RNA–PROTEIN CROSSLINKING BY REACTION WITH A SOLUBLE CARBODIIMIDE

Claude CHIARRUTINI and Alain EXPERT-BEZANÇON

Laboratoire de chimie cellulaire, Institut de biologie physico chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France

Received 14 July 1980

1. Introduction

Construction of a complete model of the *Escherichia coli* 30 S ribosomal subunit requires information about the secondary and tertiary structure of 16 S rRNA in the 30 S particle [1] and about its spatial relationships with the twenty one 30 S proteins. Numerous experimental approaches have been used in attempts, so far only partially successful, to obtain this information. UV-induced crosslinking which identifies regions of direct contact between RNA and protein is a useful technique for the analysis of the spatial relationships between the components of the ribosome but it appears to be limited for technical reasons to a small number of ribosomal proteins [2–5]. This limitation has spurred the recent development of several new bifunctional chemical [6,7] and photochemical [8,9] reagents for protein–RNA crosslinking. Here we describe the use of 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC) for RNA–protein crosslinking. This reagent possesses the advantages of commercial availability and ease of handling and when used under optimum conditions, of high efficiency.

2. Materials and methods

2.1. *Reagents*

Ribonuclease T1 (EC 3.1.27.3) and Ribonuclease A (EC 3.1.27.5) were supplied by Sigma; ED (1-ethyl-3-dimethylaminopropyl carbodiimide) was purchased from Merck.

2.2. *Preparation of ribosome and ribosomal subunits*

Unlabelled and ^{35}S-labelled ribosomes and ribosomal subunits were prepared from *E. coli* MRE 600, as in [10] and stored at –70°C in suspension in 50 mM triethanolamine, 10 mM Mg-acetate, 50 mM KCl, 10 mM 2-mercaptoethanol (pH 7.4). The specific activity of ^{35}S-labelled 30 S subunits was 3–4 × 10^7 cpm/A_{260}.

2.3. *Reaction with EDC*

Reaction was carried out in 0.5 mM sodium cacodylate buffers (pH 5.5–7) containing 50 mM KCl, and 0.5–20 mM $MgCl_2$. Suspensions of ^{35}S-labelled plus unlabelled 30 S subunits (5–20 A_{260}/ml, spec. act. 0.5–1 × 10^6 cpm/A_{260}), brought to the desired reaction conditions by dialysis (45 min, 4°C) against 200–500 vol. 0.5 mM Na-cacodylate, 50 mM KCl, 0.5 mM $MgCl_2$ (pH 6) followed by the same volume of the chosen reaction buffers, were incubated for 30 min at 40°C. Freshly prepared solutions of EDC, 0.5 M in the reaction buffers were then added to obtain the desired final concentrations of EDC and the mixtures were incubated at 25°C for 50 min. Reaction was stopped by adding Mg-acetate to 20 mM and NH_4Cl to 0.3 M (final conc.) for 1 h at room temperature against 2 × 100 vol. 0.5 mM Na-cacodylate, 20 mM Mg-acetate, 0.3 M NH_4Cl to remove excess EDC.

2.4. *Separation of crosslinked RNA-protein complexes from uncrosslinked proteins*

After dialysis to remove excess EDC reaction products were adjusted to 10 mM triethanolamine–HCl, 0.1 M LiCl (pH 7.5) by dialysis against this buffer; adjusted to 0.1% SDS and then heated for 1 min at 40°C to disrupt the 30 S subunit. Free RNA and RNA–protein complexes were separated from the bulk of uncrosslinked proteins by sedimentation through a 2.5% sucrose cushion in the same buffer (SW50 rotor, 35 000 rev./min, 15 h, 10°C). The pellet was resuspended in the bottom 200 μl of the cushion, the suspension was sedimented through a 5 ml 5–20% sucrose gradient in the same buffer (SW50 rotor, 45 000 rev./min, 5 h, 10°C), and the 16 S rRNA containing fractions of the gradient were pooled. Effi-

ciency of crosslinking was determined by measuring specific activities (cpm/A_{260}) of fractions through the 16 S peaks and expressing them as % of the specific activity of the original 30 S subunits.

2.5. *Analysis of crosslinked RNA–protein complexes*

Crosslinked 16 S rRNA protein complexes were precipitated (30 min, –20°C) from pooled sucrose gradient fractions after addition of carrier 16 S rRNA, NaCl to 0.1 M final conc. and 2 vol. ethanol. Precipitated material was recovered by centrifugation, dissolved in 5 mM Na-acetate, 2.5 mM EDTA, 6 M urea (pH 5.6) (100 μl/A_{260}) and cold carrier 30 S proteins (100–200 μg) were added followed by 15 μg each of RNase T1 and RNase A. The mixture was placed in a dialysis bag and RNA was digested for 3 h at 37°C during dialysis against 2 × 200 vol. of 5 mM Na-acetate 2.5 mM EDTA, 6 M urea (pH 5.6). Protein–oligonucleotide complexes were precipitated from the contents of the dialysis bag by addition of 5 vol. acetone, and precipitates were resuspended in 8 M urea at 5–10 mg/ml and samples containing 100 μg of carrier 30 S proteins and labelled crosslinked proteins were analysed by two-dimensional polyacrylamide gel electrophoresis as in [11].

2.6. *Autoradiography*

After electrophoresis and staining with Coomassie brilliant blue to detect carrier 30 S proteins, gels were either dried for autoradiography, or prepared for fluorography as in [12]. Radioactive spots localised by autoradiography were excised, rehydrated in 2% acetic acid, digested overnight at 60°C with 0.4 ml 110 vol. H_2O_2 and their ^{35}S content measured by scintillation counting.

3. Results

3.1. *Optimum conditions for protein–RNA crosslinking*

Because of its relatively large size access of EDC to at least some carboxylic groups in ribosomal proteins is likely to be enhanced by conditions which lead to an open ribosomal structure, i.e., a low concentration of divalent cations. In addition since it is positively charged its access to the neighbourhood of the negatively-charged phosphate backbone of ribosomal RNA will be favoured by the absence of a high concentration of positively charged counter ions. For these reasons media containing low concentrations of Mg^{2+} and K^+ were used to study the characteristics of the crosslinking reaction. Cacodylate was chosen as the buffer for 3 reasons:

(i) Its low pK permits analysis of the reaction below pH 7;

(ii) Its use is expected to minimize side reactions involving activation of the buffer which could lead to modification of protein and/or RNA;

(iii) Most importantly, carbodiimides have long half-lives in cacodylate buffer [13] e.g., the half life of 1-cyclohexyl-3-(2 morpholino-4-ethyl)carbodiimide metho-*p*-toluene sulfonate in 0.1 M Na-cacodylate (pH 6) is 1 h at 30°C, and is increased to 5 h by reducing the buffer concentration to 0.01 M.

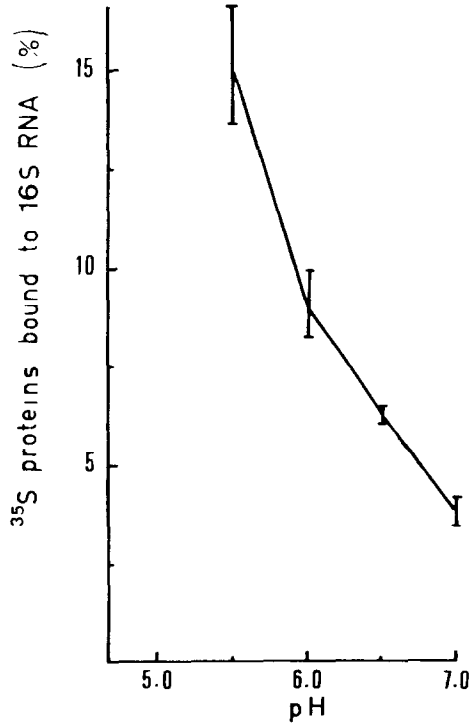

Fig.1. Effect of pH on protein–RNA crosslinking. ^{35}S-Labelled 30 S subunits were treated with 10 mM EDC in 0.5 mM Na-cacodylate; 0.5 mM Mg-acetate, 50 mM KCl buffers at the indicated pH values (section 2). After reaction, excess EDC was scavenged by addition of Mg-acetate 20 mM and methylamine 30 mM and incubation for 15 min at 20°C. Crosslinked products were then isolated and analysed as in section 2 and the % of ^{35}S-labelled proteins linked covalently to RNA was determined. The vertical bars indicate the spread of specific activity through the 16 S peaks in sucrose gradient analyses.

3.1.1. *Effect of pH*

The optimum pH for activation of a carboxyl group is close to its p*K*. Since the pH in the locality of protein carboxyl groups will vary as a function of their environments a broad pH optimum probably situated between pH 5 and 6 is expected. Fig.1 shows that the pH optimum for the crosslinking reaction is below 6. SDS–sucrose gradient sedimentation (section 2) of reaction products formed at low pH ($\leq$6) reveals the presence of large amounts of ^{35}S-labelled material sedimenting faster than 16 S rRNA (not shown). This material probably contains complexes formed by crosslinking of two or more proteins to the same 16 S rRNA and possibly complexes of the type RNA–protein–RNA formed by interparticle crosslinks. Its presence prevents accurate measurement of the specific radioactivity of material sedimenting at 16 S. To avoid this contamination without excessive loss of crosslinking efficiency further experiments were carried out at pH 6.5.

3.1.2. *Effect of cation concentration*

The effect of [Mg^{2+}] on crosslinking at pH 6.5 is shown in fig.2. As can be seen the crosslinking yield increases rapidly at $\lesssim$2 mM Mg^{2+}. Several effects of low [Mg^{2+}] probably contribute to this stimulation: unfolding of the 30 S particle improves the accessibility of protein carboxyl groups, reduced binding of Mg^{2+} phosphate groups raises the negative charge on 16 S rRNA which facilitates the approach of positively charged EDC, and reduces the local pH which increases the reactivity of carboxyl groups towards EDC. Work is in progress to determine whether or not the same 30 S proteins and the same aspartyl and glutamyl residues are involved in crosslinking at high and low [Mg^{2+}].

3.1.3. *Effect of EDC concentration*

Fig.3 shows that the overall efficiency of crosslinking is roughly proportional to [EDC] at >10 mM (pH 6.5). Control experiments have shown that high EDC concentrations do not lead to formation of large crosslinked complexes of the type observed in reaction products formed at pH <6 (section 3.1.1).

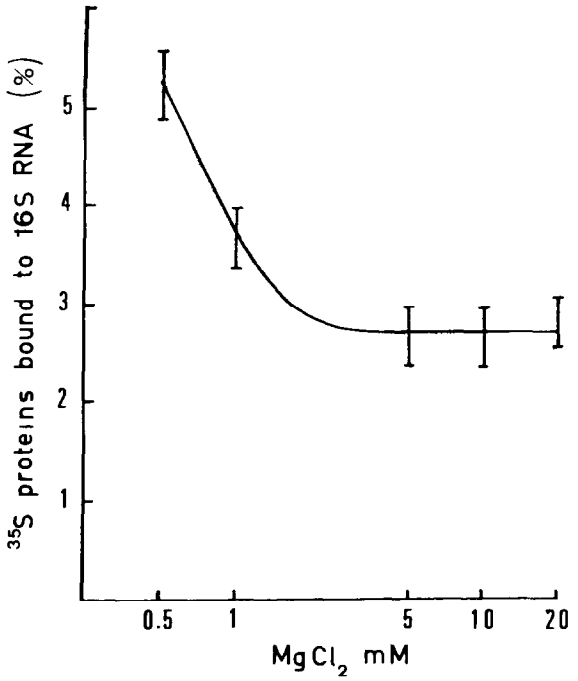

Fig.2. Effect of [Mg^{2+}] on protein–RNA crosslinking. ^{35}S-Labelled 30 S subunits were treated with 10 mM EDC in 0.5 mM Na-cacodylate, 50 mM KCl (pH 6.5) containing the indicated [$MgCl_2$]. Reaction products were analysed and the % of ^{35}S-labelled proteins crosslinked to RNA were calculated as in the legend to fig.1. The vertical bars have the same signification as in fig.1.

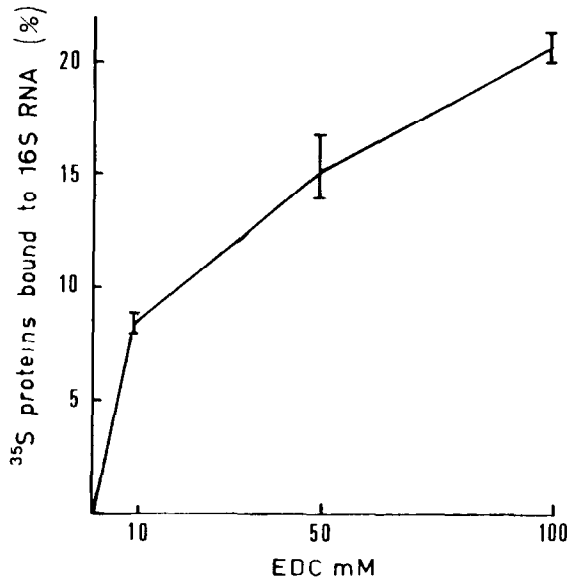

Fig.3. Effect of EDC concentration on protein–RNA crosslinking. ^{35}S-Labelled 30 S subunits were treated with the indicated [EDC] in 0.5 mM Na-cacodylate, 0.5 mM Mg-acetate, 50 mM KCl (pH 6.5). Reaction products were analysed and the % of ^{35}S-labelled protein bound to 16 S rRNA was calculated as in the legend to fig.1 (as Mg-acetate was present in the reaction mixtures, the true [EDC] during the reaction were 9, 40, and 99 mM). The vertical bars have the same signification as in fig.1.

3.2. *Analysis of crosslinked RNA–protein complexes*

The products of RNase digestion of crosslinked 16 S rRNA–protein complexes are 30 S proteins carrying small oligonucleotides and their electrophoretic properties are expected to differ slightly from those of the free proteins because of their increased negative charge (phosphate groups) and M_r. The two dimensional gel system used for their analysis (first dimension in 8 M urea (pH 4.5); second dimension in the presence of SDS) was chosen to minimize these differences. Comparison of the stained spots of carrier proteins and the radioactive spots revealed by autoradiography of the same gels permits tentative identification of the latter. To evaluate the crosslinking yield for each protein radioactive spots were cut out of the dried gel, and their radioactivity measured as in section 2. The results expressed as radioactivity/sulfur atom in the various proteins are shown in fig.4. Proteins S4, S5, S7, S13 and S18. Reaction with EDC introduces protein–protein as well as protein–RNA crosslinks. Because of the technique used to isolate protein–RNA complexes the presence of small protein–protein complexes in reaction products can be tolerated. However larger complexes of this type which are formed at high [EDC] are isolated with protein–RNA complexes and interfere with their analysis.

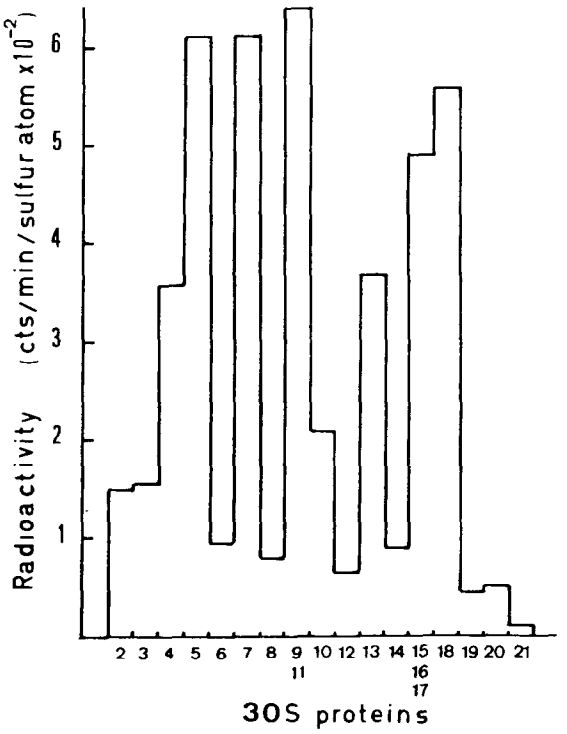

Fig.4. Efficiency of crosslinking of individual 30 S ribosomal protein. Radioactive spots detected by autoradiography of dried two-dimensional gels were cut out, rehydrated, treated with H_2O_2 and their ^{35}S contents counted as in section 2. Regions in the dried gels corresponding to the positions of 30 S proteins which did not contain sufficient radioactivity to produce visible spots on the autoradiograph were also cut out and treated in the same way. The radioactivity measured in each protein was normalised by division by the number of sulfur atoms in the protein. Protein S1 was not well resolved and the corresponding spot was not cut out, proteins S9/S11, and S15, S16, S17 were poorly resolved and were cut out of gels as single regions. The specific activity values for these regions shown in fig.4 were calculated for S9 (3 S atoms) and S15/S16 (1 S atom).

Further work is in progress to complete the identification of crosslinked proteins and to localise the sites of crosslinking in these proteins and in 16 S rRNA. This crosslinking method seems likely to yield extensive information about ribosome structure. It may also produce information concerning preferred RNA–protein interactions. The number of interactions between the carboxyl group and nucleic acid bases is limited in model systems in vitro [21,22]. EDC crosslinking studies may lead to identification of those interactions which occur in vivo.

Acknowledgement

The work described here has been supported by the Centre National de la Recherche Scientifique (ER 101), and the Delegation à la Recherche Scientifique et Technique (ACC no. 79.7.1045). We thank Dr D. H. Hayes in whose laboratory this work was performed for sustained encouragement and help in the manuscript preparation.

References

[1] Woese, C. R., Magrum, L. J., Gupta, R., Siegel, R. B., Stahl, D. A. Kop, J., Crawford, N., Brosius, J., Gutell, R., Hogan, J. J. and Noller, H. F. (1980) Nucleic Acids Res. 8, 2275–2293.

[2] Ehresmann, B., Reinbolt, J. and Ebel, J. P. (1975) FEBS Lett. 58, 106–111.

[3] Möller, K. and Brimacombe, R. (1975) Mol. Gen. Genet. 141, 343–355.

[4] Möller, K., Zwieb, C. and Brimacombe, R. (1978) J. Mol. Biol. 126, 489–506.

[5] Zwieb, C. and Brimacombe, R. (1979) Nucleic Acids Res. 6, 1775–1783.

[6] Bäumert, H. G., Sköld, S. E. and Kurland, C. G. (1978) Ew. J. Biochem. 89, 353–359.
[7] Oste, C. and Brimacombe, R. (1979) Mol. Gen. Genet. 168, 81–86.
[8] Rinke, J., Meinke, M. and Brimacombe, R. (1980) J. Mol. Biol. 137, 301–314.
[9] Millon, R., Olomucki, M., Le Gall, J. Y., Golinska, B., Ebel, J. P. and Ehresmann, B. (1980) Eur. J. Biochem. in press.
[10] Expert-Bezançon, A., Barritault, D., Milet, M., Guérin, M. F. and Hayes, D. H. (1977) J. Mol. Biol. 112, 603–629.
[11] Lutter, L. C., Ortanderl, F. and Fasold, M. (1974) FEBS Lett. 48, 288–292.
[12] Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88.
[13] Metz, D. H. and Brown, G. L. (1969) Biochemistry 8, 2312–2328.
[14] Kurzer, F. and Douraghi Zadeh, K. (1967) Chem. Rev. 67, 107.
[15] Lewis, S. D. and Shafer, J. A. (1973) Biochim. Biophys. Acta 303, 284–291.
[16] Goodfriend, T. L., Levine, L. and Fasman, G. D. (1964) Science 144, 1344–1346.
[17] Halloran, M. J. and Parker, C. W. (1966) J. Immunol. 96, 373–378.
[18] Combarnous, Y. and Hennen, G. (1974) FEBS Lett. 44, 224–228.
[19] Chang, S. E., Cashmore, A. R. and Brown, D. M. (1972) J. Mol. Biol. 68, 455–464.
[20] Hale, P., Woodward, R. S. and Lebowitz, J. (1980) Nature 284, 640–644.
[21] Hélène, C. (1977) FEBS Lett. 74, 10–13.
[22] Lancelot, G. and Hélène, C. (1977) Proc. Natl. Acad. Sci. USA 74, 4872–4875.