Magnesium Activation of Ribonuclease H. Evidence for One Catalytic Metal Ion†

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The metal ion dependence of *Escherichia coli* ribonuclease H activity has been examined by monitoring the change in absorbance of nucleotide substrate by rapid (stopped-flow) kinetic methods. Kinetic equations that fully account for enzyme activation, and substrate inhibition at high metal concentration, have been derived. Inhibition constants correlate with metal nucleotide binding affinities. Comparison of thermodynamic (Huang, H.-W.; Cowan, J. A *Eur. J. Biochem.* **1994**, *219*, 253−260) and kinetic data suggests that there is one essential catalytic metal cofactor required for ribonuclease H activation, rather than a binuclear magnesium site. This conclusion is in accord with recent crystallographic data on the Mg^{2+}-bound enzyme (Katayanagi, K.; Okumura, M.; Morikawa, K. *Proteins* **1993**, *17*, 337−346). Similar conclusions are likely to hold for the structurally homologous RNase H domains of retroviral reverse transcriptase.

Introduction

Escherichia coli ribonuclease H is a crystallographically characterized low molecular weight endonuclease ($M_r \sim 17\,580$) that hydrolytically cleaves the ribonucleotide backbone of RNA·DNA hybrids, producing 5′-phosphate and 3′-hydroxyl oligonucleotides.[1−6] Ribonuclease H activity has been implicated in bacterial plasmid replication and forms an essential catalytic domain on retroviral reverse transcriptase.[1−3] The *E. coli* enzyme is structurally homologous to the RNase H domain of HIV reverse transcriptase and shows retention of key active site residues.[3−5] Metal ions are essential for the activity of a multitude of enzymes and ribozymes in nucleic acid biochemistry,[7−11] however, a molecular understanding of their functional role is lacking. Both the molecular mechanism of ribonuclease H activation, and the stoichiometric requirement for Mg^{2+} during turnover are uncertain. A recent structural analysis of the Mn^{2+}-doped crystals of the RNase H domain of HIV-reverse transcriptase revealed two bound Mn^{2+} ions in close proximity to the catalytic site.[3] On this basis it was proposed that the enzyme most likely acts in a manner analogous to the exonuclease domain of DNA polymerase I.[9,12] The involvement of one ion during catalytic turnover has also been proposed;[1,6] however, in neither case has evidence been advanced to support metal stoichiometry during active turnover.

In this paper we describe the results of kinetic experiments designed to evaluate the stoichiometric requirement for Mg^{2+} during turnover and distinguish between these two possible reaction pathways for metal-activated enzymatic hydrolysis of a nucleotide backbone. We also illustrate the use of the change in absorbance of a nucleotide substrate to monitor rates of reaction.[12] Kinetic equations are derived that rationally account for experimental rate data. Comparison of thermodynamic and kinetic data obtained with ribonuclease H suggests that there is *one* essential catalytic cofactor, and that activation does not proceed by a two-metal-ion mechanism. The importance of these issues is underscored by current research activity in the biological chemistry of magnesium ion as it relates to the fields of ribozyme chemistry and activation of metal ion dependent enzymes in nucleic acid biochemistry.[7,9−11,13]

Materials and Methods

Stopped-Flow Instrumentation and Methods. Stopped-flow measurements were carried out using an OLIS (On-Line Instrument Systems, Inc.) stopped-flow apparatus. A broad band 75 W xenon arc lamp source (Ischio) powered by an OLIS XL150 power supply was filtered through a monochromator (model H10 by Instruments SA) with a resolving power of 8 nm/mm. A photomultiplier tube (Homatsu) was mounted linearly from the source to detect the change in absorbance of the hybrid substrate at 260 nm. The cell path length is 17 mm.

Standard reaction conditions were as follows: 50 mM Tris, pH 7.5; $[Mg^{2+}] = 10$ mM; [RNase H] = 170 nM ($\epsilon = 3.9 \times 10^4\ M^{-1}\ cm^{-1}$);[14] substrate = 0.35 mM phosphate equivalents. Typically data were taken at 37 °C. Depending on the experiment, magnesium concentrations varied up to 80 mM, and substrate concentrations up to 2.8 mM phosphate equivalents. One syringe contained ribonuclease H in reaction buffer (50 mM Tris, pH 7.5) while a second contained varying amounts of hybrid in the same reaction buffer. Each of the two syringes was loaded with the same concentration of metal cofactor (0.5−80 mM)

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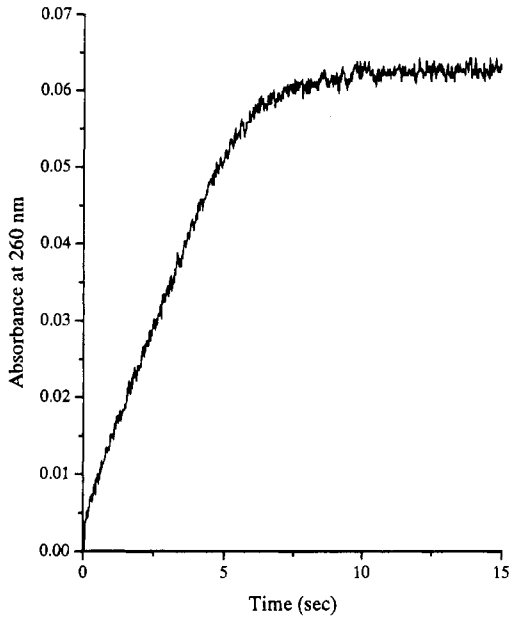

Figure 1. Stopped-flow trace showing the change in absorbance of the digested RNA·DNA hybrid. Reaction conditions are similar to the standards noted in the legend to Table 1 with $[K^+] = 100$ mM, $[Mg^{2+}] = 10$ mM, [RNase H] = 90 nM, [hybrid] = 0.35 mM phosphate equivalents. The cell path length is 17 mm.

to minimize the effects of secondary binding chemistry. The concentrations of background electrolytes were also similar in each syringe. In the absence of enzyme, no change in absorbance was observed. Reactants were preequilibrated at 37 °C prior to mixing. At least four data sets were averaged for each v_0.

Synthesis of a (A·dT)$_{20}$ Hybrid Substrate. Substrate RNA·DNA hybrid was synthesized from dT_{20} (4 mg) and poly(rA) (4 mg) (15). The components were dissolved in buffer (50 mM Tris, 100 mM KCl, pH 7.5) and annealed (42–44 °C) for 15 min. After precipitation the resulting pellet was incubated with 500 U mung bean nuclease (30 min, 37 °C). The purity of the 20-mer hybrid was verified by comparison with appropriate molecular weight markers on 20% homogenous PAGE. A single band was observed. The hybrid was stored at −20 °C. Hybrid concentration is defined by phosphate equivalents (determined from a weighed mass of substrate), since this allows us to compare inhibition constants (K_I) with previous estimates of metal affinities to polynucleotides.[16-18] This also accounts for the deviations in K_m from our previous work.[6]

Results and Discussion

Divalent magnesium ion is an essential cofactor for many enzymes in nucleic acid biochemistry. Although putative models for catalytic mechanisms have been published,[3-5] the specific details of metal activation have not been addressed. Contrary to previous speculation,[3,9] herein we provide experimental evidence that RNase H activity is promoted by a single metal cofactor rather than a binuclear metal site. This result is in accord with a recent crystallographic analysis of the magnesium-bound enzyme.[19]

Kinetic Model and Assay. We have characterized the metal ion dependence of ribonuclease H activity by stopped-flow kinetic methods, using photomultiplier detection to monitor the hyperchromic effect of an (A·dT)$_{20}$ hybrid substrate (Figure 1). The sensitivity of this methodology obviates the inaccuracies common to the use of radiolabeled substrates to measure reaction rates.[5,20] Control experiments described in the experimental section demonstrate that the change in absorbance results from substrate hydrolysis. In particular, the kinetic data obtained is consistent with published results obtained by standard radioassays,[6,14] while the magnitude of the change is in accord with the amount of substrate added. From the change in absorbance, and published extinction coefficients,[15] we estimate the amount of cleavage and strand separation to be consistent with the initial concentration of hybrid.

Scheme 1

$$E + Mg \underset{}{\overset{K_{Mg}}{\rightleftharpoons}} E\text{-}Mg$$

$$S + nMg \overset{K_I(Mg)}{\rightleftharpoons} S\text{-}Mg_n$$

$$E\text{-}Mg + S \rightleftharpoons E\text{-}Mg\text{-}S \longrightarrow E\text{-}Mg + P$$

The metal ion dependence of enzyme activity was analyzed according to the saturation model in Scheme 1. Inasmuch as the enzyme is pre-saturated with Mg^{2+}, binding of substrate (S) to metal-free enzyme (E) can be ignored. Prior Mg^{2+} NMR measurements indicated one principal Mg^{2+} binding site ($K_{Mg} \sim 0.1$ mM) on the isolated enzyme and the absence of significant secondary Mg^{2+} sites.[21] Substrate hybrid (S) can, however, bind excess Mg^{2+} which may become inhibitory at high concentrations. The inhibitory effect of metal ion binding to substrate is considered by viewing the substrate as a polyanion with multiple binding sites. The binding constant is an apparent binding constant for Mg^{2+}. This is a standard type of analysis commonly used in studies of metal ion binding to polyelectrolytes.[16,22,23] Only the metal−enzyme complex (E−Mg) is active, which we relate to the total enzyme concentration [E°] through the mass balance equation [E°] = [E] + [E−Mg] and eq 1. At

$$[E\text{−}Mg] = [E^\circ](K_{Mg}[Mg^\circ])/(1 + K[Mg^\circ]) \quad (1)$$

high metal concentration Mg^{2+} serves as a substrate inhibitor (Scheme 1 and Figure 2), forming an inhibition complex $S(Mg^{2+})$. [Note that here $S(Mg^{2+})$ is used to represent a substrate molecule with many bound metal ions (see S−Mg$_n$ in Scheme 1). Typically one considers an apparent binding affinity per Mg^{2+} ion, in this case represented by K_I.] Again, by mass balance and assuming uncompetitive inhibition (where $K_I = [S][Mg]/[S\text{-}Mg]$), relationship 2 is obtained.[4] [This

$$[S] = K_I[S^\circ]/([Mg^\circ] + K_I) \quad (2)$$

equation can also be derived from a serial expansion of the expression from Segel's text[26] $[S] = \{(([M^\circ] - [S^\circ] + K_I)^2 + 4K_I[S^\circ])^{1/2} - ([M^\circ] - [S^\circ] + K_I)\}/2$, assuming $[M^\circ] > [S^\circ]$. Equations derived assuming competitive or non-competitive inhibition do not give acceptable fits to the data. Substituting (1) and (2) into the standard equation (3) yields eq 4, which we

$$v_0 = k_{cat}[E\text{−}Mg][S]/([S] + K_m) \quad (3)$$

$$v_0 = (k_{cat}[E^\circ]K_{Mg}[Mg^\circ]K_I[S^\circ])/((1 + K_{Mg}[Mg^\circ])(K_I[S^\circ] + ([Mg^\circ] + K_I)K_m)) \quad (4)$$

routinely use to analyze the variation of reaction velocity with

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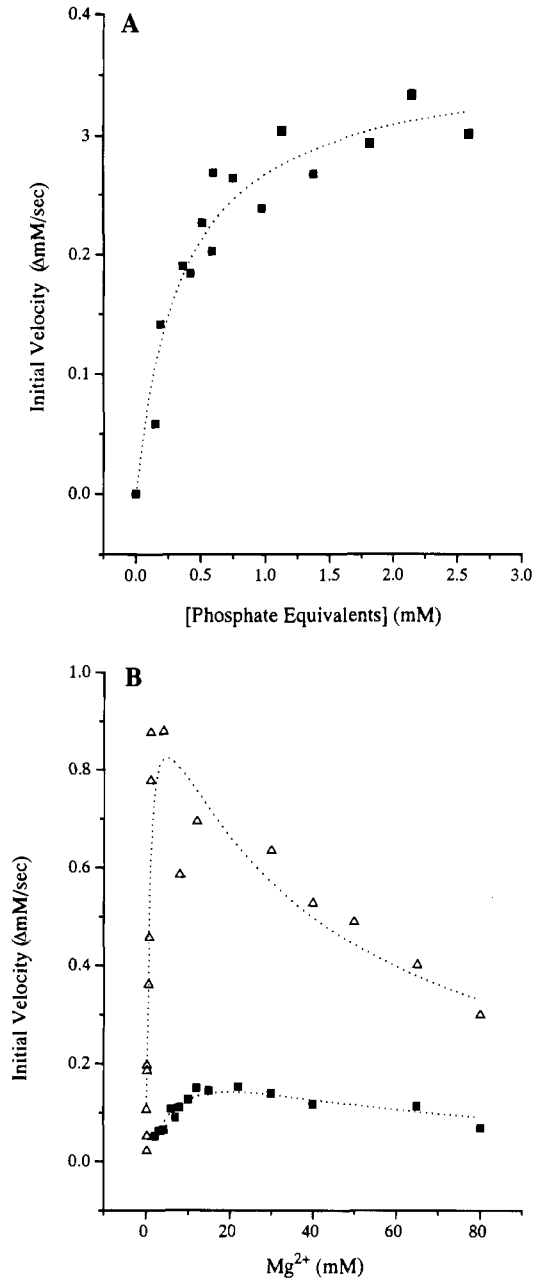

Figure 2. (A) Variation of initial velocity (v_0) with hybrid concentration ([Mg^{2+}] = 10 mM, [Na^+] = 100 mM). (B) Variation of initial velocity (v_0) with magnesium concentration. In the upper curve [Na^+] = 0 mM, while in the lower [Na^+] = 100 mM. The optimal turnover rates obtained for each of these curves are actually similar [k_{cat}(opt) in Table 1]. For (A) and (B), the general reaction buffer was 50 mM Tris, pH 7.5. Fits for (A) and (B) were obtained using eq 4, giving χ^2 errors of 0.012 and 0.008, respectively.

metal concentration. Equation 4 properly addresses the kinetic role of the cofactor since the requirement for binding at the active site of the enzyme, and the inhibitory influence of binding excess metal ion to the substrate are directly accounted for. Table 1 lists steady-state parameters, metal binding (K_{Mg}) and inhibition constants (K_I) determined from these studies. Inasmuch as the magnesium binding constant suggests lower affinity in solutions of higher salt concentration, the data demonstrates that monovalent ions (Na^+ and K^+) compete for the enzyme metal-binding site and result in at most a 5-fold decrease in k_{cat} at fixed concentrations of activating metal cofactor. Maximal rates [k_{cat}(opt) noted in Table 1], obtained by varying the concentration of metal cofactor as defined by eq 4 and fitting as in Figure 2B, are found to be relatively similar and independent of K^+ or Na^+ levels. That is, the monovalent ions simply compete for the carboxylate ligands in the active site. Otherwise these ions have only a moderate influence on substrate binding and activation (Table 1).

Quantitation of the Catalytically Active Cofactor. Previously we have demonstrated one tight binding site on RNase H for Mg^{2+}.[21] Inasmuch as data on the isolated enzyme alone does not directly indicate the number of critical metal ions required for catalytic turnover we chose to address this issue by kinetic methods. The optimal magnesium concentration for kinetic activation [$K_{Mg} \sim 0.5$ mM] is similar to the thermodynamic binding affinity of the free enzyme [$K_{Mg} \sim 0.1$ mM].[21] This provides good evidence for catalytic activation by the same metal ion that binds to the enzyme alone. Association constants for additional metals that form binuclear metal sites to effect enzymatic turnover in the enzyme–substrate complex are typically small[12,23] and would be reflected by a larger value for the binding constant (K_{Mg}) that is estimated from the kinetic measurements. Furthermore, since RNase H lacks significant secondary binding sites for metal ions,[21] the inhibitory effect of increasing [Mg^{2+}] most likely arises from binding to the negatively-charged polynucleotide. At sufficiently high concentrations of metal cofactor the excess charge can impair binding to the relatively nonpolar enzyme surface that surrounds the catalytic site.[1-3] The inhibition constants (K_I) listed in Table 1 ($K_I \sim 4.4-18$ mM) correlate well with previous estimates of binding constants to an A-conformer nucleotide ($K_d \sim 4.5$ mM) (16,18). These values are consistent with results obtained from fits of data for metal binding to nucleic acids using a simple binding model such as that assumed in Scheme 1. When analyzed in terms of the more elaborate McGhee–von Hippel model, the absolute binding constants change,[16] but this level of analysis is inappropriate for the simple comparison purposes required in this paper.

It should be noted that *a priori* our data can also be fit by assuming any number of equivalent tight binding sites on the enzyme. There is the intrinsic assumption that a second metal ion will bind with lower affinity to the active site than the existing ion. This is rather easy to justify from literature precedent. First, a binding constant on the order of 10^{-4} M is the upper limit thus far observed for Mg^{2+} binding to proteins and enzymes. Second, if we assume a model where two or

Table 1. Kinetic Parameters from Ribonuclease H Digestion of Hybrid Substrate[a]

metal cofactor	K_m (mM)	$10^{-3}k_{cat}$[b] (s^{-1})	$10^{-3}k_{cat}$(opt)[c] (s^{-1})	$10^{-3}k_{cat}/K_m$ (mM^{-1} s^{-1})	K_{Mg} (mM)	K_I (mM)
Mg^{2+} (0 mM Na^+)	0.8(±0.2)	5(±1)	6(±1)	7	0.5(±0.2)	14(±3) (4.5)[d]
Mg^{2+} (10 mM Na^+)	1.0(±0.2)	4(±1)	9(±1)	4	9(±2)	6(±3)
Mg^{2+} (100 mM Na^+)	0.4(±0.1)	2(±0.4)	4(±1)	5	31(±5)	5(±3)
Mg^{2+} (100 mM K^+)	3.8 (±1.1)	1(±0.3)	5(±1)	0.4	21(±5)	17(±3)

[a] Standard reaction conditions: 50 mM Tris, pH 7.5; [Mg^{2+}] = 10 mM; [RNase H] = 170 nM ($\epsilon = 3.9 \times 10^4$ M^{-1} cm^{-1} 14); data taken at 37 °C. For metal titrations (to determine K_{Mg} and K_I, as in Figure 2B), [substrate] = 0.8 mM phosphate equivalents, and [Mg^{2+}] varied up to 80 mM. Unit conversion for initial velocity from absorbance units to ΔmM/s was carried out using an extinction coefficient ϵ = 6800 M^{-1} cm^{-1} with 40 phosphate equivalents per substrate molecule. [b] k_{cat} values reflect the production of product in units of phosphate equivalents. [c] The value of k_{cat}(opt), determined from a fit to eq 4, reflects the maximal rates obtained in the presence of optimal concentrations of Mg^{2+} rather than the fixed cofactor concentrations ([Mg^{2+}] = 10 mM) noted in footnote *a*. [d] Value in parentheses is the affinity for thermodynamic binding of Mg^{2+} to the hybrid.[16]

more equivalent high affinity sites are required to be populated, then the apparent binding constant (K_{Mg}) determined from the fit to our data actually falls (binding is tighter) and approaches unreasonable levels ($K_{Mg} \leq 10^{-5}$ M for two sites). Literature precedent demonstrates that for contiguous metal ions (particularly alkaline earths), binding of the second metal results in a marked lowering of the observed binding affinity, presumably as a result of electrostatic interactions. For example, the Mg^{2+} site on DNA pol I is weak,[24] while recent studies of calcium channels demonstrate that while one Ca^{2+} ion binds at μM levels, a second contiguous ion results in a lowering of the affinity of both to mM levels.[25] Submillimolar binding constants are also incompatible with binding to double-strand nucleic acids, under these solution conditions.[16,23] The consistency of this body of kinetic and thermodynamic data lends support to *one* catalytically relevant metal cofactor during turnover. This is also in accord with crystallographic analyses of RNase H,[19] and other proteins and enzymes that show patterns of carboxylate residues at the metal binding site that are similar to those of RNase H.[7,21] The two bound metal ions crystallographically identified in the RNase H domain of Mn^{2+}-doped HIV-reverse transcriptase[3] may reflect either the distinct coordination chemistry of Mn^{2+} versus Mg^{2+}, or the constraints of the doping procedure as a vehicle for introducing metal cofactors to enzyme active sites.

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