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Cleavage of a 23S rRNA pseudoknot by phenanthroline–Cu(II)

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ABSTRACT

Studying the intricate folding of rRNA within the ribosome remains a complex problem. Phenanthroline–Cu(II) complexes cleave phosphodiester bonds in rRNA in specific regions, apparently especially where the rRNA structure is constrained in some fashion. We have introduced phenanthroline–copper complexes into 50S Escherichia coli ribosomal subunits and shown specific cleavages in the regions containing nucleotides 60–66 and 87–100. This specificity of cleavage is reduced when the ribosome is heated to 80°C and reduced to background when the ribosomal proteins are extracted and the cleavage repeated on protein-free 23S rRNA. It has been suggested that nucleotides 60–66 and 87–95 in E.coli 23S rRNA are involved in a putative pseudoknot structure, which is supported by covariance data. The paired cleavages of nearly equal intensity of these two regions, when in the ribosome, may further support the existence of a pseudoknot structure in the 100 region of 23S rRNA.

INTRODUCTION

Unraveling the tertiary structure of rRNA within the ribosome remains a perplexing problem. Recent efforts have utilized a number of techniques to ascertain accessible regions of rRNA (1–5), relative positions of specific regions of rRNA (6–8), and detailed interactions between regions of rRNA and various ligands, such as mRNA (9–13), tRNA (14–17), nascent protein and the ribosome (18,19) and antibiotics (20,21). Even though much information has been accumulated, details of the way in which the rRNA is folded within the ribosomal subunits remains obscure.

To help clarify these structures, we have used 1,10-phenanthroline–Cu(II) to cleave the rRNA. Phenanthroline has been used by others for RNA cleavage (22–24). We have covalently tethered it to tRNA, mRNA and DNA oligonucleotides to probe the positioning of these ligands (13.25–27) on the ribosome.

In this study we report the site-specific cleavage of a proposed pseudoknot region in Domain I of 23S rRNA. This pseudoknot was initially postulated by Leffers et al. (28) and further by Gutell et al. (29) using covariance data. We found that untethered phenanthroline, in the presence of Cu(II) and a reducing reagent, cleaves nucleotides 60–66 and 87–100 robustly and in a highly specific manner, when the rRNA was in the ribosome. When the rRNA was extracted from ribosomal proteins, some cleavage still occurred in the 87–100 region, but the cleavage in the 60–66 region disappeared. When the rRNA was heated to denature the structure, cleavage in both regions was reduced to background levels.

These results suggest that this portion of 23S rRNA, in the ribosome, has a structure which causes nucleotides in the 60–66 and 87–100 region to be very open to intercalation. This open structure, occurring mainly in the intact ribosome, is exceptionally susceptible to cleavage by untethered phenanthroline. This structure appeared to change markedly when the rRNA was extracted, and even more significantly when the rRNA was heated.

MATERIALS AND METHODS

Ribosome preparation

Ribosomes were harvested from early log phase Escherichia coli strain MRE600 using the protocol outlined by Tam et al. (30) and Lodmell et al. (31)

Isolation of 23S rRNA

Two volumes of cold ethanol were added to thawed 50S subunits. The subunits were stored in buffer (10 mM Tris–Cl pH 7.5, 100 mM KCl and 1.5 mM MgCl2). The cloudy solution was centrifuged for 20 min at 13 000 r.p.m. at 4°C and the supernatant aspirated. The resulting pellet was dissolved in 150 l CHCl3 extractions. Addition of 300 µl cold ethanol to the aqueous layer precipitated the protein-devoid rRNA. Centrifugation for 20 min at 13 000 r.p.m. at 4°C produced a pellet of rRNA. The supernatant was aspirated, the pellet dried in vacuo and dissolved in storage buffer to a final concentration of 625 µM.

Synthesis of modified phenanthrolines

5-idoacetamido-1,10-phenanthroline was synthesized by the method of Sigman et al. (32). The identity of the product was confirmed by 1H NMR (data not shown). 5-acylamide-1,10-phenanthroline (AoP) and N-acetyl-2-aminomethyl-1,10-phenanthroline (MoP) were synthesized by the method described by G.W.Muth and C.M.Thompson (in preparation). 5-nitro-1,10-phenanthroline was obtained from GFS Chemical Company.

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Cleavage reaction with free phenanthroline

In a 50 µl reaction volume water, 1× cleavage buffer (40 mM Tris–Cl pH 7.5, 150 mM KCl and 15 mM MgCl₂), 100 µM Cu(II) and 50 µM phenanthroline were combined. Intact ribosomal subunits or extracted rRNA (either native or heat denatured; 80°C, 1 h) were added (1 µM), agitation followed by a quick spin insured a homogenous solution. The addition of a reducing agent (mercaptotropionic acid 1 mM or ascorbate 1 mM) commenced the cleavage reaction which was conducted for 1 h at 37°C. If the cleavage reaction was conducted on extracted 23S rRNA addition to cleavage products. The pellet was recovered by centrifugation for 20 min at 13 000 r.p.m. at 4°C. If the cleavage reaction was conducted on 50S ribosomal subunits, the extraction procedure outlined above was conducted at this point. The supernatant was aspirated, the pellet dried in vacuo and suspended in 25 µl water to produce a solution of ~2.0 µM rRNA. This concentration was confirmed by UV absorbance readings at 260 nm.

Primer extension of cleavage sites

Primer extension of cleavage products was carried out by the method of Moazed et al. (33), using the same primer set described therein. Products of the reaction were resolved on 0.25 × 60 mm 7 M urea/6% polyacrylamide/Tris-borate gels. The gels were transferred to Whatman 3MM paper and dried. Visualization of the radioactive products was accomplished by exposure to Kodak X-OMAT film.

RESULTS

50S ribosomal subunits

Cleavage was carried out using untethered phenanthroline-Cu(II) [both 5-acylamido-1,10-phenanthroline (AoP) and N-acetyl-2-aminomethyl-1,10-orthophenanthroline (MoP); Fig. 1] on salt-washed 50S ribosomal subunits, both at 37°C and after the subunits had been heated to 80°C, using procedures outlined in the Materials and Methods. The results of this cleavage are shown in Figure 2.

To determine if heating the ribosome or the extracted RNA to 80°C prior to initiating cleavage was detrimental, two controls were run; one in which only RNA was present (lanes 2 and 6) and the other in which all other components were present except phenanthroline (lanes 3 and 7). These provided the background cleavage pattern in the RNA itself as a result of the incubation treatment. Lane 4 reflected the cleavages seen when AoP was used to cleave intact, native 50S ribosomal subunits at 37°C. Pronounced bands were seen at nucleotides 60–66 and 87–100. The intensity of these was significantly greater than those in lane 5, which was induced by MoP at the same concentration. With MoP, the cleavage occurs primarily at nucleotides 63–65 and 88–95. In both cases, the cleavage bands were much more intense than any others in that entire region of rRNA. The results of this study suggested that AoP was a much more robust cleavage agent on the 50S ribosomal subunit than was MoP. It also appeared that the AoP was more discriminating of structure than the MoP. This has been studied in considerably more detail and will be reported elsewhere (G.W.Muth, S.P.Hennelly and W.E.Hill, manuscript in preparation).

23S rRNA and denatured 23S rRNA

23S rRNA was extracted from 50S ribosomal subunits as described in the Materials and Methods. In this protein-free form, the 23S rRNA was incubated with untethered AoP and MoP in the presence of Cu(II) and ascorbate to induce cleavage. Following cleavage at 37°C for 1 h, the rRNA was purified and analyzed by primer extension as outlined in the Materials and Methods. Reactions identical to those carried out on the 50S ribosomal subunits were carried out with extracted 23S rRNA, with incubation taking place for 1 h at 80°C to denature the RNA prior to cleavage. The results of these experiments are shown in Figure 3.
Figure 2. Cleavage of 50S ribosomal subunits by C-5 substituted AoP and C-2 substituted MoP at 37°C. C and U sequencing lanes: 1, 50S (2–5 cleavage reactions upon native subunits); 2, 50S control; 3, 50S minus phenanthroline; 4, cleavage induced by AoP; 5, cleavage induced by MoP (6–9 cleavage reactions upon denatured subunits); 6, 50S control; 7, 50S minus phenanthroline; 8, cleavage induced by AoP; 9, cleavage induced by MoP.

As with the 50S subunits, two controls were run at both temperatures to provide baseline and background information (lanes 2, 3, 6 and 7). Lane 4 showed the cleavage pattern when AoP was present, showing definitive cleavage at nucleotides 92–95 and much less definitive cleavage at nucleotides 64–66. In lane 5, MoP showed essentially the same specificity.

Using heat denatured 23S rRNA (80°C, 1 h) as the sample, the cleavage of AoP at position 63–66 disappears, while that at nucleotides 92–95 was attenuated (lane 8). In the case of MoP, the cleavage pattern showed no discernible structurally related cleavage in either region (lane 9).

It was readily apparent that AoP cleaved the unheated rRNA in 87–100 region. This was constant through several preparations of rRNA, suggesting residual structure in this region. Although other sites could be seen to be cleaved preferentially as well, the 87–100 region was persistently present. What little structure remained in the protein-free 23S rRNA in the 60–66 region showed some residual cleavage.

With the denatured 23S rRNA, the AoP cleavage pattern suggested that there may be a little residual structure left in the 92–95 region, but none in the 63–66 region. With MoP, only background cleavage remained. As noted above, the background cleavage was always present, with each nucleotide being cleaved, but the residual rRNA not being entirely degraded.

Figure 3. Cleavage of 23S rRNA by C-5 substituted AoP and C-2 substituted MoP at 37°C. C and U sequencing lanes: 1, 23S rRNA; (2–5 cleavage reactions upon native 23S rRNA); 2, 23S rRNA; 3, 23S minus phenanthroline; 4, cleavage induced by AoP; 5, cleavage induced by MoP (cleavage reactions upon denatured 23S rRNA); 6, 23S rRNA control; 7, 23S minus phenanthroline; 8, cleavage induced by AoP; 9, cleavage induced by MoP.

The loss of cleavage in both the 60–66 or 87–100 region indicates that the rRNA structure that enhanced phenanthroline cleavage was removed by heating, suggesting that the proclivity of unheated 23S rRNA to be cleaved in the 87–100 region was a result of residual structure in the rRNA. This may suggest that although the pseudoknot structure itself is not present in the protein-free RNA, some structure was still intact and that a portion of the loop containing nucleotides 87–95 may still be present. Indeed, these results, when compared with those of the 50S ribosomal subunits, suggest ribosomal proteins may provide the additional stability necessary to establish the putative pseudoknot structure.

DISCUSSION

Although the rRNA cleavages observed and analyzed in this study are certainly not the only cleavages caused by untethered phenanthroline on the ribosomal subunits or rRNA, these are much more robust than any others observed. They occurred with 70S ribosomes (data not shown), as well as with 50S ribosomal subunits and 23S rRNA, as reported here. The results of this study provide substantive evidence that the reason for these robust cleavages is the presence of a strained structure, likely the postulated pseudoknot, incorporating nucleotides 60–66 and...
87–95 in *E. coli* 23S rRNA when in the 50S ribosomal subunit. The results of this study present additional evidence for the way in which phenanthroline interacts with and cleaves RNA. These cleavages are mapped in Figure 4.

In the presence of limited copper, untethered phenanthroline is generally found in a 2:1 complex composed of two phenanthroline molecules coordinately bonded in a tetrahedral fashion to one cuprous ion. In B-form DNA, the phenanthroline duplex structure is ideal to penetrate the minor groove and 'nest' along that groove (34). However, in A-form double-stranded RNA (or DNA), there is but a shallow minor groove, which does not allow the phenanthroline to 'nest' there. Thus, there is little or no cleavage by phenanthroline in the canonical A-form double-stranded RNA (34). The same is true for single-stranded A-form RNA, which would explain why single-stranded regions of rRNA are only slightly cleaved, even with extensive incubation times with phenanthroline.

However, if the A-form structure is strained, as it often is in RNA, so that the nucleotides are splayed apart then the phenanthroline may find a location in which at least a portion of the phenanthroline can intercalate. This has been dubbed 'bookmarking' by Hermann and Heumann (35) and provides a reasonable explanation for the behavior of untethered phenanthroline with RNA. Only in those regions where the structure is sufficiently strained to allow the phenanthroline to partially intercalate between the bases will cleavage occur. It would be especially robust in single-stranded regions which presented bases outward from the helical backbone in a loop or bulged region.

Phenanthroline cleaves via a proton abstraction mechanism, using a tethered hydroxyl ion or hydroxyl radical to perform the chemistry (36,37 and reviewed in 38). Under the conditions of our experiments, the hydroxyl radical would be sequestered by the copper and will not diffuse, except in conjunction with the Cu(II)–phenanthroline complex. The multiple cleavages observed in a single region, such as those that occur with nucleotides 60–66 and 87–95 in this study, suggest multiple bookmarking sites rather than hydroxyl radical diffusion. The intensity variations in these regions likely reflects the structural differences which affect the ability of the phenanthroline to dock between each set of nucleotides.

The pseudoknot postulated for this region may well give rise to a structure which promotes the strong cleavages that we observe. The evidence for a possible pseudoknot in this region came from early work by Leffers *et al.* (28), who showed that nucleotides equivalent to C61 and G93 and U65 and A89 in *E. coli* were
covariant in Desulfurococcus mobilis. They noted that this required the terminal loop of helix 7 to fold back, such that these nucleotides were in juxtaposition. Since then, further covariance has been noted by Gutell and Woese, who also showed that C66 and G88 were covariant as well (29). They noted that these two capping loops seem to contact one another, primarily through canonical base pairs. Even more recently Gutell has found additional evidence that A64-U90, and U62-U92 were coupled (R. Gutell, personal communication). Five of the six nucleotides in either capping loop appeared to be interacting in a covariant manner, suggesting possible base pairing and hydrogen bonding between these two loops.

The possible hydrogen bonding between nucleotides 61, 62, 64, 65 and 66 with nucleotides 93, 92, 90, 89 and 88 respectively, all lie within the regions which are very heavily cleaved by phenanthroline. Yet, whatever the interaction between these putative pairs, they are not canonical base pairs, since such a structure would not allow partial intercalation to occur and concomitant cleavage. Evidence for this is provided by nearby double-stranded regions having canonical base pairs, which are not cleaved.

The presence of cleavage bands of similar intensity in both loops suggest that these regions may be coupled, or at least similarly constrained, as the postulated pseudoknot structure suggests (29). As the structure is altered, the cleavage in both regions attenuates, almost concomitantly. Although the cleavage patterns themselves do not specify a particular structure, the pseudoknot folding of these two regions would explain the twin-like cleavage patterns which occurred in our experiments. Such a pseudoknot region could help provide a strained, open structure, in which the phenanthroline could readily intercalate or bookmark. The results from the heat-denatured 23S rRNA structure provide strong evidence for this, since cleavage of the heat-denatured rRNA gave essentially background cleavage throughout the entire region, with only a small amount of residual cleavage appearing in the 92–95 region when cleavage occurred using AoP (Fig. 3, lane 8). These results provide further evidence that the susceptibility of these two sites is dependent upon the structure, as constrained in the ribosome.

What constrains the structure? Since protein-free 23S rRNA showed reduced, but definitive cleavage at the 92–95 region, with but a fraction of the cleavage present in the 63–66 region, compared with the results from the 50S ribosomal subunits, the proteins are heavily implicated in creating/maintaining the pseudoknot structure. As we look for possible ribosomal proteins to constrain the structure in this region, the only proteins presently implicated as being proximal to this region are L23, L24 and L29, of which L23 is the most likely candidate for creating/maintaining this pseudoknot. Brimacombe’s group has shown that these three proteins can be crosslinked to portions of the region surrounding the pseudoknot (39).

Although crosslinking results do not mandate that the ribosomal proteins are actually bound to the rRNA regions in which the crosslinks occur, clearly the presence of a nearby protein(s) could provide stability for the pseudoknot structure. But the protein(s) is probably not covering the putatively base-paired loops, since the phenanthroline moieties have access to these sites.

What is the possible functional importance of this region of rRNA? In eukaryotes, this region is part of the 5.8S rRNA, which has been implicated in translation (40–42). Since this pseudoknot was originally postulated from studies on Drosophila, and similar structures are found in E.coli, one is tempted to suggest that identical structures may occur extensively throughout nature. But some preliminary studies on 5.8S rRNA in situ on the ribosomes from frog oocytes gave no strong cleavages in this region, suggesting that the pseudoknot structure was not present, or differed from that found in E.coli. (G.W. Muth, unpublished) It is also possible that the base pairing predicted from covariance studies may be transient in nature, occurring only at specified times in the ribosome translational cycle.

In E.coli, there is evidence that the 74–136 region participates in termination, since both sense and anti-sense fragments allow readthrough of a termination codon (43). In addition, previous cleavage results from this laboratory have shown that randomly-positioned phenanthroline on tRNA cleaves this region (25).

It is tempting to speculate that phenanthroline cleavage has special affinity for pseudoknot structures, but such generalization is not warranted. Most other proposed pseudoknots in rRNA have not shown such susceptibility to phenanthroline cleavage. It is more likely that the cleavage occurs because the structure of the rRNA in this region is constrained to a more open conformation, due to the pseudoknot and/or the proteins present. More data will be necessary to provide this information.

On a final note, in general, phenanthroline cleavage using the 5-substituted moiety AoP, NoP (5-nitro-1,10-phenanthroline) or IoP (5-iodoacetamido-1,10-phenanthroline), has shown greater reactivity with single-stranded regions of rRNA, than does the 2-substituted moiety (MoP) (G.W. Muth, S.P. Hennelly and W.E. Hill, in preparation). We have used both IoP and NoP in previous studies (25–27), and found that these, as well as phenanthroline itself, showed similar cleavage propensity for portions of the single-stranded regions of rRNA, as well as bulges and loops in the double-stranded structure.

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