Dynamic coordination of two-metal-ions orchestrates λ-exonuclease catalysis

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Metal ions at the active site of an enzyme act as cofactors, and their dynamic fluctuations can potentially influence enzyme activity. Here, we use λ-exonuclease as a model enzyme with two Mg2+ binding sites and probe activity at various concentrations of magnesium by single-molecule-FRET. We find that while MgA2+ and MgB2+ have similar binding constants, the dissociation rate of MgA2+ is two order of magnitude lower than that of MgB2+ due to a kinetic-barrier-difference. At physiological Mg2+ concentration, the MgB2+ ion near the 5’-terminal side of the scissile phosphate dissociates each-round of degradation, facilitating a series of DNA cleavages via fast product-release concomitant with enzyme-translocation. At a low magnesium concentration, occasional dissociation and slow re-coordination of MgA2+ result in pauses during processive degradation. Our study highlights the importance of metal-ion-coordination dynamics in correlation with the enzymatic reaction-steps, and offers insights into the origin of dynamic heterogeneity in enzymatic catalysis.

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**RESULTS**

**Single molecule fluorescence assay for λ-exonuclease.** λ-exonuclease, which forms a homotrimeric ring structure wrapped around the linear DNA duplex, degrades one strand of the DNA in the 5′-to-3′ direction, producing a 3′ single-stranded (ss) overhang tail (Fig. 1). The resulting product serves as a DNA intermediate, essential for homologous recombination in the bacteriophage λ system. λ-exonuclease translocation along DNA is driven by the chemical free energy released by the hydrolysis of phosphodiester bonds and is a highly processive enzyme (3,000 nucleotides (nt) per attempt). Mg²⁺, an essential cofactor for the nucleolytic activity of this enzyme, establishes catalytic coordination at the active site. The core–crystal structure of λ-exonuclease revealed that the enzyme utilizes two Mg²⁺ ions separated by 4.0 Å, both of which prefer an octahedral geometry for catalysis, typical of a classical two-metal mechanism.

To monitor the λ-exonuclease activity in real time at the single-molecule level, we designed a blunt-ended double stranded (ds) DNA substrate with a phosphate group on the 5′ end of the hydrolyzed strand (termed the 5′ strand) and a hydroxyl group on the 3′ end of the non-hydrolyzed strand (termed the 3′ strand). The FRET donor (Cy3) and acceptor (Cy5) were covalently attached to the 23rd and 43rd nucleotides of the duplex, respectively (Fig. 1b). The DNA substrate was immobilized on a polymer-coated quartz surface via biotin-streptavidin interactions (Fig. 1b). This experimental setup, used in our previous study, allowed us to monitor the processive phase of degradation in real time.

When a reaction buffer containing Mg²⁺ and λ-exonuclease was applied to DNA molecules immobilized on the surface via a flow delivery system, degradation started from the 5′ strand, with degradation-directed translocation of λ-exonuclease along the 3′ strand (Fig. 1b). The enzymatic degradation converts the rigid dsDNA substrate into flexible ssDNA, which on average shortens the distance between the two fluorophores, producing an increase in FRET (Fig. 1c, d).

As expected, upon adding Mg²⁺, we observed a gradual increase in the FRET signal (blue trace), caused by a decrease in donor intensity and an increase in acceptor intensity (green and red traces in Fig. 1c). The major peak of the FRET efficiency histogram shifted from 0.26 to 0.53 upon cleavage (Fig. 1d). Such FRET changes were not observed in the absence of Mg²⁺ (Fig. 1d, middle panel) and thus can be attributed to λ-exonuclease activity-elicited degradation of DNA. The monotonic increases in FRET without pauses indicate that λ-exonuclease is processive at physiologically relevant Mg²⁺ concentrations (>3 mM). We characterized the degradation time of the 20-nt-DNA strand between the two FRET dyes by measuring the time period over which FRET increased from the minimum to the maximum values (vertical dotted lines in Fig. 1c). The degradation rates were defined by the number of nucleotides (20 nt) divided by the degradation time.

**Mg²⁺-dependent degradation of DNA by λ-exonuclease.** During the reaction, stable metal-ion coordination is prerequisite for sustaining the persistent enzymatic activity, enabling the enzyme to translocate via continuous degradation of DNA. Thus, the dissociation of metal ions from the active site would change the enzymatic activity. To corroborate whether Mg²⁺ remains at the active site during the reaction, we examined single-molecule trajectories obtained from various Mg²⁺ concentrations and measured the degradation period over which FRET increases from the minimum to the maximum values (red lines in Fig. 2a). Surprisingly, the pattern of the FRET time trajectory (Fig. 2a) and the mean rate of degradation (Fig. 2b and Supplementary Fig. 1) were sensitive to the Mg²⁺ ion concentration. The degradation occurred more slowly at lower concentrations of Mg²⁺, and the normalized fraction of degradation along the time trajectory showed a clear decrease in the degradation rate at lower Mg²⁺ concentrations (Fig. 2c and Supplementary Fig. 2). However, the slower degradation at low Mg²⁺ concentrations was not due to the loss of cleavage activity of exonuclease because almost all DNA substrates were cleaved by the end of the reaction (Supplementary Fig. 3). The saturated enzymatic rate measured in this study (17 nt⁻¹ at 9 mM MgCl₂) was consistent with those from bulk biochemical studies (10–12 nt⁻¹) and other single-molecule studies (13–18 nt⁻¹). Remarkably, we found a significant reduction in the degradation rate with 15 mM MgCl₂ (Fig. 2b, red circle).

Interestingly, clear patterns of pauses displaying constant FRET values over an extended period of time were predominantly observed at low Mg²⁺ concentrations (e.g., blue windows in Fig. 2a). Detailed analysis reveals that the pause population and its dwell time decreased dramatically with increasing Mg²⁺ concentration (Fig. 2d, e). When the enzyme degrades the FRET-reporting region between two fluorophores, the enzyme exclusively...
**Fig. 1** Real-time measurements of processive degradation by λ-exonuclease. **a** The crystal structure of λ-exonuclease (PDB entry 1AVQ). **b** Experimental layout, depicting the DNA, protein binding to DNA, and processive degradation. **c** Schematic showing how the degradation time is measured using FRET signal. **d** Single-molecule FRET histogram obtained as in **b** (top: dsDNA only; middle: before the degradation in the absence of Mg$^{2+}$; and bottom: after the degradation in the presence of Mg$^{2+}$).

**Fig. 2** Mg$^{2+}$-dependent degradation of DNA by λ-exonuclease. **a** Representative traces showing that degradation reaction slows down at lower Mg$^{2+}$ concentrations (0 mM, 0.03 mM, 0.08 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.5 mM, 1 mM, 3 mM, 6 mM, and 9 mM Mg$^{2+}$: top to bottom) at the fixed 16 nM λ-exonuclease (trimer concentration unless otherwise stated). **b** The degradation rate (velocity) versus Mg$^{2+}$ concentrations following a Hill fit (red line) with a maximum velocity of 17.2 nt s$^{-1}$, a Km value of 0.885 mM of Mg$^{2+}$, and $n = 1.6$. Inset, blowing up at lower Mg$^{2+}$ concentrations showing fitting to a sigmoidal kinetics (red) versus a Michaelis Menten kinetics (blue). Error bars denote the standard error of the mean (SEM). The velocity at [Mg$^{2+}$] = 15 mM is highlighted in red. **c** Time-dependent fractional growth at various Mg$^{2+}$ concentrations. **d** The proportion of the pause population as a function of the Mg$^{2+}$ concentration. **e** Distribution of pause times with varying concentrations of Mg$^{2+}$. 

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**Appendix:**

| Time (s) | [Mg$^{2+}$] (mM) | Normalized fraction | Paused time (s) |
|----------|-----------------|---------------------|-----------------|
| 0        | 0.00            | 0.00                | 0.00            |
| 0        | 0.03            | 0.02                | 0.00            |
| 0        | 0.08            | 0.01                | 0.00            |
| 0        | 0.10            | 0.00                | 0.00            |
| 0        | 0.20            | 0.00                | 0.00            |
| 0        | 0.30            | 0.00                | 0.00            |
| 0        | 0.50            | 0.00                | 0.00            |
| 0        | 1.00            | 0.00                | 0.00            |
| 0        | 3.00            | 0.00                | 0.00            |
| 0        | 6.00            | 0.00                | 0.00            |
| 0        | 9.00            | 0.00                | 0.00            |

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**Fig. 3** Real-time measurements of processive degradation by λ-exonuclease. **a** The crystal structure of λ-exonuclease (PDB entry 1AVQ). **b** Experimental layout, depicting the DNA, protein binding to DNA, and processive degradation. **c** Schematic showing how the degradation time is measured using FRET signal. **d** Single-molecule FRET histogram obtained as in **b** (top: dsDNA only; middle: before the degradation in the absence of Mg$^{2+}$; and bottom: after the degradation in the presence of Mg$^{2+}$).
performs processive degradation because the enzyme is topologically engaged by the previously produced 3′ non-hydrolyzed 23-nt-long DNA strand, which is longer than the footprint of the enzyme. This molecular coupling enables ruling out the possibility that the pauses are the consequence of protein dissociation. The velocity versus protein concentration (Supplementary Fig. 4) reveals a protein-concentration independent tendency, suggesting that pauses indeed are not due to protein dissociation. The pauses are most plausibly caused by the dissociation of the Mg$^{2+}$ cofactor from the active site, as conjectured for other two-metal-ion systems. If Mg$^{2+}$ ions always remain intact at the catalytic site of the enzyme, then the pause dynamics of the enzyme should not change with Mg$^{2+}$ concentration.

**Inhibition of λ-exonuclease activity by Ca$^{2+}$.** To verify the foregoing hypothesis that the Mg$^{2+}$ ion dissociates during the reaction, we performed a kinetic poisoning experiment by introducing a catalytically inactive ion, Ca$^{2+}$, to the saturated Mg$^{2+}$ concentration of ~3 mM (Fig. 3a and Supplementary Figs. 5-7). If Mg$^{2+}$ ever dissociates from the exonuclease, Ca$^{2+}$ can replace it, causing the enzymatic reaction to pause because of competitive binding of Ca$^{2+}$ and Mg$^{2+}$ to the active site. In the presence of Ca$^{2+}$ ions, a significant number of traces demonstrate pauses (Fig. 3b, c) and backtracking (Fig. 3d, e). The analysis of single-molecule FRET time trajectories in the presence of Ca$^{2+}$ revealed three types of degradation patterns (processive, pause, and backtracking) (Fig. 3d, e), and the individual FRET time trajectories obtained from different ratios of Ca$^{2+}$ to Mg$^{2+}$ were in quantitative agreement with our conjecture. The increased Ca$^{2+}$/Mg$^{2+}$ ratio reduced the number of processive traces but increased the number of paused and backtracked traces (Fig. 3e).

Structural clues for the two-metal-ion dynamics. The active sites of the two crystal structures of exonuclease, which were co-crystallized with the DNA substrate and Mg$^{2+}$ or Ca$^{2+}$, offer molecular insight into the metal-ion coordination with the enzyme. The trimeric enzyme has three active sites and retains a PD-(D/E)XK metal-binding motif at the core of its active site consisting of two conserved acidic residues (D/E) and one positive residue (K), conserved in type II endonucleases (Supplementary Fig. 8a, zoomed-in view). The two metal ions (Mg$^{2+}$ and Mg$^{2+}^{+}$) are bridged by D119 and the scissile phosphate of DNA (red circle, left in Supplementary Fig. 8a) in octahedral geometry. One of the two water molecules, chelated to Mg$^{2+}$, nucleophilically attacks the scissile phosphate (Supplementary Fig. 8a, zoomed-in view) upon activation by K131. In contrast, Mg$^{2+}$ stabilizes the trigonal bi-pyramidal geometry, resulting in the formation of the transition state through its coordination with three water molecules, two oxygen atoms of the scissile phosphate, and D119. Among the three subunits of the trimeric ring, only the DNA-bound subunit (green, left in Supplementary Fig. 8a) possesses the two metal ions (blue balls). This result suggests that...
a DNA substrate is required to both stably bind Mg$^{2+}$ and enable the catalytically competent coordination of two Mg$^{2+}$ ions (Supplementary Fig. 8a, zoomed-in view). As observed in previous studies, bi-metal-ion catalysis becomes viable only in the presence of a substrate with which an enzymatically competent complex is formed. In contrast, in the Ca$^{2+}$-bound X-ray structure, Ca$^{2+}$ is stably coordinated even without a DNA substrate (see Ca$^{2+}$ in all the orange, blue, and green subunits: red circles around blue balls, left in Supplementary Fig. 8a). In general, the two-metal-ion catalysis of nucleic acid hydrolysis utilizes the two ions as follows: Mg$^{2+}$ binds to two oxygen atoms of the scissile phosphate$^{11}$. The coordination bonds of Mg$^{2+}$ is likely to remain intact, coordinated by three stationary residues (D119, E129 and L130) at the active site (Supplementary Fig. 8a).

The new terminal generated upon cleavage (small red dotted circle, zoomed-in view of Supplementary Fig. 8a) is translocated to the original terminal position (large green dotted circle), presumably through electrostatic attractions between the phosphate group and the positively charged pocket of the protein$^{11}$. Whereafter Mg$^{2+}$ rebinds to the original terminal position (large green dotted circle), zoomed-in view of Supplementary Fig. 8a) is translocated during each round of enzymatic turnover. In contrast, Mg$^{2+}$ is likely to dissociate from the protein as follows: Mg$^{2+}$ (mM)

\[ V([Mg^{2+}]) = \frac{k_{off}^{Mg^{2+}}}{[Mg^{2+}]} + K_B + K_B K_A [Mg^{2+}]^{-1} = \tau_1^{-1} \]

where \( K_B = k_{off}^{Mg/B} / k_{on}^{Mg/B}, K_A = k_{off}^{Mg/A} / k_{on}^{Mg/A}, \) and \( \tau_1 \) is the mean degradation time per nucleotide. The experimental data are well fitted to Eq. 1 over the entire range of [Mg$^{2+}$] (Fig. 4b, red line), yielding \( k_{off}^{Mg/A} = 18 \text{ s}^{-1}, K_B = 0.71 \text{ mM}, \) and \( K_A = 0.30 \text{ mM}, \) thus \( k_{off}^{Mg/A} = 25 \text{ s}^{-1} (\text{mM})^{-1} \) (Fig. 4b). At high [Mg$^{2+}$] (s$\text{K}_B$), Eq. 1 approximates to the standard Michaelis-Menten (MM) equation. Indeed, the experimental data were well fitted for high [Mg$^{2+}$] (e.g., [Mg$^{2+}$] > $K_B$ = 0.71 mM) but deviated significantly from MM kinetics at low [Mg$^{2+}$], ruling out the possibility of a super-stable Mg$^{2+}$ coordination. Without the two-ion dissociation, the deviation of V at low [Mg$^{2+}$] (<0.1 mM) from the MM kinetics cannot be explained. Alternatively, the Hill equation, \( V([Mg^{2+}]) = k_B^{Mg} [Mg^{2+}]^n / ([Mg^{2+}]^n + K_B^n) \) with \( n = 1.6 \) describes the data well (violet line in Fig. 4b). The Hill coefficient of \( n = 1.6, \)

**Fig. 4** Quantitative analysis of two-metal dynamics. **a** A kinetic model for the two-metal-ion dynamics consisting of three states: EMM (exonuclease$^{2+}$; Mg$^{2+}$ complex), EM (exonuclease$^{2+}$; Mg$^{2+}$ complex), and E (exonuclease only). In the model, Mg$^{2+}$ dissociates upon DNA cleavage and translocation (k$^{off}_B$, red arrow, EMM to EM) whereas Mg$^{2+}$ dissociates stochastically (k$^{off}_A$, EMM to E). The single cycle is completed upon re-binding of Mg$^{2+}$ (k$^{off}_A$, E to EM). **b** The degradation time per nucleotide (\( \tau_1 \)) versus Mg$^{2+}$ concentrations. The data are fitted to three models: (1) Michaelis Menten equation (orange line), (2) Hill equation (purple line, \( n = 1.6 \)), and (3) Eq. 1 for the model shown in **a** (red line). Inset shows velocity (\( = \tau_1^{-1} \)) versus Mg$^{2+}$ concentrations data and their fits (solid lines). Representative FRET time traces from simulations performed at three different choices of k$^{off}_A$: 0.01 s$^{-1}$ (top panel), 0.1 s$^{-1}$ (middle panel), and 1 s$^{-1}$ (bottom panel), [Mg$^{2+}$] is set to 0.03 mM. **d** Degradation rate histograms at four different [Mg$^{2+}$] conditions: 0.03 mM, 0.1 mM, 0.3 mM, and 3 mM. Gray bars represent experimental data, whereas solid lines are theoretical prediction calculated under three different choices of k$^{off}_A$: 0.01 s$^{-1}$ (black), 0.1 s$^{-1}$ (red), and 1 s$^{-1}$ (orange). **e** Mean squared error (MSE) versus k$^{off}_A$. The errors are calculated by summing the squares of the difference between the experimental and theoretically predicted results.
higher than 1, suggests that two ions bind semi-cooperatively; that is, Mg\textsubscript{\textalpha}\textsuperscript{2+} must be present in the active site for Mg\textsubscript{\textbeta}\textsuperscript{2+} to bind.

The minor difference between \( K_A \) and \( K_A' \) (\( K_{B0}/K_A = 2.4 \)), corresponding to the stability difference of \( <1 \kappa_B T \), is somewhat unexpected given that the coordination number of Mg\textsubscript{\textalpha}\textsuperscript{2+} to the surrounding residues is greater than that of Mg\textsubscript{\textbeta}\textsuperscript{2+} (i.e., 3 to 1 as in Supplementary Fig. 8a). However, very different kinetic constants can give the same thermodynamic stability. To estimate \( k_{off}^{A} \), which best explains the experimental time traces, we compare histograms of the degradation velocity (Fig. 4d) collected from experiments with those generated from the simulation at different \( k_{off}^{A} \). Representative FRET time traces from simulations are shown in Fig. 4c. We found that the simulation results obtained with \( k_{off}^{A} = 0.1 \text{ s}^{-1} \) best reproduce the trend in the experimental data (Fig. 4e and Supplementary Fig. 9). We also compared mean pause times and their histograms (Supplementary Fig. 10a, b). Again, the simulation results obtained with \( k_{off}^{A} = 0.1 \text{ s}^{-1} \) most closely match the experimental data trend (Supplementary Fig. 10c). Furthermore, simulated time traces at varying [Mg\textsubscript{2+}] with \( k_{off}^{A} = 0.1 \text{ s}^{-1} \) (Supplementary Fig. 11) show a gradual decrease in the pausing dwell time and an increase in the translocation velocity as a function of [Mg\textsubscript{2+}], which also closely resemble the experimental observations (Fig. 2b and Fig. 4b). Based on these results, we conclude \( k_{off}^{A} \approx 0.1 \text{ s}^{-1} \) and \( k_{off}^{B} \approx 0.34 \text{ s}^{-1} \) (mM\textsuperscript{-1}).

**Dynamic coupling between metal-ion stability and degradation.** A representative trajectory from the simulations at [Mg\textsubscript{2+}] = 0.03 mM shows a clear signature of pauses (black in the top panel in Fig. 5b) along with the number of cleaved nucleotides (blue in the middle panel) and the chemical states of exonuclease over time (green in the bottom panel). Comparing the three panels in Fig. 5b clarifies that the transitions from EMM to EM or between EM and E slow down the degradation process (Fig. 5b).

A careful inspection of the FRET time trajectories at varying [Mg\textsubscript{2+}] suggests that the distinct pause (pink window in Fig. 5a and top window in Fig. 5b) arises from the dissociation of both Mg\textsubscript{\textalpha}\textsuperscript{2+} and Mg\textsubscript{\textbeta}\textsuperscript{2+}, especially at low [Mg\textsubscript{2+}]. More than 95% of the observed paused states are caused by the trapping in the E state (Fig. 5c). In contrast, a slope change from steep to mild that corresponds to a decrease in translocation speed results from the binding and dissociation of Mg\textsubscript{\textbeta}\textsuperscript{2+} alone (compare the top and bottom windows in Fig. 5a, shaded in purple and green, respectively). More time trajectories based on the simulations and the analysis of different [Mg\textsubscript{2+}] are available in Supplementary Fig. 12. This simulation indicates that if Mg\textsubscript{\textbeta}\textsuperscript{2+} alone dissociates and rebinds during each round of catalysis, then long and frequent pauses would not arise; instead, degradation would be smooth. We examined a model based on simultaneous dissociation of both Mg\textsubscript{\textalpha}\textsuperscript{2+} and Mg\textsubscript{\textbeta}\textsuperscript{2+} as well, but found that it could not generate the marked pauses (Supplementary Fig. 13).

Whereas Mg\textsubscript{\textalpha}\textsuperscript{2+} and Mg\textsubscript{\textbeta}\textsuperscript{2+} bind to the active site with similar ion-coordination stability (i.e., they have similar binding constant, \( K_A = K_B \)), the Mg\textsubscript{\textbeta}\textsuperscript{2+} ion both binds and unbinds from the active site significantly more slowly because the Mg\textsubscript{\textalpha}\textsuperscript{2+} ion is coordinated by more residues than the Mg\textsubscript{\textbeta}\textsuperscript{2+} ion at the active site (Supplementary Fig. 8a). The difference between the kinetic barriers to the dissociation of Mg\textsubscript{\textbeta}\textsuperscript{2+} (\( \Delta G_{B}^{\text{1}} \)) and Mg\textsubscript{\textalpha}\textsuperscript{2+} (\( \Delta G_{A}^{\text{1}} \))
is estimated as $\Delta G_\text{A}^\text{f} - \Delta G_\text{B}^\text{f} \approx k_B T \log(k_\text{off}^\text{f} / k_\text{off}^\text{A}) \approx 5.2 k_B T$, indicating that the Mg\text{A}^{2+} ion dissociates ~200 times more slowly than Mg\text{B}^{2+}. Indeed, a comparison of the MD-simulated electrostatic interactions of Mg\text{A}^{2+} and Mg\text{B}^{2+} with their molecular environment reveals that Mg\text{A}^{2+} ions are more strongly held by the surrounding residues (Supplementary Fig. 14 and SI text), lending support to the molecular insight inferred from the crystal structure (Supplementary Fig. 8a).

A mechanistic insight into metal-ion dynamics in exonuclease activity was acquired based on single-molecule FRET time trajectories, Mg\text{B}^{2+}-bound high-resolution crystal structures, and a careful comparison of FRET data with the simulation results derived from the kinetic model. Mg\text{B}^{2+} dissociates during each round of catalysis due to the translocation, but Mg\text{A}^{2+} dissociates only occasionally. As implied by the inter-conversions of $\text{EM} \leftrightarrow \text{EMM}$ in the bottom panel of Fig. 5b (the green line), Mg\text{B}^{2+} dissociates whenever a transition is made from EMM to EM. Consequently, the rate of DNA degradation alters in a [Mg\text{A}^{2+}]-dependent manner. The occasional dissociation of Mg\text{A}^{2+} (transition from EM to E), albeit ~200-fold slower than the dissociation of Mg\text{B}^{2+} (transitions from EMM to EM), gives rise to a long pause.

As summarized in Fig. 6, metal-ion dynamics orchestrate enzymatic activity in the following manner: at low Mg\text{B}^{2+} concentrations, rebinding of Mg\text{B}^{2+} becomes rate-limiting, slowing down the overall degradation rate; at physiological Mg\text{B}^{2+} concentrations, the dissociation of Mg\text{B}^{2+} during each round of cleavage facilitates product release and the translocation, optimizing the enzymatic turnover rate for multiple rounds of hydrolysis; and Mg\text{B}^{2+} concentrations above 9 mM suppress the probability of vacancy and Mg\text{B}^{2+} dissociation (red circle in Fig. 2b), thus preventing product release and translocation for the next round of cleavage.

**Discussion**

The coordination of metal ions to the active site of $\lambda$-exonuclease displays distortion from the perfect octahedral geometry (Supplementary Fig. 8a), suggesting that they are in an energetically unfavorable state. If both metal ions ever dissociate, then it will take a longer time to re-assemble the functionally competent DNA–protein complex because the allostERIC coordination of the two ions inevitably incurs a higher kinetic penalty than that of a single ion. This explains why the two metal ions bind in a sequential manner, displaying cooperativity during the enzymatic reaction. Although the ion-coordination stabilities of Mg\text{A}^{2+} and Mg\text{B}^{2+} are comparable, there is substantial difference in their kinetic barriers associated with both binding and dissociation, as illustrated in Fig. 5d.

The difference of ~5.2 $k_B T$ between the kinetic barriers for dissociation of Mg\text{A}^{2+} and Mg\text{B}^{2+} (Fig. 5d), estimated from our theoretical model, indicates that Mg\text{A}^{2+} dissociates ~200 times more slowly than Mg\text{B}^{2+}. Thus, frequent Mg\text{B}^{2+} dissociation for fast product release while keeping Mg\text{A}^{2+} bound to prevent a large conformational change to an inactive state allows exonucleases to achieve high processivity at physiological [Mg\text{A}^{2+}] (~3–9 mM). Furthermore, the long pauses due to the dissociation of two metal ions may act as an additional regulatory mechanism at a low [Mg\text{B}^{2+}] (<0.3 mM). In other words, the dissociation of Mg\text{B}^{2+} is not random but occurs in a cleavage and translocation-dependent manner. Given that the translocation of the enzyme for the next cleavage is limited by the rate of product release (~50 ms), an excessively stable coordination of Mg\text{B}^{2+} would hinder product release, slowing down the degradation process, as evidenced by the slower translocation velocity at a high [Mg\text{B}^{2+}] (~15 mM) (Fig. 2b, red circle).

Biochemical and structural analyses 25,29,32–34 of RNase H found that after cleavage, the scissile phosphate could no longer simultaneously coordinate the two Mg\text{B}^{2+} ions, suggestive of cooperative binding right after the cleavage. The high-resolution crystal structures 16,32,37 and QM/MM MD simulations 11 of RNase H also suggest a cooperative effect of the two metal ions but this cooperation has never been kinetically demonstrated by time-resolved experiments on a relevant time scale. Our data clearly demonstrate the cooperative effect of two metal ions, as

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**Fig. 6** The mechanism of two-metal-ion dynamics. The active site of one subunit of the homotrimer is shown in green whereas two Mg\text{B}^{2+} ions and nucleotides are represented by yellow circles and rectangles in various colors, respectively. Metal-ion coordination to the catalytic active site is highly dynamic so that two Mg\text{B}^{2+} ions (Mg\text{A}^{2+} and Mg\text{B}^{2+}) can coordinate with and dissociate from the surrounding ligands. Mg\text{A}^{2+} remains stably bound to the active site, but Mg\text{B}^{2+}, which is close to the 5’ terminal side of the scissile phosphate, dissociates during every round of catalytic cleavage. As a result, the dissociation of Mg\text{B}^{2+} facilitates product release and exonuclease translocation, promoting the overall processivity of exonuclease activity. More specifically, at high [Mg\text{B}^{2+}] (≥2 mM), $\lambda$-exonuclease degrades processively and its cleavage activity is mainly controlled by Mg\text{A}^{2+} dynamics (green dashed square). The fast unbinding/rebinding dynamics of Mg\text{B}^{2+} and strict requirement of two Mg\text{B}^{2+} ions for the catalytic step yield a Mg\text{B}^{2+}-concentration-dependent exonuclease activity. Conversely at low [Mg\text{B}^{2+}] (≤0.3 mM), occasional unbinding of Mg\text{A}^{2+} from the catalytic site and slow rebinding of Mg\text{A}^{2+} stalls the exonuclease activity, giving rise to a long pause (red dashed square), thus elucidating the molecular origin of dynamic heterogeneity in exonuclease activity. The dynamic variation in the coordination states of two metal ions orchestrates the multistep process of exonuclease activity.
shown in the sigmoidal curves (Figs. 2b, 4b), which point to the allosteric conformational changes during the enzymatic activation. Furthermore, the long pause indirectly demonstrates their cooperativity and that both metal ions are definitely required to form a catalytically competent DNA–protein complex. Even though our kinetic fitting into the Hill function yields \( n = 1.6 \), suggesting that at most two metal ions participate in the function of exonuclease, there might be another transient intermediate in which a third metal ion shortly binds and leaves a site of enzymes, other than the two canonical metal-binding sites, as indicated by the recent structural25–28 and computational29–32 studies. It is widely believed that metal ion cofactors are stably bound to the catalytic sites of enzyme, and that their lifetimes are much longer than enzymatic cycles owing to the concept that the coordination with chelating ligands is thermodynamically stable. However, our study clarifies that the timing of metal-ion coordination and dissociation from the active site is one of the key factors determining the rates of the enzymatic cycle. Our finding provides experimental kinetic evidences on fluctuations of the metal-ion coordination during enzyme activity, and offers a detailed insight to the type II nuclease, opening to future dynamic studies on other two-metal-ion catalysis system. The single-molecule assay developed here enables deciphering the role of metal-ion dynamics in enzymatic cycles through the time trajectories of cleavage-coupled translocation along DNA. Based on all the data, we propose a full enzymatic cycle: (1) catalytically active DNA-protein complex formation (after initiation), (2) cleavage, (3) product and Mg\( ^{2+} \) release upon translocation with concomitant melting, (4) metal-ion rebinding, (5) then a return to step 1. Our study elucidates the full effect of Mg\( ^{2+} \) dynamics on processive activity of λ-exonuclease (Figs. 2 and 5); clarifies a molecular basis for metal-ion dynamics at the active site of the enzyme and provides mechanistic insight into the origin of the dynamic heterogeneity in enzymatic activity, which has been reported previously for λ-exonuclease23 as well as other proteins and nucleic acids41–50.

Methods

Protein expression and purification. The λ-exonuclease gene was amplified by PCR from genomic DNA of bacteriophage λ (D3654-SUN, Sigma Aldrich) by using primers designed for ligation-independent cloning25,51. The PCR product was treated with T4 DNA polymerase and cloned into the pBlu (flus)-tag-Maltose Binding Protein-TEV site vector. The cloned vector was confirmed by DNA sequencing. The vector was transformed into BL21- Star (DE3) E. coli (Thermo Fisher Scientific) and λ-exonuclease was expressed in 1 L of LB medium. Bacterial cultures were grown to an OD\(_{600} \) of 0.5, at which time IPTG was added at a final concentration of 0.3 mM. After shaking for 3.0 h at 37°C, bacteria were harvested by centrifugation at 5000 \( \times g \), re-suspended in 20 mL of buffer (50 mM Na-phosphate pH 8.0, 5 mM Tris, 300 mM NaCl, supplemented with EDTA-free protease inhibitor cocktail) and lysed by sonication. The cell lysate was clarified by centrifugation for 30 min at 35,000 \( \times g \). His-MBP-tagged λ-exonuclease was purified by nickel affinity chromatography (His-Trap FF, GE Healthcare). His-MBP-tag was removed by TEV protease, and native λ-exonuclease was collected in the flow-through by the second nickel affinity chromatography. The purified native protein was dialyzed and stored in a buffer (25 mM Tris-HCl (pH8.0), 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, and 50% glycerol).

DNA substrate preparation. DNA oligonucleotide strands used for single-molecule FRET experiments were purchased from Integrated DNA Technologies (IDT). The DNA substrate used to probe the processive phase was constructed by ligating two pieces of DNA at room temperature for 1 h using T4 ligase (P40202, New England Biolabs) and purified on a 15% PAGE gel. The sequences and modifications can be found in the supplementary information.

Single-molecule assays. DNA constructs were tied on a quartz surface coated with PEG (Laysan Bio) to minimize nonspecific surface adsorption of proteins29,32. We immobilized ~50 pM DNA molecules to the imaging chamber to achieve an appropriate density for single-molecule imaging. The reaction buffer contained 67 mM glucose-KOH (pH 9.4), various concentrations of Mg\( ^{2+} \), 20 μg/mL Blue-native, 1 mg mL\(^{-1} \) Trolox (Sigma-Aldrich) and an oxygen-scavenging system of 1 mg mL\(^{-1} \) glucose oxidase (Sigma- Aldrich), 0.04% mg mL\(^{-1} \) catalase (Sigma-Aldrich) and 0.4% (w/v) D-glucose (Sigma- Aldrich). Trolox was employed as a triplex-state quencher to avoid fluorescent photo-blinking, and glucose and glucose oxidase were used to remove oxygen, which triggers rapid photo-bleaching of fluorescent dyes39,52. The reaction began by injection of the reaction buffer containing λ-exonuclease into DNA molecules at room temperature.

Single-molecule data acquisition. FRET donor (Cy3) on the DNA was excited by a green laser (532 nm, 100 mW, Coherent Compass Laser). The fluorescence emission light was divided into donor and acceptor signals with a 635 nm dichroic mirror (Chroma) and was recorded by iXon Ultra 897 EMCCD camera (Andor). Both recorded fluorescence intensities of Cy3 and Cy5 were in an arbitrary unit (a.u.) since they were amplified by a gain factor. The data were saved in a video file format by a software written in Visual C++.

Fluorescence intensities of single molecules were extracted by IDL software and FRET efficiency was calculated as the ratio of intensities, Acceptor Intensity/(Donor Intensity + Acceptor Intensity) after amending cross-talk between the donor and acceptor channels. All data were analyzed with MATLAB codes and plotted in Origin software.

Protein-Mg\(^{2+} \) degradation simulation: mean velocity. The mean degradation time for a single nucleotide \( t_r \) of the model shown in Fig. 4a can be expressed as

\[ t_r = \frac{1}{k_{A}} + t_r + \frac{1}{k_{B}}. \]

The first term on the right is the mean transition time for EMM+EM. \( t_r \) is the average dwell time in either the EM or E state, and the final term denotes the mean transition time of EM→EMM. Competition between the two transitions EM→E and EM→EMM determines \( t_r \) which can be written as

\[ t_r = \frac{1}{k_{A}} \left( \frac{k_{eff}}{k_{off}^+} + \frac{1}{k_{off}^-} \right) + \frac{1}{k_{B}} \left( \frac{k_{off}^+}{k_{off}^-} \right)^2 + \frac{1}{k_{off}^-} \left( \frac{k_{off}^+}{k_{off}^-} \right)^3 + \ldots = \frac{k_{eff}}{k_{off}^-} \left( \frac{[Mg^{2+}]}{[Mg^{2+}] + K_{B}} \right) \]

The first term represents the escape of exonuclease to EMM from EM after only one excursion to the E state, whereas the second term describes two excursions to the E state before escaping to EMM, etc. Hence, we obtain

\[ t_r = \frac{1}{k_{A}} \left( \frac{k_{eff}^+}{k_{off}^+} + \frac{k_{eff}^-}{k_{off}^-} \right) + \frac{1}{k_{B}} \left( \frac{k_{eff}^+}{k_{off}^+} \right)^2 + \ldots \]

Finally, we obtain the expression of mean velocity \( V = 1/t_r \), which is used in the main text to fit the [Mg\(^{2+}\)]-dependent data for \( V \) (Fig. 4b).

Velocity histogram analysis and \( k_{eff}^- \) estimation. The velocity histograms show a large heterogeneity (Supplementary Fig. 9), which may originate from the presence of dynamic disorder, i.e., the previously observed heterogeneity in rate constants23. Although the rate constant of the single exonuclease itself can change within a single time trajectory, we assume that such an event is rare as the DNA sample that was degraded in this study was only 20 mL long. We rather consider the scenario of quenched disorder, i.e., the difference between the rate constants among individual time traces.

To incorporate the heterogeneity in the analysis, we assume that each rate constant can be expressed as

\[ k_{off}^- = k_{off}^- e^{-\Delta G^\ddagger} = k_{A} e^{-\Delta G^\ddagger} \]

where \( \Delta G^\ddagger \) represents the variation in the activation free energy from one molecule to another. Here, \( k_{off}^- \)’s denotes the disorder-free rate constants. \( k_{eff}^- \) and \( k_{off}^- \) are determined from the well-stirred solution of Eq. 2 once we find \( k_{eff}^- \) by using \( K_{B} \) estimated from \([Mg^{2+}]\) versus \( V \) data. Since \( \Delta G^\ddagger \), contributed by the disorder, results from the sum of many interactions between protein residues, we assume that \( \Delta G^\ddagger \) follows the normal distribution with zero mean and standard deviation \( \sigma \), Enzymes without disorder lead to \( \sigma = 0 \). To estimate \( \sigma \) of exonuclease, we fit the log \( V \) histograms to a Gaussian distribution (Supplementary Fig. 9, green lines).

Next, to estimate \( k_{eff}^- \), we first calculate the probability density function (PDF) of degradation time for 20 nts, \( t_2 \), with randomly generated \( \Delta G^\ddagger \) for a given \( k_{off}^- \). The PDF is calculated numerically by solving the following set of differential
that retain molecule-to-molecule heterogeneity, we generated 5000 random

\[
\begin{align*}
\frac{dp_{EMM}(n,t)}{dt} &= -k_{B}^{off_{EMM}} p_{EMM}(n,t) \left(1 - \frac{n}{N_{th}}\right) \\
&+ k_{P}^{off_{EMM}} p_{EMM}(n-1,t) \left(1 - \frac{n}{N_{th}}\right)
\end{align*}
\]

(6)

when the enzyme showed no degradation activity) for longer than 2 s (Supple-
mentary Fig. 10b). To compare the experimental and simulated pause times, we

\[
\begin{align*}
\frac{dp_{EM}(n,t)}{dt} &= k_{B}^{off_{EM}} p_{EM}(n,t) - \left(k_{P}^{off_{EM}} + k_{B}^{off_{EM}}\right) p_{EM}(n,t) \\
&+ k_{P}^{off_{EM}} p_{EM}(n,t)
\end{align*}
\]

(7)

to the average over them. Next, we calculated the PDF of

\[
\begin{align*}
\frac{dP(\zeta)}{d\zeta} = \tau_{20} \times \log 10 \times P(\tau_{20})
\end{align*}
\]

(9)

where \( k_{B}^{off} = k_{B}^{off_{EM}}[Mg^{2+}] \), \( k_{P}^{off} = k_{P}^{off_{EM}}[Mg^{2+}] \). \( P(n,t) \) denotes the probability that the ex-
onuclease in the state \( S \), where \( S \in \{E, EM, EMM\} \) is the n-th nucleotide at time \( t \), where \( n = 0, 1, 2, \ldots, 20 \). For the numerical integration, the exprm
function in MATLAB was employed. To calculate the probability density functions that retain molecule-to-molecule heterogeneity, we generated 5000 random numbers for \( \Delta t \) and took the average over them. Next, we calculated the PDF of

\[
\begin{align*}
\frac{\bar{v}}{V_{B}^{EMM}} = \frac{k_{P}^{off_{EMM}}}{k_{P}^{off_{EM}} + k_{B}^{off_{EM}}} + \frac{k_{P}^{off_{EMM}}}{k_{P}^{off_{EM}}}
\end{align*}
\]

(10)

where \( k_{B} = k_{B}^{off} / k_{P}^{off} \), \( k_{P} = k_{P}^{off} / k_{B}^{off} \) as before. The equation can be derived by the same approach used for model-1. Again, \( k_{B}^{off} \), \( k_{P}^{off} \), and \( k_{B}^{off} \) are estimated from the fit

Here, we assume \( k_{B}^{off} \leq k_{B}^{off_{EM}} \) based on the crystal structure. The above equations implies \( k_{B}^{off} = \frac{4k_{B}^{off_{EM}}}{(K_{A} + K_{B})^{2}} \) which mathematically supports our qualitative conclusion \( k_{B}^{off} \leq 0.1 \) in the previous paragraph.

We further test our conclusion with kinetic simulation with \( k_{B}^{off} = 32.64 \) and 128 s\(^{-1}\). Generated FRET time traces using the model-2 (Supplementary Fig. 13b), pause-time histograms (Supplementary Fig. 13c), and their mean square error to experimental data (Supplementary Fig. 13d) imply that long paused state of exonuclease is not observed in the model-2.

Futile ion binding. Frequent bindings and dissociations of Mg\(^{2+}\) ions but with many futile events are plausible. In this case, our model (model-1, Fig. 4a) can be deemed as the simplest model that describes futile binding and the occasional progress to a stable binding state by means of kinetic rate constant \( k_{B}^{off} \) and \( k_{P}^{off} \).

Data availability

Data supporting the findings of this manuscript are available from the corresponding authors upon reasonable request.

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Author contributions
G.L. wrote the manuscript. All authors contributed to the progress of the project, the experiments, kinetic simulations, and MD simulation, respectively. W.H., J.Y., C.H., and G.L. conceived this research. J.Y. and G.L. designed all experimental assays; W.H., J.Y., and C.H. designed all kinetic simulations. J.Y., W.H., and Y.L. performed FRET analysis, and interpretation of the data, and the theory and instrumental developments.

Additional information
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