THE PROTEIN NEIGHBORHOOD OF RIBOSOME-BOUND ELONGATION FACTOR Tu

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1. Introduction

The binding of an aminoacyl-tRNA to the A site of the bacterial ribosome is mediated by the protein factor EFTu [1]. Considerable evidence indicates that the 50 S ribosomal protein L7/L12 is directly or indirectly involved in the binding of the ternary complex containing EFTu, amino-acyl-tRNA and GTP [2]. A second elongation factor (EFG), which is involved in the subsequent translocation of peptidyl-tRNA [1], is apparently bound at a 50 S site also near to or containing L7/L12 [2]. Indeed, it has been possible to crosslink EFG to ribosomes and recover protein complexes containing among other components EFG as well as L7/L12 [3].

The present study was initiated to identify the ribosomal neighborhood to which EFTu is bound by bifunctional crosslinking reagents. Here, we attempted to determine the site-specificity of the complexes as well as the extent to which the proteins recovered were directly bound to EFTu. The data indicate that EFTu can be crosslinked to an extended ribosome neighborhood containing L1, L5, L7/L12, L15, L20, L30 and L33.

2. Materials and methods

β,γ-methylene-guanosine triphosphate (GDPCP) and poly(U) were obtained from Miles Laboratory: *E. coli* tRNA was obtained from Schwarz-Mann [^{14}C] formaldehyde, with a specific activity of 56 mCi/mmol came from New England Nuclear, and *p*-nitrophenyl-chloroformate from Aldrich Chem. Co. Inc.

Phe-tRNA had a charge of about 200–250 pmol Phe/mg total tRNA. 70 S 'tight couples' were prepared from MRE-600 *E. coli* cells according to the procedure of Noll et al. [4]; they were 50–60% active in EFTu-dependent Phe-tRNA binding. EFTu was prepared from MRE-600 cells according to the procedure of Arai et al. [5], but excluding the Sephadex G-100 step. Its activity was routinely measured by a [^{3}H]GDP binding assay. EFTu more than 95% pure, was labelled by reductive methylation, according to a modification of the procedure of Gualerzi et al. [6]; here, we used a ratio of 3 molecules HCHO per amino group in EFTu. The EFTu was modified at 6–7 amino groups per molecule which corresponds to 10–12% of the total amino groups. The resulting preparation had a maximum of 40% loss in activity and contained 15 000 cpm/μg.

Buffer A was 50 mM Triethanolamine, pH 8.0, 150 mM KCl and 12 mM Mg acetate. Buffer B was 6 M urea, 50 mM NaH_2PO_4 and 12 mM methylamine, pH 6.5. Buffer C was 100 mM Tris–HCl pH 8.2, 1 M LiCl and 0.4 M urea.

2.1. *Preparation of complex I*

The incubation mixture of 10–12 ml contained in buffer A: 1 mM GDCP, 2000 OD 70 S, 4 mg poly(U),

750 μg [^{14}C]EFTu, 1000 OD tRNA charged with 12 500 pmol of phenylalanine. Using these amounts, 70 S are approximately in a 2-fold excess over EFTu molecules. In terms of active molecules, the excess of 70 S and Phe-tRNA over EFTu molecules is 3–4-fold. The mixture was incubated at 30°C for 10 min and then filtered through a Sepharose 6B column (29 × 5 cm), equilibrated and eluted with buffer A, to separate unbound EFTu and Phe-tRNA. The recovered ribosomes with bound EFTu and Phe-tRNA are referred to as complex I.

2.2. *Crosslinking of complex I with* p*-nitrophenyl-chloroformate*

2.5 ml of a 0.5 M *p*-nitrophenyl-chloroformate (PNC) solution in dioxane were added to the filtered complex I mixture (250 ml). This was stirred for 2 h at 4°C and the reaction was stopped by the addition of 2.5 ml of 2 M methylamine. The solution was then filtered through CF/C paper to remove PNC precipitates. EDTA was then added to the mixture to a concentration of 11 mM, to reduce the Mg^{2+} concentration to 1 mM and prevent floculation. The pH was raised to 9 and a 30 min incubation at 37°C was carried out to inactivate the rest of the unreacted crosslinker. After lowering the pH back to 8 and making the mixture 100 mM $MgCl_2$, the complex was precipitated with 2 vol cold ethanol, which solubilizes most of the remaining reagent.

2.3. *Preparation of the EFTu crosslinked material*

The crosslinked complex, resuspended in buffer A, was centrifuged through a 40% sucrose cushion, made also in buffer A, in order to eliminate uncrosslinked EFTu. The pellet was resuspended in buffer B and extracted with 2 vol acetic acid. Then, the RNA-free protein was dialyzed into buffer B and partly purified from the bulk of single ribosomal proteins, by filtration through a Sephadex G-100 column (130 × 2.5 cm) equilibrated and eluted in buffer B. Three protein peaks come out in the eluate; the first of these contained all the radioactivity; and this peak was pooled as well as concentrated, with the aid of an Amicon membrane. In order to be able to identify the EFTu crosslinked proteins by reaction with antibodies we needed material not excluded by Biogel 1,5. Consequently, the fraction of our material eluting in the 1 000 000 mol. wt. range (about 10%) was discarded.

2.4. *Identification of the proteins crosslinked to EFTu*

The method used by Lutter et al. [7] for the fractionation of antibody–protein complexes was applied: 25–45 μg (3200–4000 cpm) of the crosslinked material freed of the high mol. wt fraction as indicated above, were incubated with the individual antisera corresponding to each of the 70 S proteins (except those for protein L17, L13, L25, L26, L31 and L32). The amount of each antiserum used was calculated after titration of each serum batch, making the tentative assumption that 20–40% of the total protein in the crosslinked material may consist of the protein in question. Moreover, we take into account that, due to the poor fractionation effect of the Sephadex G-100 step, some of the larger ribosomal proteins remain in the sample along with the crosslinked material. Therefore, correspondingly higher amounts of the homologous antisera are required. The crosslinked protein antiserum mixture was made in buffer C, plus 0.6% bovine serum albumin and 5% sucrose, and had a volume of 400 μl. The incubation was carried out at 6–8°C for 30 min. Then, the sample was applied to a Biogel 1,5 column (7.0 × 1.7 cm), equilibrated with buffer C. Fractions were collected directly in counting vials and radioactivity was estimated in Triton scintillation liquid. Radioactive material, in the absence of antibodies, elutes with the included volume of the columns. When, in the presence of a certain antibody, the radioactivity appears displaced to the excluded volume (where protein–antibody complexes elute), it is concluded that the corresponding protein is crosslinked to EFTu.

3. Results and discussion

One of the technical difficulties encountered in this study was the low yields of complex I recovered from the incubation mixtures. Thus, we generally found only 5–10% of the ribosome could be recovered with a bound EFTu and there were on the average, twice as many Phe-tRNA molecules bound as factor. One reason for the low yield of complex I is that it is unstable. In our hands the complex containing EFTu has a half life of 2 h at 20°C and 16 h at 4°C. Incubation of complex I for 30 min at 42°C left no detectable EFTu bound to the ribosomes. In addition, the presence of 1 mM GDP or dialysis

against 1 mM GDP reduced the amount of preformed complex to half of its original level. We make use of these incidental observations in experiments described below.

The choice of an appropriate crosslinking reagent also presented difficulties. Not only did we require a reagent that would crosslink EFTu to the ribosome but we wanted as little crosslinking as possible between ribosomal proteins. The latter criterion was important because it was a clear advantage to reduce the a priori probability of crosslinking one ribosomal protein to another which in turn was crosslinked to EFTu. We finally settled on PNC which creates very little crosslinking between ribosomal proteins of the isolated subunits (U. Bode, unpublished results) but could crosslink approximately 5% of the bound EFTu to the ribosomes. This reagent [8] is expected to form covalent links between lysines that are very near to each other, but the crosslink once formed is not reversible. Accordingly, we relied on immunological techniques in order to identify proteins crosslinked to EFTu.

When a typical sample of complex I crosslinked with PNC is fractionated on a sucrose gradient containing 1 mM Mg^{2+}, the radioactive EFTu is recovered in three peaks; these peaks correspond to the 30 (48% of the EFTu) and 50 S subunits (29% of the EFTu) as well as a fraction of 70 S particles (28% of the EFTu) which most probably contains the subunits crosslinked to each other. These results suggest that both subunits are involved in the binding of EFTu.

In order to test the site-specificity of the crosslinked complexes we performed control experiments to determine the extent to which EFTu is crosslinked to ribosomes under conditions unfavorable for the formation of complex I, i.e. under non permissive conditions. The idea behind these controls is that artifactual crosslinking of the factor to ribosomal proteins would be detectable under nonpermissive conditions, while the meaningful binding of factor would be reduced under these same conditions. For example, we could show a strong dependence for the formation of complex I on the presence of Phe-tRNA and its destabilizations by the presence of GDP. Accordingly, our finding that the crosslinking of EFTu to ribosomes can be reduced more than 80% by omitting Phe-tRNA from the incubation mixture and adding instead GDP, strongly suggests that most if not all the crosslinked factor is bound in a site-specific manner.

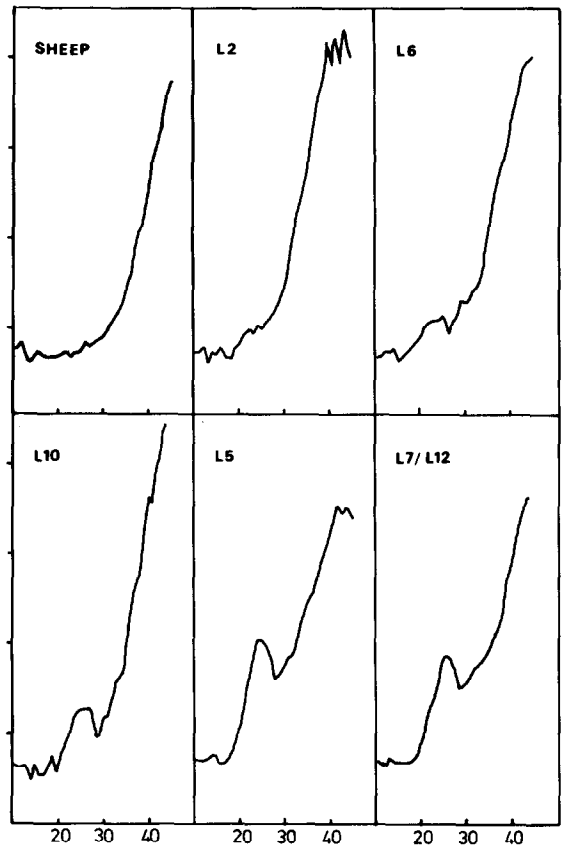

Fig.1. The displacement of radioactive EFTu by antibody raised against individual ribosomal proteins during passage through a Biogel column. The details are described in the text. Fractions 20–30 correspond to the excluded volume of the column.

A more exacting control was done to test the site specificity of the complexes recovered from the ribosomal subunits. Here, we compared the recovery of EFTu crosslinked to the 70 S particles as well as to subunits when the crosslinking was performed before and after heat inactivation of complex I. Heat inactivation reduces by more than 70% the recovery of EFTu crosslinked to 70 S and 50 S particles. However, as much as 50% of the EFTu can be crosslinked to 30 S subunits after heat inactivation. Unfortunately, this result suggests that there may be a significant amount of non-specific crosslinking to 30 S proteins, and this suspicion is reinforced by other results described below.

The identification of the proteins crosslinked to EFTu was performed by an immunological procedure that we have described earlier [7]. Here, the appearance of radioactive EFTu in the excluded volume of a Biogel column after incubation of the protein from crosslinked complex I with an antiserum raised against a particular ribosomal protein is taken as evidence that both EFTu and that ribosomal protein are members of a crosslinked complex. Some representative positive and negative results with this test are depicted in fig.1.

There are a number of aspects of the results obtained with 30 S proteins that were disquieting. First, close to two-thirds of the 30 S proteins could be recovered in crosslinked complexes with EFTu; a result which is simply difficult to accept. In addition, when the total amount of EFTu radioactivity put into the mixtures (3925 cpm/45 μg aliquot) is compared to the sum of radioactivity displaced in the columns by all the 30 S antisera (5532 cpm), we find that much more radioactivity is displaced than added in a single sample. This strongly suggests that many of the 30 S proteins are being recovered in complexes with EFTu containing more than one ribosomal protein. Accordingly, there is no way of deciding which of the 30 S proteins is directly crosslinked to EFTu. Thus, we feel that the only value of the data obtained with the antisera raised against 30 S proteins is to provide stark contrast to the much clearer data obtained with the 50 S proteins, and we will forego discussing the 30 S data in any more detail.

Twentysix antisera raised against individual 50 S proteins were tested in these experiments (table 1). Most of these 50 S proteins (L17/L26) were not recovered in significant amounts crosslinked to EFTu. The total amount of radioactivity displaced on Biogel columns by all 26 antisera (1990 cpm) was much less than the radioactivity in EFTu added in each mixture (3259 cpm/26 μg aliquot). This suggests that most of the crosslinked complexes contain EFTu and only one 50 S protein.

The data in table 1 clearly show that L1, L5, L7/L12, L15, L20, L30 and L33 are near enough to a bound EFTu to be crosslinked to the factor. We have previously shown that our samples of L8 contain an aggregate of L7/L12 and L10 [9]. Since we find that relatively small amounts of EFTu are displaced by antisera raised against L8/L9 and L10, it is possible that this effect is due solely to EFTu being crosslinked

Table 1
50 S proteins crosslinked to EFTu

Protein	EFTu-CPM
L1	242
L2	2
L3	13
L4	0
L5	224
L6	34
L7/L12	208
L8/L9	104
L10	83
L11	13
L14	31
L15	188
L16	45
L17	40
L18	36
L19	49
L20	172
L21	22
L22	36
L23	13
L24	0
L27	4
L28	49
L29	45
L30	143
L33	194

The incubation of crosslinked material with antisera raised against individual 50 S proteins was carried out as described in the text. Representative column fractionations are shown in fig.1.

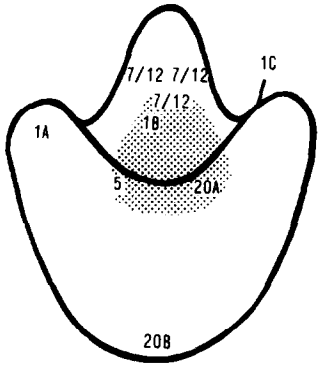

Fig.2. A diagrammatic representation of the distribution of antigenic sites for proteins from the 50 S subunit that are crosslinked to EFTu. The shaded area is the postulated binding site for EFTu.

to L7/L12 which may also be crosslinked to L10. Accordingly, we are obliged to await further information before deciding on the proximity of L10 to a bound EFTu.

The fact that considerably less is known about the spatial arrangements in situ for the 50 S proteins than for the 30 S proteins make the interpretation of the present results difficult. Nevertheless, an attractive pattern does seem to emerge when the present data is correlated with that of Stöffler et al. [10,11].

Figure 2 displays the disposition of the known antigenic sites for L1, L5, L7/L12 and L20. Although there are multiple, widely separated sites for L1 and L20, there is a single region in the center of the particle within which antigenic sites from all four proteins are clustered. Therefore, we conclude that this region of the 50 S subunit is likely to accomodate EFTu when it is bound to the ribosome, and is therefore, tentatively identified with the 50 S part of the A site.

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