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Zinc(II)-mediated inhibition of ribonuclease Sa by an *N*-hydroxyurea nucleotide and its basis

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Abstract

Ribonuclease Sa (RNase Sa) is a secretory ribonuclease from *Streptomyces aureofaciens*. Herein, 3′-*N*-hydroxyurea-3′-deoxythymidine 5′-phosphate is shown to be a competitive inhibitor of catalysis by RNase Sa. Inhibition is enhanced by nearly 10-fold in the presence of Zn^{2+}, which could coordinate to the *N*-hydroxyurea group along with enzymic residues. The carboxylate of Glu54 is the putative base that abstracts a proton from the 2′ hydroxyl group during catalysis of RNA cleavage by RNase Sa. Replacing Glu54 with a glutamine residue has no effect on the affinity of *N*-hydroxyurea **1** for the enzyme, but eliminates the zinc(II)-dependence of that affinity. These data indicate that an *N*-hydroxyurea nucleotide can recruit Zn^{2+} to inhibit the enzymatic activity of RNase Sa, and suggest that the carboxylate of Glu54 is a ligand for that Zn^{2+}. These findings further the development of a new class of ribonuclease inhibitors based on the complex of an *N*-hydroxyurea nucleotide and zinc(II).

RNA is the least stable of the biopolymers that effect information transfer in biology [1,2]. Still, the lifetime of RNA in vivo is most often determined by the rate of its enzymatic degradation [3]. Hence, ribonucleases play myriad roles that garner ever-increasing attention [4–7]. Likewise, the identification of small molecules that control ribonucleolytic activity is a worthwhile goal [8].

Recently, we described a new strategy for the inhibition of ribonucleases [9]. Our strategy uses an *N*-hydroxyurea nucleotide to deliver zinc(II) to the enzymic active site. There, the Zn^{2+} ion can be chelated by the two oxygens of the *N*-hydroxyurea group, leaving two Zn^{2+} sites open for chelation by active-site residues (Fig. 1).

We first tested our strategy with ribonuclease Bi (binase), which is a secretory ribonuclease from *Bacillus intermedius*. We found that 3′-*N*-hydroxyurea-3′-deoxythymidine 5′-phosphate (**1**) is indeed a competitive inhibitor of catalysis by binase [9]. Moreover, inhibition is enhanced in the presence of Zn^{2+}, which is consistent with our design. Here, we show that our strategy can be applied to a second ribonuclease, ribonuclease Sa (RNase Sa; Fig. 2), which is a secretory ribonuclease from *Streptomyces aureofaciens*. In addition, we use site-directed mutagenesis to identify a residue in the active site of RNase Sa that likely chelates to the Zn^{2+} ion. The results extend the demonstrated utility of *N*-hydroxyurea nucleotide·zinc(II) complexes as inhibitors of ribonucleases.

Materials and methods

Chemicals. *N*-Hydroxyurea **1** was synthesized as described previously [9]. Poly(inosinic acid) [poly(I)] was obtained from Sigma Chemical (St. Louis, MO). All other reagents were of commercial grade or better and were used without further purification.

Enzymes. Wild-type RNase Sa and its E54Q variant were prepared by C.N. Pace and coworkers (Texas A&M University). Briefly,

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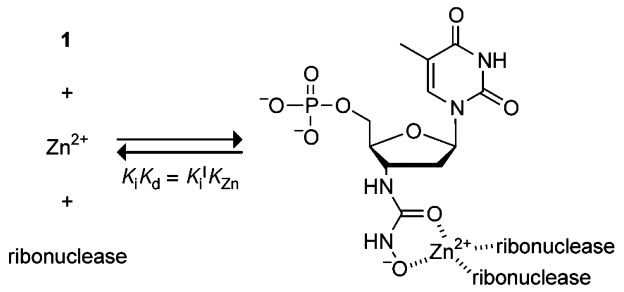

Fig. 1. Strategy for the Zn^{2+}-mediated inhibition of a ribonuclease by an *N*-hydroxyurea nucleotide.

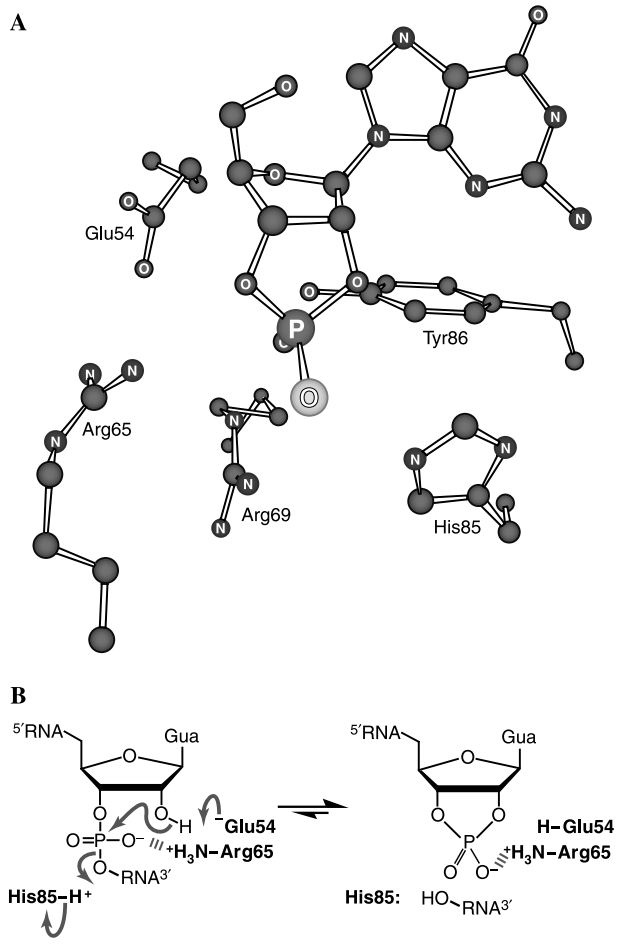

Fig. 2. Active site of RNase Sa. (A) Structure of the complex of RNase Sa and guanosine 2′,3′-cyclic phosphorothioate (PDB entry 1RSN [19]). (B) Putative mechanism of catalysis of RNA cleavage by RNase Sa.

wild-type RNase Sa was prepared as described previously [10]. Synthetic oligonucleotides for the construction of E54Q RNase Sa were obtained from Integrated DNA Technologies (Coralville, IA). A cDNA encoding the E54Q variant was constructed with the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The mutated gene was sequenced at the Gene Technologies Laboratory at Texas A&M University to confirm the introduction of the desired (but no other) mutation. E54Q RNase Sa was produced and purified as described previously [10]. The purity of the E54Q variant was confirmed by SDS–PAGE.

Enzyme kinetics. Steady-state kinetic analyses of catalysis of poly(I) cleavage by wild-type RNase Sa and its E54Q variant were performed at 25 °C in 0.10 M sodium citrate buffer, pH 6.2, containing NaCl (0.10 M). Concentrations of RNase Sa and its variant were determined by ultraviolet spectroscopy using the extinction coefficient $\varepsilon_{280} = 12{,}300\ M^{-1}\ cm^{-1}$ [11]. The poly(I) concentration was measured likewise using $\varepsilon_{248} = 10{,}000\ M^{-1}\ cm^{-1}$ at pH 7.8 [12]. Initial reaction rates were determined by recording changes in absorption at 248 nm, using the difference extinction coefficient $\Delta\varepsilon_{248} = 1330\ M^{-1}\ cm^{-1}$ at pH 6.2 [13].

Results and discussion

The Zn^{2+}-mediated inhibition of a ribonuclease by *N*-hydroxyurea **1** could occur according to the scheme in Fig. 1, as we described previously for binase [9]. Because Zn^{2+} prefers tetrahedral coordination, two enzymic functional groups likely serve as the Zn^{2+} ligands in the ternary complex. By analogy to RNase T_1, which is a homolog of RNase Sa, Glu54 is the base and His85 is the acid during catalysis of RNA cleavage [14–16]. Specifically, Glu54 abstracts a proton from the 2′ hydroxyl group, and His85 protonates the 5′ alkoxide of the adjacent nucleoside (Fig. 2B) [17]. (A triester-like mechanism has also been proposed for RNase T_1 [18].) The structure of crystalline RNase Sa has been determined by X-ray diffraction analysis [19]. Inspection of this structure suggests that the carboxylate of Glu54 and the imidazole of His85 could be the Zn^{2+} ligands (Fig. 2A). If Glu54 and His85 chelate to Zn^{2+} as in Fig. 1, then replacing either would have little effect on the affinity of RNase Sa for *N*-hydroxyurea **1** alone but would lead to a marked decrease in the affinity of the enzyme for the *N*-hydroxyurea **1**·Zn^{2+} complex.

Previously, we showed that replacing His85 of RNase Sa with another residue leads to the complete loss of measurable ribonucleolytic activity [17], making the analysis of inhibition of His85 variants problematic. In contrast, the conservative E54Q variant loses only ca. 700-fold in ribonucleolytic activity [17], which still allows for the measurement of inhibition constants. Although the side-chain amide of a glutamine residue can be a ligand for Zn^{2+}, its interaction with Zn^{2+} would be markedly weaker than that of the side-chain carboxylate of a glutamic acid residue. Accordingly, we analyzed the inhibition of RNase Sa and its E54Q variant by *N*-hydroxyurea **1** in the absence and presence of Zn^{2+}.

N-Hydroxyurea **1** is a competitive inhibitor of catalysis by both wild-type RNase Sa and its E54Q variant. Kinetic parameters for the cleavage of poly(I) by the two enzymes and its inhibition by *N*-hydroxyurea **1** were derived from the data shown in Figs. 3 and 4, and are listed in Table 1. These parameters indicate that *N*-hydroxyurea **1** has a nearly equal affinity for RNase Sa and its E54Q variant. Thus, Glu54 appears to have no role in the binding of *N*-hydroxyurea **1** to RNase Sa. In the absence of *N*-hydroxyurea **1**, no inhibition of the

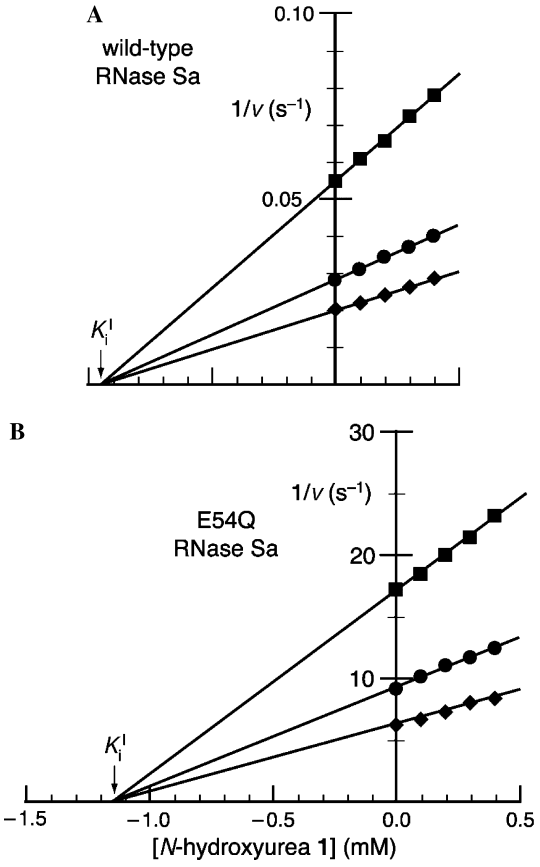

Fig. 3. Dixon plot for the inhibition of wild-type RNase Sa and its E54Q variant by *N*-hydroxyurea **1** in the absence of Zn^{2+}. (A) Wild-type RNase Sa. Assays were performed at 25 °C in 0.10 M sodium citrate buffer, pH 6.2, containing NaCl (0.10 M), wild-type RNase Sa (0.825 nM), poly(I) (■, 17 μM; ●, 34 μM; and ◆, 50 μM), and *N*-hydroxyurea **1** (0.00, 0.10, 0.20, 0.30, or 0.40 mM). (B) E54Q RNase Sa. Assays were performed at 25 °C in 0.10 M sodium citrate buffer, pH 6.2, containing NaCl (0.10 M), E54Q RNase Sa (0.50 μM), poly(I) (■, 25 μM; ●, 50 μM; and ◆, 75 μM), and *N*-hydroxyurea **1** (0.00–0.40 mM).

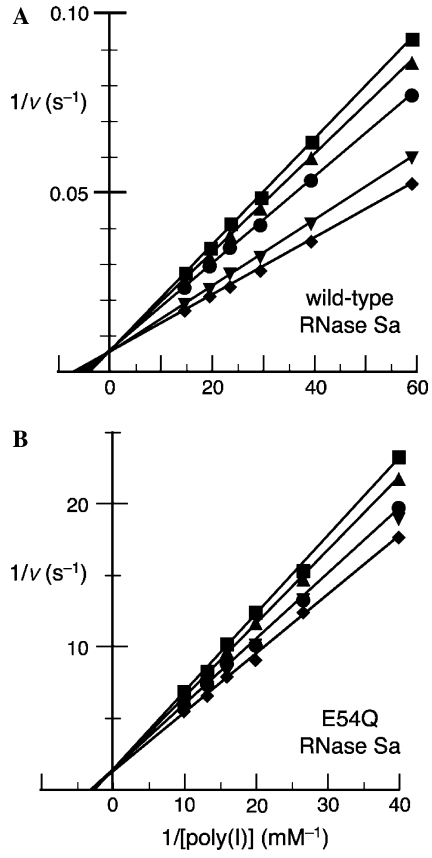

Fig. 4. Lineweaver–Burk plot for the inhibition of wild-type RNase Sa and its E54Q variant by *N*-hydroxyurea **1** in the absence and presence of Zn^{2+}. (A) Wild-type RNase Sa. Assays were performed at 25 °C in 0.10 M sodium citrate buffer, pH 6.2, containing NaCl (0.10 M), RNase Sa (0.825 nM), poly(I) (16.9–67.6 μM), *N*-hydroxyurea **1** (I), and Zn^{2+}. (B) E54Q RNase Sa. Assays were performed at 25 °C in 0.10 M sodium citrate buffer, pH 6.2, containing NaCl (0.10 M), E54Q RNase Sa (0.50 μM), poly(I) (25–100 μM), *N*-hydroxyurea **1** (I), and Zn^{2+}. ■, [I] = 0.40 mM, [Zn^{2+}] = 1.0 mM; ▲, [I] = 0.30 mM, [Zn^{2+}] = 5.0 mM; ●, [I] = 0.10 mM, [Zn^{2+}] = 5.0 mM; ▼, [I] = 0.10 mM, [Zn^{2+}] = 1.0 mM; and ◆, [I] = 0 mM, [Zn^{2+}] = 0 mM.

ribonucleolytic activity of RNase Sa or its E54Q variant by Zn^{2+} was observed, up to a Zn^{2+} concentration of 5 mM. Eqs. (1) and (2), which correspond to the scheme in Fig. 5 [9], were used to calculate inhibition constants for *N*-hydroxyurea **1** in the presence of Zn^{2+}:

$$v = \frac{[E_0][S]k_{cat}}{[S] + K_M^{obs}}, \quad (1)$$

$$K_M^{obs} = K_M\left(1 + \frac{[I]}{K_i^I} + \frac{[I \cdot Zn^{2+}]}{K_i}\right). \quad (2)$$

In Eqs. (1) and (2), K_M is the Michaelis constant for the cleavage of poly(I); K_i^I is the inhibition constant for *N*-hydroxyurea **1** alone (I); and K_i is the inhibition constant for the *N*-hydroxyurea **1**·Zn^{2+} complex ($I \cdot Zn^{2+}$). To calculate inhibition constants by Eq. (1), the [I_0] and [Zn_0^{2+}] values were used instead of [I] and [Zn^{2+}], because the enzyme concentration [E_0] was far below the [I_0] and [Zn_0^{2+}] values. A value of $K_d = 6.3$ mM was used to calculate the concentration of the $I \cdot Zn^{2+}$ complex [9].

Table 1
Parameters for catalysis of poly(I) cleavage by RNase Sa and its E54Q variant, and for its inhibition by *N*-hydroxyurea **1** at pH 6.2 and 25 °C[a]

RNase Sa	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (10^6 M^{-1} s^{-1})	K_i^I (mM)
Wild-type	189	0.151	1.25	0.95
E54Q	0.7	0.32	0.002	1.15

[a] Parameters were obtained from the data in Figs. 3 and 4.

The affinity of wild-type RNase Sa for *N*-hydroxyurea **1** increases in the presence of Zn^{2+}. Parameters that characterize the inhibition of RNase Sa by the $I \cdot Zn^{2+}$ complex are listed in Table 2. The value of K_i for RNase Sa is about 0.12 mM for the concentration product [I][Zn^{2+}] $\leqslant 1 \times 10^{-7}$ M. When the product [I][Zn^{2+}] exceeds this value, the calculated value of K_i grows. (We had observed a similar dependence of the calculated K_i

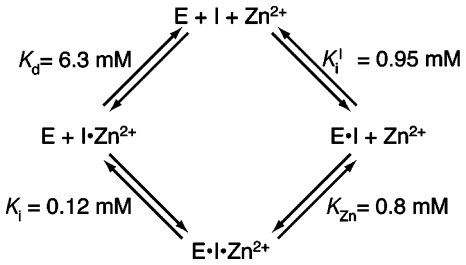

Fig. 5. Scheme for the Zn^{2+}-mediated inhibition of wild-type RNase Sa by *N*-hydroxyurea **1**.

Table 2
Parameters for inhibition of wild-type RNase Sa catalysis by *N*-hydroxyurea **1** and Zn^{2+}[a]

$[Zn^{2+}]$ (mM)	[I] (10^{-4} M)	$[I][Zn^{2+}]$ (10^{-7} M^2)	$[I \cdot Zn^{2+}]$ (10^{-5} M)	K_M^{obs} (10^{-4} M)	K_I (10^{-4} M)
0.5	1.0	0.5	0.79	1.76	1.16
0.5	3.0	1.5	2.38	2.25	1.17
1.0	1.0	1.0	1.59	1.83	1.28
1.0	2.0	2.0	3.18	2.17	1.22
1.0	3.0	3.0	4.76	2.46	1.31
1.0	4.0	4.0	6.35	2.70	1.46
2.5	1.0	2.5	3.97	2.01	1.48
2.5	3.0	7.5	11.91	2.60	2.24
5.0	1.0	5.0	7.94	2.14	2.01
5.0	3.0	15	23.81	2.62	3.55

[a] Parameters were obtained from the data in Fig. 4A.

on the $[I][Zn^{2+}]$ product with binase inhibition by *N*-hydroxyurea **1** in the presence of Zn^{2+} [9].) Most likely, this effect stems from the improper use of $[I_0]$ and $[Zn_0^{2+}]$ instead of [I] and $[Zn^{2+}]$ in calculations at high $[I][Zn^{2+}]$ values, where a complex between [I] and $[Zn^{2+}]$ could be based on the interaction of the anionic phosphoryl group of the inhibitor and a cationic zinc ion. We therefore believe that the most precise K_i value is obtained at low concentrations of *N*-hydroxyurea **1** and Zn^{2+}, and that $K_i = 0.12$ mM for wild-type RNase Sa.

The affinity of E54Q RNase Sa for *N*-hydroxyurea **1** does not increase in the presence of Zn^{2+}. The value of K_i for the E54Q variant is approximately 10-fold greater than that for the wild-type enzyme. Moreover, the K_i value for the E54Q variant nearly coincides with the K_i^I value (Tables 1 and 3). These data are indicative of a marked decrease in the affinity of the $I \cdot Zn^{2+}$ complex upon replacing Glu54 with a glutamine residue, which supports inhibition as depicted in Fig. 1 with one of the ligands being the carboxylate of Glu54.

Table 3
Parameters for inhibition of E54Q RNase Sa catalysis by *N*-hydroxyurea **1** and Zn^{2+}[a]

$[Zn^{2+}]$ (mM)	[I] (10^{-4} M)	$[I][Zn^{2+}]$ (10^{-7} M^2)	$[I \cdot Zn^{2+}]$ (10^{-5} M)	K_M^{obs} (10^{-4} M)	K_I (10^{-4} M)
1	1	1	1.59	3.50	7.7
1	2	2	3.18	3.75	12.2
1	3	3	4.76	3.98	19.2
1	4	4	6.35	4.28	14.3
5	1	5	7.94	3.46	12.5
5	3	15	23.81	4.05	11.2

[a] Parameters were obtained from the data in Fig. 4B.

RNase Sa and binase are members of the RNase T_1 family of enzymes (EC 3.1.27.3) [20]. Yet, the amino acid sequences of RNase Sa and binase are only 24% identical [21]. RNase Sa is an acidic ribonuclease with an isoelectric point of $pI = 3.5$ and highly negative net charge at pH 6.2 (where the inhibition constants were measured herein). In contrast, binase is a basic ribonuclease with an isoelectric point of $pI = 9.6$ and a highly positive net charge at pH 6.2. Yet, the constants for the inhibition of RNase Sa (as well as RNase T1, unpublished data) by *N*-hydroxyurea **1** and its complex with Zn^{2+} ion are close to those for the inhibition of binase [9], with the K_i^I values for RNase Sa and binase being 0.95 and 1.3 mM, respectively, and the K_i values being 0.12 and 0.047 mM, respectively. The active sites of RNase Sa and binase are remarkably similar [22]. This similarity is likely to be responsible for the observed similarity of the inhibition constants. The structural similarity of the active sites of microbial ribonucleases enables us to put forth nucleotide derivatives with an *N*-hydroxyurea moiety in the 3′ position as universal inhibitors of these enzymes, whose efficiency increases in the presence of zinc(II).

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