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Detection of Cleavage Sites on 5S rRNA by Methidiumpropyl-EDTA-Iron(II)

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Abstract: The affinity cleavage reagent methidiumpropyl-EDTA-Iron(II) is applied to the structural analysis of 5S rRNA. Analysis of cleavage sites induced by MPE-Fe(II) on 5S rRNA shows that MPE intercalates easily between the unstable base pairs or into the bulges, thereby it strongly cuts the nucleosides nearby. The stable helical stems A, B, D and E as well as loop d are weakly cut. Most of the single-stranded loops are not cleaved. Based on the cleavage pattern of the 5S rRNA by MPE-Fe(II) and RNase V1, we suggest that MPE-Fe(II) may be used as a potential chemical probe in searching for the unstable helical regions of RNA, and for the sequences that appear to be involved in folding and distorting 5S rRNA.
Key words: chemical nuclease, methidiumpropyl-EDTA, 5S rRNA.

Recent demonstration of the potentials of some chemical nucleases in probing the secondary and tertiary structure of RNA has drawn increased attention in the study of nucleic acid chemistry (Sigman, 1990). In contrast with chemical modification reagents such as dimethyl sulfate and diethyl pyrocarbonate, chemical nucleases can cut DNA or RNA directly under physiological conditions at all sequence positions regardless of the nucleobase linked to the deoxyribose or ribose. The sugar moieties of the nucleosides are oxidatively degraded by the action of free radicals generated by chemical nucleases. Examples of chemical nucleases being used recently in nucleic acid chemistry are the derivatives of ferrous-EDTA (Tullius and Dombroski, 1986), various metalloporphyrins (Groves and Farrell, 1989), octahedral complexes of 4,7-diphenyl-1,10-phenanthroline (Barton, 1986), bleomycin (Kozarich *et al.*, 1989), and methdiumpropyl-EDTA-Fe(II) (Hertzberg and Dervan, 1982, 1984).

The affinity cleavage reagent methidiumpropyl-EDTA (MPE) intercalates between base pairs in helical DNA and, when complexed with Fe(II), cleaves the DNA by oxidative degradation of the deoxyribose (Hertzberg and Dervan, 1982, 1984). It has been also shown that MPE-Fe(II) selectively intercalates in double-stranded regions in preference to single-stranded regions of yeast $tRNA^{phe}$ and 16S rRNA fragment (Kean *et al.*, 1985).

In this work, we mapped the MPE-Fe(II)-induced cutting sites on 5S rRNA from *Pseudomonas alcaligenes* to see if this reagent is structure-specific enough so that it could be used as a chemical probe in determining the structure of 5S rRNA. Our results show that MPE-Fe(II) can be used in characterizing the sequences that might be involved in folding or in distorting the RNA molecule.

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Materials and Methods

Materials

Methidiumpropyl-EDTA was kindly donated by Professor P. Dervan, California Institute of Technology, California, USA. Ammonium iron(II) sulfate was purchased from Aldrich. All other reagents were obtained from the Sigma Chemical Co. (St. Louis, USA).

Purification and labeling of 5S rRNA

The 5S rRNA was extracted from *Pseudomonas alcaligenes* cells by sodium dodecyl sulfate/phenol extraction at 65℃ as described previously (Kim *et al.*, 1995). The 5S rRNA was labeled at the 5′- or 3′-end using polynucleotide kinase or RNA ligase, respectively, as described by D'Alessio (1982). The RNA was renatured by heating the RNA in renaturating buffer (25 mM Tris, 100 mM NaCl, pH 7.5) to 65℃ for 5 min, followed by slow cooling to room temperature.

Cleavage by MPE-Fe(II) and RNase V1

MPE-Fe(II) reactions were carried out as described by Kean *et al.* (1985). The reaction mixture contained

25 mM Tris, pH 7.5, 100 mM NaCl, 5 μM each of MPE and ammonium iron(II) sulfate, and 2 mM DTT to initiate the cleavage and were incubated at 20℃ for 30 min. Reactions performed at different concentrations of MPE always contained equal concentrations of Fe(II).

RNase V1 reactions were carried out in the same manner as for the MPE-Fe(II) cleavage reactions except that the reaction mixture included 10 mM $MgCl_2$ to assure the activity of the enzyme. After cleavage, an equal volume of urea buffer (10 M urea, 0.03% bromophenol blue and 0.03% xylene cyanol) was added to the reaction mixture and 4 μL aliquots were loaded directly on a 10 or 12% polyacrylamide sequencing gel for the seperation of cleavage fragments.

Results and Discussion

Cleavage fragments of 5S rRNA induced by MPE-Fe(II) and RNase V1 were separated on polyacrylamide gel. Their separations are shown in Figs. 1 and 2. Cleavage sites are indicated on the secondary structure model of 5S rRNA from *P. alcaligenes* as has been proposed previously (Kim *et al.*, 1995) (Fig. 3). As shown in Fig. 3, all of the stable helical regions A, B, D and E as well as loop d are weakly cut. All the nucleosides in single-stranded loops are not cleaved, while A53 and A54 that form the the double-based bulge are weakly cut. Nucleosides at the ends of helix, such as C12, A27, C47 and G56 are strongly cut. C17 next to the single-based bulge A66, and G54 next to the double-based bulge A53A54 are also strongly cleaved. A29 located in the unstable helix next to the bulge A53A54 is also strongly cut.

It is interesting to note that all the nucleosides strongly cut by MPE-Fe(II) are located at the ends of and in unstable helical regions of 5S rRNA. In our previous proposal on the secondary structure of 5S rRNA, we have defined the unstable helical regions as follows: the unstable helical regions mean those regions in which base pair interactions are either weak or non-Watson-Crick type (Cho *et al.*, 1993a). Weak base pair interactions of this kind may cause the partial unwinding of helix. Because of this reason, unstable helical regions are prone to be attacked both by double-strand- and base-stacking stretch-specific RNase V1 and single-strand-specific nuclease S1 (Cho *et al.*, 1993a).

In our previous works using Fe(II)-EDTA and Pb^{2+} as chemical probes, and also by hybridizing loop c with deoxyhexamer, we have obtained an interesting result suggesting that the unstable b-C region and loop d might act as hinges in the folding of the 5S rRNA molecule (Cho *et al.*, 1993b; Shim *et al.*, 1994; Kim

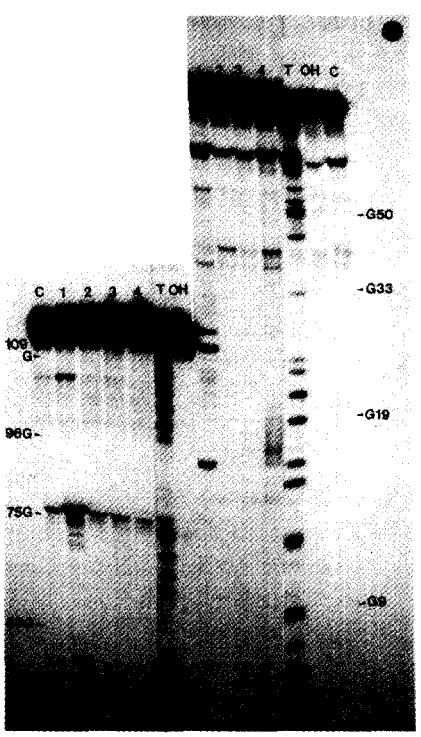

Fig. 1. 12% polyacrylamide gel electrophoresis fractionation of the 5'-end labeled 5S rRNA after MPE-Fe(II)-induced cleavage. Experimental conditions are described in Materials and Methods. Lanes 1 through 4, reactions with 5 μM, 15 μM, 45 μM, and 135 μM of MPE-Fe(II), respectively; lane C, incubation control; lanes T and OH, RNase T_1 and alkali ladders, respectively. The sequence is numbered by every G residue.

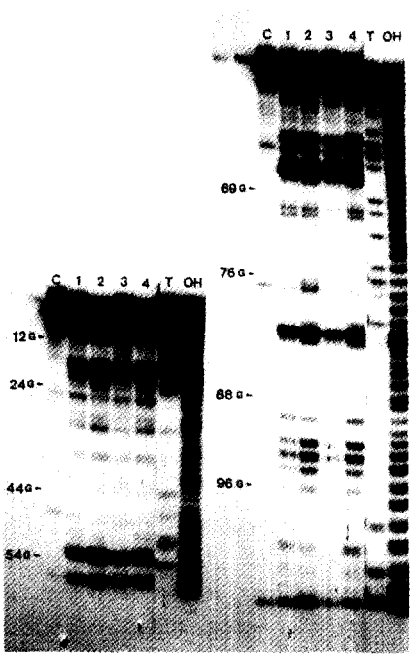

Fig. 2. 12% polyacrylamide gel electrophoresis fractionation of the 3'-end labeled 5S rRNA after digestion with RNase V1. Lanes 1 through 4, incubations with RNase V1 for 5, 10, 15, and 45 min, respectively; lane C, incubation control; lanes T and OH, RNase T1 and alkali ladders, respectively. The sequence is numbered by every G residue.

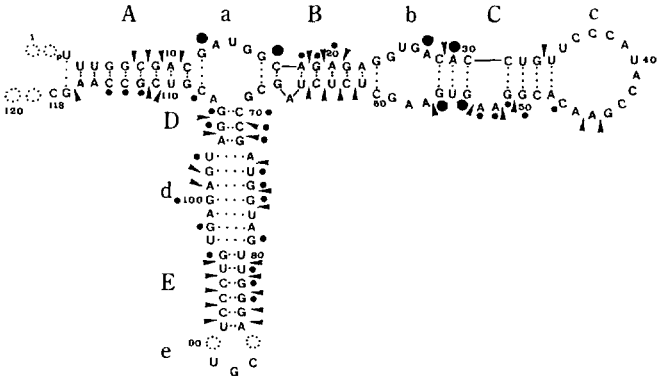

Fig. 3. Summary of MPE-Fe(II) and RNase V1 cleavage sites represented on the secondary structure of *P. alcaligenes* 5S rRNA. The secondary structure used here is that of our generalized model for procaryotic 5S rRNAs with minor modifications (Cho *et al.*, 1993a). RNase V1 cuts, (◄). MPE-Fe(II)-induced cleavages, strongly (●) and weakly (•). The dots · · and · · · · denote stable and unstable base pairs, respectively. Dotted circles at positions 1, 2, 86, 90, 119 and 120 denote the nucleotide deletions. They are added for the convenience of the numbering of the sequence.

et al., 1995). The strong cleavages at A29, G54 and G56 near the double-based bulge suggest that the conformation of base pairs C28:G56, A29:U55 and C30:G54 altered to a considerable extent and that the stacking of those base pairs is fairly broken. This alteration of conformation may have resulted from the tertiary folding of the 5S rRNA molecule. The weak cleavages at the two adenosines A52 and A53 are likely to have occurred due to the intercalation of MPE-Fe(II) in bulge A52A53. This observation suggests that a patch of C stem flanking to the bulge A52A53 has undergone a considerable conformational change and is involved in the formation of a hinge or a twist in tertiary interactions, as we have reported in our previous work (Cho *et al.*, 1993b).

As we can see in Fig. 3, loop d is weakly cut by MPE-Fe(II). Apparently, this observation is not consistent with our designation of this portion of 5S rRNA, i.e., the sequence from A73 through G79 and the sequence from U97 through U103, as loop (Cho *et al.*, 1993a). Our observations that several nucleosides of this loop are resistant to the attack of Fe(II)-EDTA and also of Pb^{2+} (Cho *et al.*, 1993b; Shim *et al.*, 1994; Kim *et al.*, 1995) in the presence of Mg^{2+}, however, suggest that these sequences are mostly buried deep in the molecule. Therefore, we can assume that weak cleavage of loop d can occur either at weak tertiary base pairs formed following the building up of a higher order structure or in base-stacked regions.

The fact that MPE-Fe(II) strongly cuts in unstable helical regions shows that MPE-Fe(II) intercalates more easily in these regions than in the stable helical regions, in which normal base pairings are possible. Many workers have suggested that intercalation of a planar molecule between the base pairs of a DNA or RNA helix requires considerable alteration of the helix conformation, with accompanying extention of the polynucleotide backbone and unwinding of the helix. Thus, this may require a considerable investment of free energy (Gralla and Crothers, 1973; Breslauer *et al.*, 1975; Kean *et al.*, 1985). Our observation that the stable helical regions of 5S rRNA are attacked only weakly is consistent with the suggestion of above-mentioned workers. We believe this observation is a support for our previous observations that stems A, B, D, and E always retain their stable double helical structures even after the 5S rRNA molecule has folded up to form a higher order structure.

Most of the single-stranded sequences are not cut by MPE-Fe(II) except at the ends of the helix(e.g. A27), at C47 in loop c, and at the two adenosines A52 and A53, although the latter three nucleosides are attacked only weakly. Obviously, this result shows that most of the single-stranded sequences are not involved in tertiary interaction; instead they are looped out of the tertiary structure of 5S rRNA. Probably, the weak cleavages at C47, A52 and A53 can be explained by assuming that they are base-paired to some distant sequence elements through tertiary interactions, or they reside in a stretch of base stacking. We have no evidence for this yet; it still remains to be proved. An investigation for this purpose is progressing in our laboratory.

We conclude that the chemical nuclease MPE-Fe(II) preferentially intercalates into the unstable double-stranded regions and cuts the nucleosides either in that region or at the ends of the helix. Therefore, MPE-Fe(II) appears to be a potential probe in characterizing the unstable double-stranded regions as well as the highly base-stacked sequences in the higher order structure of RNAs.

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