Affinity Labeling of the Ribosomal Decoding Site with an AUG-Substrate Analog†

(Initiation of Protein Synthesis/Ribosomal Proteins/mRNA Binding/Polyacrylamide Electrophoresis)

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ABSTRACT The trinucleotide AUG was condensed at the 3'-end with N-bromoacetyl-p-aminophenylphosphate. This bromoacetylated AUG analog reacted irreversibly with the mRNA binding site of Escherichia coli 70S ribosomes. After reaction of 70S ribosomes with the AUG analog, labeled 30S subunits could be isolated that were programmed for initiation-factor-dependent binding of fMet-tRNA\(^\text{Met}\). This shows that this AUG-affinity label reacted in the decoding site for fMet-tRNA\(^\text{Met}\). By combination of sodium dodecyl sulfate-, Sarkosyl-, and urea-polyacrylamide gel electrophoresis the AUG-affinity label was found to be irreversibly bound to ribosomal proteins S4, the rps gem product, and S18.

Despite recent advances in our understanding of the function of particular ribosomal proteins in protein synthesis (for a review see ref. 1), several important problems remain. Among these is the role of ribosomal proteins in decoding mRNA. This is placed in the perspective of the number of proteins necessary to initiate, propagate, and terminate translation and their function in moving along the message. One way of studying this essential aspect of the translation process is to identify the ribosomal proteins that are directly involved in this part of protein synthesis.

Recently, the technique of affinity labeling was successfully applied in several laboratories in order to elucidate the ribosomal proteins that are implicated in the peptidyltransferase center (2-4). Likewise, affinity labeling should help to answer other questions on the functions of ribosomal proteins. We have synthesized a bromoacetylated AUG analog, which irreversibly reacts at the decoding site of Escherichia coli ribosomes. After chemical linkage of this AUG analog, 30S subunits could be isolated, which were programmed for initiation-factor-dependent binding of fMet-tRNA\(^\text{Met}\). This allowed identification of ribosomal proteins associated with decoding of mRNA.

MATERIALS AND METHODS

Ribosomes of E. coli A19 were isolated and purified as described (5). They were salt-washed once with 1 M \(\text{NH}_4\text{Cl}\). About 60-70% of these ribosomes bound fMet-tRNA\(^\text{Met}\) in a puromycin-sensitive state and represented tight couples according to the nomenclature of Noll (6). fMet-tRNA\(^\text{Met}\) was prepared from purified E. coli tRNA\(^\text{Met}\) (Boehringer, Germany) according to ref. 7. It was charged with 1400-1600 pmol of \([\text{H}]\)methionine (8.2 Ci mmol; Amersham, England) per \(A_{260}\) of tRNA. The poly(U) assay was similar to the method of Nirenberg and Matthaei (8). Initiation factors IF1 and IF2 were prepared according to ref. 9.

Synthesis of 5'-\((\text{N-bromo-}[\text{\(^{14}\text{C}\}]\text{acetyl-p-aminophenyl})\phi\text{osphoryl-adenyl-(3'-5')-uridylyl-(3'-5')-guanosine}\) Nitrophenyl-pA was synthesized according to ref. 10. A 0.04 M solution of nitrophenyl-pA was then incubated together with polynucleotide phosphorylase of Micrococcus luteus (1 mg/ml), pancreatic RNase A (0.1 mg/ml) and UDP (0.05 M) for 24 hr at 37° in 3 mM MgCl\(_2\), 0.3 mM EDTA, 0.12 M Tris-HCl (pH 9.0) buffer. After treatment of the incubation mixture with alkaline phosphatase, nitrophenyl-pA-U was isolated by TEAE-cellulose column chromatography using a 0.01 M-0.2 M NaCl linear gradient. The subsequent enzymatic condensation of nitrophenyl-pA-U with GDP by polynucleotide phosphorylase in the presence of RNase T\(_1\) followed similar lines. The final nitrophenyl-pA-U-G was catalytically reduced to aminophenyl-pA-U-G and then bromoacetylated as described for the reduction and bromoacetylation of nitrophenyl-Tp (11). A detailed procedure of the synthesis of N-acetylated aminophenylphosphorylated oligonucleotides will be published elsewhere.

Affinity Labeling of Ribosomes. 70S ribosomes (7 \(\mu\)M) were incubated with a 10-fold excess of affinity label for 2 hr at 37° in 6 mM MgCl\(_2\), 80 mM \(\text{NH}_4\text{Cl}\), 50 mM Tris-HCl (pH 7.4) buffer. The reaction was stopped by 2-fold dilution with buffer containing 1.3 M 2-mercaptoethanol. Ribosomal subunits were separated on a linear 10-30% sucrose gradient in 0.3 mM MgCl\(_2\), 30 mM \(\text{NH}_4\text{Cl}\), 10 mM Tris-HCl (pH 7.4) buffer. Centrifugation was in a Spinco SW 27 rotor at 23,000 rpm for 14 hr. Fractions containing the labeled 30S subunits were collected, dialyzed against 10 mM MgCl\(_2\), 30 mM \(\text{NH}_4\text{Cl}\), 10 mM Tris-HCl (pH 7.4) buffer, and pelleted by high-speed centrifugation. The pellet was then dissolved in the above buffer to give a concentration of 250 \(A_{260}\) units of 30 S/ml. These 30S subunits were used for measuring initiation-factor-dependent fMet-tRNA\(^\text{Met}\) binding (Table 2). 23S core particles were prepared by CsCl density gradient centrifugation according to ref. 12. Extraction of proteins from 23S and 30S particles was carried out with acetic acid as described (13).

RESULTS

Fig. 1 outlines the synthesis of 5'-\((\text{N-bromo-}[\text{\(^{14}\text{C}\}]\text{acetyl-p-aminophenyl})\phi\text{osphoryl-adenyl-(3'-5')-uridylyl-(3'-5')-guanosine}\) (AUG\(^*\)). The nitrophenyl ester of 5'-adenylic acid was enzymatically condensed with UDP and subsequently with GDP. The combined use of polynucleotide phosphorylase

Abbreviations: AUG, \(^{14}\text{C}\)-labeled N-bromoacetyl-p-aminophenylphosphate-AUG; IF, initiation factor.

† Paper No. 9 on "Affinity Labeling of Ribosomes". Preceding paper: O. Pongs & R. Bald (1974), submitted for publication. Part of this paper is based on the Ph.D. dissertation of E.L.
and ribonuclease facilitated the synthesis, since it yielded only the desired oligonucleotide. The nitrophenyl ester of pA-U-G was then reduced to the aminophenyl ester of pA-U-G, which was bromoacetylated by procedures analogous to those described for nitrophenyl-dTTP (11).

Fig. 2 shows the time dependence of the AUG* binding by 70S and 30S ribosomes at 37°C. AUG* had a higher affinity for 70S than for 30S. The reaction with 70S ribosomes proceeded about four times faster, reaching a plateau after about 1.5 hr. The high affinity of AUG* for 70S ribosomes was further demonstrated by measuring the dependence of the reaction on the concentration of 70S ribosomes. Different concentrations of ribosomes were incubated with the same amount of label for 2 hr at 37°C and then analyzed for AUG* binding. As indicated by the arrow in Fig. 3, at equal molar concentrations of AUG* and 70S ribosomes, the AUG* uptake was about 80%.

The specificity of the reaction was checked in the following manner. First, the loss of poly(U)-dependent ribosomal activity was determined. Second, the labeled 70S ribosomes were analyzed on sucrose gradients. Third, the labeled subunits were isolated and their properties with respect to binding of fMet-tRNA<sub>fMet</sub> dependent on initiation factors were investigated.

70S and 30S ribosomes were incubated with AUG* for 2 hr as reported in Fig. 2. Under these conditions, about 90% of the 70S and 40% of the 30S had bound AUG*. Accordingly,

![Figure 1](image1)

**Fig. 1.** Outline of the steps in the synthesis of AUG*. **Arrow**s indicate the enzymes that are used in the reaction next to them. DCC/Py = dicyclohexylcarbodiimide in pyridine; PNPase = polynucleotide phosphorylase; AP = alkaline phosphatase.

30S and 70S particles labeled with AUG* and prepared as described in Materials and Methods are designated 30S* and 70S*. 30S (5 μM) and 70S ribosomes (6.5 μM) were incubated with a 5-10 molar excess of AUG* for 2 hr at 37°C in 5 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, 50 mM Tris·HCl (pH 7.4) buffer. Aiquots of the incubation mixtures containing 1 A<sub>260</sub> unit of 70S or 0.4 A<sub>260</sub> unit of 30S were added to a poly(U)-translation system. The 30S subunits were supplemented with 0.7 A<sub>260</sub> units of 50S. Incubation time was 7 min at 30°C. Hot 3% trichloroacetic acid was added, and the precipitable radioactivity was measured. One hundred percent AUG* bound corresponds to a 1:1 molar ratio of AUG* to ribosomes.

**Table 1.** Poly(U)-directed polyphenylalanine-synthesizing activity of E. coli ribosomes labeled with AUG*.

<table>
<thead>
<tr>
<th>Ribosomes</th>
<th>% AUG* bound</th>
<th>% Activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>30S + 50S</td>
<td>—</td>
<td>93</td>
</tr>
<tr>
<td>70S</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>30S* + 50S</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>70S*</td>
<td>90</td>
<td>25</td>
</tr>
</tbody>
</table>

30S and 70S particles labeled with AUG* and prepared as described in Materials and Methods are designated 30S* and 70S*. 30S (5 μM) and 70S ribosomes (6.5 μM) were incubated with a 5-10 molar excess of AUG* for 2 hr at 37°C in 5 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, 50 mM Tris·HCl (pH 7.4) buffer. Aiquots of the incubation mixtures containing 1 A<sub>260</sub> unit of 70S or 0.4 A<sub>260</sub> unit of 30S were added to a poly(U)-translation system. The 30S subunits were supplemented with 0.7 A<sub>260</sub> units of 50S. Incubation time was 7 min at 30°C. Hot 3% trichloroacetic acid was added, and the precipitable radioactivity was measured. One hundred percent AUG* bound corresponds to a 1:1 molar ratio of AUG* to ribosomes.

† Poly(U)-dependent activity of 1 A<sub>260</sub> unit of 70S ribosomes was determined as described (8). The control samples polymerized 60-80 mol of phenylalanine per mol of ribosomes.

![Figure 3](image2)

**Fig. 3.** Dependence of the affinity labeling with AUG* on the concentration of ribosomes. Incubations were carried out in 6 mM MgCl<sub>2</sub>, 80 mM NH<sub>4</sub>Cl, 50 mM Tris·HCl (pH 7.4) buffer for 2 hr at 37°C. Binding of AUG* was analyzed by Millipore filtration (see legend of Fig. 2). AUG* concentration was 15 μM as indicated by the arrow in Fig. 3.
factor-dependent binding of fMet-tRNA\textsubscript{Met}. The binding experiments are summarized in Table 2. It can be seen that the labeled 30S subunits possess initiator tRNA binding activity. In the absence of externally added AUG, AUG*-labeled subunits formed the initiation complex. Furthermore, fMet-tRNA\textsubscript{Met} binding required initiation factor IF2. These data indicate that AUG* had reacted in the decoding site of the ribosome, which allowed us to identify by affinity labeling ribosomal protein(s) belonging to this site.

A ribosomal protein that is chemically linked to AUG* would be expected to have different electrophoretic properties in the two-dimensional electrophoresis of Kaltschmidt and Wittmann (14). Therefore, we used a different strategy for identification of the affinity-labeled protein(s). The labeled 30S subunit was centrifuged in a CsCl density gradient. This splits the 30S subunit into a 23S core particle and split proteins (S1, S2, S3, S5, S9, S10, S14) (15).

The labeled proteins were then isolated and analyzed by a combination of polyacrylamide gel electrophoresis in urea, sodium dodecyl sulfate, and Sarkosyl. It is important to note that no significant amount of radioactivity could be detected in the 16S RNA after protein extraction. Fig. 5 shows the gel pattern of the proteins from 30S subunits and 23S core particles in the first dimension of the two-dimensional polyacrylamide gel electrophoresis carried out (14). Two radioactive bands are seen, which correspond to two basic proteins. Since electrophoresis of the 30S, 23S, and split proteins always gave the same two radioactive bands, it was likely that two basic proteins of the 23S core particle had reacted with AUG* and that the small amount of radioactivity that was found in the split protein fraction represented a contamination of the split proteins by protein(s) of the 23S core particle. We analyzed the 23S proteins and the split proteins by dodecyl sulfate gel electrophoresis in order to determine the molecular weights of the labeled proteins. Fig. 6A shows the gel pattern that was obtained for 23S core proteins. As compared to the standards of known molecular weight, the molecular weights of the two labeled proteins were determined to be 26,000 and approximately 14,000. The only protein in the 23S core particle (see also the distinct band in Fig. 6A) of molecular weight 26,000 is protein S4 (15, 16). Therefore, it is concluded that the basic protein next to the origin in Fig. 5 represents protein S4. This conclusion was confirmed by polyacrylamide–Sarkosyl gel electrophoresis (see below).

**Table 2.** Initiation-factor-dependent binding of fMet-tRNA\textsubscript{Met} to E. coli 30S subunits labeled with AUG*

<table>
<thead>
<tr>
<th>Ribosomal source in the reaction mixture</th>
<th>fMet-tRNA\textsubscript{Met} bound (cpm)</th>
</tr>
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<tbody>
<tr>
<td>30S</td>
<td>13,725</td>
</tr>
<tr>
<td>30S and 50S</td>
<td>15,068</td>
</tr>
<tr>
<td>30S minus IF2</td>
<td>751</td>
</tr>
<tr>
<td>30S*</td>
<td>6,815</td>
</tr>
<tr>
<td>30S* and 50S</td>
<td>7,408</td>
</tr>
<tr>
<td>30S* minus IF2</td>
<td>1,215</td>
</tr>
</tbody>
</table>

30S subunits labeled with AUG* and isolated as described in *Materials and Methods* are designated 30S*.

Incubation mixtures contained per 0.08 ml: 100 mM NH\textsubscript{4}Cl, 50 mM Tris-HCl (pH 7.4), 9 mM MgCl\textsubscript{2}, 1.5 mM GTP, 1.3 mM 2-mercaptoethanol, 100 pmol of fMet-tRNA\textsubscript{Met} (8.2 Ci/mmol), 20 \mu g of IF2, 2 \mu g of IF1. The reaction mixtures were supplemented either with 0.3 A\textsubscript{260} units of 30S \pm 0.6 A\textsubscript{260} units of 50S and 0.1 mM AUG or with 2 A\textsubscript{260} units of 30S*, which contained AUG*, \pm 6 A\textsubscript{260} units of 50S. After incubation for 15 min at 25 °C, the reaction mixtures were cooled to 0 °C and diluted with 1.5 ml cold incubation buffer. Filtration was carried out with Millipore filters, which were washed three times with 2 ml of buffer and then dried. Radioactivity was measured in toluene-based scintillation fluid.

**Fig. 4.** Sucrose gradient centrifugation of 70S ribosomes labeled with AUG*. 250 A\textsubscript{260} units of 70S E. coli ribosomes were layered on top of the gradient and centrifuged at 25,000 rpm for 14 hr at 4 °C in a Spinco SW 27 rotor. Fractions (1.3 ml) were collected from the bottom of the tube. Aliquots (50 \mu l) of each fraction were diluted with 0.5 ml H\textsubscript{2}O and absorbance was read at 260 nm. Radioactivity in these aliquots was monitored by mixing with 10 ml of Bray's counting solution. (●●●●) A\textsubscript{260}; (□--□) cpm.

**Fig. 5.** Radioactivity detected in ribosomal proteins labeled with AUG* (54 Ci/mol). Proteins of 30S subunits and 23S core particles were extracted, subjected to polyacrylamide gel electrophoresis in urea, and run in the first dimension of the two-dimensional electrophoresis of Kaltschmidt and Wittmann (14). The gels were sliced into 4 mm sections which were dried overnight at 60 °C and then dissolved in 0.02 ml of hydrogen peroxide. Radioactivity was measured after the addition of 1 ml of Soluene (Packard) and of 10 ml of toluene-based scintillation fluid. (A) Radioactivity in proteins extracted from 23S core particles; (B) radioactivity in proteins extracted from 30S subunits. The patterns of the gels stained with Coomassie Brilliant Blue are shown in the boxes under the histograms of the radioactive sliced gels. Radioactivity in the gel of the split protein fraction was found at the same positions as in Fig. 6.
DISCUSSION

Evidence has been presented that a bromoacetylated AUG analog preferentially and specifically reacts in the decoding site of 70S ribosomes. After the affinity labeling reaction, 30S subunits could be isolated which were programmed for binding IF2-dependent initiator tRNA, as shown by the data in Table 2. The analysis of the labeled protein material shows that two proteins that belong to the 23S core particle had reacted with AUG*. They could be unambiguously identified as proteins S4 and S18 (Figs. 5–7). Both proteins should, therefore, belong to the ribosomal mRNA binding site.

Protein S4 has very interesting properties. This protein is the ram gene product (19), which is responsible for phenotypic reversion of streptomycin dependence to independence and which is known to control translational ambiguity (20). Protein S4 binds to the 5′-end of 16S RNA (21). According to the assembly map (22), protein S12, which controls the correct initiation of bacteriophage mRNA (23), is neighboring protein S4. This is also concluded from studies on many ribosomal mutants with altered S4 proteins (24). Protein S18, on the other hand, has been implicated in aminoacyl-tRNA binding, because it could be shown that S18-specific antibody fragments interfere with elongation factor Tu-dependent poly(U)-directed Phe-tRNA binding (25).

The affinity labeling studies in this paper now directly show that proteins S4 and S18 are at or near the decoding site of the E. coli ribosome, a finding which is in good agreement with the studies just mentioned.


