**Crystallization of resurrected ancestral enzymes of haloalkane dehalogenase DbjA and DbeA**

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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 (Skovgaard *et al*., 2006). In this project, the sequences of representative members of haloalkane dehalogenase subfamily II were selected as targets for prediction of the common ancestor of haloalkane dehalogenase DbjA (Sato *et al.,* 2007) and DbeA (Chaloupkova *et al.,* 2014), AncHLD1, and additional ancestors corresponding to the deeper nodes of the branch leading towards the present-day enzymes, AncHLD2-5. The genes encoding predicted sequences were synthesized, expressed in *Escherichia coli* and the resurrected ancestral enzymes AncHLD1-5 were experimentally characterized. The ancestral HLDs exhibited significantly enhanced thermodynamic stability (Δ*T*m up to 24 °C) and higher specific activities with a preference for short multi-substituted halogenated substrates compared to extant enzymes. All crystallization trials were performed by using the sitting-drop vapor-diffusion method at 23 °C. The crystals of AncHLD2, 3 and 5 grew during the initial screening and no further optimization of the crystallization conditions was necessary. The triangular prism shaped crystals of AncHLD2 with dimensions 0.5 x 0.09 x 0.08 mm grew in condition No. 42 of JCSG consisting of 0.02 M magnesium chloride, 0.1 M Tris pH 8.5 and 20% (w/v) PEG 8000. The hexagonally shaped crystals of AncHLD3 with dimensions 0.2 x 0.1 x 0.04 mm appeared in condition No. 16 of the Wizard classic consisting of 100 mM potassium phosphate/sodium phosphate pH 6.2 and 2.5 M sodium chloride. The trigonal shaped crystals of AncHLD5 with average dimension 0.11 x 0.05 x 0.31 mm were observed in condition No. 73 of PEG suite containing 0.2 M magnesium acetate and 20 % (w/v) PEG 3350. These crystals were used for collection of X-ray diffraction data and complete diffraction data sets were collected at 1.66, 1.26 and 1.25 Å resolution for AncHLD2, AncHLD3, and AncHLD5, respectively. Obtained microcrystals of AncHLD1 and AncHLD4 were further optimized by variation of enzyme concentration, pH and precipitant concentration. Optimized crystals of AncHLD4 appeared within three days from the drop composed of 9.5 % (w/v) mix of PEG 1000, PEG 3350 and MPD, 0.1 M MOPS/HEPES-Na pH 7.0. On-going structural analysis of ancestral enzymes will provide insight into their unique catalytic properties.

**References**

Chaloupkova, R., Prudnikova, T., Rezacova, P., Prokop, Z., Koudelakova, T., Daniel, L., Brezovsky, J., Ikeda-Ohtsubo, W., Sato, Y.*,* Kuty, M., Nagata, Y., Kuta Smatanova, I. & Damborsky, J.(2014) Acta Cryst. D70, 1884-1897.

Sato Y., Natsume, J., Tsuda, M., Damborsky, J., Nagata, Y. & Senda, T. (2007) Acta Cryst. F63, 294-296.

Skovgaard, M., Kodra, J. T., Gram, D. X., Knudsen, S. M., Madsen, D. & Liberles D. A. (2006). *J. Mol. Biol.*, 363, 977–988.