Catalytic Metal Ions and Enzymatic Processing of DNA and RNA

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CONSPECTUS: Two-metal-ion-dependent nucleases cleave the phosphodiester bonds of nucleic acids via the two-metal-ion (2M) mechanism. Several high-resolution X-ray structures portraying the two-metal-aided catalytic site, together with mutagenesis and kinetics studies, have demonstrated a functional role of the ions for catalysis in numerous metallonucleases. Overall, the experimental data confirm the general mechanistic hypothesis for 2M-aided phosphoryl transfer originally reported by Steitz and Steitz (*Proc. Natl. Acad. Sci. U.S.A.* **1993** *90* (14), 6498−6502). This seminal paper proposed that one metal ion favors the formation of the nucleophile, while the nearby second metal ion facilitates leaving group departure during RNA hydrolysis. Both metals were suggested to stabilize the enzymatic transition state. Nevertheless, static X-ray structures alone cannot exhaustively unravel how the two ions execute their functional role along the enzymatic reaction during processing of DNA or RNA strands when moving from reactants to products, passing through metastable intermediates and high-energy transition states.

In this Account, we discuss the role of multiscale molecular simulations in further disclosing mechanistic insights of 2M-aided catalysis for two prototypical enzymatic targets for drug discovery, namely, ribonuclease H (RNase H) and type II topoisomerase (topoII). In both examples, first-principles molecular simulations, integrated with structural data, emphasize a cooperative motion of the bimetal motif during catalysis. The coordinated motion of both ions is crucial for maintaining a flexible metal-centered structural architecture exquisitely tailored to accommodate the DNA or RNA sugar−phosphate backbone during phosphodiester bond cleavage.

Furthermore, our analysis of RNase H and the N-terminal domain (PA$_N$) of influenza polymerase shows that classical molecular dynamics simulations coupled with enhanced sampling techniques have contributed to describe the modulatory effect of metal ion concentration and metal uptake on the 2M mechanism and efficiency. These aspects all point to the emerging and intriguing role of additional adjacent ions potentially involved in the modulation of phosphoryl transfer reactions and enzymatic turnover in 2M-catalysis, as recently observed experimentally in polymerase η and homing endonuclease I-DmoI.

These computational results, integrated with experimental findings, describe and reinforce the nascent concept of a functional and cooperative dynamics of the catalytic metal ions during the 2M-dependent enzymatic processing of DNA and RNA. Encouraged by the insights provided by computational approaches, we foresee further experiments that will feature the functional and joint dynamics of the catalytic metal ions for nucleic acid processing. This could impact the *de novo* design of artificial metallonucleases and the rational design of potent metal-chelating inhibitors of pharmaceutically relevant enzymes.

INTRODUCTION

Metallonucleases are enzymes that cleave DNA and RNA. X-ray crystallography has often depicted two divalent cations in their catalytic pocket, revealing a recurrent two-metal-ion (2M) mechanism for metal-aided enzymatic phosphoryl transfer. During bimetallonuclease catalysis, the scissile phosphodiester of the nucleic acid backbone lies between the two adjacent divalent cations, most frequently Mg^{2+}, that facilitate DNA or RNA processing.[1] While static X-ray structures are able to elucidate general mechanistic aspects, other dynamical and energetics features require the integration of experimental data with multiscale molecular modeling and simulation, enabling the detailed description of the continuous pathway from reactants to products, passing through high-energy metastable intermediates and enzymatic transition states (TS), during catalysis.

TWO-METAL-ION MECHANISM: LESSONS LEARNED FROM STRUCTURAL DATA

Metalloproteins often contain more than one single metal ion in the catalytic site, frequently used to ensure structural

Scheme 1. General Mechanism for the 2M Enzymatic Mechanism for RNase H[7a]
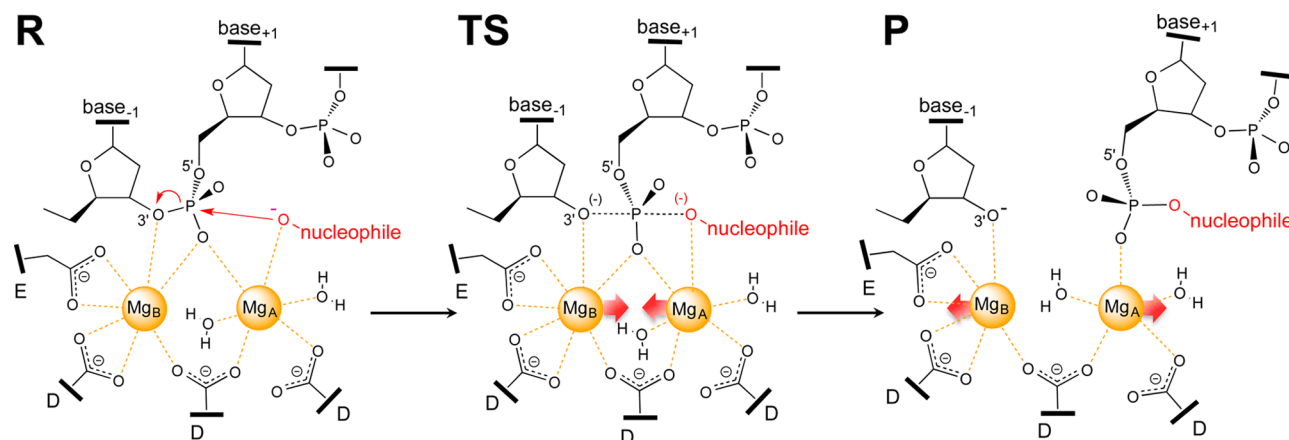
[a]Red arrows indicate the cooperative motion of the two metal ions, which from the reactants' conformation (**R**) get closer to stabilize the transition state (**TS**) and move apart to facilitate products' release (**P**).

stability, induce conformational rearrangements, and confer proper functionality among protein families.[2,3] Experimental studies in the early 1990s suggested that one, two, or even three Mg^{2+} ions could be needed for RNA enzyme activity. In 1993,[1] using a few X-ray structures sharing a similar metal-aided catalytic site architecture,[1,4] Steitz and Steitz described a general mechanism for RNA hydrolysis, suggesting that enzymatic cleavage of the phosphodiester bond is aided by a highly structured metal-containing catalytic site where two divalent metal ions are spaced ~3.9 Å apart. They correctly described several mechanistic aspects, proposing that one metal ion favors the formation of the attacking hydroxide ion, while the other helps the exit of the leaving group. Together, both metals stabilize the enzymatic TS (Scheme 1).

This general 2M mechanism has since been validated and clarified by several X-ray structures of different nucleic acid-processing metalloenzymes. Informative structural comparisons have been made among several metallonuclease structures, either wild-type or mutated, containing the endogenous substrate with or without an inhibitor or ligand bound to the catalytic site.[5,6] For example, the crystallized structures of ribonuclease H (RNase H) enzyme[7−9] and several topoisomerases[10,11] show a common metal-binding site, despite differences in the protein tertiary structure and cellular function.[5,6,12−15] Frequently, two divalent cations are ~4 Å apart, jointly coordinated to the backbone of RNA or DNA strand substrates, catalytic acidic residues, and nearby water molecules. The acidic structural motif responsible for chelating metals in the enzymatic pocket is a specific and conserved sequence, called the "DEDD motif", comprising four carboxylate moieties coordinated to one or both of the Mg^{2+} ions in the active site (Scheme 1). Together with its parent DDE motif, this highly conserved sequence characterizes a large class of nucleases, ensuring their specific metal-aided function.[5] Indeed, the 2M binding site is a recurrent structural feature in crystals of nucleic-acid-processing enzymes, suggesting the 2M mechanism as a fingerprint for all DNA and RNA polymerases, together with self-splicing ribozymes.[4,6,15−18]

■ TWO-METAL-ION CATALYSIS: IMPORTANCE OF MULTISCALE MOLECULAR SIMULATIONS

The 2M mechanism for hydrolyzing the phosphodiester bond of a DNA or RNA strand generally involves three main

chemical steps: (i) nucleophilic attack on the scissile phosphodiester, (ii) formation of a transition state showing a penta-coordinated phosphate along the phosphoryl transfer, and (iii) cleavage of the scissile bond with subsequent exit of the leaving group (Scheme 1). The efficiency and specificity of the enzymatic function depend on the energetics of the nucleophilic hydroxide ion formation, the mechanism type (concerted one-step or stepwise, with the possible formation of a stable phosphorane intermediate), the dynamic pathway and energetics related to the proton transfer events occurring during the enzymatic phosphoryl transfer, and finally the exact mechanism for TS stabilization and leaving group release. Supported by high-resolution structural data, multiscale molecular simulations are critical for understanding the role of the catalytic site architecture in modulating all these steps.

Additional mechanistic aspects that can be clarified via molecular simulations are those related to the fact that to stabilize an otherwise reactive enzyme−substrate complex, crystal structures are often obtained in conditions that differ from the catalytically native state. Catalytically relevant residues, such as those of the DDE motif, may be mutated, decreasing or fully inhibiting catalytic activity. This strategy was used, for instance, to resolve the structures of RNase H in complex with the actual substrate.[7−9] Alternatively, the native Mg^{2+} ions can be substituted using soaking strategies to obtain crystals with other divalent ions, such as Mn^{2+}, Ca^{2+}, or Zn^{2+}.[7,10] Interestingly, less stringent requirements for coordinating Mn^{2+}, compared with other divalent cations, produced Mn^{2+}-containing crystals of Mg^{2+}-dependent nucleases, which preserve the canonical 2M architecture of the native state, usually retaining low activity. Ca^{2+}, however, owing to its size, is expected to affect much more the metal−ligand interactions and overall site coordination, usually leading to complete enzyme inhibition.[17] Interestingly, Zn^{2+}, which can have both octahedral and tetrahedral coordination shells, could also affect the overall architecture of metal-centered binding sites.[3] Other non-native conditions used to facilitate crystal soaking in nucleic-acid-processing enzymes include the use of an unnatural 3′-bridging phosphorothiolate that caps a DNA strand or nucleophile residue bound to a nicked DNA, as in the recent crystal of topoisomerase II.[10] Consequently, the use of one or more of these conditions is occasionally reflected in non-canonical 2M catalytic site architectures.[4,10,19−21] This raises
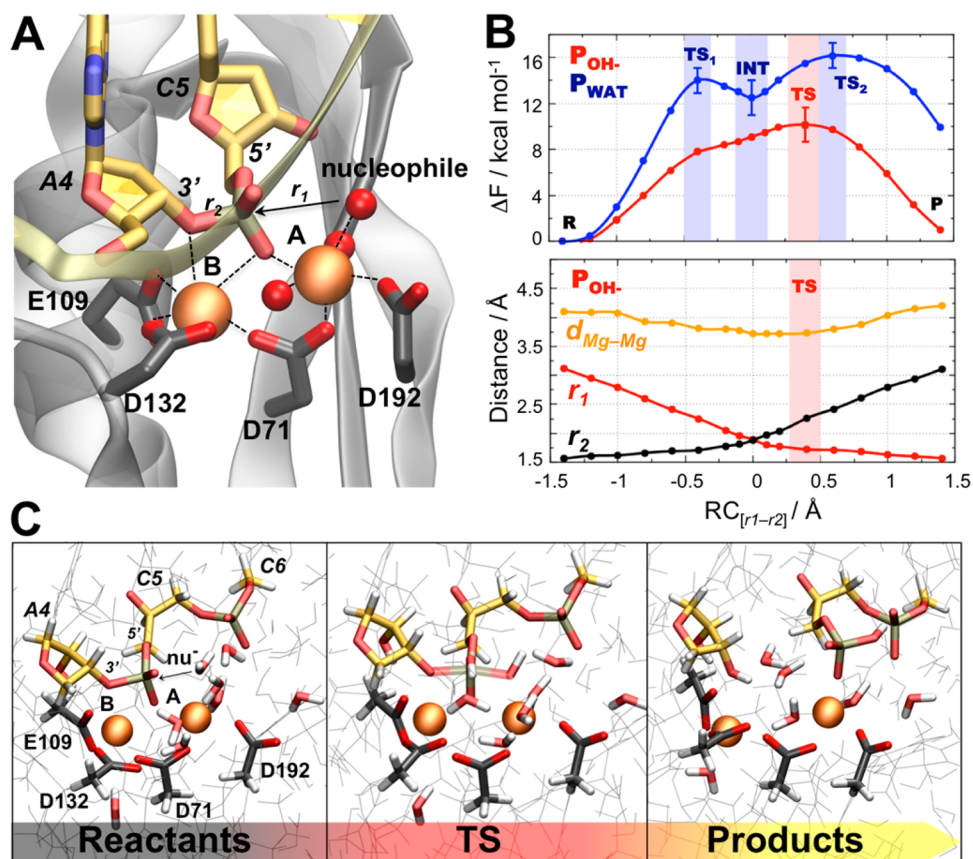
Figure 1. (A) Catalytic site of the RNase H in complex with a RNA/DNA hybrid,[7] showing a typical 2M architecture. (B) Energetic (upper graph) and structural (lower graph) properties for the RNA phosphodiester cleavage catalyzed by RNase H via the 2M mechanism, as from Car−Parrinello QM/MM MD.[35] The free energy profile, obtained through thermodynamic integration, for two investigated pathways (P_{WAT} or one hydroxide ion, P_{OH^-}) are shown. The reaction coordinate (RC) used is the difference between the forming (r_1) and breaking (r_2) bonds. Transition state (TS) and the metastable intermediate (INT) are evidenced. Average bond distances [r_1, r_2, and the metal−metal distance (d_{Mg-Mg})], along the lower free energy path (P_{OH^-}) are reported in the lower graph. (C) Representative snapshots from the QM/MM MD, showing the reactants, TS, and product states of RNase H catalysis. QM atoms are shown explicitly; MM atoms are in thin lines.

questions about how 2M architecture is affected by the experimental conditions used to obtain crystals, which inevitably differ from the actual native state of the metalloenzyme binding site.

Computational methods have the ability to address these issues by recreating, *in silico,* native enzymatic conditions. Examples reported in this Account, as numerous others in the recent literature, show how multiscale simulations can shed additional light on metal-containing enzymes. First-principles molecular dynamics (MD) within a hybrid quantum mechanics/molecular mechanics (QM/MM)[22,23] implementation can help to decipher key mechanistic details of the 2M enzymatic mechanism in the $\sim10^2-10^3$ ps time scale.[24,25] Furthermore, classical MD simulations often combined with enhanced sampling techniques, such as metadynamics,[26] are critical to sample longer time scales (i.e., $\sim10^2-10^3$ ns) and understand the effect of metal stoichiometry and dynamics during 2M catalysis.[27−29]

■ CONCERTED METAL MOTIONS DURING CATALYSIS IN RIBONUCLEASE H

Ribonuclease H (RNase H) belongs to the nucleotidyltransferase (NT) superfamily and, in the presence of two Mg^{2+} or Mn^{2+} ions, hydrolyzes the P−O3′ bond of the RNA strand, degrading RNA/DNA hybrids.[7,8,14,17] RNase H activity

in HIV reverse transcriptase (HIV-RT) is a promising target for anti-HIV drug design.[30] Yang and co-workers disclosed several high-resolution crystal structures of the homologous RNase H from *Bacillus halodurans*, revealing a bimetal architecture of the catalytic site where two divalent ions are jointly coordinated to a nonbridging oxygen of the scissile phosphodiester of the substrate RNA strand (Figure 1) and by four carboxylates (D71, E109, D132, and D192).[7,8,14,17]

We investigated the proposed mechanistic hypothesis for RNase H catalysis (Scheme 1) using Car−Parrinello QM/MM MD simulations[23] and identified possible catalytic pathways for metal-aided RNA hydrolysis. The calculated free energy ($\sim$16 kcal/mol) for the enzymatic reaction is below the experimental upper limit ($\sim$20 kcal/mol) for the catalytic rate, validating our reaction pathways. Simulations also revealed that nearby solvation water molecules play a key role in 2M catalyzed phosphoryl-transfers, providing a structured H-bond network to facilitate proton shuttle events during catalysis. These events, crucial for efficient nucleophile formation and leaving group stabilization and concomitant with the enzymatic phosphoryl transfer, could not be predicted by X-ray structures alone. This was first shown through QM/MM MD of the phosphatase activity of soluble epoxide hydrolase (sEH), where water molecules solvating the metal center could indeed facilitate the migration of protons involved in phosphoryl transfers.[31,32]
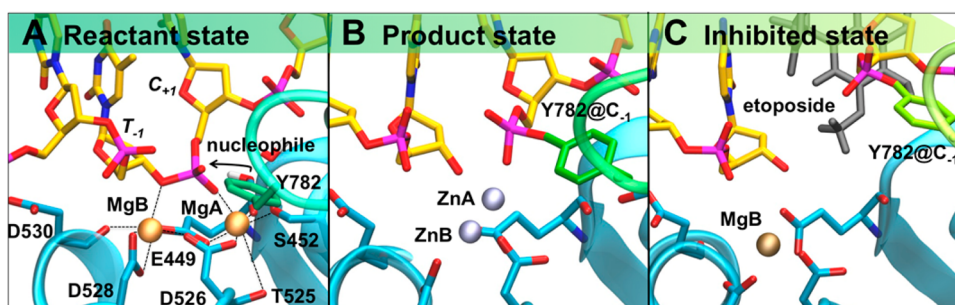
Figure 2. (A) Canonical-like 2M mechanism of the reactant state of type II topoisomerase (topoII) as emerged from multi-picosecond long QM/MM MD combined with multi-nanosecond long classical MD simulations.[37] (B) X-ray structure of topoII in complex with two Zn^{2+} ions and covalently bound to a DNA double strand (cleavage complex),[10] showing a product-like, noncanonical 2M coordination. (C) TopoII in complex with the anticancer drug etoposide (black sticks).[37,38] Y782 is green, from darker to lighter, indicating its gradual shift from the reactants (A) to a product-like (B) and inhibited (C) states.

One crucial aspect of the enzymatic reaction in RNase H revealed by DFT-based QM/MM simulations was the cooperative motion of the two Mg^{2+} ions, which act in concert to promote and facilitate nucleophile formation and leaving group stabilization. We observed an associative phosphorane-like transition state (TS) aided by the two ions, which get closer together during multi-picosecond dynamics, from the reagent to the TS geometry. While X-ray structures of RNase H showed that the internuclear distance between the two Mg^{2+} ions can vary along the reaction, molecular simulations were able to connect those static structures, quantitatively determining the concerted metal-ion motion used to bring the reactant groups closer, stabilize them in the TS through electrostatic compensation of the negatively charged scissile phosphodiester, and then facilitate the exit of the leaving group (Figure 1B,C).

More recently, Elsasser and Fels[33] and Rosta et al.[34] have also studied the 2M catalysis in RNase H using QM/MM computations; while they proposed some different proton shuttle pathways for nucleophile formation, the key dynamic role of metal ions turned out to be a common signature for all computations. On average, the distance between the ions decreases along the reaction pathway, from ∼4.1−4.2 Å in the reactants to 3.8−3.9 Å in the TS and intermediate structures (Figure 1B,C), until the products' release leads to a longer Mg^{2+}−Mg^{2+} distance ($d_{Mg} > 4.7−5$ Å), which likely precedes the release of the cleaved substrate and metal ions from the catalytic pocket.[34,35] These results are in agreement with hypotheses based on Nowotny and Yang's crystallographic data.[7,8,14,17] The distance between the two Mg^{2+} ions in the TS and intermediate states is the only difference between computational and crystallographic data (∼3.8 Å in computations vs ∼3.5 Å in X-ray "intermediate" structure). However, to impair enzymatic activity and allow the crystallographic resolution of the complex, the X-ray "intermediate" structure of RNase H lacks the scissile phosphodiester group between the two Mg^{2+} ions. On the other hand, first-principles-based calculations implicitly accounted for the steric and electronic contributions of the scissile phosphodiester between the two metal ions along the reaction pathway, which explains the longer Mg^{2+}−Mg^{2+} distance computed at the TS (Figure 1).

METAL IONS ON THE MOVE IN TYPE II TOPOISOMERASE

Type II topoisomerase (topoII) metalloenzymes control DNA topology and are important targets for clinical antibiotics and anticancer drugs.[36] TopoII cleaves and relegates DNA strands using nucleophilic tyrosyl residues. A recent high-resolution X-ray structure of the yeast topoII shows a "cleavage complex" (topoII covalently bound to cleaved DNA), in the presence of two divalent Zn^{2+} ions and a covalent link DNA-tyrosine (Y782), with a unique noncanonical coordination of the two metals in the active site.[10] This was interpreted as if only one Mg^{2+} ion could assist the catalysis of topoII, while the other metal was further afield. This surprising experimental evidence suggested a novel and unified mechanism for topoII, where only one of the two metals is actively involved in the reaction mechanism for DNA cleavage (Figure 2A).[10]

Using first-principles based QM/MM simulations, we showed that after reconstructing the topoII−DNA Michaelis complex in a catalytically competent state (i.e., detaching the DNA substrate from the nucleophilic Y782, while reconstituting the native O3′−P bond, and replacing Zn^{2+} in the crystal with the catalytically native Mg^{2+}), the active site spontaneously relaxed into a stable canonical-like 2M architecture.[37] The two metal ions shifted concertedly by ∼3.3 Å, forming octahedral coordination shells with the surrounding amino acids and the scissile phosphodiester. This suggests that a 2M mechanism can be structurally reasonable for topoII too, with both metals actively involved in the catalytic reaction.

The position of the two metal ions in our models is therefore different in the reactants (canonical-like, Figure 2A) and products (noncanonical-like, Figure 2B). This implies a gradual shift of the two metals from the reactants to the products, which was not excluded by Schmidt et al.[10] These computational findings, integrated with structural data, suggest a dynamic rearrangement of the metal ions in an extended neighborhood of the catalytic pocket.[10,38] The two metal ions move cooperatively in our simulations, getting closer together in the TS geometry, facilitating the encounter of the reactants and the stabilization of the TS. This suggests a cooperative action of the two metals during catalysis in topoII, facilitating nucleophile formation and stabilizing both transition state and leaving group, in agreement with that described for other 2M phosphodiesterases, including RNase H catalysis.

These findings on topoII need of course further corroboration by additional structural data. However, they are in agreement with other simulations on the ribozyme. Karplus et al. reported a cooperative role and movements of the two ions within the catalytic pocket, with changes in the distance between the two Mg^{2+} ions.[39] Boero et al. also probed, using first-principles MD, how each atom contributes to the catalysis in a 2M enzymatic mechanism in the ribozyme.[40] Taken
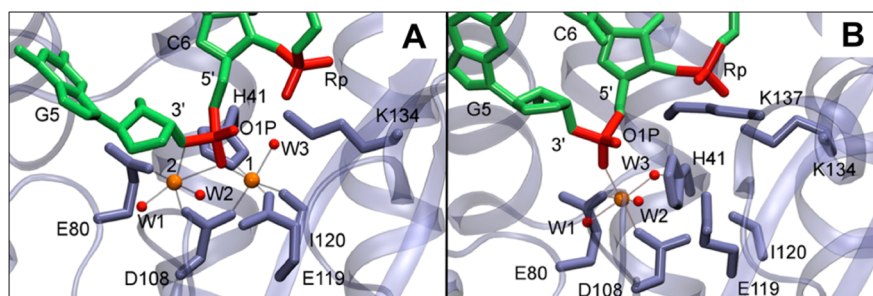
Figure 3. Active site of the PA$_N$−RNA complexes in the presence of two (A) and one (B) Mg^{2+} ions (orange spheres). The scissile phosphodiester and the adjacent 3′ phosphodiester are in red. Water molecules coordinating the metal ions are red spheres. Adapted from Xiao et al.[28]
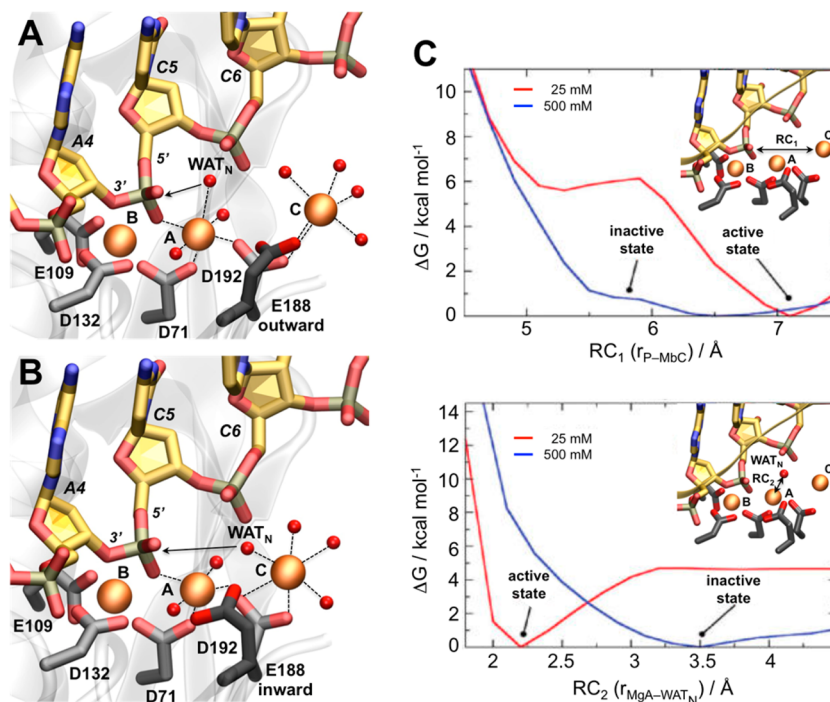
Figure 4. Active (A) and inactive (B) states of RNase H, at low (25 mM, A) and high (500 mM, B) concentrations of Mg^{2+}, respectively.[7,27] In both states, a third Mg^{2+} ion (MgC) persistently binds the second shell ligand E188.[27] In panel A, the carboxylate group of E188 points out of the active site and the nucleophilic water (WAT$_N$) binds to MgA. In panel B, WAT$_N$ coordinates MgC, while the carboxylate of E188 flips inward pointing to the phosphodiester group. (C) Free energy landscape of RNase H catalytic site, derived from the potential of mean force, using the adaptive biasing force (ABF) method, at low (red) and high (blue) concentrations of Mg^{2+}, as a function of different reaction coordinates (RC): the MgC−DNA phosphorus distance (RC$_1$, upper graph) and the WAT$_N$−MgA distance (RC$_2$, lower graph). The inactive conformation of RNase H (panel B) is more stable at higher concentrations of Mg^{2+}, leading to structures in which WAT$_N$ is far from the scissile phosphodiester.

together with structural data, these computational studies pave the way for further experimental validations of the functional dynamics of the catalytic metal ions during enzymatic phosphoryl transfer.

■ TWO-METAL-ION ENZYMATIC CATALYSIS IS MODULATED BY METAL CONCENTRATION

Metallonucleases are extremely sensitive to metal ion concentration. Slight changes may greatly affect the mechanism, efficiency, and selectivity of metal-aided catalysis.[5] Computational studies are key to understanding the molecular determinants behind this delicate metal-dependent regulation. Here we address this topic by discussing new results for influenza polymerase[41] (PA) and RNase H.[7−9] Influenza polymerase is involved in transcribing and replicating viral RNA, and thus it is an attractive target for antiviral drugs.[41] The crystallographic structures of the *holo* form of the PA N-

terminal domain (PA$_N$) of influenza polymerase, which is responsible for endonuclease activity on the viral RNA,[41] reported only one Mg^{2+} in the catalytic pocket, with a second metal-binding site either empty[42] or containing a Mn^{2+} ion.[43,44] Accordingly, influenza polymerase was suggested to use the canonical 2M mechanism.[45]

To clarify the number and nature of the metal ions involved in endonuclease activity in influenza polymerase, Xiao et al.[28] recently used classical force field-based simulations to model the PA$_N$ endonuclease in the presence of either one or two Mg^{2+} ions and with or without the mRNA substrate bound.[28,45] Overall, detailed analysis of multiple simulations, spanning hundreds of nanoseconds, indicated that binding of the second metal ion in the catalytic site depends on the presence of the RNA substrate. Importantly, the structure of the Michaelis complex with two Mg^{2+} ions (Figure 3A) is compatible with the 2M catalysis proposed for other endonucleases.[5] However, the
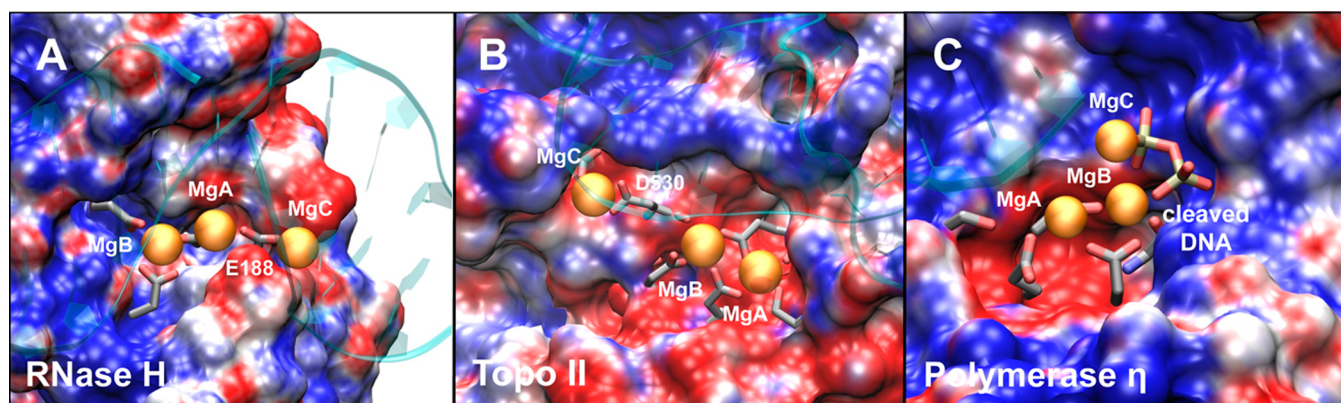
Figure 5. Metal ion active site in RNase H (A),[7] type II topoisomerase (topoII, B),[10] and polymerase η (C).[52] The electrostatic properties of the enzymatic active sites are shown. The electrostatic potential (ESP) was calculated and mapped on the protein solvent-accessible surfaces [red = negative ($-10\ kT/e$); blue = positive ($+10\ kT/e$)]. Mg^{2+} are represented as orange spheres. Panel C reports the product state of polymerase η[52] in the presence of a cleaved DNA fragment and a third Mg^{2+} ion (MgC).

significant structural flexibility sampled in MD simulations of the metal site suggests that an alternative, catalytically competent one-metal state (Figure 3B) may be present. This provided an explanation to the unexpected experimental observation of a decreased, yet significant, enzymatic efficiency at low Mg^{2+} concentration.[45] Altogether, these computations further emphasize the importance of metal stoichiometry and motion for enzymatic efficiency in 2M catalysis.

We also computationally investigated the effect of metal concentration on RNase H activity, which has been experimentally shown to change in the presence of Mg^{2+}, Mn^{2+}, and Ca^{2+} at different metal ion concentrations. RNase H has optimal activity at Mg^{2+} concentration of 10−20 mM but is inhibited at 50 mM ("attenuation effect").[7,14,46] Moreover, while Mg^{2+} and Mn^{2+} can promote the enzymatic function when in the correct concentration, Ca^{2+} inhibits RNase H activity.[17] Interestingly, Rosta et al. recently used QM/MM based free energy calculations to investigate the effect of Ca^{2+} on RNase H activity, and they indicated differences in metal−ligand charge transfers (Ca^{2+} vs Mg^{2+}) as a key factor for inhibition.[47] A previous computational study by Mordasini et al. also suggested that a large increase in the calculated energy barrier for doubly Ca^{2+}-substituted *Bam*HI endonuclease could be directly related to differences in the metal coordination geometries.[48] Recently, Lim and co-workers comprehensively studied the role of alkaline-earth-metal cations (Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}) binding to *E. coli* RNase H.[49] They integrated experimental and theoretical approaches to show that Ca^{2+} binds more tightly than Mg^{2+} to the enzyme active site and establishes strong bidentate interactions with a second-shell aspartate (D70) ligand prohibiting the direct access of water for nucleophilic attack. In contrast, Mg^{2+} activates the water attack by freeing the second-shell ligand from metal interactions. This can also explain why Ca^{2+} typically cannot catalyze the enzymatic phosphoryl transfer in RNase H.[17] Taken together, these computational studies clarify how metal−ligand charge transfers and steric and second-shell ligand effects are crucial for tuning 2M catalysis.

Effect of metal stoichiometry and concentration on RNase H activity was investigated using classical MD simulations coupled to free energy calculations (Figure 4). Our nanosecond MD simulations showed that a third Mg^{2+} ion persistently binds the second shell ligand E188.[27] Importantly, at 10−20 mM concentration of Mg^{2+}, the third metal ion does not perturb

RNase H catalytic activity (Figure 4A). Instead, at higher Mg^{2+} concentration ($\sim$50 mM and above), the third metal ion locates closer to the catalytic site, displacing the nucleophilic water from its optimal position for catalysis (Figure 4B) and hindering catalysis.[27] In addition, enhanced sampling simulations were used to characterize active and inactive conformations, returning the energetics of the transition. We found that the inactive conformation of RNase H is more stable at high concentrations of Mg^{2+}, leading to structures where the nucleophilic water is far from the scissile phosphodiester. In fact, the free energy cost to bring the nucleophilic water closer to the reactive state increases with Mg^{2+} concentration, explaining the inhibitory effect of high Mg^{2+} concentrations on RNase H activity.[27] Moreover, the E188A mutant shows no ability to engage additional Mg^{2+} ions in the vicinity of the catalytic site, supporting the MD based proposal of the crucial role of E188 for recruiting and properly localizing the third Mg^{2+}.[7] Overall, these computational insights provide a rational interpretation of the decreased catalytic activity of RNase H at high Mg^{2+} concentrations[7,14] and indicate the presence of an acidic residue in the second-shell ligand layer (a common feature in many endonucleases) as a potentially important structural motif for modulating catalytic activity.[27]

THE EMERGING ROLE OF ADDITIONAL METALS IN REGULATING TWO-METAL-ION CATALYSIS

As reported for RNase H (Figures 4 and 5A), the presence of a third metal ion located in the vicinity of the catalytic site has also been observed for the topoII−DNA complex, in which a conserved D530 residue could recruit metals to populate the topoII active site upon catalytic turnover.[10] Classical and hybrid QM/MM MD simulations have shown that D530 stably coordinates a third Mg^{2+} ion that freely accesses the active site pocket, thanks to a negatively charged connecting channel from the bulk (Figure 5B). This suggests that D530 could act as a second-shell acidic ligand, modulating the cation uptake and release at the active site upon DNA cleavage complex formation, similar to what was found for E188 in RNase H.[27]

Interestingly, other metalloenzymes have been reported to possess more than two metal binding sites. Computations of endonuclease IV,[50] an endonuclease involved in DNA repair, analyzed how three Zn^{2+} ions promote catalysis, finding that structural rearrangements in this multinuclear enzyme's active sites are essential for reaction. Additionally, the restriction

enzyme *Eco*RV presents three metal binding pockets, while only two metal ions are proposed to modulate catalysis.[20] Whether these functionally related enzymes work with two or three metal ions is still uncertain. Despite the difference in the number of observed ions in the X-ray structures, these observations suggest that a third metal ion may have a common role in promoting 2M catalysis.

The important question of the possible role of a third, solvent-exposed ion in 2M catalysis remains however puzzling, calling for more structural data to foster novel mechanistic hypotheses. One notable example is the recent structure of polymerase η, which has revealed a transient third Mg^{2+} ion at the active pocket, which might facilitate product release during phosphoryl transfer reaction (Figure 5C).[51−53] Another significant case is the recent structure of the homing endonuclease I-DmoI, where three metal ions are shown to catalyze a 2M cleavage mechanism for DNA hydrolysis.[54]

■ SUMMARY AND PERSPECTIVES

We have shown how molecular modeling and simulations covering broad temporal and spatial scales have provided new insights into the finest mechanistic details of 2M catalysis, highlighting the cooperative dynamics of the metals during RNA or DNA processing. Simulations demonstrate that indeed the two metals move in concert from their starting position, getting closer at the transition state, and moving apart when reaching the products. Simulations also indicate that correct metal stoichiometry for 2M catalysis could be greatly affected by metal ion concentration, suggesting a rational explanation for the attenuation effect observed in metal-dependent enzymes. Interestingly, computational studies have suggested the involvement of a third transient metal ion in 2M catalysis,[27] as discussed for RNase H, topoII, and PA$_N$, and recently observed experimentally in polymerase η and endonuclease I-DmoI.[51,54] The role of additional ions may be a fundamentally new and native aspect of 2M catalysis, and multiscale simulations can provide effective means to envision and dissect new mechanistic hypotheses that involve a larger and more flexible metal-centered structural architecture.[27,28,37] While this Account summarizes recent work on the 2M mechanism, we trust it will also encourage future research on this topic. Fruitful integration of experimental and theoretical techniques, such as time-resolved X-ray crystallography coupled with multiscale molecular modeling as recently reported for endonuclease I-DmoI,[54] could more broadly validate metal dynamics in binuclear metallonuclease catalysis. This in turn is poised to have important implications for the rational design of potent metal-chelating inhibitors and the de novo design of artificial enzymes with a metallo-aided function.[55,56]

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