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Affinity labelling of *Escherichia coli* ribosomes with a benzylidene derivative of $AUGU_6$ within initiation and pretranslocational complexes

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Affinity labelling of *E. coli* ribosomes with the 2′,3′-*O*-[4-(*N*-2-chloroethyl)-*N*-methylamino]benzylidene derivative of $AUGU_6$ was studied within the initiation complex (complex I) obtained by using fMet-$tRNA_f^{Met}$ and initiation factors and within the pretranslocational complex (complex II) obtained by treatment of complex I with the ternary complex Phe-$tRNA^{Phe}$·GTP·EF-Tu. Both proteins and rRNA of 30 S as well as 50 S subunits were found to be labelled. Sets of proteins labelled within complexes I and II differ considerably. Within complex II, proteins S13 and L10 were labelled preferentially. On the other hand, within complex I, multiple modification is observed (proteins S4, S12, S13, S14, S15, S18, S19, S20/L26 were found to be alkylated) despite the single fixation of a template in the ribosome by interaction of the AUG codon with fMet-$tRNA_f^{Met}$.

Affinity labeling *Ribosome* *Ribosomal protein* *mRNA-binding site* *Initiation complex* *Oligonucleotide derivative* *Pretranslocational complex*

1. INTRODUCTION

Previously, investigation of the structural organization of the mRNA-binding center of *E. coli* ribosomes was performed by using oligouridilate derivatives bearing different reactive groups attached to either the 3′- or 5′-end of the oligonucleotide moiety [1–5]. In first investigations, deacylated $tRNA^{Phe}$ was used and it is clear now that it occupied preferentially the P-site [6,7]. Multiple modification of the ribosomes within the complexes with mRNA analogs and $tRNA^{Phe}$ was observed in [1–4]. Direct crosslinking between heptauridilate and *E. coli* ribosomes caused by a water-soluble carbodiimide within a complex with a vacant A-site and a complex stabilized by codon-anticodon interaction at both the A- and P-sites has been studied [5]. It was shown that one of the causes of the multiple modification is the lack of codon-anticodon interaction at the A-site. Highly selective modification was observed within the complexes stabilized by codon-anticodon interaction at both A- and P-sites [5]. The other cause of the multiple modification is probably concerned with the multiplicity of location of the oligouridilate derivative in the ribosomal decoding site. For example, for oligouridilate derivatives containing more than 3 nucleotide residues with codon-anticodon interaction at the P-site only, as well as for derivatives containing more than 6 nucleotide residues with codon-anticodon interaction at both A- and P-sites, one can propose the formation of several types of complexes differing in the location of a reactive group towards the ribosome. Recently, we have proposed the use of benzylidene derivatives of oligoribonucleotides containing initiation codon AUG at their 5′-end for studying ribosomal components interacting

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with a template at different steps of translation [8].

Here, we have investigated affinity labelling of *E. coli* ribosomes with the 2′,3′-*O*-[4-(*N*-2-chloroethyl)-*N*-methylamino]benzylidene derivative of $AUGU_6$ both within the ribosomal initiation complex obtained by use of fMet-$tRNA_f^{Met}$ and initiation factors and within the pretranslocational complex obtained by treatment of the initiation complex with the ternary complex Phe-$tRNA^{Phe}$·GTP·EF-Tu to occupy the A-site. Selective labelling of proteins S13 and L10 was observed within the pretranslocational complex. On the other hand, within the initiation complex with a vacant A-site, despite the unequivocal fixation of mRNA analog in the ribosome, a rather large set of modified proteins was found.

2. MATERIALS AND METHODS

$tRNA_f^{Met}$ and $tRNA^{Phe}$ were purchased from Boehringer Mannheim; unfractionated tRNA from *E. coli* and elongation factors EF-Tu·Ts from Soyuzreactive (USSR). tRNA enriched up to 30% in $tRNA_f^{Met}$ was isolated according to [9]. [^{3}H]Methionine (spec. act. 15 Ci/mmol) was from Amersham; [^{14}C]phenylalanine from UVVVR (Czechoslovakia); fMet-$tRNA_f^{Met}$ (1200 pmol/A_{260} unit) and Phe-$tRNA^{Phe}$ (1500 pmol/A_{260} unit) were prepared as in [8]. Ribosomal subunits were isolated from *E. coli* MRE-600 as in [10]. Ribosomes obtained by reassociation of 30 S and 50 S subunits had 100% activity in poly(U)-dependent binding of Phe-$tRNA^{Phe}$. Initiation factors IF1, IF2 and IF3, ribooligonucleotide $AUGU_6$ and its 2′,3′-*O*-[4-(*N*-2-chloroethyl)-*N*-methylamino]benzylidene derivative ($AUGU_6$CHRCl) with specific activity 25 Ci/mol were prepared as in [8]. The ribosomal initiation complex 70 S ribosome·fMet-$tRNA_f^{Met}$·$AUGU_6$CHRCl (or $AUGU_6$) was obtained as in [8] and the ternary complex EF-Tu·GTP·Phe-$tRNA^{Phe}$ according to [11]. Formation of fMetPhe after treatment of the initiation complex fMet-tRNA·70 S ribosome·$AUGU_6$CHRCl with the ternary complex EF-Tu·GTP·Phe-$tRNA^{Phe}$ was tested according to [12]. Buffer A (pH 7.4) contained 50 mM Tris-HCl, 50 mM NH_4Cl, 5 mM $MgCl_2$, 1 mM dithiothreitol; buffer B (pH 7.5) comprised 50 mM Tris-HCl, 100 mM NH_4Cl, 0.5 mM $MgCl_2$.

3. RESULTS

To obtain the initiation complex mixture of 16.2 nmol of 30 S subunits, 15.8 nmol of 50 S subunits, 160 nmol of [^{14}C]$AUGU_6$CHRCl, 20 nmol fMet-$tRNA_f^{Met}$, 140 μg IF1, 220 μg IF2, 180 μg IF3 and 2 μmol GTP were incubated in 10 ml buffer A at 37°C for 10 min. This resulted in the formation of the complex ribosome·[^{14}C]$AUGU_6$CHRCl·fMet-$tRNA_f^{Met}$ (complex I). To evaluate the level of binding of fMet-$tRNA_f^{Met}$ with ribosomes in a separate experiment complex I was obtained using [^{3}H]fMet-$tRNA_f^{Met}$. It was found that 0.33 mol fMet-$tRNA_f^{Met}$ was bound per mol 70 S ribosomes. In another experiment 18 nmol Phe-$tRNA^{Phe}$ in the form of a ternary complex with EF-Tu and GTP was added to complex I and the mixture incubated at 37°C for 10 min. To determine the level of Phe-$tRNA^{Phe}$ binding in a parallel experiment the same complex was obtained using unlabelled fMet-$tRNA_f^{Met}$ and [^{14}C]Phe-$tRNA^{Phe}$. The level of Phe-$tRNA^{Phe}$ binding was found to be 0.30 mol per mol 70 S ribosomes. Occupation of the A-site with Phe-$tRNA^{Phe}$ directed by EF-Tu and GTP resulted in transpeptidation in 90% complexes as judged from experiments using either [^{3}H]fMet-$tRNA_f^{Met}$ and Phe-$tRNA^{Phe}$ or fMet-$tRNA_f^{Met}$ and [^{14}C]Phe-$tRNA^{Phe}$. The data are listed in table 1. Transpeptidation led to formation of the complex ribosome·[^{14}C]$AUGU_6$CHRCl·$tRNA_f^{Met}$(P-site)·fMetPhe-$tRNA^{Phe}$(A-site) designated as complex II. For affinity labelling of ribosomes complexes I

Table 1

Formation of fMetPhe in pretranslocational complex II (30 pmol of 70 S ribosomes were taken in each experiment)

Aminoacyl-tRNA	Binding (pmol)		fMetPhe (pmol)
	f[^{3}H]Met-$tRNA_f^{Met}$	[^{14}C]Phe-$tRNA^{Phe}$	
f[^{3}H]Met-$tRNA_f^{Met}$ Phe-$tRNA^{Phe}$	9.9	–	8.4
fMet-$tRNA_f^{Met}$ [^{14}C]Phe-$tRNA^{Phe}$	–	9.0	8.5

Table 2

Extent of modification of the ribosomal subunits with [^{14}C]AUGU$_6$CHRCl within complexes I and II (mol covalently attached reagent residues per mol subunit)

Complex	AUGU$_6$	Extent of modification	
		30 S	50 S
I	–	0.095	0.025
II	–	0.065	0.060
I	+	0.015	0.006

and II were incubated at 37°C for 1 h (at this time 35–40% of [^{14}C]AUGU$_6$CHRCl is converted into a reactive intermediate ethyleneimmonium cation [13]). After incubation ribosomes were separated into subunits by sucrose gradient (10–40%) centrifugation in 0.5 mM Mg^{2+} and the radioactivity bound to ribosomes was counted. The distribution of ^{14}C label between the subunits is given in table 2. In separate experiments complexes I and II were subjected to the same procedures in the presence of a 50-fold excess of AUGU$_6$ (over [^{14}C]AUGU$_6$CHRCl) and with complex I in the absence of fMet-tRNA$_f^{Met}$. Covalent attachment of [^{14}C]AUGU$_6$CHRCl was observed in both complex I and II. Both 30 S and 50 S subunits were labelled. Modification of 30 S and 50 S subunits within complexes I and II was inhibited practically in the presence of AUGU$_6$. Thus, modification of both 30 S and 50 S subunits may be considered as affinity labelling. Both rRNA and proteins were modified, rRNA being labelled preferentially within complex I. Labelling of both rRNA and proteins was inhibited practically by a 50-fold molar excess of AUGU$_6$ over the reagent (table 3). For identification of the labelled proteins they were extracted from ribosomes alkylated within complex I or II with 67% acetic acid. After hydrolysis of the benzylidene bond in the residues of covalently bound reagent, proteins were separated by two-dimensional polyacrylamide gel electrophoresis [1]. The data are presented in fig.1. It is seen that the results of modification of ribosomes within complexes I and II differ considerably. In the case of complex I, 8–9 proteins were labelled to a comparable extent whereas in complex II only proteins S13 and L10 were labelled to a significant degree. In the control experiment with complex I carried out in the absence of fMet-tRNA$_f^{Met}$, no labelled proteins were found and radioactivity in the protein spots was no higher than 20–30 cpm (background values for this gel were 70–80 cpm).

4. DISCUSSION

Incubation of a complex of [^{14}C]AUGU$_6$CHRCl with ribosomes bearing fMet-tRNA$_f^{Met}$ at the P-site or tRNA$_f^{Met}$ at the P-site and fMetPhe-tRNA^{Phe} at the A-site (complexes I and II, respectively) results in covalent attachment of AUGU$_6$CHRCl to ribosomes. The level of modification is rather high. In complex I as well as in complex II, 80% of the bound reagent is consumed in the alkylation of ribosomes (taking into account the extent of conversion of the reagent into the reactive in-

Table 3

Relative modification extents of proteins and rRNA from ribosomal subunits modified with [^{14}C]AUGU$_6$CHRCl within complexes I and II

Complex	Subunit	Amount of radioactivity[a] (cpm)			
		Proteins		rRNA	
		– AUGU$_6$	+ AUGU$_6$	– AUGU$_6$	+ AUGU$_6$
I	30 S	1200	180	3000	450
I	50 S	100	50	900	150
II	30 S	1930	–	1380	–
II	50 S	2460	–	660	–

[a] Assigned to 1000 pmol of the subunit in each case

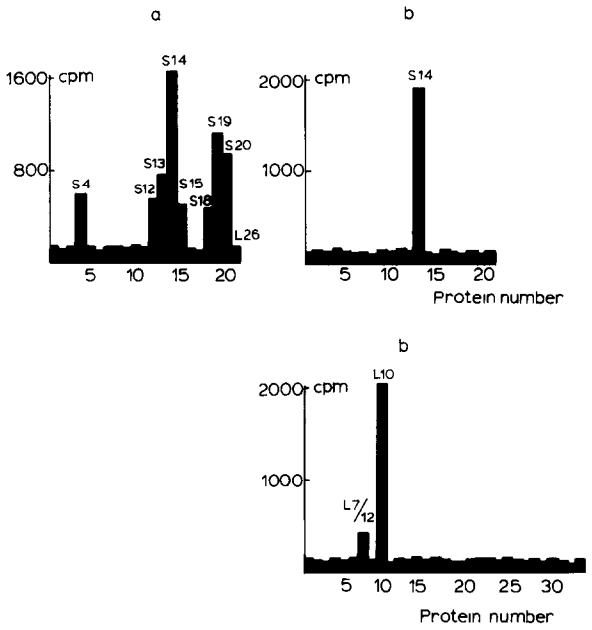

Fig.1. Distribution of label among the ribosomal protein modified $AUGU_6CHRCl$ within initiation (a) and pretranslocational (b) complexes.

termediate [13]). As is evident from the competition experiments between free $AUGU_6$ and $AUGU_6CHRCl$, labelling of proteins and rRNA in both 30 S and 50 S ribosomal subunits is specific. The sets of ribosomal proteins labelled within complexes I and II differ significantly. In complex I (A-site vacant) a number of proteins are found to be labelled: S4, S12, S13, S14, S15, S18, S19, S20/L26. In complex II (both A- and P-sites occupied) proteins S13 and L10 are labelled preferentially. Protein S13 is found in the set of proteins labelled within complex I whereas proteins L10 and L7/L12 were not labelled in complex I.

Hence, in spite of the fact that $AUGU_6CHRCl$ is definitely fixed in the ribosome by interaction of the AUG codon with $fMet\text{-}tRNA_f^{Met}$ (in contrast to previously used mRNA analogs – derivatives of oligouridilates [1–5]) the set of proteins labelled in the 30 S subunit within the 70 S initiation complex is rather large. This may be due to the fact that the reactive group of the reagent is not strictly fixed towards the ribosome because of a rather long free moiety of the reagent which is not fixed in the ribosome by the codon-anticodon interaction. Stabilization of this complex by the additional codon-anticodon interaction at the A-site reduces the 'free' oligonucleotide moiety of the reagent which results in increased selectivity of modification. Hence, the existence of the codon-anticodon interaction at the A-site is of decisive significance for the correct localization of oligoribonucleotides, both homogeneous [5] and bearing an initiation codon at the 5′-end in the decoding center.

Systematic study of the mRNA-binding center of *E. coli* ribosomes was previously performed in a nonenzymatic system using alkylating derivatives of oligouridilates bearing the *p-N*-2-chloroethyl-*N*-methylaminophenyl group (RCl) attached to either the 3′- or 5′-end [1,2]. In most cases a large amount of proteins is labelled. In some cases significant changes are observed when the oligouridilate moiety is elongated by only one uridilate residue. Proteins S4, S13, S14, S15 and S18 which were found to be labelled with $AUGU_6CHRCl$ were found previously to be labelled with $p(Up)_{n-1}UCHRCl$ [1]; proteins S4, S13 and S19 were also labelled with $ClRCH_2NH(pU)_n$ [2]. Moreover, proteins S4, S12, S13 and S18 were found by Pongs et al. [14] in the decoding region from affinity labelling studies with the use of an analog of the initiation codon AUG with a reactive group at the 5′-end. However, the existence of multiple labelling in the case of the derivatives of oligonucleotides [1,2,14], as well as for $AUGU_6CHRCl$ in complex I, hampers the assignment of some definite proteins to the decoding region. Nevertheless, the labelling of protein S13 in complex II is in good agreement with other data indicating that S13 belongs to the decoding region [15,16]. Hence, in the course of investigation of affinity labelling of ribosomes with $tRNA^{Phe}$ derivatives bearing an arylazido group on a guanine residue it was shown that labelling of the single protein S13 takes place in the presence of poly(U) whereas labelling of proteins S5, S9, S11, S12, S19 and S21 proceeds when the $tRNA^{Phe}$ derivative is bound to the ribosome in either the presence or absence of poly(U) [15]. Besides, it was shown previously that the binary complex poly(U)·S13 possesses a strongly expressed ability of codon-selective binding of tRNA. The affinity of $tRNA^{Phe}$ to the binary complex poly(U)·S13 is about 3 orders of magnitude higher than for poly(U) alone [16].

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