**Structural analysis of resurrected ancestral enzymes of haloalkane dehalogenases**

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Ancestral sequence reconstruction is a powerful approach allowing the resurrection of ancient enzymes based on sequences predicted by a phylogenetic analysis[1]. In this project, we predicted five ancestral enzymes (AncHLD1-5) of haloalkane dehalogenase belonging to subfamily II and representing the ancestors of the thoroughly characterized dehalogenases DbjA[2], DbeA[3], DhaA[4], DmxA[5] and DmmA[6]. Resurrected enzymes were overexpressed in *Escherichia coli*, purified to homogeneity, concentrated to 10 mg/ml and used for crystallization experiments. All crystallization trials were performed by using the sitting-drop vapor-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, 1.26 and 1.8 Å resolution, respectively. Obtained microcrystals of AncHLD1 and AncHLD4 are currently optimized by variation of enzyme concentration, pH and precipitant concentration. The structure of AncHLD2, AncHLD3 and AncHLD5 was solved by molecular replacement. The structure of haloalkane dehalogenase DbjA (PDB ID 3AFI)[7] was used as a search model. Similarly to thoroughly characterized descendant haloalkane dehalogenase enzymes, 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. The first chloride anion occupied the product-binding site and interacts with two conserved halide-stabilizing residues. The second chloride anion was located about 10 Å from the product-binding site and is buried deep in the protein core. The second halide-binding site was previously observed in the structure of DbeA, the closely related descendant counterpart of AncHLD2. Comparison of structures of ancestral enzymes with structures of descendant haloalkane dehalogenases accompanied by dynamic simulations could provide detailed insight into their catalytic properties.

**References**

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