**Protonation states of key residues observed during in situ ADPRase reaction**

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ADPR-ribose pyrophosphatase (ADPRase), an enzyme classified into the Nudix family, catalyzes the hydrolysis reaction of ADP-Ribose (ADPR) to AMP and ribose-5'-phosphorus (R5'P) in the presence of divalent metal cations such as Mn2+, Zn2+, and Mg2+. Crystal structures of ADPRases from four species, human, M. tuberculosis, E. coli, and Thermus thermophilus HB8 (Tt) have been studied in parallel as well as points of mutual-putants and their reaction kinetics in solution. However, the reaction mechanism has been under discussion. This is the first presentation referring to protonation states of key residues based on in situ observation of TtADPRase hydrolysis reaction in the crystalline-state at atomic resolutions around 1.0 Å.

We collected X-ray diffraction data from the apo-form crystal of TtADPRase at an ultra-high resolution of 0.91 Å at BL31B1 of SPring-8, Japan. Crystals of ADPR-ADPRase binary complex were prepared by soaking the apo-form crystals into ADPR solution over night at room temperature. The ADPR hydrolysis reaction did not progress in the binary complex crystals because of absence of divalent metal cations. The hydrolysis reaction in crystals was triggered by soaking the binary complex crystals into an ADPR solution including Mn2+ at room temperature and stopped at different reaction times by flash-cooling with N2 gas stream at 90K. In total, diffraction data sets were collected from 7 crystals of Mn2+-ADPR-ADPRase ternary complex, and each crystal structure was solved independently at resolutions beyond 1.3 Å. In order to discuss the protonation states for key residues of ADPRase reaction, we compared two C-O bond lengths of Glu and Asp carboxyl side chains with considering their standard deviation after the unrestrained anisotropic refinement with SHELX-97 as Ahmed discussed previously [1].

The time-resolved crystallographic analysis revealed; (i) the first Mn2+ was introduced into the reaction cavity of ADPRase and coordinated to Glu86, changed ADPR conformation to the intermediate form, (ii) the second Mn2+ altered Glu82 conformation to the active form, and bound the intermediate ADPR, the active Glu82, and a water molecule, (iii) Glu82 may deprotonate this water molecule to a hydroxide ion in the coordination sphere of the second Mn2+, and (iv) the hydroxide ion may attack to the α-phosphorus atom of ADPR to cleave the pyrophosphate bond. Glu86 and Glu82 are highly-conserved residues among the Nudix family proteins and considered as the most important key residues. Glu86 was in an equilibrium state between protonated and deprotonated states throughout the reaction, but Glu82 may be deprotonated before the ADPR hydrolysis and be protonated after that. Glu85, the highly-conserved residue as well as Glu82 and Glu86, changed the protonation states also from deprotonated to protonated ones along the reaction time. Glu85 may receive a proton from the nucleophilic water molecule via Glu82 through a hydrogen bond network. In the conference, the results obtained for Glu73, Glu77, and Asp107 will also be presented and discussed.

**Keywords:** copper, protein, biochemistry

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**Versatile loops in mycocypins inhibit three protease families**

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Mycocypins, clitocepins and macrocyepins are cysteine protease inhibitors isolated from the mushrooms Clitocybe nebularis and Macrolypea procerca. Lack of sequence homology to other families of protease inhibitors suggested that mycocypins inhibit their target cysteine protease by a unique mechanism and that a novel fold may be found. The crystal structures of the complex of mycocypin with the papain-like cysteine protease cathespins V and of macrocyepin and clitocepin alone have revealed yet another motif of binding to papain like-cysteine proteases, which in a yet unrevealed way, occludes the catalytic residue. The binding is associated with a peptide-bond flip of glycine that occurs prior to or concurrently with the inhibitor docking. Mycocypins possess a beta-trefoil fold, the hallmark of Kunitz type inhibitors. It is a tree-like structure with 2 loops in the root region, a stem comprising a six-stranded beta-barrel, and two layers of loops (6+3) in the crown region. The two loops that bind to cysteine cathepsins belong to the lower layer of the crown loops, while a single loop from the crown region can inhibit trypsin or asparaginyl endopeptidase, as demonstrated by site directed mutagenesis. These loops present a versatile surface with the potential to bind to additional classes of proteases. When appropriately engineered, they could provide the basis for possible exploitation in crop protection.

**Keywords:** cathepsins, mycocypins, inhibition

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**Identification and structural studies of novel bacterial chitinases**

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Bacterium Clostridium paraputrificum 34, inhabiting the human gastrointestinal tract, produces a spectrum of extracellular enzymes including several types capable of hydrolysis of chitin at various levels of degradation steps. Endo- and exochitinase as well as β-N-acetylglucosaminidase activities have been detected in the extracellular extracts of this bacterium grown on a chitin-containing medium [1]. Catalytic digestion of chitin supports the anaerobic bacterium by supply of the final product of the degradation cascade – monosaccharides and can also substantially enhance human immunity by attacking pathogenic fungi with chitin-containing cell walls.

In the frame of a complex study we have identified a chitina B homologous to chitase B from C. paraputrificum M-21 [2], and an endochitinase and an exochitinase with molecular weights 61.2 and 62.7 kDa, respectively, as well as other chitinolytic enzymes produced