Time-resolved X-ray crystallography of enzymatic reaction that degrades a mutagenic nucleotide

Time-resolved X-ray crystallography is a methodology to observe intermediate structures during enzymatic reactions and reveals reaction mechanisms at atomic resolution. To perform the time-resolved X-ray crystallography of enzymes, it is essential to establish reaction conditions in a crystalline state, i.e., to prepare crystals of an enzyme-substrate complex in its active form and control the entire reaction process on a time scale that can be followed while maintaining its crystalline state. We previously followed the nucleotidyl-transfer reaction in DNA polymerase [1,2]. The reaction in the crystals of DNA polymerase was initiated by soaking the crystals in a solution containing Mg^{2+} ions, active metal ions for the reaction, and stopped by freezing the crystals at desired time points. The intermediate structures captured by this freeze-trap method allowed us to observe the process of phosphodiester bond formation by DNA polymerase at a neutral pH of 7.0 in a time scale of 40 to 300 s. In this study, we have followed the course of the enzymatic reaction of *E. coli* MutT, which degrades a mutagenic nucleotide 8-oxo-dGTP and suppresses transversion mutations. We have visualized the entire reaction process of *E. coli* MutT by the freeze-trap method with a lower reaction rate at a lower pH of 5.8 [3].

8-Oxo-dGTP, which is produced by the oxidation of dGTP, is a mutagenic nucleotide because it is misincorporated into DNA by a DNA polymerase and the 8-oxoguanine (8-oxoG) base can mispair with adenine (Fig. 1). *E. coli* MutT hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP and pyrophosphate (PPi) through the nucleophilic attack by a water molecule in the presence of Mg^{2+} or Mn^{2+} [4]. We previously determined the crystal structure of MutT in a complex with 8-oxo-dGMP (a product) and revealed the recognition mechanism of the 8-oxoG nucleotide through the large conformational change of MutT [5]. To follow the hydrolytic reaction of 8-oxo-dGTP by MutT, the complex of MutT and 8-oxo-dGTP was crystallized. The complex structure showed that 8-oxo-dGTP is bound to the active site (enzyme-substrate complex structure: ES, Fig. 2), and the hydrolysis of 8-oxo-dGTP during crystallization without Mg^{2+} or Mn^{2+} ions was not observed. The crystals of ES were soaked in Mg^{2+} or Mn^{2+} solutions to trigger the hydrolytic reaction and then incubated for several hours. We confirmed that 8-oxo-dGTP is hydrolyzed to 8-oxo-dGMP (enzyme-product complex structure: EP) while maintaining its crystalline state. Next, the reaction conditions were screened to capture intermediate structures including an important step: nucleophilic attack by a water molecule. More than a hundred crystals were prepared under different reaction conditions of Mn^{2+} concentration (1 to 20 mM), reaction time (minutes to hours), and pH (5.6 to 6.0), and X-ray diffraction data were collected at SPring-8 **BL44XU** and **BL41XU**. The Mn^{2+} ion was used as an active metal ion because the anomalous signal derived from Mn^{2+} is used for the calculation of its occupancy, which is important information for the refinement of the intermediate structures. The reaction condition of 5 mM Mn^{2+} was suitable for observing the reaction processes immediately before nucleophilic attack, i.e., the sequential binding of metal ions, the structural change of the active site for catalysis, and the motion of nucleophilic water, whereas the reaction condition of 20 mM Mn^{2+} was suitable for visualizing the gradual hydrolysis of 8-oxo-dGTP to 8-oxo-dGMP and the release of PPi and Mn^{2+} ions. Finally, six types of intermediate structure (including ES) were determined at 1.36–1.90 Å resolutions.

MutT belongs to the Nudix hydrolase superfamily, which is widely distributed in all species, and is one of the most studied Nudix hydrolases. A number of studies of the Nudix hydrolases including MutT indicated that the Nudix hydrolases require two or three metal ions for catalysis. The intermediate structures in this study have clearly shown that MutT requires three metal ions for catalysis (Fig. 3). In the structure of the MutT and 8-oxo-dGTP complex (ES), 8-oxo-dGTP binds to the active site through Na^{+} ions and a water molecule, and the triphosphate moiety

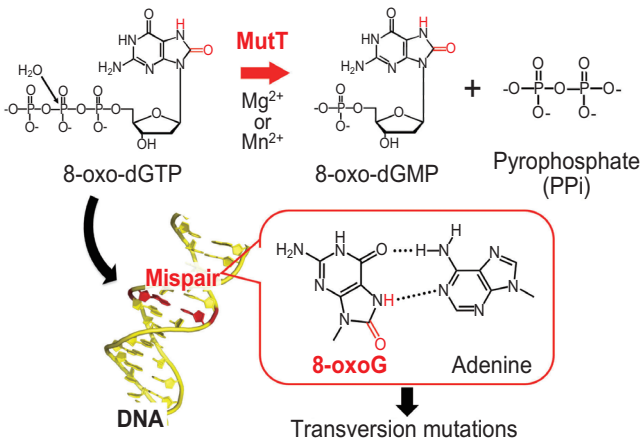

Fig. 1. MutT hydrolyzes 8-oxo-dGTP and prevents transversion mutations caused by the mispairing of 8-oxoG in DNA.

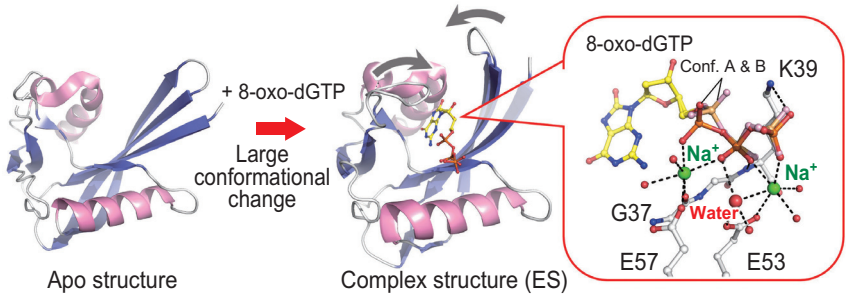

Fig. 2. Crystal structure of the MutT and 8-oxo-dGTP complex.

of 8-oxo-dGTP shows two alternate conformations (Fig. 2). Upon soaking in a solution containing Mn^{2+} ions, three Mn ions bind to the active site, and the triphosphate moiety is aligned for the reaction (left and middle of Fig. 3). Nucleophilic water bridges the two Mn^{2+} ions, at a position appropriate for nucleophilic attack on 8-oxo-dGTP (a red circle and an arrow in the middle of Fig. 3). After nucleophilic attack, 8-oxo-dGTP is hydrolyzed to 8-oxo-dGMP and PPi, and the generated PPi is released with two Mn^{2+} ions (right of Fig. 3). These intermediate structures suggested an activation mechanism of the nucleophilic water. When three Mn^{2+} ions bind to the active site, new electron densities are observed around the Mn^{2+} binding site (pink mesh on the left of Fig. 3). The electron densities would indicate the motion of the water molecule for nucleophilic attack, that is, a water molecule bound to Glu53 in ES (Fig. 2) moves to the position of nucleophilic attack by bridging the two newly arrived Mn^{2+} ions (left and middle of Fig. 3). The motion of the water molecule suggests that Glu53 is involved in the deprotonation of water before nucleophilic attack. Previous kinetic studies also suggested that Glu53 is a general base for catalysis. Alternatively, there is a possibility that water molecules in the bulk solvent are involved in the deprotonation of the nucleophilic water molecule. To further discuss the hydrolytic reaction mechanism in detail, the protonation/deprotonation states in the active site should be investigated by neutron crystallography and/or ultrahigh-resolution X-ray crystallography. In conclusion, MutT hydrolyzes 8-oxo-dGTP via nucleophilic attack by a water molecule activated through the sequential binding of three metal ions. The reaction mechanism of MutT proposed in this study would be shared among some of the Nudix hydrolases.

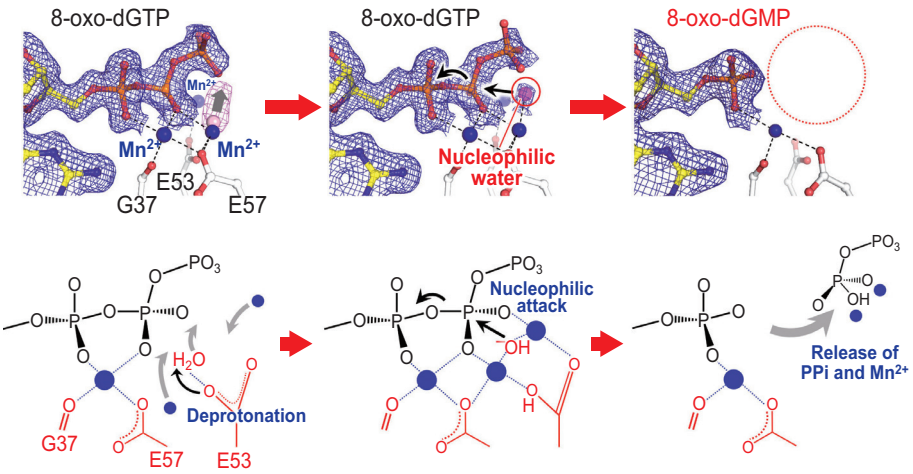

Fig. 3. Structures and electron density maps during the hydrolytic reaction of 8-oxo-dGTP and the proposed mechanism.

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