Direct cross-linking of heptauridilate to *E. coli* ribosomes by water-soluble carbodiimide in the complex stabilized by codon-anticodon interaction at both A- and P-sites

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Affinity labelling of *E coli* ribosomes is performed by treatment with water-soluble carbodiimide of the complex of ribosomes with $(pU)_7$, $tRNA^{Phe}$ at the P-site and with Phe-$tRNA^{Phe}$ (complex I) and without Phe-$tRNA^{Phe}$ (complex II) at the A-site. The extent of modification is, respectively, 0 06 and 0 026 mol $(pU)_7$ per mol ribosomes Protein S3 is found as a single labelled protein in complex I, whereas S7, S8, L25 are modified in complex II Thus, in the absence of a large spacer group within the complex stabilized by codon-anticodon interactions at both A- and P-sites, a highly selective modification occurs

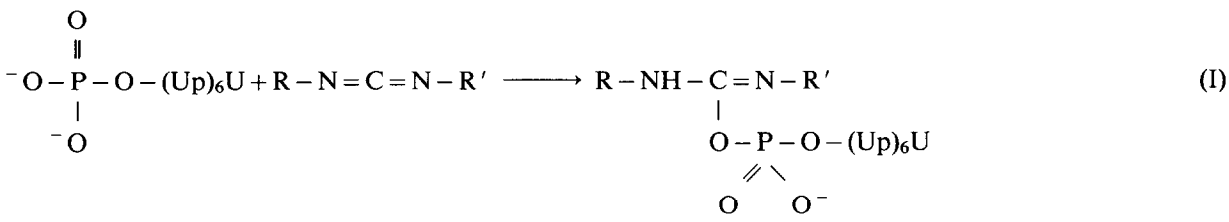
mRNA analog *Affinity labeling* *Ribosome* *Soluble carbodiimide* *Ribosomal protein* *mRNA binding area*

1. INTRODUCTION

The functional topography of ribosomes is being intensively studied by affinity labelling. To elucidate the mRNA binding area, reactive oligouridilate derivatives of different length were used. The reagents bearing an alkylating *p*-*N*-2-chloroethyl-*N*-methylaminophenyl moiety attached to either the 3′- or 5′-end [1,2], photoreactive arylazido derivatives [3], oligouridilate with a 5′-terminal triphosphate group activatable with *N*-cyclohexyl-*N*′-*β*-(4-methylmorpholinium)ethylcarbodiimide (CMEC) [4] were prepared and studied as affinity labels. In each case a set of ribosomal proteins was found to be labelled with alkylating derivatives, moreover, the modification of rRNA usually predominated. The multiple modification impeded referal of definite proteins and rRNA domains to the mRNA binding area. Multiple modification of ribosomes may be explained by two reasons: (i) the use of reagents with rather large spacer, separating the recognized moiety of mRNA analog and the reactive group; (ii) the strong dependence of the modification results on the functional state of ribosomes. In previous experiments, excess $tRNA^{Phe}$ was used to saturate both A- and P-sites of ribosomes. Recent studies have shown that *E. coli* ribosomes in the presence of poly(U) have 3 sites for deacylated $tRNA^{Phe}$ binding, i.e., P-, E-, A-sites [5,6] (respectively association constants equal to 2.2×10^{11}, 3×10^{7}, 1×10^{6} M^{-1} at 10 mM Mg^{2+} and 0°C [6]). It can be seen that A-site binding is the weakest. With short oligonucleotides the association constants for tRNA binding are even lower [7,8] Therefore, it seems highly probable that all previous experiments were performed in the absence of $tRNA^{Phe}$ at the A-site.

Here we have investigated affinity labelling of *E. coli* ribosomes with oligouridilate $(pU)_7$ in the complex obtained in the presence of $tRNA^{Phe}$ to saturate the P-site and treated with the ternary complex EF-Tu · GTP · Phe-$tRNA^{Phe}$. To activate $(pU)_7$, CMEC was added which is believed to form the highly reactive *O*-phosphorylisourea derivative I [9]:

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(I)

This addition resulted in the selective modification of protein S3 of the small ribosomal subunit.

2. MATERIALS AND METHODS

N'-Cyclohexyl-*N'*-β-(4-methylmorpholinium) ethylcarbodiimide *p*-toluenesulfonate was produced at a pilot plant of the Institute of Organic Chemistry, Novosibirsk. Polynucleotide kinase and *E. coli* $tRNA^{Phe}$ (1420 pmol/A_{260}) were from Boehringer Mannheim, RNase A from Sankyo (Japan), [^{14}C]phenylalanine (360 Ci/mol) from UVVVR (Czechoslovakia), NaB[^{3}H]H_4 (2 9 Ci/mol) and [γ-^{32}P]ATP (> 1000 Ci/mmol) from VO Isotope (USSR), 70 S ribosomes with 85% activity in the poly(U)dependent binding of [^{14}C]Phe-$tRNA^{Phe}$ were obtained as in [10], and complex EF-Tu EF-Ts from VO Soyuzreaktiv (USSR). Ternary complex EF-Tu Phe-$tRNA^{Phe}$ was prepared as described in [11]. Buffer A (pH 6.0) contained 50 mM NH_4Cl, 50 mM sodium cacodylate, 10 mM $MgCl_2$. Ribosome and tRNA preparations were stored in liquid nitrogen and activated in buffer A at 37°C for 5–10 min prior to use. Radioactivity of the probes was counted in a Mark III (Nuclear Chicago, USA). The probes containing ^{32}P radioactivity were counted by the Cerenkov method.

3. RESULTS

In the main experiment 1.5 nmol of 70 S ribosomes were incubated with 14 nmol [^{32}P](pU)$_7$ and 1 8 nmol $tRNA^{Phe}$ in 1 4 ml buffer A at 37°C for 15 min. Then, ternary complex EF-Tu GTP Phe-$tRNA^{Phe}$ was added at 0°C and incubation allowed to proceed for 15 min This resulted in the formation of the complex ribosome·(pU)$_7$ $tRNA^{Phe}$ (P)·Phe-$tRNA^{Phe}$(A), hereinafter referred to as complex I. In a parallel experiment, a similar complex was prepared using unlabelled (pU)$_7$ and [^{14}C]Phe-$tRNA^{Phe}$. This complex contained 0 5 mol Phe-$tRNA^{Phe}$ per mol ribosomes

In the control experiment, a similar complex (referred to as complex II) was prepared using Phe-$tRNA^{Phe}$ in a free state instead of in a ternary complex with EF-Tu and GTP. In this case, Phe-$tRNA^{Phe}$ binding did not exceed 0.1 mol per mol ribosomes. Therefore, complex II is further considered as a complex lacking Phe-tRNA at the A-site

Phosphate activation was carried out by treatment with 5 mM CMEC at pH 6 and 20°C for 10 min After this treatment, the subunits were separated by ultracentrifugation in a sucrose gradient (10–30%) in 0.5 mM Mg^{2+}. The distribution label is presented in table 1 In a separate experi-

Table 1

Modification extent of ribosomal subunits with [^{32}P](pU)$_7$ in the presence of CMEC (spec act of reagent 500 cpm/pmol)

Complex	U(pU)$_6$	Modification extent			
		30 S		50 S	
		cpm/100 pmol	mol/mol 30 S	cpm/100 pmol	mol/mol 50 S
I	–	2400	0 05	480	0 01
I	+	0	0 00	480	0 01
II	–	860	0 018	380	0 008

ment the same procedure was carried out with complex I using a 50-fold excess of $(Up)_6U$ lacking the 5′-terminal phosphate.

It can be seen that both in the case of complex I and complex II, ribosomes are modified and labelling of the 30 S subunit significantly predominates. The covalent attachment of oligouridilate is nearly 3-times greater in the case of complex I. Labelling of the 30 S subunit is nearly completely suppressed by competition with $U(pU)_6$. Labelling of the 50 S subunit is not changed in the presence of the same competitor. Thus, only modification of the 30 S subunit may be considered as affinity labelling. Only proteins were found to be modified with heptauridilate.

Proteins from modified ribosomes were subjected to DEAE-cellulose chromatography [1]. This procedure separates proteins containing the negatively charged uridilate moiety. Unmodified acid proteins S1, S2, S6 and L4, L9, L7/L12, L10 and L21 are present in the same fraction and, therefore, the level of modification of these proteins cannot be estimated in the subsequent treatment. The proteins separated by this procedure were treated with RNase A and alkaline phosphomonoesterase to cleave the oligonucleotide moiety and additionally labelled by reductive methylation with formaldehyde and $NaB[^3H]H_4$ [1]. ^3H-labelled proteins were analyzed by two-dimensional gel electrophoresis [1]. The data are presented in fig.1. The results strongly differ for two complexes. In the case of complex II, 3 proteins are found to be labelled to a comparable extent, namely S7, S8 and L25 In the case of complex I, only protein S3 is modified to a significant extent. In the control experiment performed with complex I in the presence of both $[^{32}P](pU)_7$ and $U(pU)_6$, the radioactivity of the proteins was at the background level.

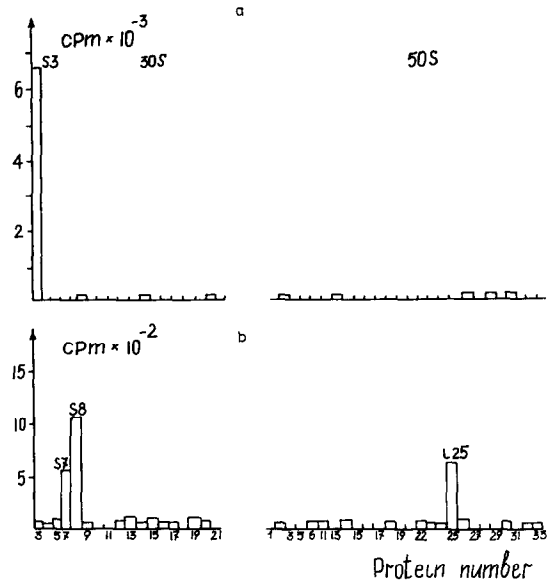


Fig.1 Distribution of the ^3H label among ribosomal proteins modified by $[^{32}P](pU)_7$ in the presence of CMEC in complexes I (a) and II (b)

4. DISCUSSION

It is seen that the treatment of the complex of $(pU)_7$ with ribosomes containing either $tRNA^{Phe}$ at the P-site or $Phe\text{-}tRNA^{Phe}$ at the A-site and $tRNA^{Phe}$ at the P-site (complexes II and I, respectively) results in the covalent attachment of oligouridilate to ribosomes It should be emphasized that no cross-linking between proteins and nucleic acids was observed in the presence of $(Up)_6U$. This means that in the conditions used there is no reaction of proteins with tRNA and rRNAs This fact does agree with the data indicating that the minimal amount of crosslinks rRNA-protein (less than 3% of the total amount of the ribosomal protein) is formed when *E. coli* ribosomes have been treated with 1-ethyl-3-dimethylaminopropylcarbodiimide under the same conditions [12]. The extent of modification is rather low. This seems quite reasonable due to the extreme lability of the hypothetical intermediate I in aqueous medium. Location of $Phe\text{-}tRNA^{Phe}$ at the A site increases the level of modification nearly 3-fold. This means that the contact of 5′-phosphate with the ribosome is significantly closer in this case. Although both subunits are found to be modified, only 30 S ribosomes may be considered as being affinity labelled, as revealed by the competition experiments.

The modification patterns differ drastically for the two types of complexes under investigation Only protein S3 is found to be modified within complex I with occupied A- and P-sites When $Phe\text{-}tRNA^{Phe}$ is absent at the A-site, labelling of S3 disappears and 3 proteins S7, S8 and L25 predominate among labelled proteins. This means that the presence of aminoacyl-tRNA at the A-site is of extreme significance for the location of an mRNA

analog in a complex

Recently, systematic investigations of the mRNA binding area of *E. coli* ribosomes were carried out using alkylating derivatives of oligouridilate of different length bearing reactive groups attached to either the 3′- or 5′-terminal nucleotide residue The proteins found to be labelled are presented in table 2. In most cases a sufficiently large number of proteins are modified. Dramatic changes are sometimes observed with a simple increase in the oligonucleotide moiety by one uridilate residue. The number of labelled proteins decreases on average with decrease in the length of the oligonucleotide part of the reagent and with decrease in the spacer dimension (cf. data for $ClRCH_2NH(pU)_7$, $pp(pU)_7$ and $(pU)_7$).

As table 2 shows, protein S3 has yet to be found among the proteins labelled with oligo(U) derivatives bearing the reactive group at the 3′-end In contrast, half of the results obtained with oligo(U) derivatives containing the reactive group at the 5′-end show that S3 is present among the labelled proteins.

The participation of S3 in the formation of the mRNA binding area is in agreement with the results obtained by other methods [13,14].

It is worth mentioning that, for eukaryotic ribosomes, the labelling of a single protein with similar oligo(U) derivatives was found to take place without the use of ternary complex EF-Tu·GTP Phe-tRNA^{Phe} to locate Phe-tRNA^{Phe} at the A-site. Only protein S26 was found among the modified proteins of rat liver ribosomes when $ClRCH_2NH(pU)_7$ was used [15]. With $(pU)_8>CHRCl$ a single labelled protein was identified as S3/S3a [16].

ACKNOWLEDGEMENTS

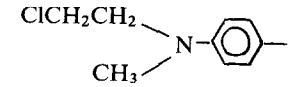
We are very grateful to Dr A G. Venyaminova for heptauridilate as well as to Dr V N Ankilova for phenylalanyl-tRNA-synthetase

Table 2

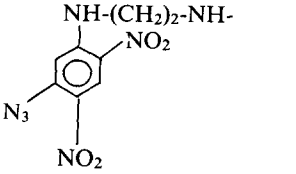
Survey of the ribosomal proteins found to be modified with oligo(U) derivatives

Reagent	Modified proteins	Reference
$ClRCH_2NH(pU)_4$	S3, S4, L7/L12	[2]
$ClRCH_2NH(pU)_5$	S13, S14, S19, L7/L12,L13,L32	[2]
$ClRCH_2NH(pU)_6$	S3, S9, S11, S13, L23, L25, L32	[2]
$ClRCH_2NH(pU)_7$	S5, S11, S13, L2, L7/L12, L31	[1]
$(pU)_5>CHRCl$	S5, S9, S14	[1]
$(pU)_6>CHRCl$	S4, S7, S9, S15, S18, S21, L1, L7/L12, L19	[1]
$(pU)_7>CHRCl$	S9, S18	[1]
$(pU)_8>CHRCl$	S4, S7, S9, S13, S15, S18, S21, L6, L19, L32	[1]
$N_3X(pU)_4$	S3, S11, S14, L2, L32	[3]
$N_3X(pU)_7$	S3, S12, S17, L2, L32, L33	[3]
$N_3X(pU)_8$	S4, S9, S19, S20, L13, L16, L27	[3]
$pp(pU)_7$ (in the presence of CMEC)	S4, S5, S9, L2	[4]
$(pU)_7$ (complex II + CMEC)	S7, S8, L25	This paper
$(pU)_7$ (complex I + CMEC)	S3	This paper

ClR —



N_3X —



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