A photoaffinity labelling study of the messenger RNA-binding region of Escherichia coli ribosomes

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ABSTRACT

A photoaffinity labelling study of the messenger RNA-binding region of E. coli ribosomes has been made, using oligoadenylic acids as mRNA analogs. The oligonucleotides, of chain length 6 to 8 and thus several nucleotides longer than oligonucleotides previously employed for this purpose, carried a radioactive photolabile aromatic azide reagent bound covalently to the 3' terminal ribose moiety. The synthesis of the reagent, p-azidobenzoyl-(3H)-glycylhydrazide, is described. The derivatized oligonucleotides were shown to be functional messengers. They stimulated the binding of the cognate aminoacyl-tRNA, lysyl-tRNA; their binding was reciprocally stimulated by lysyl-tRNA; and they competed with underivatized oligoadenylates for ribosomal binding sites. When the 70 S ribosomal binding complex was irradiated, the photolabile reagent reacted covalently with both RNA and proteins of the 30 S subunit and with tRNA, but not with the 50 S subunit. The 16 S RNA appeared to be labelled at more than one site. Of the proteins, S3 and S5 reacted with the reagent with high specificity; and the possibility was not eliminated that S4 may have been labelled to a minor degree. Functional studies in other laboratories have implicated S3 and S5 in the decoding process, but these proteins were not labelled by any of the previously reported mRNA affinity labelling analogs. The results reported here therefore indicate that S3 and S5 not only affect the decoding process, but are located in the mRNA-binding region of the ribosome, presumably to the 3' side of the decoding site.

INTRODUCTION

A number of ribosomal binding sites are currently under investigation by means of affinity labelling techniques (1-3). The mRNA binding region of E. coli ribosomes has been probed with oligonucleotide analogs containing a single reactive group at a defined position, or with homopolynucleotides. The homopolynucleotides have the potential of reacting, on irradiation, at any position along the chain and thus of tracing the entire -- possibly quite extended -- binding region of mRNA, but yield no information about the site of labelling relative to a reference point on the ribosome, such as the decoding site. Such information can be obtained with reactive oligonucleotide analogs.
All probes used to date have labelled components of the 30 S subunit exclusively, in accordance with the current concept of mRNA translation. Whether ribosomal protein or RNA becomes labelled depends to a large degree on the chemical properties of the probe. Thus, haloacetyl derivatives of tri- and tetranucleotides have labelled a limited number of proteins (4-7), while a glyoxal oligonucleotide derivative reacted with an RNA region that could be precisely localized (8). Polynucleotides have reacted under ultraviolet irradiation with both protein and RNA (9-14).

In this communication we describe experiments with oligonucleotides containing 6, 7 or 8 adenine residues and thus several nucleotides longer than the oligonucleotides previously employed for this purpose. They were derivatized at the 3'-end with a photolabile reagent of the aromatic azide type. The radioactive reagent, p-azidobenzoyl-glycylhydrazide (ABGH)**, is easily attached to periodate-oxidized oligonucleotides and, when irradiated, would be expected to react unselectively and without the need of a particular reactive group with protein or RNA at a region 3' to the decoding (aminoacyl-tRNA binding) site. A similar though nonradioactive reagent, p-azido-2-nitrobenzoylhydrazide, has previously been used for the synthesis of a GTP analog (15).

** MATERIALS **

The following materials were used: 2-(H)-glycine and uniformly labelled (14C)-lysine (Amersham), E. coli tRNA (Boehringer), polyadenylic acid (Schwarz-Mann), silica gel thin layer plates (Riedel-DeHaen), Porapak type Q (Supelco, Inc., Bellefonte, Pa.), nitrocellulose filters (Sartorius, 0.45 μ). All solvents were analytical grade. Specific activities: (3H)-A₅-ABGH, 3.4 Ci/mmole; (14C)-Lys-tRNA, 318 mCi/mmole.

** METHODS **

Buffers

MTA 20-50-150: 20 mM magnesium acetate - 50 mM Tris - 150 mM NH₄Cl, finally adjusted to pH 7.4 with HCl. MTA 0.5-10-100: 0.5 mM magnesium acetate - 10 mM Tris - 100 mM NH₄Cl, finally adjusted to pH 7.4 with HCl. MTK 20-30-330: 20 mM MgCl₂ - 30 mM Tris-HCl (pH 7.4) - 330 mM KCl.

Synthesis of p-azidobenzoyl-(3H)-glycylhydrazide (ABGH)

2.35 μmoles of (3H)-glycine (3.4 Ci/mmole) in water were taken to dryness under a stream of dry air with warming. 200 μl of absolute methanol were added and dry HCl
was introduced for 10 min, keeping the reaction vessel at about 60°. The solution was evaporated to dryness by switching from dry HCl to a stream of dry air, and the residue was dissolved in 100 μl of absolute ethanol. The extent of reaction was determined by chromatography on silica gel thin layer plates developed with H2O–ethanol (1:1, v:v) and sprayed with ninhydrin for detection. Non-radioactive glycine methyl ester and glycine were used as markers. Rf: glycine, 0.7; glycine methyl ester, 0.31. Yield: 85% by radioactivity.

Synthesis of p-azidobenzoic acid

40 mmoles of p-aminobenzoic acid were dissolved in 8 ml of concentrated HCl, diluted with 20 ml of water, and chilled. 40 mmoles of sodium nitrite in 10 ml of water were added, keeping the temperature between -5° and 0°. After 45 min the solution was filtered in the cold and 40 mmoles of sodium azide in 10 ml of water were added dropwise, keeping the temperature at about 0°. Ethyl acetate was added during the reaction to prevent excessive foaming due to nitrogen evolution. Chloroform was added and the organic phase was concentrated by evaporation. The crystals that separated out were recrystallized from ethanol/water. Yield: 12.3 mmoles, 31%. Thin layer chromatography on silica gel in chloroform-methanol (95:5, v:v) Rf: p-azidobenzoic acid, 0.61; p-aminobenzoic acid, 0.39. Infrared spectrum vKBr (cm⁻¹): 2100 (~N3), 1680, 1600. The compound has also been prepared by reacting the diazonium salt with hydroxylamine (16).

Synthesis of the N-hydroxysuccinimide ester of p-azidozoenoic acid

4.4 mmoles of p-azidobenzoic acid, 5.2 mmoles of N-hydroxysuccinimide and 3.6 mmoles of dicyclohexylcarbodiimide were dissolved in 9 ml dioxane. After 2 hours at room temperature, 20 ml of ethyl acetate were added, and the precipitate was filtered off and washed with 10 ml of ethyl acetate. The combined filtrates were concentrated by evaporation to about 5 ml and were quickly extracted in the cold with 1 M KOH, 1 M HCl and water. The organic phase was evaporated, leaving a slightly yellowish residue (2 mmoles). Thin layer chromatography on silica gel: Rf = 0.44 in chloroform, 0.63 in ethyl acetate, 0.93 in chloroform-methanol (8.5:1.5, v:v). The compound has recently been described (17).

Synthesis of p-azidobenzoyl-(3H)-glycine methyl ester

3.5 μmoles of the N-hydroxysuccinimide ester of p-azidobenzoic acid were dissolved in 100 μl of dioxane and added to an equal volume of ethanol containing about 2 μmoles of (3H)-glycine methyl ester. 30 μl of dioxane containing 12.5% triethylamine
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and 3% acetic acid by volume were added. After 2 days at room temperature, 1 ml of chloroform was added and the organic phase was extracted 3 times with 2 ml of water and concentrated to about 20 µl.

A non-radioactive batch was prepared similarly from 1 mmole of active ester, 2 mmoles of glycine methyl ester hydrochloride and 2.2 mmoles of triethylamine in 2 ml of dioxane-ethanol (1:1, v:v) yielding a slightly yellowish oil after evaporation of the solvent.

Synthesis of p-azidobenzoyl-(3H)-glycylhydrazide (ABGH)

The concentrated solution of p-azidobenzoyl-(3H)-glycine methyl ester was diluted with 200 µl of 2.5% hydrazine hydrate (about 100 µmoles) in ethanol. The solution was left overnight at room temperature, diluted with about 1 ml of chloroform-methanol (9:1.5, v:v), applied to a preparative silica gel thin layer plate (0.2 x 20 x 20 cm), and developed in chloroform-methanol (9:1.5, v:v). The area corresponding to ABGH was located under an ultraviolet lamp by comparison with a non-radioactive sample run on the same plate. It was scratched off into a centrifuge tube and extracted four times with water by suspending and precipitating the silica gel powder. Yield 1.06 µmoles (45% based on glycine).

A larger amount of non-radioactive ABGH was prepared similarly except that the hydrazide was precipitated with dioxane (under cooling) and recrystallized from methanol. Thin layer chromatography on silica gel: Rf = 0.27 in chloroform, 0.5 in chloroform-methanol (9:1.5, v:v). Infrared spectrum ν-KBr (cm⁻¹): 2100 (-N₃), 1620, 1580, 1500. Ultraviolet absorption: maximum, 270 nm; molar absorbance, 18,200.

Coupling of ABGH to periodate-oxidized oligoadénylic acids

Oligoadénylic acids of chain length 4 to 8 nucleotides were prepared by the partial alkaline hydrolysis of polyadénylic acid and separated by paper chromatography according to Asteriadis et al. (18). Monoesterified phosphate at the 3' termini was removed with bacterial alkaline phosphatase, and the oligonucleotides were purified by paper chromatography as above, eluted with water, filtered to remove lint, and lyophilized. The 2',3' diol of the 3'-terminal ribose moiety was then oxidized to the dialdehyde at a concentration of about 0.5 mM oligonucleotide in 10 µl of 20 mM sodium acetate (pH 5) by adding sodium periodate to a final concentration of 2 mM. After 1 hour at room temperature in the dark, sodium thiosulfate was added to a final concentration of 20 mM.
(*H)-ABGH in 2 to 3 times the molar amount of dialdehyde was taken to dryness and dissolved in the solution containing the dialdehyde. Incubation was at room temperature in the dark for about 24 hours. To remove excess reagent, the oligonucleotides were adsorbed on a DEAE-cellulose column (about 1 ml, previously washed with 2 M NaHCO₃ and water), washed with 5 ml of 100 mM NH₄Cl (adjusted to pH 8 with NH₄OH), and eluted with 2 ml of 2 M NH₄Cl. To remove underivatized polynucleotides and salt, the eluate was passed through a column (about 0.3 ml) of polystyrene resin, Porapak Q, which adsorbs aromatic compounds such as ABGH and its derivatives (19). The column was washed with 5 ml of water and eluted with 1 ml of 50% ethanol (v:v). Yields were around 75%, based on radioactivity.

Assay: binding of oligoadenylate-ABGH to 70 S ribosomes

Ribosomes were prepared from E. coli MRE 600 by standard procedures. Bulk E. coli tRNA was charged with lysine (20). Reversible (non-covalent) binding was determined in MTA 20-50-150 buffer containing 70 S ribosomes, Lys-tRNA, oligoadenylate-ABGH (A₇-ABGH), and ethanol, in concentrations specified for each experiment. Where not specified, the ethanol concentration was 15% by volume. Reactions were started by the addition of ribosomes. After 1 hour at 0° the samples were diluted with 2 ml of MTA 20-50-150 in 20% ethanol at 0°, filtered on nitrocellulose filters (previously soaked 1 hour in MTA 20-50-100 without ethanol), and washed twice with 2 ml MTA 20-50-150 in 20% ethanol. Radioactivity was determined in 4 ml of toluene-based scintillator at 19-22% efficiency.

Irradiation

An ACE-Hanovia high pressure quartz-mercury vapor lamp of 450 watts was used as the light source. Samples in Pyrex test tubes were irradiated for 4 min at 0° at a distance of 8 cm from the center of the lamp. The tubes were shaken manually at 1 min intervals.

Sucrose gradient centrifugation

All gradients were exponential and approximately isokinetic (21). The sucrose concentration was 5% at the top. Centrifugation was at 5° in a Beckman SW 41 rotor run at 40,000 rpm or an SW 50.1 rotor run at 48,000 rpm.

Polyacrylamide gel electrophoresis

Four analytical systems were used: a. Two-dimensional: first dimension, 7.5% acrylamide, 6 M urea, pH about 4.5; second dimension, 20% acrylamide, 6 M urea, pH about 4.5 (22). b. One-dimensional: the second dimension of system a. c. One-
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dimensional: 8% acrylamide, 6 M urea, pH about 8.6 (23). d. One-dimensional: 10% and 15% acrylamide, sodium dodecyl sulfate and buffers according to Laemmli (24). a, b and c were run in a horizontal slab gel apparatus previously described (22). d. was run in vertical slab gels 1.4 mm thick and of length 10 cm (separation gel) plus 3 cm (stacking gel).

The gels were stained with 0.2% Coomassie blue in 5% methanol - 7% acetic acid and destained in the same solvent to locate the proteins. For the determination of radioactivity, the stained spots were excised from the 2-dimensional gel, while the 1-dimensional gels were cut into uniform slices. These were dried, oxidized in a sample oxidizer (Packard Tri-Carb, model 306), and counted.

RESULTS

Non-covalent binding of An-ABGH to ribosomes

Before proceeding with photoaffinity labelling experiments, it was necessary to show that the affinity labelling mRNA analogs, An-ABGH, actually function as messengers, and to work out favorable conditions for forming the ternary ribosome-messenger-aminoclyl-tRNA binding complex. The reaction mixture was not irradiated in these experiments, so that the An~ABGH species would function as conventional oligonucleotide messengers and form non-covalent reversible binding complexes.

In early experiments the binding of An~ABGH to ribosomes was erratic, being often very low and not enhanced by Lys-tRNA. This was eventually found to depend on whether the An~ABGH was used as eluted from the polystyrene column, in which case it contained ethanol, or had been freed of ethanol by evaporation; and it was shown to be necessary to include ethanol in the incubation mixture (Fig. 1). Other experiments showed that 70 S ribosomes were about five times more effective than 30 S ribosomes in forming the ternary binding complex. Subsequent experiments were therefore carried out with 70 S ribosomes in the presence of ethanol.

The results of a binding experiment are shown in Table 1. As expected for the formation of a ternary binding complex, the presence of the messenger analog A7-ABGH stimulated the binding of its cognate aminoclyl-tRNA Lys-tRNA, and vice versa. When the unlabelled oligonucleotide A7 was added at the same time, the binding of A7-ABGH was reduced appreciably, indicating competition. Once bound, however, An-ABGH exchanged with its competitor A7 only very slowly (Fig. 2). This firmness of binding was not due to covalent attachment to the ribosome, since the bound An~ABGH was detached.
Fig. 1. Enhancement of non-covalent binding of A7-ABGH by ethanol. Quantities in 20 μl: 70 ribosomes, 13.3 pmoles (0.5 A260 unit); (3H)-A7-ABGH, 5.1 pmoles; Lys-tRNA, none or 0.36 A260 units; ethanol, as indicated. See Methods for conditions.

Table 1: Mutual stimulation of Lys-tRNA and A7-ABGH non-covalent binding to 70 S ribosomes.

<table>
<thead>
<tr>
<th>Ribosomes</th>
<th>Lys-tRNA</th>
<th>A7-ABGH</th>
<th>A7</th>
<th>pmoles bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>2.7</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>3.5</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Quantities in 20 μl: 70 S ribosomes, 13.3 pmoles; (14C)-Lys-tRNA, 17 pmoles of lysine; (3H)-A7-ABGH, 3.8 pmoles; A7, 66 pmoles; ethanol, 15% (v/v). A7 was added together with A7-ABGH. See Methods for conditions.

when the 70 S ribosomes were dissociated into subunits. Fig. 3 shows complex formation as a function of A7-ABGH concentration. Lys-tRNA stimulated the binding over the entire range tested. The shape of the curve suggests that saturation would be achieved at about 0.2-0.25 moles of A7-ABGH bound per mole of ribosome.

Irradiation-induced covalent binding of A7-ABGH to ribosomes

The preceding experiments showed that the messenger analogs A7-ABGH were functional messenger RNA's. In the following experiments the reaction mixture was irradiated (Methods) so as to induce covalent bond formation. This proceeded rapidly (Fig. 4) and, like non-covalent binding, was stimulated by Lys-tRNA, inhibited by competing non-derivatized oligoadenylic acid, and required time prior to irradiation for the
Fig. 2. Formation and stability of the binding complex with A\textsubscript{6}-ABGH.

Quantities in 210 \mu l: 70 S ribosomes, 350 pmoles; \( ^{3}\text{H}\)-A\textsubscript{6}-ABGH, 20 pmoles; \( ^{14}\text{C}\)-Lys-tRNA, 238 pmoles of lysine; ethanol, 10\% (v:v). After 60 min at 0\º, 2500 pmoles of A\textsubscript{6} ("cold template") were added in 5 \mu l. 20 \mu l aliquots were removed at the indicated times and assayed.

Fig. 3: Enhancement of non-covalent binding of A\textsubscript{7}-ABGH by Lys-tRNA.

Quantities in 20 \mu l: 70 S ribosomes, 13.3 pmoles; \( ^{3}\text{H}\)-A\textsubscript{7}-ABGH, as indicated; Lys-tRNA, none or 0.36 A\textsubscript{260} units; ethanol, 15\% (v:v).

Formation of the non-covalent complex (Table 2). Even in the absence of ribosomes but in the presence of Lys-tRNA, TCA-precipitable radioactivity above background was recovered. Since this was reduced by the addition of competing A\textsubscript{6} (not shown), the formation of a non-ribosomal (Lys-tRNA-(A\textsubscript{6}-ABGH) covalent complex may be indicated.

When the covalently labelled ribosome was separated into subunits and analyzed by sucrose gradient centrifugation, the attached A\textsubscript{7}-ABGH was found on the 30 S but very little or none on the 50 S subunit (Fig. 5, Table 3). A complex with tRNA was also seen. When the binding complex was not irradiated, no TCA-precipitable radioactivity was recovered along the gradient, showing that no non-covalently bound A\textsubscript{7}-ABGH remained on the subunits (Table 3).
Fig. 4: Covalent bond formation on irradiation.

Quantities: 70 S ribosomes, 53.5 pmoles; Lys-tRNA, none or 1.4 A260 units; (\(\text{H}\))-A\(_6\)-ABGH, 45 pmoles. The non-covalent binding complex was allowed to form in 80 \(\mu\)l of MTA 20-50-150 containing 15% ethanol and was then diluted to 900 \(\mu\)l with the same buffer lacking ethanol. 15 \(\mu\)l aliquots were irradiated (see Methods) for the indicated times, after which 1 ml of 0.2 M EDTA (pH 7.4) was added. After 5 min at room temperature, cold 5% trichloroacetic acid was added and the precipitates was collected on glass fiber filters (Whatman, GF/C), washed and counted. In this experiment the non-covalent binding was 0.13 pmoles A\(_6\)-ABGH/pmole ribosome in the presence and 0.02 pmoles/pmole in the absence of Lys-tRNA.

Table 2: Covalent binding of A\(_6\)-ABGH: Stimulation by Lys-tRNA and competition with A\(_6\).

<table>
<thead>
<tr>
<th>Ribosomes</th>
<th>Lys-tRNA</th>
<th>A(_6)-ABGH</th>
<th>A(_6) (pmoles bound)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+ (zero time)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+ (zero time)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Quantities in 40 \(\mu\)l: 70 S ribosomes, 26.7 pmoles; Lys-tRNA, 0.72 A260 units; (\(\text{H}\))-A\(_6\)-ABGH, 6.8 pmoles; A\(_6\), 80 pmoles; ethanol, 10% (v:v). The mixtures were irradiated after 1 hour at 0\(^\circ\) or immediately after the addition of ribosomes (zero time). Covalent binding was assayed by dilution with 40 \(\mu\)l of 20 mM EDTA followed, after 5 min at room temperature, by precipitation with cold 10% TCA. Where absent, ribosomes were added as carrier after irradiation. A background value of 0.55 pmoles (pre-irradiated A\(_6\)-ABGH) was subtracted from all values. Non-covalent binding was 2.31 pmoles (+Lys-tRNA) and 0.79 pmoles (-Lys-tRNA).
Fig. 5: Localization of covalent binding on ribosomal subunits.

70 S ribosomes, 532 pmols; Lys-tRNA, none or 16 A_{260} units; and (3H)-A6-ABGH, 92 pmols were incubated in the presence of 5% ethanol to allow complex formation, irradiated, and dissociated by dialysis against MTA 0.5-10-100. Subunits were separated by sucrose gradient centrifugation in the same buffer for 90 min at 48,000 rpm. (Methods). 30 fractions were collected. 50 \mu l aliquots were diluted to 350 \mu l, measured for optical density at 260 nm, precipitated with cold 5% TCA, and counted. Bovine serum albumin was added as carrier.

Table 3: Labeling of subunits: effect of irradiation.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Counts per 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Irradiated</td>
</tr>
<tr>
<td>50 S</td>
<td>272</td>
</tr>
<tr>
<td>30 S</td>
<td>1944</td>
</tr>
<tr>
<td>Top (tRNA)</td>
<td>671</td>
</tr>
</tbody>
</table>

Quantities in 200 \mu l: 70S ribosomes, 173 pmols; Lys-tRNA, 36 A_{260} units; (3H)-A6-ABGH, 20 pmols; ethanol, 5% (v/v). After 1 hour incubation at 0\degree, the mixture was diluted with 1 ml of MTA 20-50-150 and divided into two equal portions. One portion was irradiated and the other was not. The ribosomes were dissociated by dialysis against MTA 0.5-10-100 and separated by sucrose gradient centrifugation as in Fig. 5. Peak fractions by absorbance were pooled, precipitated with cold 5% TCA, and counted.

One preliminary experiment was done with 30 S ribosomes carrying covalently bound A6-ABGH, to test whether the covalently bound oligonucleotide was still functional. The labelled subunits were isolated by sucrose gradient centrifugation, reactivated (25), and tested for their ability to bind (14C)-Lys-tRNA. Binding was stimulated by an amount, above background, that corresponded to about 15% of the covalently attached oligonucleotide. This is not unreasonable, considering that 30 S subunits are less effective than 70 S.
ribosomes in the binding assay.

**Labelled ribosomal components**

As shown above, irradiation of the 70 S ribosomal binding complex caused the radioactive messenger RNA analog to bind covalently to tRNA and to the 30 S subunit. Dissociation of the subunit into its components showed that the RNA and protein moieties were about equally labelled.

**Labelled 30 S ribosomal proteins**

**Technical**

The identification of the radioactively labelled proteins was based on their mobilities in four different polyacrylamide gel electrophoresis systems, one 2-dimensional and three 1-dimensional. The proteins were liberated by destroying the ribosomal RNA with a mixture of RNase A and RNase T1 in urea. The low levels of radioactivity precluded the use of radioautography as a means of detection, and it was necessary to section the dried gels and burn them (Methods). One dimensional analysis was preferable to 2-dimensional, since it allowed analysis of the entire gel, ensuring that labelled components with altered mobilities would not be missed, as might occur in 2-dimensional gels where analysis of the entire gel is too cumbersome. In addition, it is easier in uniformly sliced 1-dimensional gels to assess the possibility of radioactive contamination of one protein by a neighboring one. In some experiments the proteins were extracted from isolated 30 S subunits, and in others directly from the 70 S ribosomes. Both procedures gave the same radioactivity pattern.

Since a covalently linked oligonucleotide would be expected to alter the electrophoretic mobility of a protein, it was felt necessary to remove the oligonucleotide before electrophoresis. Control experiments with A_n-ABGH showed that this could be accomplished by a brief incubation at pH 9 and 60°, which removed ABGH from the oligoadenylate, presumably by hydrolysis of the hydrazide-dialdehyde bonds and perhaps also by β-elimination. Crude E. coli RNase I (26, 27), a non-specific ribonuclease, was also effective in hydrolyzing the oligoadenylate moiety, which is not attacked by RNase A or RNase T1. Unexpectedly, neither treatment affected the mobilities of the labelled proteins. We suspect that the oligoadenylate part of the messenger analog was cleaved off during the work-up of the proteins, either spontaneously or by contaminating nucleases in the RNase A or T1 preparations used. Nevertheless, the extracted proteins were routinely incubated at pH 9 and 60° before electrophoresis.
Results
Table 4 shows the results of a 2-dimensional analysis. Four proteins -- S2, S3, S4, and S5 -- were labelled above the background labelling observed when Lys-tRNA was omitted from the binding mixture. Two others -- S20 and S21 -- were too faint in this gel to be analyzed properly.

The 1-dimensional analyses (Figs. 6, 7, 8) confirmed the labelling of S3 and S5. S20 and S21 were eliminated by the absence of significant radioactivity in their locations in all the gels, and the 8% basic system (Fig. 7) excluded S2. The 20% acidic system (Fig. 6) indicated that S4 was not labelled, but did not fully eliminate the possibility, since S4 migrates close to S3 or S5 in all the systems employed.

Taken together, the results show that S3 and S5 were the major labelled proteins, with S4 not excluded as a possible minor product.

Labelled RNA
As mentioned above, both tRNA and the ribosomal 16 S RNA became covalently labelled when the ternary binding complex was irradiated. The location of the label on the tRNA molecule has not been investigated, and only a preliminary exploratory experi-

Table 4: Two-dimensional gel electrophoresis of labelled proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>cpm</th>
<th>Protein</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+tRNA</td>
<td>-tRNA</td>
<td>+tRNA</td>
</tr>
<tr>
<td>S1</td>
<td>12</td>
<td>14</td>
<td>S8</td>
</tr>
<tr>
<td>S2</td>
<td>45</td>
<td>4</td>
<td>S9/11</td>
</tr>
<tr>
<td>S3</td>
<td>76</td>
<td>11</td>
<td>S10</td>
</tr>
<tr>
<td>S4</td>
<td>24</td>
<td>6</td>
<td>S12, S13</td>
</tr>
<tr>
<td>S5</td>
<td>93</td>
<td>25</td>
<td>S14, S15, S16, S17</td>
</tr>
<tr>
<td>S6</td>
<td>14</td>
<td>14</td>
<td>S18</td>
</tr>
<tr>
<td>S7</td>
<td>4</td>
<td>7</td>
<td>S19</td>
</tr>
</tbody>
</table>

A binding complex formed in 15% ethanol from 267 pmoles 70 S ribosomes, 77 pmoles ($^3$H)-A7-ABGH, and 8 A260 units or none of Lys-tRNA was irradiated, dissociated into subunits, and separated by sucrose gradient centrifugation (as in Fig. 5). The pooled 30 S subunits were precipitated with cold 5% TCA, redissolved in 6 M urea - 0.1 M Tris-HCl (pH 7.4) - 20 mM EDTA, and digested with 2 μg RNase A and 1 μg RNase T1 at 40° for 1 hour (final vol., 30 μl). The digest was made 0.5 M in ammonium acetate (pH 9) and heated 10 min at 60° (see text). It was dialyzed against 10 mM HCl and lyophilized. The proteins were dissolved in 6 M urea and subjected to 2-dimensional gel electrophoresis (22).
Fig. 6: One-dimensional gel electrophoresis of labelled proteins (pH 4.5, urea, 20% acrylamide).
A binding complex from 53 pmoles of 70 S ribosomes, 15 pmoles of [(H)-A7-ABGH, and 1.4 A260 units or none of Lys-tRNA in 80 µl (containing 15% ethanol) was irradiated. 80 µl of 10 M urea and 10 µl of 0.2 M EDTA were added, and the mixture was incubated with 2 µg RNase A and 0.2 µg RNase T1 for 2 hours at 40°C. The proteins were then treated as described in Table 4 and subjected to electrophoresis (22).
A: Complete binding mixture. B: Lys-tRNA omitted from binding mixture. C: Positions of 30 S proteins in stained gel.

Fig. 7: One-dimensional gel electrophoresis of labelled proteins (pH 8.5, urea, 8% acrylamide).
See legend of Table 4 for details. Electrophoresis was according to Kaltschmidt and Wittmann (23). A: Complete binding complex. B: Lys-tRNA omitted from binding mixture. C: Position of 30 S proteins in stained gel.

Labelled 16 S RNA was cleaved into two unequal fragments by limited digestion with RNase T1 (29). The 12 S fragment comprises the original 5' end and the 8 S fragment, most of the rest of the molecule. When analyzed by sucrose gradient centrifugation, both fragments were found to be labelled, roughly in proportion to their optical density. The same results were obtained with A7-ABGH and A8-ABGH.
Fig. 8: One-dimensional gel electrophoresis of labelled proteins (SDS, 10% or 15% acrylamide).

A binding complex formed in 15% ethanol from 80 pmoles of 70S ribosomes, 15 pmoles of (3H)-A7-ABGH, and 2.4 A260 units of Lys-tRNA in 120 μl was irradiated. 30 μl of 0.2 M EDTA, 50 μl of 1 M sodium acetate, 4 μg of RNase A, 2 μg of RNase T1 and 0.5 units of RNase T2 were added. The mixture was incubated overnight at 37°, dialyzed against 10 mM HCl, and lyophilized. Proteins were taken up in sample buffer containing SDS, heated 10 min at 100°, and separated on SDS gels according to Laemmli (24). 30 S proteins were identified by comparison with published data (28). A: 15% SDS gel. B: 10% SDS gel (only part of the gel was analyzed for radioactivity).

Fig. 9: Labelling of 16 ribosomal RNA

A: Two A260 units of 16 S RNA extracted from (3H)-A8-ABGH affinity-labelled 30 S sub-units were heated for 30 min at 40° in reconstitution buffer (MTK-20-30-330), incubated for 20 min at 0° with 0.2 μg of RNase T1 in a volume of 220 μl (29), and centrifuged in a sucrose gradient.

B: Control, 0.7 A260 units of 16 S RNA were treated similarly, but without enzyme. Sucrose gradient centrifugation was for 11.5 hours at 40,000 rpm (see Methods). 30 fractions were collected and their optical density was measured. The RNA was then precipitated with cold 5% TCA and its radioactivity determined.
DISCUSSION

In these experiments a family of photolabile mRNA analogs was used to label the binding region of the ribosome to the 3' side of the decoding site. The analogs consisted of a photolabile aromatic azide bound to an oligoadenylate of chain length 6 to 8. The adenylate codons are known to be among the most effective of the trinucleotide messengers (30), and their effectiveness is further increased by increasing the chain length (31).

To assess the specificity of the labeling, it was demonstrated for the non-covalent (not irradiated) binding complex that the analog stimulated the binding of the cognate Lys-tRNA, that its binding was reciprocally stimulated by Lys-tRNA, and that the non-derivatized oligonucleotide was a competitor. The covalent binding reaction, induced by irradiation, was also stimulated by Lys-tRNA and inhibited by nonderivatized oligonucleotide. Since the exchange of the unactivated analog with a competitor was very slow, it was inferred that the activated analog would react covalently while still at its original binding site; and this supposition received tentative support from a preliminary experiment which indicated that the covalently bound oligonucleotide had messenger activity.

Specific and efficient formation of the reversible complex could be achieved only in the presence of ethanol. Whatever the reason for this, it is known that alcohols stimulate several other ribosomal activities, among them the fragment reaction assay for peptidyl transferase activity (32); thermal activation of ribosomal subunits (20); and elongation factor-dependent GTPase activity (33).

In the presence of Lys-tRNA the analog labelled both the protein and RNA moieties of the 30 S subunit. tRNA was also labelled, but the 50 S subunit was not. In the absence of tRNA only the 30 S subunit was labelled, but less than in the complete system.

The labelled proteins were identified by polyacrylamide gel electrophoresis in four different systems. The results showed clearly that two proteins, S3 and S5, were labelled and that their labelling depended on the presence of Lys-tRNA. A third protein, S4, which migrates close to S3 or S5 in all the systems, could not be excluded as a minor labelled product; however, the evidence for its labelling was not conclusive.

 Proteins S3 and S5 have not been labelled by previously reported mRNA affinity labelling analogs (Table 5). This may be due to the fact that our analog is longer than the oligonucleotide analogs employed in other laboratories and, possibly, that the reactive group is attached to the 3' terminal ribose moiety (Table 5). There is, however,
<table>
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<tr>
<td>4</td>
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<td>(Br-CH₂-CO-NH-[O]-0-P₂-O-)-5'-AUG</td>
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<td>8</td>
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<tr>
<td>13</td>
<td>Poly (Br₅-U)</td>
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<td>34</td>
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<tr>
<td></td>
<td>by Rose Bengal</td>
<td>S1, S2, S3, S5</td>
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<tr>
<td>35</td>
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<td>36</td>
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<td>from Rose Bengal oxidation</td>
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<td>40</td>
<td>from tryptic digestion</td>
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Structural and functional evidence from other sources that S3 and S5 are involved in the binding and handling of mRNA by the ribosome. Reconstitution experiments have indicated that mRNA binding is impaired by the absence or alteration of these proteins and is then enhanced by their addition (34, see Table 5). Indirect functional evidence comes from the influence of S3 and S5 on the interaction between the 30 S ribosome and streptomycin, an antibiotic which affects the translation of mRNA on the ribosome. Thus, protein-deficient subunits (core particles) lacking S3 and S5 are unable to bind streptomycin, and
regain the ability to do so when these proteins are added back (41). Also, a mutant strain of E. coli requiring streptomycin for growth (the mutation is in protein S12) may revert to streptomycin independence through a subsequent mutation in S5 (42). In addition, protein–protein crosslinking studies in a number of laboratories have shown that S5 neighbors on S4 in the ribosome (43, 44 and references cited therein). Crosslinks have also been reported between S3 and S5, S3 and S4, S3 and S12, and S5 and S13 (45). S4, S12 and S13 have been labelled by mRNA affinity analogs (Table 5). The literature contains other reports implicating proteins S3 and S5 in the ribosomal binding of tRNA, but in these cases it is difficult to tell if the results were due to an effect on mRNA binding.

The results cited in the preceding paragraph indicate that S3 and S5 are involved in the interaction between ribosomes and mRNA. The affinity labelling results reported here indicate that these proteins are located in the region of the mRNA binding site.

The labelling sites of the 16 S RNA have not been identified. Mild nuclease digestion produced two large fragments of unequal size with roughly equal specific radioactivity. Such a pattern might result if labelling occurred in a region where distant sections of the 16 S RNA come into close contact by three-dimensional folding. There is some support for this in the literature, since different sections of the RNA have been labelled by other mRNA affinity analogs (Table 5). A less likely possibility is that the enzymatic cleavage point happened to coincide with the center of a single labelled section. A third possibility, that the affinity reagent reacted indiscriminately with the entire RNA chain, seems virtually excluded by the high specificity of the protein labelling.

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**ABBREVIATIONS

ABGH, p-azidobenzoylglycylhydrazide; An, an oligoadenylic acid containing n adenosine residues and no 3'-terminal phosphomonoester (e.g., A4 is ApApApA); An-ABGH, An with ABGH bound covalently to its 3'-terminal ribose moiety; mRNA, messenger RNA; tRNA, transfer RNA; Lys-tRNA, bulk E. coli tRNA charged with lysine; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.
REFERENCES
