the covalent reaction must depend on the modification of the Lys-tRNA. Exposure of εBrAcLys-tRNA to 0.5 M mercaptoethanol 15 min prior to incubation with EF-Tu and GTP decreased the covalent reaction by 97%. Hence, the reaction probably involves replacement of the bromine by an EF-Tu nucleophile.

Alkylation of EF-Tu is achieved only in a complete incubation mixture, as shown in Fig. 2. $(BrAc)_2$Lys-tRNA, a reactive analog of peptidyl-tRNA that does not form a ternary complex (6), does not alkylate EF-Tu. This shows that there is no non-

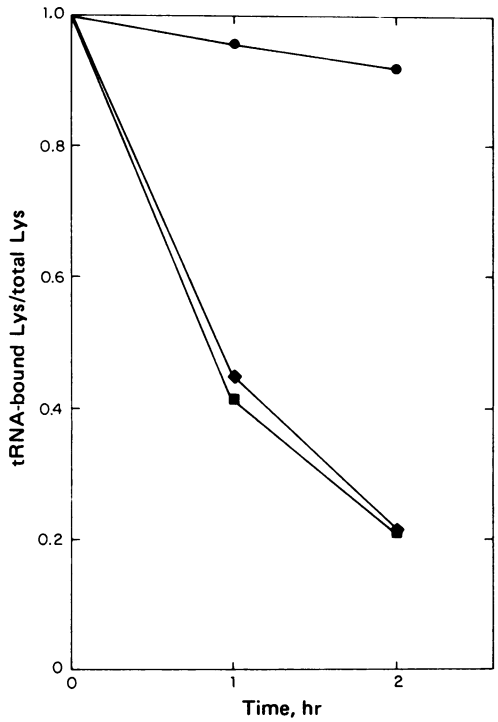

FIG. 1. Ternary complex formation inhibits εBrAcLys-tRNA deacylation. Parallel incubations at 37° contained, in 0.5 ml: 10 mM magnesium acetate, 50 mM Hepes (pH 7.4), 50 mM NH_4Cl, 10 μM GDP, 100 μM dithiothreitol, 3.9 units of pyruvate kinase (Sigma), 1 mM phospho*enol*pyruvate (except ■), 60 μg of EF-Tu·GDP (except ♦), and 1.48 A_{260} units of εBrAc[^{14}C]Lys-tRNA (77 pmol of [^{14}C]Lys; 601 cpm/pmol Lys). Analysis of the εBrAcLys-tRNA solution by paper electrophoresis after alkaline hydrolysis (8) yielded 73% εBrAcLys-tRNA, 8% $(BrAc)_2$Lys-tRNA, 11% Lys-tRNA, 3% N^{α}-BrAcLys-tRNA, and 5% unhydrolyzed adducts of lysine species and tRNA; a minimum of 80% of the bromoacetyl groups reacted with 2-mercaptoethanol. Aliquots (35 μl) were assayed for cold trichloroacetic acid-insoluble radioactivity at the times indicated.

specific covalent reaction between EF-Tu and tRNA-bound bromoacetyl moieties. Alkylation by small molecules does not occur because there is no alkylation in the −PEP incubation without phospho*enol*pyruvate, even though that solution contains more free εBrAcLys (due to deacylation; Fig. 1) than does the complete incubation. Neither EF-Tu·GTP nor EF-Tu·GDP reacts covalently with tRNA-bound bromoacetyl groups, as shown by the $(BrAc)_2$Lys-tRNA and the phospho*enol*pyruvate-free data, respectively, in Fig. 2. Pyruvate kinase is not the protein that is alkylated, because there was no alkylation in the EF-Tu-free incubation. The lack of alkylation in the control incubations of Fig. 2 demonstrate that the covalent reaction requires ternary complex formation.

The yield of covalent product in the affinity labeling incubation was exceptionally high (Fig. 2). After 1 hr of incubation the alkylation was generally 40–50% but has ranged from 30 to 70%, relative to the amount of εBrAcLys-tRNA added. The actual yields are higher because a percentage (up to 20%) of the added εBrAcLys-tRNA lacks reactive bromines. The extent of crosslinking increased with pH; the optimal pH seems to be above 8.

Inhibition of Affinity Labeling by Lys-tRNA. If the alkylation of EF-Tu is binding site-specific, the presence of unreactive AA-tRNA in the affinity labeling incubation should decrease the amount of covalent reaction by competing with εBrAcLys-tRNA for EF-Tu binding sites. In fact, addition of unmodified Lys-tRNA to the mix dramatically decreased the amount of crosslinking (Table 1). This indicates that the

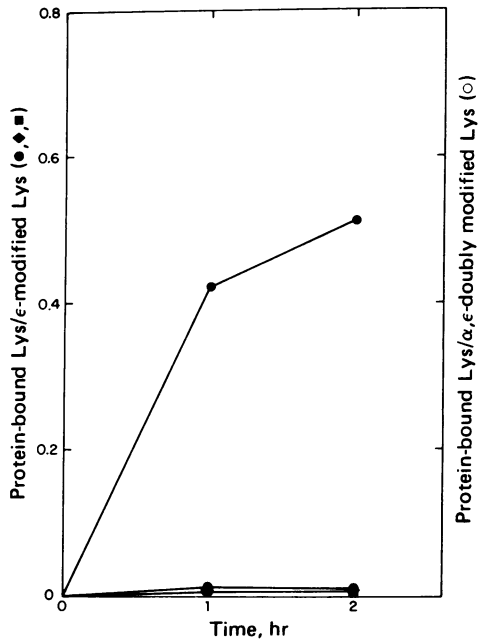

FIG. 2. Affinity labeling of EF-Tu by ϵBrAcLys-tRNA. Aliquots (35 μl) from the incubations of Fig. 1 were assayed for lysine moieties covalently attached to protein by measuring the hot (10 min, 80°) trichloroacetic acid-insoluble radioactivity. Ordinate values for these incubations (complete, ●; without EF-Tu, ♦; without phospho*enol*pyruvate, ■) were calculated as a function of the amount of ϵBrAcLys-tRNA originally present. Data obtained from a complete incubation that contained, instead of ϵBrAcLys-tRNA, 1.50 A_{260} units of $(BrAc)_2$[^{3}H]Lys-tRNA (81 pmol of [^{3}H]Lys; 2470 cpm/pmol Lys) are given by the open circles (○); in this case, ordinate values were calculated as a function of the amount of $(BrAc)_2$Lys-tRNA originally present and corrected for the crosslinking expected from the ϵBrAcLys-tRNA impurity in the $(BrAc)_2$Lys-tRNA solution.

alkylation is binding site-specific and therefore constitutes true affinity labeling.

Unacylated tRNA also inhibited the alkylation. However, the binding constant for the association of EF-Tu·GTP and unacylated tRNA was considerably less than that for normal ternary complex formation: approximately 200 times as many unacylated tRNA molecules as Lys-tRNA molecules were required to inhibit alkylation to the same extent. An interaction between EF-Tu and unacylated tRNA has been detected by using NMR spectroscopy (12).

Table 1. Effect of unmodified tRNA on the affinity labeling of EF-Tu*

	Unacylated tRNA, A_{260} units	Lys-tRNA, pmol	Alkylation, % of control†
Control	0.5	3	100
+ tRNA (× 6)	3.0	3	78
+ Lys-tRNA (× 7)	3.0	120	17
+ tRNA (× 21)	10.5	3	29
+ Lys-tRNA (× 26)	10.5	474	4

* Parallel 175-μl incubations contained 50 mM Hepes (pH 7.4), 50 mM NH_4Cl, 10 mM magnesium acetate, 10 μM GTP, 1 mM phospho*enol*pyruvate, 3.1 units of pyruvate kinase (Sigma), 5 μg of EF-Tu·GDP, 18 pmol of ϵBrAc[^{14}C]Lys-tRNA (the same solution as in Fig. 1), and unacylated tRNA and Lys-tRNA as indicated. The mixtures were incubated for 10 min at 37° without tRNA and then cooled in ice for 5 min. Unacylated tRNA or Lys-tRNA was added to a mixture immediately prior to the addition of ϵBrAcLys-tRNA and incubation at 37° for 60 min. Aliquots were assayed for hot trichloroacetic acid-insoluble radioactivity.

† Calculated as in Fig. 1. In the control 44% of ϵBrAcLys-tRNA reacted covalently with EF-Tu.

Purification of Crosslinked Ternary Complex. Purification of the covalent ternary complex necessitated its separation from noncovalent ternary complex, uncomplexed EF-Tu, and uncomplexed tRNA species. Because ternary complexes require Mg^{2+} for stability, removal of Mg^{2+} results in dissociation of the noncovalent ternary complex. Gel filtration in the absence of Mg^{2+} can then separate the crosslinked ternary complex from the resulting mixture of uncomplexed EF-Tu and tRNA. Such a separation is presented in Fig. 3. The crosslinked ternary complex eluted from the column in fraction 47, well ahead of the uncrosslinked ternary complex, EF-Tu, or Lys-tRNA. The earlier elution of crosslinked ternary complex both at 10 mM Mg^{2+} (data not shown) and at 0.1 mM Mg^{2+} (Fig. 3) presumably results from the nondissociable nature of the complex. The material eluting in fraction 70 is an uncomplexed Lys-tRNA species, probably N^{α}-bromoacetyl-Lys-tRNA and $(BrAc)_2$-Lys-tRNA. At a Mg^{2+} concentration of 0.1 mM, these species emerged earlier, at fraction 59. The cause of this shift to a larger apparent molecular size is unknown. Perhaps the low Mg^{2+} concentration induces the tRNA to unfold.

We have not routinely isolated crosslinked complexes completely free of uncrosslinked complexes. However, the degree of contamination may be less than indicated by the ratio of hot to cold trichloroacetic acid-insoluble radioactivity (Table 2), because the hot precipitation may be incomplete and the cold acid-insoluble radioactivity may include tRNA aggregates.

Nucleotide Exchange. During gel filtration in 0.1 mM Mg^{2+}, GTP was released and separated from the covalent complex. An active ternary complex was regained upon addition of Mg^{2+} and GTP to the pooled fractions of crosslinked protein–nucleic acid. Crosslinked ternary complexes containing [γ-^{32}P]GTP or [^{14}C]GTP were prepared in this way. It is also possible, by using this method, to prepare crosslinked complexes containing the nonhydrolyzable analog guanylylimidodiphosphate instead of GTP.

After gel filtration at low Mg^{2+} concentration, crosslinked ternary complex bound to Millipore filters. The absence of the nucleotide allowed the protein to bind to the filter despite the presence of the tRNA moiety, suggesting that the crosslinked

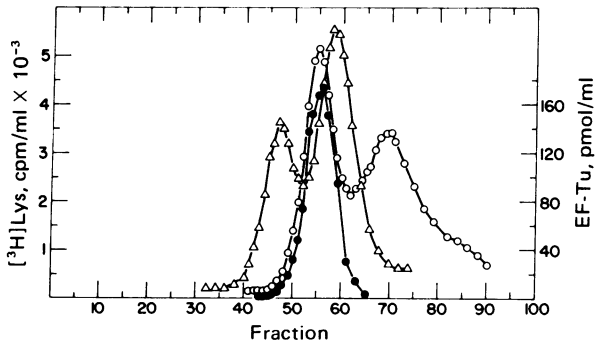

FIG. 3. Gel filtration analysis of normal and crosslinked ternary complexes. Crosslinked ternary complex, prepared with ϵBrAc-[^{3}H]Lys-tRNA, was subjected to gel filtration chromatography on a 1.6 × 60 cm column of Ultrogel AcA44 equilibrated with 20 mM Tris·HCl, pH 7.4/0.1 mM $MgCl_2$/100 mM NH_4Cl/1 mM dithiothreitol (△). For comparison, a normal (uncrosslinked) ternary complex was prepared and chromatographed under the same conditions except that (*i*) the 37° crosslinking incubation was omitted and (*ii*) the $MgCl_2$ concentration in the chromatography buffer was increased to 10 mM. The column effluent was assayed for both [^{3}H]Lys (○) and EF-Tu·GDP (●).

Table 2. Binding of crosslinked EF-Tu·ϵBrAc[^{3}H]Lys-tRNA·[γ-^{32}P]GTP to ribosomes*

	Total (−)poly(A)	Total (+)poly(A)†	Crosslinked (+)poly(A)†
[^{3}H]Lys bound, pmol	0.39	0.85	0.56
[γ-^{32}P]GTP bound, pmol	0.01	0.01	0.00

* Incubations were as described in *Materials and Methods*, except that these 50-μl incubations contained 25 μM GTP and one-fourth as much poly(A) (except as indicated), tRNA, and ribosomes. The crosslinked ternary complex solution was purified over Ultrogel but not through Millipore filters. Each incubation received 0.93 pmol of crosslinked ternary complex (3620 ^{3}H cpm/pmol Lys), 0.30 pmol of uncrosslinked species, 0.21 pmol of disrupted crosslinked ternary complex, and 10 pmol of [γ-^{32}P]GTP (1070 cpm/pmol). A ribosome-free blank containing 0.21 pmol of ^{3}H and 0.01 pmol of ^{32}P has been subtracted from all of the values.

† Four parallel (+)poly(A) incubations were assayed by filter binding. Then two of the filters were immersed in 10% trichloroacetic acid at 80° for 5–10 min three times, washed twice with ethanol, and dried in air. The radioactivity remaining on the filters resulted from crosslinked ternary complex that bound to ribosomes (radioactivity bound directly to the filter was subtracted out). Control filters containing noncovalent ternary complex·ribosome·poly(A) complexes were processed together with the above two filters, and only 6% of the radioactivity originally bound to the filters remained at the end of this procedure.

EF-Tu and tRNA moieties do not associate correctly, if at all, without GTP.

Ribosome Binding of Crosslinked Ternary Complex. The ability of the covalent complex to bind to ribosomes was assayed both by filter binding and by gel filtration chromatography. The filter binding results in Table 2 show that the [^{3}H]lysine binding is message-dependent. The insolubility of most of the radioactivity on the filter in hot trichloroacetic acid shows that crosslinked ternary complex is binding to ribosomes. This is also shown by the fact that the total amount of poly(A)-dependent binding (0.46 pmol) is greater than the total amount of nonprotein-bound radioactivity added to the incubation. Thus, the crosslinked ternary complex binds to ribosomes, and its binding is message-specific.

To confirm that the ^{3}H radioactivity bound to the filter was ribosome-bound, the binding mixture was analyzed by gel filtration chromatography (Fig. 4). Elution positions, determined by single-component chromatography, were as follows. Ribosomes were excluded from the Sepharose 6B gel and eluted at the void volume; this corresponds to fractions 8–11 in Fig. 4. Ternary complex, EF-Tu, and tRNA all were partially included in the gel and eluted between fractions 14 and 21. Small molecules were totally included and eluted at fractions 19–26. It is clear from Fig. 4 that about half of the ^{3}H was eluted in the void volume, and hence crosslinked ternary complex is binding to ribosomes, corroborating the filter assay results.

The rates at which the normal ternary complex and the covalent complex bound to ribosomes are compared in Table 3. By themselves, the complexes bound to ribosomes at similar rates (0.79 and 1.05 pmol in 2 min), with a similar dependence upon poly(A). When the two complexes were mixed together with ribosomes, each bound at a decreased rate, which indicates that the complexes compete for a limited number of active ribosomes. The fractional binding rate was roughly proportional to the amount of each complex in the mixture, which implies that both complexes have similar affinities for ribosomes.

Hydrolysis of GTP in Crosslinked Ternary Complex. Because the covalent ternary complex binds to ribosomes, it is natural to ask whether the crosslinking interferes with the GTP hydrolysis that normally accompanies the binding. In incubations containing crosslinked EF-Tu·ϵBrAc-[^{3}H]Lys-tRNA·[γ-^{32}P]GTP complexes, ^{3}H, but not ^{32}P, was bound to ribosomes (Table 2; Fig. 4). Either the GTP is hydrolyzed after association of the covalent ternary complex with a ribosome, or the GTP is released intact from the ribosomal complex.

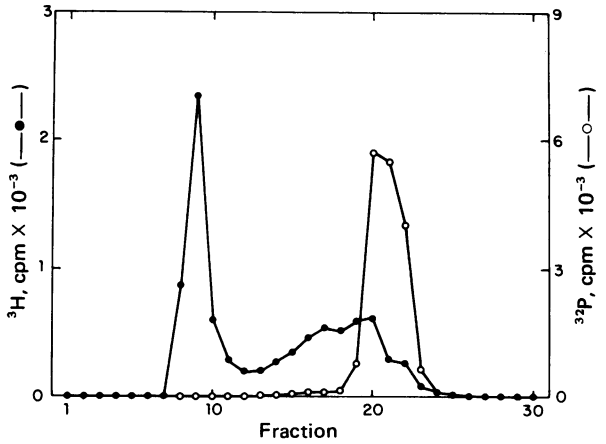

FIG. 4. Gel filtration analysis of the binding to ribosomes of crosslinked EF-Tu·ϵBrAc[^{3}H]Lys-tRNA·[γ-^{32}P]GTP. The 200-μl binding incubation contained 3.7 pmol of crosslinked ternary complex (4020 ^{3}H cpm/pmol of Lys), 1.2 pmol of uncrosslinked species, 0.8 pmol of disrupted crosslinked ternary complex, and 40 pmol of [γ-^{32}P]GTP (810 cpm/pmol).

This ambiguity was resolved by gel filtration analysis of a ribosome-binding incubation utilizing crosslinked EF-Tu·ϵBrAc[^{3}H]Lys-tRNA·[^{14}C]GTP complexes. In this case, both [^{14}C]GDP and [^{3}H]Lys-tRNA coeluted from the column with ribosomes (Fig. 5). The molar quantity of ^{14}C was less than that of ^{3}H in fractions 7–10 due to noncovalent ternary complex binding to ribosomes, which leaves the [^{3}H]Lys-tRNA species associated with the ribosome while EF-Tu·[^{14}C]GDP is released.

DISCUSSION

The affinity labeling of the protein EF-Tu with ϵBrAcLys-tRNA demonstrates that chemical crosslinking between two macromolecules can proceed in high yield. It also shows that the covalent reaction need not impair the normal function of the complex, except for the inability of the crosslinked components to separate. In addition, it provides a unique opportunity to study not only the basis of interaction between the nucleic acid and the protein in the complex itself but also the mechanism of interaction of the ternary complex with the

Table 3. Competition between the covalent complex and normal complex for binding to ribosomes

Mixture	[^{3}H]Lys-tRNA bound, pmol	Covalent complex bound, pmol
Complete*	0.63	0.24
−TC	—	0.79
−XLTC	1.05	—
−poly(A), −TC	—	0.17
−poly(A), −XLTC	0.20	—

* The complete mixture contained 2 pmol of ^{14}C-labeled ternary complex (XLTC, about 50% crosslinked) and 8 pmol of [^{3}H]Lys-tRNA·EF-Tu·GTP (TC) in the ribosome-binding mixture described in *Materials and Methods*. The final incubation was conducted at 0° for 2 min. Covalent complexes were determined by measuring the radioactivity bound to filters after hot trichloroacetic acid treatment.

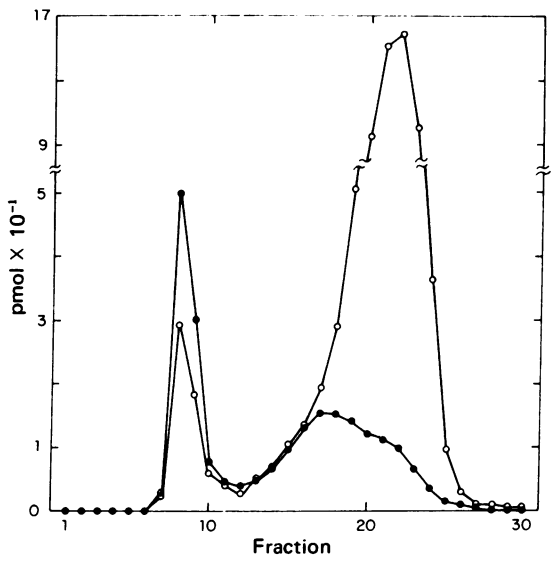

FIG. 5. Gel filtration analysis of the binding to ribosomes of crosslinked EF-Tu-ϵBrAc[^{3}H]Lys-tRNA-[^{14}C]GTP. The 200-μl binding incubation, detailed in *Materials and Methods*, contained 2.2 pmol of crosslinked ternary complex (4010 cpm/pmol of Lys), 2.4 pmol of uncrosslinked species, 0.7 pmol of disrupted crosslinked ternary complex, and 14.9 pmol of [^{14}C]GTP (560 cpm/pmol). ●, ^{3}H; ○, ^{14}C.

ribosome. For example, because the covalent complex binds to ribosomes as readily as the normal complex, it seems likely that the ternary complex is the actual intermediate in the binding reaction and not just an artifact in rapid equilibrium with the true intermediate.

Some conclusions can already be made about the topology of the ternary complex. The reacting EF-Tu nucleophile must be accessible and on the surface of the protein. The nucleophile is less than 9 Å (the fully extended length of the ϵBrAcLys side chain) along the surface of the protein from the position of the lysine α-carbon in the ternary complex. The nucleophile is not involved in the binding of either AA-tRNA or GTP, nor is it covered by either of these ligands in the ternary complex. Because the crosslinking does not prevent GTP exchange, the GTP binding site is not blocked by the crosslink. The nucleophile is not required for binding to the ribosome or for GTP hydrolysis, and immobilization of the lysine side chain does not interfere with normal function. This suggests that the ternary complex interactions with the ribosome involve regions of the complex removed from the site of crosslinking.

Because radioactivity from [^{14}C]GTP, but not [γ-^{32}P]GTP, is found associated with ribosomes after incubation with covalent ternary complex, the GTP in the complex must be hydrolyzed to GDP, which then remains bound to the EF-Tu. Fig. 5 shows that crosslinked EF-Tu is still bound to the ribosome after GTP hydrolysis, as expected. Presumably, this EF-Tu GDP is now associated with the ribosomal complex only, or primarily, through the covalent bond between the EF-Tu and the ϵBrAcLys-tRNA; but we have no direct evidence for this. An obvious question is whether this ribosome-bound crosslinked complex can function in peptide bond formation. Our attempts to resolve this point have so far yielded ambiguous results. Ribosomes have been affinity labeled with ϵBrAcLys-tRNA under EF-Tu-dependent binding conditions, and no significant crosslinking to EF-Tu has been observed (ref. 6; unpublished data). This is explained both by the transfer of ϵBrAcLys-tRNA from ternary complex to ribosomes during the incubation and by a molar ratio of EF-Tu to ϵBrAcLys-tRNA that is less than 1/10 the molar ratio in the crosslinking incubations described here.

The ability of EF-Tu to complex many different AA-tRNAs indicates that there is no distinct binding site on EF-Tu for the amino acid side chain. Yet the modified lysine side chain is capable of associating with EF-Tu in a specific manner. This is suggested by the exceptionally high efficiency of covalent reaction between EF-Tu and ϵBrAcLys-tRNA under conditions in which no reaction occurs between uncomplexed chemically reactive tRNA and EF-Tu. This view is further supported by preliminary results that indicate that only one (or perhaps two) tryptic peptide(s) of EF-Tu is radioactive after crosslinking.

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