**Structure-function relationships of ancestral haloalkane dehalogenases**

**P. Babková1,2, M. Marek1, E. Šebestová1, J. Brezovský1,2, R. Chaloupková1,2,**

**J. Damborský1,2**

1 Loschmidt Laboratories, Department of Experimental Biology and Research Centre for Toxic Compounds in the Environment RECETOX, Masaryk University, Kamenice 5/A13, 625 00 Brno, Czech Republic.2 International Clinical Research Center, St. Anne's University Hospital Brno, Pekarska 53, 656 91 Brno, Czech Republic. Petra.Babkova01@gmail.com

Ancestral sequence reconstruction represents a powerful approach for empirical testing structure-function relationships of the evolutionary intermediates of diverse proteins[1]. We employed the reconstruction to predict sequences of five ancestral haloalkane dehalogenases (EC 3.8.1.5, HLDs) from the subfamily HLD-II. HLDs are hydrolytic enzymes catalyzing conversion of halogenated aliphatic hydrocarbons to their corresponding alcohols and halide anions[2]. The genes encoding inferred ancestral enzyme sequences were synthesized and expressed in *Escherichia coli* and the resurrected ancestral enzymes AncHLD1-5 were experimentally characterized. All enzymes were correctly folded, revealed significantly enhanced thermodynamic stability (Δ*T*m up to 24 °C) and higher specific activities with preference for short multi-substituted halogenated substrates compared to extant HLDs. The multivariate statistical analysis uncovered a shift in the substrate specificity profiles of AncHLD1 and AncHLD2. The crystallization trials were performed by using sitting-drop vapour-diffusion method at 23 °C. The crystals of AncHLD2, AncHLD3 and AncHLD5 grown during the initial screening, were used for X-ray diffraction data collection and a complete data sets were collected to a resolution of 1.7, 1.3 and 1.8 Å resolution, respectively. Obtained microcrystals of AncHLD1 and AncHLD4 were optimized by variation of enzyme concentration, pH and precipitant concentration. The structures of AncHLD2, AncHLD3 and AncHLD5 were solved by the molecular replacement using the structure of DbjA (PDB ID 3AFI)[3] as a search model. Similarly to descendant HLDs, ancestral enzymes consists of two domains, the conserved main domain formed by eight-stranded β-sheet with β2 lying in an antiparallel orientation with respect to the direction of the β-sheet surrounded by 6 α-helices. The second variable cap domain consists of five α-helices. During refinement of the crystal structure of AncHLD2, two chloride anions were detected in the vicinity of the enzyme active site similarly as in the structure of modern HLD DbeA[4]. The CHES molecule was detected in the active site of AncHLD3 and large active site cavity was identified in the structure of AncHLD5. Comparison of structures of ancestral enzymes with structures of descendants accompanied by dynamic simulations will provide detailed insight into their catalytic properties.

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