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Metal-ion stoichiometry of the HIV-1 RT ribonuclease H domain: evidence for two mutually exclusive sites leads to new mechanistic insights on metal-mediated hydrolysis in nucleic acid biochemistry

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Abstract Crystallographic studies of the Mn^{2+}-doped RNase H domain of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) [1] have revealed two bound Mn^{2+} separated by approximately 4 Å and surrounded by a cluster of four conserved carboxylates. *Escherichia coli* RNase H is structurally similar to the RNase H domain of HIV-1 RT, but requires one divalent metal cation for its activity [2, 3], implying either that the HIV-1 RT RNase H domain contrasts in its ability to bind two divalent metal ions, or that the crystallographic data reflect specific use of Mn^{2+} and/or the doping technique employed. Metal binding stoichiometry has been determined for Mn^{2+} and the biologically more relevant Mg^{2+} cation by solution calorimetric studies of native and recombinant p66/p51 HIV-1 RT. Three Mn^{2+} ions bind to HIV-1 RT apo-enzyme: one at the DNA polymerase and two at the RNase H catalytic center, the latter being consistent with crystallographic results. However, only one Mg^{2+} ion is bound in the RNase H catalytic center. Several mechanistic implications arise from these results, including the possibility of mutually exclusive Mg^{2+} binding sites that might be occupied according to the specific reaction being catalyzed by the multifunctional RNase H domain. The occurrence of distinct binding stoichiometries for Mg^{2+} and Mn^{2+} to multifunctional enzymes has previously been reported [4].

Key words Human immunodeficiency virus type 1 reverse transcriptase ribonuclease H domain · Calorimetry · Metal binding · Mechanism · Stoichiometry

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Introduction

Reverse transcription of the single-stranded viral genomic RNA is an essential step in the life cycle of all retroviruses, and is catalyzed by a multifunctional viral reverse transcriptase (RT) [5, 6]. The human immunodeficiency virus type 1 (HIV-1) enzyme has been the subject of numerous reports as a result of its critical role in viral infectivity and its potential as a drug target [7, 8]. HIV-1 RT possesses structurally separated DNA polymerase and ribonuclease H (RNase H) domains, located at the N- and C-termini of the catalytically competent p66 subunit of the p66/p51 heterodimer, respectively. While the RNase H activity of the simpler 18 kDa *Escherichia coli* enzyme mediates only exonucleolytic cleavage of the RNA strand of an RNA-DNA hybrid substrate, the RNase H domain of retroviral RT is designed to mediate a variety of highly specialized catalytic pathways related both to hydrolysis of RNA strands and formation and removal of primer sequences for DNA polymerase activity. Inasmuch as metal ions are prerequisite cofactors for the catalytic activities of both the DNA polymerase and RNase H domains, it is of great importance to develop an understanding of the mechanistic and structural roles of these catalytic centers.

Crystallographic studies of the Mn^{2+}-doped RNase H domain of HIV-1 RT [1] have revealed two bound Mn^{2+} separated by approximately 4 Å and surrounded by a cluster of four conserved carboxylates (D443,

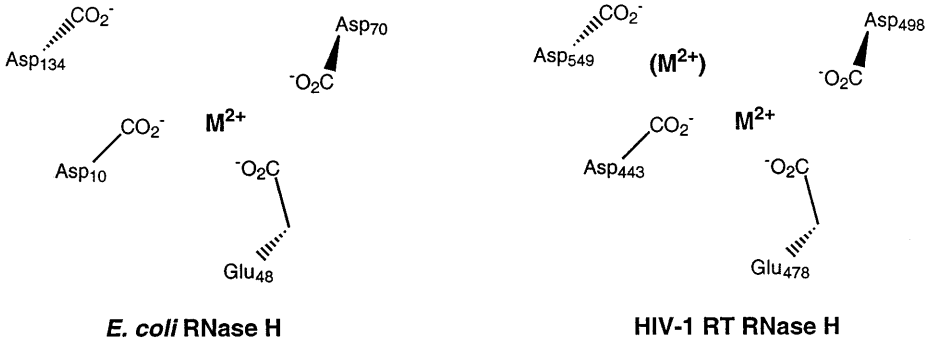

Fig. 1 Comparison of metal binding residues for the RNase H domains of *E. coli* ribonuclease H and HIV-1 RT

E478, D498, and D549) that are among seven conserved residues found in all bacterial and retroviral RNase H domains (Fig. 1). The metal binding pockets are formed by D443 and D549, and D443, E478, and D498, respectively, and are populated in a Mn^{2+}-doped crystal following treatment with 45 mM Mn^{2+} [1]. However, equivalent studies with the biologically more relevant Mg^{2+} cofactor have not been reported. The Mn^{2+} binding stoichiometry that we had obtained for *E. coli* RNase H from solution studies (one site) [2] contrasts with this crystallographic model obtained for the structurally homologous RNase H domain of HIV-1 RT. Inasmuch as the aforementioned carboxylate residues are four of the seven highly conserved residues found in all bacterial and retroviral RNase H domains, including the *E. coli* enzyme, either the RNase H domain of HIV-1 RT does indeed bind two divalent metal ions, or this result reflects the specific use of Mn^{2+} and/or the doping technique employed. It is therefore important to determine the cofactor stoichiometry for the latter: (1) with the biologically relevant Mg^{2+} and (2) in the context of the parental p66/p51 heterodimer. Prior to a recent report from one of our laboratories [4], the possibility of distinct metal binding stoichiometries, according to the selection of cofactor, had not been clearly demonstrated for a metallonuclease enzyme. The crystallographic results obtained for the RNase H domain of HIV-1 RT with a Mn^{2+} analog are not necessarily a good indicator of Mg^{2+} binding chemistry, especially in view of the fact that the intracellular concentration of Mn^{2+} is submicromolar, which is significantly lower than the doping concentrations used in X-ray crystallography experiments, while Mn^{2+} may be an intrinsically poor mimic for Mg^{2+} [9]. In this regard, Cirino et al. [10] have reported different effects for Mg^{2+} and Mn^{2+} binding to HIV-1 RT bearing a mutation in its RNase H domain. To resolve this issue, a detailed study of metal ion binding has been carried out by direct isothermal titration calorimetry experiments on several variants of the recombinant p66/p51 HIV-1 RT enzyme. Mutants of residues in both the DNA polymerase (D185, D186) and RNase H (E478, D549) domains were targeted in these studies. Mutations in either domain resulted in loss of that specific activity, but retention of the other.

Materials and methods

Reverse transcriptase mutants

The p66/p51 heterodimer form of RT, produced through dual expression of p66 HIV-1 RT with the retroviral protease [11], was used throughout these studies. Thus, DNA polymerase active site mutations D185N and D186N [12] were present in both the p66 and p51 subunits ($p66^{D185N,D186N}/p51^{D185N,D186N}$ RT). While the DNA polymerase activity of this mutant was completely eliminated, RNase H activity was unimpaired. Since the heterodimeric enzyme contains a single RNase H domain, mutations in this catalytic center, i.e. E478Q [13] and D549A [14], were only represented once on $p66^{E478Q}/p51$ and $p66^{D549A}/p51$ RT, respectively. RNase H mutants were likewise demonstrated to have unimpaired DNA polymerase function. All recombinant plasmids expressing these RT variants described herein were maintained in the *E. coli* strain M15::pDMI.1 under inducible control of *lac* regulatory elements [15].

Protein isolation

Enzyme isolation was routinely performed on 200–300 g biomass, produced at the biofermentation facilities of The Ohio State University, and the Center For AIDS Research at Case Western Reserve University. A three-column purification procedure, involving Ni^{2+}-NTA-Sepharose, DEAE-Sepharose, and Mono-S Sepharose [6] was used, and yielded enzymes with a 1:1 subunit stoichiometry and purity exceeding 95%. All enzymes were free of contaminating *E. coli* RNase H activity, and stored at –20 °C in a 50% glycerol-containing buffer [16] until required.

Calorimetry experiments

To a solution of recombinant enzyme was added 1 mM dithiothreitol (DTT) and 2 mM EDTA (final concentrations). This solution was then dialyzed three times (6 h each) against 3.5 L of 20 mM Tris base buffer (pH 7.3 at room temperature) at 4 °C, using dialysis tubing obtained from Spectra/Por (molecular weight cutoff: 12,000–14,000 Da). After dialysis, the enzyme was concentrated by ultrafiltration using a 30K centricon unit (Amicon). The concentration of enzyme was determined from the absorption at 280 nm, using an extinction coefficient $(280\ nm) = 260{,}450\ cm^{-1}\ M^{-1}$ [17]. Solutions of divalent metal ions (Mg^{2+}, Mn^{2+}) were prepared by dissolving an appropriate amount of the respective dichloride salt in a solution of Tris buffer (20 mM, pH 7.3 at room temperature). Immediately prior to calorimetric measurement, DTT (1 mM final concentration) was added to both the metal ion and enzyme solutions.

Data were collected on a Microcal OMEGA ultrasensitive titration calorimeter [18]. The enzyme solution (~0.1 mM) was loaded into the reaction cell and calorimetry experiments were carried out with a stirring speed of 200 rpm. Divalent metal cation

solution (4–6 mM, depending on the concentration of enzyme) was injected as 16 aliquots of 15 μL from a 250 μL syringe over a 10-s time interval at 25 °C, with 7 min between injections to allow complete equilibration. Concentrations of magnesium stock solutions were accurately determined by atomic absorbance measurements and exactly reflected expected concentrations based on the mass of $MgCl_2$ used. Manganese concentrations were based on measured mass. Volume changes associated with the titration were automatically corrected for. Data were fit using Microcal Origin software (v. 4.1) and data from two or three experiments were averaged. Fitting models have been described in detail elsewhere [18]. In this instance, a model that assumed non-identical and non-interacting sites gave the best fits to the experimental data. In all cases, fits to data that assumed metal ion stoichiometries differing from those described herein led to an increase in χ^2 (the sum of the squares of deviations of experimental points from the fitted line) by almost an order of magnitude.

Results

Isothermal titration calorimetry provides the most direct method for a complete evaluation of the thermodynamic profile for metal binding to HIV-1 RT. To distinguish metal binding to the RNase H and DNA polymerase domains, a series of mutant enzymes were studied and compared with results obtained from the recombinant wild-type enzyme. These include a D185N/D186N double mutant [12], predicted to remove metal binding at the DNA polymerase site, and E478Q [13] and D549A RNase H mutants [14] which should perturb binding in the vicinity of each of the Mn^{2+} sites identified in the isolated HIV-1 RNase H polypeptide by X-ray crystallography [1].

As detailed below, experiments with Mn^{2+} proceeded relatively smoothly with well-defined binding isotherms for each protein sample. In each case, binding was strongly exothermic. However, in general it did not prove feasible to follow Mg^{2+} binding by direct measurement. The calorimetry experiment requires a net enthalpy change (either exothermic or endothermic) to produce a measurable response. While we had not previously encountered a situation where Mg^{2+} binding gave rise to a small binding enthalpy, this was found to be the case with HIV-1 RT. For wild-type p66/p51, $p66^{E478Q}$/p51, and $p66^{D549A}$/p51 RT, the response was too weak to provide reliable binding parameters. The one exception to this was the DNA polymerase active site mutant $p66^{D185N,D186N}/p51^{D185N,D186N}$, which gave rise to a modest enthalpy change. For the other enzymes, the heats of binding at the DNA polymerase and RNase H sites were either equal, but opposite, or the enthalpy was truly small. As explained later, the latter is the most likely scenario.

With this difficulty, we sought to evaluate Mg^{2+} binding by a Mn^{2+} competition experiment. In these experiments, HIV-1 RT solutions were pre-saturated with Mg^{2+} (0.5 mM). Subsequently, Mn^{2+} was titrated as usual. Prior binding of Mg^{2+} to specific metal binding sites would preclude Mn^{2+} binding at those sites, and only those metal binding sites that were not populated by Mg^{2+} would be capable of taking up Mn^{2+} at the concentrations of Mn^{2+} used. The difference in Mn^{2+} binding stoichiometry for experiments carried out in the presence and absence of Mg^{2+} would then give a direct measure of Mg^{2+} binding stoichiometry, although no additional thermodynamic information could be reliably obtained as a result of the complexity of the system. The number of bound Mg^{2+} would then be given by $n–x$, where n is the number of Mn^{2+} ions bound in the absence of Mg^{2+} and x is the number of Mn^{2+} ions bound in the presence of Mg^{2+}.

Wild-type p66/p51 HIV-1 RT

Table 1 summarizes the results of thermodynamic parameters obtained from isothermal titration calorimetric experiments by addition of Mn^{2+} to solutions of native p66/p51 HIV-1 RT. For Mn^{2+} the binding stoichiometry clearly indicates three bound ions. Inasmuch as the data is incapable of accurately discriminating the thermodynamic parameters from sites of similar binding affinity, we have analyzed the binding isotherms in terms of two classes of sites (stronger and weaker) and

Table 1 Thermodynamic constants[a] for Mn^{2+} binding to p66/p51, $p66^{E478Q}$/p51, $p66^{D549A}$/p51, and $p66^{D185N,D186N}/p51^{D185N,D186N}$ HIV-1 RT in the absence of added Mg^{2+}

	No. of Mn^{2+}	K_a (M^{-1})	ΔH (kcal/mol)	ΔG (kcal/mol)
p66/p51 wild-type				
rnh1	1	$4.2 \pm 0.7 \times 10^4$	–6.55	–6.3
poly/rnh2	2	$4.1 \pm 0.5 \times 10^3$	–4.38	–4.9
$p66^{E478Q}$/p51				
poly	1	$6.8 \pm 0.9 \times 10^3$	–6.63	–5.2
rnh2	1	$7.2 \pm 1.2 \times 10^2$	–2.16	–3.9
$p66^{D549A}$/p51				
rnh1	1	$5.6 \pm 0.4 \times 10^4$	–5.05	–6.5
poly	1	$4.0 \pm 0.8 \times 10^3$	–3.63	–4.9
$p66^{D185N,D186N}/p51^{D185N,D186N}$				
rnh1	1	$1.9 \pm 0.4 \times 10^4$	–5.03	–5.8
rnh2	1	$4.4 \pm 0.8 \times 10^3$	–4.5	–5.0

[a] The general metal binding positions for the poly, rnh1, and rnh2 sites are indicated in Fig. 4. The error in the metal ion stoichiometry is ±10% from the average of at least two measurements

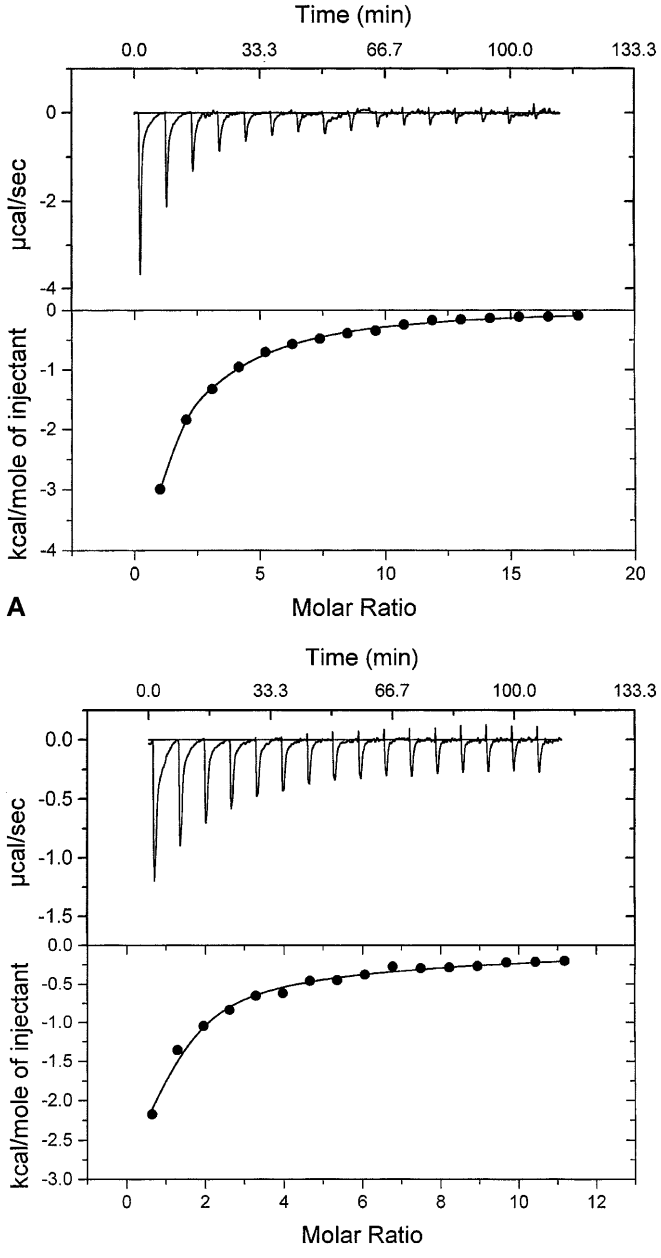

Fig. 2 Calorimetric traces for Mn^{2+} binding to **A** p66/p51 wild-type RT and **B** $p66^{E478Q}$/p51 RT**.** Both raw and integrated data are shown

have evaluated the binding parameters and metal ion stoichiometry for each (Fig. 2A, Table 1). Since crystallographic studies with the isolated C-terminal RNase H domain [1] account for two of these, the current data indicate that the apo-enzyme accommodates a single Mn^{2+} ion in the DNA polymerase catalytic center. Fits that assumed only one class of site, or that assumed two classes but each binding one cofactor, either did not yield reasonable fits or gave unreasonable binding parameters. Also, the values of χ^2 obtained from such fits were significantly larger than that obtained for the fit reported herein (in this case $\chi^2 = 4136$ and 3353 versus 326, respectively). The thermodynamic parameters that we have determined for these sites (summarized in Table 1) are consistent with data that we have obtained with other enzymes [4].

Mg^{2+} binding was evaluated through competition experiments with Mn^{2+}, and binding parameters for Mn^{2+} in the presence of 0.5 mM Mg^{2+} are summarized in Table 2. Typically, a 0.08 mM solution of wild-type HIV-1 RT containing 0.5 mM Mg^{2+} was titrated with aliquots from a 10 mM stock solution of Mn^{2+}. One Mn^{2+} ion was found to bind. The thermodynamic parameters are similar to those found for Mn^{2+} binding alone to the weaker class of site (Table 1), although the binding affinity is further reduced, most likely as a result of a neighboring Mg^{2+} with a higher charge density than Mn^{2+}. Several experiments were performed in the presence of 5 mM Mg^{2+}. While the binding affinity for Mn^{2+} was slightly lower, the results concerning Mg^{2+} stoichiometry were unchanged. These results indicate that the K_a for the Mg^{2+} site is greater than that for Mn^{2+}, a conclusion that is directly supported by the data shown in Fig. 3 and Table 2. As described earlier, comparison of the data in Tables 1 and 2 suggests that the parental HIV-1 apo-enzyme binds two Mg^{2+} ions: one at the polymerase site and the other at the RNase H catalytic center.

$p66^{E478Q}$/p51 RT

Manganese binding parameters for the RNase H-deficient enzyme, $p66^{E478Q}$/p51, are summarized in Table 1. For Mn^{2+} binding, one high-affinity site and one weaker binding site ($K_a \sim 720\ M^{-1}$) were identified. The weaker site was not fully populated within the concentration range used and gave rise to a non-zero endpoint (Fig. 2B). This result indicates that one of the two metal binding sites in the RNase H domain has been destroyed, while the other is significantly weakened (presumably the D443/D549 site). The similarity in binding parameters for the high-affinity site, relative to those determined for the native enzyme, suggests that this corresponds to the DNA polymerase site. It is theoretically possible that this mutation within the RNase H domain leads to structural perturbation of the DNA polymerase domain such that the latter cannot accommodate metal ions; that is, the higher-affinity site observed in the Mn^{2+} binding experiment lies in the RNase H domain. However, this scenario seems unlikely in view of studies indicating that both the RNA- and DNA-dependent DNA polymerase activities of $p66^{E478Q}$/p51 RT are unaffected on a variety of nucleic acid substrates [12, 19, 20].

As found for native enzyme, Mg^{2+} binding could not be readily measured directly by titration. However, one Mn^{2+} was observed to bind in a competition experiment when the enzyme solution was pre-saturated with Mg^{2+}. Accordingly, the number of Mg^{2+} binding sites is presumed to be one, corresponding to the DNA

Table 2 Thermodynamic constants[a] for Mn^{2+} binding to p66/p51, $p66^{E478Q}$/p51, $p66^{D549A}$/p51, and $p66^{D185N,D186N}$/$p51^{D185N,D186N}$ HIV-1 RT with added Mg^{2+}

	No. of Mn^{2+}	K_a (M^{-1})	ΔH (kcal/mol)	ΔG (kcal/mol)	No. of Mg^{2+}
p66/p51 wild-type					
rnh2	1	$3.5 \pm 0.9 \times 10^3$	–6.38	–5.1	2
$p66^{E478Q}$/p51					
rnh2	1	$2.4 \pm 0.8 \times 10^3$	–5.66	–4.6	1
$p66^{D549A}$/p51					
–	0	No binding			2
$p66^{D185N,D186N}$/$p51^{D185N,D186N}$					
rnh2	1	$6.0 \pm 1.0 \times 10^3$	–7.0	–5.2	1

[a] The position of the rnh2 site is indicated in Fig. 4. Direct titration of the $p66^{D185N,D186N}$/$p51^{D185N,D186N}$ mutant with Mg^{2+} yields an isotherm (Fig. 3B) that is fit to a stoichiometry of 1 bound Mg^{2+}, $\Delta H \sim 1.25$ kcal/mol, and $K_a \sim 1.1 \pm 0.3 \times 10^4$ M^{-1}. Error limits are described in the legend to Table 1

polymerase domain (Table 2). The single point mutation E478Q, which removes a putative magnesium binding ligand, thus appears to completely eliminate Mg^{2+} binding within the RNase H catalytic center (but see the later discussion for circumstances under which this might be populated).

$p66^{D549A}$/p51 RT

Binding parameters for $p66^{D549A}$/p51 RT are summarized in Table 1. As found for the $p66^{E478Q}$/p51 mutant, there is a reduction in the number of bound Mn^{2+} ions relative to native enzyme. However, for $p66^{D549A}$/p51 RT the stoichiometry is reduced by only one. The binding parameters for the site with $K_a = 4.0 \times 10^3$ M^{-1} are again similar to those associated with the DNA polymerase catalytic center for wild-type p66/p51 and $p66^{E478Q}$/p51 enzymes, while the remaining Mn^{2+} with $K_a = 5.6 \times 10^4$ M^{-1} presumably binds at the E478 site. In contrast to $p66^{E478Q}$/p51 RT, there remains one strong binding site in the RNase H domain, with a K_a similar to that of the native site.

Competition experiments with titration of Mn^{2+} to solutions of $p66^{D549A}$/p51 RT/Mg^{2+} (0.5 mM) provide no evidence for additional Mn^{2+} binding (Table 2), and so by difference there exists two Mg^{2+} binding sites on the mutant enzyme, presumably distributed between the DNA polymerase domain and the site involving residues D443, E478, and D498. There is no indication that Mg^{2+} can bind to the D443/D549 site identified crystallographically for Mn^{2+} [1], except in a mutually exclusive fashion in the presence of an appropriate substrate (see later discussion).

$p66^{D185N,D186N}$/$p51^{D185N,D186N}$ RT

Residues D110, D185, and D186 of HIV-1 RT have been implicated in metal binding at the DNA polymerase catalytic center [21]. Thus, a D185N/D186N double mutant was expected to nullify metal binding at this site in both the p66 and p51 subunits of $p66^{D185N,D186N}$/$p51^{D185N,D186N}$ RT (in addition, alternative folding of the p51 subunit [22] would also prevent metal ion coordination). This hypothesis is supported by the data

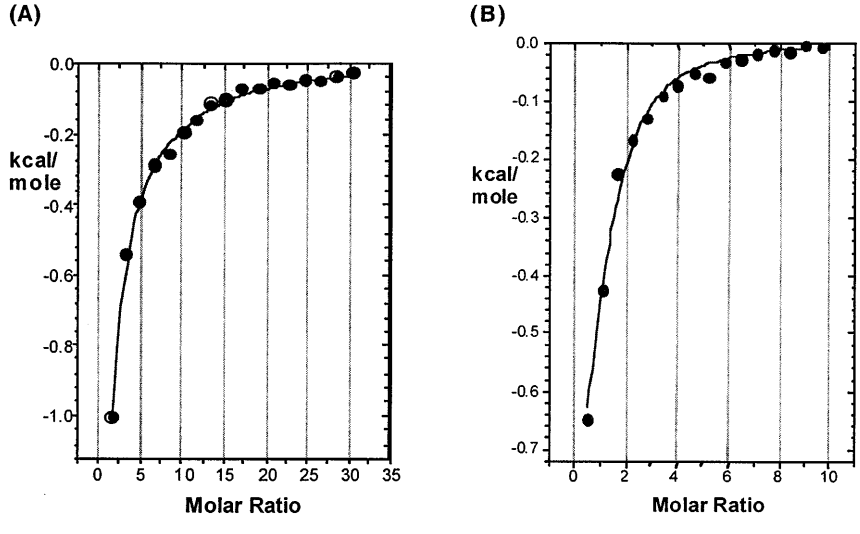

Fig. 3 Calorimetric traces for **A** Mn^{2+} and **B** Mg^{2+} binding to $p66^{D185N,D186N}$/$p51^{D185N,D186N}$

summarized in Table 1. Direct titration by Mn^{2+} (Fig. 3A) gave evidence of two bound Mn^{2+}, a reduction of one relative to the wild-type heterodimer, presumably through loss of the cofactor at the DNA polymerase catalytic center. The thermodynamic parameters for the remaining two ions compare favorably with data obtained for Mn^{2+} binding to the wild-type enzyme (Table 1).

Competition experiments with Mg^{2+}-saturated enzyme give evidence for one bound Mn^{2+} (Table 2), indicating the presence of only one Mg^{2+} site in the double mutant. Since (1) metal ion binding at the DNA polymerase catalytic center has been eliminated and (2) $p66^{D185N,D186N}/p51^{D185N,D186N}$ RT retains full RNase H activity [12], this single Mg^{2+} site presumably lies in the RNase H domain. This result is confirmed by direct titration of the enzyme with Mg^{2+}, which in this case yields an observable response with a binding stoichiometry of one divalent magnesium (Fig. 3B, legend to Table 2).

Discussion

The stoichiometry of the metal cofactor that is required to mediate phosphate ester hydrolysis is an important variable for the mechanistic understanding of metallonucleases. This has been the subject of much debate as a result of conflicting evidence from solution and crystallographic studies [23–29]. While binuclear metalloenzymes are well characterized for both redox and hydrolytic enzymes, the evidence for binuclear magnesium in nucleic acid hydrolysis is less convincing. A critical review that discusses the problems in this field has recently appeared [27].

Based on prior crystallographic studies indicating the presence of two Mn^{2+} ions in the RNase H domain of HIV-1 RT [1], it has been proposed that this enzyme most likely acts in an analogous fashion to the exonuclease domain of DNA polymerase I [23]. A similar analogy for *E. coli* RNase H proved inconsistent with crystallographic work on the magnesium-bound enzyme [3], and other related enzymes (including CheY and retroviral integrase [30, 31]), and was not supported by solution kinetic, NMR, and calorimetric studies, for either Mg^{2+} or Mn^{2+} binding [2, 4, 32]. Inasmuch as metal occupation is carried out by doping at extreme concentrations of divalent metal ion and background electrolyte (typically ammonium sulfate), this might lead to population of sites that are mere artifacts from the conditions used. Moreover, the use of Mg^{2+} analogs such as Mn^{2+} may also give rise to binding at sites otherwise not populated by Mg^{2+}, since several Mn^{2+} binding sites identified crystallographically show pentacoordinate geometries that would not support Mg^{2+} coordination [23]. Such analogs are used since the natural Mg^{2+} cofactor has a low electron density and is difficult to distinguish from oxygen in water. Increasingly, however, with more sensitive detection the natural Mg^{2+} cofactor is employed, making use of the strong pattern of octahedral coordination by metal-bound H_2O to distinguish Mg^{2+} from general solvent water [33].

In general, the consensus of solution measurements seems to support a single divalent magnesium ion as the sole catalytic requirement to promote nuclease activity [2, 9, 24, 25, 27, 29, 34]. While two Mg^{2+} ions have been shown to bind to the bacteriophage T4 RNase H [33], these were not contiguous (7 Å separation) and served distinct roles. In particular, this structure shows a structural magnesium ion that is surrounded by six H_2O and is held in an outer sphere mode, while the other catalytic metal ion is ligated by five H_2O and one carboxylate residue.

An important caveat to consider is that simple binding experiments do not necessarily indicate the number of metal ions required for catalytic turnover. While it is possible that an additional metal binding site is found in the enzyme-substrate complex, there is no evidence from these studies for such a site on the enzyme. However, in this work our calorimetric data do reproduce the binding stoichiometry for Mn^{2+} observed by crystallography [1]. The more significant result is that the manganese stoichiometry is different from that of the biologically more relevant magnesium ion. Furthermore, our finding of a single catalytically active Mg^{2+} is supported both by solution studies [2, 4, 24, 25, 28, 29] and crystallographic studies of other nuclease enzymes [33–36], and is consistent with the significantly increased charge density of Mg^{2+} relative to Mn^{2+}. Finally, the requirement for two metal ions to mediate efficient phosphate ester hydrolysis is undermined by the prevalence of various calcium-dependent nucleases that employ a single low charge density Ca^{2+} to effi-

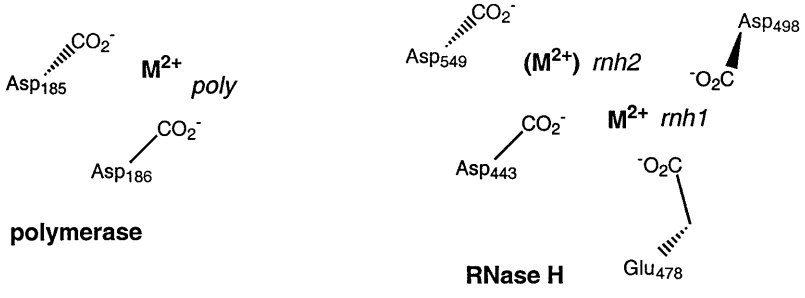

Fig. 4 Schematic illustration of the metal binding sites for wild-type and mutant HIV-1 RT. The site in parentheses would not normally be associated with Mg^{2+} binding by the native enzyme in the absence of a substrate (but see [38]). The positions of the poly, rnh1, and rnh2 sites described in Tables 1 and 2 are indicated

ciently catalyze such reactions [37]. Accordingly, there is no evidence from solution studies for the involvement of two natural Mg^{2+} cofactors for RNase H activity.

Also, it is noteworthy that these calorimetric experiments do not define the coordination mode of the bound metal ions. While the crystallographic structure of the Mn^{2+} complex with native RT provides insight on the coordination sites for the bound cations, the only structural data reported for Mg^{2+} coordination is with a mutant RT [38]. The illustration shown in Fig. 4 should be viewed with this fact in mind. Finally, it should also be noted that some recent structural studies of magnesium binding proteins show explicit coordination of Mg^{2+} by amide sidechains [39]. However, the loss of metal binding for the mutant proteins described herein suggests that amide binding, if it occurs, must be weak and is in accord with our assumptions. Furthermore, the influence of structural change arising from these mutations is unlikely to be significant inasmuch as the enzyme retains a sub-set of the catalytic activities promoted by the RNase H domain, suggesting retention of core structural elements in the RNase H domain.

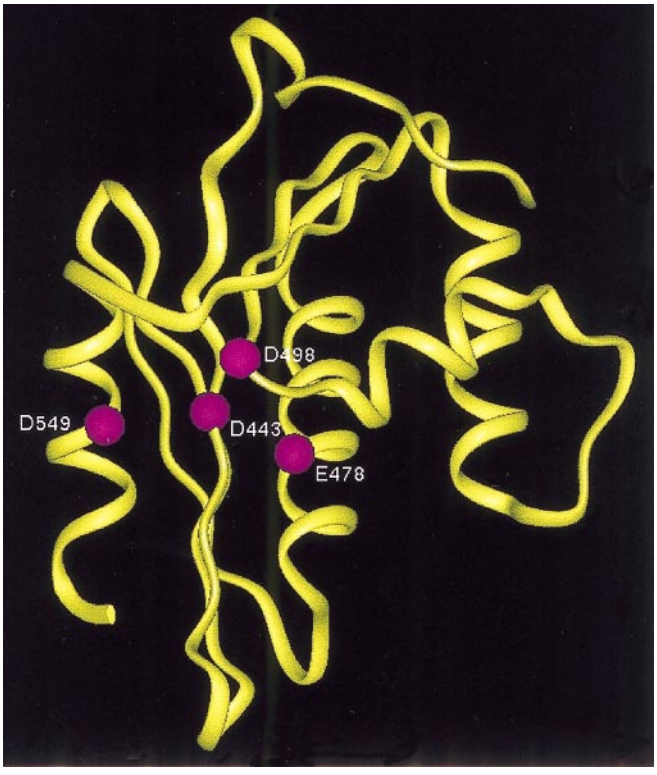

Fig. 5 Illustration of the crystallographically characterized metal binding residues in the RNase H catalytic center of HIV-1 RT

Mutually exclusive binding sites

An illustrative summary of the binding data obtained for Mg^{2+} and Mn^{2+} to wild-type and mutant HIV-1 RT is presented in Fig. 4. By engineering mutations to the carboxylate core of the RNase H domain (Fig. 5), it has proven possible both to evaluate the metal binding stoichiometry for Mg^{2+} versus Mn^{2+} and also to identify specific binding loci. Further mutations in the DNA polymerase catalytic center of the p66 subunit have also allowed verification of metal binding stoichiometries deduced for the RNase H domain.

By solution calorimetry, three Mn^{2+} ions bind to wild-type p66/p51 HIV-1 RT, namely one within the p66 DNA polymerase catalytic center and two at the RNase H domain. The latter conclusion is consistent with crystallographic results [1], which show the binding pockets to be formed by D443/D549 and D443/E478/D498, respectively. However, our solution calorimetric results indicate that only one Mg^{2+} ion is bound to the RNase H domain, apparently at the site formed by D443, E478, and D498. These observations raise the intriguing possibility that the RNase H domain of HIV-1 RT (Fig. 5) might possess two distinct metal binding domains, each of which can be simultaneously populated by Mn^{2+}, but which can be occupied by Mg^{2+} only in a mutually exclusive mode. This is readily understandable in terms of the distinct charge densities of the two ions ($r_{Mg}=0.65$ Å; $r_{Mn}=0.85$ Å), with a significantly increased repulsion for two contiguous Mg^{2+} ions. Such a model is supported by a recent crystallographic analysis of a trapped catalytic complex of HIV-1 RT with a DNA template-primer [38]. The E478Q mutant used in this structural study shows a Mg^{2+} ion bound by D443, D549, and the *n*-17 phosphate of the bound DNA duplex. While this bound Mg^{2+} is not detected in our calorimetric study (most likely the binding affinity is too weak), it is seen in the crystallographic analysis as a result of an enhanced binding constant brought about by phosphate coordination, and use of higher concentrations of Mg^{2+}. The possibility that this second site can be populated in a mutually exclusive manner is thereby established.

Conclusions

Our results have important mechanistic implications. In contrast to the simpler *E. coli* enzyme, the RNase H domain of retroviral RT is designed to mediate a variety of highly specialized catalytic pathways, including: (1) polymerization-independent hydrolysis of the RNA template from the point of initial endonucleolytic cleavage to within eight nucleotides of the primer terminus; (2) RNase H cleavage of the 3′-OH of the polypurine tract to generate the (+) strand primer; and finally, (3) removal of the (+) and (–) strand primers from the nascent DNA to preserve the integrity of the 5′ and 3′ long terminal repeat units [7]. An appealing idea that we have developed to explain the difference with the metal binding behavior of the *E. coli* RNase H domain is that the two Mn^{2+} ions identified in the crys-

tal structure might represent two distinct and mutually exclusive Mg^{2+} binding sites, depending on the reaction that is catalyzed, with only one metal site populated for any specific reaction. Such a hypothesis would be consistent with data we have documented on the mutant $p66^{E478Q}$/p51, which lacks both subsets of RNase H activity (viz., endonucleolytic cleavage and directional processing) in the presence of Mg^{2+}. However, substitution of Mn^{2+} for Mg^{2+} results in quantitative, but selective, recovery of endonuclease activity with a specificity equivalent to wild-type HIV-1 RT, suggesting similar coordination modes [10].

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