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Differences in 23 S rRNA-protein neighbourhood in *Escherichia coli* 70 S ribosomes and 70 S initiation complex

Probing by bifunctional Pt(II)-containing reagent

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rRNA-protein cross-links in free *E. coli* ^{35}S-labeled 70 S ribosomes and in the initiation complex ^{35}S-labeled 70 S ribosome · $AUGU_6$ · fMet-$tRNA_f^{Met}$ were studied with the aid of a new type of binuclear Pt(II) compound – dichlorotetraammine(1,6-hexamethylenediaminediplatinum) dichloride. The use of this reagent allowed us to reveal differences in the rRNA-protein neighbourhood in free 70 S ribosomes and in the initiation complex. Proteins L3, L6, L23 and L25 were shown to cross-link to 23 S rRNA only in the initiation complex, whereas proteins L1, L13, L14, L16, L17, L18, L22, L28 and S1 did so in both free ribosomes and the complex. 16 S rRNA was found to be cross-linked preferentially to a single protein, S1, in both states of the ribosomes.

RNA, ribosomal; Protein, ribosomal; Initiation complex; Platinum(II) reagent; Crosslinking, cleavable

1. INTRODUCTION

In the study of ribosomal topography, Pt(II) compounds are rather promising [1–4]. The reagent {dichloro[*N,N,N',N'*-tetrakis(2-aminoethyl) - 1,6 - hexamethylenediaminediplatinum(II)]} dichloride (Pt_2AmCl_4) has been used in investigation of rRNA-protein cross-links in free 70 S ribosomes and in the initiation complex 70 S ribosome · $AUGU_6$ · fMet-$tRNA_f^{Met}$ [4]. Differences in rRNA-protein neighbourhood between these two states of ribosomes were found to be insignificant. Here, we describe an investigation of rRNA-protein cross-links with the aid of a new type of binuclear Pt(II) compound: dichlorotetraammine(1,6-hexamethylenediamine)diplatinum dichloride (Pt_2amCl_4). This reagent differs from Pt_2AmCl_4 in the structure of the spacer moiety between the two Pt complex ions (see fig.1). Pt_2amCl_4 appears to be a more sensitive (as com-

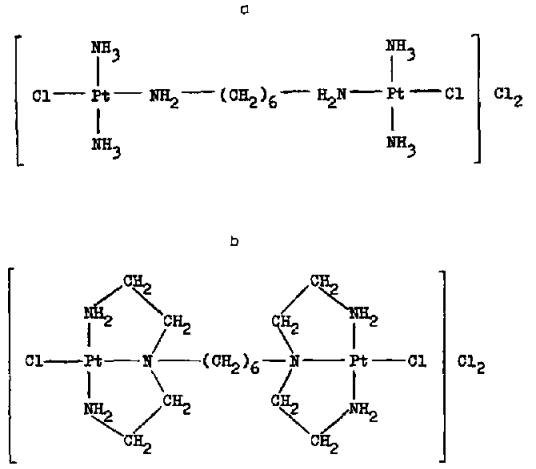

Fig.1. Structural formulae of the reagents: (a) Pt_2amCl_4 and (b) Pt_2AmCl_4.

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pared to Pt_2AmCl_4) probe of alterations in the rRNA-protein neighbourhood in free ^{35}S-labeled 70 S ribosomes and in the initiation complex ^{35}S-labeled ribosomes · $AUGU_6$ · fMet-$tRNA_f^{Met}$. Using Pt_2amCl_4 we show that proteins L3, L6, L23 and L25 are capable of cross-linking to 23 S rRNA only in the initiation complex.

2. MATERIALS AND METHODS

$tRNA_f^{Met}$ was purchased from Boehringer Mannheim; ^{35}S-labeled 30 S and 50 S ribosomal subunits were isolated as in [5]. f[^{35}S]Met-$tRNA_f^{Met}$ was obtained as described [6]. 1 A_{260} unit of $tRNA_f^{Met}$ corresponded to 1200 pmol methionine residues and 900 pmol formyl groups. $AUGU_6$ was synthesized according to [6]. Pt_2amCl_4 was a kind gift from Professor A.I. Stezenko. Unlabeled 30 S and 50 S subunits were a kind gift from Dr V.I. Makhno. Activity of ribosomes in poly(U)-dependent synthesis of diphenylalanine was determined as in [5].

The complex ^{35}S-labeled 70 S ribosome · $AUGU_6$ · fMet-$tRNA_f^{Met}$ (henceforth referred to as the initiation complex) was obtained as in [4]. Free 70 S ribosomes and the initiation complex were treated with 1 mM Pt_2amCl_4 at 20°C for 50 min. Treated ribosomes were divided into subunits according to [2,4]. rRNA-protein cross-links were isolated as in [2,4] and reversed by treatment with 2 M thiourea for 1 h at 37°C and pH 3.5. Ribosomal proteins were analysed by two-dimensional polyacrylamide gel electrophoresis as described in [4].

3. RESULTS AND DISCUSSION

To produce rRNA-protein cross-links, ^{35}S-labeled 70 S ribosomes and the initiation complex were treated with Pt_2amCl_4 in a buffer which contained 10 mM $Mg(NO_3)_2$, 100 mM KNO_3 and 20 mM Tris-HNO_3 (pH 7.5). In a parallel experiment with unlabeled ribosomes it was shown that 0.5 mol f[^{35}S]Met-$tRNA_f^{Met}$ was bound per mol ribosomes (in the absence of template, binding of fMet-$tRNA_f^{Met}$ was not observed). Treatment of the complex with Pt_2amCl_4 did not result in its appreciable dissociation. Moreover, preincubation of ribosomes with the reagent does not lead to their significant inactivation [at least in poly(U)-dependent synthesis of $(Phe)_2$]. Therefore, the level of Phe-$tRNA^{Phe}$ binding to control and treated ribosomes was 1.77 and 1.60 mol per mol ribosomes, respectively; the level of $(Phe)_2$ formation on treated ribosomes was about 90% of that of the controls. Hence, the functional activity of ribosomes in both codon-dependent binding of aminoacyl-tRNA and the transpeptidation reaction is practically retained on treatment with Pt_2amCl_4.

Reaction mixtures treated with Pt_2amCl_4 were centrifuged in sucrose gradients (15–30%) under conditions for dissociation into subunits (1 mM Mg^{2+}). In all cases, about 10% of ^{35}S-labeled ribosomes remained undissociated, evidently due to intersubunit cross-links. To isolate rRNA-protein cross-links, subunits were centrifuged in sucrose gradients (5–20%) in the presence of SDS and EDTA (conditions for dissociation into rRNA and proteins [4]). The cross-links were detected as labeled material of sedimentation coefficient somewhat higher than 16 S (23 S); in control experiments with untreated ^{35}S-labeled subunits these fractions did not contain the label. The extent of covalent attachment of labeled proteins to rRNA in both free ribosomes and the initiation complex was approx. 10% (calculated as a percentage of label contained in fractions of rRNA-protein cross-links from the total amount of label in the corresponding treated subunit).

rRNA-protein cross-links were reversed by treatment with 2 M thiourea at pH 3.5 according to [4]. The results obtained from an analysis of ^{35}S-labeled proteins cross-linked to rRNA are shown in figs 2 and 3. A typical electropherogram of proteins stained with Coomassie is shown in fig.4.

Raising the concentration of Pt_2amCl_4 from 1 to 5 mM did not practically affect the cross-linking patterns in fig.2; however, the fraction of ribosomes remaining undissociated due to intersubunit cross-links (see above) increased to 30% in both free ribosomes and the initiation complex. Therefore, we suggest that in Pt_2amCl_4-treated ribosomes, rRNA-protein (unlike protein-protein) cross-links are produced preferentially; a proportion of the ribosomes become undissociable due to 16 S–23 S rRNA intersubunit cross-links. In fact, the positive charge of Pt_2amCl_4 should result in attraction towards the negatively charged RNA ribose-phosphate backbone which is unshielded by ribosomal proteins. Hence, the reagent must firstly be attached to rRNA via the first active group, and then to the protein by the second. The suggested scheme resembles the affinity labeling approach when a reactive group is primarily introduced into RNA and reacts with a protein after formation of a specific ribonucleoprotein complex.

A number of proteins capable of being cross-

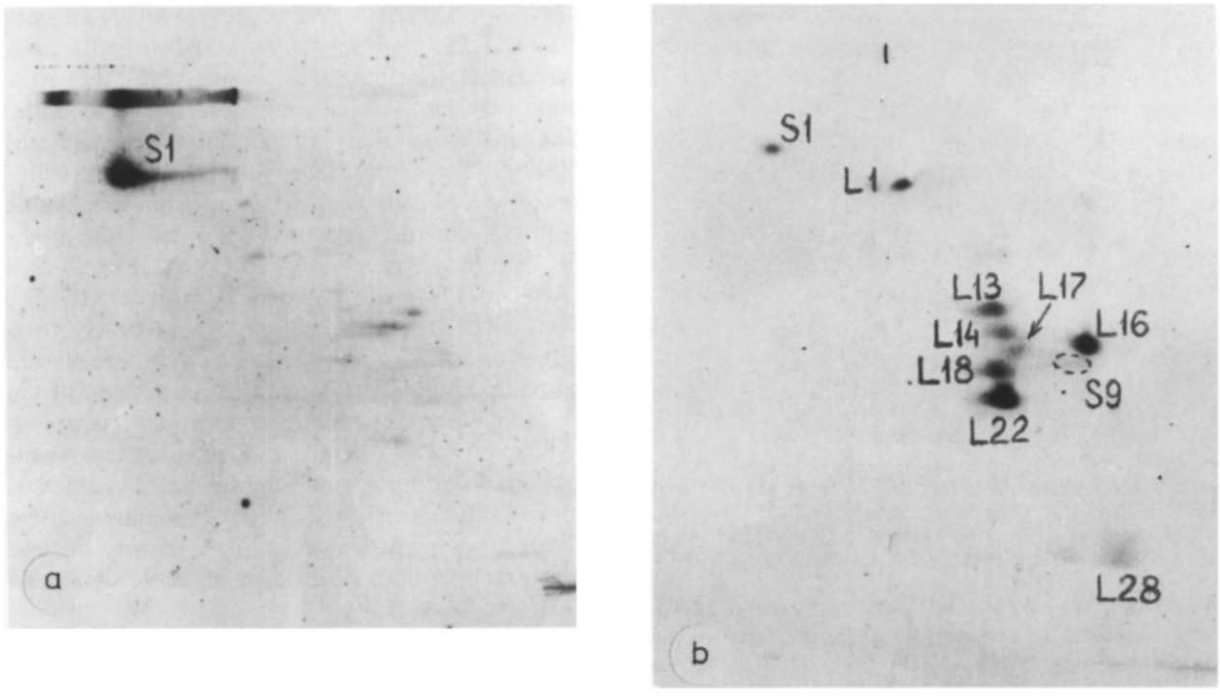

Fig.2. Electropherogram of ^{35}S-labeled ribosomal proteins cross-linked to rRNA after treatment of 70 S ribosomes with Pt_2amCl_4: (a) 30 S subunit; (b) 50 S subunit. Autoradiography of the gel.

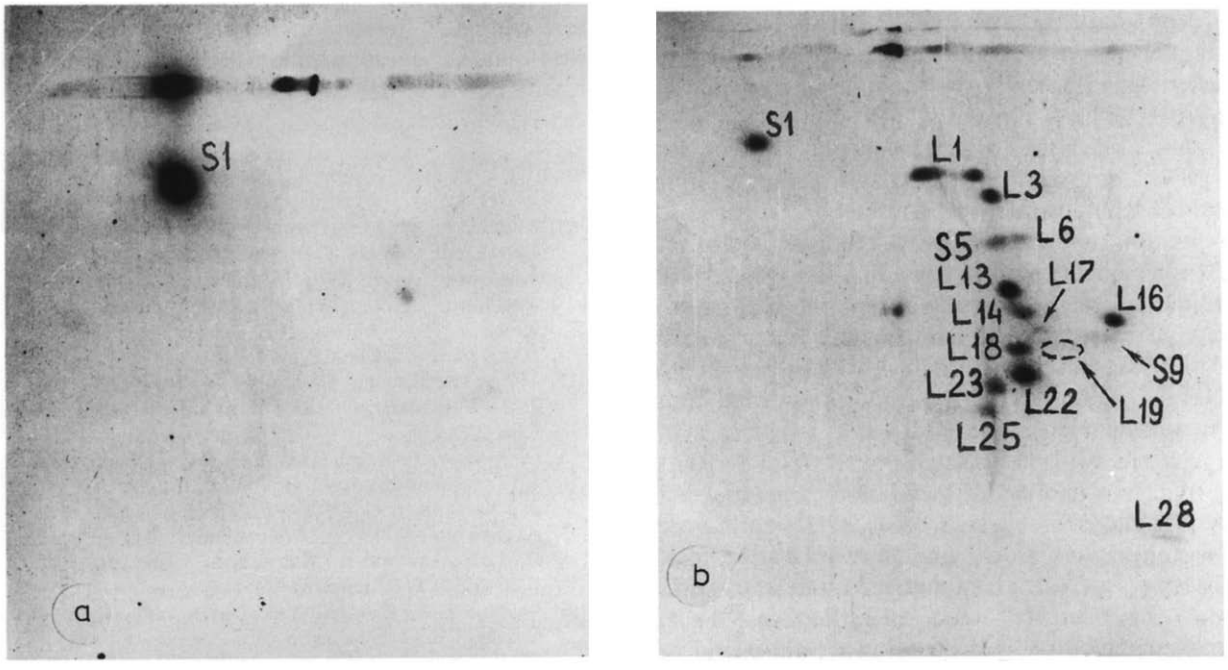

Fig.3. Electropherogram of ^{35}S-labeled ribosomal proteins cross-linked to rRNA after treatment of the 70 S initiation complex ^{35}S-labeled 70 S ribosomes·$AUGU_6$·fMet-$tRNA_f^{Met}$ with Pt_2amCl_4: (a) 30 S subunit; (b) 50 S subunit. Autoradiography of the gel.

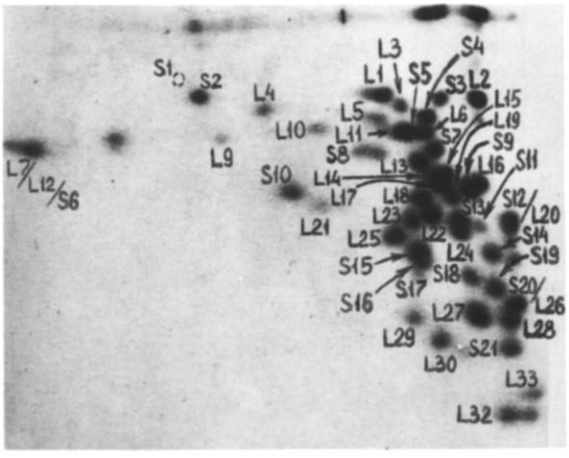

Fig.4. Typical electropherogram of ribosomal proteins after Coomassie staining.

linked to 16 S and 23 S rRNA by Pt_2amCl_4 have been included in the corresponding cross-linking patterns obtained previously with another Pt(II) binuclear reagent, Pt_2AmCl_4 [4]; the sets of proteins cross-linked to rRNA by Pt_2amCl_4 are much narrower, especially in the case of 16 S rRNA. This may be due to structural differences between the reagents (see fig.1). Pt_2AmCl_4 is more hydrophobic than Pt_2amCl_4 due to a greater number of $-CH_2-$ groups, and hence is capable of penetrating deeper into ribosomes and producing more cross-links. Secondly, the two chelate rings in Pt_2AmCl_4 produce a stronger bond between the two Pt complex ions and correspondingly more stable rRNA-protein cross-links as compared to Pt_2amCl_4. In the latter case, the replacement of the Cl^- coordinated to the platinum by a strong *trans*-labilizing group of a protein (e.g. $-S-CH_3$ or -S-) may result in destruction of the binuclear structure of the platinum reagent (scission occurring at the $-NH_2-Pt-$ bond), the corresponding cross-link thus becoming undetectable.

The sets of proteins cross-linked to 23 S rRNA in free ribosomes and the initiation complex differ significantly. Proteins L3, L6, L23, L25 and S5 are able to form cross-links only in the initiation complex (see figs 2,3). The above-mentioned 50 S proteins have never been identified as being cross-linked to tRNA in the donor tRNA binding center [7–10]. Therefore, it appears unlikely that these proteins would be able to cross-link to 23 S rRNA via $tRNA_f^{Met}$. We suggest proteins L3, L6, L23 and L25 as being cross-linked to 23 S rRNA directly in the initiation complex. On the other hand, protein S5 was found previously to cross-link to fMet-$tRNA_f^{Met}$ or AcPhe-$tRNA^{Phe}$ in the 30 S initiation complex or its analog [11,12]. This protein was also labeled with photoactivated $tRNA_f^{Met}$ derivatives in the 70 S initiation complex [9]. Therefore, cross-linking of protein S5 to 23 S rRNA by Pt_2amCl_4 may be effected via $tRNA_f^{Met}$. Differences between the sets of proteins cross-linked to rRNA in free ribosomes and the initiation complex may reflect structural alterations in the ribosome as a consequence of the interaction with tRNA and mRNA. Previously, rRNA-protein contacts in the 30 S subunit had been demonstrated as undergoing alterations on tRNA binding; these changes even displayed a dependence on the presence of aminoacyl residues at the tRNA 3′-end [13].

To summarize, the use of binuclear Pt compounds as bifunctional cross-linking reagents and ^{35}S-labeled ribosomes with a high degree of labeling: (i) enabled us to obtain more detailed information on the rRNA-protein neighbourhood in different functional states; (ii) permitted the discovery of new rRNA-protein cross-links [4]; and (iii) led to the demonstration of alterations in ribosomal topography caused by template and initiator tRNA in the corresponding complex.

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