Activation/Attenuation Model for RNase H
A ONE-METAL MECHANISM WITH SECOND-METAL INHIBITION

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Ribonucleases H (RNases H) comprise a family of metal-dependent enzymes that catalyze the hydrolysis of the 3'-O-P bond of RNA in RNA-DNA hybrids. The mechanism by which RNases H use active-site metal(s) for catalysis is unclear. Based upon the seemingly contradictory structural observations of one divalent metal bound to Escherichia coli RNase HI and two divalent metals bound to the HIV RNase H domain, two models explaining RNase H metal dependence have been proposed: a one-metal mechanism and a two-metal mechanism. In this paper, we show that the Mn²⁺-dependent activity of E. coli RNase HI is not consistent with either of these mechanisms. RNase H activity in the presence of Mn²⁺ is complex, with activation and inhibition of the enzyme at low and high Mn²⁺ concentrations, respectively. Mutations at Asp-134 result in a partial loss of this inhibition, with little effect on activation. Neutralization of His-124 by mutation to Ala results in an enzyme with a significantly decreased specific activity and an absolute loss of Mn²⁺ inhibition. Inhibition by high Mn²⁺ concentrations is shown to be due to a reduction in kcat; this attenuation has a critical dependence on the presence of His-124. Based upon these results, we propose an “activation/attenuation” model explaining the metal dependence of RNase H activity where one metal is required for enzyme activation and binding of a second metal is inhibitory.

The ribonuclease H (RNase H) family consists of ubiquitous, metal-dependent enzymes that catalyze the hydrolysis of RNA in RNA-DNA hybrids (for review, see Ref. 1). RNases H are unusual among well studied ribonucleases in that they require active-site divalent metals for activity, a property commonly observed in deoxyribonucleases. RNases H are also unusual in their specificity for leaving free 3'-hydroxyl products after phosphodiester hydrolysis. By contrast, the more familiar RNase A or RNase T1 leaves 3'-phosphate products, using the RNA 2'-hydroxyl as a nucleophile. RNases H are thought to employ an hydroxyl ion (activated water) as the attacking nucleophile, but the manner in which RNases H use active-site metal(s) to catalyze this specific ribonuclease reaction remains unclear.

RNases H and related enzymes have emerged as important therapeutic targets because RNase H activity is absolutely required for proliferation of HIV and other retroviruses. Mutations in the RNase H domain of HIV reverse transcriptase that reduce activity result in a loss of virulence (2), making RNase H an attractive target for anti-HIV therapies. Understanding the cofactor requirements for RNase H should therefore aid in the development of such drugs. Moreover, the three-dimensional structures of a number of proteins with structural homology to RNase H have recently been solved by x-ray analysis, all with metal-dependent nucleic acid-modifying functions (reviewed in Refs. 3–5). This superfamily of proteins, termed “polynucleotide transferases,” includes RNase H (6–9), resolvase (10), integrase (11, 12), transposase (13), and exonuclease III (14). These enzymes have homologous active sites and are therefore likely to share a common mechanism for catalysis. Hence, an understanding of the metal dependence of the RNase H mechanism should aid in understanding the mechanism of other members of this superfamily.

Two classes of catalytic mechanisms have been proposed to explain the metal-dependent nature of the RNase H reaction, a one-metal (15, 36) and a two-metal mechanism (8). The one-metal RNase H mechanism is similar to that proposed for DNase I (17), with amino acid side chains serving as both a general base and proton shuttle and with a single divalent metal stabilizing the hydrolysis intermediate. Alternatively, the two-metal RNase H mechanism is analogous to the proposed mechanism for the Klenow fragment 3'-5'-exonuclease domain (18, 19), where one metal serves to stabilize the phosphorane intermediate and a second metal assists in activating the nucleophilic water (generalized in Ref. 20). Neither the one-metal nor the two-metal mechanism, however, can entirely explain the wealth of structural and mutagenic information that has been compiled for the RNase H family of enzymes.

Mechanistic analyses of the RNase H family of enzymes have been complicated by the use of different divalent metals ions in functional studies and by the assumption that different metals affect the enzyme in the same way. While it has been known for over 20 years that either Mg²⁺ or Mn²⁺ can activate RNase H (21), there are significant differences between the effects of these two divalent metals. First, mutagenesis studies on the RNase H domains of retroviral reverse transcriptases have shown that some active-site mutations result in a complete loss of Mg²⁺-dependent activity but only a specific reduction in Mn²⁺-dependent activity (22, 23). Second, the HIV RNase H domain is inactive when expressed independently of the rest of reverse transcriptase, but a variety of mutations that activate the isolated domain do so in a strictly Mn²⁺-dependent manner (24–27). Third, inhibitors of HIV RNase H activity affect Mg²⁺- and Mn²⁺-dependent activities differently, implying a mecha-
nistic difference in the presence of different divalent metals (28). Finally, whereas two Mn$^{2+}$ ions bind to HIV Rnase H (8), only one Mg$^{2+}$ ion binds Escherichia coli Rnase HI (6, 16, 33, 38) (Fig. 1). With these results in mind, we have initiated studies using E. coli Rnase HI as a model system to probe the general metal requirements of the family as well as the distinctions between Mg$^{2+}$- and Mn$^{2+}$-dependent Rnase H activities.

E. coli Rnase HI requires significantly lower concentrations of Mn$^{2+}$ than Mg$^{2+}$ for activation and is inhibited upon the further addition of Mn$^{2+}$ (29, 30). One interpretation consistent with these data is that, in the presence of Mn$^{2+}$, E. coli Rnase HI is activated by a single metal but can be inhibited upon subsequent binding of a second metal, an “activation/attenuation” model. In this paper we demonstrate that this model is supported by a panel of E. coli Rnase HI mutant proteins designed to impair the putative second metal-binding site. Furthermore, we note that the activation/attenuation model is consistent with the previous structural observations of both one and two metals binding to RNases H.

**EXPERIMENTAL PROCEDURES**

**Materials**—All buffer components were from Sigma or Fisher unless specified. Ribonucleotides (Boehringer Mannheim), [α-32P]CTP (Amerham Pharmacia Biotech), acetylated bovine serum albumin (BSA) (U. S. Biochemical Corp.), heparin-Sepharose (Amersham Pharmacia Biotech), guanidine HCl (Pierce), and restriction enzymes (New England Biolabs) were used in accordance with the suppliers’ recommendations. Synthetic oligonucleotides were made on an Applied Biosystems 392 RNA/DNA synthesizer. Purified E. coli Rnase HI polymerase was kindly provided by Michael Chamberlin (University of California, Berkeley). MnCl$_2$ stock solutions were made by serial dilution of a 1 M MnCl$_2$ stock solution in 1% nitric acid, with final diluted stocks in 0.1% nitric acid.

*Creation, Expression, and Purification of Mutant Proteins*—pKH502 is a T7 expression, low copy plasmid vector that bears a synthetic gene encoding the wild type E. coli Rnase HI sequence (30). Site-directed mutations of pKH502 were made by standard polymerase chain reaction mutagenesis, with a mismatched mutagenic oligonucleotide and a universal primer used to amplify a portion of the gene. These polymerase chain reaction products were subcloned into the E. coli Rnase HI gene, resulting in the creation of specific point mutants of the gene (plasmid names and mutations are listed in Table I). All genes were sequenced by standard methods.

Overexpression and purification of wild type and mutant Rnase H proteins were performed identically. E. coli BL21 (DE3) pLysS cells transformed with the specified plasmid (see Table I) were grown at 37 °C in Luria-Bertani (LB) medium plus 200 μg of ampicillin and 25 μg of chloramphenicol per ml. Cells at mid-log phase (A$_{600}$ 0.5) were induced to overexpress Rnase H with 1 mM isopropyl-β-D-thiogalactopyranoside and were harvested after an additional 3 h of growth. Cell pellets were frozen at −80 °C followed by thawing via resuspension in 50 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol (buffer A) with 1 mM phenylmethanesulfonyl fluoride and were further lysed by sonication. The soluble fraction was loaded onto a heparin affinity chromatography column (Amersham Pharmacia Biotech) and developed with a linear NaCl gradient from 20 to 420 mM.

**RESULTS**

**Design and Construction of E. coli Rnase HI Point Mutants**—A comparison of the Mn$^{2+}$-binding sites observed in the HIV Rnase H domain crystal structure with the crystal structure of E. coli Rnase HI demonstrated that two metals could, in principle, bind the E. coli homolog in an analogous fashion to the binding mode observed in the HIV Rnase H domain (Fig. 1). One metal-binding site is formed by Asp-10 and Glu-48, as seen in the Mg$^{2+}$-co-crystal of E. coli Rnase HI (33). The exact placement of the metal ion bound by Asp-10 and Glu-48 varies by ∼1 Å between the E. coli Mg$^{2+}$-co-crystal and the HIV Mn$^{2+}$-co-crystal, and we have not drawn any conclusions on which site represents the true metal positioning during catalysis. In our model, we propose that a second metal-binding pocket is formed by the conserved residues Asp-134 and possibly His-124, perhaps with contributions from Asp-10.

To test whether Asp-134 and His-124 form a second metal-binding site, we constructed point mutants of E. coli Rnase HI that served to partially neutralize Asp-134 (mutating it to His or Asn) or completely neutralize His-124 (mutating it to Ala) (Table I). These mutations were chosen to impair affinity at the putative second metal-binding site. Plasmids encoding the mutant enzymes were constructed, and Rnase H variants were overexpressed and purified to apparent homogeneity as described under “Experimental Procedures.” The activity of these mutants has been studied previously but only in the presence of Mg$^{2+}$ (35–37). In Mg$^{2+}$, mutations in Asp-134 retain essentially wild type activity, whereas H124A shows a ∼100-fold reduction. X-ray crystallography (of D134H, D134N) and circular dichroism studies (of H124A) have shown very minimal deviations in these mutant proteins from the topology and stability of the wild type enzyme (35, 37, 43).

Mn$^{2+}$ Dependence of Rnase H Asp-134 and His-124 Mutants—Wild type E. coli Rnase HI activity shows strong inhibition in the presence of Mn$^{2+}$ at concentrations greater than 5 μM (Fig. 2), consistent with our previous observations of Mn$^{2+}$-dependent E. coli Rnase HI activity (29, 30). In order to test the activation/attenuation model, we examined the dependence of Rnase H activity on Mn$^{2+}$-concentration for these mutant proteins.
enzymes. Upon mutation of Asp-134 to either His or Asn, the Mn\(^{2+}\) dependence of activity changes significantly (Fig. 2A).

Both variants are activated by Mn\(^{2+}\) similarly to the wild type enzyme (requiring very low micromolar concentrations), but the inhibition observed at higher concentrations of Mn\(^{2+}\) is clearly diminished upon mutation of Asp-134. Reduced Mn\(^{2+}\) inhibition leads to a shift in the concentration of MnCl\(_2\) required for maximal activity (5, 30, and 100 \(\mu M\) Mn\(^{2+}\) for wild type, D134H, and D134N, respectively). The degree to which these mutations relieve inhibition correlates with the expected decrease in polarity at the \(\delta\)-position of the side chain: Asp (-0.706 electronic units (e.u.)) < His (-0.527 e.u.) < Asn (-0.470 e.u.) (34).

The H124A mutation has previously been shown to lower \(k_{cat}\) values -100-fold with an increase in \(K_m\) values -3-fold in a Mg\(^{2+}\)-dependent RNase H assay (36). In low Mn\(^{2+}\) concentrations, we find a similar reduction in activity (-50-fold) (Fig. 2B), and the amount of Mn\(^{2+}\) required for activation of H124A is comparable to that for wild type RNase H. In contrast to the other RNase H variants tested, inhibition of H124A is not observed in higher Mn\(^{2+}\) concentrations. The activity of H124A at 1 mM MnCl\(_2\) is equivalent to the attenuated form of the wild type protein.

In the presence of Mg\(^{2+}\), where presumably only one metal binds (as suggested by extensive affinity studies (33, 38)), we observed slight increases in the amounts of Mg\(^{2+}\) required to activate the mutant RNases H relative to wild type (data not shown). Similar effects in Mn\(^{2+}\) activation are apparent in D134N and H124A (Fig. 2), and hence, slight alterations in the structure and electrostatic environment of the active site likely account for these changes.

**Michaelis-Menten Kinetics of Wild Type and H124A RNase H as a Function of Mn\(^{2+}\)**—To probe the mechanism of inhibition in high Mn\(^{2+}\) concentrations, we determined the kinetic parameters for catalysis by RNase H assuming steady-state kinetics.

In the simplest case, Mn\(^{2+}\) could attenuate activity by either inhibiting substrate binding (an increase in \(K_m\)) or slowing catalysis at the level of the rate-limiting step (a decrease in \(k_{cat}\)). In 3 \(\mu M\) Mn\(^{2+}\) (approximately the optimum for activity), the observed \(k_{cat}\) is 321 min\(^{-1}\) for wild type with a \(K_m\) of 0.030 \(\mu M\) (Fig. 3A). Upon addition of inhibitory concentrations of Mn\(^{2+}\), \(k_{cat}\) and \(K_m\) are reduced to values of 13 min\(^{-1}\) and 0.003 \(\mu M\), respectively.

Given the striking differences between the Mn\(^{2+}\) dependence of wild type and H124A RNase H activities (Fig. 2B), we wanted to determine the effects of Mn\(^{2+}\) concentration on \(K_m\) and \(k_{cat}\) in H124A. Fig. 3B shows that although higher concentrations of Mn\(^{2+}\) lead to a reduction in \(K_m\), \(k_{cat}\) remains constant in all Mn\(^{2+}\) concentrations for the H124A mutant. Reduction of the \(K_m\) for H124A is -2-fold from 3 to 10 \(\mu M\) MnCl\(_2\). These data are in contrast to that of the wild type where both
Activation/Attenuation Model for RNase H

Crystal structures of two homologous RNase H enzymes have been solved with divalent metals bound at their active sites as follows: *E. coli* RNase HI with a single Mg$^{2+}$ ion (33) and the HIV RNase H domain with two Mn$^{2+}$ ions (8). Using these structural observations, two models for RNase H activation have been proposed.

The first model for RNase H activation is a one-metal mechanism analogous to the proposed mechanism for DNase I activity (17). In this model, a single divalent metal is bound by the conserved residues Asp-10 and Glu-48 (15, 36) and serves to stabilize the transition state intermediate of the hydrolysis reaction. Generation of a nucleophilic hydroxyl ion is hypothesized to occur by deprotonation of water, with Asp-70 acting as a general base. In further analogy to the proposed DNase I mechanism, deprotonation of Asp-70 is thought to be facilitated by a proton transfer to His-124 which then rapidly loses the proton into bulk solvent (35). This mechanism is supported by extensive mutagenesis and structural experiments utilizing Mg$^{2+}$ as a cofactor for activity (16, 33, 35–39).

The second model for RNase H activation is a two-metal mechanism similar to the proposed mechanism of the Klenow fragment 3′-5′-exonuclease domain (8). In this model, two metals are required for RNase H activation as follows: the first metal stabilizes the phosphorane intermediate (as in the single-metal mechanism), and the second metal orders and activates the water that serves as the attacking nucleophile in hydrolysis (generalized in Ref. 20). The first metal is positioned in an analogous position to the single metal observed in *E. coli* RNase HI, whereas the second is hypothesized to be liganded by Asp-134 and possibly His-124. To date there has been no reported structure of an active RNase H with two metals bound in its active site, and therefore these residues are proposed based on the co-crystal structure of the homologous but inactive HIV RNase H domain complexed with Mn$^{2+}$ (8).

The observed dependence of RNase H activity on Mn$^{2+}$ fits neither of these mechanisms. Activation of the enzyme by Mn$^{2+}$ occurs at very low concentrations of metal (<5 μM), but inhibition is detected at higher metal concentrations (Refs. 29 and 30 and see Fig. 2A). Furthermore, we have noted a similar activation/attenuation profile using a poly(rA)·(dT) substrate at much higher RNA·DNA hybrid concentrations (data not shown). Because Mn$^{2+}$ can also inhibit the Mg$^{2+}$-dependent activity of *E. coli* RNase HI (data not shown), second-metal attenuation is likely relevant to catalysis in the presence of the presumed physiological divalent cofactor. Based on these observations, we hypothesize that one metal activates RNase H and a second metal of weaker affinity can bind but inhibits enzyme activity, an “activation/attenuation model.”

Mutagenesis of Residues Involved in a Putative Inhibitory Metal-binding Site—In this paper we have tested the activation/attenuation model of RNase H activity by assaying a panel of *E. coli* RNase HI mutants in which the presumed inhibitory metal-binding site has been impaired. We postulated that a second metal binds in a site analogous to that observed in the HIV RNase H crystal structure and that Asp-134 and perhaps His-124 ligand this metal (Fig. 1). Previous work has shown that *E. coli* RNase HI does not require Asp-134 or His-124 for activity (35–36, 42) unlike active-site residues Asp-10, Glu-48, and Asp-70 (37). Although Asp-134 is invariant in the RNase H family, conservative mutations in this residue retain essentially wild type activity in Mg$^{2+}$ (35). Why, then, is this residue faithfully conserved throughout RNases H? One possibility is that it is needed to regulate activity via second-metal inhibition.

Our hypothesis that Asp-134 is involved in the formation of an inhibitory Mn$^{2+}$-binding site is supported by the result that

![Graph](image-url)

**Fig. 3.** Steady-state kinetic parameters of RNase H activity as a function of Mn$^{2+}$ concentration. A. $K_n$ (open circles) and $k_{cat}$ (closed circles) values as a function of Mn$^{2+}$ for wild type *E. coli* RNase HI in 3, 10, 30, or 100 μM MnCl$_2$, as determined by analysis of the substrate dependence of initial rates of reaction. B. $K_n$ (open boxes) and $k_{cat}$ (closed boxes) values as a function of Mn$^{2+}$ for His-124 → Ala RNase HI in 3, 10, or 30 μM MnCl$_2$, as determined by analysis of the substrate dependence of initial rates of reaction. Assays were carried out in 50 mM Tris·HCl, pH 8.0, 50 mM NaCl, 1.5 mM BSA, 1 mM DTT, 1–100 nM (base pairs) RNA·DNA hybrid with indicated MnCl$_2$ concentration. Enzyme concentrations were 10–25 pm (wild type RNase HI) and 25–50 pm (His-124 → Ala RNase H). Steady-state parameters were determined using the LEONORA fitting program (32).

$K_m$ and $k_{cat}$ are reduced in high Mn$^{2+}$ concentrations.

Metal Binding by *E. coli* RNase HI—We attempted to determine Mn$^{2+}$ binding constants for *E. coli* RNase HI using titration calorimetry to compare the concentration requirements for activation and inhibition in *vitro*. However, at pH values greater than 7.5, Mn$^{2+}$ causes enzyme precipitation at high protein concentrations (data not shown), thus precluding the possibility of accurate determination of stoichiometry or affinity constants. High concentrations of Mn$^{2+}$ had no effect on the enzyme concentration dependence of the activity assays, however, demonstrating that inhibition in our activity assays did not occur by a trivial mechanism such as enzyme precipitation (data not shown).

**DISCUSSION**

Models for RNase H Activation by Divalent Metals—Currently, the mechanism by which the RNase H family of enzymes utilizes divalent metal to catalyze the hydrolysis of RNA in RNA·DNA hybrids is unclear. Although *E. coli* RNase HI can utilize both Mg$^{2+}$ and Mn$^{2+}$ as cofactors, we have observed considerable differences in the optimal concentrations needed for activity (~1 mM for Mg$^{2+}$ and ~5 μM for Mn$^{2+}$ (29)). The
neutralizing mutations in this residue result in a loss of the inhibitory effect of high Mn$^{2+}$ concentrations. Activation of the Asp-134 mutant enzymes in the presence of Mn$^{2+}$ is very similar to that for the wild type E. coli RNase HI, but inhibition is quite different (Fig. 2A). As residue 134 becomes less polar (polarity: Asp > His > Asn (34)), inhibition by Mn$^{2+}$ is impaired in a parallel fashion. This strongly supports the involvement of Asp-134 in the formation of an inhibitory metal-binding site.

Interestingly, these same mutations have little effect on Mg$^{2+}$-dependent activity of E. coli RNase HI (50–90% of wild type activity at 10 mM MgCl$_2$ (35)). However, because the enzyme has weaker affinity for Mg$^{2+}$ than Mn$^{2+}$, maximum activity in Mg$^{2+}$ is not achieved unless millimolar concentrations of MgCl$_2$ are supplied, at which point simple nonspecific, salt inhibition complicates potential studies of second-metal attenuation. It is therefore unclear whether a second Mg$^{2+}$ ion can bind and inhibit the enzyme. The observation that inhibition can be altered by mutations in the enzyme demonstrates that Mn$^{2+}$ inhibition in the wild type enzyme is not due to the effects of metal binding to the substrate but instead must be due to metal binding to RNase H.

The mutant H124A is activated in low Mn$^{2+}$ concentrations but has no inhibitory phase at higher metal concentrations. Taken alone, these data could indicate that neutralizing His-124 abolishes binding of the inhibitory metal. Alternatively, they may suggest that the mechanism of inhibition in high Mn$^{2+}$ concentrations involves His-124, and the second metal may still bind the H124A mutant but is no longer inhibitory. Interestingly, at high MnCl$_2$ concentrations, both the wild type enzyme and the H124A mutant have the same level of activity. This suggests that the maximum attenuation of activity by Mn$^{2+}$ is equivalent to neutralization of His-124.

Michaelis-Menten Kinetic Parameters as a Function of Mn$^{2+}$ Concentration—What is the mechanism for inhibition of RNase H in high Mn$^{2+}$ concentrations? Two possibilities could account for the observed attenuation as follows: an increase in $K_m$ (reduction of substrate binding affinity) or a reduction of $k_{cat}$ (inhibition of the rate-limiting step for catalysis). In the presence of Mn$^{2+}$, E. coli RNase HI activity is maximal at low micromolar concentrations, and presumably this observed activity is due primarily to the single metal-bound enzyme. Upon the addition of inhibitory concentrations of Mn$^{2+}$, both the $k_{cat}$ and $K_m$ are reduced significantly (25- and 10-fold, respectively). Inhibition, therefore, is not a function of decreased substrate affinity. Instead, the reduction in $K_m$ as a function of Mn$^{2+}$ implies that the second metal actually strengthens the association between RNAse H and RNA-DNA hybrid and, reciprocally, that substrate helps form the second metal-binding site.

Based upon these data, we suggest two possible mechanisms for Mn$^{2+}$ inhibition of E. coli RNase HI. First, since both $k_{cat}$ and $K_m$ values are reduced in a parallel fashion (Fig. 3A), perhaps RNase H catalyzes the cleavage of RNA at the same rate with a second metal bound but cannot release the reaction products due to the stabilization of the enzyme-product complex by the second metal ion. Alternatively, product release may not be inhibited upon second metal binding but, instead, the catalytic rate of cleavage could be attenuated. We favor the notion that binding of a second metal inhibits the rate of catalysis rather than via limiting product release because the H124A mutant lacks inhibition of activity (Fig. 2B) but still shows a reduction of $K_m$ as a function of Mn$^{2+}$ (Fig. 3B). This is consistent with a second metal binding to H124A in a manner that stabilizes the enzyme-nucleic acid complex but with the second metal no longer being inhibitory.

Model for Activation and Inhibition of E. coli RNase HI by Mn$^{2+}$—Our current model for the metal dependence of RNase H activity takes aspects of the single-metal mechanism (Asp-70 as general base and His-124 as a proton shuttle (16, 36)) and adds a second, inhibitory metal liganded in part by Asp-134. Mutation of His-124 to Ala lowers the activity of the mutant approximately the same amount as the maximum Mn$^{2+}$ inhibition seen in the wild type enzyme (Fig. 2B). Two simple models could explain Mn$^{2+}$ inhibition, both of which rely on the
role of His-124 in the RNase H active site (Fig. 4). First, perhaps His-124 acts as a liganding element for the second metal. The effect of liganding a metal would be to lower the pK_a of His-124 and thus down-regulate the efficiency of deprotonation of Asp-70. The maximum effect of this attenuation mechanism would be equivalent to abolishing His-124 as a conduit for protons from Asp-70. A second mechanism consistent with our data is that second metal binding may stabilize a conformation of the RNase H active site where His-124 is improperly positioned to accept a proton from Asp-70. Indeed, structural studies of E. coli RNase HI have demonstrated that His-124 can assume multiple conformations (6, 7), allowing for the possibility that His-124 could be frozen into a position where it would be unlikely to act as a proton shuttle. Second-metal binding appears linked to a decrease in K_m values in the wild type enzyme. If His-124 is an important liganding element for the second metal-binding site, we would therefore expect a greater Mn^{2+} requirement for the reduction in K_m (i.e., a reduction in Mn^{2+} binding affinity). However, the Mn^{2+} concentration at which a reduction in K_m is observed is similar for both wild type RNase H and the H124A mutant (Fig. 3B) supporting the second hypotheses. Nonetheless, a structural investigation of E. coli RNase H bound to Mn^{2+} will be required to distinguish between these two potential mechanisms for metal inhibition of RNase H activity.

The activity of T. thermophilus RNase H also shows Mn^{2+} and Mg^{2+} dependence that is very similar to that of E. coli RNase HI (data not shown) suggesting that second-metal attenuation occurs in this homolog as well. It remains to be seen, however, if second-metal inhibition is common to eukaryotic RNase HI (19). These observations are consistent with our findings in E. coli RNase HI suggesting that only one metal is required for activity. It remains an open question whether second-metal attenuation plays an important role in the regulation and/or specificity of RNase H.

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