NATIVE AND DENATURED STRUCTURES WITHIN THE 5'-REGION OF 16 S RIBOSOMAL RNA FROM *ESCHERICHIA COLI*

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1. Introduction

Native and denatured forms of small RNA molecules have been well-characterised, especially for the 5 S RNA of *Escherichia coli* [1,2]. These RNA forms can be readily distinguished by their electrophoretic properties [2], and by their ribosomal protein binding capacities [3]. There have also been reports of different conformational forms of the larger bacterial ribosomal RNAs that can be separated electrophoretically. However, in most of these studies the RNA was examined in the presence of EDTA and no criterion for the nativity of a conformation was applied, such as the specific interaction with ribosomal proteins [4–7]. Since it is known that magnesium ions are essential for the 'native' conformations of the RNA sites of ribosomal proteins [8,9], these results probably only reflect different denatured states of 16 S RNA.

In the present work, we show that RNA extracted by a phenol-dodecylsulphate procedure contains a mixture of three conformational forms that can be resolved electrophoretically in the presence of magnesium. We have denoted them 'native' (N), intermediate (I) and denatured (D) forms of 16 S RNA. The 'native' form was distinguishable from the denatured form in that it contained the complex RNA tertiary structure in the 5'-half of 16 S RNA that constitutes the RNA binding site of protein S4 (S4-RNA). The latter could be isolated, directly, by digestion of the 16 S RNA with carrier-bound pancreatic ribonuclease. The native and denatured forms also showed different binding properties with the fluorescent dye ethidium bromide.

Although 16 S RNA isolated by the acetic acid–urea procedure [11] could not be resolved into different conformers electrophoretically, ribonuclease digestion studies demonstrated that, as for phenol-extracted RNA, only a minor fraction of the freshly-prepared RNA molecules contained the native RNA tertiary structure in their 5'-ends.

2. Materials and methods

2.1. *16 S RNA, S4-RNA, protein S4 and an S4–16 S RNA complex*

16 S RNA was prepared from 30 S subunits of *Escherichia coli*, strain A19, by a standard phenol procedure described earlier [10], except that 0.5 mM magnesium chloride and 0.05% dodecylsulphate were included in the first phenol-extraction step. 16 S RNA was also prepared by the acetic acid–urea procedure of Hochkeppel et al. [11]. S4-RNA was obtained from renatured 16 S RNA (see below) using carrier-bound pancreatic ribonuclease as described earlier [12]. Protein S4 was prepared by a standard procedure [13] in collaboration with Dr E. Schiltz.

The protein S4-16 S RNA complexes were prepared as described earlier such that the unbound protein was separated from the complex [8].

2.2. *Denaturation and renaturation of 16 S RNA*

16 S RNA was denatured by dissolving 60 μg 16 S RNA in 20 μl TMK buffer (30 mM Tris–HCl, pH 7.4, 20 mM Mg chloride, 0.35 M KCl and 6 mM 2-mercaptoethanol), incubating for 5 min at 60°C and rapidly cooling in liquid nitrogen. The RNA was renatured by incubating the RNA solution at 60°C for 5 min and subsequently maintaining it at 40°C for 55 min and then cooling slowly in a water bath to 0°C. These procedures closely correspond to those used for denaturing and renaturing 5 S RNA [1].

2.3. *Compound agarose–polyacrylamide gel electrophoresis*

The RNA was electrophoresed in gels containing 0.5% agarose and 3% polyacrylamide as described earlier [14]. The running buffer was 40 mM Tris-acetate, pH 8, and 5 mM Mg acetate. The gel was maintained at 1°C by a cryostat; it was prerun. The RNA was electrophoresed for 8 h at 35 V and 15 mA. It was stained for RNA with toluidine blue and exclusively for protein with Coomassie brilliant blue [8].

2.4. *Fluorescence measurements*

Binding of ethidium bromide to 16 S RNA was monitored at 20°C by its fluorescence in a single photon counting unit essentially as described earlier [16]. Excitation and emission wavelengths were, respectively, 540 nm and 610 nm. The affinity constant of the dye for the RNA and the number of intercalating sites were deduced from the Scatchard equation [15] assuming that the sites are independent:

$$\frac{r}{c} = Kn - Kr,$$

where r is the concentration of the dye bound per nucleotide, n is the number of binding sites per nucleotide, c is the concentration of free dye, K is the affinity constant of the complex. A linear plot of r/c versus r giving a slope of $-K$ and an intercept on the r axis of n, was drawn. The biphasic Scatchard plots were interpreted in terms of two independent sets of independent sites; each set was assumed to follow the preceding Scatchard equation. The number of ethidium bromide binding sites was calculated assuming that the 16 S RNA contains 1600 nucleotides (e.g. [22]). All experiments were done in TMK reconstitution buffer (see above) or in TEN buffer (20 mM Tris–HCl, pH 7.4, 5 mM EDTA and 40 mM NH_4 chloride). To 1.5 μg 16 S RNA in 1 ml buffer, increasing amounts of ethidium bromide (0–10 μg) were added gradually. Three independent measurements were done for each point. Appropriate correction for serial dilution of the dye was applied.

3. Results

When the phenol-16 S RNA was electrophoresed in compound polyacrylamide–agarose gels, two or three distinct bands were resolved that could be interconverted by heat treatment. The results are shown in fig.1. In denatured samples (heated and rapidly cooled) two bands were resolved (Gel a); a major component (D), slower migrating and diffuse and a minor sharper band (I). Renaturing this RNA at 40°C and 60°C (Gels b and c, respectively) produced in addition to these two components a faster migrating component (N). The reversibility of these conformational transitions was demonstrated by denaturing the renatured sample by heating to 60°C and rapidly cooling; the resultant band pattern in Gel d is indistinguishable from that of the denatured sample in Gel a. When the RNA was extensively dialysed against water the RNA migrated exclusively in band D (see also [9]). When the renatured RNA was treated with EDTA, and electrophoresed in the presence of EDTA, a broad diffuse RNA band was observed (Gel e). The broadness of the latter band suggests that multiple denatured conformations may co-exist. In order to show that there was no concentration dependence of the migration of the RNA components in Gels a–d, samples in the range 30–100 μg RNA were co-electrophoresed and the migration differences shown in fig.1 were reproduced.

Freshly prepared acetic acid–urea extracted 16 S RNA, that had not been heated above 4°C, migrated similarly to band N in Gel a except that marked trailing occurred back to the position of band D and significant amounts of aggregates were observed. Incubating the RNA for 1 h at 30°C or 40°C yielded a

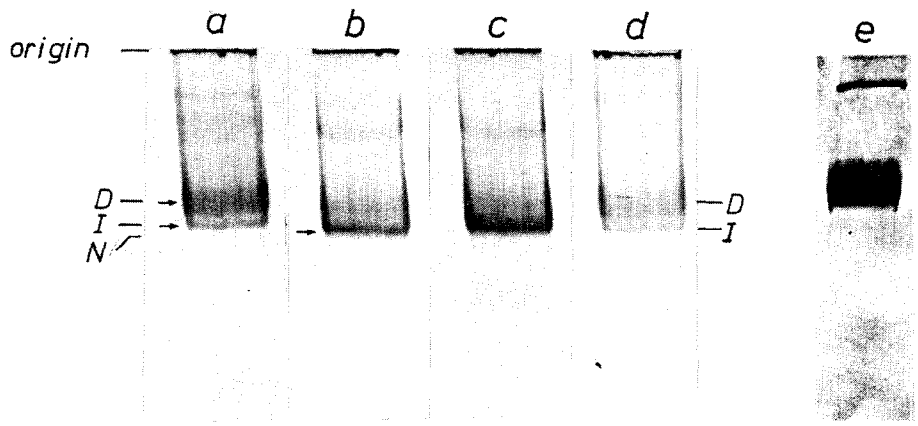

Fig.1. Electrophoretic resolution of 16 S RNA conformations. 67 μg denatured phenol-RNA was preincubated at different temperatures in 20 μl TMK buffer and then electrophoresed in a polyacrylamide–agarose gel containing 40 mM Tris-acetate, pH 8, 5 mM Mg acetate (see Methods). Samples were preincubated at (a) 0°C, (b) 40°C and slowly cooled to 0°C, (c) 60°C and slowly cooled to 0°C and (d) 60°C and rapidly cooled in liquid nitrogen. The denatured (D), intermediate (I) and native (N) forms are indicated. In sample (e) the RNA was dialysed against 20 mM Tris–HCl, pH 7.4, 2.5 mM Na EDTA and 50 mM Na chloride preincubated at 60°C, slowly cooled and electrophoresed in the following buffer: 20 mM Tris–acetate, pH 8, 2.5 mM Na EDTA and 50 mM NH_4 chloride. The sharp band migrating near the origin is an aggregate.

band that was indistinguishable from band N. However, even after this treatment significant amounts of aggregates were present in the gel.

3.1. *Criteria for the 'native' RNA conformation*

3.1.1. S4-protein binding

The RNA conformations were tested for nativity by the criterion of specific protein binding. Protein S4 was incubated at 0°C with (a) denatured, (b) renatured 16 S RNA. The results are shown in fig.2. For the denatured sample (Gel a/b) no bound protein was detected on band D, and only very weak binding occurred to the intermediate band I that cannot be seen in fig.2b. Optimal protein binding occurred, however, to the renatured RNA (Gel c/d).

3.1.2. Isolation of the S4-RNA site

We checked, directly, for the presence of the correct tertiary structure in the 5′-region of the 16 S RNA in the different RNA conformations by digesting phenol-RNA, that had been renatured at increasing temperatures, with carrier-bound pancreatic ribonuclease. The results, shown in fig.3, demonstrate that as the optimal renaturation conditions are approached, the yield of S4-RNA increases.

Acetic acid–urea 16 S RNA was also treated in the same way as the phenol–RNA. Very similar results were obtained in that increasing yields of S4-RNA were produced at increasing temperatures. Gels e and f show the S4-RNA yields after incubating at 0°C (Gel e) and 60°C (Gel f).

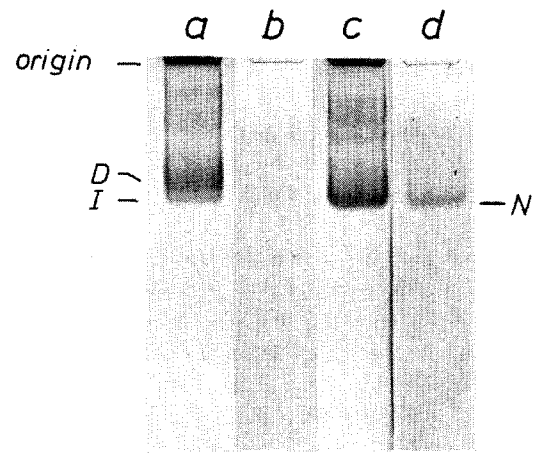

Fig.2. Protein S4 binding. Denatured 16 S RNA was incubated with protein S4 for 1 h at 0°C (see Methods) and stained (a) for RNA and (b) for protein. Renatured 16 S RNA was incubated with S4 and 0°C and stained (c) for RNA and (d) for protein S4.

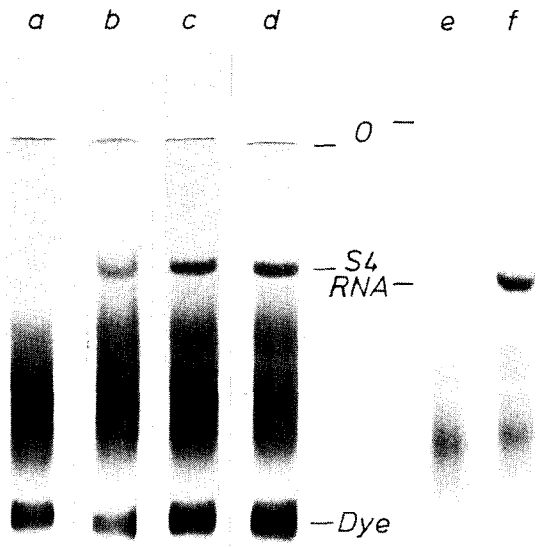

Fig.3. Degradation of 16 S RNA after incubating at different temperatures. Phenol-RNA was incubated in TMK buffer at (a) 0°C, (b) 20°C, (c) 40°C and (d) 60°C for 1 h, slowly adjusted to 20°C and degraded with carrier-bound pancreatic ribonuclease. The hydrolysate was electrophoresed in a 5% polyacrylamide gel containing 20 mM Tris–HCl, pH 7.4, 5 mM Mg chloride, and stained for RNA. In sample (e) and (f) acetic acid–urea RNA was dissolved in TMK buffer, preincubated at 0°C and 40°C, respectively, adjusted slowly to 20°C and digested as for phenol-RNA.

The yields of the S4-RNA obtained from both RNAs were variable. If the 16 S RNAs contained no hidden breaks and care was taken not to overdigest, then average yields were in the range 20–40% at 0°C increasing to 80–100% at 60°C.

3.1.3. Binding studies with ethidium bromide

We investigated whether the RNA conformations of the phenol-RNA could be distinguished by some physical-chemical property. A number of properties were examined. Whereas no clear differences were detected in ultraviolet hyperchromicity effects, or in sedimentation rate, small but significant differences were found in the capacities of the two conformations to enhance the fluorescent properties of bound ethidium bromide.

Equal amounts of the two conformations were titrated with ethidium bromide, and Scatchard plots were prepared. A similar experiment was performed with EDTA-treated 16 S RNA. The results are shown in fig.4. The number of dye-binding sites, and their respective binding constants are listed in table 1.

The results show that whereas the two conformations have the same number of dye-binding sites, the native form has the smaller number of strong binding sites, namely 40, compared with 51 in the denatured

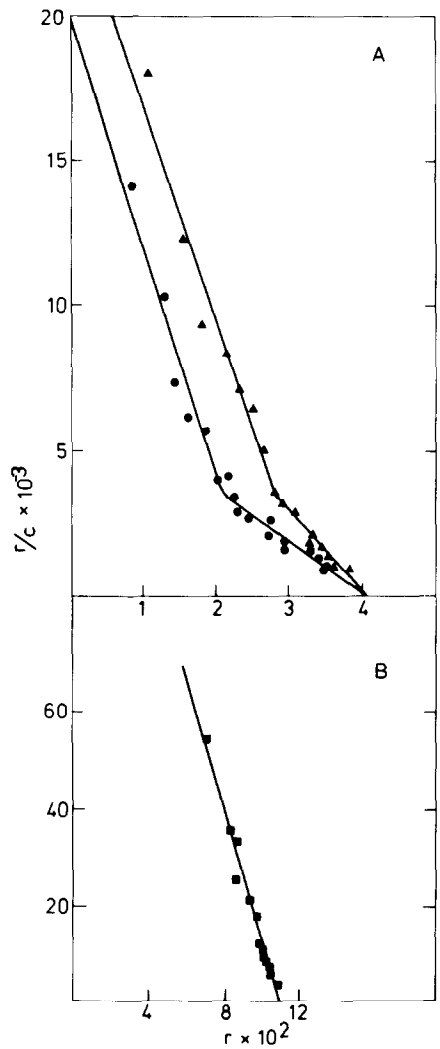

Fig.4. Scatchard plots of ethidium bromide binding to phenol-16 S RNA. A: (-●-●-) native 16 S RNA, (-▲-▲-) denatured 16 S RNA; and B: (-■-■-) EDTA-treated 16 S RNA. r = concentration of dye bound per nucleotide and c = concentration of free dye.

Table 1
Ethidium bromide binding sites in 16 S RNA

16 S RNA	Strong sites		Weak sites	
	Sites/16 S RNA molecule	K	Sites/16 S RNA molecule	K
Native	40	8×10^5	24	1.7×10^5
Denatured	51	8×10^5	13	3×10^5
EDTA-treated	176	14×10^5		

form. Both have considerably less than the EDTA-treated RNA which has 176. These results suggest that the native conformation contains a more compactly folded structure.

A comparison with the acetic acid–urea RNA was not practicable because it always contained significant amounts of aggregates that might have exhibited different ethidium bromide binding properties. Although denatured phenol-RNA readily forms aggregates, none were detected by analytical ultracentrifugation at the RNA concentrations and ionic conditions used in the above study.

3.2. *The effect of protein S4 on the RNA conformation*

It has been argued that protein S4 may induce the 'native' RNA conformation in its 16 S RNA binding site [17]. In order to test whether protein S4 increases the yield of the native RNA conformation the 16 S RNA was renatured in the presence and absence of protein S4. For both samples the ratios of bands N/D (see figs 1 and 2) were estimated at 1 (± 0.5) : 1 for a 40°C renaturation and 3.5 (± 0.3) : 1 for a 60°C renaturation. Therefore, no change in the amount of renaturation occurred, and it was concluded that protein S4 does not appreciably induce the binding conformation in the RNA.

4. Discussion

We demonstrated earlier that the specific RNA-binding site of protein S4 is compactly folded into a tertiary structure, and that this RNA region can be isolated from free 'native' 16 S RNA by treatment with carrier-bound ribonuclease A [12,20]. Evidence has also been provided for the following:

(i) The RNA is folded into a tertiary structure as a consequence of interactions between widely separated sequence regions [18,19].
(ii) This tertiary structure is ribonuclease A resistant [12,18].
(iii) The structure requires the presence of magnesium ions [8].
(iv) The conformations of the RNA region and the protein are not markedly altered on complex formation [12].
(v) The shape of the RNA corresponds to a flattened disc structure [20].

In the present study we have shown that this structure can be reversibly renatured by heat treatment. Both the latter result, and those listed above demonstrate, unequivocably, that the main conformational change in the 16 S RNA occurs on heating and precedes protein S4 binding. This result is in agreement with those of a similar study by Muto and Zimmermann [9], but disagrees with the conclusion of Hochkeppel and Craven [21] that protein S4 induces a slower migrating conformation in the 16 S RNA.

In this study, we have concentrated on the more stable and more readily characterisible region in the 5′-half of the 16 S RNA, but have not investigated whether any tertiary structure that may exist in the 3′-half of the 16 S RNA is reversibly disruptible. There is, however, some evidence that within this RNA region interactions do occur between widely separated sequence regions in both the free RNA [22] and the 30 S subunit [23]. Moreover, Muto and Zimmermann [9] have also indicated that the RNA structure in this region can be readily disrupted such that protein S7 cannot bind. This may provide an explanation for the intermediate (I) band in fig.1 in that the 16 S RNA structure is only renatured in one of its halves.

Recently, a metastable RNA conformation was characterised by Craven et al. [11] in the acetic acid–urea RNA that binds proteins which do not normally attach directly to phenol-RNA. This conformation may be related to pH-dependent metastable RNA structures previously described [24,25]. Our results suggest that the 5′-region of this RNA closely resembles that of phenol-RNA; when freshly prepared it is denatured in a large fraction of the molecular population and it can be renatured by heating. Therefore, any fundamental differences in the conformations of the two 16 S RNAs probably reside in the central or 3′-regions.

The RNA binding site of protein S4 is highly conserved in many bacteria [26], and since it is partially or completely disrupted in denatured 16 S RNA, it is very likely that such native and denatured conformational forms will be found in other bacterial RNAs. In this context, there is also an important correlation with eukaryotic ribosomal RNA structure. Singh and Keller [27] demonstrated that two interconvertible conformations of 28 S RNA could be resolved on MAK columns and that the faster sedimenting component was favoured at higher magnesium concentrations. The two forms also exhibited small differences in their physico-chemical properties. This suggests that such magnesium-dependent structures may be a general feature of all small and large ribosomal RNAs.

In conclusion, the results presented demonstrate that the 5′-region of 16 S RNA can exist in both native and denatured forms in the presence of magnesium ions, independent of the preparation method, and that these two forms can be distinguished by their protein S4 binding capacity, their sensitivity to ribonuclease degradation and by their fluorescent properties. In these respects, this part of the 16 S RNA behaves analogously to the smaller 5 S RNA.

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