FLUORESCENCE POLARIZATION ASSAY TO QUANTIFY BINDING OF SELECTED FLUORESCENT LIGANDS TO HALOALKANE DEHALOGENASES WITH MODIFIED TUNNELS

Shubhangi KAUSHIK[1], Jiri DAMBORSKY[1], Radka CHALOUPKOVA[1]*

[1]*Loschmidt Laboratories, Department of Experimental Biology and Research Centre for Toxic Compounds in the Environment RECETOX, Masaryk University, Brno, Czech Republic*

**radka@chemi.muni.cz*

Abstract

Fluorescence polarization (FP) is a method for rapid and non-destructive quantitative analysis of the interaction of small fluorescent ligands with a larger biomolecules in a real time [1]. Here we describe FP assay for analysis of binding kinetics of selected HaloTag ligands into haloalkane dehalogenase enzymes with modified tunnels. A set of biochemically characterized variants of haloalkane dehalogenase DhaA [2-4] and LinB [5] with modified access tunnels were studied in the present work to analyze their ability to accommodate various fluorescence ligands by FP method.

1. INTRODUCTION

Haloalkane dehalogenases are enzymes that catalyze hydrolytic cleavage of the carbon-halogen bonds in halogenated aliphatic hydrocarbons releasing a halide ion, a corresponding alcohol and a proton as the reaction products. Active sites of these enzymes are deeply buried inside the protein interior and connected with surrounding environment by access tunnels, which play important role in functionality of the enzymes [6]. In order to assess how introduced mutations in the access tunnels of haloalkane dehalogenase affect their ability to accommodate small ligands, FP method was used to determine binding kinetics of selected enzymes with various HaloTag ligands. All studied variants of haloalkane dehalogenase carry the mutation in the catalytic histidine (His272Phe) resulting in formation of an ester bond between the nucleophile and the substrate, which cannot be further hydrolyzed, providing the covalent alkyl-enzyme intermediate [2].

2. MATERIALS AND METHODS

Mutagenesis of catalytic histidine to phenylalanine at position 272 in studied haloalkane dehalogenases was carried out using QuikChange™ Site-directed mutagenesis kit (Stratagene,

USA). The nucleotide sequences of the mutants were confirmed by DNA sequencing (GATC, Germany). The genes encoding mutants of selected haloalkane dehalogenases were transformed into expression host and protein expression was induced by addition of isopropyl β-D-1-thiogalacto-pyranoside (IPTG) in culture media. The proteins were purified using metallo-affinity chromatography and their proper folding was determined by circular dichroism (CD) spectroscopy. FP analysis was conducted at room temperature using Infinite F500 plate reader (Tecan, Switzerland) equipped with polarizers for excitation and emission. The purified enzymes were reacted with selected HaloTag ligands in phosphate-buffered saline containing 0.01% CHAPS detergent to minimize the non-specific interactions. MALDI-TOF MS spectra were recorded for selected haloalkane dehalogenases on an Ultraflextreme instrument (Bruker Daltonics, Germany) operated in the linear mode with detection of positive ions to confirm formation of covalent complex between studied enzymes and HaloTag ligands.

3. RESULTS AND DISCUSSION

The constructed histidine-substituted variants of haloalkane dehalogenases with modified tunnels were successfully overexpressed in *E.coli* host cells and purified to homogeneity. CD spectroscopy in far-UV region revealed that His272Phe substitution has no effect on overall secondary structure of the enzymes. Obtained results from FP analysis indicated that the method is sufficiently sensitive to monitor differences in binding kinetics of HaloTag ligands to various haloalkane dehalogenases based on the nature of mutation present in their access tunnels. The differences in binding kinetics were detected even when a single point mutation was introduced into the access tunnel. Moreover, the binding kinetics was found to be influenced by the type of fluorescent ligand, employed during the binding reaction. The MALDI-TOF MS analysis confirmed successful binding of fluorescence ligands into haloalkane dehalogenases with large tunnel opening and no formation of covalent complexes between the ligands and haloalkane dehalogenases carrying the bulky substitutions introduced into their tunnels.

4. CONCLUSIONS

The present work used haloalkane dehalogenase as model proteins, but FP method is broadly applicable for monitoring binding kinetics with other proteins where acyl enzyme intermediate could be trapped. This method thus has a good potential for analysis of accessibility of tunnels and active sites in enzymes forming the covalent intermediates.

5. ACKNOWLEDGEMENTS

This work was supported by the Grant of the Ministry of Education of the Czech Republic (CZ.1.07/2.3.00/30.0037).

6. REFERENCES

[1] Perrin F: Journal de Physique et le Radium, 7 (1926), 390-401

[2] Los GV, et al.: ACS Chemical Biology 3 (2008), 373-382

[3] Pavlova M, et al.: Nature Chemical Biology 5 (2009), 727-733

[4] Liskova V, et al.: ChemCatChem 7 (2015), 648-659

[5] Chaloupkova R, et al.: Journal of Biological Chemistry 278 (2003), 52622-52628

[6] Prokop Z, et al.: Protein Engineering Handbook (2012), 421-464