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Entire-Dataset Analysis of NMR Fast-Exchange Titration Spectra: a Mg^{2+} Titration Analysis for HIV-1 Ribonuclease H Domain

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Abstract

This article communicates our study to elucidate the molecular determinants of weak Mg^{2+} interaction with the ribonuclease H (RNH) domain of HIV-1 reverse transcriptase in solution. Since the interaction is weak (a ligand-dissociation constant > 1 mM), non-specific Mg^{2+} interaction with the protein or interaction of the protein with other solutes that are present in the buffer solution can confound the observed Mg^{2+} -titration data. To investigate these indirect effects, we monitored changes in the chemical shifts of backbone amides of RNH by recording NMR ^1H - ^{15}N single-quantum coherence (HSQC) spectra upon titration of Mg^{2+} into an RNH solution. We performed the titration in three different conditions: (1) in the absence of NaCl, (2) in the presence of 50 mM NaCl, and (3) at a constant 160 mM Cl^- concentration. Careful analysis of these three sets of titration data, along with molecular dynamics simulation data of RNH with Na^+ and Cl^- ions, demonstrates two characteristic phenomena distinct from the specific Mg^{2+} interaction with the active site: (a) weak interaction of Mg^{2+} , as a salt, with the substrate-handle region of the protein, (b) overall apparent lower Mg^{2+} -affinity in the absence of NaCl compared to that in the presence of 50 mM NaCl. A possible explanation may be that the titrated MgCl_2 is consumed as a salt and interacts with RNH in the absence of NaCl. In addition, our data suggest that Na^+ increases the kinetic rate of the specific Mg^{2+} interaction at the active site of RNH. Taken

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Supporting Information. A file that contains the following data is available free of charge: Table S1–S3, description of sample preparation; Figure S1, Comparison of δ values at 2, 5, 10, 20, and 50 mM Mg^{2+} versus those at 80 mM Mg^{2+} concentration; Figure S2, Plots of δ at the entire Mg^{2+} concentration range vs. the low Mg^{2+} concentration range; Figure S3, Comparison of HSQC spectral changes upon Mg^{2+} titration for the RNH residues at active site; Figure S4, K_D determined from the $\text{MgCl}_2(50\text{NaCl})$ and $\text{MgCl}_2(\text{const. Cl}^-)$ titration data.

together, our study provides biophysical insight into the mechanism of weak metal interaction on a protein.

Graphical abstract

Introduction

Weak, transient molecular interactions are critically important in many biological processes, where they regulate low frequency events in cellular signaling as well as enzyme dynamics. For example, carbohydrates often weakly interact with proteins^{1–3}, the binding affinities of metals to protein, some of which are very weak, vary in individual systems^{4–6}, and weak ion interactions with a protein's surface provide protein stability^{7–9}. Precise analysis of protein-protein interactions, as well as protein-ligand interactions, is crucial for understanding such physiologically relevant mechanisms and is also important in drug discovery^{10–14}.

Nuclear magnetic resonance (NMR) spectroscopy is an extremely sensitive method to gain insight into structural, dynamical, and chemical changes of individual sites in macromolecules and is often used to characterize ligand-protein interactions by monitoring either protein or ligand signals^{15–24}. In particular, ligand-titration experiments in which protein signals are monitored, mostly by recording ¹H-¹⁵N single-quantum coherence (HSQC) spectra or ¹H-¹³C multi-quantum coherence (HMQC) spectra, are useful to identify both the affinity of ligands and their binding sites on proteins¹⁵. When a ligand-protein interaction is weak, migration of protein chemical shifts as a function of ligand concentration is detected in the NMR spectra and exhibits a fast exchange régime. Analysis is typically straightforward using established approaches^{15, 25–28}.

However, when the ligand-protein interaction is extremely weak, with a ligand-dissociation constant > 1 mM, the analysis of ligand titration data is not straightforward because confounding effects, such as non-specific interaction of the ligand or interaction from other solutes in the solution, may complicate the analysis. Indeed, it is well known that solute composition influences NMR relaxation data recorded for proteins in aqueous solution^{29–31}. Although a method to investigate weak Mg²⁺ interactions, using a chelator diphosphate, has been described³², this method does not provide information for the ligand binding site. Moreover, the method cannot be used with nucleotide binding proteins that may interact with the diphosphate. Thus, we propose a protocol that involves inspection of the entire dataset obtained from NMR fast-exchange titration to identify effects other than those from

specific ligand-protein interactions. In addition, we also propose that data be acquired at different salt concentrations to allow better interpretation of the data.

Here, we revisit the Mg^{2+} interaction with the ribonuclease H (RNH) domain of HIV-1 reverse transcriptase (RT) (Figure 1A), which was studied by us and other groups previously^{33–37}. The RNH domain is located at the C-terminus of RT and degrades the viral RNA from the RNA/DNA hybrid that forms during reverse transcription, an essential step in replication of the retrovirus^{38, 39}. RNH requires Mg^{2+} ion(s) for its activity and forms the metal binding site with four acidic groups, D443, E478, D498, and D549 (Figure 1A)^{40–45}. A two-metal interaction model is generally accepted in the respect of structure of RT, since in RT, binding affinity of a second divalent ion is increased in the quaternary complex, with a substrate, in physiological conditions^{46–50}. In contrast, in the absence of substrate in solution, only one Mg^{2+} ion is weakly coordinated at the RNH active site in RT, at $K_D \sim 0.1$ mM³⁶. Further, while Mg^{2+} ion interaction with the isolated RNH domain in solution is qualitatively explained with a single Mg^{2+} -binding model ($K_D \sim 10$ mM), the previous titration data itself is better expressed with a two- Mg^{2+} interaction model ($K_D \sim 3$ mM and 35 mM)^{34, 35}. As a next step of the studies, we endeavored to answer a biophysical question about whether Mg^{2+} ions non-specifically interact with RNH in solution and how such non-specific interaction affects titration data.

To investigate the molecular determinants of Mg^{2+} interaction with the isolated RNH domain, we monitored changes in the chemical shifts of RNH backbone amides by recording NMR ^1H - ^{15}N single-quantum coherence (HSQC) spectra upon Mg^{2+} titration in three different conditions: (1) in the absence of NaCl, (2) in the presence of 50 mM NaCl, and (3) at a constant 160 mM Cl^- concentration, the latter achieved by a two-fold reduction in Na^+ concentration when Mg^{2+} was titrated. Our study demonstrated two effects of Mg^{2+} , in addition to its interaction with the active site: (a) weak interaction of Mg^{2+} , as a salt, with the substrate-handle region that is located adjacent to the active-site residues, and (b) an influence of NaCl on the effective Mg^{2+} concentration. We think that the latter observation may be due to the consumption of Mg^{2+} as a salt by the substrate-handle region and perhaps by other regions in a non-specific manner. Consistent with our NMR observations, MD simulation data indicate weak localization of cations on the substrate handle region and the active site. Our study elucidates non-specific interactions of Mg^{2+} ions and provides biophysical insight into the determinants of weak metal-protein interactions.

Experimental and Theoretical Methods

Protein Preparation

The wild type RNH used for this experiment is the same construct used previously and was expressed and purified as reported⁵¹. In brief, cDNA encoding residues 427–560 of HIV-1 reverse transcriptase was expressed by inserting it into pE-SUMO vector (LifeSensors, Malvern, PA) with a six histidine tag (His6-) at the N-terminus of the SUMO-fusion construct. The protein was expressed in *E. Coli* Rosetta 2 (DE3) cells using IPTG for induction. Soluble RNH was extracted from the cell lysate and purified using HisTrap HP columns (GE Healthcare, Piscataway, NJ) and gel filtration on a Superdex75 column (GE Healthcare, Piscataway, NJ). After removal of the N-terminal His₆-SUMO fusion by

digestion with ULP1 enzyme, the RNH was separated from His-tagged proteins using a HisTrap column (GE Healthcare, Piscataway, NJ). Purified RNH was stored at -80°C with a buffer containing 25 mM Sodium Phosphate, 100 mM NaCl and 0.02% NaN_3 , at pH 7.0. The buffers for NMR experiments were exchanged as described below.

NMR samples

A total of 27 NMR samples were prepared to conduct three different Mg^{2+} titration experiments: (I) MgCl_2 titration in a 20 mM bisTris buffer (pH 7.2) in the absence of additional NaCl, referred to as $\text{MgCl}_2(0\text{NaCl})$, (II) MgCl_2 titration in a 20 mM bisTris buffer in the presence of 50 mM NaCl, $\text{MgCl}_2(50\text{NaCl})$, (III) Mg^{2+} titration in a 20 mM bisTris buffer at constant Cl^- concentration (160 mM), referred as $\text{MgCl}_2(\text{const. Cl}^-)$. In each titration experiment, nine samples were made to vary Mg^{2+} concentrations at 0, 2, 5, 10, 20, 30, 50, 70, and 80 mM. We prepared these samples in the following way.

First, RNH protein, ca. 3 mL at 180 μM , sufficient for the entire titration experiment, was dialyzed in a 20 mM bisTris buffer at pH 7.2 and diluted with D_2O to reach 7.5% D_2O concentration. Second, stock solutions of 240 mM MgCl_2 (99.9% trace metals, Sigma-Aldrich), 5 M NaCl, and 480 mM NaCl (99.5 % Bioextra, Sigma-Aldrich) were prepared using the same bisTris buffer as that used for protein. Last, each NMR sample for a 3 mm NMR tube, total volume of 150 μL , was prepared by mixing 100 μL of the protein solution with 50 μL of bisTris buffer containing NaCl and/or MgCl_2 , as described below. A 5 M NaCl stock solution was used to prepare (II) $\text{MgCl}_2(50\text{NaCl})$ titration samples, while a 480 mM NaCl stock solution was used to prepare (III) $\text{MgCl}_2(\text{const. Cl}^-)$ titration samples. A 240 mM MgCl_2 stock solution was used in all three titrations. In (III), constant Cl^- was achieved by decreasing the amount of NaCl when increasing MgCl_2 , maintaining the Cl^- concentration at 160 mM. The final protein concentration for each NMR sample was 114 μM , containing 5% D_2O . Details are described in Tables S1–S3.

NMR experiments and titration data analysis

The magnesium titrations into RNH were monitored by recording ^1H - ^{15}N HSQC data at 20°C on a Bruker Avance 900 MHz spectrometer (Bruker BioSpin, Billerica, MA, USA). NMR spectra were processed and analyzed using NMRPipe²¹ and CcpNmr⁵². Note, three samples among the total 27 MgCl_2 titration samples do not contain Mg^{2+} and have different NaCl concentrations. NMR spectra acquired for these Mg^{2+} -free samples make a set of NaCl titration data: 0, 50 mM, and 160 mM NaCl concentration.

Chemical shift perturbation, δ^i , for each step i of the titration series was calculated as normalized chemical shift differences of ^1H and ^{15}N .

$$\Delta\delta_{obs}^i = \sqrt{\left(\delta_H^i - \delta_H^{free}\right)^2 + \left[\left(\delta_N^i - \delta_N^{free}\right) \times \frac{\gamma_N}{\gamma_H}\right]^2} \quad (1)$$

where γ_N and γ_H are gyromagnetic ratios of nitrogen and proton, respectively. Since relative changes are analyzed, the spectra were referenced through internal deuterium lock⁵³.

$$\sigma_{uncert.}^i = \frac{\sqrt{(\delta_H^i - \delta_H^{free})^2 \cdot ((\sigma_H^i)^2 + (\sigma_H^{free})^2) + \left(\frac{\gamma_N^2}{\gamma_H^2}\right)^2 \cdot (\delta_N^i - \delta_N^{free})^2 \cdot ((\sigma_N^i)^2 + (\sigma_N^{free})^2)}}{\Delta \delta_{obs}^i}$$

(2)

Uncertainty, $\sigma_{uncert.}^i$, of δ_{obs}^i was estimated using Eq. (2) for each titration point. Chemical shift errors for proton and nitrogen, σ_H^i and σ_N^i , were estimated from the peak position uncertainty at Nmrpipe. Using a 1:1 interaction model with a K_D of a ligand, δ^i is calculated in Eq. (3).

$$\Delta \delta_{obs}^i = \Delta \delta_{\infty} \cdot \frac{([P]_{total} + [L]^i + K_D) + \sqrt{([P]_{total} + [L]^i + K_D)^2 - 4 \cdot [P]_{total} \cdot [L]^i}}{2 \cdot [P]_{total}} \quad (3)$$

Here, $[L]^i$ and $[P]_{total}$ stand for total ligand at titration point i , and protein concentration is uniform throughout the titration. The two unknown parameters, namely K_D and the magnitude of chemical shift change at saturation, $\delta_{\infty} = \delta_{bound} - \delta_{free}$, were optimized by minimizing the difference between the experimentally measured δ_{obs} and the δ_{cal} for each titration curve, as described previously³³. In this step, uniform shift uncertainty, by taking the average of δ_{uncert}^i for each titration curve, was used.

Calculation of ion density from an MD simulation trajectory

Previously, a 200 ns MD simulation of the isolated RNH was performed⁵¹. In this simulation, each structure was surrounded with TIP3 water in a rhombic dodecahedral box, allowing a 12 Å margin on all sides of the protein with a total of 21 Na⁺ and 18 Cl⁻ ions, equivalent to ca. 100 mM salt concentration at neutral pH. The simulation did not include any Mg²⁺ ions. Based on the MD trajectories, we calculated the density of Na⁺ and Cl⁻ ions in the following manner. Previously recorded frames, every 1 ps, were first aligned by backbone RMSD against the saved minimized starting structure. Then, the ions in each frame were re-centered, placing each ion at the periodic image closest to the protein. The number density (i.e. concentration, not charge density) was calculated using the volmap command in VMD⁵⁴, on a grid of 0.5 Å resolution. Each ion was represented by a normalized Gaussian distribution with a width equal to its van der Waals radius, and the resulting scalar fields were averaged over all of the frames in the trajectory. The resulting scalar field was portrayed as an isosurface using VMD software.

Results and discussion

Overview of Mg^{2+} titration to RNH by monitoring ^1H - ^{15}N HSQC spectra

We titrated MgCl_2 in three different conditions: $\text{MgCl}_2(0\text{NaCl})$, $\text{MgCl}_2(50\text{NaCl})$, and $\text{MgCl}_2(\text{const. Cl}^-)$. In all three Mg^{2+} titration experiments, the recorded spectra change as a function of Mg^{2+} concentration. δ values were calculated for 87 detectable backbone amide ^1H - ^{15}N cross peaks using Eq. (1). The average uncertainty of δ , calculated using the propagation of error formula for ^1H and ^{15}N chemical shifts, was 0.00094 ± 0.00090 ppm in $\text{MgCl}_2(0\text{NaCl})$, 0.00042 ± 0.00050 ppm in $\text{MgCl}_2(0\text{NaCl})$, and 0.00092 ± 0.00097 ppm in $\text{MgCl}_2(0\text{NaCl})$. Although pipetting error and sample instability may be larger than these noise uncertainties, such additional error was estimated to be at a level similar to the noise error obtained by examining the fluctuation of the smallest δ changes of the data (Figure S1). As expected, overall profiles of the chemical shift perturbations, obtained by comparing the δ values at 80 mM MgCl_2 , were very similar to one another among the three titration datasets (Figure 2). Several residues, particularly those close to the active site of RNH (*i.e.* residues D443, E478, D498, D549), exhibited large δ , over 0.2 ppm (approximately two-standard deviation above the average shift change) at 80 mM Mg^{2+} concentration. Significant changes in δ , over one-standard deviation above the average shift change, were observed in the regions surrounding the active site (Figure 1A). Similar chemical shift changes at and around the active site residues upon Mg^{2+} interaction were reported previously^{33–35}.

Even though the relative δ profiles, as a function of amino-acid residue number, for all three titrations are similar to one another (Figure 2), there were slight differences in δ values among the three data sets. In particular, two notable differences were observed upon comparison of δ magnitudes between $\text{MgCl}_2(0\text{NaCl})$ and $\text{MgCl}_2(50\text{NaCl})$ (Figure 3A–3C). First, at a low, ~5 mM, MgCl_2 concentration, δ values in the $\text{MgCl}_2(0\text{NaCl})$ titration were, in general, smaller than those in the $\text{MgCl}_2(50\text{NaCl})$ titration, except for those of residues in the RNH substrate-handle region (residues 503 to 527),⁴² (Figure 3A). Second, at high, 80 mM, MgCl_2 concentration, δ values of the residues in the substrate-handle region were greater in the $\text{MgCl}_2(0\text{NaCl})$ titration than the $\text{MgCl}_2(50\text{NaCl})$ titration (Figure 3C). We further analyzed these observations as described below.

Salt interaction at the substrate handle region

Since the Mg^{2+} titration experiments were recorded at different NaCl concentrations, the combined first data points of each series (that is, at 0 mM MgCl_2) generate a NaCl titration series at 0, 50 mM and 160 mM. Using these data, the effect of NaCl on RNH was qualitatively characterized by comparing the three spectra. As expected, the average chemical shift change for each residue upon 160 mM NaCl titration was small, less than 0.02 ppm (Figure 3D). Such a small NaCl effect, although 20 times larger than the chemical shift uncertainty, may be ignored in practical applications. However, in the substrate-handle region, the observed NaCl effect was significant, $\delta > 0.05$ ppm, at comparable points in the Mg^{2+} titration series (Figure 3D). Interestingly, the pattern of δ at 160 mM NaCl, Figure 3D, is very similar to $\delta(0\text{NaCl}) - \delta(50\text{NaCl})$ at 80 mM MgCl_2 concentration (Figure 3C). Thus, this comparison of the chemical shift changes indicates that the difference in the δ

changes observed between $\text{MgCl}_2(0\text{NaCl})$ and $\text{MgCl}_2(50\text{NaCl})$ in the substrate-handle region is mainly due to a NaCl effect.

The salt effect observed in the substrate-handle region was further confirmed by comparing the chemical shift trajectories of $\text{MgCl}_2(0\text{NaCl})$ with those of $\text{MgCl}_2(\text{const. Cl}^-)$ in more detail (Figure 2). For residues K512, E514, and S515, located in the substrate-handle region, the directions of chemical shift changes upon $\text{MgCl}_2(0\text{NaCl})$ and $\text{MgCl}_2(50\text{NaCl})$ titration differ from those of the $\text{MgCl}_2(\text{const. Cl}^-)$ titration but are similar to those of the NaCl titration (Figure 4). These residues also exhibit larger chemical shift changes in the $\text{MgCl}_2(0\text{NaCl})$ compared to the $\text{MgCl}_2(50\text{NaCl})$ (Figure 4). Consistent with these observations, titration curves, i.e., plots of δ value as a function of MgCl_2 concentration, were far from saturation even at 80 mM MgCl_2 for the residues in the substrate-handle region (Figure 5, dash and dot lines). In contrast, build-up of the titration curves of the $\text{MgCl}_2(\text{const. Cl}^-)$ are much weaker than those of $\text{MgCl}_2(0\text{NaCl})$ and $\text{MgCl}_2(50\text{NaCl})$ (Figure 5, solid lines).

The substrate-handle region is known to be mobile in the isolated RNH domain^{55, 56}, and this region interfaces with the p51 domain in the p66 subunit in RT^{57, 58}. The region also contains five negatively charged and two positively charged residues. Unlike *E. Coli* RNH⁵⁹, there are no Trp residues in this region. Indeed, in our calculation of ion density using MD simulation trajectory, the charge distribution exhibits physically expected behavior, showing that Na^+ is slightly localized at the substrate handle region (Figure 1B). For reference, this isovalue corresponds to a local salt concentration of 282 mM. The localization of solutes on a protein surface is a generally known phenomenon⁷⁻⁹. Considering these structural characteristics of the substrate-handle region of HIV-1 RNH along with the solvent charge distribution calculated from the MD simulation trajectory, it is not surprising that this region experiences significant salt effect as observed in the NaCl titration data.

Weak salt effect to the entire protein

In contrast to the specific salt effect observed in the substrate-handle region, the remainder of residues in the protein exhibited an apparent weak Mg^{2+} interaction profile in $\text{MgCl}_2(0\text{NaCl})$, compared to the $\text{MgCl}_2(50\text{NaCl})$ condition (Figure 3A and 3B). This is not a one-point titration anomaly because the entire titration curve for each residue shows lower affinity binding in the $\text{MgCl}_2(0\text{NaCl})$ condition, compared to the $\text{MgCl}_2(50\text{NaCl})$ condition even for residues that exhibited δ values greater than two standard deviation above the average and did not overlap with other peaks throughout the titration: A445, A446, G453, T477, S499, Q500, A502, W535, V536, I542, G543 (Figure 6). This observation is not likely due to systematic error of the titrated amount of MgCl_2 for three reasons. As described in the Experimental and Theoretical Methods, we used the same stock solution to prepare samples in parallel for the $\text{MgCl}_2(0\text{NaCl})$ and the $\text{MgCl}_2(50\text{NaCl})$ titrations but saw consistent lower affinity curves (Figure S2). Second, the δ values in the substrate-handle region show the opposite tendency and, thus, are not likely due to systematic depletion of the titration curves (Figure 3C). Last, we repeated the Mg^{2+} titration experiments, using a few

NMR samples, and reproduced the low-affinity Mg^{2+} titration effect in the $\text{MgCl}_2(0\text{NaCl})$ condition compared to the $\text{MgCl}_2(50\text{NaCl})$ condition (data not shown).

To further verify this apparent low-affinity MgCl_2 response in the absence of NaCl, we inspected the individual trajectories of the NMR signals in the $\text{MgCl}_2(0\text{NaCl})$ and the $\text{MgCl}_2(50\text{NaCl})$ titration data sets, especially, for residues that exhibited significant δ s and that did not overlap with other peaks throughout the titration (see Figure 6); among these, A445, A446, G453, and T477 are located in the RNH β -sheet region, S499, Q500, and A502 are close to the substrate-handle region, and V536, I542, and G543 are at the C-terminal α -helix (Figure 7, 8, 9, respectively). W535 was not included in the trajectory inspection because side chain motion, in addition to the Mg^{2+} interaction, may affect the trajectory (due to ring-current shift). At low Mg^{2+} concentration, as indicated by red and black arrows, the apparent low-affinity titration response in the $\text{MgCl}_2(0\text{NaCl})$ condition, compared to $\text{MgCl}_2(50\text{NaCl})$ and $\text{MgCl}_2(\text{const. Cl}^-)$ conditions, was evident for most of the residues. However, in contrast to the observed salt effect for residues in the substrate-handle region (Figure 4), the orientations of the trajectories for each residue in the $\text{MgCl}_2(0\text{NaCl})$ titration were not necessarily the same as those in the NaCl titration (Figure 7, right). Therefore, the apparent low-affinity Mg^{2+} build-up for these residues (Figure 6) was not due to a weak salt effect but instead caused by a specific Mg^{2+} interaction. The apparent low-affinity build-up was not due to the pH shift caused by the different salt concentrations either (discussed in the section of the Mg^{2+} dissociation constant). Since the apparent low-affinity build-up is significant, particularly at low Mg^{2+} concentration, some of the titrated MgCl_2 may interact with sites other than the active site, such as the substrate-handle region, and, thus, decrease the free Mg^{2+} concentration: as a result, the apparent build-up curve in the $\text{MgCl}_2(0\text{NaCl})$ titration might become weaker, compared to the calculated molar concentration of MgCl_2 in the $\text{MgCl}_2(0\text{NaCl})$ titration.

Salt effects on the exchange rate of the Mg^{2+} interaction

When the on-rate of a ligand is within the molecular diffusion limit ⁶⁰ (typically, $10^8 - 10^{10} \text{ s}^{-1}/\text{M}$) and K_D is $\sim 1 \text{ mM}$, the off-rate is $10^5 - 10^8 \text{ s}^{-1}$, which is most likely much faster than the difference in the chemical shifts between the free and the ligand-bound form, δ_∞ , at 900 MHz resonance frequency. In such a condition (*i.e.*, in the fast exchange régime), NMR signals are typically sharp throughout the titration without exchange signal-broadening. However, even though Mg^{2+} is a very weak binder to RNH, the titration spectra in both $\text{MgCl}_2(0\text{NaCl})$ and $\text{MgCl}_2(50\text{NaCl})$ titration experiments exhibited severe signal broadenings, observed as low signal intensity, at the mid-range, 20 – 60 mM, titration data points (Figure 7 and 8), indicating that the off-rate is not sufficiently large and is compatible to δ_∞ ²⁶. In contrast, the $\text{MgCl}_2(\text{const. Cl}^-)$ titration did not produce such broadening for most residues (Figure 7 and 8). Titration data obtained in the $\text{MgCl}_2(\text{const. Cl}^-)$ condition, containing 160 mM Cl^- , also showed a saturation pattern and reached a plateau at $> 50 \text{ mM}$ Mg^{2+} concentration (Figure 6). These data suggest that an abundance of cations, not limited to Mg^{2+} , affect the exchange rate of the Mg^{2+} interaction at the active site of RNH. In the ion density calculations, the highest Na^+ density was observed at the active site (a primary metal binding) region (Figure 1B). Although the Na^+ effect does not shift K_D (described below), it may affect the on/off rate of the Mg^{2+} interaction.

Possible effects of conformational change on the second Mg^{2+} interaction

Residues V536, I542, and G543 are located in the last α -helix in RNH (helix E), and are known to undergo internal motion^{55, 56, 61}. The helix contains one of the active site residues, D549, and was previously suggested to be stabilized upon Mg^{2+} interaction⁶¹. Consistent with previous observations, in all three titrations, $\text{MgCl}_2(0\text{NaCl})$, $\text{MgCl}_2(50\text{NaCl})$, and $\text{MgCl}_2(\text{const. Cl}^-)$, these residues showed broadening of signals and were difficult to track (Figure 9) compared to other residues shown above (Figure 7 and 8). These residues, located in the last α -helix, exhibited significant non-linear chemical shift changes above 50 mM Mg^{2+} concentration in the $\text{MgCl}_2(50\text{NaCl})$ and the $\text{MgCl}_2(\text{const. Cl}^-)$ titration spectra (Figure 9, curved arrows), and, in a less pronounced manner, in the $\text{MgCl}_2(0\text{NaCl})$ titration spectra. Directions of the changes of chemical shifts at high Mg^{2+} concentration (> 50 mM) were quite different from those at low Mg^{2+} concentration (< 50 mM), suggesting that the second event happens only at high Mg^{2+} concentration. This observation is consistent with previous NMR titration data³⁵. Although titration profiles for the active-site residues should be analyzed to clarify whether the observed rate event is due to binding of the second Mg^{2+} , sufficient data was not obtained due to severe signal broadening for the active-site residues. Only two of the active-site residues, E478 and D498, exhibited partial titration profiles (Figure S3).

T477 and Q500 also exhibited significant δ upon Mg^{2+} titration, but with non-linear, i.e., curved, migration patterns in the spectra (Figure 7 and 8). The non-linear migration pattern of δ for T477, especially ^1H shift above 50 mM Mg^{2+} concentration, is towards low-field in the $\text{MgCl}_2(0\text{NaCl})$ titration but towards high-field in the $\text{MgCl}_2(\text{const. Cl}^-)$ titration. Such a difference in the titration patterns suggests that NaCl concentration or ionic strength affects the Mg^{2+} titration profile of T477. Our previous simulation studies suggested that T477 forms a transient hydrogen bond network with T472 or T473 to support the core of the protein⁵¹. T477 is also adjacent to one of the active-site residues, E478, which coordinates metal ions. Based on these data and the previous result, the Mg^{2+} titration profile of T477 may reflect both changes in the hydrogen bond network as well as the effect of Mg^{2+} coordination at the active site. The non-linear, slight curved, migration pattern of δ was observed for Q500 in all the Mg^{2+} titration experiments. Since Q500 shows significant NaCl dependence of chemical shifts, the direction of which is similar to those of Mg^{2+} titration data, Q500 may sense both Mg^{2+} interactions at the active site and salt effects in the substrate handle region. Both T477 and Q500 are located close to the active site residues, E478 and D498, respectively. Through these inspections, it is apparent that even residues that exhibit significant δ upon Mg^{2+} titration are influenced by factors other than the direct Mg^{2+} interaction at the active site.

Mg^{2+} dissociation constant obtained by NMR

We selected 20 residues that satisfy the following criteria to evaluate K_D : (A) those for which chemical shift changes at 80 mM Mg^{2+} were one-standard deviation larger than the average value among all the residues and (B) all data points in each titration curve that were detected without signal overlap (Figure 1A). K_D s were calculated for these selected residues using a fit as described in the Experimental and Theoretical Methods (Table 1). The average K_D for the residues in the $\text{MgCl}_2(0\text{NaCl})$ titration, 29.8 ± 5.0 mM, was significantly greater

than those for the $\text{MgCl}_2(50\text{NaCl})$ and $\text{MgCl}_2(\text{const. Cl}^-)$ titrations (10–15 mM). Although practical error caused by pipetting error and sample instability were not taken into account, this larger K_D in the $\text{MgCl}_2(0\text{NaCl})$ titration is consistent with the observed apparent low-affinity build-up of the Mg^{2+} -bound form in the $\text{MgCl}_2(0\text{NaCl})$ titration compared to the others (Figure 6). In both $\text{MgCl}_2(0\text{NaCl})$ and $\text{MgCl}_2(50\text{NaCl})$ titrations, samples were prepared using the same 20 mM Tris buffer at pH 7.2. The 50 mM NaCl increases the pH by 0.04–0.05 unit (confirmed theoretically and experimentally), which would increase the K_D of a divalent metal ion and is opposite to the observed larger K_D s obtained for the $\text{MgCl}_2(0\text{NaCl})$ condition compared to the $\text{MgCl}_2(50\text{NaCl})$. Thus, the apparent low-affinity build up in the $\text{MgCl}_2(0\text{NaCl})$ titration is not due to a pH shift caused by the lack of NaCl. Indeed, the K_D s obtained in our $\text{MgCl}_2(50\text{NaCl})$ and $\text{MgCl}_2(\text{const. Cl}^-)$ titration experiments, 10 – 15 mM (Figure S4), are similar to the values reported previously^{34, 35}. The slightly larger K_D s in our data, compared to others, are reasonably explained by a difference in the experimental pH: our experiments were performed at pH 7.2 whereas the experiments by the London and Maegley groups were carried out at pH 6.8 and pH 6.5, respectively^{34, 35}.

The observed K_D s are higher than those obtained for the RNH domain in the full-length RT ($K_D \sim 0.1$ mM³⁶). There are two possible reasons for it. (I) The RNH structure is more rigid when it is incorporated as a part of the RT heterodimer^{57, 58, 61}, and, therefore, Mg^{2+} interaction with the full-length RT may be much stronger than the isolated RNH. (II) salt effects are not sufficiently separated from the effects of a specific Mg^{2+} interaction with RNH, resulting in a larger K_D with the isolated RNH compared to full-length RT. In either case, this domain-level study is biologically significant since the RNH domain is exposed to the solvent in a RT precursor form⁶². Our findings of various non-specific Mg^{2+} interaction effects precluded us from analyzing the titration data using a two-metal binding model.

Conclusions

Our aim in this study was to investigate molecular determinants of weak Mg^{2+} interaction with the protein. We successfully elucidated multiple factors that affect Mg^{2+} interaction to RNH, by comparing three different Mg^{2+} titration data sets. Careful analysis of these three sets of titration data, along with molecular dynamics simulation data of RNH with Na^+ and Cl^- ions, demonstrated two characteristic phenomena distinct from the specific Mg^{2+} interaction with the active site: (a) weak interaction of Mg^{2+} , as a salt, with the substrate-handle region of the protein, (b) overall apparent lower affinity in the Mg^{2+} titration in the absence of NaCl compared to those in the presence of 50 mM NaCl. Taken together, these observations may suggest that the titrated MgCl_2 is consumed as a salt and interacts with RNH not only specifically at the active site, but also in an unspecific fashion which modulates the RNH response. Therefore, in providing an overall landscape of a weak ligand interaction with a protein, the approach presented here may be widely applicable to usefully refine characterization of other complex ligand binding patterns, for instance in the context of drug discovery efforts and cell signaling studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

RNH	ribonuclease H
HSQC	single-quantum coherence
MgCl₂(0NaCl)	MgCl ₂ titration in a 20 mM bisTris buffer (pH 7.2) in the absence of additional NaCl
MgCl₂(50NaCl)	MgCl ₂ titration in a 20 mM bisTris buffer (pH 7.2) in the presence of 50 mM NaCl
MgCl₂(const. Cl⁻)	Mg ²⁺ titration in a 20 mM bisTris buffer at constant Cl ⁻ concentration

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Figure 1.

Ribbon representations of the structure of the RNH domain and the metal binding site (yellow side chains), (A) alone and (B) with isosurface overlay showing ion densities of Na^+ (red) and Cl^- (blue) calculated using MD trajectories. In (A), red highlights 20 selected residues, for which chemical shift changes at all the Mg^{2+} concentrations were detected without signal overlap and those at 80 mM Mg^{2+} were one-standard deviation larger than the average values among all the residues (described in the Results). In (B), residues at the RNH substrate-handle region (residues 503 to 527, ⁴²) are highlighted as green ribbons with stick representation of the side chains. The isosurfaces are at an isovalue of $1.7 \times 10^{-4} (\text{\AA}^{-3})$, corresponding to a salt concentration of approximately 282 mM (described in the Results). In panel B, there is a region of increased Na^+ density near the substrate handle region. Note the bottom region includes Cl^- density surrounded by Na^+ density. The structure was drawn using PDB 3K2P.

Figure 2.

Chemical shift perturbation, δ (ppm), at 80 mM Mg^{2+} concentration detected in (a) $\text{MgCl}_2(0\text{NaCl})$, (b) $\text{MgCl}_2(50\text{NaCl})$, and (c) $\text{Mg}^{2+}(\text{const. Cl}^-)$ titration experiments. Error bars represent the uncertainty of δ . Asterisks (*) indicate unassigned or overlapped backbone NH amides, and P indicates prolines. Active site residues (D443, E478, D498, and D549) of RNH are labeled and indicated by arrows. Approximately two-standard deviations above the average shift change (~ 0.2 ppm) is shown by dashed line. A horizontal arrow in the top panel indicates the location of the substrate handle-region (residues 503–527).

Figure 3.

Differences in chemical shifts between $\text{MgCl}_2(0\text{NaCl})$ and $\text{MgCl}_2(50\text{NaCl})$, labeled as $\delta(0 - 50 \text{ NaCl})$, at 5 mM (A), 20 mM (B), and 80 mM (C) MgCl_2 , and (D) chemical shift perturbation, $\delta(\text{ppm})$, at 160 mM NaCl concentration detected using the 0 MgCl concentration points of $\text{MgCl}_2(0\text{NaCl})$ and $\text{Mg}^{2+}(\text{const. Cl}^-)$. A horizontal arrow in the top panel indicates the location of the substrate handle-region (residues 503–527). Asterisks (*) indicate unassigned or overlapped backbone NH amides, and P indicates prolines.

Figure 4.

Changes in chemical shifts in ^1H - ^{15}N HSQC spectra upon Mg^{2+} titration and NaCl titration for RNH residues at the substrate-handle region, (A) K512, (B) E514, and (C) S515. Four panels are shown for each residue: from left to right, $\text{MgCl}_2(0\text{NaCl})$, $\text{MgCl}_2(50\text{NaCl})$, $\text{Mg}^{2+}(\text{const. Cl}^-)$, and NaCl. Spectra at 0 (black) and 80 mM (red) MgCl_2 concentrations were compared in the $\text{MgCl}_2(0\text{NaCl})$, $\text{MgCl}_2(50\text{NaCl})$, $\text{Mg}^{2+}(\text{const. Cl}^-)$ titration data. Spectra at 0 (black) and 160 mM (red) NaCl concentrations were compared in the NaCl titration data.

Figure 5.

Plots of $\Delta\delta$ as a function of Mg^{2+} concentration for residues (A) K512, (B) E514, and (C) S515 at the substrate-handle region of RNH. Experimental data points for $\text{MgCl}_2(0\text{NaCl})$, $\text{MgCl}_2(50\text{NaCl})$, and $\text{Mg}^{2+}(\text{const. Cl}^-)$ are represented by open square, closed triangle and cross symbols, respectively. Fit curves calculated assuming 1:1 interaction are shown by dotted, dashed, and solid lines for $\text{MgCl}_2(0\text{NaCl})$, $\text{MgCl}_2(50\text{NaCl})$, and $\text{Mg}^{2+}(\text{const. Cl}^-)$, respectively.

Figure 6.

Plots of δ as a function of Mg^{2+} concentration for residues that exhibited δ values greater than two standard deviations above the average. The symbols used for the titration data points are the same as those in Figure 5.

Figure 7.

Changes in chemical shifts in ^1H - ^{15}N HSQC spectra upon Mg^{2+} titration and NaCl titration for RNH residues in the β -sheet region, (A) A445, (B) A446, (C) G453, and (D) T477. Four panels are shown for each residue: from left to right, $\text{MgCl}_2(0\text{NaCl})$, $\text{MgCl}_2(50\text{NaCl})$, $\text{Mg}^{2+}(\text{const. Cl}^-)$, and NaCl. Overlay of ^1H - ^{15}N HSQC signals were made for $\text{MgCl}_2(0\text{NaCl})$, $\text{MgCl}_2(50\text{NaCl})$, and $\text{Mg}^{2+}(\text{const. Cl}^-)$ titrations at 0 mM (black), 2 mM (pink), 5 mM (teal), 10 mM (purple), 20 mM (green), 30 mM (navy), 50 mM (orange), 70 mM (skyblue), and 80 mM (red) Mg^{2+} concentrations. Overlays of ^1H - ^{15}N HSQC signals were made for NaCl titration at 0 (black), 50 mM (blue), and 160 mM (red). Arrows show the direction of chemical shift perturbation. Scales on each plot (black bars) represent 1 ppm for ^{15}N and 0.1 ppm for ^1H dimension.

Figure 8.

Changes in chemical shifts in ^1H - ^{15}N HSQC spectra upon Mg^{2+} titration and NaCl titration for RNH residues close to the substrate-handle region, (A) S499, (B) Q500, and (C) A502. The same color coordination as in Figure 7 was used.

Figure 9.

Changes in chemical shifts in ^1H - ^{15}N HSQC spectra upon Mg^{2+} titration and NaCl titration for RNH residues (A) V536, (B) I542, and (C) G543, in the last α -helix in RNH. The same color coordination as in Figure 7 was used. Trajectories of chemical shifts above 50 mM Mg^{2+} concentrations in the $\text{MgCl}_2(50\text{NaCl})$ and the $\text{MgCl}_2(\text{const. Cl}^-)$ titration spectra are outlined with curved arrows

Table 1

Average Mg^{2+} dissociation constants determined in three titration experiments.^a

	Titration experiments		
	$\text{MgCl}_2(0\text{NaCl})$	$\text{MgCl}_2(50\text{NaCl})$	$\text{Mg}^{2+}(\text{const. Cl}^-)$
$K_D(\text{mM})$	29.8 ± 5.0	13.6 ± 2.5	12.3 ± 4.5

^a K_D values were determined for 20 residues which satisfy two conditions: chemical shift changes at 80 mM Mg^{2+} were (A) above one-standard deviation of the average δ and (B) detected without signal overlap in the three datasets.