AN APPROACH TO SPECIFIC LABELLING OF RIBOSOME IN THE REGION OF PEPTIDYL-TRANSFERASE CENTER USING *N*-ACYLAMINOACYL-tRNA WITH AN ACTIVE ALKYLATING GROUPING

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Received 16 August 1971

1. Introduction

An important problem in studies of the mechanism of ribosome-catalyzed protein synthesis is elucidation of the functional topography of its peptidyl-transferase center. A promising approach to this seems to be to apply the affinity labelling technique that has been elaborated for enzyme studies which is based on specific labelling of the enzyme active center with a substrate analog bearing a chemically reactive grouping.

N-Chlorambucylyl-^{14}C-phenylalanyl-tRNA (chlorambucylyl-^{14}C-phe-tRNA)* was synthesized for this purpose since it was expected that it would specifically bind to ribosomes as do other *N*-acyl-aminoacyl-tRNA's thus directing the bis-(2-chloroethyl)-amino group of the chlorambucylyl residue to the region of the peptidyl-transferase center. The chlorambucylyl residue was chosen as the reactive grouping since its bis-(2-chloroethyl)-amino moiety is known to react with nucleic acid components as well as with a wide variety of functional groups present in proteins.

The purpose of the present study was to elucidate (1) the ability of chlorambucylyl-^{14}C-phe-tRNA to form a stable specific complex with ribosomes in the presence of poly U, and (2) the ability of chlorambucylyl-^{14}C-phe-tRNA to alkylate ribosomes in the bound state.

* Chlorambucyl is *p*-bis-(chloroethyl)-aminophenylbutyric acid.

2. Materials and methods

Chlorambucylyl-^{14}C-phe-tRNA was obtained by alkylation of ^{14}C-phenylalanyl-tRNA (^{14}C-phe-tRNA) of *E. coli* MRE 600 with chlorambucyl *N*-hydroxy-succinimide ester according to Lapidot et al. [1]. The yield of chlorambucylyl-^{14}C-phe-tRNA determined by the method of Schofield and Zamecnik [2] was 90–95% of the starting ^{14}C-phe-tRNA. *N*-Acetyl-^{14}C-phenylalanyl-tRNA (Ac-^{14}C-phe-tRNA) was obtained as described earlier [3]. The specific radioactivity of ^{14}C-L-phenyl-alanine (Chemapol, Czechoslovakia) was about 80 Ci/mole.

Because of the considerable adsorption of chlorambucylyl-^{14}C-phe-tRNA onto nitrocellulose filters the extent of its binding with ribosomes was determined by gel filtration on Sephadex G-100. The composition of the reaction mixture and the conditions of formation of the complex and of its isolation are given in a footnote to table 1. Analysis of the extent of alkylation of ribosomes was performed as described in the legend to fig. 2.

Sucrose gradient (5–20%) centrifugation was performed on a Spinco L-2 ultracentrifuge, rotor SW-25 at 22,000 rpm for 9.5 hr.

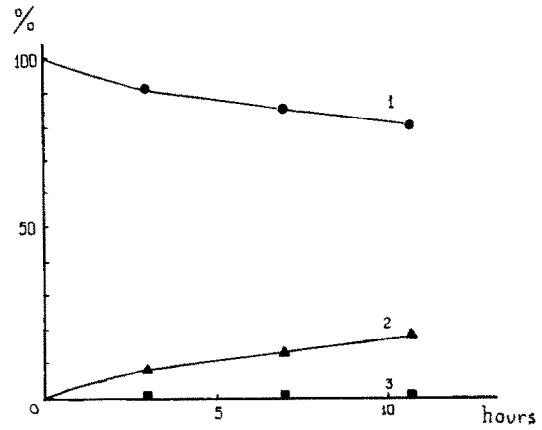

Fig. 1. Kinetic curves of the decrease of the content of chlorambucylyl-^{14}C-phe-tRNA in the ternary complex with ribosomes and poly U (curve 1), of accumulation of free chlorambucylyl-^{14}C-phenylalanine (curve 2) and of free chlorambucylyl-^{14}C-phe-tRNA (curve 3). Incubation of the complex was in 0.1 M triethanolamine-HNO_3 buffer pH 7.2, 0.05 M KNO_3, 0.04 M $Mg(NO_3)_2$ at 25°. Aliquots of the incubation mixture (0.2 ml) were removed at time intervals and subjected to gel filtration as described in the footnote to table 1. The contents of chlorambucylyl-^{14}C-phe-tRNA in the complex, of free chlorambucylyl-^{14}C-phe-tRNA and of free chlorambucylyl-^{14}C-phenylalanine were estimated from the radioactivity of the corresponding peaks and expressed as percent of the total radioactivity of effluent.

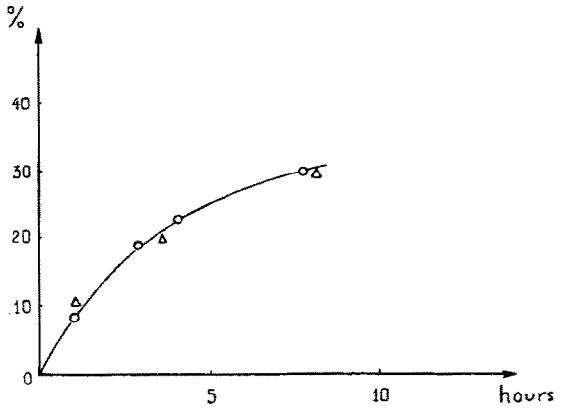

Fig. 2. Kinetics of the "intra-complex" alkylation of ribosome with chlorambucylyl-^{14}C-phe-tRNA (data of two separate experiments). The incubation conditions are the same as in fig. 1. Aliquots were removed at time intervals from the reaction mixture (about 5×10^3 cpm of total radioactivity per aliquot) and polymer precipitated with two volumes of ethanol in the presence of 2% sodium acetate, pH 5.0. The precipitate, after centrifugation, was dissolved in 0.6 ml 7 M urea with 0.1 M cysteine, 2.5% sodium dodecylsulphate and 0.1 M hydroxylamine, pH 10, heated for 30 min at 37° and subjected to gel filtration on Sephadex G-25 (medium, 1 × 15 cm) to separate polymers from the chlorambucylyl-^{14}C-phenylalanine hydroxamate formed. The extent of alkylation was calculated as the ratio of the radioactivity in the polymer fraction to total radioactivity (%).

3. Results and discussion

Table 1 shows the extent of binding of chlorambucylyl-^{14}C-phe-tRNA with ribosomes in the presence of poly U. For comparison, the extent of binding of Ac-^{14}C-phe-tRNA that is well known to form a specific complex with ribosomes is also given. It is seen that in the presence of poly U, chlorambucylyl-^{14}C-phe-tRNA binds to ribosomes to the same extent as Ac-^{14}C-phe-tRNA and that the binding is specific since it practically does not take place in the presence of poly A.

The following experiments were performed to demonstrate the stability of the chlorambucylyl-^{14}C-phe-tRNA–ribosome–poly U complex under alkylation conditions. The specific complex was isolated by gel filtration on Sephadex G-100 and incubated in buffer solution (see legend to fig. 1). Analysis of the reaction products arising in the course of incubation of the complex was performed by the same method since it provides good separation of the complex from unbound chlorambucylyl-^{14}C-phe-tRNA and of the product of hydrolysis of the ester bond between the *N*-acylaminoacyl residue and tRNA-*N*-chlorambucylyl-^{14}C-phenylalanine. The results are presented in fig. 1. It is seen that during incubation in our conditions the content of chlorambucylyl-^{14}C-phe-tRNA in the complex gradually decreases to a small extent (about 20% after 11 hr of incubation, curve 1) with the simultaneous accumulation of chlorambucylyl-^{14}C-phenylalanine (curve 2). No free chlorambucylyl-^{14}C-phe-tRNA appears (curve 3). It is necessary to emphasize that the free chlorambucylyl-^{14}C-phenylalanine accumulated in the reaction mixture cannot alkylate ribosomes to any appreciable extent since its concentration is very small (cf. [4]) and therefore any alkylation of ribosomes under the above conditions should be specific.

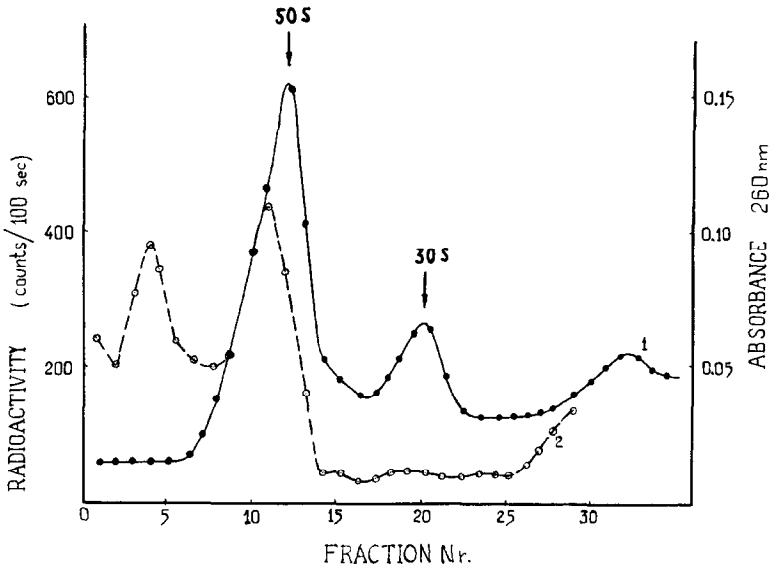

Fig. 3. Sedimentation profiles of optical density (1) and radioactivity (2) of 1.5 mg of complex ribosome–poly U–chlorambucylyl-^{14}C-phe-tRNA, incubated 7 hr at 25° after centrifugation in sucrose gradient with 1 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl buffer pH 7.5. Prior to centrifugation the complex was dialysed against the same buffer.

Table 1
Binding of *N*-acyl-^{14}C-phenylalanyl-tRNA with ribosomes.

N-Acyl-^{14}C-phe-tRNA	tRNA bound into complex (%) in the presence of Poly U	Poly A
Chlorambucylyl-^{14}C-phe-tRNA	90–95	10–12
Ac-^{14}C-phe-tRNA	85	9

Composition of reaction mixture (0.2 ml): 5 A_{260} units of ribosomes, *E. coli* MRE 600; 0.8 A_{260} units of chlorambucylyl-^{14}C-phe-tRNA or Ac-^{14}C-phe-tRNA (about 5 × 10^3 cpm); 0.04 mg of poly U or poly A; 0.1 M triethanolamine-HNO_3 buffer pH 7.2; 0.05 M KNO_3; 0.02 M $Mg(NO_3)_2$; 20 min incubation at 25°. Analysis: Mixtures were subjected to gel filtration on Sephadex G-100 (0.8 × 15 cm) in the above buffer solution. Flow rate 6 ml/hr, fraction volume 0.2 ml, temperature during gel filtration 4°. The extent of binding is the ratio of the radioactivity of the first peak (ribosomes) to total radioactivity of effluent (%).

In fig. 2 is shown the kinetics of alkylation of ribosomes with chlorambucylyl-^{14}C-phe-tRNA bound in the complex. It is seen that after 7 hr of incubation about 30% of the chlorambucylyl-^{14}C-phe-tRNA forms covalent bonds with ribosome components. This suggests that inside the ternary complex the efficiency of alkylation is very high. The fact of alkylation and of its high efficiency suggests that in the region of the peptidyl-transferase center of the ribosome there exist groupings capable of being alkylated.

It is known that the peptidyl-transferase center is localised at the 50 S ribosomal subunit [5]. Therefore it was expected that after specific alkylation followed by dissociation of the ribosomes, label would be found in the 50 S peak. To prove this suggestion the ternary complex incubated for 7 hr at 25° was put on a sucrose gradient with 1 mM Mg^{2+} and the distribution of the label was investigated after sedimentation. In agreement with our suggestion the ^{14}C-label was found in the 50 S particles and was absent in the 30 S particles (fig. 3). Besides this, nearly half the ^{14}C-label was found in the 70 S component. Control experiments with chlorambucylyl-^{14}C-phe-tRNA incubated prior to complex formation at pH 5.5, 25° for 15 hr to hydrolyze the active C–Cl bond, showed that after the same procedure ribosomes dissociate completely and no radioactivity is found in the 70 S region of the sucrose gradient.

The data obtained suggest that a part of the ribosomes is alkylated in the region of the peptidyl-transferase center in the 50 S subunit, whereas a part of the subunits remain bound together. This may be due to bifunctionality of the alkylating group, permitting simultaneous alkylation by the same bis-(2-chloroethyl)-amino residue of the peptidyl-transferase center and to some extent of a neighbouring region of the 30 S subunit.

These results demonstrate that chlorambucylyl-^{14}C-phe-tRNA is a promising affinity labelling reagent applicable to investigations of the ribosomal peptidyl-transferase center. At present, studies are in progress concerned with the elucidation of the distribution of label between ribosomal components.

Acknowledgements

The authors would like to thank Prof. A.S. Spirin for active interest and support of this work and valuable discussions, Dr. M.B. Garber for participation in experiments with ribosomal subunits and Dr. M.I. Rivkin for synthesis of chlorambucyl *N*-hydroxysuccinimide ester.

References

[1] Y. Lapidot, N. de Groot, S. Rappoport and A.D. Hamburger, Biochim. Biophys. Acta 149 (1967) 532.
[2] P. Schofield and P. Zamecnik, Biochim. Biophys. Acta 155 (1968) 410.
[3] D.G. Knorre, L.P. Senzhenko and N.M. Teplova, Molekul. Biol. 4 (1970) 749.
[4] A.M. Belikova, T.E. Vakhrusheva, V.V. Vlasov, N.I. Grineva, V.F. Zarytova, D.G. Knorre and N.M. Teplova, Molekul. Biol. 4 (1970) 30.
[5] R.E. Monro, J. Mol. Biol. 26 (1967) 147.