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Binding thermodynamics of metal ions to HIV-1 ribonuclease H domain

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Abstract

Metal-protein interactions are not necessarily tight in many transient biological processes, such as cellular signaling, enzyme regulation, and molecular recognition. Here, we analyzed the binding thermodynamics and characterized the structural effect of divalent metal ions, i.e. Mn^{2+} , Zn^{2+} , and Mg^{2+} , to the isolated ribonuclease H (RNH) of human immunodeficiency virus (HIV) using isothermal titration calorimetry (ITC) and circular dichroism. The binding thermodynamics of Mg^{2+} to RNH was determined using competition ITC experiments, and the binding affinity of Mg^{2+} was found to be about 40- and 400-times lower than those of Mn^{2+} and of Zn^{2+} , respectively. The structural analysis showed that Mg^{2+} binding had little effect on the thermal stability of RNH, while Zn^{2+} and Mn^{2+} binding increased the stability. The thermodynamic characteristics of RNH metal binding, compared to intact HIV reverse transcriptase, and a possible mechanism of conformational change induced upon metal ion binding, in correlation with the structure-function relationship, are discussed.

Keywords

circular dichroism; co-factor; isothermal titration calorimetry; metal-protein interaction; RNase H

Introduction

Metal ions play important roles in molecular interactions and functions in biology: their thermodynamics studies have provided critical insights into the molecular mechanism [1–5]. Human immunodeficiency virus-1 reverse transcriptase (HIV-RT) possesses both nucleotide

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Electronic Supplementary Material

A pdf file that contains the following data is available free of charge: **Fig. S1**, ITC trace of Mg^{2+} binding to RNH or to the experimental buffer (B), and **Fig. S2**, ITC traces of Mn^{2+} and Zn^{2+} injection into the experimental buffer.

polymerization and ribonuclease H (RNH) activities, the latter of which degrades RNA of an RNA-DNA hybrid. Both activities require a divalent cation as a cofactor in the catalysis. Under physiological conditions, Mg^{2+} is the cofactor for RNH activity, and some divalent cations, such as Mn^{2+} and Zn^{2+} , have been shown to regulate the Mg^{2+} -dependent RT activity [6–8]. In the case of the mature RT heterodimer, which comprises p66 and p51 subunits, various crystal structures show one or two Mg^{2+} bound to the RNH domain, depending on the crystallographic condition [9–12], presumably due to the weak binding to the RNH domain. Analysis of Mg^{2+} binding to the intact RT, by isothermal titration calorimetry (ITC), indicates binding to the RNH domain of the heterodimer at a single site with an association constant, K_a , of $1.1 \times 10^4 \text{ M}^{-1}$ [7].

The isolated RNH domain of RT, folded by itself, is not catalytically active, due to lack of the substrate handle region, but exhibits a native-like protein fold and has been used to test RNH-inhibitor interactions for therapeutic applications [13–17]. The weak but critical binding of Mg^{2+} to isolated RNH has been analyzed using NMR titration experiments, showing the K_a value to be $3.1 - 3.7 \times 10^2 \text{ M}^{-1}$ for the A site and $\sim 29 \text{ M}^{-1}$ for the B site [18]. Our recent NMR analysis of Mg^{2+} binding to the isolated RNH demonstrated that Mg^{2+} titration to RNH shows a saturated titration curve at a constant 160 mM NaCl condition, while it does not exhibit a saturated titration curve at a low NaCl condition, the latter of which results in a 2.4 times weaker apparent $K_a = 33.9 (\pm 5.7) \text{ M}^{-1}$ than the constant NaCl condition [19]. Consistent to the NMR titration data, MD simulation indicates clustering of Na^+ on one area of the protein surface as well as the metal-coordinating site [19]. To understand whether these weak metal interactions are truly nonspecific, an analysis of the underlying thermodynamics of such weak metal interactions is important.

In this study, we analyzed the binding thermodynamics of Mn^{2+} , Zn^{2+} , and Mg^{2+} to isolated RNH using ITC. More specifically, we carried out a *relative* comparison of these metal ions in unique experimental conditions, i.e., thermodynamically detected metal-protein interaction in a background that metal-buffer interaction may exist [20–23]. In contrast to the previous study for direct binding of Mg^{2+} to RT [7], little heat was observed for direct binding to isolated RNH, presumably due to its weak binding affinity. Instead, Mg^{2+} binding was evaluated by a competition experiment either using Mn^{2+} or Zn^{2+} [24]. The respective metal ion binding was compared, and the results support the notion that the biologically critical binding of Mg^{2+} to RNH is weaker than binding of other metals. To assess structural perturbation in RNH induced upon metal ion binding, the thermal stability of RNH in the absence or presence of metal ions was evaluated using circular dichroism (CD). The results clearly showed that Mg^{2+} binding had little effect on the stability of the protein, while Mn^{2+} and Zn^{2+} binding increased the stability.

Experimental

Materials

Metal chlorides, MnCl_2 , ZnCl_2 , and MgCl_2 , (99.99% purity, purchased from Sigma Aldrich) were first solubilized in H_2O to prepare a 500 mM solution, which was subsequently diluted in 20 mM Bis-Tris buffer (pH 7.2). RNH was overexpressed in *Escherichia coli* and purified as reported previously [19]. The purified RNH was dialyzed and concentrated in 20 mM Bis-

Tris buffer (pH 7.2). The protein concentration was spectrophotometrically determined using a molar absorption coefficient of $1.46 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm.

ITC measurements

ITC measurements were carried out using a PEAQ-ITC calorimeter (Malvern). Metal ion solutions were titrated into the RNH solution (200 μL) using a 40 μL syringe. To determine the binding thermodynamics of Mg^{2+} , competition ITC experiments were carried out. Mn^{2+} (6.0 mM) or Zn^{2+} (1.7 mM) was titrated into the RNH solution (0.1 mM) in the presence of Mg^{2+} (5.6 mM). Both metal ion and RNH solutions were prepared in 20 mM Bis-Tris buffer (pH 7.2). Each titration consisted of a preliminary 0.5 μL injection followed by 19 subsequent 2 μL additions at 293 K. The raw data were plotted by normalizing a constant control heat to zero. The heat generated was integrated and divided by the moles of metal ion injected. Thermodynamic parameters were determined by using the fitting procedure supplied by the manufacturer. Since a model that assumes two different metal binding affinities did not show a significant fit, we analyzed all data using a n -site binding model with a uniform affinity.

CD measurements

Far-UV CD spectra were measured in 10 mM Tris-HCl buffer (pH 8.0) at 293 K on a Jasco J-810 spectropolarimeter. The buffer conditions slightly different from those used in our ITC experiments were chosen for two reasons: (a) Bis-Tris buffer has its own CD in the far-UV region and (b) the pH of Tris-HCl buffer changes from 8.0 at 293 K to 7.2 at 333 K, i.e., around the transition temperature of RNH [25]. The RNH concentration was 0.04 mg mL^{-1} . CD spectra were obtained under the following condition: optical path length of 1 cm, scan rate of 20 nm min^{-1} , response time of 1 sec, and bandwidth of 1 nm.

The melting curves were recorded for RNH in the absence or presence of Zn^{2+} (0.29 mM), Mn^{2+} (0.73 mM), and Mg^{2+} (0.73, 3.7, 11 mM), in temperature scanning mode at 222 nm, from 293 to 353 K with a heating rate of 1.0 K min^{-1} . The analysis of the transition curve to determine the transition temperature (T_m) and the van't Hoff enthalpy change (ΔH_{VH}) was performed on the basis of a two-state transition model assuming that the heat capacity change is zero, as described previously [26,27].

Results

Binding thermodynamics of Mn^{2+} and Zn^{2+} to RNH

The binding thermodynamics of Mg^{2+} to RNH was difficult to determine directly using ITC (Fig. S1), due to its weak binding affinity. In contrast, binding of Mn^{2+} and Zn^{2+} to RNH were readily observed (Figs. 1A and 1C), and the binding thermodynamics could be determined with reasonable reproducibility (Table 1). The binding affinity of Mn^{2+} was lower than that of Zn^{2+} . Both reactions were entropy driven, possibly due to dehydration effects. The binding stoichiometry of Mn^{2+} was 2, while that of Zn^{2+} was 1. In the case of Mn^{2+} binding, the fitting procedure was carried out with a one-site binding model, assuming two indistinguishable Mn^{2+} binding events occurred.

Binding thermodynamics of Mg^{2+} to RNH

To determine the binding thermodynamics of Mg^{2+} to RNH, competition-based ITC experiments were carried out [24]. In the presence of Mg^{2+} , at an excess molar concentration relative to RNH (5.6 mM Mg^{2+} and 0.1 mM RNH), the binding thermodynamics of high-affinity metal ions, Mn^{2+} and Zn^{2+} , to RNH were determined (Figs. 1B and 1D, Fig. S2, Table 1). Based on the respective results for Mn^{2+} and Zn^{2+} binding, either in the absence or presence of Mg^{2+} , the binding affinity and enthalpy change of Mg^{2+} were determined (Table 1). The binding affinity of Mg^{2+} was much lower than that of Mn^{2+} or Zn^{2+} , and the reaction was entropy driven, similar to the reactions carried out in the absence of Mg^{2+} . The overall binding thermodynamics of each metal ion analyzed in this study are summarized in Fig. 2. The binding affinity of each metal ion is in good correlation with the favorability of entropy change, partially compensated by the unfavorable enthalpy change.

Effects of metal ion binding on RNH stability

Far-UV CD spectra of the isolated RNH in the absence or presence of metal ions, Zn^{2+} , Mn^{2+} , and Mg^{2+} , were similar to previously reported spectra (Fig. 3A) [28]. The results indicate that metal binding induces little change in the secondary structure of RNH, with each condition exhibiting almost identical CD profiles to the others. The effects of metal ions on the thermal stability of RNH were examined by monitoring the CD value at 222 nm in the context of increasing temperature (Fig. 3B). Since RNH may fold a kinetically trapped dimer [16], CD experiments were performed at a low protein concentration, 0.04 mg mL⁻¹, as much as possible.

The unfolding transitions of RNH in the absence or presence of Mn^{2+} or Mg^{2+} were reversible, making it possible to determine the melting temperature, T_m , on the basis of a two-state transition model (Table 2). In the case of the low affinity binder, Mg^{2+} , ca. 75% of RNH is estimated to be in the Mg^{2+} -bound form in the presence of Mg^{2+} (11 mM) from the above determined K_a at 293 K. Based on our experience, dialysis of Mg^{2+} -RNH complex in denaturant removes Mg^{2+} from RNH (data not shown). Assuming that the observed thermal transition results primarily from the metal-bound RNH in the presence of metal ion, we can conclude that Mn^{2+} binding increases the thermal stability of RNH while Mg^{2+} binding has little effect on it (Table 2). RNH thermal unfolding in the presence of Zn^{2+} was irreversible, possibly due to the aggregation of RNH. Since Zn^{2+} often leads protein misfolding or aggregation [29], its irreversibility may not be surprising. Although the T_m in the presence of Zn^{2+} can not be quantitatively determined, thermal transition curves, recorded at a 1.0 K min⁻¹ rate, suggest that Zn^{2+} binding qualitatively increases the stability of RNH (Fig. 3B).

Discussion

In this study, we analyzed the binding thermodynamics of the metal ions, Zn^{2+} , Mn^{2+} , and Mg^{2+} , to isolated RNH, and determined the effects of these ions on the protein's thermal stability. The binding affinity of Mg^{2+} , a biologically relevant metal for HIV-1 RNase H activity [7,8], was lower than those of other metal ions. Previous NMR experiments, in the absence of NaCl, were performed at conditions similar to those used for ITC: 114 μM RNH in 20 mM Bis-Tris buffer at pH 7.2 and 293 K [19]. These NMR experiments did not exhibit

a saturated titration profile, with a tentative $K_a = 33.9 (\pm 5.7) \text{ M}^{-1}$, higher than the presently determined ITC-derived K_a value. Previously, London's group fit the NMR data assuming two association constants, $K_a = \sim 29 \text{ M}^{-1}$ and $3.1 - 3.7 \times 10^2 \text{ M}^{-1}$ [18], which agreed with our previous NMR data, $33.9 (\pm 5.7) \text{ M}^{-1}$, and the current ITC data, $\sim 3 \times 10^2 \text{ M}^{-1}$, respectively. Given that the present ITC experiments could only detect specific Mg^{2+} binding to RNH in a competitive manner with Mn^{2+} or Zn^{2+} binding, the weaker Mg^{2+} binding detected by NMR, 33.9 M^{-1} , may indicate nonspecific binding events.

The binding affinity of Mn^{2+} to the isolated RNH, $1.23 (\pm 0.26) \times 10^4 \text{ M}^{-1}$, is comparable to previous results, $1.9 (\pm 0.4) \times 10^4 \text{ M}^{-1}$ and $0.44 (\pm 0.08) \times 10^4 \text{ M}^{-1}$, obtained using the full-length RT heterodimer and recorded using similar experimental conditions, 20 mM Tris buffer at pH 7.3 and 298 K [7]. Here, we did not assume a two-sites binding model for the Mn^{2+} -RNH interaction because statistically significant results were not obtained using a two binding model. Importantly, the present result of ΔH for RNH, 5.8 kJ mol^{-1} , is endothermic, while those in the full-length RT, $-20.9 \text{ kJ mol}^{-1}$ and $-18.8 \text{ kJ mol}^{-1}$, are exothermic [7], indicating an effect of the protein regions surrounding RNH on RNH stability. In principle, metal ion binding would result in the release of bound water molecules into the solvent, resulting in an unfavorable ΔH and favorable ΔS . This entropy gain induced upon metal ion binding would overcome the entropy loss resulting from the decreased flexibility of isolated RNH. In contrast, the RNH domain in RT interacts with the connection domain in the p66 subunit and thumb domain in the p51 subunit [9–12]. The favorable ΔH and unfavorable ΔS of Mn^{2+} binding to the RNH domain in RT may be due to enhanced structural ordering conferred by tertiary contacts to the surrounding domains in RT.

We obtained a binding stoichiometry of 1 for Zn^{2+} binding to RNH, in contrast to Mn^{2+} binding stoichiometry. It should be noted that exothermic heat was observed even after 1:1 binding of Zn^{2+} (Figs. 1C and 1D). The exothermic heat was reproducible for Zn^{2+} injection into RNH, and little heat was observed for Zn^{2+} injection into buffer (Fig. S2). Zn^{2+} at low concentration ($\sim 25 \mu\text{M}$) catalyzes RNH activity similar to Mg^{2+} , but Zn^{2+} at high concentration ($\sim 100 \mu\text{M}$) inhibits the activity [8]. The enthalpy-driven event may be due to additional Zn^{2+} binding to RNH, relating to such functionality, or reflect differences in metal-buffer interaction or metal coordination in the protein [30,31]. Since a propensity of the thermal unfolding of RNH in the presence of Zn^{2+} , detected by CD, is qualitatively different from that in the presence of Mn^{2+} or Mg^{2+} , the observed differences in ITC data more likely relate to the difference of functionality in the RNH activity. Indeed, NMR study by London's group has demonstrated that Zn^{2+} titration to RNH shows different non-linear titration curves for some residues, compared to Mg^{2+} titration [32]. Such effect may be due to an additional conformational change effect as well as the metal coordination effect, and may cause the observed exothermic heat.

Conclusions

We conducted relative comparisons of the divalent metal interaction with isolated RNH probed by ITC and CD. Although a buffer effect may contribute to the absolute binding thermodynamics [20–23], specific thermal and structural features of Zn^{2+} -binding were obtained, consistent with a previous study by DeStefano's group [8]. Further, Mg^{2+} binding

thermodynamics to RNH were determined using conditions similar to those applied in previous NMR studies. By combining the current ITC data with the previous NMR results [18,19], we could distinguish the nonspecific metal-protein interactions from the specific metal interactions at the RNH active-site.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

ITC profiles of metal ion binding to RNH at 293 K. A solution of MnCl_2 (A, B) or ZnCl_2 (C, D) was injected into 0.1 mM RNH in the absence of MgCl_2 (A and C) or in the presence of 5.6 mM MgCl_2 (B and D). In A and B, concentrations of the injected MnCl_2 solution were 4.0 mM and 6.0 mM, respectively. In C and D, concentrations of the injected ZnCl_2 were both 1.7 mM. The data points were obtained by integration of the peaks in the raw data (upper), and plotted against the molar ratio, metal-ion/protein (lower).

Fig. 2.

Thermodynamics of metal ion binding to RNH at 293 K. The grey, white, and black bars indicate the results for Mn²⁺, Zn²⁺, and Mg²⁺, respectively. Binding thermodynamics of Mg²⁺ are the averaged values for those determined from Mn²⁺ and Zn²⁺ competition experiments (Table 1).

Fig. 3. CD analysis. (A) Far-UV CD spectra of RNH in the absence or presence of Zn²⁺ (0.29 mM), Mn²⁺ (0.73 mM), or Mg²⁺ (11 mM) at 293 K. The spectra are superimposed, showing that the CD profiles of the three are almost identical. (B) Thermal denaturation curves of RNH in the absence (black line) or presence of 0.29 mM Zn²⁺ (grey line).

Table 1Thermodynamic parameters for metal ion binding to RNH in the absence or presence of Mg^{2+} at 293 K.

n^a		K_a (M ⁻¹)	G (kJ mol ⁻¹)	H (kJ mol ⁻¹)	$T \Delta S$ (kJ mol ⁻¹)
<i>Metal ion binding to RNH</i>					
in the absence of Mg ²⁺					
Mn ²⁺	1.9 (± 0.2) ^b	1.23 (± 0.26) × 10 ⁴ ^b	-22.9	5.8 (± 0.8) ^b	28.7
Zn ²⁺	1.0 (± 0.1) ^b	1.37 (± 0.26) × 10 ⁵ ^b	-28.8	13.7 (± 0.5) ^b	42.5
in the presence of Mg ²⁺					
Mn ²⁺	3.1 (± 0.6) ^c	4.78 (± 0.25) × 10 ³ ^c	-20.6	1.5 (± 0.1) ^c	22.1
Zn ²⁺	1.0 (± 0.1) ^b	4.70 (± 1.05) × 10 ⁴ ^b	-26.2	7.8 (± 0.5) ^b	34.0
<i>Mg²⁺ binding to RNH determined by competition experiments</i>					
Mg ²⁺ (Mn ²⁺) ^d		2.80 (± 0.6) × 10 ² ^e	-13.7	7.1 (± 3.8) ^e	20.8
Mg ²⁺ (Zn ²⁺) ^d		3.42 (± 3.1) × 10 ² ^e	-14.2	9.0 (± 3.9) ^e	23.2

^aBinding stoichiometry, metal ion / RNH.^bValues averaged from two experiments with fitting error shown.^cValues averaged from three experiments with standard deviation shown.^dThe metal ion shown in parenthesis was used as the high-affinity binder.^eUncertainty of the Mg^{2+} binding thermodynamics was estimated from the error propagation in the competitive equations [33].

Table 2Thermal stability of RNH in the absence or presence of Mn^{2+} or Mg^{2+}

Metal ion concentration (mM)	T_m (K) ^a	H_{vH} (kJ mol ⁻¹) ^a
0	334.7 ± 0.4	235 ± 9
Mn^{2+}		
0.73	339.0 ± 0.8	235 ± 15
Mg^{2+}		
0.73	334.3 ± 0.4	242 ± 10
3.7	333.8 ± 0.4	236 ± 10
11	333.3 ± 0.4	221 ± 9

^aFitting error shown.