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Keywords: copper, protein, biochemistry

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

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ADP-ribose pyrophosphatase (ADPRase), an enzyme classified into the Nudix family, catalyses the hydrolysis reaction of ADP-Ribose (ADPR) to AMP and ribose-5'-phospholus (R5'P) in the presence of divalent metal cations such as Mn^{2+}, Zn^{2+}, and Mg^{2+}. 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 ones of point-mutants 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 *Tt*ADPRase 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 *Tt*ADPRase at an ultra-high resolution of 0.91 Å at BL38B1 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 Mn^{2+} at room temperature and stopped at different reaction times by flash-cooling with N_2 gas stream at 90K. In total, diffraction data sets were collected from 7 crystals of Mn^{2+}-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 hydrolysis 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 Mn^{2+}, introduced into the reaction cavity of ADPRase and coordinated by Glu86, changed ADPR conformation to the intermediate form, (ii) the second Mn^{2+} 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 Mn^{2+}, 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 ones 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.

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Keywords: time resolved crystallography, ultra high resolution, proton transfer

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Versatile loops in mycocypins inhibit three protease families

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Mycocypins, clitocypin and macrocypins are cysteine protease inhibitors isolated from the mushrooms Clitocybe nebularis and Macrolepiota procera. 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 clitocypin with the papain-like cysteine protease cathepsin V and of macrocypin and clitocypin 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* J4, 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 chitinase B homologous to chitinase 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

by the bacterium. Purification from the supernatants of the crude bacterial extracts by a combination of ultrafiltration, chromatography on EDTA-modified DOWEX-1x2 anion exchanger and size exclusion chromatography (Superdex 200) leads to separation of the 62.7 kDa enzyme. N-terminal sequencing of the first 30 residues confirms the enzyme to be a chitinase with the closest sequence homologue a chitinase D–like enzyme from *Bacillus thuringiensis* serovar *finitimus* YBT-020 [3], even if the overall domain organisation differs and has not been observed as yet.

Quality of the 62.7 kDa exochitinase samples has reached the level suitable for structural studies. The first crystallisation experiments and further sequencing and characterisation have been performed.

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Keywords: enzyme, structure-function studies, chitinase

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Structural insights into autoactivation mechanism of p21-activated protein kinase

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The p21-activated kinases (PAKs) are serine/threonine protein kinases defined by their interaction with the small G proteins, and play important roles in diverse cellular processes including cytoskeletal dynamics, growth/apoptotic signal transduction and regulation of transcription factors. All PAKs contain an N-terminal regulatory domain and a C-terminal kinase domain. Full activation of PAKs requires autophosphorylation of a critical threonine/serine (Thr423 in PAK1) located in the activation loop of kinase domain. A large body of experimental evidence shows that phosphorylation of PAK1 Thr423 is a trans-autophosphorylation reaction that is wholly dependent on dimerization of PAK1 (i.e. between two identical kinase molecules). Here, we report the crystal structures of phosphorylated and unphosphorylated PAK1 kinase domain. The phosphorylated PAK1 kinase domain has the conformation typical of all active protein kinases. Interestingly, the structure of unphosphorylated PAK1 kinase domain reveals an unusual dimeric arrangement expected in an authentic enzyme-substrate complex, in which the activation loop of the putative 'substrate' is projected into the active site of 'enzyme'. The 'enzyme' is bound to AMP-PNP and has an active conformation, whereas 'substrate' is empty and adopts an inactive conformation. Thus, the structure of asymmetric homodimer mimics a trans-autophosphorylation complex, and suggests that the unphosphorylated PAK1 could dynamically adopt both the active and inactive conformations in solution.

Keywords: p21-activated kinase, pre-existing equilibrium, trans-autophosphorylation

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Site directed mutagenesis to the rescue: unravelling conformational changes of Inositol 1,3,4,5,6 pentakisphosphate 2-kinase

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Inositol polyphosphates are a wide group of second messengers, involved in key cellular events. In particular, Inositol Pentakisphosphate 2-Kinase (IP5 2-K) converts inositol pentakisphosphate (IP5) to inositol hexakisphosphate or phytic acid (IP6). IP5 2-K is the unique enzyme that phosphorylates the axial position (2-OH) of the inositide ring. IP6 is present in all eukaryote cells and plays an essential role in processes such as lymphocyte development or apoptosis. In addition, IP6 tends to accumulate in plant seeds, what may have detrimental effects in human health and environment. Briefly, phytic acid is a potent chelator agent, contributing to malnutrition in populations where the diet is grain-based. We are undertaken structural studies by X-ray Crystallography to understand the mechanism underlying the function and regulation of this key enzyme in cell biology.

We have very recently crystallized and solved the first structure of an IP5 2-K from *A. thaliana*, in complex with substrates (IP5, IP5 plus AMP-PNP) and products (IP6, IP6 plus ADP) [1], [2]. The enzyme presents an αβfold, being divided in two lobes, a N lobe, conserved from protein kinases, and a C-terminal lobe. The N lobe and some residues from the C lobe are implicated in the nucleotide binding Also, a big part of this C-terminal lobe (the CIP-lobe) forms a novel structural region to bind the inositol phosphate. Despite all this knowledge, many obscure aspects remains around the catalytic mechanism and conformational changes of the enzyme. In order to elucidate this questions, and after a deep analysis of existing structural information, we are combining X-ray crystallography with other techniques, principally site directed mutagenesis. This approach has been very successful to improve the crystallizability of our samples as well as to gain insights into the no bonded (apo) and AMP-PNP bonded forms of IP5 2-K. All these findings represent an important tool to design inhibitors for the enzyme, what have potential applications in biomedicine and animal feed staff industry, for example in designing crops with low phytate levels.

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Structural basis of methylglucose lipopolysaccharide biosynthesis in mycobacteria

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