RNA structure analysis using methidiumpropyl-EDTA·Fe(II): A base-pair-specific RNA structure probe

(yeast tRNA^{Phe}/RNA secondary structure/base pair specificity)

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ABSTRACT Methidiumpropyl-EDTA·Fe(II) [MPE·Fe(II)] in the presence of dithiothreitol, is shown to cleave phenylalanine-accepting tRNA (tRNA^{Phe}) in a structure-specific fashion. Molar ratios of MPE·Fe(II) to tRNA^{Phe} of <1 preferentially cleave phosphodiester bonds known to occur in double-stranded regions of the tRNA^{Phe} molecule. Microdensitometric analysis of autoradiograms of MPE·Fe(II) cleavage products following gel electrophoresis reveals a correspondence between preferred sites of MPE·Fe(II) cleavage and sites in tRNA^{Phe} most sensitive to cobra venom ribonuclease, a double-strand-specific endoribonuclease. Conversely, sites of cleavage by the single-strand-specific S1 nuclease correspond to those nucleotides that are least susceptible to MPE·Fe(II) hydrolysis. Sensitive helical regions in tRNA^{Phe} include the dihydrouracil and the "TΨC" stems, which cannot be detected by cobra venom ribonuclease because of steric constraints. Phosphodiester bonds within the TΨC and dihydrouracil loop regions, which are not detected by S1 nuclease under rigorously controlled digestion conditions, are revealed by inference from their relative insensitivity to MPE·Fe(II). These results demonstrate the utility of MPE·Fe(II) as a general small molecular weight probe of RNA structure, having a greater accessibility to base-paired regions than do the more bulky enzymic probes.

Structure-specific enzymic probes of RNA structure, including single-strand-specific S1 nuclease and base-pair-specific cobra venom RNase (CVRase), have been used to obtain detailed information on the solution structure of RNA molecules. Digestion under partial ("single hit") kinetic conditions with the enzymic probes, of 5'- or 3'-end-labeled RNA molecules, provides direct experimental data on the accessibility of phosphodiester bonds to the enzymes, thus revealing their status as being in either single- or double-stranded regions, respectively, under specific conditions of pH, ionic strength, and temperature (1). This approach has been used to obtain detailed structural information on many RNA molecules including tRNAs (2), ribosomal RNAs from various sources (ref. 3 and unpublished data), and mouse (4) and rabbit globin mRNAs (5, 6, 7). While enzymic probes of RNA structure provide reliable and highly selective structure information on large RNA molecules, the majority of phosphodiester bonds in a given molecule are often inaccessible to the enzymes because of steric constraints imposed by the large size of the probe and the compact nature of the native RNA molecule.

Small molecular weight chemical probes offer, in principle, a solution to the problem of steric exclusion. A number of small molecular weight chemical probes, including diethyl pyrocarbonate, hydrazine, and dimethyl sulfate (7) have been available for some time. Their size allows access to more hindered regions of RNA molecules and afford the

ability to study RNA structure within a ribonucleoprotein complex (8, 9). Because these compounds react with the base moieties of an RNA, they show considerable sequence specificity, thereby restricting the sampling to particular subsets of bases. Such restrictions necessitate the use of several probe molecules to maximize the information available for a detailed structural analysis of a large RNA molecule. There have been no previous reports of a small molecular weight RNA structure probe having double-strand specificity of cleavage.

We here report the use of methidiumpropyl-EDTA·Fe(II) [MPE·Fe(II)] to directly map the helical regions and, by inference, single-stranded regions in yeast phenylalanine-accepting tRNA (tRNA^{Phe}). MPE·Fe(II) provides a useful double-strand-specific complement to the single-strand-specific chemical probes listed above. As a result of the apparent lack of sequence specificity, including modified bases, MPE·Fe(II) cleavage gives information on the relative sensitivity of virtually every base in the RNA molecule. Quantitation of the cleavage products generated by the action of MPE·Fe(II) on tRNA^{Phe} has been accomplished by scanning microdensitometric analysis of autoradiograms, followed by conversion of the data from transmittance to absorbance to facilitate estimation of the relative frequencies of cleavage at all sites in the molecule.

MATERIALS AND METHODS

Materials. Yeast tRNA^{Phe} was purchased from Boehringer Mannheim. S1 nuclease and CVRase were purified and characterized as described (1). T4 RNA ligase and T4 polynucleotide kinase were purchased from Bethesda Research Laboratories. [³²P]ATP (8000 Ci/mmol; 1 Ci = 37 GBq) and cytidine 3',5'-[5',³²P]bisphosphate ([³²P]pCp; 2000 Ci/mmol) were purchased from Amersham. MPE was a gift from P. Dervan (Department of Chemistry, California Institute of Technology, La Jolla).

Preparation of End-Labeled tRNA^{Phe}. Yeast tRNA^{Phe} was dephosphorylated by using calf intestinal alkaline phosphatase and subsequently was labeled at the 5' or 3' terminus by using [³²P]ATP and polynucleotide kinase (10) or [³²P]pCp and RNA ligase (11), respectively. Labeled RNAs were purified by preparative gel electrophoresis, isolated, and stored in the presence of carrier RNA in 70% ethanol/0.3 M NaOAc at -20°C (1).

Enzymatic Digestion Conditions. Aliquots of end-labeled tRNA^{Phe} (10,000–50,000 Cerenkov cpm) were digested with the sequence-specific enzymes T1 RNase and U2 RNase under denaturing conditions to provide sequence information (12) and with S1 nuclease and CVRase to establish regions of single-stranded and double-stranded conformation as has been described (13). All digestions were allowed to proceed

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Abbreviations: MPE·Fe(II), methidiumpropyl-EDTA·Fe(II); CVRase, cobra venom ribonuclease; tRNA^{Phe}, phenylalanine-accepting tRNA; [³²P]pCp, cytidine 3',5'-[5',³²P]bisphosphate.

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until no more than 30% of the full-length molecule had been cleaved to ensure that digestion does not exceed one cleavage event per tRNA molecule. CVRase digestions were conducted in buffer containing 50 mM Tris·HCl (pH 7.5), 2 mM MgCl_2 , and 100 mM NaCl.

MPE·Fe(II) Digestion Conditions. Stock solutions of MPE·Fe(II) were prepared at a concentration of approximately 0.85 mM, verified spectrophotometrically by using an extinction coefficient (at 480 nm) of $5680 \text{ M}^{-1}\cdot\text{cm}^{-1}$ (14). To a standard reaction buffer identical to the CVRase buffer containing 100 mM NaCl, 2 mM MgCl_2 , and 50 mM Tris·HCl (pH 7.5) was added 5'- or 3'-end-labeled tRNA^{Phe} (10,000–50,000 Cerenkov cpm), 3.2 μg of carrier tRNA, (total tRNA, 125 pmol), 100 pmol of MPE, and 80 pmol of ferrous ammonium sulfate in a final volume of 25 μl . After preincubation of the mixture at 37°C for 5 min, the reaction was initiated by the addition of dithiothreitol to a final concentration of 5

mM. Aliquots (5 μl) were taken at various times, and the reactions were terminated by addition of an equal volume of a solution containing 0.1% bromphenol blue and xylene cyanol dyes, 10 M urea, 20 mM EDTA, and 20 mg of carrier tRNAs per ml, followed by storage on dry ice. Reactions were analyzed by electrophoresis on standard denaturing polyacrylamide gels as described (1).

Autoradiography and Densitometry. Gels were autoradiographed with Kodak X-Omat R5 x-ray film for 10–30 hr at -20°C . Care was taken to expose gels so that bands (other than full length) had an optical density no greater than 1.0 in order to insure a linear relationship between optical density and cpm. Autoradiograms were scanned using a Jarrell-Ash model 23-100 scanning microdensitometer with slit dimensions of $2.0 \text{ nm} \times 10 \mu\text{m}$. This device has been interfaced to an NEC-APL microcomputer, and data analysis was performed with newly written software. The optical transmit-

FIG. 1. Cleavage of RNA by MPE·Fe(II). Reaction mixtures containing 100 mM NaCl, 2 mM MgCl_2 , 50 mM Tris·HCl (pH 7.5), and 10,000–50,000 cpm (Cerenkov) of 5'-end-labeled tRNA^{Phe} (100–150 pmol) were incubated for 10 min at 0°C (lane 1) or at 37°C (lane 2–9) with no further additions (lanes 1 and 2) or with the following additions: 100 pmol of MPE, 80 pmol of ferrous ammonium sulfate, and 5 mM dithiothreitol (lane 3); 80 pmol of ferrous ammonium sulfate and 100 pmol of MPE (lane 4); 80 pmol of ferrous ammonium sulfate and 5 mM dithiothreitol (lane 5); 100 pmol of MPE and 5 mM dithiothreitol (lane 6); 100 pmol of MPE alone (lane 7); 80 pmol of ferrous ammonium sulfate alone (lane 8); and 5 mM dithiothreitol alone (lane 9). The molar ratio of MPE to tRNA is 0.7–0.8 in lanes 3, 4, and 6. The digestion products were resolved on a 20% denaturing polyacrylamide gel as described.

FIG. 2. Titration of Fe(II) with increasing concentrations of MPE. Reactions were conducted as described except that the amount of MPE was varied at constant levels of ferrous ammonium sulfate (80 pmol), tRNA (100 pmol), and dithiothreitol (5 mM). Lanes: 1, T1 ribonuclease (5×10^{-4} units per μg of RNA) at 60°C for 5 min; 2, alkaline hydrolysis in 50 mM $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer (pH 9) at 90°C for 10 min; 3–8, molar ratios of MPE to tRNA^{Phe} of 0.16, 0.24, 0.4, 0.8, 4.0, and 8.0 and to Fe(II) ions of 0.2, 0.3, 0.5, 1.0, 5.0, and 10, respectively, at 37°C for 10 min.

tance data were smoothed by using a gaussian-curve-fitting algorithm. The peak areas for each band were calculated after conversion of the data from percent transmittance to absorbance. Areas calculated in this fashion have been shown to be proportional to cpm and concentration (unpublished data).

RESULTS

Fig. 1 shows the result of incubation of 5'-end-labeled tRNA^{Phe} in the presence of MPE, ferrous ammonium sulfate, and dithiothreitol. Virtually every position in the tRNA^{Phe} molecule appeared to be sensitive at some level to MPE·Fe(II)-catalyzed cleavage. All single and binary controls shown in Fig. 1 were negative with the exception of the ferrous ammonium sulfate/dithiothreitol reaction. This combination of reagents produced two distinct cleavages at positions 19 and 20 in the "TΨC" loop of tRNA^{Phe}. Titration with increasing amounts of MPE at a constant level of fer-

rous ion showed that the ferrous-specific cleavages seen in Fig. 1 lost prominence in the presence of excess MPE (Fig. 2). Fig. 3 relates the positions of cleavage of 5'-end-labeled tRNA^{Phe} to the positions of S1 nuclease- and CVRase-catalyzed hydrolysis. Quantitation of the structure-specific reaction products of S1 nuclease, CVRase, and MPE·Fe(II) cleavage could be accomplished by analysis of microdensitometric scans of autoradiograms. Results of scanning lanes 3, 4, 5, and 6 of Fig. 3A are shown in Fig. 3B, bottom scan to top, respectively. The data in Fig. 3B are presented as absorbance. Therefore, the areas under the individual bands represent the relative concentrations of individual cleavage products. Nucleotides that showed CVRase sensitivity were among those nucleotides that showed maximal MPE·Fe(II) sensitivity. Conversely, the S1 nuclease-sensitive nucleotides in the anticodon loop at positions 35 and 36 were among the least-sensitive positions in tRNA^{Phe} towards MPE·Fe(II). Certain nucleotides that showed maximal sensitivity to MPE·Fe(II) showed no sensitivity to CVRase (e.g., posi-

FIG. 3. MPE·Fe(II)-specific cleavage products of 5'-end-labeled [³²P]tRNA^{Phe}. (A) lanes: 1, T1 ribonuclease at 60°C for 5 min; 2, alkaline digestion at 90°C for 10 min; 3, S1 nuclease (0.08 units per μ g of RNA) at 37°C for 2 min; 4, CVRase (8×10^{-4} units per μ g of RNA) at 37°C for 5 min; 5, 80 pmol of ferrous ammonium sulfate and 5 mM dithiothreitol at 37°C for 10 min; 6 and 7, 100 pmol of MPE, 80 pmol of ferrous ammonium sulfate, and 5 mM dithiothreitol at 37°C for 1 min and 3 min, respectively. (B) Microdensitometric analysis of selected lanes in the autoradiogram in A. Absorbance (ordinate) is plotted versus the direction of migration. Scans from the top: MPE·Fe(II) digestion products (A, lane 6), Fe²⁺/Fe³⁺/dithiothreitol digestion products (A, lane 5); CVRase digestion products (A, lane 4); and S1 nuclease digestion products (A, lane 3). The horizontal bar in the S1 nuclease scan indicates the anticodon loop bands at positions adenosine-35 and -36 in tRNA^{Phe}.

tions 8–11 in Fig. 3*B*). Also, regions of minimal MPE·Fe(II) cleavage (positions 15–18 in Fig. 3*B*) are known to be single-stranded in the tRNA^{Phe} three-dimensional structure (15, 16) but are not easily detected by S1 nuclease (1). Fig. 3*B* also shows the position of the unique cleavages produced in tRNA^{Phe} generated by the action of Fe(II) in the presence of dithiothreitol.

Fig. 4 shows the results of the cleavage of 3'-end-labeled tRNA^{Phe} by MPE·Fe(II). Densitometric analysis of lanes 4, 5, and 6 of the autoradiogram in Fig. 4*A* is shown in Fig. 4*B*, bottom scan to top, respectively. The data confirm and extend the results obtained for the 5'-end-labeled tRNA^{Phe}. Once again, the positional correlation of MPE·Fe(II) and CVRase cleavage was observed, as was the correspondence relating S1 nuclease sensitivity to MPE·Fe(II) resistance. However, the failure of S1 nuclease to detect the TΨC loop

contrasts with the inferred presence of single-strandedness. The observed relative resistance of this region to MPE·Fe(II), corresponding to nucleotides 56–59 in Fig. 4*B* *Top*, is consistent with the known structure of tRNA^{Phe}.

DISCUSSION

This paper reports the use of MPE·Fe(II) as a probe of base-paired structure in RNA. MPE, synthesized by Dervan *et al.* (17), has been shown previously to cleave double-helical DNA with no apparent sequence specificity and has been used to probe drug–DNA interactions (18). The compound is thought to bind to double-helical DNA by an intercalative mechanism similar to that proposed for the closely related compound ethidium bromide (19). We show that a molar ratio of MPE·Fe(II) to tRNA considerably less than 1 results

FIG. 4. (A) MPE·Fe(II) cleavage products of 3'-end-labeled tRNA^{Phe}. Reaction conditions were identical to those described in the legend to Fig. 3. Lanes: 1, incubation for 10 min minus reagents; 2, T1 nuclease; 3, alkaline digestion; 4, S1 nuclease; 5, CVRase; 6–10, MPE·Fe(II) cleavage reactions for 0.5, 1.0, 1.5, 2.0, and 2.5 minutes, respectively. (B) Microdensitometric analysis of the S1 nuclease, CVRase, and MPE·Fe(II) cleavage products of 3'-end-labeled tRNA^{Phe}. Absorbance (ordinate) is plotted versus the direction of migration. The lanes scanned are from the autoradiograms shown in A and correspond to MPE·Fe(II) (A, lane 6) (*Top*), CVRase (A, lane 5) (*Middle*), and S1 nuclease (A, lane 4) (*Bottom*).

in the cleavage of tRNA^{Phe} in a helix-specific fashion. The number of sites available for binding of MPE is not known at this time. Some controversy exists as to the mode of binding of ethidium bromide to tRNA^{Phe}. Earlier work has suggested that an intercalative binding site exists at the base of the amino acid acceptor stem stacked over bases uridine-7 and adenosine-66 (20). Subsequent crystallographic evidence supports a model in which a nonintercalative mode of binding occurs. In the crystal, the ethidium moiety appears to be located in the P-10 loop of tRNA^{Phe}, with its exocyclic amino groups hydrogen bonded to backbone phosphates P-8 and P-15 (21).

Recent work (16) suggests that the mechanism of binding of ethidium bromide to RNA is intercalative in nature. It seems unlikely that all helical regions in the molecule could be cleaved by MPE·Fe(II) with the uniformity observed in Figs. 3 and 4, if the only site of binding were restricted to either of those cited above. We cannot exclude the possibility that the free-radical species that initiate the cleavage reaction after the addition of dithiothreitol may diffuse from a single binding site. However, this seems unlikely since radical scavenging agents have little effect on the reaction specificity and rate (unpublished results). Data obtained over a total of 300 nucleotides, from studies on *Tetrahymena thermophila* 5S rRNA and rabbit globin mRNA, confirm the correspondence between CVRase sensitivity and MPE·Fe(II) cleavage and an inverse relationship between MPE·Fe(II) cleavage and S1 nuclease sensitivity (unpublished observations). Therefore, when used at molar levels that are less than or equal to the levels of the RNA molecule under study, and in conjunction with supporting evidence provided by enzymic probes of demonstrated selectivity, MPE·Fe(II) is a valuable small molecular weight probe of RNA structure that can provide extensive information on helical domains within RNA molecules.

Data presented above indicate the utility of ferrous ammonium sulfate as a sensitive probe of metal ion binding sites in RNA. The positions guanosine-18 and -19 cleaved by ferrous ammonium sulfate in the presence of dithiothreitol in tRNA^{Phe} are identical to the bases involved in Mg²⁺ coordination, as determined by crystallographic analysis of yeast tRNA^{Phe} (21), and to the location of a samarium ion isomorphic replacement in crystals of tRNA^{Phe} (22). Fe²⁺-dithiothreitol-catalyzed cleavage does not occur in the anticodon loop where another Mg²⁺ ion is believed to bind. These results indicate that ferrous ions can be used to detect some metal ion binding sites in naturally occurring RNA molecules. It is interesting that at similar concentrations of Fe²⁺, 5S rRNA and rabbit globin mRNA show no cleavage products (unpublished observation).

MPE·Fe(II) cleavage data, following quantitation by densitometry, can be used directly as input to a RNA-folding computer-based algorithm developed by M. Zuker (personal communication) and modified by Vary and Vournakis. This algorithm predicts secondary structures that are consistent with MPE·Fe(II)-sensitivity data. Other experiments have shown that MPE·Fe(II) can penetrate ribosomal structures

and, in the presence of dithiothreitol, cleave the rRNAs *in situ* (unpublished observations). These results suggest an important potential role for the use of MPE·Fe(II) to study RNA structure in naturally occurring ribonucleoprotein complexes and ribosomes, in addition to native RNA.

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