enzymes has been proposed, details of the reaction process are still not clear. This paper presents findings from a study that is aimed at understanding the mechanism of action in amidases, particularly the contribution of each of the catalytic residues in the reaction process.

The four catalytic residues (C165, E61, E139 and K131) have been mutated individually in the *Nesterenkonia* sp. amidase (NitN). In each case, the mutant was reacted with a range of amide substrates and the results analyzed by mass spectroscopy and X-ray crystallography. Mutation of the catalytic cysteine has allowed visualization of non-covalently bound substrates in the active site. However, unexpected reactions resulting in unusual adducts at the reactive cysteine have been observed with the mutants of the two glutamates and the lysine.

Mutation of the two active site glutamates and the lysine resulted in unstable mutants that lacked biochemical activity. While the reaction of E61Q/L mutants with some amide substrates resulted in thioester acyl-enzyme intermediates, 'abnormal' reactions have been observed with fluoroacetamide (FAE) and acrylamide (ACR). These include an S_N2 reaction which displaces the fluorine of FAE and a Michael addition of ACR at the catalytic cysteine, both of which have been visualized in the E61L/Q mutants. E139Q mutant does not exhibit any reactions with most of the amide substrates except with ACR where a Michael addition is observed. A cacodylate adduct at the catalytic cysteine has been visualized in the E139Q active site. The K131Q/H mutants showed no reactions, adducts or intermediates with the tested amide substrates, but a crystal structure of K131Q has revealed an adipamide reaction intermediate covalently attached to the catalytic cysteine. The trapped intermediate is likely to be of an *E.coli* metabolite that reacted with the mutants during protein expression.

The occurrence of unusual reactions with the NitN mutants highlights the importance of the catalytic residues (particularly the glutamates) in ensuring optimal positioning of the substrates in the active site and has also given insights into the critical balance of forces necessary for enzymatic catalysis in amidases.

Keywords: catalysis, mechanism, adduct

MS93.P34

Acta Cryst. (2011) A**67,** C780

Structural investigation of *Pseudomonas aeruginosa* glyoxalase i enzymes

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Methylglyoxal is a naturally produced cytotoxic electrophile. The toxicity of methylglyoxal is thought to be due to attack of nucleophilic centres of macromolecules including DNA, RNA and proteins. The glyoxalase enzyme system serves to detoxify methylglyoxal via a two-step pathway dependent on cellular glutathione. The first step is the conversion of the non-enzymatically formed glutathione-methylglyoxal hemithioacetal to the corresponding thioester by the metalloenzyme Glyoxalase I. Glyoxalase II cleaves the thioester to produce D-lactate and regenerate glutathione.

Pseudomonas aeruginosa is a multi-host, opportunistic pathogen. It is implicated in the pathology of pneumonia, septic shock, and other types of infection. *P. aeruginosa* uniquely possesses three separate enzymes with glyoxalase I activity, GloA1, GloA2 and GloA3. Other organisms only contain a single glyoxalase I enzyme. Interestingly, GloA1 and GloA2 contain a Ni^{2+} catalytic centre and are inactive when bound to Zn^{2+} unlike GloA3, which is active when bound to Zn^{2+} [1]. Having more than one enzyme with different metal centres may equip the bacteria with greater chemical flexibility allowing the microbe to inhabit more environments, i.e. areas that are deficient in Zn^{2+} but not Ni^{2+}. The presence of three different glyoxalase I proteins within the same organism provides the unique opportunity to compare both metal ion activation classes of this enzyme. X-ray crystallographic studies can provide detailed structural information about how the protein binds to the metal ion as well as metal ion-substrate interactions.

We report the crystal structures of GloA2 with Ni^{2+} and with Zn^{2+} bound at the active site. Anomalous data were collected to confirm the identity of the metal ion. The coordination geometries of the Zn^{2+} and Ni^{2+} centered *P. aeruginosa* GloA2 enzyme are almost identical as seen in the *E. coli* glyoxalase I enzyme [2], which is also only active with an octahedrally coordinated metal group [3].

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Keywords: glyoxalase I, metalloenzyme, coordination

MS93.P35

Acta Cryst. (2011) A**67,** C780

Structural basis for increased thermal stability of adenylate kinase variants

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Engineering proteins for higher thermal stability is an important and difficult challenge. We describe a comprehensive approach to mutate proteins to be more stable, and report structural analysis of the redesigned protein variants.

First, we identified mutations for thermal stabilization of our model, adenylate kinase based on a variety of experimental and computational techniques. Then, we designed variants by combining the individual stabilizing mutations together. In the experiment using differential scanning calorimetry, the adenylate kinase variants displayed considerable increases in their thermal stabilities.

We determined crystal structures of the variants to confirm the structural basis for their thermal stabilization. The structures showed that the resulting variants have mutation(s) for extra electrostatic interactions by newly added ion pairs, additional hydrophobic interaction and optimized local structural entropy suggesting their contribution to thermal stabilization of the variants.

Keywords: thermostable, mutagenesis, enzyme

MS93.P36

Acta Cryst. (2011) A**67,** C780-C781

Structure of the β-galactosidase from *Kluyveromyces lactis*

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β-galactosidase (EC 3.2.1.23) is the enzyme responsible of the hydrolysis of the disaccharide lactose into glucose and galactose. It is

present in microorganisms, plants and animals. From a biotechnological point of view, *Kluyveromyces lactis* β-galactosidase is suitable for many applications due to its neutral optimum pH and for the fact that *K. lactis* is a GRAS organism (Generally Recognized As Safe). Interestingly, β-galactosidases are being used in lactose intolerance treatments and in food industry. Moreover, yeast expressing this enzyme can be used to improve the valorization of the cheese whey, a cheese industry byproduct by coupling the degradation of lactose with ethanol production, biomass production, etc. [1].

On the basis of their sequence, β-galactosidases are classified within families 1, 2, 35 and 42 of glycosyl hydrolases in the CAZy database [2]. Those from eukaryotic organisms are grouped into family 35 with the only exceptions of *K. lactis* and *K. marxianus* β-galactosidases which belong to family 2, together with the prokaryotic β-galactosidases from *Escherichia coli* and *Arthrobacter* sp. Whereas the structures of these last two prokaryotic enzymes have been determined [3], [4], none of the eukaryotic β-galactosidase structures have been reported to date. Although their sequence similarity with the prokaryotic enzymes is significant (48% vs. *E. coli* and 47% vs. *Arthrobacter*) there are many differences, particularly some long insertions and deletions, which play an important role in protein stability and in substrate recognition and specificity.

Gaining insight into the structural features that determine its stability and understanding the specificity determinants and catalytic mechanism should lead to improvements of its biotechnological applications by rational protein engineering. In this study, we describe X-ray crystallographic studies and an analysis of *Kluyveromyces lactis* β-galactosidase structure.

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Keywords: yeast, glycosidase, biotechnology

MS93.P37

Acta Cryst. (2011) A**67,** C781

Structural studies of β–D-xylosidase from *Streptomyces thermoviolaceus* OPC-520

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Xylan is one of the major hemicellulose components of plant cells and is the second most abundant resource after cellulose. *Streptomyces thermoviolaceus* OPC-520 produces relatively high xylanase activity when grown in a medium containing xylan as a carbon source.

An intracellular β–D-xylosidase (BxlA) from *Streptomyces thermoviolaceus* OPC-520, together with a extra-cellular BxlE and the integral membrane proteins BxlF and BxlG, constitutes a xylanolytic system that participates in the intracellular transport of xylan degradation products and the production of xylose. To elucidate the hydrolytic mechanism of xylooligosaccarides to xylose at the atomic level, we attempted X-ray structural analysis of BxlA. The recombinant BxlA protein (molecular weight: 82kDa) was crystallized by the hanging-drop vapor-diffusion method at 293 K. The crystals belonged to the monoclinic space group *C*2, with unit-cell parameters *a*=141.0, *b*=129.5, *c*=100.4 Å, β=120.1°, and contained two molecules per asymmetric unit (V_M=2.47 Å^3/Da). Diffraction data were collected to a resolution to 2.2 Å on the BL26B1 beam line at the SPring-8. The brilliant light source present at SPring-8 was necessary to obtain useful data from these very weakly diffracting crystals. The initial structure of BxlA was determined by MAD method using the SeMet-labeled crystal. The structure of BxlA was built from typical catalytic domain and additional c-terminal domain. We now progress the refinement of structure to elucidate the detailed mechanism of glycoside hydrolysis of BxlA at the atomic level.

Keywords: xylosidase, MAD phasing, crystallographic structure

MS93.P38

Acta Cryst. (2011) A**67,** C781

Structural study of the mutants of FDTS from *T. maritima*

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Like thymidylate synthase (TS) in eukaryotes, the flavin-dependent thymidylate synthase (FDTS) is essential for cell survival of many prokaryotes for the *de novo* synthesis of thymidylate (dTMP). TS and FDTS inhibition stops DNA production, eventually leading to cell death. The two enzyme families present no structural or sequence homology. While both convert dUMP to dTMP, in contrast to the classical TS enzymes, the FDTS enzymes employ 5,10-methylene-5,6,7,8-tetrahydrofolate (CH2H4folate) as the methyl donor but not as reducing agent. Furthermore, recent studies showed that, in contrast to TS, FDTS doesn't use enzymatic nucleophile for the reaction and that the catalytic mechanism of TSs and FDTSs is substantially different [1]. These differences offer the possibility of developing specific FDTS inhibitors as antibiotic drugs with low toxicity.

We report the structural study of five mutants of FDTS from *Thermotoga maritima*. All of the mutants crystallize in the orthorhombic space group $P2_12_12_1$ with unit cell dimensions of a=~55Å, b= ~116Å, and c=~141Å. A comparison of the structures with the wild type enzyme and a comparison of the structures of the mutant enzymes with and without the substrate dUMP show several features that are important for the FDTS reaction. The structural information is also compared with the activity data to get a better understanding of the reaction mechanism.

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Keywords: flavin, thymidylate, synthase

MS93.P39

Acta Cryst. (2011) A**67,** C781-C782

Structure determination of levan fructotransferase

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Levan is fructan composed of linear and branched polymers of