CHLORAMBUCILYL [^{3}H]PHENYLALANYL-tRNA CROSSLINKING TO 50 S RIBOSOMAL SUBUNIT PROTEINS L4, L26–27 and L18–20

Eric WICKSTROM, Keith K. PARKER, Deborah A. HURSH* and Richard L. NEWTON+
Department of Chemistry, University of Denver, Denver, CO 80208, USA

Received 10 October 1980; revised version received 8 December 1980

1. Introduction

In the last decade studies with chemically modified tRNAs have contributed to the understanding of ribosomal topography [1–5]. The bifunctional alkylating reagent chlorambucil has been successfully attached to the 3′-end of $tRNA^{Phe}$ via the α-amine of phenylalanine [6,7] and has been shown to alkylate the 23 S RNA of the 50 S subunit of *Escherichia coli* ribosomes [6]. Here, we have covalently labeled the 50 S subunit from *E. coli* ribosomes with CAB [^{3}H]Phe-$tRNA^{Phe}$ under poly(U)-directed, puromycin-sensitive conditions. The subject of 50 S ribosomal topography was then examined by two-dimensional gel electrophoretic fingerprinting of radiolabeled proteins. We report here that significant crosslinking was observed to 50 S ribosomal proteins L4, L26–27 and L18–20. Analysis of radiolabeled 50 S ribosomal RNAs crosslinking studies with CAB oligoprolyl [^{3}H]Phe-tRNA [7] are in progress.

2. Materials and methods

70 S ribosomes were prepared as in [8] from early log-phase MRE 600 *E. coli* (Grain Processing, Inc.) and stored in liquid nitrogen until use. 70 S ribosomes sedimented in sucrose gradients containing 6 mM Mg^{2+} were shown to be comprised almost exclusively of tight couples [9]. *E. coli* $tRNA^{Phe}$ (Plenum Scientific) was aminoacylated with [^{3}H]Phe (4000–15 000 Ci/mol, New England Nuclear; 76 000 Ci/mol, Radiochemical Centre) and [^{3}H]Phe-$tRNA^{Phe}$ was reacted with 80 mM CAB *N*-hydroxysuccinimide ester as in [7,10]. Ribosomes were alkylated for 3 h at 37°C in 900–2600 μl reaction mixtures containing 0.3 μM 70 S ribosomes, 50 nM CAB [^{3}H]Phe-$tRNA^{Phe}$, 0.5 mM poly(U), 100 mM Tris–HCl (pH 7.4), 9 mM $Mg(OAc)_2$, 50 mM LiCl, and 0.1 mM DTT. Filter assays indicated that 90% of the CAB [^{3}H]Phe-$tRNA^{Phe}$ bound to ribosomes remained bound after interaction with puromycin.

Following alkylation, ribosomes were precipitated in 0.8 vol. cold EtOH, centrifuged for 10 min at 12 000 × *g* at 4°C, and redissolved in 100–600 μl buffer A [20 mM Tris–HCl (pH 7.4), 100 mM NH_4Cl, 1 mM DTT, 1 mM $Mg(OAc)_2$]. The ribosomes were then sedimented through 0.2–0.8 M sucrose density gradients in buffer A, in a Beckman SW 50.1 rotor at 4°C for 2.25 h at 300 000 × *g*. Gradient tubes were fractionated by upward displacement with 40% (w/v) sucrose and the fractions were monitored by A_{260}, using in ISCO UA-5 monitor with gradient accessory. Fractions were analyzed for radioactivity by liquid scintillation counting in 3 ml 10% (v/v) Bio-Solv BBS-3 (Beckman), 0.45% (w/v) 2,5-diphenyloxazole in toluene.

Ribosomal proteins were extracted in 67% acetic acid and dialyzed extensively against 8 M urea, 10 mM DTT, 0.01 M bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane(bis-Tris)-HOAc (pH 5.0) [11]. Protein samples were electrophoresed in two dimensions on polyacrylamide gels [12,13] and radioactive spots were visualized by scintillation autography [14], after soaking the gels in EN^{3}HANCE (New England Nucle-

Abbreviations: CAB, chlorambucil; DTT, dithiothreitol; Tris, tris (hydroxymethyl) amino methane

* Present address: Department of Biology, University of Indiana, Bloomington, IN 47401, USA

+ Present address: School of Medicine, University of Mississippi, Jackson, MI 39216, USA

ar). Following autography, radioactivity in ribosomal proteins was quantified by cutting out Coomassie blue-stained spots, rehydrating in 100 μl H_2O, incubating for 4 h at 43°C in 4.4 ml Lipofluor:Solulyte, 10:1 (J. T. Baker), and counting by liquid scintillation. Protein concentrations were determined by reaction with Coomassie blue (Bio-Rad) [15].

3. Results

Table 1 demonstrates the dependence of CAB-[^{3}H]Phe-tRNA^{Phe} alkylation of ribosomes on [Mg^{2+}]. A number of important pieces of information about this experiment should be considered.

(i) The total amount of CAB[^{3}H]Phe-tRNA^{Phe} bound to 50 S subunits increases dramatically with increasing [Mg^{2+}].

(ii) The ratio of CAB[^{3}H]Phe-tRNA^{Phe} bound to 30 S ribosomes in the presence of messenger to that bound in the absence of messenger declines with increasing [Mg^{2+}]. As a result, less CAB-[^{3}H]Phe-tRNA^{Phe} is bound in the presence of messenger than in the absence of messenger at higher [Mg^{2+}]. In the case of messenger-dependent binding, the ratio of tRNA bound to the 50 S subunit compared to the 30 S subunit increases with increasing [Mg^{2+}].

Thus, considering only the 50 S subunit, binding at 20 mM Mg^{2+} is optimal in that the greatest amount of CAB[^{3}H]Phe-tRNA^{Phe} is bound at this concentration. However, only ~50% of the tRNA bound to the 50 S subunit in the presence of messenger can interact with 2.5 mM puromycin (not shown). On the other hand, all of the messenger-dependent binding to the 50 S subunit can interact with 2.5 mM puromycin at 9 mM Mg^{2+} (fig.1). Therefore, binding at 9 mM Mg^{2+} represents the greatest amount of binding that is totally sensitive to puromycin. Binding at 7 mM Mg^{2+} (not shown) was intermediate to that at 5 mM and 9 mM Mg^{2+} and gave less consistent results than were observed at 9 mM Mg^{2+}. Binding at 5 mM Mg^{2+} showed the same initiation factor dependence, messenger dependence, and puromycin susceptibility as with formyl[^{3}H]Met-tRNA and *N*-acetyl[^{3}H]Phe-tRNA (not shown).

Following alkylation of 50 S subunits at 9 mM Mg^{2+}, ribosomal proteins were extracted [11] and separated by two-dimensional gel electrophoresis [12,13]. Radioactivity detected in the proteins, as determined by liquid scintillation counting, is shown in table 2. Qualitatively similar results were obtained when radioactivity was detected by scintillation autography. Proteins L4, L26–27 and L18–20 were significantly alkylated under these conditions.

4. Discussion

The results presented in table 1 and fig.1 illustrate the crucial role that Mg^{2+} plays in the binding of CAB-[^{3}H]Phe-tRNA^{Phe} to the 50 S subunit of *E. coli* ribosomes. The increased binding at higher [Mg^{2+}] may represent a similar effect to that seen for the 30 S

Table 1
Amount of CAB[^{3}H]Phe-tRNA^{Phe} (fmol) covalently bound to ribosomes at various Mg^{2+} levels

[Mg^{2+}] (mM)	30 S Subunit			50 S Subunit		
	−Poly(U)	+Poly(U)	R^a	−Poly(U)	+Poly(U)	R^a
5	140	180	1.29	0	50	–
9	290	200	0.69	20	90	4.50
20	230	160	0.70	90	520	5.78

[a] *R*, ratio of fmoles bound in the presence of poly(U) to fmoles bound in the absence of poly(U)

70 S ribosomes (300 pmol) were reacted with CAB[^{3}H]Phe-tRNA^{Phe} for 3 h at 37°C as in section 2 except that [Mg^{2+}] was as above. Reacted ribosomes (60 pmol) were then precipitated in 0.8 vol. cold ethanol, pelleted by centrifugation, and redissolved in 100 μl buffer A. 30 S and 50 S subunits were separated by sucrose density gradient sedimentation, and aliquots of the separated fractions were counted for radioactivity

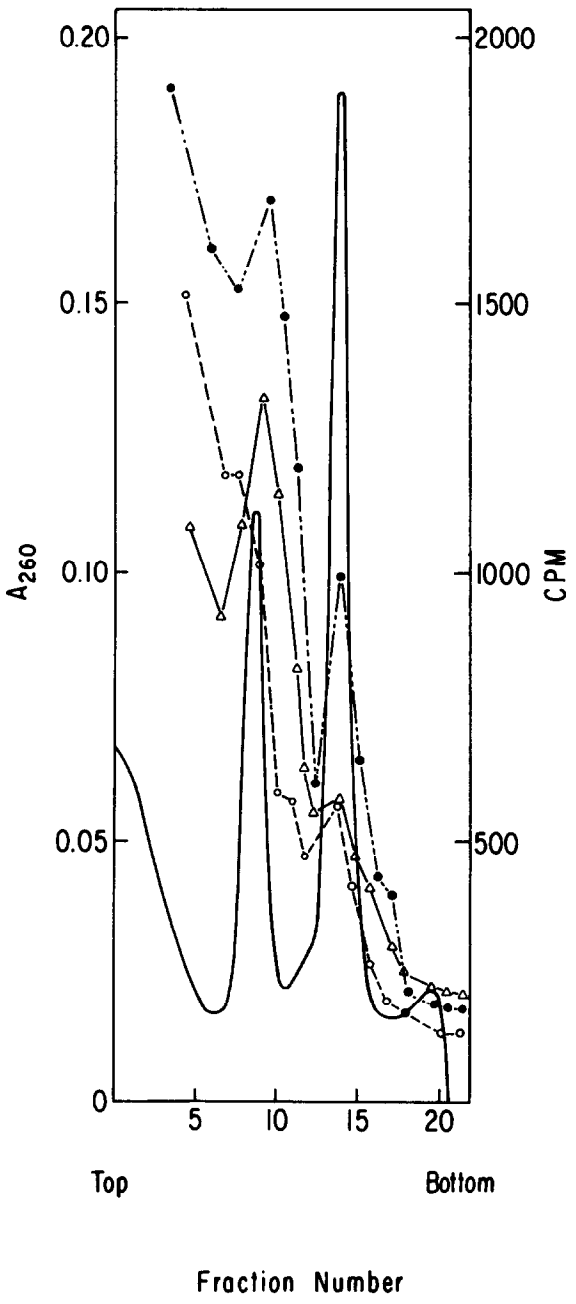

Fig.1. Covalent binding of CAB[^{3}H]Phe-tRNA^{Phe} to ribosomes at 9 mM Mg^{2+}. Experimental conditions were as in section 2 and in table 1. In the case where puromycin was present, 2.5 mM puromycin was added prior to the addition of CAB[^{3}H]Phe-tRNA^{Phe}. Separation of 30 S and 50 S subunits was by sucrose density gradient sedimentation as described (——) A_{260}; (○——○) – poly(U); (·—·) + poly(U); (△——△) + poly(U); + puromycin. Disappearance of A_{260} at the bottom of the gradient is due to collection of some of the dense sucrose chase solution.

Table 2
Labeling of 50 S ribosomal proteins following crosslinking of 70 S ribosomes with CAB[^{3}H]Phe-tRNA^{Phe}

Protein	R^a (± 10%)
L1	1.04
L2	1.03
L3	0.88
L4	1.25
L5	0.92
L6	0.89
L7	1.06
L8	1.02
L9	0.90
L10	1.06
L11	1.03
L12	0.93
L13	0.90
L14	0.96
L15,16	1.02
L17	1.04
L18–20	1.31
L23–24	1.05
L25	1.10
L26–27	1.22
L28–29	1.06

[a] R ratio of cpm in ribosomal protein spots crosslinked in the presence of poly(U) to ribosomal protein spots crosslinked in the absence of poly(U)

70 S ribosomes were reacted with CAB[^{3}H]Phe-tRNA^{Phe} as in section 2 (except that control preparations did not contain 0.5 mM poly(U)). 50 S subunits were isolated by sucrose density gradient sedimentation, proteins were extracted and separated by two-dimensional gel electrophoresis and Coomassie blue-stained spots were cut out and counted by liquid scintillation spectroscopy, as in section 2. Under these reaction conditions, ~0.15% of the 50 S subunits are crosslinked with CAB[^{3}H]Phe-tRNA^{Phe}

subunit where *N*-acetyl-Phe-tRNA^{Phe} binds with higher affinity at higher [Mg^{2+}] [15]. The observation with 30 S subunits that less CAB[^{3}H]Phe-tRNA^{Phe} is bound in the presence of poly(U) than in the absence of poly(U) at 9 and 20 mM Mg^{2+} (table 1) is unexplained at this point; however, this phenomenon may be due to the fact that the crosslinking reaction was performed with 70 S ribosomes as opposed to separated 30 S subunits [16].

We also found during initial studies that the ionic composition of the reaction medium greatly influenced the extent and specificity of covalent binding. For example, in 10 mM Tris–HCl (pH 7.4), 30 mM NH_4Cl, 20 mM $Mg(OAc)_2$, 1 mM DTT, binding was only slightly stimulated in the presence of messenger, and binding

was not significantly stimulated in the presence of messenger if NH_4Cl was increased to 100 mM and that of $Mg(OAc)_2$ decreased to 5 mM. Therefore, in this study the P site specificity at 9 mM Mg^{2+} is probably also dependent on the 100 mM Tris–HCl (pH 7.4), 50 mM LiCl present in the reaction medium, just as P site binding of *N*-acetyl-Val-tRNA is optimal at 75 mM KCl, 75 mM NH_4Cl [17].

The alkylation of proteins in the vicinity of the P site as presented in this study shows some similarities to, as well as some differences from, previous results. Eight 50 S proteins (L2, L11, L14, L15, L16, L18, L23, L27) constitute a group that various affinity labels have placed near the 3′-OH domain of the P site [2–4]. Our crosslinking reagent overlaps with this group to the extent that we see significant labeling at L26–27 and L18–20. However, our identification of L4 does not overlap with previous results. L4 is in the immediate vicinity of L2, L16, and L27 on the outer face of the 50 S subunit, as determined by immunoelectron microscopy [18]. Both the different chemical affinity and reactivity of our alkylating agent, and its length (13 Å), compared to reported agents, may account for the particular set of proteins labeled here. It should be noted that the results presented here are based on low percentage yields of crosslinked ribosomes, as is often the case with affinity crosslinking studies. Thus, the functional relevance of such experiments must be viewed cautiously.

In conclusion, we have identified L4 as a potential candidate for localization near the P site. We are currently undertaking similar studies with rigid, variable length chlorambucilyl oligoproline molecular rulers [7,19], which may contribute to our knowledge regarding the precise topographical relationships of 50 S ribosomal proteins and RNAs.

Acknowledgements

We thank Drs James Ofengand and Arthur Johnson for useful discussions. This work was supported by the US National Institutes of Health, grant GM 24128.

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