Proc. Nat. Acad. Sci. USA
Vol. 71, No. 1, pp. 230-234, January 1974

Proteins at the tRNA Binding Sites of *Escherichia coli* Ribosomes

(affinity label/2-D gel electrophoresis/50S ribosomal proteins/antibodies)

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Communicated by Hewson Swift, September 7, 1973

ABSTRACT ***p*-Nitrophenylcarbamyl-phenylalanyl-tRNA binds to ribosomes in response to poly(U) and reacts with ribosomal proteins via covalent bond formation. Ribosomal proteins of *Escherichia coli* were characterized by two-dimensional gel electrophoresis and by reaction with specific antibodies. The affinity label is shown to react with the 50S proteins L27, L15, L2, L16, and L14. We therefore conclude that these particular proteins are located at or near tRNA-binding sites within the 50S ribosomal subunit.**

Knowledge of the topography of the ribosomal components and of the ribosomal binding sites for tRNA and translational factors is required for the development of a molecular model or ribosomal function. Several approaches have been utilized to unravel these relationships. Some information on the spatial arrangement of ribosomal RNA and proteins has come from renaturation experiments and from studies of specific complexes between ribosomal RNAs and proteins (1, 2). More recently, several groups have described bifunctional reagents for cross-linking ribosomal proteins located in adjacent positions (3–5). Determination of the nucleotide sequence of the messenger RNA fragment protected by the ribosome against ribonuclease digestion has been employed to locate the site of condon–anticodon recognition within the ribosome (6, 7). Recently, analogues of antibiotics have been employed as affinity labels for ribosomal proteins (8).

A different approach to identify ribosomal proteins at the tRNA binding sites has been taken by Pellegrini *et al.* (9) and by Czernilofsky and Kuechler (10). In both these studies, derivatives of aminoacyl-tRNA carrying reactive side groups attached to the aminoacyl moiety were synthesized as affinity labels for the ribosome. Reaction of the affinity label resulted in the formation of a covalent bond between the tRNA derivative and ribosomal proteins. Following digestion of tRNA by ribonuclease the labeled proteins were partially characterized by one-dimensional polyacrylamide gel electrophoresis. Pellegrini *et al.* (9) reported that bromoacetylphenylalanyl-tRNA reacts with only one ribosomal protein on the 50S subunit. In contrast, *p*-nitrophenylcarbamyl-phenylalanyl-tRNA used by our group was shown to label at least two 50S ribosomal proteins (10). However, due to the limited resolution of the one-dimensional gels the labeled proteins could not be unambiguously identified.

In the present investigation the labeled ribosomal proteins were characterized by two-dimensional polyacrylamide gel electrophoresis and by specific antigen–antibody reaction with antibodies directed against individual ribosomal proteins. These experiments indicate that proteins L27, L15, L2, L16, and L14 are located in the proximity of the peptidyltransferase center of the *Escherichia coli* ribosome.

MATERIALS AND METHODS

Buffers. Buffer A: 30 mM NH_4Cl, 50 mM Tris·HCl (pH 7.8), 10 mM magnesium acetate, 1 mM EDTA, and 6 mM 2-mercaptoethanol. Buffer B: 0.5 M NH_4Cl, 10 mM Tris·HCl (pH 7.4), and 0.1 mM magnesium acetate. Buffer C: same as buffer B, but 10 mM magnesium acetate. Buffer D: 30 mM NH_4Cl, 10 mM Tris·HCl (pH 7.4), and 10 mM magnesium acetate. Buffer E: 60 mM KCl, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2), and 20 mM magnesium acetate. Buffer F: 0.5 M LiCl, 0.5 M urea, 50 mM Tris·HCl (pH 8.2), and 10 mM 2-mercaptoethanol.

Preparation of Ribosomes. Frozen cells of *E. coli* strain D 10 were ground with alumina and suspended in buffer A. Following removal of alumina and cell debris, ribosomes were pelleted at 60,000 rpm for 3 hr in a Spinco ultracentrifuge with a 65 rotor. Ribosomes were subsequently washed sequentially in buffer B and buffer C. After the final centrifugation the pellet was resuspended in buffer D and the ribosomes were stored at −70°.

Synthesis of the Affinity Label. *p*-Nitrophenylcarbamyl-[^{3}H]phenylalanyl-tRNA was synthesized by reacting *p*-nitrophenylchloroformate (Fluka AG) with [^{3}H]phenylalanyl-tRNA as described previously (10, 11). [3-^{3}H]Phenylalanine (12.8 Ci/mmol) was obtained from New England Nuclear Corp.

Reaction of the Affinity Label with Ribosomes. *p*-Nitrophenylcarbamyl-[^{3}H]phenylalanyl-tRNA (0.17 mg at 1.5×10^7 cpm/mg) was added to 1 ml of a mixture containing 4 mg of 70S *E. coli* ribosomes and 0.2 mg of poly(U) in buffer E. Incubation, separation of ribosomal subunits, and extraction of 50S proteins were carried out as described previously (10). The proteins were dialyzed, lyophilized, and identified as described by Kaltschmidt and Wittmann (12). The specific activities were between 5 and 6×10^4 cpm/mg of total 50S protein.

Gradient Centrifugation of Antigen–Antibody Complexes. Antisera against single 50S ribosomal proteins were prepared according to Stöffler and Wittmann (13) and Stöffler, G., Tischendorf, G. W., Hasenbank, R., and Wittmann, H. G. (unpublished results). Immunoglobulins were purified as described by Stöffler and Wittmann (13, 14) and Maschler, R.,

Hasenbank, R., and Stöffler, G. (unpublished results). The method of Lutter *et al.* (5) was modified. Sedimentation on sucrose gradients (15), rather than gel filtration, was employed for separation of the antigen–antibody complexes. Labeled ribosomal protein (0.25 mg) was incubated with 6 mg of specific immunoglobulin in 1.2 ml of buffer F at 0° for 30 min. The mixture was layered on to a gradient of 5–20% (w/w) sucrose in buffer F and centrifuged in a Spinco ultracentrifuge with an SW 27 rotor for 33 hr at 27,000 rpm at 2°. Absorbance was measured at 280 nm in a Gilford spectrophotometer. Fractions were counted in Triton X 100-toluene in a scintillation counter.

RESULTS

Effect of Puromycin on the Affinity Labeling Reaction. Previous experiments (10) with the affinity label *p*-nitrophenylcarbamyl-phenylalanyl-tRNA have demonstrated that the formation of a covalent bond between the tRNA derivative and ribosomal protein is completely dependent on the presence of poly(U). In order to obtain further evidence for specific labeling at the active center of the ribosome, the influence of puromycin known to act at the peptidyltransferase was investigated.

Ribosomes were reacted with the affinity label in the presence of puromycin. The effects on the labeling of total 50S proteins are shown in Table 1. Puromycin at 0.8 mM concentration inhibits the reaction by 52%. Raising the puromycin concentration to 4 mM increases the inhibition to 64%.

Analysis of Two-Dimensional Gels. For purposes of identification, the labeled proteins were subjected to two-dimensional polyacrylamide gel electrophoresis (Fig. 1). Protein spots stained with amidoblack were removed and the radioactivity determined (Fig. 2, *solid bars*). It can be seen that most of the radioactivity occurs in the spots corresponding to proteins L27 and L16 and in the region of proteins L13–L14–L15, which are not readily enough separated to allow a discrimination of the radioactivity within the stained spots from the radioactivity in the surrounding area (Fig. 1, *insert*). Other protein spots contain only small amounts of radioactivity.

In order to check for radioactivity outside of the stained protein spots, adjacent areas were cut into four equal sections as indicated by the grid on the insert in Fig. 1 (for explanation, see below). The distribution of radioactivity between the

TABLE 1. *Inhibition of the affinity labeling reaction by puromycin*

Puromycin concentration (mM)	Radioactivity incorporation (cpm)	Inhibition (%)
0	2,648	0
0.8	1,271	52
4.0	948	64

The reaction mixture (total volume 0.75 ml) contained 0.02 mg of *p*-nitrophenylcarbamyl-[^{3}H]phenylalanyl-tRNA, 1.6 mg of *E. coli* ribosomes, 0.1 mg of poly(U) in buffer E, and puromycin as indicated. Incubation, fractionation of ribosomal subunits on sucrose gradients, digestion by ribonuclease, and precipitation with trichloroacetic acid were carried out as described previously (10). The radioactivity represents total precipitable material in the 50S region of the sucrose gradient.

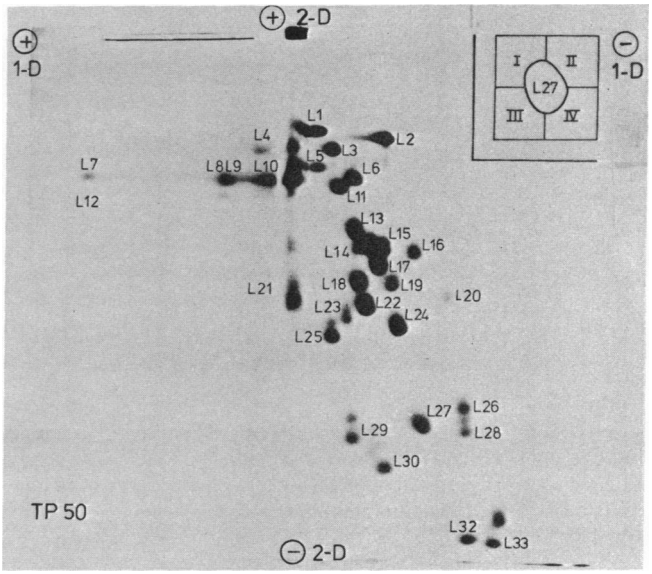

FIG. 1. Two-dimensional electrophoretogram of 50S proteins. The insert shows the grid used to remove sections from the gel. The radioactivity in each of the protein spots *L2*, *L16*, and *L27* and in sections around each spot designated *I*, *II*, *III*, and *IV* is given in Table 2. Stained protein spots not numbered are occurring independently of the affinity label and have been shown to correspond to nonribosomal proteins (24). No radioactivity was found in these unnumbered spots and in that part of the gel which does not contain stained spots.

stained spots and their adjacent regions is illustrated in Table 2. Most of the radioactivity in the region of L27 is in fact not found within the stained spot but in section I. A small amount of radioactivity is also recovered in section III. In contrast, sections II and IV show only background radioactivity. The distribution of radioactivity in the region of L16 is very similar (Table 2). This displacement of the labeled protein relative to the unlabeled protein is to be expected as a result of the alteration in the charge caused by the affinity label (see *Discussion*). We have therefore added the radioactivity recovered in sections I and III (Fig. 2, *open bars*) to the radioactivity found in the respective stained protein spots (Fig. 2, *solid bars*). All other protein spots and the areas surrounding them were cut out according to the same grid. It should be emphasized that all radioactivity recovered from the two-dimensional gel can be accounted for in the manner shown in Figs. 1 and 2. For reasons so far not explainable, there is a higher amount of radioactivity in the sections I and III corresponding to proteins L2 and the L32–L33 region, respectively (Fig. 2, *open bars*).

TABLE 2. *Distribution of radioactivity around stained protein spots*

Protein	Stained spot	Radioactivity (cpm) Sections I	II	III	IV
L2	8	328	7	265	65
L16	376	505	45	269	149
L27	1,492	2,340	186	529	234

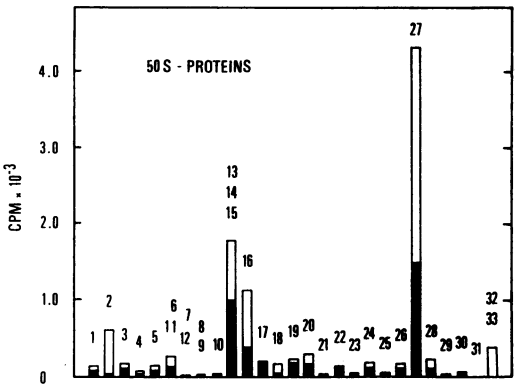

FIG. 2. Distribution of radioactivity on the two-dimensional electrophoretogram. *Solid bars* represent radioactivity in stained protein spots. Radioactivity in areas adjacent to the respective stained spots is represented by *open bars*. The recovery of radioactivity was 31%. (The numbers on the ordinate have been multiplied by 10^{-3}.)

Due to the observed shift, the identification of a labeled protein within groups of proteins having similar electrophoretic mobility is somewhat difficult. For this reason the radioactivity found in the region of L13–L14–L15 could not be correlated with any single protein by electrophoretic separations. An identification of these labeled proteins can only come from an immunological method.

Immunological Identification of Labeled Ribosomal Proteins. Straightforward immunoprecipitation is not practicable with these labeled ribosomal proteins because of their tendency to aggregate. According to Lutter *et al.* (5), the sensitivity of the immunological assay can be increased by working under conditions of antibody excess, which prevents precipitation of the antigen–antibody complexes. The complexes can be separated from the bulk of radioactively labeled ribosomal proteins by molecular sieve chromatography (5) or centrifugation. We have compared both methods and have found centrifugation to give lower backgrounds of aggregated ribosomal proteins. The complete mixtures containing total labeled ribosomal proteins and excess antibody were layered onto sucrose gradients and centrifuged (Fig. 3). The absorbance at 280 nm represents 7S immunoglobulin and serves as a sedimentation marker. As illustrated in Fig. 3, anti-L27 antibody shifts part of the radioactivity from the top to the gradient into fast sedimenting material. The same amount of immunoglobulin from a nonimmunized rabbit causes no shift of radioactivity into material sedimenting at 7S or faster.

This experiment has been repeated with antibodies directed against different proteins and the results obtained are listed in Table 3. Antibodies against L27, L15, L2, L16, and L14 shift radioactively labeled protein into rapidly sedimenting material. Antibodies against L7 and L13 yield results similar to immunoglobulin from nonimmunized rabbits. The results are consistant with the distribution of radioactivity found by two-dimensional gel electrophoresis.

TABLE 3. *Sucrose gradient centrifugation of antigen–antibody complexes using various specific immunoglobulins*

Ribosomal protein specificity of IgG preparation	Radioactivity (cpm)	
	Shifted	Not shifted
L27	1,296	8,850
L15	421	10,882
L2	375	11,205
L14	210	11,211
L16	179	11,409
L13	79	10,035
L7	44	11,309
Pre-immune	45	8,365

DISCUSSION

Affinity labeling with reactive derivatives of phenylalanyl-tRNA has been employed as a tool to identify proteins at the tRNA binding sites of the bacterial ribosome. By analogy with the binding of *N*-acetylphenylalanyl-tRNA at high Mg concentration, the affinity label would be expected to bind equally well to the donor- and the acceptor-site on the ribosome (16).

According to Savelyev *et al.* (17) puromycin reacts with ureido-derivatives of *p*-nitrophenylcarbamyl-phenylalanyl-tRNA bound to the donor-site of the ribosome. Puromycin is therefore expected to decrease labeling of donor-site proteins by reacting with the affinity label before covalent attachment to a ribosomal protein can occur. Labeling of acceptor-site proteins, on the other hand, might also be inhibited by direct competition for the same ribosomal site between puromycin and the terminal aminoacyladenosin of the affinity label (18). Therefore, the use of puromycin does not allow discrimination between labeling of proteins at the donor-site and at the acceptor-site. Nevertheless, the inhibition of labeling observed in the presence of puromycin supports our previous suggestion that the labeled proteins are part of the peptidyltransferase center. Further experiments employing different antibiotics should help to distinguish between labeling of proteins at the donor-site and at the acceptor-site.

Binding of the phenylalanyl-oligonucleotide fragment obtained from phenylalanyl-tRNA to ribosomes is reduced when the amino-group of phenylalanine is acylated (19). This indicates that the free amino-group of the amino acid contributes to the binding of aminoacyl-tRNA. In the case of *p*-nitrophenylcarbamyl-phenylalanyl-tRNA the reactive side group is attached to the amino-group of phenylalanine. Even though the tRNA derivative is bound specifically by codon–anticodon interaction, the aminoacyl moiety—at a distance of 82 Å from the anticodon (20)—might not be fixed to any one protein. This would allow for some mobility of the aminoacyl-terminus of tRNA which would in turn permit the affinity label to react with several proteins in the environment of its binding site. The extent of labeling would then be determined by the three-dimensional arrangement of the proteins around the active center and by the availability of reactive groups. Heterogeneity of ribosomes with regard to the number of proteins attached and differences in ribosomal conformation may also influence the labeling patterns.

The affinity label reacts primarily with amino-groups, most likely with ϵ-amino-groups of lysine (10). In the reaction the ester group of the *p*-nitrophenylcarbamyl moiety is cleaved with release of *p*-nitrophenol resulting in acylation of the amino-group. Subsequent ribonuclease treatment degrades

tRNA leaving [^{3}H]phenylalanyladenosine covalently attached to the protein by a urea linkage. Thus, the introduction of the affinity label causes acylation of a strongly basic amino-group with concomitant loss of one positive charge on the ribosomal protein. In addition, adenosine might be released by spontaneous hydrolysis during the purification of the ribosomal proteins leaving phenylalanine with a free carboxyl group bound to the protein.

The loss of positive charge results in a decreased electrophoretic migration towards the cathode which causes a slight shift in the position of the labeled protein compared to that of the unlabeled protein in the two-dimensional gel pattern (Fig. 1). From the specific activity of the labeled 50S protein it can be calculated that only one out of 250 to 300 ribosomes reacts with the affinity label. It is, therefore, impossible to detect the shift of the modified proteins in the stained pattern.

A shift caused by a difference of one positive charge is also observed in the case of proteins L7 and L12, which differ only by one acetyl-group on the N-terminal serine (Fig. 1, ref. 21). Since the α-amino-group of a seryl peptide is largely uncharged at pH 8.6 (the pH of the buffer for the first dimension), no separation of L7 and L12 occurs in the first dimension of the gel. Separation is observed only in the second dimension at pH 4.3. Because of the high pK_a value of the ϵ-amino-group of lysine (pK 9–10), acylation of one lysine residue should express itself as a change in electrophoretic mobility in the first *and* the second dimension. As shown in Table 2, the radioactively labeled protein indeed migrates slightly more slowly than the unlabeled protein in both directions. Therefore, labeled proteins such as L13, L14, and L15, which have similar electrophoretic mobility, cannot be unambiguously identified by the two-dimensional gel technique.

This obstacle is circumvented by the use of an immunological assay. Under conditions of antibody excess, antigenic sites are saturated and precipitation is largely prevented. Most ribosomal proteins have small molecular weights. The attachment of immunoglobulin molecules results in the formation of complexes which sediment rapidly and can be separated from the bulk of labeled ribosomal proteins on sucrose gradients.

Although the antigen–antibody complexes are formed under conditions of antibody excess, the equilibrium is shifted towards dissociation as soon as the complexes enter into the sucrose gradient. Dissociation is further enhanced by the presence of urea in the buffer. This most certainly accounts for the fact that the sum of radioactive protein shifted by the antibodies does not add up to the total amount of radioactive material layered on to the gradient. Nevertheless, a good correspondence is observed between the results of the "antibody-shift" experiments (Table 3) and the pattern obtained by two-dimensional polyacrylamide gel electrophoresis (Fig. 2). However, it should be emphasized that the amount of radioactive protein shifted on the sucrose gradient also depends on the binding constant of the particular antibody. This introduces a slight variance which does not allow an exact quantitative comparison of the results obtained by the two techniques.

Little is known so far about the functions of the ribosomal proteins labeled in this experiment. Protein L16 has been identified as the protein important for the binding of chloramphenicol (8, 22, 23). L16 is also labeled in our experiments

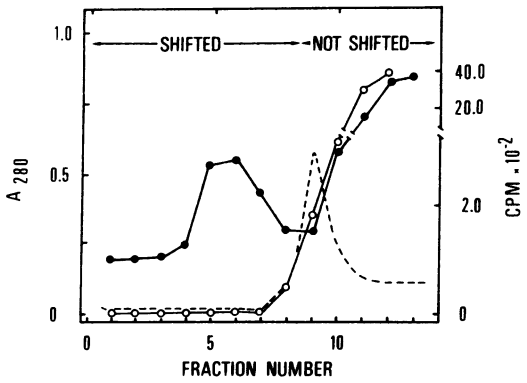

FIG. 3. Sucrose gradient centrifugation of the antigen–antibody complex using IgG specific for L27. Total labeled 50S protein was incubated with anti-L27 antibody (●——●) and centrifuged on a 5–20% (w/w) sucrose gradient. In the control experiment, immunoglobulin G from a nonimmunized rabbit (O——O) was employed. The peak at A_{280nm}(– – –) indicates the position of 7S immunoglobulin. (The numbers on the right ordinate have been multiplied by 10^{-2}.)

which emphasizes the importance of this protein as part of the peptidyltransferase center. L27 shows the greatest extent of reaction with the affinity label. Protein L14 is labeled to a much smaller extent. Both of these proteins seem to function in nonenzymatic binding of phenylalanyl-tRNA, as demonstrated by antibody inhibition experiments. The inhibition is observed only when the monovalent antibody-fragments are bound to 50S subunits before the addition of 30S subunits (unpublished experiments). This result suggests that the peptidyltransferase center of the ribosome is located close to the interface between the large and the small subunit.

Our investigations (10), as well as those of other groups (8, 9), prove affinity labeling to be a suitable means for the exploration of the ribosomal structure. Additional studies are expected to allow the determination of the precise location of the labeled proteins within the ribosome.

We thank Drs. C. G. Kurland, H. Tuppy, and H. G. Wittmann for discussions and suggestions. This work was supported by a grant from the "Fonds zur Föderung der wissenschaftlichen Forschung." A.P.C. is a recipient of an EMBO Short-Term Fellowship.

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