Karin van Wijk for competent secretarial work.

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Affinity Labeling of *Escherichia coli* Ribosomes with a Covalently Binding Derivative of the Antibiotic Pleuromutilin†

G. Högenauer,* H. Egger, C. Ruf, and B. Stumper

ABSTRACT: Reaction of an alkylating pleuromutilin derivative with *E. coli* ribosomes led to the binding of the compound to both proteins and RNA. If ribosomes of the *E. coli* strain MRE600 were used, mainly S18 and L2 became labeled. Ribosomes from *E. coli* D10 bound the reagent to S18 and frequently to L27 instead of L2. Possibly a slight difference in the structure of these ribosomes exposes different, although closely neighboring, L proteins to the reagent. The simultaneous labeling of L and S proteins seems to reflect the presence of two binding sites for the antibiotic and indicates that the binding sites are located at the interphase region between large and small ribosomal subunits. Analysis of the RNA showed that the affinity label is mainly attached to the 23S species. These data are in good agreement with the known effects of pleuromutilin derivatives on ribosomal functions.

Tiamulin, a derivative of the antibiotic pleuromutilin, has been shown to inhibit *E. coli* ribosomes by blocking their assembly into functional initiation complexes (Hodgin & Högenauer, 1974; Dornhelm & Högenauer, 1978). The antibiotic binds very strongly, yet reversibly, to 70S ribosomes. The presence of two specific binding sites has been shown (Högenauer & Ruf, 1981), and at least one overlaps with that of the antibiotics chloramphenicol and puromycin. The binding sites for other protein synthesis inhibitors, like lincomycin, erythromycin, and thiostrepton, do not extend into the pleuromutilin-specific binding region (Högenauer, 1975; G. Högenauer, unpublished experiments). The fact that the nucleotides CpA and CpCpA reduce the binding of the pleuromutilin derivative is taken as evidence that the binding sites of the drug are located at or near the region for the attachment of the 3′-terminus of tRNA.

A further characterization of the binding sites for the antibiotic by identification of the proteins at this particular ribosomal location seemed desirable. This was achieved by the

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affinity labeling technique in which a bromoacetyl derivative of pleuromutilin was covalently attached to the ribosome. With the aid of this compound which is chemically very similar to tiamulin, 23S RNA and essentially three ribosomal proteins were labeled and are thus indicated as being at or near the binding sites for this antibiotic.

Materials and Methods

Bacterial cell paste of *E. coli* MRE600 and D10 was purchased from Merck (Darmstadt, Federal Republic of Germany). Radioactive bromoacetic acid, either ^3H or ^{14}C labeled, was obtained from The Radiochemical Center (Amersham, England). Acrylamide, bis(acrylamide), *N,N,N′,N′*-tetramethylethylenediamine (Temed), and pancreatic RNase were products of Serva (Heidelberg, Federal Republic of Germany). T1 RNase was obtained from Calbiochem (Luzern, Switzerland), and "Soluene" was from Packard (Vienna, Austria). Royal X-Omat X-ray films (Kodak) were used for fluorography.

Synthesis of Affinity Labeling Reagent (Affinity Label). The synthesis of the ^{14}C-labeled derivative is described. ^3H-labeled and nonradioactive derivatives were synthesized in an analogous procedure.

A 1.1-mg (0.096 mmol) sample of *N*-hydroxysuccinimide in 40 μL of absolute dioxane, 20 μL of a dioxane solution of ^{14}C-labeled bromoacetic acid (1.2 mg, 0.085 mmol) with specific activity of 50 mCi/mmol, and 2 mg (0.097 mmol) of solid dicyclohexylcarbodiimide were mixed to give a clear solution. To this mixture was added 20 μL of a solution of 4 mg (0.09 mmol) of the (aminoethyl)mercapto derivative of pleuromutilin (structure Ib), synthesized in our laboratories according to published procedures (Egger & Reinshagen, 1976), and the reaction mixture was allowed to stand at room temperature for 1 h. A white precipitate of dicyclohexylurea was formed. At the end of the reaction, 200 μL of ethyl acetate was added, and the precipitate was removed by centrifugation and washed once more with 200 μL of ethyl acetate. The combined ethyl acetate solution was extracted with an equal amount of water. The organic phase was evaporated in vacuo and the remaining solid dissolved in 50 μL of chloroform and applied to a silica gel thin-layer plate. The chromatogram was developed in benzene–ethyl acetate (2:1), and the spots of reference materials, run on a parallel plate, were made visible by spraying the plate with a solution of 2% ceric sulfate in 10% aqueous sulfuric acid and subsequently heating it to 120 °C. The radioactive spots were located on the plate with a radioactivity scanner for thin-layer plates (Berthold), the silica gel at the location of the desired substance was scraped off the plate and extracted with chloroform, and the chloroform was removed in vacuo. The solid material was dissolved in ethanol and used for affinity labeling experiments.

Preparation of Ribosomes. Ribosomes were prepared by the method of Nirenberg (1963), including a preincubation step but omitting the dialysis after the preincubation of the 30000*g* supernatant. The ribosomes were washed three times with 0.5 M ammonium chloride containing standard buffer.

Affinity Labeling Experiments. In typical experiments, 300 OD units (7.2 nmol) of ribosomes were incubated with 40 nmol or less of affinity labeling reagent (structure Ic), dissolved in either dioxane (earlier experiments) or ethanol. The final ethanol concentration of the incubation mixture was approximately 2%. The reaction mixture was incubated for 1 h at 37 °C in the medium which was used for dissolving the ribosomes, i.e., 10 mM Tris-HCl, pH 7.5, 60 mM KCl, 10 mM magnesium acetate, and 6 mM β-mercaptoethanol (standard buffer). Afterward, the ribosomes were pelleted through a 10% sucrose shelf in standard buffer at 100000*g* for 20 h. The pellet was dissolved in 1 mL of standard buffer. In earlier experiments, the buffer system in which the ribosomes were dissolved was replaced, prior to affinity labeling reaction, by 50 mM *N*-2-(hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid (Hepes), pH 7.5, 60 mM KCl, and 10 mM magnesium acetate, but since the results of the two buffer systems gave the same labeling pattern, both quantitatively as well as qualitatively, the majority of the experiments reported were performed by using the Tris- and mercaptoethanol-containing buffer.

The ribosomal proteins were extracted from the dissolved ribosomes by the method of Czernilofsky & Küchler (1972). The ribosomal pellet, dissolved in 1 mL of standard buffer, was mixed with 0.6 mL of 8 M urea and 0.2 M EDTA, pH 7.5. To this solution were added 1.12 mg of dithioerythritol, 32 μg of pancreatic ribonuclease, and 6.4 μg of T1 ribonuclease. The mixture was incubated for 2 h at 37 °C, and the proteins were precipitated by adding an equal volume of 10% trichloroacetic acid. The protein precipitate was collected by centrifugation and dissolved in 500 μL of 10 M urea. This solution was then dialyzed (1 h against 1 L of 10% acetic acid, 2–3 h against 1 L of 1% acetic acid) and subsequently lyophilized. The protein obtained was dissolved in 100 μL of 1-D buffer (Howard & Traut, 1974) which was 9 M in urea.

2-D Electrophoresis of Ribosomal Proteins. Two-dimensional electrophoresis was performed by the method of Kaltschmidt & Wittmann (1970) with the modification of Howard & Traut (1974). In typical electrophoresis experiments, 750 μg of ribosomal proteins was applied. After completion of the second dimension, the slab gel was stained with a 2.5% Coomassie brilliant blue R-250 solution and destained by applying an electric current perpendicular to the gel surface. After this procedure, the radioactivity of the protein spots was determined.

Determination of Radioactivity. In earlier experiments, the spots were cut out with a razor blade in the form of rectangles. These gel pieces normally included unstained gel surrounding the oval protein spots. The gel squares were treated with 500 μL of Soluene (2 h, 56 °C) and subsequently mixed with Bray's solution (Bray, 1960). In this form, the radioactivity of the samples was determined. In the majority of the experiments, direct fluorography (Bonner & Laskey, 1974) of the destained gels was used in order to locate radioactivity on the gels. For quantitative determination, radioactivity of collected CO_2 was determined following combustion of dried gel pieces in a Packard sample oxidizer.

Affinity Labeling and Extraction of Ribosomal RNA. Affinity labeling of *E. coli* MRE600 ribosoms was performed as described above. After the ribosomes were pelleted and dissolved, triisopropylnaphthalenesulfonate was added to give a final concentration of 0.36%. This solution was then shaken vigorously for 25 min at room temperature with twice the volume of water-saturated phenol, containing 10% (w/v) *m*-cresol (Loening, 1969). After phenolization, the water phase was removed and made 2% in sodium acetate. Finally, the ribosomal RNA was precipitated with twice the volume of ethanol, and the RNA was collected by centrifugation, washed once with ethanol, and then dried and dissolved in 1 mL of 0.1 M sodium acetate, pH 5.1, and 0.05 M NaCl. The RNA was then layered on top of a sucrose gradient (15–30%) in the sodium acetate–NaCl solution and centrifuged for 21 h at 82000*g*. The gradient was pumped through a recording photometer, set for 260 nm, and fractionated. The radioactive RNA present in the fractions was measured following precipitation with trichloroacetic acid (in the presence of serum

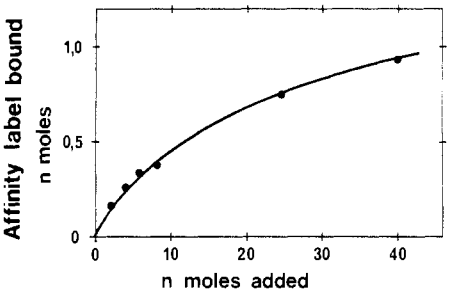
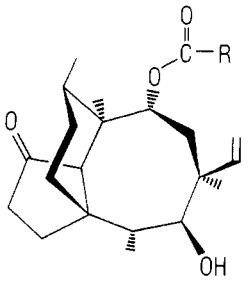


FIGURE 1: Affinity labeling of ribosomes. A 0.28-nmol sample of *E. coli* D10 ribosomes was incubated with varying concentrations of ^{14}C-labeled bromoacetyl derivative (Ic) for 1 h at 37 °C. The samples were mixed with 1 mL of ethanol, filtered on nitrocellulose filters, and washed five times with 3 mL of 80% ethanol. After the samples were dried, the filters were counted in a toluene-based scintillation fluid.

albumin) and collection on nitrocellulose filters.

Results

Covalent attachment of the pleuromutilin moiety to *E. coli* ribosomes was possible if ribosomes were exposed to a bromoacetyl derivative of this antibiotic. Since most of the work on the mode of action of pleuromutilin was carried out on the semisynthetic derivative tiamulin, a compound containing an aliphatic thioether side chain (structure Ia), the bromoacetyl



a) R = $CH_2SCH_2CH_2N(C_2H_5)_2$
b) R = $CH_2SCH_2CH_2NH_2$
c) R = $CH_2SCH_2CH_2NHCOCH_2Br$

I

derivative used for affinity labeling studies was designed to be structurally related to tiamulin. The starting material for the synthesis of the affinity label was the (aminoethyl)-mercaptoacetoxy derivative (structure Ib) which was reacted with bromoacetic acid and dicyclohexylcarbodiimide to give compound Ic. In order to obtain the radioactive bromoacetyl derivative, ^{14}C- or ^{3}H-labeled bromoacetic acid was incubated with the aminoethyl thioether derivative of pleuromutilin.

When the radioactive affinity label was incubated with *E. coli* ribosomes at 37 °C for 1 h and the excess of unbound material removed from the ribosomes by ethanol precipitation and subsequent washing with ethanol, some radioactivity remained attached to the ribosomes. Both *E. coli* D10 and MRE600 ribosomes gave virtually the same result. The amount of bound material increased with increasing concentrations of affinity label in the reaction mixture. At higher concentrations of affinity label, apparently more than the expected two molecules per ribosome became bound because the curve in Figure 1 did not reach a saturation plateau at a 2:1 stoichiometry. As this was probably due to unspecific attachment of the drug, a low bromoacetyl derivative to ribosome ratio was used in later experiments. That this reaction indeed occurred at the desired, tiamulin-specific ribosomal location was deduced from the results of two experiments: (1) a partial inhibition of the covalent binding by competing tiamulin and chloramphenicol and (2) the similar inhibition of ribosomal functions achieved with tiamulin and the affinity label.

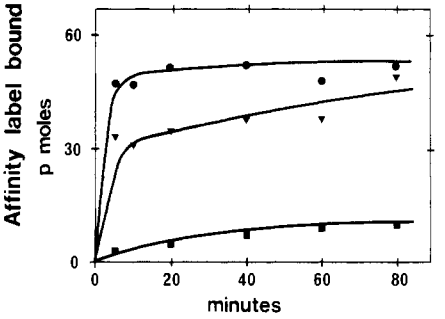


FIGURE 2: Competition for the affinity labeling reaction. A 0.28-nmol sample of *E. coli* MRE600 ribosomes was incubated with 0.32 nmol of affinity reagent and incubated at 37 °C in the presence of 80 nmol of tiamulin (■), 40 nmol of chloramphenicol (▼), or in a control experiment without any addition (●). The samples were filtered, and ethanol was washed as described under Materials and Methods.

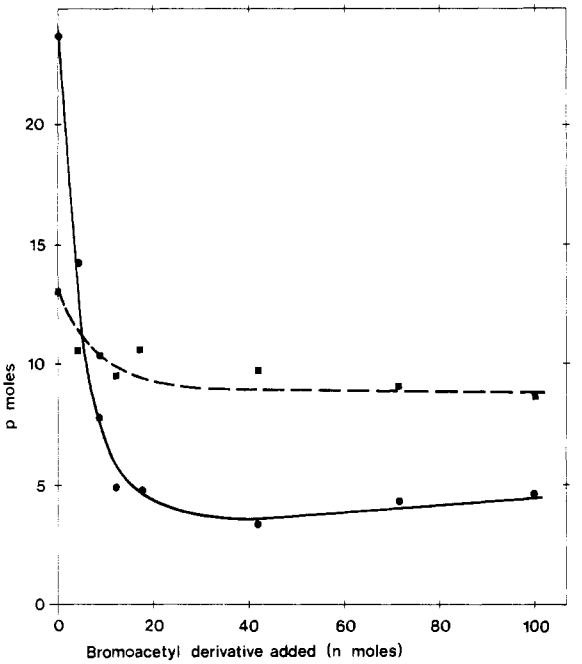


FIGURE 3: fMet-tRNA binding and puromycin reaction on affinity labeled ribosomes. A 2.4-nmol aliquot of each ribosome sample was treated with varying amounts of nonradioactive bromoacetyl derivative (Ic) for 1 h at 37 °C. Subsequently, 30 μL of β-mercaptoethanol was added, and the samples were dialyzed for 4 h against 2 L of standard buffer with two buffer changes. A 48-pmol portion of each ribosome sample was programmed with poly(AUG) and tested for initiation factor catalyzed formylmethionyl-tRNA binding as described earlier (Hodgin & Högenauer, 1974). In parallel experiments, the puromycin reaction was measured by adding to the initiation complexes puromycin at a final concentration of 3 mM. Formylmethionyl puromycin was extracted with 2 mL of ethyl acetate. The radioactivity in an aliquot of the organic phase was determined. (●) fMet puromycin formed; (■) fMet-tRNA bound.

The first inhibition experiment was based on the time course by which the labeled bromoacetyl derivative became covalently linked to ribosomes in the presence of a 70-fold molar excess of tiamulin. Figure 2 shows the large effect produced by this competition on the covalent binding reaction in comparison to the uninhibited control. Since chloramphenicol shares a binding site with tiamulin (Högenauer, 1975), this drug was also tested as a competitor of the alkylation reaction by using a 35-fold excess. Again, a slowing of the reaction kinetics could be observed although the inhibition occurred to a lesser extent.

In the second control experiment, *E. coli* D10 ribosomes were incubated with increasing concentrations of nonradioactive bromoacetyl derivative. After removal of the excess

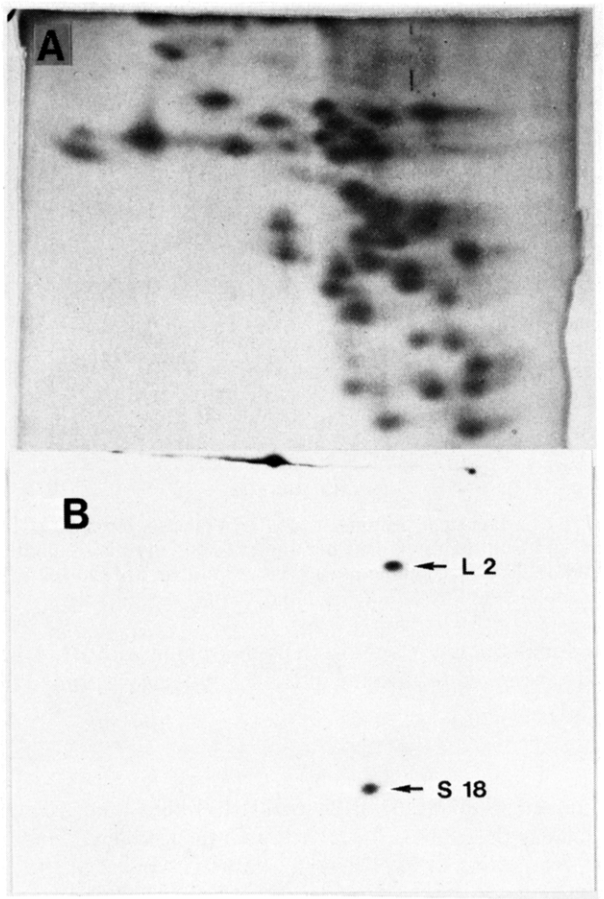


FIGURE 4: Analysis of electrophoretically separated ribosomal proteins from *E. coli* MRE600. (A) Photograph of the stained two-dimensional gel. (B) Radiofluorography of the dried gel.

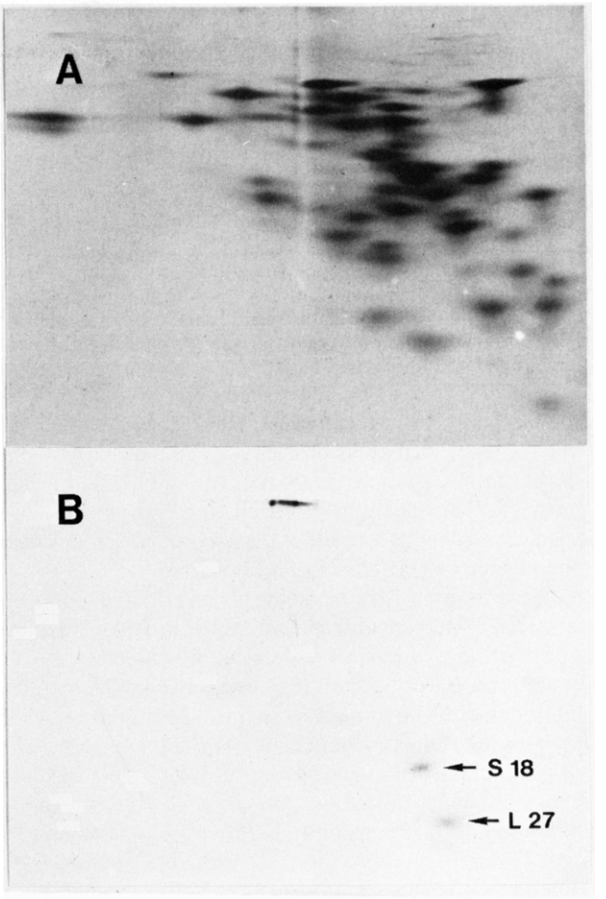


FIGURE 5: Analysis of electrophoretically separated ribosomal proteins from *E. coli* D10. (A) Photograph of the stained two-dimensional gel. (B) Radiofluorography of the dried gel.

reagent by dialysis, the ribosomes were tested for their ability to bind enzymatically fMet-tRNA in the presence of poly(AUG). The puromycin reaction of the P-site bound fMet-tRNA was also tested by using standard techniques (Figure 3). Treating the ribosomes with the affinity label even to high concentrations only slightly affected fMet-tRNA binding while the transfer of the formylmethionine residue to puromycin was strongly inhibited.

For all affinity labeling experiments which were aimed at a subsequent identification of the labeled ribosomal components, an affinity label to ribosome ratio of 5.5:1 or less was used. In a typical experiment, 40 nmol of the reagent, either ^{3}H or ^{14}C labeled, was incubated with 7.2 nmol of ribosomes for 1 h at 37 °C. In earlier experiments, it was ascertained that the labeling of ribosomal proteins was independent of the presence or absence of Tris ions and β-mercaptoethanol in the reaction medium. Since dialysis of ribosomes is detrimental for the reversible binding of tiamulin (Högenauer & Ruf, 1981), it was decided to treat the ribosomes with the reagent in the original Tris- and β-mercaptoethanol-containing buffer rather than to change the ionic milieu by dialysis.

When 7.2 nmol of *E. coli* D10 70S ribosomes was treated with 40 nmol of the affinity reagent, the label was bound to both subunits. This was shown by separating the ribosomal subunits in a sucrose gradient centrifugation at a low magnesium ion concentration and the subsequent determination of the radioactivity associated with the subunits. Prior to the radioactivity measurements, the subunits were pelleted in the ultracentrifuge and redissolved in 1 mL of standard buffer. A 1-mol sample of the 30S particle had 0.12 mol of affinity label bound while 1 mol of 50S subunit had 0.13 mol of affinity label bound.

In order to determine the labeling of single proteins, the ribosomes were pelleted after incubation with the reagent and the proteins prepared as described by Czernilofsky & Küchler (1972), followed by an acetic acid dialysis step. The proteins were then separated by a two-dimensional electrophoresis (Howard & Traut, 1974), the gel was stained, and the stained protein spots were analyzed for radioactivity. In earlier experiments, the stained protein spots were cut out from the gel, and the radioactivity was measured by counting in a liquid scintillation spectrometer. In later experiments, the radioactivity was determined by autofluorography (Bonner & Laskey, 1974). The results obtained by the two methods were in good agreement except that the method of cutting the protein spots indicated a labeling of S14 which by direct fluorography could not be detected. Probably the S14-containing gel slice was contaminated with the strongly radioactive protein S18, an immediate neighbor of S14 in the two-dimensional electrophoresis.

The results of the fluorography are shown in Figure 4. Ribosomes from *E. coli* MRE600 were labeled in proteins L2 and S18, and in some experiments slight radioactivities of other proteins were observed. Since these proteins were only faintly blackening the X-ray film, gave inconsistent patterns in various experiments, and were undetectable at low-affinity reagent concentrations, we ascribe their labeling to side reactions. Consequently, we considered only the reproducible and dominant spots as reaction products from properly positioned affinity reagent. Ribosomes from the *E. coli* strain D10 were predominantly labeled in L27 and S18 (Figure 5) although

Table I: Concentration Dependence in the Labeling of L2 and S18[a]

affinity label added (nmol)	radioactivity (cpm) L2	S18	ratio
5	310	211	1.5
10	644	425	1.5
20	833	901	0.9
40	1007	2372	0.42

[a] A 7.2-nmol sample of *E. coli* MRE600 ribosomes was incubated with various amounts of affinity label by the standard procedure. Isolation of ribosomal proteins, separation, autoradiography, and determination of radioactivity were carried out as described under Materials and Methods.

in some experiments a preferential labeling of L2 was observed. While the blackened areas on the X-ray film exactly coincided with the stained spots of the proteins L2 and L27, the radioactivity of S18 was slightly shifted to the upper part of the gel, indicating that as a result of the alkylation of S18 a change in the charge of this protein had occurred.

If tiamulin-insensitive ribosomes from the resistant mutant *E. coli* D10 C were incubated with the affinity reagent at room temperature, no radioactive spots could be detected. An incubation at a lower temperature had to be used because tiamulin binding of the mutant ribosomes is restored at elevated temperatures (G. Högenauer, W. Kotzmann, and F. Turnowsky, unpublished experiments). Lowering the incubation temperature does not affect the affinity labeling reaction significantly as has been shown with sensitive ribosomes from *E. coli* D10.

In a further experiment, 2.4 nmol of 30S ribosomal subunits was incubated with 16.5 nmol of the affinity reagent in the standard experimental system, and 100 μg of the isolated proteins was separated by two-dimensional gel electrophoresis. Direct fluorography did not show any zone of radioactivity on the dried gel. By combustion analysis of the S18-containing segment, 19 cpm were determined. As a positive control, 70S ribosomes were treated with the affinity reagent in the appropriate molar ratio. A 230-μg aliquot of the proteins was applied to two-dimensional gel electrophoresis. When analyzed for radioactivity, S18 contained 1040 cpm. It is thus concluded that none of the 30S proteins become labeled when the small subunit is exposed to the affinity reagent.

For a quantitative assessment of the affinity labeling reaction, gel pieces containing proteins L2 and S18 were cut out after fluorography, and the radioactive content was estimated. The extent of labeling varies with the concentration of affinity label used. The concentration dependence is shown in Table I, where 5, 10, 20, and 40 nmol of affinity label were incubated with each 7.5 nmol of ribosomes. Clearly, at low concentrations, the radioactivity of affinity label bound to L2 was proportionally higher than that attached to S18. S18 became more labeled at intermediate concentrations of the affinity label while at high concentrations of the compound the label attached to S18 exceed that of L2 (Table I).

In other experiments, the radioactivity in the RNA of affinity labeled ribosomes was analyzed. The RNA was extracted by a phenol procedure from *E. coli* MRE600 70S ribosomes, which had been incubated with the affinity reagent in the same manner as described for the labeling of the ribosomal proteins. A total of 34% of the sum of radioactive counts associated with the ribosomal proteins was found in the total RNA. When the RNA was separated by sucrose gradient centrifugation, the majority of the counts were found to sediment with the 23S RNA (Figure 6), only a small fraction of the radioactivity being associated with 16S RNA.

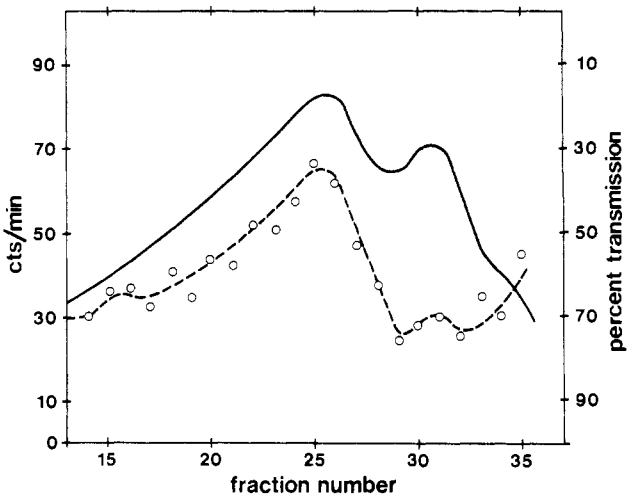


FIGURE 6: Gradient centrifugation of affinity labeled ribosomal RNA. The light absorbance at 260 nm and radioactivity distribution of ribosomal RNA sedimented in a sucrose gradient are shown. (—) Transmission at 260 nm; (- - -) radioactivity.

The small amount of radioactivity associated with RNA has so far prevented sequencing of the RNA segment around the affinity label.

Discussion

The technique of affinity labeling has been used in order to identify the proteins associated with the ribosomal binding sites for protein biosynthesis inhibitors (Pongs et al., 1973; Pongs & Erdmann, 1973; Sonnenberg et al., 1973; Cooperman et al., 1975). A similar approach was chosen for the characterization of the ribosomal pleuromutilin specific binding site. In a first attempt, an imido ester derivative of pleuromutilin was tested, but because of an unstable attachment of the drug, which is believed to occur through amidine residues, this compound was replaced by a bromoacetyl derivative of pleuromutilin. This substance is structurally related to tiamulin, the most effective of the semisynthetic pleuromutilin derivatives (Drews et al., 1975), in that the terminal diethylamino residue of the side chain of tiamulin is replaced by a bromoacetylated amino group. The radioactively labeled bromoacetyl derivative was stably incorporated into *E. coli* ribosomes, attachment occurring presumably through a covalent alkylation reaction involving SH or NH_2 groups of proteins. Tiamulin, a reversibly binding derivative, attached very strongly to two ribosomal binding sites (Högenauer & Ruf, 1981). By assuming a similar behavior for alkylating derivatives, one could expect a relatively specific binding reaction at low concentrations of the compound. That this is indeed the case is shown by the successful competition of the covalent attachment of the affinity label to ribosomes by both tiamulin and chloramphenicol, which shares a binding site with tiamulin. Also, the correct positioning of the affinity label is indicated by the fact that *E. coli* ribosomes, treated with nonradioactive bromoacetyl derivative, showed the functional defects which were known from earlier studies (Hodgin & Högenauer, 1974) on tiamulin: a slight decrease of enzymatic fMet-tRNA binding but a strong inhibition of the puromycin release of P-site bound fMet-tRNA.

When ribosomes, after treatment with the radioactive affinity label, were separated into their components, the major part of the radioactivity was found associated with the protein fraction, and some could be located on the RNA. The pattern of labeling of the electrophoretically separated protein was not uniform with different types of ribosomes. Proteins from

ribosomes of the *E. coli* strain D10 carried the label mainly on the proteins L27 and S18 while *E. coli* MRE600 ribosomes preferentially incorporated the affinity label into L2 and S18. In some cases, a slight labeling of S21 was seen, both with D10 and with MRE600 ribosomes. The effect on L27 could not always be shown with *E. coli* D10 ribosomes. In some experiments, L2 carried the label instead of L27. We have been unable to establish which conditions lead to the two labeling patterns.

When the binding of radioactive tiamulin to *E. coli* ribosomes was determined with the aid of the equilibrium dialysis technique and the data were arranged in a Scatchard plot, a nonlinear graph was obtained. This curve indicates a positve cooperative binding behavior of tiamulin. Furthermore, the extrapolation of the linear portion of the curve showed the presence of two ribosomal, tiamulin-specific binding sites (Högenauer & Ruf, 1981). When the concentration of affinity label was increased, the labeling of the MRE600 ribosomal proteins changed. While at low concentration of the reagent the label was predominantly incorporated into L2, the ratio reversed at high concentrations where mainly S18 became linked to the reagent. The variation in the affinity labeling pattern seems to reflect the binding of the compound to these two different ribosomal sites. Probably the affinity label in the first binding site reacts with L2, and, as the second binding site becomes occupied, S18 is the target for the alkylation. S21 never became a main target for the affinity label. Hence, this protein probably lies near the tiamulin-specific binding site but is not as closely or as favorably positioned as L2 and S18.

RNA was also labeled, albeit to a lesser extent. The labeling of the RNA occurred almost exclusively in the 23S species. This, of course, means that unprotected parts of the 23S RNA are near the tiamulin-specific binding site. RNA had been described to become alkylated by halogenated acetic acid residues (Pan & Bobst, 1973).

Tiamulin is believed to become attached close to the peptidyl transferase because it inhibits the puromycin reaction (Hodgin & Högenauer, 1974) and shares its binding site with chloramphenicol, a known inhibitor of peptidyl transferase (Högenauer, 1975). The peptidyl transferase seems to be located at the interface of the large subunit because all proteins associated with this function have been identified in this area [for review, see Stöffler (1974)]. The proposition that the tiamulin binding site is near the peptidyl transferase center is thus in accord with the affinity labeling of L2 and L27. Both these proteins have been shown to be arranged at the attachment site of the 3′ terminus of tRNAs at the P site close to the peptidyl transferase (Traut et al., 1974).

Radioactive tiamulin binds to the isolated 50S but not the 30S ribosomal subunits (Högenauer & Ruf, 1981). Thus, the affinity labeling of two 30S proteins, S18 and, to a much lesser extent, S21, means that the binding site for pleuromutilin is at the interface between large and small subunits and that in the undissociated ribosome the small subunit comes in such close contact with the drug bound to the large particle that a covalent reaction occurs across the cleft between the two ribosomal subunits. Another example for a peptidyl transferase specific antibiotic to become linked to a 30S protein has been reported by Grant et al. (1979). When they analyzed the results of a photoinduced incorporation of puromycin, the authors identified L23 and S14 as the main binding proteins.

The fact that in the ribosomes from the strains MRE600 and D10 either L2 or L27 reacts covalently with the pleuromutilin derivative seems to indicate a slightly different molecular arrangement on the ribosomes of these strains. These subtle structural differences in the ribosome do not influence other parameters of drug binding like the binding strength or the number of binding sites. Also, the binding kinetics of the affinity label and its competitive inhibition by tiamulin and chloramphenicol appear almost indistinguishable in these two ribosomal preparations.

Sonnenberg et al. (1973) found that a chloramphenicol derivative attaches covalently to L2 and L27. Since chloramphenicol and tiamulin partially share their binding sites, this result is in accord with the affinity labeling of pleuromutlin.

The protein S18 contains a very reactive SH group (Pongs et al., 1973). It is conceivable that the reaction of the pleuromutilin affinity label with S18 occurs without prior attachment to the tiamulin binding site. In order to rule out such an unspecific reaction of S18, ribosomes from a tiamulin-resistant mutant, which bind tiamulin poorly, were incubated with this reagent. No radioactivity was associated with any of the ribosomal proteins, showing that the labeling of all proteins observed with sensitive ribosomes occurs through a specifically attached bromoacetyl derivative. Also, 30S subunits from sensitive ribosomes were not labeled by the affinity reagent. Hence, the involvement of the particularly reactive SH residue of S18 in the covalent binding site of the affinity label may be ruled out. Furthermore, since the radioactive spot on the two-dimensional gel did not fully coincide with the stain of S18, one may conclude that the alkylation altered the charge of the protein. Since an alkylation of the SH group of S18 would not be expected to result in a different charge of the protein, we take this as evidence that this particular sulfhydryl group is not involved in the affinity labeling reaction.

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Correct Codon–Anticodon Base Pairing at the 5′-Anticodon Position Blocks Covalent Cross-Linking between Transfer Ribonucleic Acid and 16S RNA at the Ribosomal P Site†

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ABSTRACT: The codon-dependent covalent cross-linking between the 5′-anticodon base of certain tRNAs bound in the ribosomal P site and a pyrimidine residue of 16S RNA is highly specific for codons which do not base pair with the 5′-anticodon base (c)mo^{5}U. Thus, with tRNA$_1^{Val}$ (anticodon cmo^{5}UAC), pGpUpU, GpUpU, and pGpU allowed high-yield (64–95%) cross-linking, and pGpUpC was about equivalent to poly(U_2,G) in allowing moderate (45–60%) cross-linking. No cross-linking occurred when the normal codon, GpUpA, or the "wobble" codon, GpUpG, was used, although, as expected, they were the best codons in noncovalent ribosomal binding. There was no cross-linking in the absence of codons. Oligonucleotide-induced cross-linking was like that induced by poly(U_2,G) in that only 16S RNA was cross-linked and the covalently bound acetylvaline could be released from the ribosome by reaction with puromycin. Thus all cross-linking occurred at the P site. The rate of photolysis at 254 nm of the cyclobutane dimer which has been shown to be the structure of the cross-link between the tRNA and 16S RNA [Ofengand, J., & Liou, R. (1980) *Biochemistry 19*, 4814–4822], was also the same, again indicating a similar or identical structure in all cases. Covalent cross-linking of AcAla-tRNA (anticodon cmo^{5}UGC) was dependent on the presence of poly(I,C,U) but was not stimulated (<15%) by poly(I,C,A). Thus the inhibitory effect of forming the base-pair cmo^{5}U-A was also found with this tRNA. These results provide the first direct evidence that codon–anticodon interaction is maintained at the ribosomal P site. They also provide evidence that positional changes occur in the anticodon loop of P-site bound tRNA upon interaction of the 5′-anticodon base with its cognate codon residue.

Previous studies from this laboratory have shown that certain tRNAs can be cross-linked to 70S ribosomes when irradiated with light >300 nm. The reaction only occurs when the correct codon is present and when the tRNA is bound in the ribosomal P site. Irradiation of A-site bound tRNA does not result in cross-linking. A tRNA with either a cmo^{5}U[1] or mo^{5}U residue at the 5′-anticodon position is required, and the cross-link is exclusively to 16S ribosomal RNA (Schwartz & Ofengand, 1978; Ofengand et al., 1979). The cross-link is directly between the tRNA and 16S RNA as neither a protein (Zimmermann et al, 1979) nor mRNA (Ofengand & Liou, 1980) linker is involved. The structure of the cross-link has been shown to by a cyclobutane dimer between the 5′-anticodon base, (c)mo^{5}U, and a pyrimidine of the 16S RNA, based upon a number of photochemical properties usually described as being characteristic of such dimers (Ofengand & Liou, 1980).

For most of these studies, poly(U_2,G) was used as mRNA, since it supplied the potential codons, GUU and GUG, and empirically proved to be effective. However, various preliminary experiments, performed with polymers of different base compositions and ratio as a source of codons in cross-linking experiments with different tRNAs, led us to suspect that the nature of the residue paired with the 5′-anticodon base of the tRNA influenced the ability of this base to cross-link to 16S RNA. In particular, the results seemed to suggest that either A or G base-pairing with (c)mo^{5}U strongly *decreased* the cross-linking efficiency. There was no reason to believe that correct codon–anticodon base pairing should negatively influence the ability to form a cyclobutane dimer since cyclobutane dimers can be formed between adjacent thymine residues in native DNA. These residues must have been base-paired to A residues on the complementary DNA strand before dimer formation occurred. This consideration suggested that some special aspect of the mRNA–ribosome–tRNA interaction in the decoding region of the P site might be responsible for the apparent codon influence on cross-linking. It seemed, therefore, worthwhile to investigate the effect in a more rigorous way by the use of defined codons. In this paper, we describe such a detailed study and its conclusions.

Experimental Procedures

Chemicals. Ac[^{3}H]Val-tRNA$_1^{Val}$ (*E. coli*), Ac[^{3}H]Val-tRNA^{Val} (*B. subtilis*), tight couple ribosomes, and poly(U_2,G) were prepared or obtained as described previously (Ofengand et al., 1979). Ac[^{3}H]Ala-tRNA (*E. coli*) was prepared by

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[1] Abbreviations used: mo^{5}U, 5-methoxyuridine; cmo^{5}U, 5-(carboxymethoxy)uridine; AcVal- and AcAla-, *N*-acetylvalyl- and *N*-acetylalanyl-, respectively; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid; NaDodSO$_4$, sodium dodecyl sulfate.

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