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Crosslinking of ribosomal protein S18 to 16 S RNA in *E.coli* ribosomal 30 S subunits by the use of a reversible crosslinking agent: *trans*-diamminedichloroplatinum(II)

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We have previously developed [(1987) Biochemistry 26, 5200–5208] the use of *trans*-diamminedichloroplatinum(II) to induce reversible RNA-protein crosslinks in the ribosomal 30 S subunit. Protein S18 and, to a lesser extent, proteins S13/S14, S11, S4 and S3 could be crosslinked to the 16 S rRNA. The aim of the present work was to identify the crosslinking sites of protein S18. Three sites could be detected: a major one located in region 825–858, and two others located in regions 434–500 and 233–297. This result is discussed in the light of current knowledge of the topographical localization of S18 in the 30 S subunit and of its relation with function.

Ribosome; rRNA-protein interaction; Reversible crosslinking; Protein S18

1. INTRODUCTION

The ability of *trans*-diamminedichloroplatinum(II) (*trans*-DDP) to induce reversible RNA-protein crosslinks has been detailed in [1]. *trans*-DDP has a square planar geometry and contains two chlorines spanning a distance of 7 Å. Both chlorines can easily be substituted by stronger nucleophilic groups. In ribonucleoprotein complexes, platinum coordinates on the RNA side primarily to position N7 of guanines and to a lesser extent to N1 of adenines and N3 of cytosines. On the protein side, it binds to the sulfur atom of cysteines and methionines and to the unprotonated imidazole ring of histidines. At neutral pH, these crosslinks can either remain stable or be reversed by the addition of stronger nucleophilic groups. This crosslinking agent has been used successfully in our laboratory to induce RNA-protein crosslinks between tRNA and aminoacyl-tRNA synthetase [1], tRNA and elongation factor EF-Tu in the ternary EF-Tu · GTP · tRNA complex [2], and between initiation factor IF3 and 16 S rRNA in the 30 S subunit · IF3 complex [3]. A detailed study of *trans*-DDP crosslinking in *E. coli* ribosomal 30 S subunits has been made in [1]. This study allowed us to establish standard conditions for the crosslinking reaction and demonstrated its specificity. Only proteins S18 (in 15% of the treated subunits), S13/S14 (8%), S3 (5%), S11 (4%) and S4 (2%) could be crosslinked to the 16 S rRNA. Here, we focused on the identification of the crosslinking sites of protein S18 on the 16 S rRNA.

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2. MATERIALS AND METHODS

2.1. *Preparation of ribosomal subunits*

Ribosomes were prepared from *E. coli* MRE 600 and dissociated into subunits as in [1]. The collected 30 S subunits were precipitated with ethanol (0.65 vol., 30 min, 0°C), and resuspended in 20 mM Na phosphate (pH 7.4), 5 mM Mg acetate, 25 mM NaCl (buffer A).

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2.2. *Crosslinking conditions*

1–2 nmol 30 S subunits were renatured at 37°C in buffer A at a concentration of 2 μM for 30 min, then shifted to 20°C and diluted to 0.5 mM in buffer A. *trans*-DDP was added at a final concentration of 0.3 mM and platination was conducted for 1 h at 20°C in the dark.

2.3. *Isolation of the crosslinked oligonucleotide-protein complexes*

At the end of the platination reaction, the 30 S subunits were deproteinized by LiCl-SDS treatment and the crosslinked 16 S RNA-protein complexes were isolated from non-crosslinked proteins by centrifugation on sucrose gradient in the presence of LiCl-SDS as described [1]. The crosslinked 16 S rRNA-protein complexes were precipitated with ethanol and dissolved in water. The protein moiety contained in the crosslinked complexes was then labeled with [^{125}I]iodine, according to [4]. To eliminate excess radioactive iodine, labeled complexes were extensively dialyzed and reisolated on a sucrose gradient as in the above conditions. The complexes were then subjected to RNase T_1 digestion (0.001 U/μg RNA, 15 min, 0°C), and precipitated with ethanol in the presence of 0.3 M Na acetate. The resulting ^{125}I-protein-oligonucleotide complexes were fractionated by one-dimensional electrophoresis on a 15.6% acrylamide/0.4% bisacrylamide gel in the presence of 0.1% SDS [5] and visualized by autoradiography. Migration was on a 16 × 14 × 0.1 cm^3 slab gel at 20 mA for 4 h. The labeled bands were cut off, and the material eluted from the gel by incubation in 100 μl of 500 mM NH_4 acetate, 10 mM Mg acetate, 0.1% SDS, 0.1 mM EDTA (overnight, 37°C), precipitated twice with 3 vols ethanol in the presence of 50 μg pCp as carrier, and dissolved in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 300 mM NaCl. Contaminating non-crosslinked oligonucleotides were eliminated by filtration on glass filters (Whatman GF-C, 2.5 cm diameter) as in [6]. Complexes retained on the filter were washed with 20 ml of the same buffer, then eluted twice from the filter with 100 μl of 10 mM Tris-HCl (pH 7.5), 1% SDS, and precipitated with 3 vols ethanol in the presence of 0.3 M Na acetate and pCp as carrier. The complexes were then resuspended in buffer A before protein and oligonucleotide analysis.

2.4. *Identification of crosslinked material*

Crosslinked ^{125}I-protein-oligonucleotide complexes were treated with thiourea (1 M final concentration, 30 min, 37°C) to reverse the crosslinks. For analysis of the protein moiety, 70 μg unlabeled 30 S subunits were added to an aliquot (1:5 of the material), and the proteins extracted with acetic acid [7], precipitated with 5 vols acetone, resuspended in the appropriate buffer and fractionated on the two-dimensional polyacrylamide gel electrophoresis system of Mets and Bogorad [8]. After electrophoresis, the gel was stained with Coomassie blue for localization of the reference 30 S proteins. The gel was then dried and subjected to autoradiography for identification of iodinated proteins.

To determine the oligonucleotide content, oligonucleotides liberated by the thiourea treatment were precipitated with 3 vols ethanol in the presence of 0.3 M Na acetate at −80°C for 30 min. The oligonucleotides were labeled at their 5′-end in the presence of T_4 polynucleotide kinase and 200 μCi [γ-^{32}P]ATP, according to [9]. The 5′-labeled fragments were fractionated by electrophoresis on a 15% polyacrylamide (1/20 bis-)/8 M urea slab gel. After autoradiography, the corresponding bands were excised, the oligonucleotides eluted [10], precipitated twice with ethanol in the presence of 10 μg tRNA as carrier, dissolved in 10 μl water and sequenced using RNase statistical hydrolysis. Digestion was with RNase T_1 (0.025 U/μg RNA), RNase U_2 (0.5 U/μg RNA), RNase Phy M (0.5 U/μg RNA) and RNase from *B. cereus* (0.5 U/μg RNA). Incubation was at 55°C for 15 min in 0.02 M citrate (pH 7.5), 1 mM EDTA buffer, in the presence of 8 M urea for RNases T_1, U_2 and Phy M, and in the absence of urea for RNase from *B. cereus*. Analysis of the digests was by electrophoresis on 20% polyacrylamide/8 M urea slab gels. The 5′-terminal nucleotide of each fragment was identified separately as follows: the 5′-end-labeled fragments were hydrolysed for 12 h at 37°C with RNase P_1 (0.5 μg) in 50 mM NH_4 acetate (pH 5.3) in the presence of 5 μg tRNA as carrier, and chromatographed on a thin-layer cellulose plate with HCl/2-propanol/H_2O (17.6:68:14.4) as solvent.

3. RESULTS

3.1. *Formation and isolation of crosslinked complexes*

Standard crosslinking conditions used here were those described by Tukalo et al. [1]. Under these platination conditions, the authors showed that no

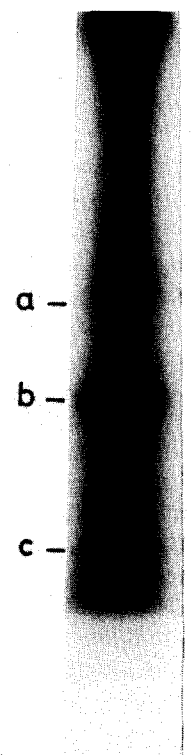

Fig.1. Fractionation by SDS-polyacrylamide gel electrophoresis of the crosslinked ^{125}I-protein-oligonucleotide complexes.

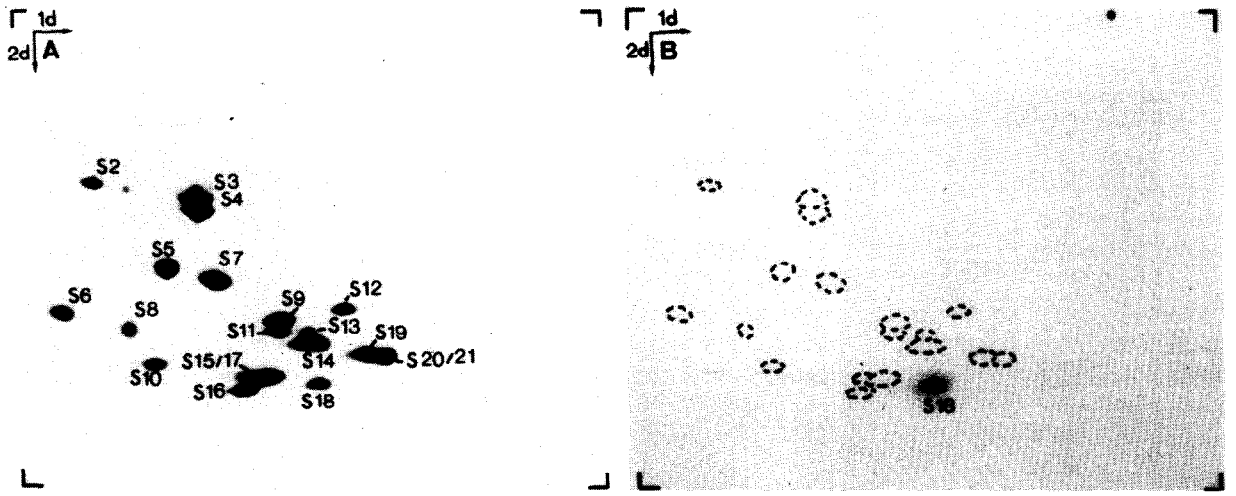

Fig.2. Identification on two-dimensional polyacrylamide gel electrophoresis of the ^{125}I-labeled protein moiety contained in the crosslinked complex eluted from band c (fig.1). Fractionation pattern of the 30 S proteins visualized by Coomassie blue staining (A). Autoradiography of the same gel (B).

RNA aggregates or degradation could be detected. The resulting ^{125}I-protein-Pt-oligonucleotide complexes were fractionated by one-dimensional polyacrylamide gel electrophoresis in the presence of SDS. As shown in fig.1, three major bands could be detected. The protein moiety of each band was identified as described in section 2. In all three bands, the autoradiograms clearly showed that a single protein was present, identified as protein S18 (see a typical experiment in fig.2).

3.2. *Sequence analysis of crosslinked oligonucleotides*

The 5′-end-labeled oligonucleotides contained in the different S18 complexes were fractionated as reported in fig.3. Each fragment was eluted and sequenced as described in section 2. Typical examples of the sequencing gels are presented in fig.4, and the results are summarized in fig.5. Our results show that the free fragments (825–858, 825–859, 825–861) found crosslinked in the major S18 crosslinking band (band b, fig.1) are subfragments of fragment 819–880 isolated from the complex corresponding to band a. Band c contains two different RNA fragments, encompassing nucleotides 434–500 and 233–297. Since exclusively protein S18 was the crosslinked protein (see fig.2), two alternative possibilities can be proposed: both fragments are crosslinked to the same protein, or band c contains two different S18-oligonucleotide

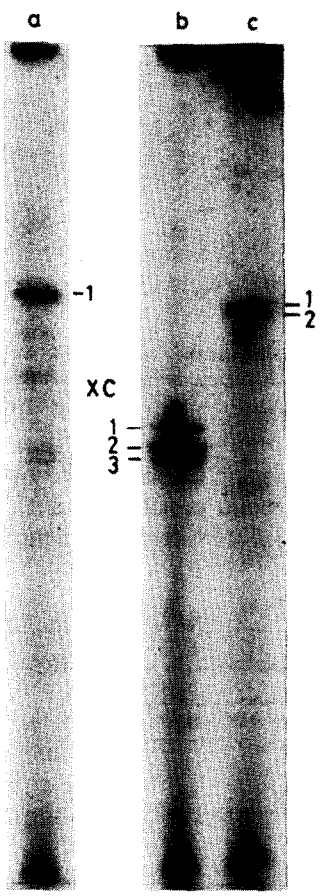

Fig.3. Fractionation by polyacrylamide/urea gel electrophoresis of the oligonucleotide content of bands a–c isolated in fig.1.

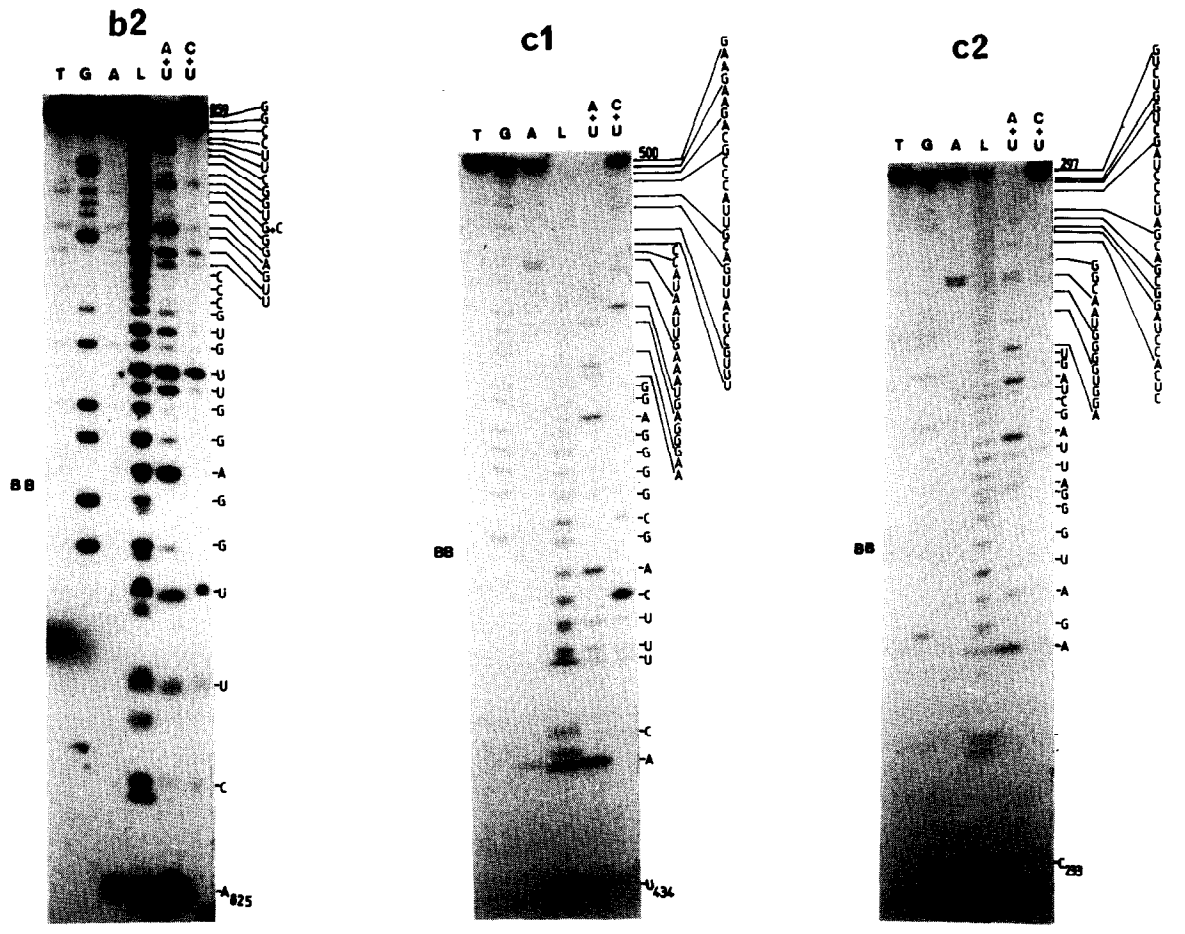

Fig.4. Sequence analysis of the crosslinked RNA fragments b1, c1 and c2. Statistical digestions were with RNases T_1 (lane G), U_2 (lane A), Phy M (lane A + U) and from *B. cereus* (lane C + U). The ladder (lane L) was obtained by limited alkaline digestion. A control (lane T) was run in parallel in the absence of enzyme.

complexes migrating at the same position in the SDS gel. The second possibility would be in keeping with the size of both fragments (see fig.5).

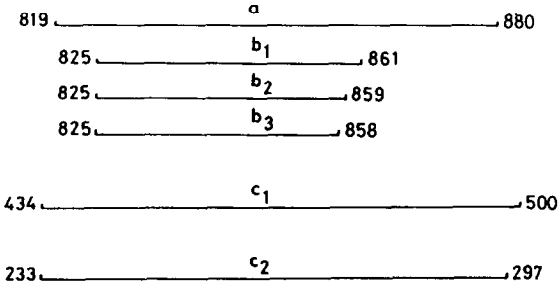

Fig.5. Schematic diagram of the different fragments crosslinked to protein S18.

However, the migration of band c in the SDS gel (fig.1) poses a puzzling problem, compared to the migration of bands a and b. If fractionation of the ribonucleoproteins in such a gel is only based on the size of the RNA content (the protein moiety being the same in all three bands), one would expect the complex(es) found in band c to migrate at the level of band a, or even lower if both fragments are crosslinked to the same protein. Up to now, we have no explanation for this phenomenon.

3.3. *Controls*

To test the specificity of the crosslinking reaction used here, we devised several control experiments. In the first, unplatinated 30 S subunits were subjected to the same procedure as in the

standard assay (deproteinisation, iodination, RNase T_1 digestion, SDS gel electrophoresis). It was shown that no protein remained unspecifically bound to RNA during the isolation procedure. In the second, isolated 16 S rRNA was mixed with [^{125}I]iodinated 30 S proteins and subjected to RNase T_1 digestion. In this case again, no protein could be detected on the SDS gel. In the third, we ensured that no contaminating RNA fragments co-migrated with the crosslinked complexes: 16 S rRNA (same amount as in a standard assay) was subjected to the same hydrolysis conditions and fractionated on the SDS gel in parallel to the platination assay. Bands were cut off from the control at the same migration position as bands a–c, and then eluted and submitted to the post-labeling procedure. Under these conditions, no labeled RNA fragments could be detected, even after long exposure times. Finally, we ensured that platinated proteins could not be unspecifically crosslinked to 16 S rRNA during the different isolation steps: total 30 S proteins (corresponding to the same amount of 30 S subunits in a standard assay) were treated with *trans*-DDP, precipitated with acetone, resuspended and incubated with the corresponding amount of 16 S rRNA in the dissociation buffer containing LiCl-SDS. After sucrose gradient fractionation, the material contained in the 16 S rRNA peak was precipitated and subjected to iodination. The absence of detectable radioactive proteins on the SDS gel unambiguously indicates that no protein was unspecifically crosslinked to 16 S rRNA or to a fragment of RNA.

4. DISCUSSION AND CONCLUSION

Protein S18 presents a particularly interesting object for study. It has been located by immuno-electron microscopy and neutron-scattering studies in the platform of the subunit [11–13] which is known to contain the decoding site [14]. Crosslinking and affinity-labeling approaches suggest that S18 is involved in mRNA binding [15] and has been designated as an A-site protein [16]. Crosslinking experiments have also demonstrated that S18 is in the close neighborhood of other proteins involved in the formation of the initiation complex such as S1, S21 and IF3 [17,18].

Here, we identified three different regions of the 16 S rRNA located in the close neighborhood of protein S18 within the 30 S subunit by crosslinking with *trans*-DDP: 825–859, 434–500, 233–297. The major crosslink occurred within nucleotides 825–859 located in the central part of the RNA molecule. This result fully agrees with the location of protein S18 in this region as observed in in vitro reconstitution and nuclease protection of a complex between 16 S rRNA and proteins S6, S8, S15 and S18 [19,20], site-directed mutagenesis in the central domain of 16 S RNA [21], and other crosslinking experiments [22]. It is interesting to note that region 825–859 is adjacent to region 861–889 found crosslinked to protein S1 [23], and also contains a crosslinking site for IF3 in the 30 S-IF3 complex [3]. The close proximity of proteins S18, S1 and IF3, all involved in the binding of the messenger, has also been shown by protein-protein crosslinking experiments. The crosslinking of region 434–500 to protein S18 might also be related to the involvement of S18 in the binding of the mRNA on the subunit, since this region contains sequences 458–462 and 475–481 which have been found to be crosslinked to an analogue of an mRNA [24]. The third crosslinked region (233–297) together with the other two provides interesting information about the tertiary folding of the 16 S RNA around protein S18 within the 30 S subunit. Immuno-electron microscopy and neutron-scattering data have located protein S18 in the close neighborhood of proteins S6, S8, S11, S15, S17 and S21 [11–13]. In vitro assembly experiments have shown that the binding of S6 and S18 is induced by that of S15 [25] and probably of S8 [20]. It is interesting to note that different regions of the 16 S rRNA molecule can be folded near these proteins. Protein S6 was located in a region containing the major S18 crosslinking site 819–859 [19,20]. Protein S17 was crosslinked by others [22] in region 233–297 and near the binding region of proteins S8 and S15 [26,27]. Crosslinked protein S21 [22] and protein S11 [28] were also found near the S8/S15-binding sites. Note that protein S21 which has been crosslinked to S18 [17] was also found crosslinked to the 3′-terminus of 16 S rRNA [28], a region which is essential in mRNA binding. This provides further evidence for the proximity of the 3′-terminus with the other regions involved in messenger binding on the 20 S subunit around S18. Finally, it should be mentioned that crosslinking data are exclusively topo-

graphical data, providing information about close neighborhoods (in the present case at a distance of 7 Å), and do not reflect physical interactions between the interacting molecules. This should explain why crosslinking data are not systematically superposable with other studies, such as footprinting experiments, but are obviously complementary.

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