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Detection of reaction intermediates in Mg^{2+} -dependent DNA synthesis and RNA degradation by time-resolved X-ray crystallography

Nadine Samara^{#1,2}, Yang Gao^{#1}, Jinjun Wu¹, and Wei Yang^{1,4}

¹Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, MD 20892

²Biological Chemistry Section, NIDCR, National Institutes of Health, Bethesda, MD 20892

[#] These authors contributed equally to this work.

Abstract

Structures of enzyme-substrate/product complexes have been studied for over four decades but have been limited to either before or after a chemical reaction. Recently using *in crystallo* catalysis combined with X-ray diffraction, we have discovered that many enzymatic reactions in nucleic-acid metabolism require additional metal-ion cofactors that are not present in the substrate or product state. By controlling metal ions essential for catalysis, the *in crystallo* approach has revealed unprecedented details of reaction intermediates. Here we present protocols used for successful studies of Mg^{2+} -dependent DNA polymerases and ribonucleases that are applicable to analyses of a variety of metal ion-dependent reactions.

Keywords

catalysis; transition state; phosphoryl and nucleotidyl transfer; metal ions

1. Introduction

Enzymatic reactions are intrinsic to all biological processes. How enzymes achieve their extraordinary catalytic power has been intensively investigated for many decades. Enzyme kinetic studies, which measure the concentration-dependent rates of substrate conversion to product, and mathematical modeling, which fits kinetic data under different conditions to a reaction pathway, have generated a wealth of information about requirements for enzyme catalysis, intermediate steps and their rates of occurrence (Schechter, 1970; Johnson, 1992). To obtain kinetic parameters of intermediate steps of a reaction, various strategies have to be developed and applied to alter reaction processes and isolate the intermediate steps (Patel, Wong&Johnson, 1991; Kati, Johnson, Jerva&Anderson, 1992). Complementary to kinetic approaches, structural methods, especially X-ray crystallography, have yielded atomic details of molecular arrangement. When substrates are visualized in the active site poised for catalysis, the reaction mechanism is routinely inferred. However, to obtain structures of

⁴Correspondence: wei.yang@nih.gov, Tel: (301) 402-4645, Fax: (301) 496-0201.

enzyme-substrate complexes, chemical reactions are often manipulated and stopped by using non-reactive substrate mimics, enzyme inactivation by mutagenesis, or non-permissible cofactors (Nowotny, Gaidamakov, Crouch&Yang, 2005; Nowotny&Yang, 2006; Biertumpfel *et al.*, 2010), all of which may perturb the native active site configuration. Furthermore, crystallization requires homogeneous samples and an identical conformational state, and the resulting static X-ray structures are inadequate to fully describe dynamic processes of chemical reactions. Without high-resolution structural information about a complete reaction process, the physical basis of enzyme catalysis and the nature of intermediate steps remain elusive.

Time-resolved crystallography (Moffat, 1989; Stoddard, 2001), which follows the catalytic process *in crystallo*, can potentially yield a complete picture of enzyme catalysis with atomic details. As early as 1926, James B. Sumner discovered that crystallized urease can catalyze the breakdown of urea to ammonium and carbon dioxide (Sumner, 1926). Following that, various enzymes have been proven to be active in crystalline forms, albeit with a reduced catalytic rate (Makinen&Fink, 1977; Mozzarelli&Rossi, 1996). To obtain high-resolution structures of intermediate states during catalysis, one needs to inhibit the reaction during crystal growth and rapidly initiate synchronous reactions *in crystallo*. For light driven reactions, the reaction can be triggered with a laser pulse and followed by time-resolved X-ray diffraction data collection (Srajer *et al.*, 1996; Genick *et al.*, 1997; Schotte *et al.*, 2003; Nango *et al.*, 2016; Pande *et al.*, 2016). With the Laue method, a complete data set can be collected within 100 ps (Hajdu *et al.*, 1987). Data collection with recently developed X-ray free electron lasers (XFEL) can be accomplished for micron-size crystals at a femtosecond (fs) timescale (Emma *et al.*, 2010). These time-resolved structures have illuminated early events in light-driven reactions and provided insights to conformational changes associated with these processes.

However, the majority of enzymatic reactions are light independent. To circumvent this issue, light sensitive caged compounds have been developed (Corrie *et al.*, 1992). Caged compounds, which are inactive precursors of substrate, can be used during crystallization, and reactions are initiated by light to remove the cages. With light-sensitive caged compounds, a number of reactions have been investigated *in crystallo* (Schlichting *et al.*, 1990; Stoddard *et al.*, 1991; Stoddard *et al.*, 1998). However, the synthesis of inert caged compounds is not always possible. Diffusion triggered *in crystallo* reactions is another strategy that has been used to study many enzymatic reactions (Hajdu *et al.*, 1987; Schlichting&Goody, 1997; Williams *et al.*, 1997; Burzlaff *et al.*, 1999; Wilmot *et al.*, 1999; Schlichting *et al.*, 2000; Johnson, Taylor&Beese, 2003; Perkins *et al.*, 2016). The ample solvent volume inside protein crystals allows diffusion of substrates as large as cytochrome C (Tegoni, Mozzarelli, Rossi&Labeyrie, 1983). However, diffusion, especially for larger substrates, may be slow compared to the chemical reaction, and short-lived intermediate states have only been successfully resolved with diffusions of small ligands, such as molecular oxygen (Williams, Fulop *et al.*, 1997; Burzlaff, Rutledge *et al.*, 1999; Wilmot, Hajdu *et al.*, 1999; Schlichting, Berendzen *et al.*, 2000). The diffusion-triggered *in crystallo* reaction can be either stopped by flash freezing at various time points for later data collection (Hajdu, Acharya *et al.*, 1987; Williams, Fulop *et al.*, 1997; Burzlaff, Rutledge *et al.*, 1999; Wilmot, Hajdu *et al.*, 1999; Schlichting, Berendzen *et al.*, 2000; Johnson, Taylor *et al.*, 1999; Wilmot, Hajdu *et al.*, 1999; Schlichting, Berendzen *et al.*, 2000; Johnson, Taylor *et al.*, 1999).

al., 2003; Perkins, Parsonage et al., 2016) or followed directly by Laue (Bolduc *et al.*, 1995) or XFEL (Stagno *et al.*, 2017) diffraction measurements.

Magnesium/Manganese ion-dependent enzymes play fundamental roles in nucleic acid metabolism (Cowan, 1998; Yang, Lee&Nowotny, 2006). The small size of Mg^{2+}/Mn^{2+} ensures a fast diffusion rate *in crystallo*. In recent years, we have developed a general diffusion-and-freeze trapping strategy to study Mg^{2+}/Mn^{2+} -dependent reaction processes *in crystallo* (Nakamura *et al.*, 2012; Gao&Yang, 2016). We have successfully visualized reaction intermediates for DNA polymerase and ribonucleases and discovered that previously unobserved metal ions that are transiently bound during catalysis play essential roles in DNA synthesis and RNA hydrolysis.

2. DNA Polymerase η

DNA polymerases catalyze phosphoryltransfer reactions that incorporate dNTPs (A, G, T and C) one at a time into a DNA primer according to the template sequence (Fig. 1A). The S_N2 type reaction involves the 3'-OH at a primer end and the α -phosphate of an incoming dNTP. The reaction requires deprotonation of the 3'-OH and protonation of pyrophosphate leaving group and thus exhibits bell-shaped pH dependence (Castro *et al.*, 2007). All DNA polymerases depend on metal ions Mg^{2+} or Mn^{2+} for catalysis. Two metal ions have previously been observed in non-reactive DNA polymerase ternary complexes (Pelletier *et al.*, 1994; Doubie *et al.*, 1998; Huang, Chopra, Verdine&Harrison, 1998; Ling, Boudsocq, Woodgate&Yang, 2001; Biertumpfel, Zhao et al., 2010). The Mg^{2+} ions are proposed to (1) neutralize the catalytic carboxylates and triphosphates of dNTP, (2) align substrates in the active site, and (3) facilitate deprotonation of the 3'-OH for the DNA synthesis reaction. Recently, we have discovered an additional divalent cation that has to be captured en route to production formation and may drive the reaction from breaking the phosphodiester bond between the α and β phosphates in dNTP (Gao&Yang, 2016) (Fig. 1B). Many DNA polymerases undergo dNTP-dependent large conformational changes, while some do not (Rothwell&Waksman, 2005; Yang, 2014). Extensive kinetic and FRET studies have led to the conclusion that the large conformational changes in DNA polymerases are faster than the chemical reaction itself and thus are not reaction rate-limiting (Shah, Li, Anderson&Sweasy, 2001; Showalter&Tsai, 2002; Bakhtina *et al.*, 2005; Rothwell, Mitaksov&Waksman, 2005; Zhang *et al.*, 2007). But these studies cannot exclude the possibility that subtle conformational changes in the active site are involved and essential for DNA synthesis. After decades of investigations, it remains uncertain how the DNA synthesis reaction is initiated and whether the rate-limiting step of DNA synthesis is chemical or conformational (Joyce&Benkovic, 2004; Johnson, 2008; Tsai, 2014).

Although sharing the same catalytic mechanism, DNA polymerases are diverse in amino acid sequence, secondary and tertiary structures as well as biological function (Rothwell&Waksman, 2005; Yang&Woodgate, 2007). Accordingly, DNA polymerases are divided into seven families (A, B, C, D, X, Y, RT). DNA polymerase η (Pol η) belongs to the Y family, which includes many polymerases specialized in bypassing damaged DNA bases and translesion DNA synthesis (Yang, 2014). Pol η is specific for bypassing UV-induced cyclobutane pyrimidine dimers and related base lesions. Inactivation of POLH (the

gene encoding pol η in humans leads to extreme UV sensitivity and predisposition to skin cancers, a syndrome known as the variant form of Xeroderma Pigmentosum (XPV) (Johnson, Kondratik, Prakash&Prakash, 1999; Masutani *et al.*, 1999). Pol η has a preformed and solvent exposed active site that is evolved to accommodate the UV lesion (Biertumpfel, Zhao et al., 2010). Crystal structures of polymerase η complexed with a non-hydrolyzable incoming nucleotide (dNMPNPP) and normal or damaged DNA template have been solved at 1.8Å (Biertumpfel, Zhao et al., 2010). We herein use Pol η as a model system to illustrate the *in crystallo* reaction approach.

2.1 Sample preparation

2.1.1 Expression of catalytic core of Pol η

Equipment

- Incubator/shaker (Innova 44, New Brunswick Scientific)
- Spectrophotometer (Nanodrop 2000c, Thermo Scientific)
- Centrifuge (Avanti JXN-26, Beckman Coulter)

Reagents and solutions

- Human Pol η (aa 1–432) with an N-terminal His₆ tag and PreScission Protease cleavage site is cloned into pET28a, and the resulting plasmid is called pWY2132 (Biertumpfel, Zhao et al., 2010).
- *E. coli* BL21 (DE3) pLysS component cells (New England Biolabs).
- LB media
- 50 mg/ml kanamycin stock, filtered with a 0.22 μ m syringe filter (ThermoFisher).
- 1 M IPTG stock, filtered with a 0.22 μ m syringe filter.

Procedure

1. Transform pWY2132 into *E. coli* BL21 (DE3) pLysS component cells.
2. Inoculate a single colony in 100 ml LB media with 50 μ g/ml kanamycin at 37°C overnight.
3. Transfer 10 ml of cells to 1 L LB media in 2 L flasks (six in total) each with 50 μ g/ml Kanamycin, and grow at 37°C to an OD₆₀₀ of 0.6–0.8.
4. Add IPTG to a final concentration of 0.5 mM to induce protein expression at 16°C for 20 h.
5. Collect cells by centrifuging at 4,000 rpm for 30 min at 4°C. Store cell paste at –80°C.

2.1.2 Purification of the catalytic core of Pol η —Purification of Pol η follows the steps outlined in Fig. 2A. SDS-PAGE gel of a typical run of Pol η purification is shown in Fig. 2B.

Equipment

- Q500 sonicator with a 3/4'' horn and solid tip (Qsonica sonicators)
- Ultra high-speed centrifuge, Beckman Optima XL-100 K
- High speed refrigerated micro centrifuge Tomy MX-307 (Digital Biology)
- EP-1 Econo peristaltic pump (Bio-Rad)
- ÄKTA purifier (GE Healthcare)
- Spectrophotometer (Nanodrop 2000c, Thermo Scientific)
- HisTrap HP column (5 ml, GE healthcare)
- NAP-25 desalting column (GE healthcare)
- MonoS-10/100 (GE healthcare)

Reagents and solutions

- Complete™, EDTA-free Protease Inhibitor Cocktail Tablets (Roche)
- 100 mM PMSF stock dissolved in isopropanol
- 50 mg/ml DNase I
- Ni-A buffer: 20 mM Tris (pH 7.5), 1 M NaCl, 20 mM Imidazole, and 5 mM β -mercaptoethanol (BME).
- Ni-B buffer: 20 mM Tris (pH 7.5), 1 M NaCl, 50 mM Imidazole, and 5 mM BME
- Ni-C buffer: 20 mM Tris (pH 7.5), 1 M NaCl, 300 mM Imidazole, and 3 mM DTT (dithiothreitol)
- Protease buffer: 20 mM Tris (pH 7.5), 500 mM KCl, 10% glycerol, 0.1 mM EDTA and 3 mM DTT
- PreScission Protease (home-made), 2 mg/ml stock
- MonoS-A buffer: 20 mM MES (pH 6.0), 250 mM KCl, 10% glycerol, 0.1 mM EDTA and 3 mM DTT
- MonoS-B buffer: 20 mM MES (pH 6.0), 1 M KCl, 10% glycerol, 0.1 mM EDTA and 3 mM DTT
- Stock buffer: 20 mM Tris (pH 7.5), 500 mM KCl, 20% glycerol, and 3 mM DTT

Procedure

1. Re-suspend the cell paste from 2 L LB media in 40 ml Ni-A buffer. Add one protease inhibitor tablet, 1 mM PMSF and 50 μ g/ml DNase I.
2. Break cells by sonication (3s on, 6s off at 80% amplitude for 3 min) at 4 °C. Clarify cell lysate at 4 °C by centrifugation at 35,000 rpm for 1h. Filter cleared supernatant using a 0.22 μ m syringe filter.

3. Equilibrate a 5 ml HisTrap HP column with 50 ml Ni-A buffer using a peristaltic pump. Load the filtered supernatant solution onto the column at 1 ml/min. Wash the column with 500 ml Ni-B buffer. Elute the proteins (500 μ L per fraction) with Ni-C buffer.
4. Combine the protein fractions according to OD₂₈₀ (usually over 20 mg proteins can be recovered at this step). Buffer-exchange the protein sample into the protease buffer using NAP-25 columns, following manufacturer's instructions. Add PreScission Protease at a mass ratio of 1:50 (protease to protein) to cleave the N-terminal histidine tag at 4 °C overnight.
5. Buffer-exchange the protein sample into MonoS-A buffer using a NAP-25 column. Load the protein sample onto MonoS-A buffer (5 mg sample per run) pre-equilibrated MonoS column on an AKTA purifier. Wash the column with 20 ml MonoS-A buffer. Elute the proteins with a gradient elution of 0–100% MonoS-B buffer in 100 ml at a flow rate of 1 ml/min.
6. Combine the protein fractions (usually, ~8–10 mg proteins can be obtained after this step). Buffer-exchange the protein sample to the stock buffer using NAP-25 columns. Concentrate the protein sample to ~2 mg/ml in a 50 ml Amicon device (Millipore) and aliquot the protein sample to 500 μ L per tube. Flash freeze the samples in liquid nitrogen (LN₂) and store at –80 °C.

2.1.3 Engineering crystal lattices by varying DNA length—DNA length plays a critical role in determining lattice contacts of protein-DNA co-crystals, and unfavorable lattice contacts may disrupt native protein-DNA interactions. We screened DNA length from 8 to 20 bps to obtain Pol η –DNA co-crystals that preserve native enzyme-substrate interactions in the active site and allow the reaction to occur readily. Our initial crystals of Pol η with a 13 bp dsDNA (Type I, Fig. 3A) are formed by hydrophobic contacts between adjacent protein molecules (Fig. 3B). Due to the lattice contacts, Pol η and the protein DNA interactions are distorted (Biertumpfel, Zhao et al., 2010). But with the DNA substrate shortened to 8 bp, new lattice contacts between protein and DNA substrate are formed, and the native protein-DNA interactions are restored (Type III, Fig. 3C-D) (Biertumpfel, Zhao et al. (2010). These crystal lattice contacts remain intact during the substrate to product transition.

2.1.4. DNA substrate preparation

Equipment

- Thermocycler (BioRad)
- Spectrophotometer (Nanodrop 2000c, Thermo Scientific)

Reagents and solutions

- Annealing buffer: 10 mM Tris (pH 8.0), 50 mM NaCl (without EDTA to avoid competing Ca²⁺ away from protein-DNA complexes).

Procedure

1. DNA oligos (Fig. 3) were synthesized chemically (Integrated DNA Technologies, IDT, Coralville, IA USA) and used after simple buffer exchange in NAP-25.
2. Dissolve DNA substrates in the annealing buffer at a concentration of 1 mM as determined by OD₂₆₀.
3. Mix 50 µl DNA primer and 50 µl DNA template in a PCR tube and heat the tube to 90°C for 1 min followed by cooling to 4°C at a 1°C/min rate in a thermocycler.

2.2 Co-crystallization of WT DNA pol η, DNA substrate and dATP

To prepare WT enzyme-substrate complexes (ES), we choose to use Ca²⁺ instead of Mg²⁺ because Ca²⁺ supports ternary complex formation but is a potent inhibitor of the DNA synthesis reaction (Nakamura, Zhao et al., 2012). However, all commercially available Ca²⁺ salts contain a trace amount of Mg²⁺ and Mn²⁺, which may trigger product formation during the period of crystal growth. Thus, when preparing crystals, Ca²⁺ was added at a 1:1 molar ratio to the polymerase, which is necessary for the incoming dNTP to bind to the active site, but insufficient for catalysis even with Mg²⁺ and Mn²⁺ contaminants because at least two Mg²⁺/Mn²⁺ per active site are needed for the reaction. The catalytic rate of Pol η is extremely low at pH 6.0 and rises with increasing pH from 6.0 to 8.0 (Nakamura, Zhao et al., 2012). We thus chose a pH 6.0 buffer to further inhibit the reaction during crystallization and used 100 mM MES (pH 6.0) buffer to overcome the 20 mM Tris (pH 7.5) in the protein-substrate stock solution. As expected, product did not form during crystal growth and only one Ca²⁺ was found within each protein–DNA–dATP complex in the ES structure (Nakamura, Zhao et al., 2012).

2.2.1 Preparation of wildtype enzyme-substrate complexes

Equipment

- ÄKTA purifier (GE Healthcare)
- Superdex 75 10/300 GL column (GE Healthcare)
- High speed refrigerated micro centrifuge Tomy MX-307 (Digital Biology)

Reagents and solutions

- SD buffer: 20 mM Tris (pH 7.5), 450 mM KCl, and 3 mM DTT
- Low salt buffer: 20 mM (pH 7.5) and 3 mM DTT
- 1.0 M CaCl₂
- 100 mM dATP

Procedure

1. Take one tube of protein stock from –80°C storage and thaw it on ice. Spin it at 13,000 rpm for 10 min to remove any aggregates.

2. Load the 0.5 ml protein samples onto a Superdex 75 column that has been equilibrated with the SD buffer and elute at a speed of 0.5 ml/min. Combine protein peak fractions (usually 1.5 ml at ~0.4 mg/ml).
3. Mix protein and 0.5 mM annealed DNA substrate at a molar ratio of 1:1.1. Add CaCl_2 and dATP to a final molar ratio of 1:1:1.5 of Pol η : CaCl_2 :dATP. Keep on ice for 10 min.
4. Add the low salt buffer to a final salt concentration of 150 mM KCl. Add dATP to a final concentration of 1 mM. High salt will weaken protein-DNA interactions.

2.2.2 Crystallization

Equipment

- Leica M205 C microscope
- High speed refrigerated micro centrifuge Tomy MX-307 (Digital Biology)
- Refrigerated incubator
- EasyXtal 15-well tool (Qiagen)

Reagents and solutions

- Precipitant solution: 14 to 20% PEG 2000 MME, 100 mM MES (pH 6.0).

Procedure

1. Concentrate the freshly prepared protein-substrate complexes in a 0.5 ml Amicon device (Millipore) to ~4 mg/ml according to the Bradford method (standardized based on Pol η concentration determined at OD_{280}). The molar concentrations of Pol η , CaCl_2 , and dATP are ~90 μM , ~90 μM and 1 mM, respectively. Spin it at 13,000 rpm for 10 min to remove any aggregates.
2. Set up crystallization drops using the hanging drop vapor diffusion method by mixing 2 μl of protein and 2 μl precipitant solutions from 14 to 20% PEG 2000 MME (in 1% increments) and equilibrate against wells of the same precipitant solutions in a hanging-drop plate at 22 °C. Keep the rest of protein samples on ice.
3. Check crystal growth after ~5 h to determine an optimal PEG 2000 MME concentration for crystal growth.
4. Set up hanging drop plates by mixing 2 μl of protein sample and 2 μl precipitant solution containing optimized concentrations of PEG 2000 MME (with 0.1% increment). This method produces high quality crystals (single crystals with sharp edges and dimensions ~200 $\times$ 80 $\times$ 80 μm) ideal for *in crystallo* reactions. Crystals are ready for harvesting after 2–3 days.

2.3 DNA synthesis reaction *in crystallo*

2.3.1 pH variation—To initiate the *in crystallo* reaction quickly and synchronously, we need to first raise the pH and then replace Ca^{2+} with Mg^{2+} . We tried crystal stabilization buffers at pH 6.0, 6.5, 6.8, 7.2 or 7.5 and found that beyond pH 7.2, X-ray diffraction quality of crystals decayed with reduced resolution and increased diffuse scattering. pH affects not only lattice contacts and deprotonation of the 3'-OH, but also the metal ion-binding environment. In the absence of additional divalent metal ions, the A site is increasingly occupied by monovalent cations Na^+ (and less so by K^+) with increasing pH (Nakamura, Zhao et al., 2012). To maintain diffraction quality, we choose to use MES buffer at pH 7.0 for our *in crystallo* reactions.

Equipment

- Leica M205 C microscope
- Crystal loops, 0.2–0.3 mm (Hampton Research)
- EasyXtal 15-well tool (Qiagen)

Reagents and solutions

- Stabilization buffer: 20% PEG2000 MME, 3 mM DTT and 100 mM MES-K at pH 6.0
- PH buffer: 20% PEG2000 MME, 3 mM DTT and 100 mM MES-NaOH or MES-K at pH 6.5, 6.8, and 7.0 or 100 mM HEPES-K pH 7.2 and 7.5
- Cryo buffer: PH buffer plus 20% glycerol

Procedure

1. Place 150 μl of each of the stabilization buffer, PH buffer, and cryo buffer into three adjacent wells in a crystal plate (EasyXtal 15-well tool, Qiagen).
2. Transfer each crystal with a crystal loop into the stabilization buffer at 22 °C and incubate with wells covered for 30 min.
3. Transfer the crystal to the PH buffer and incubate for 5 min at 22 °C.
4. Cryo-protect by dipping the crystal into the cryo buffer and flash-freeze the crystal in LN_2 .
5. Test X-ray diffraction.

2.3.2 Standard protocol for *in crystallo* reaction—The scheme of *in crystallo* reaction is shown in Fig. 4A. We use 1 mM Mg^{2+} to initiate the reaction and vary the reaction time to follow metal-ion binding and reaction processes. We find that diffusion of metal ions into the active site of all protein molecules occurs within 30s, but reaction products do not form for 60s and reach a plateau after 230s (Fig. 4B-D). Flash-freezing of crystals in LN_2 stops chemical reactions.

Equipment (same as in Section 2.3.1)

Reagents and solutions

- Equilibration buffer: 20% PEG2000 MME, 100 mM MES-KOH (pH 7.0), 3 mM DTT
- Reaction buffer: 20% PEG2000 MME, 100 mM MES-KOH (pH 7.0), 3 mM DTT and 1 mM MgCl_2
- Cryo buffer: Reaction buffer plus 20% glycerol

Procedure

1. Place 150 μl of each of the equilibration buffer, reaction buffer and cryo buffer into three adjacent wells in a hanging-drop crystal plate.
2. Use a crystal loop to transfer single crystals to the equilibration buffer. Incubate at 22 °C for 30 min to allow equilibration to pH 7.0 and removal of non-crystal form of polymerase-DNA- Ca^{2+} -dATP complexes and excess dATP.
3. Transfer each crystal to the reaction buffer to initialize the reaction and let the reaction proceed for a certain time period (30 to 600s). During the reaction time course, gently stir the buffer with a crystal loop without touching the crystal for rapid equilibration.
4. After a desired reaction time period, quickly dip the crystal in the cryo buffer and flash-freeze in LN_2 .
5. Collect X-ray diffraction data with 4- to 7-fold redundancy at the APS synchrotron radiation source (BM22 or ID22). A detailed procedure for data processing and structure analysis is described in Section 5.

2.4 Applications in studying catalytic mechanism of Pol η

We can readily modify the buffer composition and reaction conditions described above to further investigate the catalytic mechanism.

2.4.1 Determination of the number of metal ion binding sites—Two metal ions have been proposed to be necessary and sufficient for the DNA synthesis reaction (Steitz, 1999). However, a third Mg^{2+} binding site has been observed during our *in crystallo* reaction (Nakamura, Zhao et al., 2012) (Fig. 1B, 4D). To determine the number of functional metal-ion sites and their binding affinities, we carried out an *in crystallo* metal-ion titration experiment with Mn^{2+} . Both Mg^{2+} and Mn^{2+} support DNA polymerase reactions, but Mn^{2+} is more electron-rich than Mg^{2+} and can be easily detected even at low occupancy. Mn^{2+} titration *in crystallo* was carried out similarly as the reaction with Mg^{2+} (Section 2.3.2), except that 0.5, 1, 2, 4, 6, 10, or 15 mM Mn^{2+} were used in the reaction and cryo buffers. The reaction times were 90s, 180s, 300s, and 600s. We find that when A- and B-sites are saturated with Mn^{2+} but C site is empty (at 1 mM Mn^{2+}), no reaction product is detected for up to 30 min. We therefore deduce that binding of the 3rd $\text{Mg}^{2+}/\text{Mn}^{2+}$ is essential for and probably the rate-limiting step of the DNA synthesis reaction (Gao&Yang, 2016).

The *in crystallo* observations can be verified by *in solution* titration studies as described below.

Equipment

- Thermo incubator (Thermo Fisher)
- Gel box and power supply for electrophoresis (Thermo Fisher)
- Typhoon imaging system (GE Healthcare)

Reagents and solutions

- DNA substrate (primer: 5'-GTGCCTAGCGTAA-3'; template: 5'-GAGTCATGTTTACGCTAGGCAC-3'). The primer is FAM labeled at the 5'-end. Substrates are from IDT and HPLC purified.
- 1.0 mM dATP stock
- 1.0 M MgCl₂
- 20% Acrylamide-Urea gel (National Diagnostics)
- Protein dilution buffer: 500 mM KCl, 20 mM Tris (pH 7.5), 3 mM DTT, 20% glycerol
- 5X Reaction buffer: 375 mM KCl, 200 mM Tris (pH 7.5), 15 mM DTT, 0.5 mg/ml BSA, 5% glycerol
- 2X Quench buffer: 80% formamide, 50 mM EDTA, 0.01% bromophenol blue

Procedure

1. Prepare DNA substrate according to the described procedures (Section 2.1.4). Dilute Pol η with the protein dilution solution to a final concentration of 50 nM.
2. Mix Pol η , DNA, 5X Reaction buffer with water to form a 2X MasterMix (60 μ l in total) with final concentrations of 10 nM of Pol η , 10 μ M of DNA, and 2X Reaction buffer. Incubate the mixture at 37°C for 5 min.
3. Prepare a series of 5- μ l 2X dATP.Mg²⁺ or dATP.Mn²⁺ solutions. Each contains 100 μ M dATP and 0, 0.2, 0.4, 0.8, 1.6, 3, 6, 12, 20, 40 mM of Mg²⁺ or Mn²⁺. Incubate them at 37°C for 5 min.
4. Initiate reactions by adding 5 μ l 2X MasterMix into 5 μ l dATP.Mg²⁺/Mn²⁺ solution. Allow the reaction to proceed at 37°C for 4 min before quenching with an addition of 10 μ l 2X quench buffer. Heat the reaction mixtures at 95°C for 3 min and immediately chill on ice.
5. Pre-run a 20% Acrylamide-Urea gel at 300 V for 30 min. Load 5 μ l of each quenched reaction mixtures and run at 300 V at room temperature until the bromophenol blue dye reaches the end of gel. Scan gel with the Typhoon imaging system and quantitate the product amount with ImageJ.

2.4.2 Characterization of R61A mutant Pol η —Residue R61 of Pol η forms bifurcated salt bridges with the α and β phosphates of the incoming dNTP in the ground state. The guanidium group of R61 would overlap with the C-site metal ion in the polymerase-product complexes if R61 did not change its rotamer conformation (Fig. 4B-D). To probe the role of the R61 sidechain, we changed R61 to alanine and characterized its catalytic properties with the *in crystallo* reaction procedure. The *in crystallo* reaction of R61A mutant Pol η were analyzed in the same way as wild-type (Section 2.3), with either 1 mM Mg^{2+} or 10 mM Mn^{2+} in the reaction buffer and cryo buffer. We find that the reduced reaction rate of R61A mutant Pol η is due to its retarded C-site metal ion binding (Gao&Yang, 2016).

2.4.3 Reaction with sulfur-substituted dNTP analog—All DNA polymerases exhibited reduced catalytic rates when an incoming dNTP is substituted with sulfur in the pro-Sp position (Sp-dNTP α S) (Patel, Wong et al., 1991; Joyce&Benkovic, 2004). As pro-Sp oxygen atom is one of the two non-water ligands of the third metal ion (C-site) (Fig. 4D), sulfur substitution of Sp-dNTP α S is expected to retard the third metal ion binding and reduce the reaction rate. To find out how Sp-dNTP α S is incorporated by Pol η , we used *in crystallo* reaction analysis. The *in crystallo* reactions were carried out in the same fashion as with dATP (Section 2.3) except that: (1) concentrations of Mg^{2+} or Mn^{2+} were raised to 20 mM because *in solution* studies showed that higher concentrations of Me^{2+} are needed for Pol η to incorporate Sp-dNTP α S (Gao&Yang, 2016), and (2) 10 mM Sp-dNTP α S (Jena science) was used instead of dATP.

2.4.4 A two-step reaction to determine the C-site metal-ion preference—Because four of six C-site ligands are water molecules, it is possible that a variety of divalent cations can bind the C site and support Pol η mediated catalysis. To find out which divalent cation the C site prefers, we developed a two-step reaction protocol to separate binding of A- and B-site metal ions from that of C-site and product formation.

Reagents and solutions

- Equilibration buffer: 20% PEG2000 MME, 100 mM MES-KOH (pH 7.0), 1mM $MnCl_2$, 3 mM DTT
- Reaction buffer: 20% PEG2000 MME, 100 mM MES-KOH (pH 7.0), 10 mM $MgCl_2$, $MnCl_2$, $ZnCl_2$, $CdCl_2$, or $CaCl_2$, and 3 mM DTT
- Cryo buffer: reaction buffer plus 20% glycerol

Procedure

1. Incubate crystals in 150 μ l equilibration buffer at 22 °C for 30 min. At 1 mM Mn^{2+} , A and B-sites are saturated with Mn^{2+} , but C site is empty and hence no products are detected during the incubation.
2. Transfer crystals to 150 μ l reaction buffer for 1 min.
3. Cryo-protect and flash-freeze each crystal.
4. Take diffraction data and analyze structures

2.4.5 Thermal energy requirement for C-site Mg^{2+} binding and reaction—To understand the effects of temperature on the metal ion-binding site (C site, in particular) formation and catalytic rate, we carried out the two-step reaction at different temperatures using the following procedure.

Reagents and solutions

- Equilibration buffer: 20% PEG2000 MME, 100 mM MES-KOH (pH 7.0), 1mM MnCl_2 , 3 mM DTT
- Reaction buffer: 20% PEG2000 MME, 100 mM MES-KOH (pH 7.0), 5 mM MnCl_2 , and 3 mM DTT
- Cryo buffer: reaction buffer plus 20% glycerol

Procedure

1. Incubate a hanging drop plate with 1 ml equilibration, reaction and cryo buffer in adjacent three wells in an incubator of desired temperature (4, 14, 22, 30, or 37 °C) for 10 min.
2. Incubate crystals in the pre-cooled/warmed 1 ml equilibration buffer at desired temperature (4, 14, 22, 30, or 37 °C) for 30 min.
3. Transfer each crystal to the corresponding 1ml pre-cooled/warmed reaction buffer and incubate for 1 min. This step can be conducted at room temperature (~22°C) because the temperature of 1 ml buffer changes less than one degree in the first minute at room temperature.
4. Cryo-protect and flash-freeze these crystals.
5. Take diffraction data and analyze structures.

2.4.6 Engineering crystal contacts for DNA translocation—After each round of nucleotide incorporation, the DNA product usually translocates away from the active site by one base pair to be ready for the next round of incorporation. Because the DNA end distal to the active site in the Pol η crystals (Type III) is involved in crystal lattice contacts (Fig. 3C), product translocation along the double helical axis does not occur (Nakamura, Zhao et al., 2012). To follow the translocation process *in crystallo*, we have engineered a mismatched base pair at the lattice contact (TG substrate, Fig. 3C) to weaken the DNA duplex stability. The TG substrate not only allows the Pol η -substrate complex formation and *in crystallo* reaction, but also promotes DNA product translocation (Nakamura, Zhao et al., 2012).

3. Ribonuclease H1

Ribonuclease H1 (RNase H1) recognizes and hydrolyzes the RNA strand in an RNA/DNA hybrid. The reaction occurs in a $\text{Mg}^{2+}/\text{Mn}^{2+}$ dependent and sequence non-specific manner (Cerritelli&Crouch, 2009; Tadokoro&Kanaya, 2009). RNase H1 participates in replication by cleaving RNA primers in Okazaki fragments (Cerritelli&Crouch, 2009; Tadokoro&Kanaya, 2009) and is also a part of HIV-1 Reverse Transcriptase, where it cleaves and removes viral RNA in the process of reverse transcription (Champoux&Schultz,

2009). RNase H1 is homologous to the catalytic domains of DNA recombinase RAG1, HIV-1 Integrase, Holliday Junction Resolvase RuvC, and RNA processing enzyme Argonaute/PIWI (Yang&Steitz, 1995; Nowotny, 2009; Majorek *et al.*, 2014). The active site of RNase H1 contains four catalytic carboxylates (DEDD), which coordinate two divalent cations in the A and B sites, and these divalent cations in turn coordinate the substrate and the nucleophilic water for RNA cleavage (Nowotny, Gaidamakov et al., 2005; Nowotny *et al.*, 2007) (Fig. 5A).

RNA cleavage is fundamentally a reverse of DNA synthesis, so it is not surprising that RNase H1 and DNA polymerase η share a similar active site configuration. Both enzymes are activated by two Mg^{2+}/Mn^{2+} that bind to the A and B sites in the active site. Like DNA polymerase, RNase H1 is inhibited by Ca^{2+} , which supports binding of RNA/DNA hybrids to RNase H1, but does not allow RNA hydrolysis (Rosta, Yang&Hummer, 2014). Based on these similarities, it is predicted that DNA polymerase and RNase H1 share a similar reaction mechanism, known as two-metal-ion catalysis (Steitz & Steitz, 1993, Yang et al., 2006), with parallel roles for the A and B site metals. Recent *in crystallo* studies of the DNA synthesis reaction have revealed the presence of a third metal ion in the active site (Me^{2+}_C), which is essential for product formation (Nakamura, Zhao et al., 2012, Gao&Yang, 2016). It is unknown whether Me^{2+}_C occurs in the active site of RNase H1 during the hydrolysis reaction, and whether the requirement for a third metal ion is conserved.

Crystal structures of catalytically inactive mutant *B. Halodurans* RNases HC (containing the C-terminal catalytic domain) bound to an RNA/DNA substrate revealed the active site configuration and two Mg^{2+} in the catalytic center (Nowotny, Gaidamakov et al., 2005). However, mutant enzymes were crystallized in pre- and post-reaction states without revealing the dynamic process of catalysis. To determine RNA hydrolysis intermediates in the reaction, we have adapted the diffusion-and-freeze trapping method developed for DNA polymerase to observe the reaction process of RNase H1 hydrolyzing RNA *in crystallo*.

3.1 Sample preparation and RNA/DNA substrate design

3.1.1. Expression of the catalytic domain of *B. Halodurans* RNase H1 (RNase HC)—*Note:* Bh-RNase HC (aa 59–196) is cloned into pET15b with an N-terminal His₆-tag and Thrombin cleavage site (pWY2042); the E188A (pWY2063) and D192N (pWY2060) mutant RNases H1 are generated by site-directed mutagenesis from pWT2042 (Nowotny, Gaidamakov et al., 2005). The K196A mutant (pWY2780) is generated for this study. The expression and purification procedure of Bh-RNase HC is described briefly below.

Equipment

- Incubator/shaker (Innova 44, New Brunswick Scientific)
- Centrifuge (Avanti JXN-26, Beckman Coulter Life Sciences)

Reagents and solutions

- RNase HC plasmids
- *E. coli* BL21 (DE3) pLysS competent cells (New England Biolabs)

- Luria Broth (LB) agar plates containing 50–100 µg/ml ampicillin
- Luria Broth (LB) liquid media
- Ampicillin (100 mg/ml stock solution)
- IPTG (1.0 M stock solution)
- Buffer A: 40 mM NaH₂PO₄, 300 mM NaCl, 5% glycerol, 1.4 mM β-mercaptoethanol (BME), 10 mM imidazole, pH 7.0
- 100 mM PMSF (stock solution, made in ethanol)
- Complete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche)

Procedure

1. Inoculate a colony of BL21 (DE3) pLysS cells transformed with a RNase HC expression plasmid into 10 ml LB containing 100 µg/ml ampicillin, and grow the cells overnight at 37 °C.
2. Dilute the overnight culture 100X into 1 L of LB media containing 100 µg/ml ampicillin in a 2 L shake flask and grow to an OD₆₀₀ of ~0.6–1.0.
3. Induce the cells by adding IPTG to a final concentration of 0.5 mM and continue to grow at 37 °C for 4 h.
4. Collect cultured cells by centrifugation and re-suspend them in 30 ml freshly prepared and chilled (4 °C) buffer A.
5. Add PMSF to a final concentration of 1 mM and 1 protease inhibitor tablet to the cell suspension, transfer the cells to a 50 mL conical tubes and flash freeze in LN₂ for storage at –80 °C.

3.1.2 Purification of *Bh* RNase HC

Equipment

- Microfluidizer (M-110Y, Microfluidics)
- Ultra high-speed centrifuge (Beckman Optima XL-100 K, Beckman Coulter Life Sciences)
- 5 ml HisTrap column (GE Healthcare Lifesciences)
- Peristaltic pump (EP-1 Econo Pump, Bio-Rad)
- FPLC (ÄKTA purifier, GE Healthcare Lifesciences)
- Spectrophotometer (Nanodrop 2000c, Thermo Scientific)
- NAP-25 desalting columns (GE Healthcare Lifesciences)
- 0.45 µm filter and syringe (25 or 50 ml)
- Superloop, 150 ml, complete (GE Healthcare Lifesciences)
- HiPrep Phenyl HP 16/10 (GE Healthcare Lifesciences)

- 3 kD cutoff Centriprep (Millipore)

Reagents

- Thrombin (Sigma)

Buffers: Note: All buffers are freshly prepared and cooled to 4 °C immediately prior to use.

- Buffer A: 40 mM NaH₂PO₄, 300 mM NaCl, 5% glycerol, 1.4 mM BME, 10 mM imidazole, pH 7.0
- Buffer B: 40 mM NaH₂PO₄, 300 mM NaCl, 5% glycerol, 1.4 mM BME, 300 mM imidazole, pH 7.0
- Buffer C: 40 mM NaH₂PO₄, 150 mM NaCl, 5% glycerol, 2 mM DTT, 0.5 mM EDTA, pH 7.0
- Buffer D: 80 mM NaH₂PO₄, 4 M (NH₄)₂SO₄, 5 mM DTT, pH 7.0
- Buffer E: 40 mM NaH₂PO₄, 2 M (NH₄)₂SO₄, 5 mM DTT, 0.5 EDTA, pH 7.0
- Buffer F: 40 mM NaH₂PO₄, 5 mM DTT, 0.5 EDTA, pH 7.0
- Buffer G: 20 mM HEPES, 75 mM NaCl, 5% glycerol, 0.5 mM EDTA and 2 mM DTT, pH 7.0

Procedure

1. Thaw cells on ice and lyse them by passing through the microfluidizer 3X.
2. Clear the lysate by ultracentrifugation at 35,000 rpm for 30 min at 4 °C and load onto a 5 ml HisTrap column pre-equilibrated with buffer A. Wash the column with 20 column volumes (CV) of buffer A and elute the protein by increasing imidazole concentrations (buffers A and B).
3. Exchange protein peak fractions into buffer C, add 500– 700 units of thrombin to the protein, and incubate for ~3.5 hours at 21°C. Add an equal volume of buffer D to the sample and remove precipitate with a 0.45 µm syringe filter.
4. Load sample onto a HiPrep Phenyl HP 16/10 column pre-equilibrated with buffer E and elute RNase H1 with a 2.0 to 0 M (NH₄)₂SO₄ gradient (buffers E and F) over 10 CV.
5. Pool the peak fractions, exchange protein into buffer G, and determine the final concentration by measuring OD₂₈₀. Aliquot the pure protein and store at –20°C. The yield is ~40 mg of protein/L of cells.

3.1.3 Designing RNA/DNA substrates for *in crystallo* catalysis—We took the following into consideration when designing RNA/DNA hybrid substrates for *in crystallo* reactions. Firstly, RNase H1 is not sequence specific, and it can slide on an RNA/DNA hybrid. Previous co-crystals exhibited two alternative registers of enzyme to substrate (Nowotny, Gaidamakov et al., 2005; Nowotny&Yang, 2006), which results in heterogeneity in the crystal (Fig. 5B) and potentially makes assignment and refinement of reaction

intermediates difficult. To limit RNase H1 binding to an RNA/DNA substrate in a single register in the crystal, we designed an RNA/DNA substrate (Fig.5A) containing the minimum number of four ribonucleotides (Ohtani *et al.*, 1999) required for RNase H1 binding and hydrolysis. The number of deoxyribonucleotides flanking the four ribonucleotides in the “RNA” strand can be varied to optimize crystal lattice contacts. Secondly, co-crystals of RNase H1-RNA/DNA substrate should diffract to high resolution (ideally better than 1.8 Å) for us to observe reaction intermediates and additional elements transiently recruited during *in crystallo* catalysis. These transiently associated co-factors often exhibit low occupancies, and reaction substrate, intermediates and product often co-exist as a mixed population in a single crystal. Finally, interactions between the substrate and catalytic center should be native and unperturbed by crystal lattice contacts so that the observed *in crystallo* reaction represents what occurs in solution.

3.1.4 RNA/DNA hybrid preparation

Equipment

- Spectrophotometer (Nanodrop 2000c, Thermo Scientific)
- Thermocycler (PTC-200, MJ Research)

Reagents: Note: Oligonucleotides are chemically synthesized (Integrated DNA Technologies, Coralville, IO USA) and used without further purification.

- “RNA” oligo: 5’- ArCrArUrCG - 3’
- DNA oligo: 3’- TG TAGC - 5’

Buffers

- TE (10 mM Tris (pH 8.0) and 1 mM EDTA)

Procedure

1. Re-suspend RNA and DNA in TE to make 2–4 mM stock solutions and accurately determine final stock concentrations by measuring OD₂₆₀.
2. Anneal RNA and DNA strands by mixing 1.5 mM RNA with 1.5 mM DNA and warming to 65°C in a thermocycler. Decrease the temperature by 1°C/min to 4°C and leave the reaction on ice until use or store at –20°C.

3.2 Co-crystallization of WT RNase H1 with RNA/DNA hybrid

The RNA/DNA hybrid shown in Fig. 5A results in a unique binding site for RNase HC and an unperturbed active site of the enzyme-substrate in the co-crystal that can grow in the presence of Ca²⁺ and diffract X-rays up to 1.18Å. In this section, we describe the assembly and co-crystallization of the *Bh*-RNase HC-RNA/DNA complex.

Equipment

- VDX48 and VDX24 plates with sealant (Hampton Research)
- 12 mm and 18 mm thick siliconized glass cover slides (Hampton Research)

Buffers—Note: Buffers are freshly prepared immediately prior to use.

- Drop buffer: 14 % PEG3350, 20% glycerol, 0.2 M KI and 25 mM CaCl₂
- Well buffer: 12 % PEG3350, 20% glycerol, 0.2 M KI, and 25 mM CaCl₂

3.2.1 Complex assembly—To ensure that all active sites contain substrate, we mix *Bh*-RNase HC and RNA/DNA at a 1.1:1 molar ratio in the presence of buffer G (see section 3.1.2) to final concentrations of [RNA/DNA] = 330 μM and [RNase HC] = 300 μM, and incubate the mixture at 21°C for 30 min. An optimal complex concentration for crystallization is determined based on crystal size and quality.

3.2.2 Crystallization of the *Bh*-RNase HC-RNA/DNA complex in Ca²⁺

Procedure

1. Use the hanging drop diffusion method to produce crystals.
2. Mix 1 μl of *Bh*-RNase HC-RNA/DNA and 1 μl of the drop buffer and equilibrate 1–4 such drops over each well of 200 μl to 400 μl well buffer.
3. Crystals (usually 1–2 crystals per drop) grow to a final size of ~300 μm in 3–4 days.
4. Freeze and store crystals directly in LN₂ for future data collection.

3.3 RNase HI catalysis *in crystallo*

Equipment

- CrystalCap (Hampton Research)
- CryoLoops, 0.3–0.4 mm (Hampton Research)

Stock solutions

- 1.0 M HEPES, pH 7.0
- 50% PEG3350
- 50 mM EGTA
- 50% glycerol
- Monovalent salts: 1.0 M KI, 1.0 M RbCl, 1.0 M LiCl
- Divalent salts: 100 mM CaCl₂, 1.0 M MgCl₂, 1.0 M MnCl₂

Buffers—Note: Buffers are freshly prepared immediately prior to use.

- Stabilization buffer: 18–23% PEG-3350, 50 mM HEPES (pH 7.0), 0.2 M mM KI, 0.5 mM EGTA, 20% glycerol
- Reaction buffer: 18% PEG-3350, 50 mM HEPES (pH 7.0), 0.2 M mM KI, 2 mM MgCl₂

- Cryo-protection buffer: 18% PEG-3350, 50 mM HEPES (pH 7.0), 0.2 M mM KI, 2 mM MgCl₂, 30% glycerol

3.3.1 Removing excess Ca²⁺—Following complex crystallization, we set out to establish the conditions for *in crystallo* catalysis. First, we soak crystals in a pre-reaction stabilization solution containing the crystallization mother liquor, with the addition of the following critical components (Fig. 4A):

- *Add a pH 7.0 buffer to fix the reaction pH:* The crystallization solution does not contain additional buffer to what was in the *Bh*-RNase HC-RNA/DNA complexes. Crystals of *Bh*-RNase HC-RNA/DNA are most stable in a stabilization solution with a pH 7.0–7.5 and crack or dissolve at a pH higher than 7.5.
- *Add EGTA to facilitate replacement of Ca²⁺ with Mg²⁺/Mn²⁺ for catalysis:* Mg²⁺ does not readily replace the A and B site Ca²⁺ if crystals are soaked in solutions containing MgCl₂. Adding EGTA to the stabilization buffer helps to extract Ca²⁺ from the active site and remove excess Ca²⁺ in the solvent. With EGTA in the stabilization buffer, monovalent cations replace Ca²⁺ in the A and B sites and are readily replaced by Mg²⁺/Mn²⁺ in the reaction buffer.

3.3.2 Reaction *in crystallo*—After stabilization, crystals are ready for the RNA cleavage reaction. We remove glycerol from the reaction buffer because it appears to inhibit the reaction, presumably by reducing the Mg²⁺/Mn²⁺ diffusion rate into crystals. The procedure for *in crystallo* catalysis is outlined in Fig. 4A. Transfer individual crystals to a drop containing the reaction buffer, let the reaction proceed for t=40s, 80s, 120s, 160s, or 480s at 21°C, and then dip crystals in the cryo-protection buffer for 10s before freezing in LN₂ to stop the reaction.

3.4 Scenarios for mechanism investigation

3.4.1 Verification of monovalent cation requirement—Structures of the *Bh*-RNase HC-RNA/DNA complex reveal unexpected density in the active site consistent with the coordination sphere of a potassium ion. Since rubidium is a monovalent cation like potassium but has a unique anomalous diffraction signature, we use rubidium to unambiguously verify the presence of potassium in the active site of RNase HC after confirming that Rb⁺ can replace K⁺ and support the RNase H1 reaction in solution. We perform the Rb⁺ substitution experiments essentially according to the protocol described in section 3.3, except for the replacement of KI with RbCl in the stabilization, reaction and cryo buffers. We have successfully adapted this protocol to study reactions in other monovalent cations.

3.4.2 K⁺ titration *in crystallo*—To investigate the binding affinity of monovalent cations in the active site, we perform potassium concentration-titration experiments *in crystallo*. We include repeated wash steps of crystals in a stabilization buffer containing 5 mM K⁺ only (45–60 min for the first time and 5 min the second) to deplete potassium ions carried over from the crystallization buffer. We perform the titration experiment in the

reaction and cryo buffers containing 5, 25 50, 100 to 300 mM KCl with a reaction time of 120s (21 °C). The extent of product formation is dependent on the K⁺ concentrations.

3.4.3 Mn²⁺ titration *in crystallo*—To understand the effects of increasing divalent cation concentrations on the rate of product formation and to determine the number of divalent cation binding sites and their affinities for metal ions, we perform the *in crystallo* analyses according to the procedure described in section 3.3 with varying concentrations of Mn²⁺ in the reaction and cryo-buffers.

3.4.4 Studies of variants of RNase HC *in crystallo*—Three distinct variants of RNase HC (E188A, D192N and K196A) exhibit reduced catalytic activity in solution. However, it is likely that they play different roles in RNase H1 catalysis because of their diverse charge nature and distinct locations in the active site. Studying these mutant proteins provides additional information on the catalytic mechanism of WT RNase H1. We purify variant RNases HC and co-crystallize them with RNA/DNA hybrids according to the procedure described in section 3.2. Analyses of these enzymes *in crystallo* require some changes in Mg²⁺/Mn²⁺ concentrations as well as adjustment of reaction time from the protocol described in section 3.3.

Using a combinatorial approach of K⁺/Rb⁺, Mg²⁺/Mn²⁺ and RNase H1 variants, we discover that RNase H1, like DNA pol η, indeed requires a third divalent cation for RNA hydrolysis. In addition, monovalent cations play critical roles in RNase H1 catalysis.

4. Endonuclease V

Endonuclease V (EndoV) was initially discovered in *E. coli* as a DNA endonuclease, which cleaves damaged DNA *in vitro* (Gates&Linn, 1977; Demple&Linn, 1982; _S1_Reference91 Yao&Kow, 1994; Yao&Kow, 1996). This enzyme was later characterized as a deoxyinosine specific endonuclease (Yao, Hatahet, Melamed&Kow, 1994), which cleaves the second phosphodiester bond 3' to a deoxyinosine. *E. coli* mutants lacking EndoV activity have increased mutation frequencies (predominantly AT to GC and GC to AT) when exposed to nitrous acid, suggesting that EndoV plays an important role in the repair of deaminated purine bases *in vivo* (Guo&Weiss, 1998; Schouten&Weiss, 1999). It was proposed that EndoV initiates the alternative excision repair (AER) pathway by making an incision after certain DNA lesions in bacteria (Weiss, 2008). Surprisingly, eukaryotic homologues of EndoV cleave RNA containing an inosine *in vitro*, but not DNA, indicating that they may be involved in RNA metabolism (Morita *et al.*, 2013; Vik *et al.*, 2013). EndoV belongs to the RNase H-like nuclease superfamily, which includes a large number of enzymes participating in nucleic acid metabolism, for example, RNase H, RuvC, DNA transposases, integrase, UvrC and Argonaute (Yang&Steitz, 1995; Nowotny, 2009; Majorek, Dunin-Horkawicz *et al.*, 2014). Like RNase H, EndoV contains a carboxylate quartet in the catalytic center, which coordinates two divalent cations essential for catalysis (Nowotny, Gaidamakov *et al.*, 2005). We applied the *in crystallo* analysis method to capture reaction intermediates of EndoV catalysis.

4.1 Preparation of eukaryotic Endonuclease V and RNA substrate

4.1.1 Protein expression

Equipment

- Incubator/shaker (Innova 44, New Brunswick Scientific)
- Spectrophotometer (Nanodrop 2000c, Thermo Scientific)
- Centrifuge (Avanti JXN-26, Beckman Coulter)

Reagents and solutions

- Expression plasmid containing eukaryotic EndoV (pWY2669)
- *E. coli* BL21 (DE3) pLysS component cells (New England Biolabs)
- LB media
- 50 mg/ml kanamycin stock, filtered with a 0.22 µm syringe filter
- 1 M IPTG stock, filtered with a 0.22 µm syringe filter

Procedure

1. Transfect pWY2669 into *E. coli* BL21 (DE3) pLysS component cells.
2. Inoculate a single colony in 5 ml LB media containing 50 µg/ml kanamycin. Grow the cells in an incubator/shaker at 37°C overnight.
3. Scale up the cell culture to 200 ml culture, and then transfer 30 ml of cells to 1 L LB media in 2 L flasks (six in total) each with 50 µg/ml kanamycin, and grow them at 37°C to an OD₆₀₀ of ~0.6.
4. Add IPTG to the cell culture to a final concentration of 0.5 mM to induce protein expression and let the cells grow at 16°C overnight.
5. Spin down the cells at 4000 rpm for 20 min at 4°C. Re-suspend cell paste from 1 L culture in 30 ml PBS buffer, transfer to a 50 ml conical tube, and spin down again. Store cell paste at -80°C.

4.1.2 Protein purification

Equipment

- Q500 sonicator with a 3/4'' horn and solid tip (Qsonica sonicators)
- IKA T-25 homogenizer (ULTRA-TURRAX)
- Beckman Optima XL-100 K ultra high-speed centrifuge
- Steriflip-GP (0.22 µm, EMD Millipore)
- High speed refrigerated micro centrifuge Tomy MX-307 (Digital Biology)
- EP-1 Econo Pump (Bio-Rad); ÄKTA purifier (GE Healthcare)
- HisTrap HP (5 ml column, GE Healthcare)

- NAP-25 (GE Healthcare)
- HiTrap Heparin HP (5 ml column, GE Healthcare);
- Spectrophotometer (Nanodrop 2000c, Thermo Scientific)
- Amicon ultra-4 centrifugal filter (10 kDa cutoff, EMD Millipore)

Reagents and solutions

- Binding buffer: 20 mM Tris-HCl (pH 8.0), 40 mM imidazole, 500 mM NaCl
- Wash buffer: 20 mM Tris-HCl (pH 8.0), 80 mM imidazole, 1 M NaCl
- Elution buffer: 20 mM Tris-HCl (pH 8.0), 300 mM imidazole, 500 mM NaCl
- Low salt buffer: 20 mM HEPES (pH 7.0), 1 mM DTT, 1 mM EDTA, 100 mM NaCl
- High salt buffer: 20 mM HEPES (pH 7.0), 1 mM DTT, 1 mM EDTA, 2.0 M NaCl
- Annealing buffer: 10 mM Tris-HCl (pH 8.0), 50 mM NaCl
- Glycerol

Procedure

1. Resuspend ~10 grams cell paste (from 3 L cell culture) in 60 ml binding buffer using a homogenizer.
2. Sonicate cell suspension on ice (5s on, 10s off at 80% amplitude for 4 min).
3. Divide the cell lysate into two tubes and spin in a Ti45 rotor at 35,000 rpm at 4°C for 45 min. Transfer the supernatant to clean 50 ml tubes.
4. Filter the supernatant with steriflip-GP and load the filtered lysate at 1 ml/min onto a 5ml HisTrap HP connected to a Biorad EP-1 Econo Pump pre-equilibrated with binding buffer.
5. Wash at 3 ml/min the column with at least 10 column volumes (CV) of the binding buffer and then wash with at least 20 CV of the wash buffer.
6. Elute at 1.5 ml/min with 3 CV of the elution buffer and collect the fractions (0.5 ml each).
7. Combine peak fractions containing protein and exchange into the low salt buffer using a NAP-25 column. Apply the sample at 1 ml/min to a 5 ml Heparin column connected to an ÄKTA purifier pre-equilibrated with the low salt buffer.

Note: add glycerol to a final concentration of 5% (v/v) to the desalted protein sample to prevent precipitation when the sample is kept overnight.
8. Wash the column with the low salt buffer for 10 CV and then run a 50 ml 0.1–1.0 M NaCl gradient using the low and high salt buffer at 2.5 ml/min and collect 1.0 ml fractions.

9. Pool peak fractions containing the target protein (~4–5 ml) and concentrate in an Amicon ultra-4 centrifugal filter unit (10 kDa cutoff) at 6,000 g to ~0.2 ml (~8 mg/ml). Dilute the concentrated sample in the Amicon filter unit with 2 ml glycerol-less storage buffer (20 mM HEPES (pH 7.0), 1 mM DTT, 100 mM NaCl, 0.1 mM EDTA) and concentrate down and repeat the procedure again to exchange buffer. Measure the protein concentration using a Nanodrop by absorption at 280 nm under the molar extinction coefficient mode.
10. Add glycerol to the concentrated sample to a final concentration of 30% (v/v) and measure the protein concentration again. Aliquot protein (50 µl each) and store at –25°C.

4.1.3 RNA substrate preparation—Note: Use RNase away to clean pipet and bench to remove RNases.

1. Add the annealing buffer (section 4.1.2) to dissolve RNA oligos (Fig. 6A) with a deoxyinosine (purchased from IDT), clean with a NAP-25 desalting column, and centrifuge at 13,000 rpm for 5 min to get rid of particles or aggregates.
2. Measure the concentration using Nanodrop by absorption at 260 nm with a custom extinction coefficient (~400 µM). Make aliquots (50 µl) and store them at –20°C. These oligos are used as RNA substrates.

4.2 Co-crystallization of eukaryotic EndoV-substrate

4.2.1 Substrate design—One challenge we have faced is to obtain co-crystals of EndoV bound to a substrate with the scissile phosphate in the active site and poised for catalysis. At an early stage of our studies, we found that single-stranded oligos designed to form complexes with EndoV tend to self-associate during crystallization and form hetero or homoduplexes (Fig. 6A). Similarly, in the structures of *T. maritima* EndoV-DNA complexes, two EndoV molecules are in an asymmetric unit and each binds at one end of a heteroduplex with a deoxyinosine (Dalhus *et al.*, 2009). Although the DNA substrate designed for crystallization is a perfect duplex containing one single lesion (Dalhus, Arvai *et al.*, 2009), the DNA duplex is dissociated and the lesion-containing strands re-associate to form a heteroduplex. Our attempts to trap a well-aligned scissile phosphate in the active site of EndoV by varying the length of oligos in search of favorable crystal lattice contacts were largely unsuccessful. After trying multiple EndoV homologues and different length of oligos, we find a eukaryotic EndoV and an RNA oligo with a deoxyinosine can form a stable enzyme-substrate complex and co-crystallize in a manner amenable for the *in crystallo* reaction (Fig. 6A).

4.2.2 Crystallization protocol

Equipment

- Mosquito LCP (TTP Labtech Ltd);
- Rock Imager 182 (Formulatrix Inc)
- Greiner 96 Well Hanging Drop Vapor Diffusion Plates (Hampton Research)

- Leica M205 C microscope
- APS SER-CAT BM- and ID-22
- Amicon Ultra 0.5 mL centrifugal filter (NMWL, 10 KDa, EMD Millipore)
- EasyXtal 15-well tool (Qiagen)

Solutions and reagents

- Complex formation buffer: 20 mM HEPES (pH 7.0), 1 mM DTT, 2 mM CaCl₂, 100 mM NaCl, 0.1 mM EDTA
- Crystallization kits: JCSG core I and JCSG+ suites (Qiagen)
- Reservoir solution: 0.2 M potassium sodium tartrate, 10–25% (w/v) PE3350

Procedure

1. Mix purified EndoV (either freshly purified protein or stored at –25 °C, ~6 mg/ml) with an RNA substrate (~400 μM in Annealing buffer, Fig. 6A) at a molar ratio of 1 : 1.1 (protein : substrate). Add the substrate to a freshly made complex formation buffer first, mix well and then add the protein to a final concentration of ~1 mg/ml. Adjust the glycerol concentration to ~5% (v/v) if needed.
2. Incubate the mixture for 15 min at room temperature and then on ice for at least 15 min.
3. Transfer the mixture to an Amicon Ultra 0.5 mL centrifugal filter unit and centrifuge for ~20 min at 13,500 g to concentrate the sample by ~ 6-fold to a protein concentration of ~6 mg/ml, which is confirmed by the Bradford assay.
4. Mix the concentrated sample thoroughly and transfer it to a clean tube. Spin at 14,000 g for 10 min at 4°C to get rid of any debris. The concentrated sample is stable at 4°C for 2–3 weeks.
5. Set up crystallization screens with JCSG core I and JCSG+ suites in the hanging-drop format by a Mosquito LCP robot. Each drop contains 100 nl of sample and 100 nl of crystallization buffer, and each well contains 80 μl of crystallization buffer. Incubate and survey the crystal screens in a Rock Imager 182 at 22 °C.
6. Reproduce the hits from the initial screening and test X-ray diffraction using the in-house X-ray source or at APS Beamline 22.
7. Screen crystals and select those diffracting X-rays to better than 3.0 Å with low diffusion scattering for data collection at the APS ID-22. Solve structures and determine whether the active center contains a scissile phosphate. If the scissile phosphate does not appear to be poised for catalysis, design a new oligo based on the structure solved, and repeat step 1–7 to get a potentially active ES complex.
8. The best crystallization hit occurs with 0.2 M potassium sodium tartrate and 20% (w/v) PEG3350. The initial crystals are small (< 50 μm in the longest dimension). To get bigger crystals, mix freshly prepared EndoV-RNA complexes

with the reservoir solution at a volume ratio of 1–2: 1 (0.5 or 0.75 or 1 μ l of sample + 0.5 μ l reservoir solution for each well) and set up hanging drops using the EasyXtal 15-well tool. The reservoir solution (400 μ l) contains 10, 15, 20 or 25% (w/v) PEG3350. Incubate the crystallization plate with hanging drops in a 20°C incubator.

9. Check the hanging drops under the microscope the next day, and set up more drops using the condition that result crystals appearing overnight. Crystals usually grow with 20–25% (w/v) PEG3350 and mature in 3–6 days.

4.3 Analysis of *in crystallo* EndoV catalysis

4.3.1 A Ca^{2+} bound EndoV-RNA complex structure—Although 2 mM Ca^{2+} is included in the EndoV-RNA (ES) complex, initially we observed little electron density in the expected metal-binding A and B sites in the F_o-F_c map. We suspect that the Ca^{2+} ions are chelated by 0.1 M tartrate in the crystallization buffer. As a result, the scissile phosphate is improperly positioned in the active site and not aligned for catalysis (Fig. 6B, colored yellow). To test if the active site of EndoV can bind two Ca^{2+} in the canonical A and B sites and align the scissile phosphate for *in crystallo* catalysis, we transferred stabilized crystals of EndoV-RNA complexes to a pre-reaction buffer without tartrate but containing 50 to 300 mM Ca^{2+} and determined crystal structures after soaking for hours to days. Soaking in 50 mM Ca^{2+} for 90 min does not lead to metal ion binding in the A and B sites. However, both metal ion-binding sites are occupied by Ca^{2+} after soaking EndoV-RNA co-crystals in 100 mM Ca^{2+} for 3 days. We suspect that the need for extensive soaks in high concentrations of Ca^{2+} is probably due to the crystal packing, which distorts the configuration of active center and prevents metal ions from binding to A and B sites. With Ca^{2+} bound, the scissile phosphate along with the RNA strand moves ~ 1.0 Å compared to the structure without a bound Ca^{2+} ion and becomes aligned properly relative to the catalytic center for the reaction (Fig. 6B).

Solutions and reagents

- Stabilization buffer: 0.1 M potassium sodium tartrate, 15–25% (w/v) PEG3350
- Pre-reaction buffer: 0.1 M HEPES (pH 7.5), 0.1 M NaCl, 25% (w/v) PEG3350

Protocol

1. Transfer crystals ($\sim 200 \times 50 \times 50$ μm) to 10 μL drop of the stabilization buffer containing 15% (v/v) PEG3350 on an EasyXtal screw cap and incubate at 21°C for 5–10 min over a well containing 400 μl of the same stabilization buffer. Then add another 10 μL of the stabilization buffer to the drop for further incubation. During incubation, screw and fasten the cap onto the plate to minimize evaporation.
2. After stabilization soak in 15% PEG3350, repeat the step 1 using the stabilization buffer containing 20% PEG3350, and then again to 25% PEG3350.
3. Transfer individual crystals into the pre-reaction buffer and incubate at 21°C for half an hour (to remove as much tartrate as possible).

4. Transfer these crystals one each into the pre-reaction buffer plus 10 to 300 mM Ca^{2+} and incubate at 20°C for up to 3 days.
5. Freeze and store crystals in LN_2 .
6. Collect diffraction data and analyze structures.

4.3.2 EndoV catalysis *in crystallo*—After obtaining the appropriate crystals of EndoV-RNA complexes, we set out to understand the catalytic mechanism by using the *in crystallo* reaction and time-resolved crystallography method that was successfully used in Pol η and RNase H1 analyses. Binding of Mn^{2+} in both A and B sites is achieved after soaking the crystals in 10 mM Mn^{2+} for 1 min, occurring much faster than Ca^{2+} , and the resulting structure is highly similar to the Ca^{2+} -bound EndoV-RNA complex, which we term the ground state (GS). Interestingly, a longer (60 min) soak in 1 mM Mn^{2+} is not enough to occupy both sites. The hydrolysis reaction is observed to take place after 2 min of soaking in 10 mM Mn^{2+} . There are two RNA bound EndoV in each asymmetric unit (Fig. 6A), and both hydrolyze RNA *in crystallo*. The reaction appears to occur slightly faster in one EndoV than the other due to different crystal lattice contact environment.

Well-diffracting crystals (1.8 Å or better resolution, as of Pol η and RNase H1) permit the detection of low-occupancy intermediate states during *in crystallo* reactions. EndoV diffracts X-rays between 1.9 to 2.3 Å only, but we find that partially occupied Mn^{2+} ions can be readily detected and refined even when both the substrate and product state co-exist in one structure. In the EndoV-RNA complex structures, water molecules coordinating the Mn^{2+} ions in the active site are also clearly observed. The protocol for *in crystallo* catalysis is described below.

Solutions and reagents

- Reaction buffer: 0.1 M HEPES (pH 7.5), 100 mM NaCl, 25% (w/v) PEG3350, 10 mM MnCl_2 or MgCl_2

Procedure

1. Follow steps 1 and 2 in the protocol of section 4.3.1.
2. Transfer crystals into reaction buffers of pH 7.0 and 7.5 to test whether crystals are stable at neutral pH or above in preparation for efficient *in crystallo* catalysis. Crystals soaked in the pH 7.0 and 7.5 buffer both diffract X-rays as well as the crystals from the original crystallization drop. Therefore, we examine *in crystallo* reactions at pH 7.5.
3. The crystals soaked in the reaction buffer with 10 mM MnCl_2 are more stable than those with 10 mM MgCl_2 . Many crystals soaked in MgCl_2 crack regardless of PEG3350 concentrations. The resolution limit (2.4 Å) of crystals soaked in MgCl_2 is lower than those in MnCl_2 (1.9 Å). Thus, we perform the time-course of *in crystallo* reaction in MnCl_2 .

4. Transfer pH stabilized crystals into the reaction buffer with 10 mM MnCl_2 to initiate the reaction. Stop the reaction at a desired time point (2 min to 180 min) by flash-freezing and store the crystals in LN_2 .
5. Collection X-ray diffraction data and analyze structure.

Taking the *in crystallo* reaction approach, we find that EndoV also requires a third divalent cation for RNA hydrolysis just like RNase H1.

5. X-ray data processing

Most procedures used for X-ray diffraction data collection and structure refinement here are routine. An initial structural model of enzyme-substrate complex in a ground state (GS) is presumed to exist already for each project. This section focuses mainly on procedures unique for *in crystallo* reaction data analysis to determine structural changes in intermediate states.

Equipment

- X-ray home-source (Rigaku MicroMax-007HF, Saturn A200 CCD detector)
- X-ray synchrotron source (BM-22 or ID-22 at APS, Argonne, Illinois)
- Unix computers (Mac or Linux)

Software

- HKL2000 (Otwinowski&Minor, 1997)
- XDS and XSCALE (Kabsch, 2010)
- CCP4: TRUNCATE, CAD, SCALEIT, SFALL, FFT, SHELX, REFMAC (Winn *et al.*, 2011)
- COOT (Emsley, Lohkamp, Scott&Cowtan, 2010)
- SHELX: ShelxC, Anode, Shelx2map (Sheldrick, 2010)
- PHENIX (Adams *et al.*, 2010)

5.1 X-ray data scaling and analysis

Diffraction data are routinely indexed and scaled using HKL2000 and SCALEPACK or XDS and XSCALE. Resolution limit is usually estimated by $I/\sigma I (> 1.5\text{--}2.0)$ and $> 90\%$ coverage in the highest resolution shell. We rely on unbiased $F_o\text{--}F_c$ map to follow reaction progresses, which are manifested in substrate disappearance and product formation. The following protocol also allows us to estimate occupancies and B-factors of active-site metal ions (Fig. 7A).

1. Edit datasets in a time-course or titration series in a text editor to the same averaged unit cell parameters because crystals are isomorphous.
2. Reduce each dataset and convert it to mtz files using TRUNCATE (CCP4). The averaged unit cell parameters are entered for all datasets.

3. Combine all datasets in the experimental series into a single mtz file using CAD (CCP4).
4. Scale datasets from the CAD output to the F_c 's of a reference structure (usually a well-refined GS structure of the enzyme-substrate complex) and to the same high-resolution limit based on the lowest resolution data in the set using SCALEIT (CCP4). For example, for Pol η , we use the ground state structure of PDB 4ECQ (Fig. 4B) as a reference for F_c calculation, and the resolution limit is set to 2.0 Å in SCALEIT. Data beyond the resolution cutoff are not used for the structural comparison described below.
5. To find changes around the active center during the reaction process, generate an omit reference model by removing the scissile phosphate, and sometimes the A and B metal ions and associated water molecules, from the GS reference structure. Remove active site residues from the reference structure if conformational changes occur. Calculate F_o - F_c omit maps for each dataset using F_o (SCALEIT output) and F_c of the omit reference model using SFALL and FFT (CCP4) (Fig. 7B).
6. Examine and analyze the maps in COOT. Measure peak heights of substrate, product, and metal ions using python script "density_at_point" or the peak search function in COOT and normalize to the RMSD of each F_o - F_c map.
7. Plot values of peak height (electron density) at points of interest, such as metal ion sites, appearing product and disappearing substrate, over time points or co-factor concentrations for the experiment series. Estimate trends of metal ion binding/exchange and extent of reactions based on these plots (Nakamura, Zhao et al., 2012; Gao&Yang, 2016).

5.2 Anomalous data collection and analysis

We use Rb^+ and Mn^{2+} , respectively, to confirm monovalent and divalent cation binding. X-ray data are collected on crystals that have been soaked in Rb^+ (substituting for K^+ or Na^+) or Mn^{2+} (substituting for Mg^{2+}) at an appropriate wavelength to locate these anomalous scatters. We use 0.8 Å for Rb^+ (above the absorption edge) and 1.5 Å for Mn^{2+} . To maximize anomalous signals over noise, highly redundant data (> 8-fold redundancy) are collected. ShelxC is used for anomalous data preparation, Anode for anomalous signal calculation, and Shelx2map for making anomalous maps.

5.3 Structure refinement

In our experience, refining macromolecular structures (protein and nucleic acid) for *in crystallo* reaction series is mostly trivial and not different from normal refinement. In contrast to the structural comparisons described above, diffraction data are used without any resolution cutoff. One precaution we take is not to over interpret data. Although the electron densities for a mixture of the substrate and product state in a phosphoryltransfer reaction resemble that of the hypothesized pentacovalent intermediate state, we refrain from modeling the elusive and transiently existing intermediate and choose to build a mixed substrate and product state instead (Fig. 4D).

The most difficult part in refinement of reaction intermediates is to determine the extent of reaction (amount of product versus substrate), and occupancies and B factors of metal ions necessary for catalysis. The procedure described below works for the three projects reported here.

1. Estimate the metal ion and product occupancies based on the unbiased F_o-F_c omit maps (as described in 5.1.6).
2. Test a range of occupancies (in 10% increment or descent) by editing the occupancies of atoms of interest in a coordinate file by assigning guessed occupancies to these atoms, and refine individual B factors of the entire complex.
3. Whether an appropriate occupancy has been assigned is judged by the result of B-factor refinement. A correctly estimated occupancy leads to no significant electron density peak in $2F_o-F_c$ and F_o-F_c maps around the atom of interest, e.g. a metal ion, and the metal ion has a B factor similar to its coordinating ligands.

6. Applications and future prospects

Here we present studies of DNA polymerase η , RNase H1 and EndoV as examples of successful applications of *in crystallo* catalysis and time-lapse X-ray crystallography that uncovered novel details of reaction intermediates and catalytic mechanisms. The methods described above can be adapted to studying the majority of nucleic acid and phosphoryltransfer enzymes as well as enzymes, which typically require specific divalent cations for catalysis. This includes all DNA and RNA polymerases, helicases, nucleases, ligases, as well as ATP and GTP kinases and beyond. The method may be applied to studying enzymes that are not involved in nucleotide and nucleic acid metabolism, for example, (1) GalNAc:polypeptide GalNAc-transferases (GalNAc-Ts), which catalyze the Mg^{2+} -dependent transfer of GalNAc from UDP-GalNAc to a serine or threonine residue in the first step of mucin O-linked glycosylation (Bennett *et al.*, 2012), (2) Ketol-Acid Reductoisomerases (KARI), which require two Mg^{2+} ions for the isomerization step in branched amino acid synthesis (Tadrowski *et al.*, 2016), and (3) the radical SAM enzyme QueE, which utilizes Mg^{2+} for radical-mediated ring contraction (Dowling *et al.*, 2014).

An intrinsic impediment to studying reactions *in crystallo* is potential large conformational changes associated with catalysis that may be restricted by crystal packing as shown in the studies of myoglobin and PYP (Genick, Borgstahl *et al.*, 1997; Schotte, Lim *et al.*, 2003). Furthermore, large conformational changes may occur asynchronously and lead to blurred or even absent electron density and thus non-interpretable results. Fortunately, the three phosphoryltransfer reactions we have examined using the diffusion-and-freezing trapping method occur synchronously *in crystallo* without major conformational changes outside of the reaction center. We anticipate limited conformational changes in similar enzymatic reactions.

A general approach for *in crystallo* reactions of these enzymes is to (1) obtain co-crystals of protein-substrate complexes in the presence of non-permissive metal ion cofactors, (2) check that lattice contacts allow native protein-substrate interactions and product formation, and (3) initiate the reaction by addition of cognate metal ion co-factors and let the reaction

proceed for various amounts of time before freezing crystals for X-ray analysis. Crystals should diffract X-rays reasonably well to yield information about intermediate states and be robust and homogeneous enough to survive multiple buffer transfers, *in crystallo* catalysis, and reproducible structural comparison.

Our current time resolution using diffusion-and-freezing trap method works for relatively slow reactions, but is likely not fast enough for many other reactions. It may fail to capture transition states and other transient elements that exist for less than a few seconds. Reducing temperature usually slows down reactions, but it may lead to asynchronous reactions. Laue diffraction and time-resolved X-ray techniques have been used to study (1) CO binding to myoglobin in a time scale of 100 ps to 10 μ s and (2) the photocycle of photoactive yellow protein, both of which are initiated by laser pulses while crystals are exposed to X-rays (Srajer, Teng et al., 1996; Schotte, Lim et al., 2003). The diffusion method can be troublesome for large crystals, but problems arising from slow and uneven diffusion may be circumvented by using micro- or nano-crystals (Chapman *et al.*, 2011).

X-ray free electron lasers (XFEL), which produce coherent and high intensity X-ray pulses in the fs time scale can overcome many limitations of our current approaches (Neutze&Moffat, 2012; Neutze, 2014). The intensity of XFEL allows high-resolution diffraction from tiny crystals, which make sub-millisecond diffusion feasible. Using XFEL and micro-size crystal diffraction, each crystal is exposed to X-ray for fs only, and even at room temperature data are collected before the crystals decays. A liquid jet can be used to deliver micro to nanometer-sized crystals to the X-ray source at RT, thus maintaining the integrity of the active site and minimizing radiation damage. High quality and high-resolution data can be collected using femtosecond XFEL (Boutet *et al.*, 2012). Since the XFEL technique is still in its infancy, many exciting applications are still to come. Recently, this method has been successfully applied to capture ligand-induced conformational changes of a riboswitch RNA (Stagno, Liu et al., 2017).

Although a powerful method to determine atomic structures, X-ray crystallography rarely allows observation of hydrogen atoms, which are key elements in acid and base catalyzed reactions. Hydrogen atoms can be observed in ultra high-resolution diffracting crystals (Blakeley, Hasnain&Antonyuk, 2015; Ogata, Nishikawa&Lubitz, 2015). Alternatively neutron diffractions may be employed to gain information of position and mobility of protons, hydrogen and polarized hydrogen atoms (Niimura, 1999). In conclusion, following *in crystallo* enzymatic reactions with conventional X-ray, XFEL and neutron diffraction methods presents exciting opportunities to reveal intermediate states and catalytic mechanism with unprecedented detail.

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A **B** **Fig 1.**

DNA synthesis. (A) DNA polymerases catalyze phosphoryltransfer reactions that incorporate dNTPs (A, G, T, and C) one at a time into the DNA primer according to the template sequence. (B) The three-metal-ion mechanism for DNA synthesis based on the *in crystallo* reaction and X-ray crystallographic studies.

Fig 2.

Purification of Pol η . (A) A diagram of purification steps. (B) Coomassie stained SDS-PAGE gel showing the purification process. The lanes from left to right are MW markers (M), cell lysate (Lys), flow through of the HisTrap column (FT), elution from HisTrap (Ni), PreScission cleavage (cut), and elution from MonoS (S).

Fig 3.

DNA substrate designed for *in crystallo* catalysis by Pol η . (A) DNA substrate of 12–13 bp in length leads to Type I crystals of Pol η . (B) Lattice contacts in Type I crystals. The palm, thumb, finger, and little finger (LF) domains of Pol η , template and primer are shown in cartoon diagram and colored in red, green, blue, purple, orange, and yellow, respectively. The symmetry related molecule is shown in gray. The crystal lattice contacts between the two LFs lead to distorted Pol η -DNA interactions. (C) DNA substrate of 8–9 bp in length leads to Type III crystals. (D) Lattice contacts in type III crystals are between protein and DNA of adjacent complexes. Protein and DNA are shown in the same color scheme as in (B).

Fig 4.

In crystallo reaction procedure. (A) Scheme of a general *in crystallo* reaction procedure.

EGTA is only added to the stabilization buffer when excess Ca²⁺ are present in ES crystals.

(B)-(D) Representative structures of different reaction times. At *t* = 0s, (before soaking in the Mg²⁺ reaction buffer), there is one Ca²⁺ bound in the B site. At *t* = 40s (soaking in the reaction buffer for 40s), both A and B sites are occupied by Mg²⁺, and DNA and dATP (yellow) are aligned for catalysis. But no product is observed. At *t* = 230s, half of substrate is converted to product (blue). A 3rd Mg²⁺ emerges in the C site during product formation and replaces the R61 sidechain.

Fig. 5. RNase H1 cleaves the RNA strand in an RNA/DNA hybrid. (A) A diagram of RNase H1 (green oval) bound to RNA/DNA hybrid (DNA in light yellow, RNA in orange). The scissile phosphate is in the active site (red atoms), and two divalent atoms A and B mediate the interactions between the DEDD catalytic residues of RNase H1 and RNA substrate. (B) RNase H1 is a sequence non-specific ribonuclease and may “slide” on its substrates, which leads to binding with different registers in crystal forms. Dark and light green symbols represent two alternative binding modes of Bh-RNase H1 on a 12 bp RNA/DNA hybrid.

Fig. 6. EndoV catalyzed RNA hydrolysis. (A) A diagram of the self-complementary RNA substrate complexed with two EndoV molecules in an asymmetric unit. “T” stands for deoxyinosine. D1, E2, D3 and D4 represent the 1st, 2nd, 3rd and 4th carboxylate in the conserved DEDD motif, respectively. M_A and M_B are metal A and B, respectively. The duplex portion of RNA substrate is highlighted in yellow and boxed. The EndoV cleavage site is marked by the scissors. Both the duplex portion and single-stranded overhang have been varied in length, sequence, and deoxyribose versus ribose during crystallization screening. (B) Comparison of the Ca²⁺ bound (blue) and metal ion-free (yellow) EndoV-RNA complex structures. After

superposition of the two structures (left, red atoms are catalytic carboxylates), the active center is shown in ball-and-sticks (right). The arrow indicates the difference of the scissile phosphate in the two structures.

Note: ¹ Run TRUNCATE with the same averaged unit cell parameters for all datasets.

² F_{Rc} is calculated using a well-refined ground-state reference structure.

³ All dataset are scaled to the F_{Rc} and truncated to the high-resolution limit of the lowest diffracting dataset in the series.

Fig. 7.

Structure determination of reaction intermediates. (A) A flow chart from data processing to structure refinement. It outlines (1) procedure for calculating nonbiased F_0-F_c maps of a time course or titration experiment, (2) procedure for anomalous map calculation to determine metal ion binding sites, and (3) procedure of determining B-factors and occupancy during structure refinement. (B) An example of the unbiased F_0-F_c maps in the time-course of Pol catalysis. All data are scaled and truncated as depicted in (A), and the scissile phosphate (the α -phosphate of dATP) and the 3'-OH at the primer end are removed from the reference structure for F_c calculation. The black arrows point at the newly formed phosphodiester bond, and black circles highlight the 3rd Mg^{2+} .