Structural differences between active and inactive 30 S ribosomal subunits revealed by RNA–protein crosslinking

Claude Chiaruttini, Michèle Milet and Donal Hayes*

Laboratoire de Chimie Cellulaire, Institut de Biologie Physico Chimique, 75005 Paris, France

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30 S protein–16 S rRNA crosslinking by reaction with 1-ethyl-3-dimethylaminopropylcarbodiimide is more efficient in the active than in the inactive form of the *E. coli* 30 S ribosomal subunit. This difference is particularly striking in the case of protein S8.

30 S ribosomal subunit *16 S rRNA* *Crosslinking* *Protein S8*

1. INTRODUCTION

Escherichia coli 30 S ribosomal subunits exposed to low concentrations of Mg^{2+} during their isolation and purification display low activity in in vitro protein synthesis and in tests of functions related to this process, but can be reactivated by brief incubation at 30–50°C in the presence of 10 mM Mg^{2+} (lysozyme synthesis in vitro [1]; non-enzymatic binding of aminoacyl tRNA [2,3]; association with 50 S subunits [4,5]). In contrast, 30 S subunits isolated in the presence of high concentrations of Mg^{2+} possess high activity in vitro (lysozyme synthesis [1]; association with 50 S subunits [6]). Reactivation of inactive 30 S subunits by heating in appropriate media indicated that the active and inactive forms of this particle differ in conformation and this conclusion has been supported by the demonstration of differences in the chemical reactivity of specific residues in the proteins and 16 S rRNAs of active and inactive subunits (reaction of protein -SH groups with *N*-ethylmaleimide [7] and of guanine residues in 16 S rRNA with kethoxal [8]). The latter study showed that inactive 30 S subunits contain twice as many (≃40) reactive guanine residues as active subunits, and that the residues which are reactive in active subunits become 10–20-times more reactive in inactive subunits. These results were interpreted as evidence that the inactive 30 S subunit possesses a more 'open' structure than the active 30 S subunit as a result of conformational changes in 16 S rRNA and/or disruption of protein–RNA interactions and/or conformational changes in 30 S ribosomal proteins [8]. Recent observations [9] have demonstrated the existence of conformational differences between 16 S rRNA in inactive and reactivated 30 S subunits. Here we present evidence for the existence of differences in protein–RNA proximity relationships in the two forms of the 30 S subunit.

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

2.1. *Reagents*

Ribonuclease T1 (EC 3.1.27.3) and ribonuclease A (EC 3.1.27.5) were obtained from Sigma, 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC) from Merck and carrier-free $H_2{}^{35}SO_4$ from Amersham.

2.2. *Buffers*

Buffers used were: (1) 10 mM Tris–HCl, 10 mM Mg acetate, 60 mM NH_4Cl, 6 mM 2-mercaptoethanol (pH 7.4); (2) 10 mM Tris–HCl, 10 mM Mg

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acetate, 400 mM NH_4Cl, 6 mM 2-mercaptoethanol (pH 7.4); (3) 10 mM Tris–HCl, 0.1 mM Mg acetate, 60 mM NH_4Cl, 6 mM 2-mercaptoethanol (pH 7.4); (4) 10 mM triethanolamine–HCl, 10 mM Mg acetate, 50 mM KCl, 10 mM 2-mercaptoethanol (pH 7.4); (5) 10 mM triethanolamine–HCl, 10 mM Mg acetate, 400 mM NaCl, 10 mM 2-mercaptoethanol (pH 7.4); (6) 10 mM triethanolamine–HCl, 0.1 mM Mg acetate, 50 mM KCl, 10 mM 2-mercaptoethanol (pH 7.4); (7) 10 mM triethanolamine–HCl, 20 mM Mg acetate, 60 mM NH_4Cl, 10 mM 2-mercaptoethanol (pH 7.4).

2.3. *Preparation of ribosomes and ribosomal subunits*

E. coli MRE 600 was grown, labelled with ^{35}S, and harvested as in [10]. Subsequent operations were carried out at 0–4°C using triethanolamine-containing buffers to isolate subunits for crosslinking experiments and Tris-containing buffers for all other preparations. Labelled cells were disrupted by grinding with alumina and unlabelled cells by the use of a French pressure cell as in [1].

(i) Active subunits: alumina ground cell homogenates were extracted with buffer 1 or 4; extracts of non-radioactive cells were prepared from suspensions of bacteria in these buffers. Ribosomes were isolated from the crude extracts by centrifugation (150000 × *g*, 3 h) and dissociated by resuspension in buffer 2 or 5. Subunits were separated by centrifugation of the suspensions on 5–20% linear sucrose gradients prepared in the same buffers and concentrated from pooled gradient fractions by precipitation with polyethylene glycol (see section 1 for details).

(ii) Inactive subunits: as above, except that buffers 3 or 6 were used for dissociation of ribosomes and separation of subunits.

Subunit pellets prepared by either method were resuspended in buffer 1 or 4 in the case of active subunits and in buffer 3 or 6 in the case of inactive subunits at 200 A_{260}/ml and stored at −70°C. The specific activity of ^{35}S-labelled 30 S subunits was 3.7×10^7 cpm/A_{260}.

2.4. *Preparation of 30 S ribosomal proteins*

30 S subunits were treated with acetic acid [11]. Proteins were stored at 20°C in solution in 8 M urea, 1 mM dithiothreitol at 10 mg/ml.

2.5. *RNA–protein crosslinking, purification of crosslinked 30 S subunits and isolation of 30 S protein–16 S rRNA complexes*

Active and inactive 30 S subunits were crosslinked under the same conditions using a scaled down version of the procedure in [12]. 30 S subunit suspensions in buffers 1 or 4 in the case of active subunits and in buffers 3 or 6 in the case of inactive subunits (10 A_{260}, spec. act. 5×10^6 cpm/A_{260}) prepared by mixing samples of ^{35}S-labelled and unlabelled subunits were dialysed for 2 h and then overnight at 4°C against 500 vols crosslinking buffer (1 mM Na cacodylate, 0.5 mM $MgCl_2$, 50 mM KCl, pH 6.5). The concentration of 30 S subunits in the dialysed suspensions was adjusted to 10 A_{260}/ml with crosslinking buffer, EDC was added to a final concentration of 50 mM, the pH was adjusted to 6.5 with 0.1 N HCl and the mixtures were stirred at 25°C for 25 min. Reaction was stopped by adding 0.1 vol. of 1 mM Na cacodylate, 0.22 M Mg acetate, 1.74 M NH_4 acetate, 1.56 M NH_4Cl (pH 6.5) and incubating the resulting mixture at 25°C for 1 h. Monomeric crosslinked 30 S subunits purified from the reaction products by sucrose gradient centrifugation (removal of dimers and higher aggregates) were dissociated by treatment with SDS at 40°C and free 16 S rRNA and covalently linked 30 S protein–16 S rRNA complexes were isolated together by sedimentation of the dissociation products on SDS-containing sucrose gradients (see [12] for details).

2.6. *Analyses of crosslinked RNA–protein complexes*

^{35}S-labelled crosslinked protein–16 S rRNA complexes recovered from pooled SDS sucrose gradient fractions (2 A_{260}, 0.75×10^6 cpm ^{35}S in the case of complexes derived from active subunits and 2 A_{260} and 0.53×10^6 cpm ^{35}S in the case of complexes derived from inactive subunits) were dissolved in 10 mM Tris–HCl, 1 mM EDTA, 6 mM 2-mercaptoethanol. RNase T1 (13 units) and RNase A (3.2 μg) were added and the mixtures were incubated at 37°C for 1 h. Protein–oligonucleotide complexes were precipitated from the reaction mixture by addition of 5 vols acetone, collected by centrifugation, reprecipitated several times from solution in 8 M urea and finally dissolved in 8 M urea, 1 mM dithiothreitol (60 μl).

Unlabelled total 30 S proteins (50 μg) were added and the mixtures were analyzed by two-dimensional polyacrylamide gel electrophoresis [13]. After electrophoresis gels were stained with Coomassie brilliant blue, dried, and autoradiographed.

Table 1

Association of active and inactive 30 S subunits with active 50 S subunits

Subunit mixture	Percentage of input A_{260} recovered in 70 S	
	Before heat activation	After heat activation
Active 30 S + active 50 S	40	85
Inactive 30 S + active 50 S	8	70

Active 50 S subunits (10 A_{260} nm) and active or inactive 30 S subunits (5 A_{260}) were mixed in buffer 7 (1 ml) at 0°C and samples (200 μl, 3 A_{260}) were incubated for 30 min at 0°C (control) or 40°C (heat activation) and analysed by centrifugation on 5–20% linear sucrose gradients prepared in buffer 7 (18000 rpm, 17 h, 4°C, SW25 rotor with 3 place adapters [15]). The distribution of A_{260} nm absorbance in the centrifuged gradients was recorded and the percentage of input A_{260} present as 70 S particles was calculated

3. RESULTS

3.1. *Capacity of active and inactive 30 S subunits to form 30 S–50 S couples*

It has been shown that the capacity to form 30 S–50 S couples is one of the most stringent criteria for complete activity of ribosomal subunits [5,6,14]. The results in table 1 show that, prepared as described here, active and inactive 30 S subunits differ by a factor of 5 in their content of particles competent for couple formation and that most of the incompetent particles present in either preparation acquire the capacity to form 30 S–50 S couples after heat activation.

3.2. *Proteins crosslinked to 16 S rRNA in active and inactive 30 S subunits*

Under the reaction conditions used here higher yields of crosslinked 16 S rRNA–protein complexes are obtained from active than from inactive 30 S subunits (7.5 *vs* 5.3% of total 30 S protein). Qualitative comparison of the crosslinked products (fig.1,2) shows that this difference in

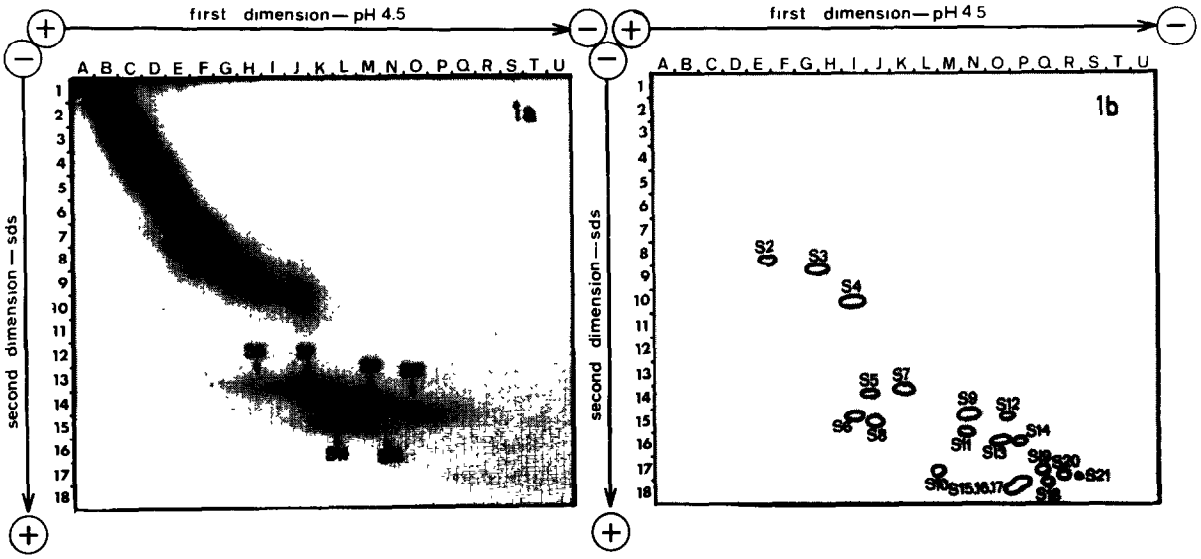

Fig.1. Two-dimensional polyacrylamide gel electrophoretic analysis of crosslinked protein–oligonucleotide complexes isolated from ^{35}S-labelled inactive 30 S subunits. (a) Autoradiograph of ^{35}S-labelled protein–oligonucleotide complexes. Darkened zones corresponding to proteins in the group S10, S15, S16, S17, S18, S19, S20, S21 were faintly visible in the original autoradiograph; (b) positions of stained spots of unlabelled control 30 S proteins in the dried gel slab.

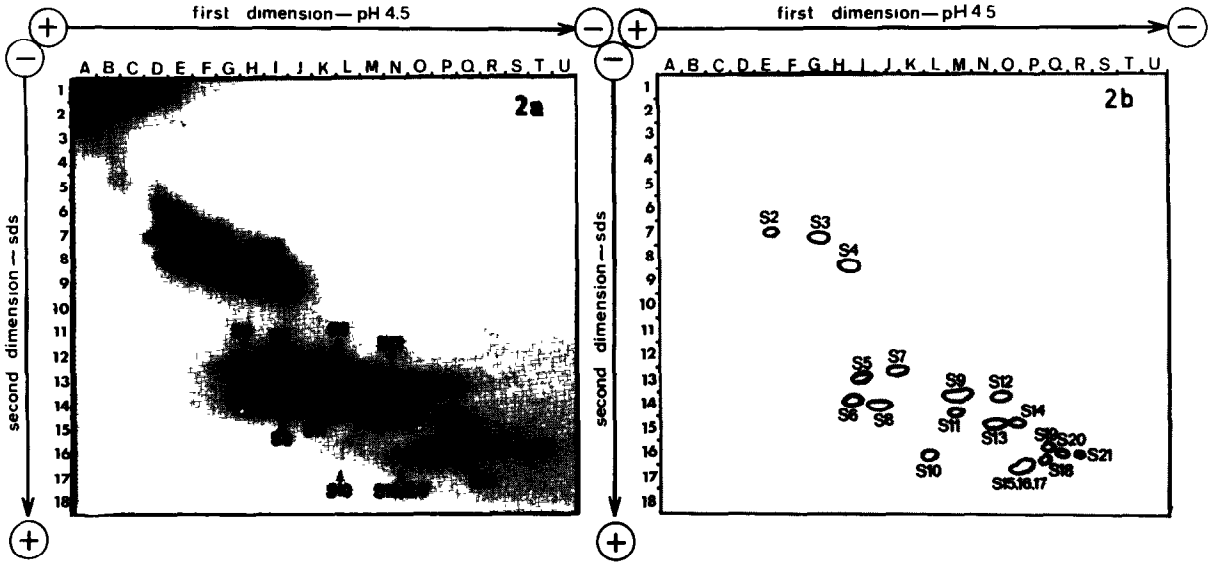

Fig.2. Two-dimensional polyacrylamide gel electrophoretic analyses of crosslinked protein oligonucleotide complexes isolated from ^{35}S-labelled active 30 S subunits. (a) Autoradiograph of ^{35}S-labelled protein–oligonucleotide complexes, (b) positions of stained spots of unlabelled control 30 S proteins in the dried gel slab.

crosslinking yield is not uniformly distributed among 30 S proteins. Complexes containing proteins S5, S7, S9, S11, S12 and S13 are formed in similar relative amounts by reaction of either form of the 30 S subunit with EDC whereas formation of complexes containing proteins S10, S15, S16, S17, S18, S21 and especially S8 is more efficient in the case of active than of inactive subunits.

4. DISCUSSION

Protein–RNA crosslinking by EDC proceeds via activation of protein carboxyl groups which then react with neighbouring amino groups in nucleic acid bases. For the latter reaction to be possible the amino and activated carboxyl groups must be in close proximity (amide C–N bond length 1.3 Å).

The lower overall yield of protein–RNA crosslinking by EDC in inactive than in active 30 S subunits suggests that the conformational change which occurs during inactivation increases the average separation between RNA and protein in the 30 S particle and may therefore involve decrease or loss of interaction between these two components of the ribosomal subunit. It has been proposed [8] that the increased accessibility of 16 S rRNA to kethoxal in inactive *vs* active 30 S subunits may be explained at least in part by disruption of protein–RNA interactions.

The difference in efficiency of crosslinking of protein S8 in active and inactive 30 S subunits under the experimental conditions used here is striking. The binding site for S8 in 16 S rRNA has been defined with considerable accuracy [16–18] as a stable base-paired structure formed by interaction of sequences 586–602 and 634–652 in 16 S rRNA. Two studies have produced evidence for conformational differences between 16 S rRNA in active and inactive 30 S subunits involving nucleotides close to these sequences: (i) guanosine at position 605 in 16 S rRNA which does not react with kethoxal in active 30 S subunits, is reactive in inactive subunits [8]; (ii) psoralen crosslinking between residues near position 500 and the 3-terminus of 16 S rRNA, indicative of interaction between these regions, is much less efficient in active than in inactive 30 S subunits [9].

Identification of the site of EDC-induced crosslinking of S8 to 16 S rRNA in active 30 S subunits may permit correlation of these results.

ACKNOWLEDGEMENTS

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