

## Structural characterization of substrate complexes of Class 3 L-asparaginase

K. Pokrywka<sup>1</sup>, M. Grzechowiak<sup>1</sup>, J. Sliwiak<sup>1</sup>, P. Worsztynowicz<sup>1</sup>, J. Loch<sup>2</sup>, M. Gilski<sup>1,3</sup>, M. Jaskolski<sup>1,3</sup>

<sup>1</sup>Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland, <sup>2</sup>Jagiellonian University, Faculty of Chemistry, Krakow, Poland, <sup>3</sup>Department of Crystallography, Faculty of Chemistry, Adam Mickiewicz University, Poznan, Poland

*kpokrywka@ibch.poznan.pl*

ReAV, a Class 3 L-asparaginase from *Rhizobium etli*, is structurally and sequentially distinct from other L-asparaginases, making it an attractive candidate for the development of new antileukemics that could replace the immunogenic Class 1 asparaginases currently used in the treatment of acute lymphoblastic leukemia. While the enzyme may employ a unique catalytic mechanism for L-asparagine hydrolysis, the precise mode of action of ReAV remains unknown. To investigate the catalytic apparatus of ReAV, we carried out mutagenesis of the conserved Lys138 residue from the zinc coordination sphere, generating K138A and K138H variants with significantly reduced turnover numbers. Kinetic analyses revealed that replacing the native Zn<sup>2+</sup> cation with Cd<sup>2+</sup> significantly reduced the activity of both the wild-type enzyme and its two variants, while increasing at the same time the substrate affinity. The Cd<sup>2+</sup> containing K138A and K138H variants, together with the WT protein were co-crystallized with L-Asn as stable enzyme-substrate complexes, revealing the mode of substrate binding. Moreover, the two structures of the K138A variant, with and without the metal cation, highlight the importance of the metal for ensuring correct substrate positioning within the active site for effective reaction progression. Analysis of the optimal trajectory of the nucleophilic attack led to the identification of Ser48 as the catalytic nucleophile in the L-asparaginase reaction of ReAV. We propose a double-displacement mechanism, where an acyl-enzyme intermediate is hydrolyzed by an activated water molecule in the second step of the reaction. In the highest resolution structure of the K138A variant (1.3 Å) supplemented with Cd<sup>2+</sup> cations, we identified a potential allosteric site near the dimer interface. The hydrogen bond network and B-factor analysis confirmed the presence of L-Asp, the product of the L-asparaginase reaction, in this site. Work supported by National Science Centre (NCN, Poland) grant 2020/37/B/NZ1/03250.