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Location of protein S1 of Escherichia coli ribosomes at the 'A'-site of the codon binding site. Affinity labeling studies with a 3'-modified A-U-G analog.

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ABSTRACT.

An affinity analog with a 5-bromoacetamido uridine 5'-phosphate moiety bonded to the 3' end of A-U-G has been prepared with the aid of polynucleotide phosphorylase. This 3'-modified, chemically reactive A-U-G analog was used to probe the ribosomal codon binding site. The yield of the reaction depended strongly on the ribosomal source and was sensitive to salt-washing ribosomes. The major crosslinking product was identified to be protein S1. Since the reaction of this 3'-modified A-U-G programmed ribosomes for Met-tRNA$_M^{Met}$ binding, it is concluded that protein S1 is located at or near the 3'-side of the ribosomal codon binding site.

INTRODUCTION.

Recently, the codon binding site of Escherichia coli ribosomes has been successfully explored by using a 5'-modified bromoacetamidophenyl derivative of the initiation codon A-U-g as affinity probe (1,2,3). The results of these studies showed that proteins S4 and S18 are part of the 'A'-site and that proteins S11 and S12 are part of the 'P'-site of the ribosomal codon binding site (3). Now, we have synthesized a 3'modified A-U-G by linking 5-bromoacetamido-uridine 5'-diphosphate to A-U-G and have studied the reaction of this new A-U-G affinity probe with E. coli ribosomes. The results of these studies are reported in this paper.

MATERIAL AND METHODS.

5-aminouridine was synthesized according to (4). It was phosphorylated by the same method, which was described for phosphorylation of 3'-amino-3'-deoxyadenosine (5). Adenylyl-(3'-5')-uridylyl-(3'-5')-guanylyl-(3'-5')-5-aminouridine (A-U-G-5-N-U) was synthesized from A-U-G and 5-aminouridine 5'-diphosphate

by the use of polynucleotide phosphorylase. Incubation conditions were as those described previously for the synthesis of *A-U-G †)(2). Bromoacetylation of A-U-G-5-N-U was carried out with α,α'-bromo-[2-^{14}C]acetic anhydride (22.6 Ci/Mol) under the same conditions, which were used for bromoacetylation of *A-U-G (2). A-U-G* was isolated by paperchromatography on Whatman 3MM paper with n-butanol/H_2O/glacial acetic acid (5:3:2) as solvent system (developing time 50 h; mobility relative to 5'-uridylic acid, 0.60). The structure of A-U-G* was verified by RNase T_1 digestion (followed by treatment with alkaline phosphatase) to A-U-G and to 5-bromoacetamido uridine.

70s ribosomes of E.coli A19 mid-log cells were isolated by alumina grinding, purified and salt washed as described(6). Ribosomal subunits were separated from salt washed 70S ribosomes by centrifugation on SW27 linear 10-30% sucrose gradients in 0.3mM $MgCl_2$, 30 mM NH_4Cl, 10 mM Tris-HCl (pH 7.8) buffer containing 3 mM 2-mercaptoethanol. Crude initiation factors (IF) and E.coli f[^{3}H]Met-tRNA$^{Met}_F$ (8.2 Ci/mMol) were prepared as described (7,8). E.coli [^{3}H]Met-tRNA$^{Met}_M$ (4.6 Ci/mMol) was a generous gift of U. Petersen, Institut de Biochimie et Biophysique, Paris, France. Elongation factor Tu was kindly provided by Dr. A. Parmeggiani, Universite de Paris, France.

Typical incubation mixtures for initiation complex formation contained per 0.08 ml: 100 mM NH_4Cl, 50 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 1.5 mM GTP, 1.3 mM 2-mercaptoethanol, 10 µg IF, 70S ribosomes as indicated in Tables 1 and 2, 100 pmol fMet-tRNA$^{Met}_F$ and 50 µM A-U-G. After incubation for 15 min at 30 °C reaction mixtures were chilled, diluted with 1.5 ml cold incubation buffer and were then assayed for initiation complex formation by Millipore filtration.

Incubation mixtures for Met-tRNA$^{Met}_M$ binding contained per 0.08 ml: 160 mM NH_4Cl, 10 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 50 mM Tris-HCl(pH 7.8), 1.2 mM GTP, 5 µg EF-Tu, 27 pmol Met-tRNA$^{Met}_M$, 50 µM A-U-G and ribosomes as indicated in Table 3. Standard labeling reaction mixtures contained in 1 ml 6 µM ribosomes, 60 µM A-U-G*(22.6 Ci/Mol), 6 mM $MgCl_2$, 50 mM Tris-HCl(pH 7.4), 80 mM NH_4Cl. The mixture was incubated at 37 °C for 2 h. The reaction mixture was chilled and ribosomes were

ethanol precipitated before electrophoretic analyses of labeled proteins. 7.5% polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out according to (9) and 15% polyacrylamide gel electrophoresis in Sarkosyl according to (10). The technique of autoradioimmunodiffusion has been previously described (3). Purified ribosomal proteins S4, S13 and S18 were kindly given to us by Dr. H. G. Wittmann, Berlin, and ribosomal protein S1 by Dr. J. Van Duin, Wallenberg-Laboratory, University of Uppsala, Sweden.

RESULTS.

Recently, the synthesis of bromoacetylated U-U-U-5-N-U has been reported (11). Similarly, A-U-G-5-N-U was synthesized by use of polynucleotide phosphorylase with A-U-G as primer and 5-aminouridine 5' diphosphate. The subsequent bromoacetylation of A-U-G-5-N-U was achieved with bromoacetic anhydride using conditions, which had been employed before to bromoacetylate *A-U-G. This gave in our hands better results than a bromoacetylation with haloacetylchloride (2,11).

A-U-G* was recognized by ribosomes as well as A-U-G during initiation complex formation. The data of Table 1 demonstrates that the modification of A-U-G to A-U-G* does not effect the template properties of A-U-G for binding fMet-$tRNA_F^{Met}$ to ribosomes.

Table 1

Comparison of A-U-G with A-U-G* in Initiation

Experiment	Template	IF	fMet-$tRNA_F^{Met}$ bound (pmol)
1	A-U-G	-	1.6
2	A-U-G	+	13.6
3	A-U-G*	-	1.1
4	A-U-G*	+	12.5

Experimental conditions are described in Material and Methods. Ribosome concentration was 0.3 µM (22 pmol/assay). Concentrations of A-U-G and A-U-G* were 50 µM.

The kinetics of the reaction of A-U-G* were very similar to that of *A-U-G with 70S ribosomes (2), and, therefore, are

not shown. At a 10:1 ratio of label to 70S ribosome, the reaction reached a plateau within 2 h at 37 °C and yielded 0.20 mol A-U-G* bound per mol ribosome. The affinity of A-U-G* to 70S ribosomes, however, was very low as determined by the dependence of A-U-G* binding on ribosome concentration (Fig. 1). At equimolar concentrations of A-U-G* and of salt-washed

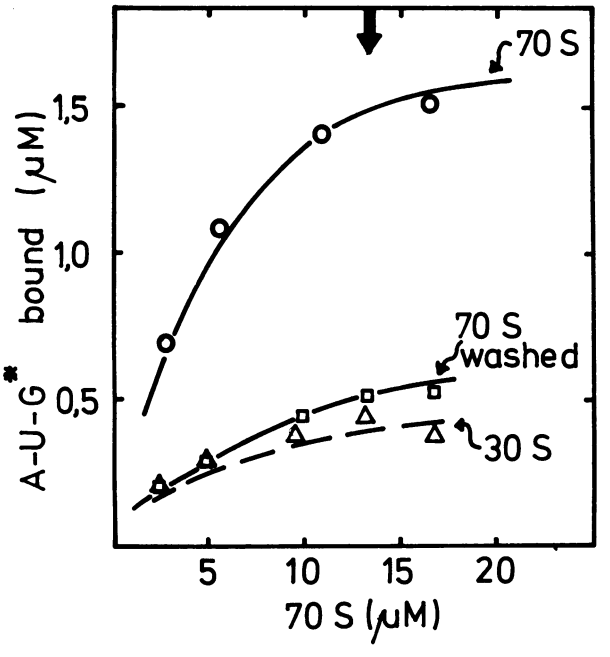

Fig. 1. Dependence of the reaction of A-U-G* with ribosomes on ribosome concentration. Incubations were carried out for 2 h at 37°C as described in Material and Methods. Binding of A-U-G* was analyzed by Millipore filtration (3). A-U-G* concentration was 14 μM as indicated by the arrow.

70S ribosomes or of 30S subunits, only 0.04 mol A-U-G* bound per mol ribosome. Ribosomes, which had not been washed, showed a threefold higher affinity, i.e. about 0.12 mol A-U-G* bound per mol ribosome. This indicated that the major target of the A-U-G* labeling reaction was only loosely associated with ribosomes and dissociated easily off the ribosome. This is also demonstrated by the sucrose gradient analysis of the reaction of A-U-G* with ribosomes, shown in Fig. 2.

After having reacted 70S ribosomes with A-U-G*, the incubation mixture was immediately layered on top of a linear 10-30% sucrose gradient in low Mg^{2+}. Ribosomal subunits were then separated by centrifugation and isolated. As can be seen from the data in Fig.2, only small amounts of A-U-G* label

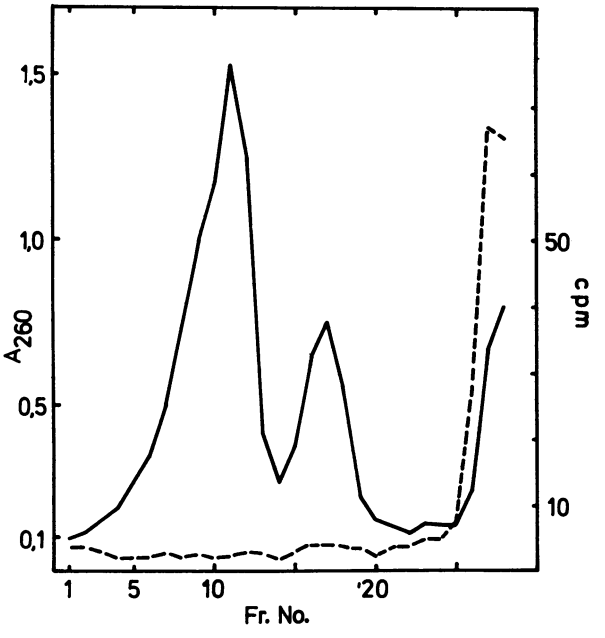

Fig. 2. Sucrose gradient centrifugation of 70S ribosomes labeled with A-U-G*. 250 A_{260} units of 70S E. coli ribosomes were layered on top of a linear 10-30% sucrose gradient in 0.3 mM $MgCl_2$, 30 mM NH_4CL, 10 mM Tris-HCl (ph 7.8) buffer, containing 3 mM 2-mercaptoethanol, and centrifuged at 23,000 rpm for 14 h at 4°C in a Spinco SW27 rotor. 1.3 ml fractions were collected from the bottom of the tube. Aliquots of each fraction (50 µl) were diluted with 0.5 ml H_2O. Absorbance was read at 260 nm. Radioactivity in these aliquots was monitored after adding 10 ml of Bray's scintillation cocktail. (-) A_{260}; (---) [^{14}C]-cts/min.

cosedimented with the 30S subunit. The majority of label stayed at the top of the gradient. Since A-U-G* labeled protein had probably been split off the subunits during sucrose gradient centrifugation, the radioactivity containing fractions at the top of the gradient, were collected, exhaustively dialyzed against 2% acetic acid and then lyophilized.

The lyophilized protein material was investigated by

electrophoresis on 7.5% polyacrylamid-sodium dodecyl sulfate gel slabs as shown in Fig.3. The gel pattern indicates that a

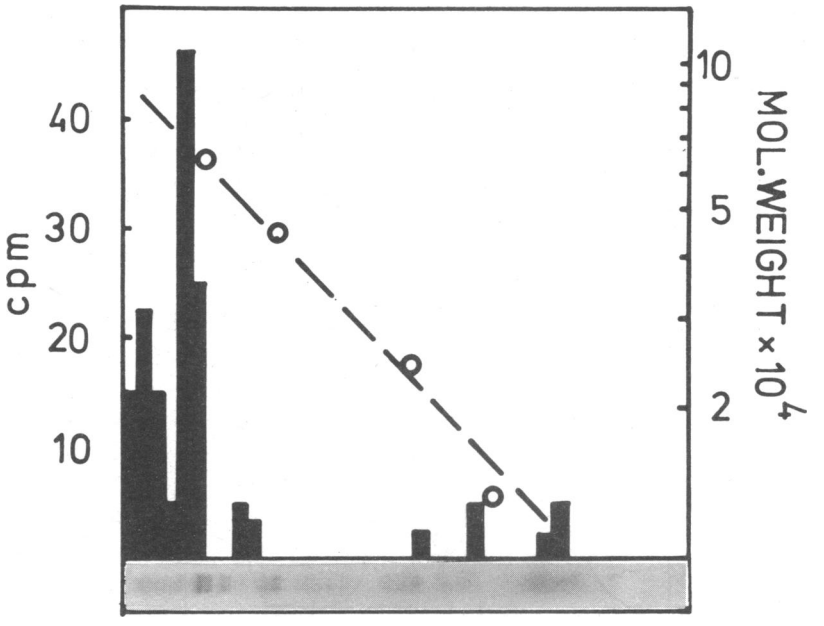

Fig. 3. Semilog plot of molecular weight versus relative migration distance for A-U-G* labeled proteins isolated from the top of the sucrose gradient shown in Fig. 2. The 7.5% polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out as described (9). Standard proteins were (--o--) ribonuclease A (mol. weight 14,000), chymotrypsinogen (27,000), ovalbumin (42,000), bovine serum albumin (67,000). Gels were sliced and analyzed for radioactivity as described (1). The staining pattern of the sample gel is shown in the box below the radioactivity contained in gel slices.

number of proteins came off during sucrose gradient centrifugation at low Mg^{2+}. The stained gel was sliced and analyzed for radioactivity of label. The results of this analysis are represented by the bars on top of the staining pattern in Fig.3. The data indicates that there is one major band, which contains A-U-G* labeled protein. Its apparent molecular weight of 70,000 daltons corresponds to that of proteinS1(12).

Previously, we have been able to characterize *A-U-G labeled ribosomal proteins by polyacrylamide gel electrophoresis in Sarkosyl (1). Accordingly, A-U-G* labeled 30S sub-

units were isolated from the sucrose gradient shown in Fig.2 and 30S ribosomal proteins were separated on 15% polyacrylamid-Sarkosyl gel slabs (Fig.4). The major amount of radio-

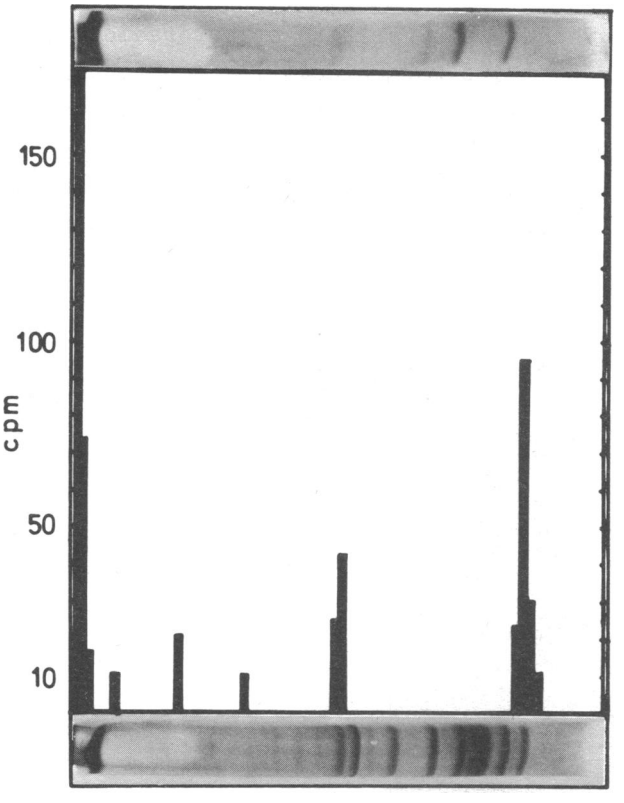

Fig. 4. Separation of A-U-G* labeled 30S ribosomal proteins on polyacrylamide-Sarkosyl gels (3,10). Reference proteins (box on top of Fig. 4) were, in the order of migration from left to right, proteins S4, S11, S13 and S18. Gels were sliced and analyzed for radioactivity as described (3).

activity of label stayed on top of the gel. It tentatively belongs to A-U-G* labeled protein S1, which would not enter the Sarkosyl-gel (10). Two protein bands of the gel contained some radioactivity, which was about 10 to 20 times less than the amounts found previously with*A-U-G labeled proteins (3). As indicated in Fig.4, one band corresponds to protein S4, which is a single band on polyacrylamide-Sarkosyl gels (3,10) and which was electrophoresed as reference in paralell. The

other band tentatively corresponds to protein S18.

The identification of the A-U-G* labeled proteins as protein S1, which was the major target of the labeling reaction, and protein S18 according to the gel electrophoretic data of Fig.3 and 4 was verified by autoradioimmunodiffusion (3). Labeled proteins were placed for double diffusion into the center well of Ouchterlony plates and enriched antisera, specific against individual proteins, were placed into the peripheral wells. The Ouchterlony plates were autoradiographed after immunodiffusion in order to detect radioactivity of label in the corresponding precipitine bands. The majority of A-U-G* label was again detected in protein S1 (data are not shown). Some label was detected in the immunoprecipitate of protein S18, which can be seen as a faint band in Fig.5. The

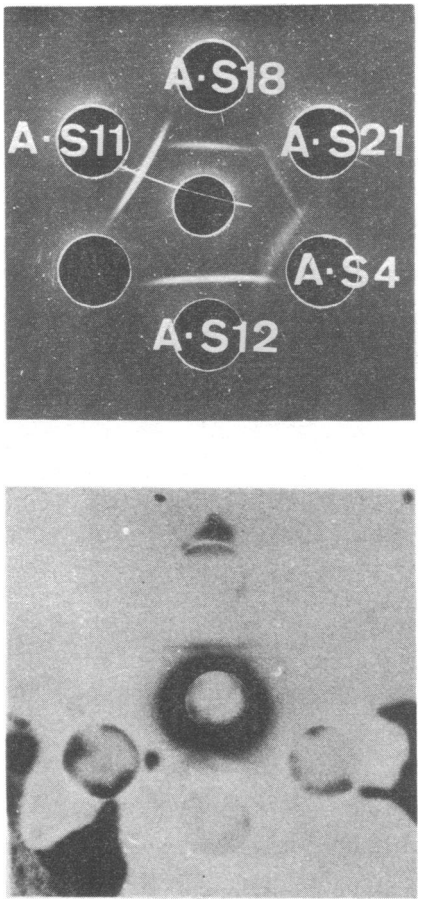

Fig. 5. Radioimmunodiffusion tests of 70 S ribosomal proteins, isolated after a reaction of A-U-G* with 70 S ribosomes. The center well contained 180 μg of the labeled 70 S proteins; the peripheral wells contained the following anti-single protein sera as indicated: 100 μl two times enriched anti-S18, 100 μl two times enriched anti-S21, 100 μl anti-S4, 75 μl anti-S12 and 100 μl anti-S11. Ouchterlony double diffusion tests (top) and auto-radiography (bottom) were carried out as previously described (3).

immunoprecipitate of protein S4 did not contain detectable radioactivity.

A-U-G* reacted ribosomes should be programmed either for binding fMet-tRNA$^{Met}_{F}$ or for binding Met-tRNA$^{Met}_{M}$, if A-U-G* had labeled a protein at the ribosomal codon binding site. The data of Table 2 (experiment 2 versus experiment 5) show that A-U-G* reacted ribosomes bind fMet-tRNA$^{Met}_{F}$ in the presence of initiation factors only to a small extent. This bound fMet-tRNA$^{Met}_{F}$, however, was not puromycin reactive (data are not shown). More interesting are the results of experiment 6 of Table 2. If A-U-G was added to the incubation mixtures with labeled ribosomes, it could not stimulate fMet-tRNA$^{Met}_{F}$ binding as much as in the control experiment 3. A comparison of the data indicates that about 25% of the labeled ribosomes were not active. This is similar to the amount of ribosomes (20%), to which A-U-G* was crosslinked.

Table 2

Initiation complex formation with 70S ribosomes, which contain A-U-G* covalently bound

Experiment	Ribosomes	A-U-G	IF	fMet-tRNA$^{Met}_{F}$ bound (pmol)
1	70S	-	-	1.1
2	70S	-	+	2.5
3	70S	+	+	13.5
4	70S-A-U-G*	-	-	0.8
5	70S-A-U-G*	-	+	3.1
6	70S-A-U-G*	+	+	10.0

Initiation complex formation was assayed as described in Material and Methods except that in experiments 4-6 70S ribosomes (22 pmol) were replaced by A-U-G* labeled ribosomes (22 pmol), which contained 4.5 pmol A-U-G* covalently bound. Isolation of labeled ribosomes, free of non-covalently bound A-U-G*, was carried out as described (3).

The stimulation of Met-tRNA$^{Met}_{M}$ binding to labeled ribosomes by EF-Tu is summarized in Table 3. In the absence of A-U-G, control ribosomes exhibited very little Met-tRNA$^{Met}_{M}$

binding activity. This is stimulated ninefold by addition of A-U-G to the incubation mixture. Labeled ribosomes also bound Met-tRNA$_M^{Met}$. This binding was stimulated about twofold as shown by the results of experiments 4 and 5. If one takes into consideration that in experiment 4 only 20% of the ribosomes carried crosslinked A-U-G*, then the amounts of Met-tRNA$_M^{Met}$, which bound to control ribosomes or to A-U-G*-labeled ribosomes, are compatible. This is further demonstrated by experiment 5, where the amount of labeled ribosomes was increased and accordingly, the amount of bound Met-tRNA$_M^{Met}$. Therefore, it is concluded from the data of Table 2 and 3 that A-U-G* reacted in the 'A'-site of the ribosomal codon binding site.

Table 3

Met-tRNA$_M^{Met}$ binding to A-U-G* labeled 70S ribosomes

Experiment	Ribosomes	A-U-G	EF-Tu	Met-tRNA$_M^{Met}$ bound (pmol)
1	70S	-	+	1.2
2	70S	+	+	10.1
3	70S-A-U-G*	-	-	0.9
4	70S-A-U-G*	-	+	2.0
5	70S-A-U-G*	-	+	4.0

Met-tRNA$_M^{Met}$ binding was assayed as described in Material and Methods. Ribosome concentration was 0.3 µM (22 pmol/0.08 ml). In experiments 3 and 4 22 pmol ribosomes contained covalently bound 4.5 pmol A-U-G* and in experiment 5 44 pmol 70S ribosomes contained 9.0 pmol A-U-G*.

DISCUSSION.

Reactions of *A-U-G with 70S ribosomes had yields between 0.75 and 1.10 mol *A-U-G crosslinked/mol salt washed ribosome (3). This contrasts to the low yields of the labeling reactions with A-U-G*, which were in the order of 0.1 mol crosslinked/mol salt washed ribosome. This low reactivity is apparently due to the fact that protein S1, which was found to be the major crosslinking product, is a fractional ribosomal protein and is not present on each ribosome (13,14). Since protein

S1 readily dissociates off the ribosome, the affinity of A-U-G* to ribosomes was decreased by washing ribosomes with 1M NH_4Cl. It is important to note that in the absence of ribosomes isolated protein S1 did not react with A-U-G* under the conditions of the labeling reactions.

5'-modified *A-U-G preferentially reacted in the 'A'-site of the ribosomal codon binding site with protein S18 (3), whereas 3'-modified A-U-G* reacted with protein S1. Photochemical crosslinking of poly(U) to ribosomes resulted in the attachment of poly(U) to proteins S1, S18 and S21 (15). Proteins S18 and S21 are close neighbours in the ribosome (16); proteins S21 and S1, on the other hand, bind both to the 3'-end of 16S RNA (17). These data combined then suggest that protein S18 is located close to the 5'-side of the 'A'-site of the codon binding site; next to protein S18 would be protein S21 and protein S1 would be located at the 3'-side of the 'A'-site. However, it is difficult to reconcile this observation with the known function of protein S1 in binding mRNA. Protein S1 is involved in the recognition of mRNA, which is prerequisit to initiation of protein synthesis in the 'P'-site of the ribosome (18).

The results of the experiments, which are reported in Tables 2 and 3, show another interesting feature. The reaction of A-U-G* in the 'A'-site of the ribosomal codon binding site inhibits A-U-G stimulated fMet-tRNA$^{Met}_F$ binding. Similar data had previously been obtained with *A-U-G labeled ribosomes (1,3), but at that point, it was not clear, whether the inhibition of additional A-U-G binding was due to the structure of *A-U-G or not. This possibility can now be excluded, since A-U-G* is modified at the 3'-side, which should not be in contact with the 'P'-site. This suggests that a reaction of label in one codon binding site inhibits the function of the other.

†) Abbreviations: *A-U-G = 5'-{4(bromoacetamido)phenylphospho}adenylyl-(3'-5')-uridylyl-(3'-5')-guanosine; A-U-G* = adenylyl-(3'-5')-uridylyl-(3'-5')-guanylyl-(3'-5')-5-bromo-[2-^{14}C]acetamido-uridine.

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REFERENCES.

1. Pongs, O. & Lanka, E.(1975) Proc.Nat.Acad.Sci.USA 72, 1505-1509.
2. Pongs, O. & Lanka, E. (1975) Hoppe Seyler's Z. Physiol. Chem. 356, 449-458.
3. Pongs, O., Stoffler, G. & Lanka, E. (1975) J.Mol.Biol. 99, 301-315.
4. Friedland, M. & Visser, D.W. (1961) Biochim.Biophys.Acta 51, 148-152.
5. Kula, M.R. (1974) Tetrahedron Lett. 1, 23-24.
6. Erdmann, V.A., Fahnestock, S., Higo, K. & Nomura, M. (1971) Proc.Nat.Acad.Sci.USA 68, 2932-2936.
7. Dubnoff, J.S. & Maitra, U. (1971) Meth.Enzymol. 20, 248-261.
8. Hershey, J.W.B. & Thach, R.E. (1967) Proc.Nat.Acad.Sci.USA 57, 759-763.
9. Studier, F.W. (1973) J.Mol.Biol. 79, 237-248.
10. Morgan, J. & Brimacombe, R. (1973) Eur.J.Biochem. 37, 472-480.
11. Luhrmann, R., Schwarz, V. & Gassen, H.G. (1973) FEBS Lett. 32, 55-59.
12. Dzionara, M., Kaltschmidt, E. & Wittmann, H.G. (1970) Proc.Nat.Acad.Sci.USA 67, 1909-1914.
13. Weber, J. (1972) Mol.Gen.Genet. 119, 233-237.
14. Duin, van J. & Knippenberg, van P. (1974) J.Mol.Biol. 84, 185-197.
15. Fiser, I., Margaritella, P. & Kuechler, E. (1975) FEBS Lett. 52, 281-283.
16. Lutter, L.C., Zeichhardt, H., Kurland, C.G. & Stoffler, G. (1972) Mol.Gen.Genet. 119, 357-362.
17. Czernilofsky, A.P., Kurland, C.G. & Stoffler, G. (1975) FEBS Lett. 58, 281-284.
18. Dahlberg, A.E. & Dahlberg, J.E. (1975) Proc.Nat.Acad.Sci.USA 72, 2941-2944.