Substrate masking: binding of RNA by EGTA-inactivated micrococcal nuclease results in artifactual inhibition of RNA processing reactions

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ABSTRACT

Inhibition of an RNA processing reaction after treatment with the Ca^{2+}- dependent micrococcal nuclease (MN) is often used as a criterion for the presence of a required RNA or ribonucleoprotein component in the system. Following MN digestion, the nuclease is inactivated with EGTA and radiolabeled substrate is added to assay for remaining RNA processing activity. We found previously that inhibition of RNA processing by MN need not involve RNA hydrolysis: EGTA-inactivated MN can suppress RNA processing if the assay is performed in the absence of carrier RNA. We now demonstrate both by native gel electrophoresis and by nitrocellulose filter retention that EGTA-inactivated MN forms a complex with free RNA which can be dissociated by addition of synthetic polynucleotides or heparin. In the absence of Ca^{2+}, nuclease binds to precursor tRNA with an apparent $K_D \simeq 1.4 \times 10^{-6}$ M, comparable to its reported affinity for DNA. In an assay for endonucleolytic tRNA maturation, inactivated MN bound to radiolabeled pre-tRNA physically blocks the sites of endonuclease cleavage and prevents tRNA processing. We call this phenomenon 'substrate masking'. Addition of excess carrier RNA competes with pre-tRNA for MN binding and restores normal processing.

INTRODUCTION

Micrococcal nuclease (**MN**; also called *S. aureus* nuclease, staphylococcal nuclease or nuclease V8) has become one of the most thoroughly characterized enzymes (1, 2). It is a monomeric polypeptide of 149 amino acids (1, 3) which behaves as a globular protein in aqueous solution. It has binding sites for 3 nucleotides, and its physical dimensions of 30×30×40 Å cover or protect about 4 nucleotides of unstacked, single-stranded nucleic acid (1). MN is a non-specific nuclease and can digest both DNA and RNA in endo- and exo-nucleolytic fashion. The enzyme prefers single-stranded DNA over duplex DNA (1,4,5).

MN is a metalloenzyme which requires Ca^{2+} for catalysis (1). The crystal structure of an MN·Ca^{2+} complex with the competitive inhibitor thymidine 3′,5′-bisphosphate (pdTp) has been elucidated at 1.5Å resolution (6). Site-directed mutagenesis of MN (2) has recently allowed separate identification of residues involved in metal binding, in nucleic acid binding, or in catalysis. These studies together led to a catalytic mechanism which involves nucleophilic attack on phosphorus by a water molecule from the second coordination sphere of Ca^{2+}. Ca^{2+} is coordinated by Asp-21, Asp-40, the amide carbonyl of Thr-41, two water molecules, and the oxyanion of the scissile phosphodiester bond. This phosphodiester phosphate is also coordinated by Arg-35, and, in the trigonal transition state, by Arg-87 (2). The attacking water is activated by Glu-43 acting as a general base (6).

Its broad substrate specificity and divalent cation selectivity have made MN a useful tool for probing nucleic acid structure. Studies on RNA processing enzymes (e.g., 7–9), and small nuclear ribonucleoproteins (e.g., 10,11) have shown that nucleic acid components can selectively be digested by MN treatment. Because of its absolute ion dependence, the nuclease activity can be inactivated by chelating agents. For investigations of Mg^{2+}-dependent RNA processing reactions, it is convenient to chelate Ca^{2+} with ethylene glycol N,N,N′,N′-tetraacetate (EGTA) (12), whose affinity for Mg^{2+} is 10^3-fold lower than that for other divalent cations (13). A common procedure for testing the MN sensitivity of a suspected ribonucleoprotein enzyme consists of two steps: a preincubation with active MN in the presence of Ca^{2+} followed, after addition of EGTA, by an assay for remaining enzyme activity. Following this protocol, the presence of a required RNA component was inferred, for example, in mitochondrial RNase P from HeLa cells (14) and nuclear RNase P from veal heart (15), *Sc. pombe* (16), and *S. cerevisiae* (17).

We previously examined the effect of MN treatment on RNA processing activities from spinach chloroplasts (18). We found that exhaustive MN digestion of partially purified tRNA processing fractions completely inhibited both 5′ and 3′ cleavage activities. Furthermore, and in agreement with Ryner and Manley (19), we showed that processing activity was fully restored by

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the addition of a synthetic carrier polynucleotide (polyadenylate) following MN treatment. Finally, we were able to demonstrate that MN inhibition of RNA processing did not require RNA hydrolysis because suppression of activity was equally effective with active MN or with EGTA-inactivated MN if the enzyme fraction tested had first been freed of endogenous RNA (18; see below). Reversible inhibition of RNA processing by MN treatment had previously been interpreted to indicate that the processing enzyme(s), or the substrate·enzyme complex, was in some way 'activated' or stabilized by bulk RNA (19). Another hypothesis was that the enzyme fraction itself contained non-specific RNA binding proteins which could bind added RNA substrate to block RNA processing once endogenous RNA had been removed with MN (W. Keller, personal communication). We propose an alternative explanation, namely that EGTA-inactivated MN itself is the non-specific RNA binding protein which binds directly and with high affinity to the RNA substrate, thus blocking access by the processing enzymes. This phenomenon is an example of substrate occlusion or 'masking' in which formation of an inactive enzyme·substrate complex precludes utilization of the substrate by a second enzyme. In this communication, we have directly tested this hypothesis by using nitrocellulose filter retention and electrophoretic retardation analyses to measure formation of a complex between EGTA-inactivated MN and RNA.

MATERIALS AND METHODS

Precursor RNA substrates

Precursors to transfer RNAs were transcribed *in vitro* from cloned tRNA genes with phage T7 RNA polymerase as described (18). A 200 nucleotide (nt) long 5′-mature, 3′ extended precursor to *E. coli* $tRNA^{Phe}$ was transcribed from PvuII-cut plasmid p67CF0 (a gift from Dr. O. C. Uhlenbeck). Maize chloroplast $tRNA^{Phe}$ transcripts have been described (18): pNXPhe/HindIII transcripts are 333 nt long, whereas pNBPhe/BamHI transcripts are 231 nt in length. A 111 nt yeast mitochondrial pre-$tRNA^{Asp}$, 5′-extended and 3′-mature, was produced from BstNI-cleaved plasmid pAsp31 (a gift from Drs. M. J. Hollingsworth and N. C. Martin). RNAs were labeled with one or with all four [α^{32}P]rNTPs to 0.072−0.84 μCi/pmol for pre-$tRNA^{Asp}$ and 0.165−1.24 μCi/pmol for pre-$tRNA^{Phe}$.

Polymers

Polyadenylate (poly(A)), polyuridylate (poly(U)), and polycytidylate (poly(C)) (Pharmacia, Boehringer or Calbiochem) were dissolved in sterile water to 20− 50 mg/ml and stored at −20°. Heparin and yeast $tRNA^{Phe}$ were obtained from Sigma and stored in sterile water at −20°C. Yeast Na·RNA was purchased from BDH and further treated by digestion with 0.1 mg/ml proteinase K/ 10 mM Na·EDTA/ 1% SDS for 15 min at 55°C. RNA was then extracted twice with phenol:chloroform and precipitated twice with ethanol from 0.2M Na·acetate (pH 5.5). The final RNA pellet was dissolved at 20 mg/ml in sterile water.

Estimation of MN Binding Sites

The number of potential micrococcal nuclease (MN) binding sites (L) on an RNA molecule can be estimated in two ways. The size of a single MN binding site is 4 nt (1). Since on an unoccupied polynucleotide any tetramer is a potential binding site, the total number of available sites (L_T) on a polynucleotide N nt long is $N-4+1$ (see ref. 20). The maximum number of contiguous sites (L_C) is $N/4$. The number of MN binding sites on a precursor tRNA is likely to be less than that on an unstructured RNA species, since we anticipate that MN will bind to the mature tRNA domain (76 nt) less well than to the relatively unstructured precursor regions. The number of contiguous single-stranded sites (L_S) on an end-extended tRNA precursor is thus roughly $(N-76)/4$. As discussed by McGhee and von Hippel (20), however, the actual occupancy of contiguous sites will always be less than the maximum.

For pAsp31 transcripts, $L_C=28$ and $L_S=9$; for p67CF0/PvuII RNA, $L_C=50$ and $L_S=31$, and for pNXPhe/HindIII, $L_C=83$ and $L_S=64$. For synthetic polymers we derived L_C ($=L_S$) from the average $S_{w,20}$ supplied by the manufacturer. For poly(A) (average mol wt, 112 kDa), $\hat{N}=323$ nt, $L_C=81$; for poly(C) (~156 kDa), $\hat{N}=483$ nt, $L_C=121$; and for poly(U) (~40 kDa), $\hat{N}=124$ nt, $L_C=31$. We also estimated the number of binding sites on heparin by assuming that each sulfated sugar residue of average mol. wt. 273 (see ref. 28) binds equivalently as does one phosphoribose group. Hence for heparin (~14 kDa), $\hat{N}\simeq 51$ and $L_C=13$.

RNase P preparation

Fraction III spinach chloroplast processing activities were prepared by hypotonic lysis and $(NH_4)_2SO_4$ precipitation (18), followed by CsCl gradient centrifugation essentially as described previously for *E. coli* extracts (18). An RNase P-containing nuclear extract from *Saccharomyces cerevisiae* (21) was generously provided by Greg Raymond and Jerry Johnson.

Micrococcal nuclease treatment

Micrococcal nuclease (MN) (15,000 U/mg) was purchased from Boehringer and dissolved at 30−300 U/μl in 20 mM Hepes-KOH (pH 8.0), 60 mM KCl, 1 mM Na·EDTA (pH 8). Serial dilutions were made in the same buffer. Preincubation of tRNA processing enzymes was done in 5−7 μl by mixing 1−2 μl of MN dilutions (2−300 U/μl) with 1 μl of $CaCl_2$ (5−20 mM) or of Na·EGTA (pH 8; 50−250 mM) on ice and adding 1−4 μl of RNase P preparation. After 30 min at 37°C, the reaction mixture was chilled on ice and EGTA (1 μl, as above) was added to those reactions lacking it. Poly(A), poly(C) or carrier RNA, 10−20 μg each, was added as indicated. The assay for remaining tRNA processing activity was done in 20 μl final volume by adding a substrate mix containing [^{32}P]pre-$tRNA^{Phe}$ (pNXPhe/HindIII), 5% glycerol and sufficient 10× concentrated processing buffer (200 mM Hepes-KOH [pH 8], 500 mM KCl, 150 mM $MgCl_2$, 2 mM Na·EDTA [pH 8], 20 mM DTT, and 50% glycerol) for 1× final. The mixture was incubated 30 min at 37°C. Reactions were terminated and analyzed by denaturing gel electrophoresis as described (18).

Filter binding assays

Typically, one microliter of MN (30 U/μl in 50 mM EGTA) was added to tRNA precursor in 19 μl of 1× processing buffer. Final concentrations were 5.88 μM MN and 2.5 mM EGTA. At successive times duplicate incubation mixtures were applied with mild vacuum to a nitrocellulose filter in a BRL Hybri-Dot-Blot apparatus (6 mm diameter wells). Each well was then washed twice with 200 μl of washing buffer (processing buffer minus DTT), dried, and exposed to X-ray film. Individual sections of the filter were excised using the autoradiogram as a guide and counted in toluene-based scintillation fluid. No correction was

made for unequal counting efficiency of RNA on filters (bound RNA) *vs.* input RNA in solution (total RNA).

To measure complex dissociation (Fig. 2B), a scaled up binding reaction (as above) was incubated at 37°C for 40 minutes. Poly(A) was added to 0.95 μg/μl, giving 8.5 μM polymer. At the indicated times after poly(A) addition, 21 μl were withdrawn and applied to nitrocellulose filters. In the competitive dissociation assay (Fig. 3), EGTA-treated MN was mixed with pAsp31 RNA in processing buffer and incubated on ice for 20 min. Final conditions were 2.35 μM nuclease, 1.1 mM EGTA, and ca. 2 nM [^{32}P]pre-tRNA^{Asp} (containing 18 nM (=L_S) or 55 nM (=L_C) nuclease binding sites as described above). Serial dilutions of poly(A), poly(U), poly(C), heparin, or yeast tRNA^{Phe} were made in 50 μl of water and an equal volume of MN/pre-tRNA mixture was then dispensed into each tube of competitor. These MN/pre-tRNA/competitor solutions were incubated at room temperature for 20 min and the amount of MN/RNA complex remaining was determined by nitrocellulose filter retention (as above). The number of nuclease binding sites on each competitor was calculated as described above.

Electrophoretic retardation assay

The electrophoretic retardation assay was performed similarly to the protocol of Dorn et al. (22). Each reaction contained 0.36 pmol [^{32}P]pre-tRNA^{Phe} pNXPhe/HindIII, equivalent to 23 (=L_S) or 30 (=L_C) pmol nuclease binding sites. RNA was incubated for 30 min at 22°C in processing buffer with 4.8 mM EGTA and 0−1.5 U/μl (0−118 pmol total) micrococcal nuclease. Some reactions also contained heparin (0.25−0.5 μg; equivalent to 260−520 pmol nuclease binding sites) or poly(A) (0.25−1.0 μg; 186−740 pmol nuclease sites). After incubation, each reaction was mixed with 5 μl of 3× loading mix (18) lacking urea and loaded directly onto a non-denaturing 5% polyacrylamide gel (acrylamide:bisacrylamide, 30:1) buffered with 0.5× TBE (1× TBE is 89 mM Tris, 89 mM boric acid, 2.5 mM Na·EDTA). Gels were run below 35 mA at room temperature and analyzed by autoradiography.

Analysis of binding data

The binding experiments shown in Figure 4 were most easily performed by titrating a fixed amount of nuclease with increasing amounts of [^{32}P]RNA. Unfortunately, traditional analyses of protein·ligand interactions cannot be used to determine a dissociation constant because multiple proteins (MN) bind to one RNA molecule. Effectively, RNA is the 'receptor' and MN is the 'ligand'. McGhee and von Hippel (20) have provided a lucid treatment of the situation in which a small protein ('ligand') binds randomly to multiple identical and overlapping sites on a linear polymer ('one-dimensional lattice'). Application of this treatment, however, requires that the amount of bound protein be determined. (If the number of binding sites per polynucleotide were constant and known, the treatment of Faus and Richardson (23) could be applied.) Both of these models require information which was not available. We therefore derived an exact binding equation from the equilibrium equation which defines K_D. We are interested in dissociation of an enzyme·ligand complex in which many molecules of enzyme (micrococcal nuclease) are bound to a single molecule of ligand (RNA). The enzyme:ligand ratio, n, equals $[MN_b]/[MN \cdot RNA]$. (The subscripts *b*, *f*, and *t* denote bound, free, and total species respectively.) Since each complex contains one ligand (RNA) molecule, $[MN \cdot RNA]=[RNA_b]$. In the present situation, we did not determine the efficiency of filter retention nor did we correct for differential counting efficiency of input *vs.* bound RNA. We therefore use the superscript * for apparent values, and define n* such that $n^*[RNA_b]^* = n[RNA_b]$. By substitution into the equilibrium definition of K_D, and solving for $[RNA_b]$, we derive

$$[RNA_b]^* = \frac{[MN_t]\,[RNA_f]}{(K_D^* + n^*[RNA_f])} \qquad \text{(Equation I).}$$

Linearizing gives

$$\frac{[RNA_b]}{[RNA_f]} = \frac{[MN_t]}{K^*_D} - \frac{n^*}{K^*_D}[RNA_b] \qquad \text{(Equation II).}$$

Values of K_D * and n* were determined by direct non-linear regression fitting of Eqn. I to the plot of $[RNA_b]$ *vs.* $[RNA_f]$, using the program ENZFITTER (R.J. Leatherbarrow; distributed by Biosoft, Cambridge, UK). Robust weighting was used with explicit weights (standard deviations) of 0 for each point; this gave optimal fit to the data and was necessary for convergence of the Marquardt iterative curve-fitting algorithm. Initial estimates of K_D* and n* were obtained within ENZFITTER using linear regression on Eqn. II.

From the derivation of Eqn. I, it is apparent that binding constants could also be derived from a titration of [^{32}P]RNA with MN. This format would have the advantage that interpretation of side effects such as cooperativity is more straightforward. The disadvantage is that the experiments require considerably more mass of labeled RNA. We are interested only in estimating K_D, for which purpose the present data are adequate.

RESULTS

Artifactual Nuclease Sensitivity of Spinach Chloroplast RNase P

During our investigations of chloroplast transfer RNA processing, we wished to determine whether chloroplast tRNA 5′-processing endonuclease resembled its eubacterial counterpart, RNase P, in possessing a required RNA subunit. We assayed pre-tRNA 5′ and 3′ processing by a crude enzyme preparation after treatment of the enzymes with micrococcal nuclease and inactivation of the nuclease with EGTA.

Figure 1A, lanes 2−4, demonstrates that chloroplast tRNA-specific 5′ and 3′ endonuclease activities in a partially purified enzyme preparation are suppressed by preincubation with Ca^{2+}-activated micrococcal nuclease (MN). As a control to indicate whether the inhibition by MN resulted from hydrolysis of specific RNA components, the MN-treated samples were assayed in the presence of synthetic polyadenylate. Figure 1A, lanes 5−7, shows the surprising result: tRNA processing activity was completely restored to control levels. This enzyme fraction had largely been freed of endogenous nucleic acids by CsCl density gradient ultracentrifugation (see Materials and Methods). Figure 1B demonstrates that RNase P activity of this fraction can be suppressed either by active MN or by EGTA-inactivated MN, and that RNase P activity is restored in both instances by addition of poly(A). Treatment of this fraction with increasing amounts of EGTA-inactivated MN, as seen in lanes 2−4 of Figure 1A and lanes 10−12 of Figure 1B, suppresses RNase P activity and results in progressively greater electrophoretic

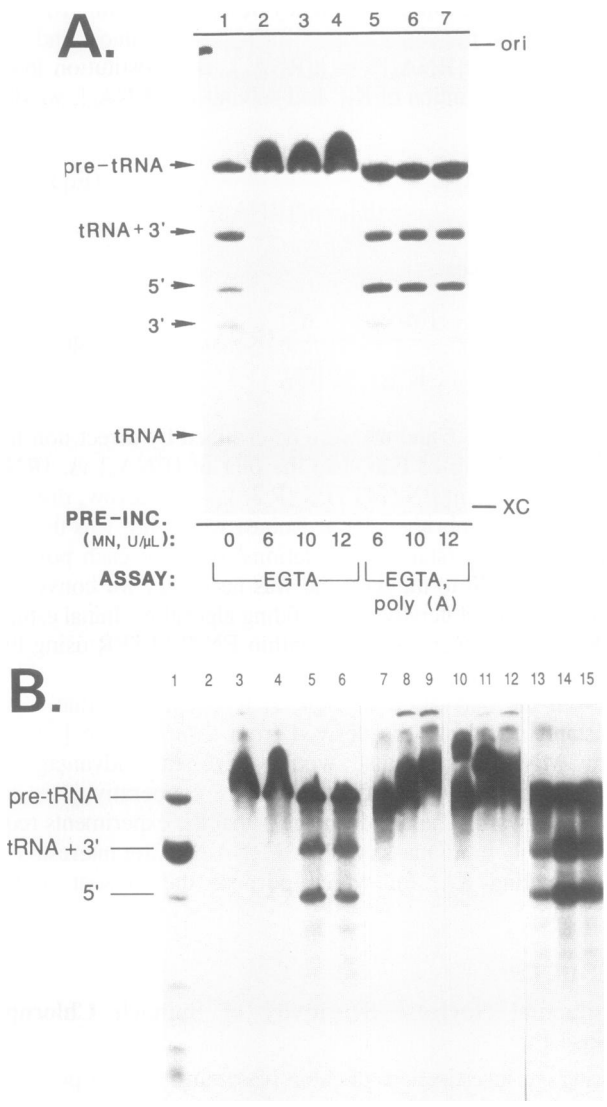

Figure 1. Suppression and restoration of chloroplast transfer RNA processing activities following micrococcal nuclease treatment. **A.** *Restoration of activity with poly(A).* Spinach chloroplast protein (Fraction III, 1 μl) was incubated, following Materials and Methods, in 5 μl containing 1 mM $CaCl_2$ and micrococcal nuclease at 0, 6, 10, or 12 U/μl. Reaction was terminated with EGTA to 5 mM. tRNA processing was then assayed in the absence (*lanes 1–4*) or in the presence (*lanes 5–7*) of 1 μg poly(A)/μl. **B.** *Both active and inactive MN suppress RNase P activity.* The enzyme source was 4 μl chloroplast Fraction III activity (18), which contains little endogenous RNA. *Lanes 1 and 2* are controls for RNase P and for MN activity, respectively. *Lanes 3 to 6*: RNase P was preincubated with 50 U/μl MN plus 3.3 mM $CaCl_2$ and assayed in the presence of 12 mM EGTA and 0, 0.5, 1.6 or 2.1 μg/μl poly(A). *Lanes 7 to 15*: the enzyme fraction was treated with MN at 4.2 U/μl (*lanes 7,10,13*), 6.7 U/μl (*lanes 8,11,14*), or 16.7 U/μl (*lanes 9,12,15*). During preincubation, MN was active (+ 3.3 mM $CaCl_2$) in *lanes 7 to 9* and inactive (+ 11 mM EGTA) in *lanes 10 to 15*. RNase P then was assayed in the absence of carrier RNA, *lanes 7 to 12*, or in the presence of 1 μg/μl of poly(A), *lanes 13 to 15*.

retardation of the [^{32}P]RNA substrate. When poly(A) is added, as in lanes 5–7 of Figure 1A or lanes 13–15 of Figure 13, electrophoretic retardation is relieved and RNase P activity is restored essentially to control levels.

EGTA-Inactivated Micrococcal Nuclease Can Bind pre-tRNA

To quantify the binding of EGTA-inactivated MN to RNA, we used a nitrocellulose filter retention assay. Labeled RNA was

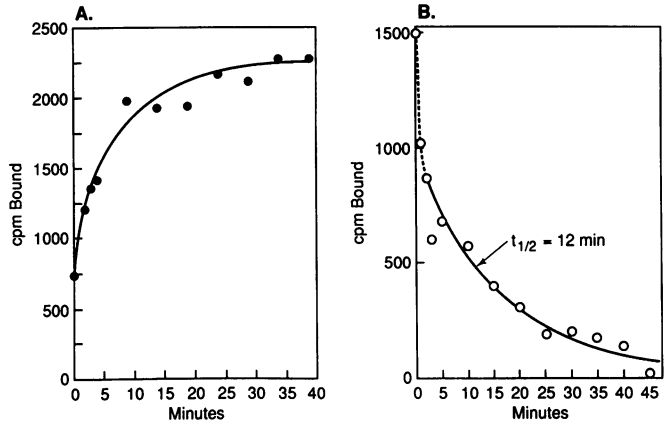

Figure 2. Kinetics of interaction between pre-tRNA and EGTA-inactivated MN. (A) *Time course of association.* Duplicate binding mixtures (see Methods) containing [^{32}P]p67CF0 RNA were incubated at 22°C for the indicated times and nitrocellulose filter retention of pre-tRNA was measured by liquid scintillation spectrometry. (B) *Dissociation of MN from pre-tRNA by polyadenylate.* A scaled-up binding reaction like that of panel A was challenged with poly(A) as described in Materials and Methods. Samples were withdrawn at the indicated times. The exponential decay constant was determined by non-linear curve-fitting.

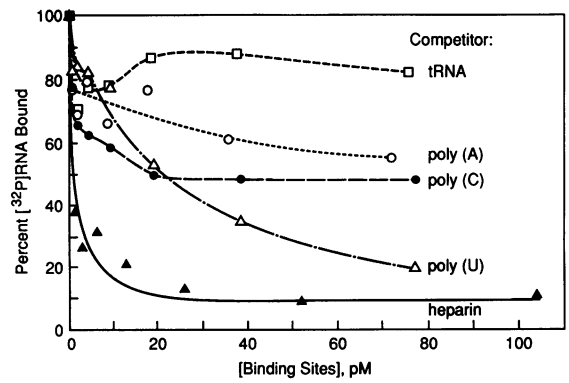

Figure 3. Dissociation of pre-tRNA·MN complex by various polymers. Binding between MN and pre-tRNA was measured according to Materials and Methods. The concentration of competitors is expressed as concentration of contiguous binding sites (see Methods). Assays in which no competitor was present were taken as 100% binding. An average of duplicate assays is shown.

retained on the membrane only in the presence of EGTA-treated MN. RNA alone was not bound, nor was binding detected in the presence of BSA, poly(A), or of heparin (data not shown). Figure 2A shows the kinetics of complex formation between EGTA-inactivated MN and [^{32}P]pre-tRNA. Figure 2B demonstrates that the complex, once formed, can be dissociated by addition of a polynucleotide such as poly(A). The kinetics of dissociation by poly(A) are revealing, since after an initial rapid release they fit an exponential decay curve. This indicates that dissociation is a stochastic process, consistent with the random, non- specific, and primarily non-cooperative nature expected for MN binding.

To increase our understanding of MN·RNA interactions, we tested a variety of polynucleotide or polyanion competitors over a wide range of concentrations. Figure 3 illustrates that an increase in competitor concentration results in proportional

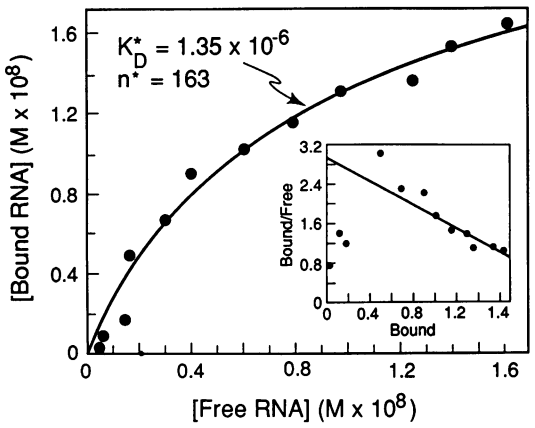

Figure 4. Equilibrium binding of MN to pre-tRNA. [^{32}P]pre-tRNA^{Asp} bound to MN was measured by the filter retention assay. MN (3.92 μM in 20 μl of 1.5X processing buffer and 2.5 mM EGTA) was mixed with 0.8125 nM to 32.5 nM RNA in 10 μl water. The mixture was incubated 20 min at 22°C and MN·RNA complexes were measured by nitrocellulose filter binding. Each point is an average of duplicate determinations. The hyperbolic curve represents the best fit to the binding equation, Eqn. I (Materials and Methods), with K_D *=1.35×10^{-6} M and n* = 163. The *inset* shows a linear transformation of the same data; the solid line is a plot of Eqn. II (Materials and Methods) using the constants derived above.

reduction of nitrocellulose filter-bound pre-tRNA. From the IC_{50} values (concentration giving 50% inhibition of binding) of these compounds, we can rank the relative affinity of these polymers as heparin > poly(U) > poly(C) ≥ poly(A) >> yeast tRNA^{Phe}. Indeed, yeast tRNA^{Phe} is unable to alleviate suppression of RNase P activity by MN in an assay like that of Figure 1A (data not shown). This observation suggests that MN binds poorly to mature tRNA, consistent with the finding that MN prefers single-stranded nucleic acid as substrate (1,4).

To estimate the binding affinity of MN for RNA, we titrated a fixed amount of MN with [^{32}P]pre-tRNA^{Asp} (pAsp31) and determined filter-bound RNA. At the maximum, close to 75% of the input RNA formed complexes that could be retained on the filter. Figure 4 shows the results of a titration performed at 3.92 μM MN and 0.8125 nM to 32.5 nM RNA. This range corresponds to 0.007 μM to 0.29 μM binding sites, given that for pAsp31 RNA, the likely number of binding sites (L_s) is 9. The data were fitted to equation I by non-linear regression as described in Materials and Methods, yielding an apparent dissociation constant K_D*=1.35×10^{-6} M±0.147×10^{-6} M. We also calculated K_D* directly from the equilibrium ratio at each data point using the assumptions that either a 1:1 or a 10:1 protein·RNA stoichiometry or 'loading ratio' is sufficient for filter retention. The average value so determined for K_D* at both loading ratios was 2.86×10^{-6} M±1.06×10^{-6} M (range, 1.3×10^{-6} to 5.3×10^{-6}). K_D* shows little dependence on the exact amount of MN bound to RNA, for the following reason. The total concentration of MN in this titration is 10^2 to 10^3-fold greater than the concentration of total RNA. The concentration of bound MN is probably no greater than L_S, and must be less than L_C times the concentration of bound RNA. Since L_S = 9 and L_C = 28 for pAsp31, it follows that $[MN]_{free} \approx [MN]_{total}$. Our experimental K_D* values agree well with K_D determined for metal-free MN binding to DNA of 12.8×10^{-6} M 0.5×10^{-6} M (2). (Our binding experiments were conducted in the presence of Mg^{2+}, which might be expected to reduce K_D. K_D for pdTp of the enzyme complex with Ca^{2+} or Mn^{2+} is lower than K_D of the enzyme alone (2)). The magnitude of K_D* is consistent with our observations of efficient but reversible binding at high [MN], as illustrated in Figures 2 and 3.

We asked whether a protein with this K_D could realistically cause substrate occlusion or 'masking' *in vitro* by competing with RNase P for pre- tRNA. K_M values for eubacterial RNase Ps are from 10^{-8} M to 10^{-7} M (24), and the apparent K_D of yeast (*S. cerevisiae*) nuclear RNase P for pre-tRNA is ~1×10^{-9} M (25). Thus, in our experimental nuclease treatment protocols micrococcal nuclease successfully competes against RNase P for binding to pre- tRNA because MN binding is driven by the high concentration of MN and by the high ratio of free MN to available binding sites.

Direct Visualization of MN·RNA Complexes

Another approach to characterize protein·nucleic acid binding is detection of the complex by non-denaturing gel electrophoresis. This method was used for the experiments shown in Figure 5. Below an MN: binding-site ratio of ~1.5:1, a modest amount of complex 1 (**C1**) accumulates as seen in lanes 2 and 3. Lane 4 indicates that, at a ratio of ~4:1, most of the pre-tRNA is found in complex **O** which is excluded from the gel. The remainder forms a diffuse complex **C2**. The amount of RNA in the excluded complex **O** is sometimes substoichiometric, as seen in lane 7, possibly because the complex can be lost from the top of the gel during post-electrophoresis handling.

Figure 5, lanes 5 and 6, demonstrates that addition of excess heparin effects essentially complete release of [^{32}P]RNA from the MN·RNA complexes **O** and **C2**. We estimated the concentration of micrococcal nuclease binding sites in heparin by assuming that MN could cover 4 sugar residues in heparin just as in nucleic acids. In these experiments, the MN binding sites in heparin were added in 8.7 or 17.3-fold molar excess over binding sites on [^{32}P]RNA; this represents a 2.2-fold or 4.4-fold molar excess over molecules of MN.

We also tested poly(A) for its ability to compete for binding MN. Figure 5, lanes 8–10, shows that molar excess of poly(A) substantially reduces but does not eliminate the MN·[^{32}P]RNA complex **O**. The ratio of poly(A) to MN, expressed as (moles MN binding sites):(moles MN), ranges from 1.6:1 to 6.3:1 in lanes 8–10. This represents a 6.2 to 24.7-fold excess of binding sites on poly(A) over sites on [^{32}P]RNA. At the lower levels of poly(A), the [^{32}P]RNA migrates at a position, denoted **C0** in Figure 5, somewhat slower than free RNA. This material may represent [^{32}P]RNA bound to only a few molecules of MN. The relative efficiencies of heparin and poly(A) as competitors in the gel retardation experiment of Fig. 5 are consistent with their effectiveness measured by the nitrocellulose filter retention assay of Fig. 3.

Nuclease Resistance of Yeast Nuclear RNase P

Having demonstrated the artifactual nature of MN inhibition of tRNA processing in the chloroplast system, we were curious to learn whether other reported examples of MN-sensitive RNA processing enzymes might not also be attributable to the substrate masking phenomenon described here. Nuclear RNase Ps from human cells, budding yeast (*Saccharomyces cerevisiae*), and from fission yeast (*Schizosaccharomyces pombe*) contain an RNA subunit, as determined by biochemical purification (9,17,26). The *S. cerevisiae* activity seems not to require the RNA component for binding pre-tRNA substrate: MN treatment of a partially purified enzyme preparation was shown not to alter the apparent K_D for RNase P·pre-tRNA association (25).

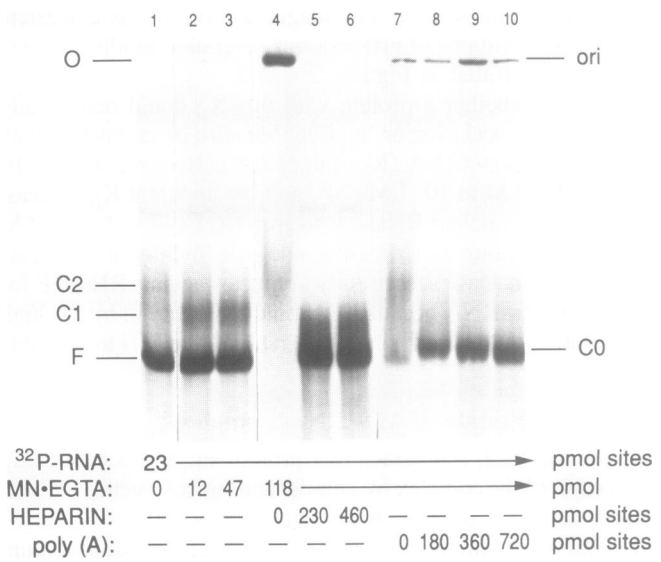

Figure 5. Electrophoretic retardation of MN·RNA complex. Incubation and electrophoresis were as described in Materials and Methods. The positions of MN·pre-tRNA complexes are indicated above free pre-tRNA. *Lane 1* shows a control incubation without MN. *Lanes 2 to 4* contain increasing amounts of MN. *Lanes 5 to 9* contain the maximal amount of MN. *Lanes 5 and 6* show complex dissociation by increasing amounts of heparin, and *lanes 7 to 9* demonstrate dissociation by increasing amounts of poly(A). Concentrations of nuclease binding sites were calculated according to Materials and Methods.

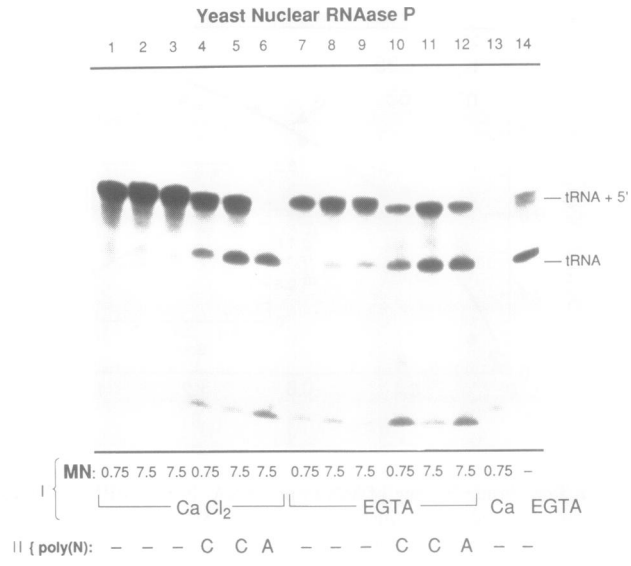

Figure 6. Apparent Inhibition of Yeast Nuclear RNase P by Micrococcal Nuclease. Yeast nuclear extract (2 μl) was incubated with 0.75 or 7.5 U MN per μl, as indicated below each lane. $CaCl_2$ (2.5 mM) was present in *lanes 1–6* and *13*, and EGTA (11.6 mM) was present in *lanes 7–12* and *14*. Following incubation and subsequent addition of EGTA to *lanes 1–6*, [^{32}P]pre-tRNA^{Asp} was added and RNase P cleavage was assayed. During the assay, poly(C) or poly(A), as indicated, was present in *lanes 4–6* and *10–12*. *Lane 13* is a positive control for MN activity, and *lane 14* is a positive control for RNase P.

RNase P activity can be assayed in lysates of isolated yeast (*S. cerevisiae*) nuclei (27). We treated *S. cerevisiae* nuclear lysate with micrococcal nuclease, inactivated the nuclease with EGTA, and assayed for RNase P both in the absence and in the presence of carrier polynucleotides. Figure 6, lanes 1–3, indicates that yeast nuclear RNase P activity is completely suppressed by treatment with 7.5 U/μl MN when assayed without carrier RNA. Lanes 4–6 and 10–12 of Figure 6 demonstrate that activity is restored to control levels by addition of poly(C) to 0.625 mg/ml or poly(A) to 0.5 mg/ml. Yeast nuclear RNase P activity thus appears to be completely refractory to digestion by micrococcal nuclease. We note, however, that Lee and Engelke, following a protocol similar to that of Fig. 6, were unable to restore activity to MN-treated samples of substantially purified *S. cerevisiae* nuclear RNase P (17). The reason for this discrepancy awaits further investigation.

DISCUSSION

Formation of RNA·MN complexes

We conclude, from the results of gel electrophoretic retardation and of nitrocellulose filter retention experiments, that EGTA-inactivated MN can bind RNA in a stable complex. Our results are consistent with previous determinations that the DNA binding domain is independent of the catalytic site (1,2,6), and that Ca^{2+} enhances but is not required for substrate binding (6). However, since Ca^{2+}, Mn^{2+}, and Co^{2+} all increase binding of pdTp to MN (6), it is possible that the Mg^{2+} present in our assays likewise strengthens the interaction between MN and RNA.

The binding between ribonucleic acid and EGTA-inactivated MN is consistent also with the substrate specificity of this enzyme. Much of this binding is directed towards repeating anionic substituents rather than towards specific sugars or nucleotide bases. As shown in Figures 3 and 5, heparin, a highly sulfated mucopolysaccharide composed of derivatives of D-glucosamine and D-uronic acids (28), can strongly displace RNA from the MN·pre-tRNA complex. On the other hand, an uncharged polysaccharide like glycogen, or the uncharged polymers Ficoll and polyethylene glycol, were ineffective at displacing labeled pre-tRNA (data not shown).

One striking suggestion of the strength of the MN·RNA interaction is seen in Figure 1B, lanes 7–12. With increasing amounts of MN present in the reaction, there is an increasing extent of electrophoretic retardation of the [^{32}P]RNA substrate. This progressive retardation is routinely observed in electrophoresis of MN-containing reactions (18, 25, and this work), but it is never observed in such reactions performed in the presence of poly(A) (ref. 18, this work, and unpublished data). It was this phenomenon which first alerted us to the possibility that a specific MN·RNA complex might exist. Detection of such a complex in the gels of Figure 1 is unexpected because the reaction mixture was digested with proteinase K in the presence of SDS and EDTA prior to electrophoretic separation at ~40°C in a polyacrylamide gel containing 7 M urea. These observations are consistent, however, with the formation of a protease- and urea-insensitive protein·RNA complex. DNase I, a Ca^{2+}-metalloenzyme similar to micrococcal nuclease, is protected from proteinase K digestion when complexed with Ca^{2+} (29). Micrococcal nuclease is resistant to heat denaturation when bound to Ca^{2+} or to denatured DNA (4). Thus, we might expect micrococcal nuclease to be at least partially proteinase K-resistant and denaturation-resistant when bound to RNA.

Artifactual Inhibition of RNA Processing by Inactive Micrococcal Nuclease

Ryner and Manley (19) demonstrated that mRNA 3′ cleavage and polyadenylation activity could be restored to an MN-treated extract of HeLa cell nuclei by adding carrier *E. coli* RNA. Their

explanation was that the processing activities had a general requirement for RNA mass. Other groups have observed similar findings, and have sometimes attributed the inhibition to non-specific RNA binding proteins released from endogenous RNA by MN treatment. We offer a molecular explanation termed substrate masking which we find is supported by direct biochemical measurements. Micrococcal nuclease, from which Ca^{2+} has been removed by chelation with EGTA, retains the capacity to bind RNA tightly but reversibly. The sites for processing on the RNA are thus occluded or masked and are unavailable for enzymatic interaction. Addition of excess competitive ligand, namely carrier RNA, displaces MN from substrate RNA and allows normal RNA processing. If the protein fraction to be tested for MN sensitivity contains significant endogenous RNA, then substrate masking will be observed only after hydrolysis of this RNA, i.e., only when MN is active during the preincubation. Protein fractions lacking RNA will be subject to substrate masking both by active as well as by inactive MN. This phenomenon is documented in Figure 1B. It is important to choose a carrier RNA which does not inhibit the RNA processing reaction under investigation. For example, RNase P activities of chloroplasts, *E. coli*, wheat embryo, and yeast nuclei are not inhibited by poly(A), but are strongly inhibited by poly(U) and (at least for chloroplast and yeast RNase P) by heparin (unpublished observationıs). RNase P activity in HeLa cell nuclear extracts, on the other hand, is strongly (>95%) inhibited by all synthetic polynucleotides we have tested, and only slightly less so by carrier yeast RNA (unpublished observations).

Previous experiments with nuclear RNase P indicated that its activity was suppressed by treatment with MN (16,17). Our results indicate that the yeast nuclear activity may not intrinsically be sensitive to nuclease digestion. Eukaryotic nuclear RNase P activities co-purify extensively with large RNA molecules, but there is currently no independent evidence to indicate that these RNAs are required for enzyme activity. We suggest that further work is needed to clarify the relationship between micrococcal nuclease inhibition and RNA function in nuclear RNase P. The RNA component of nuclear RNase Ps might be bound to protein in such a way as to be inaccessible to, or resistant to, nuclease attack. The low buoyant density of these ribonucleoproteins is also consistent with a model in which the RNA is sequestered by protein, thus reducing its interaction with Cs^+ ions. These considerations, combined with our experimental evidence, argue for careful experimental design when using micrococcal nuclease in ribonucleoprotein analysis, and for caution in the interpretation of such data.

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