the specific activities of all prepared enzymes were determined for twelve different substrates (1-chlorobutane, 1-chlorohexane, 1-bromobutane, 1-iodobutane, 1,2-dichloroethane, 1,2-dibromoethane, 1,3-diidopropane, 1,2-dichloropropane, 1,2,3-trichloropropane, chlorocyclohexane, bromocyclohexane and 3-chloro-2- methyl propene) and statistically analysed by Principal Component Analysis. The first and only important component explained 45.8% of the data variance. Catalytic activity of mutant protein correlated mainly with the size of amino acid introduced to the position 177.

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STUDY OF ENZYME CATALYSIS USING TRANSIENT KINETIC AND MICROCALORIMETRY TECHNIQUES

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Kinetic studies in enzymology deals with elucidation of enzymatic reaction pathway, identification of reaction intermediates and with specification of the steps that limit the rate of turnover. The kinetic analysis of an enzyme mechanism often begins by analysis of the steady-state kinetics. The steady-state kinetic parameters, K_m and k_{cat}, provide information sufficient to describe a minimal kinetic scheme. Conclusions that can be derived by steady-state analysis are considered preliminary. Because the steady-state kinetic parameters are complex functions of all the reactions occurring during enzymatic catalysis, individual reaction steps are buried within these terms and cannot be resolved. These limitations are overcome by examination of the reaction pathway by transient kinetic methods, where the enzyme is examined as a stoichiometric reactant, allowing individual steps in pathway to be established by direct measurement. Steady-state and transient-state kinetic studies complement each other in elucidation of enzymatic reaction pathway. Analysis in the steady-state should be a prelude to the proper design and interpretation for more detailed transient-state experiments [1].

Both steady-state and transient-state kinetic methods were applied to solve reaction pathway, to identify reaction intermediate and to specify the rate limiting step of catalytic action of haloalkane dehalogenase LinB from bacterial strain *Sphingomonas paucimobilis* UT26 [2]. Steady-state experiments involved direct monitoring of LinB activity by isothermal titration calorimetry and initial rate of product formation measurements by gas chromatography. Transient-state kinetics used stopped-flow fluorescence and rapid-quench-flow techniques. Additional steady-state inhibition experiments and transient-state binding experiments were employed to find out leaving ability of both products (a halide and a alcohol) during dehalogenation reaction.

The results showed that export of products as well as import of substrates into the active site of LinB are fast processes reaching rapid equilibrium. This fast exchange of the ligands between the active site and bulk solvent can be explained by wide opening of the entrance tunnel and large active site of LinB. In contrary, the release of the halide ion from narrow active site after the reaction was found to be slow rate limiting step for another haloalkane dehalogenase, enzyme DhlA from *Xanthobacter autotrophicus* GJ10 [3]. The actual cleavage of the carbon-halogen bond was found to be fast step in both enzymes.

The rate of cleavage of C-Br bound is faster than cleavage of C-Cl bound, which is in agreement with bromide being a better leaving group in biomolecular nucleophilic substitution than chloride. This observation correlates with the lower affinity of LinB for chloride compared to bromide. Further the results confirmed, that the reaction proceeds via a covalent alkyl-enzyme intermediate. Using bromocyclohexane, chlorocyclohexane and 1-chlorohexane as model substrates, hydrolysis of this intermediate was found to be the slowest step in the catalytic cycle of LinB. The alkyl-enzyme complex was highly accumulated due to the fast dehalogenation step following the slow hydrolyses of this intermediate. The study provides a basis for the analysis of kinetic steps in hydrolysis of environmentally important substrates by the action of LinB.

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