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Transhalogenation Catalysed by Haloalkane Dehalogenases Engineered to Stop Natural Pathway at Intermediate

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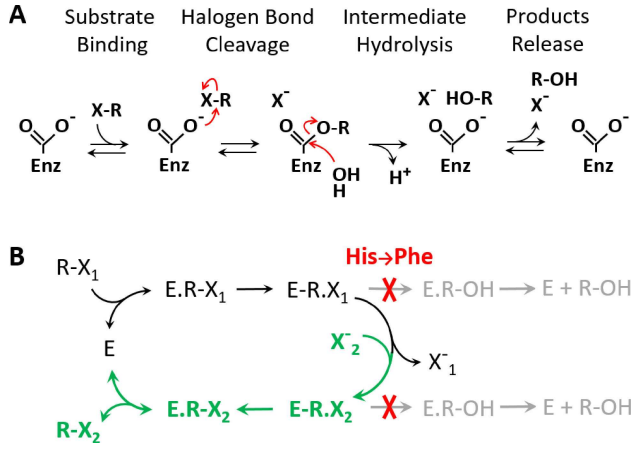
Abstract: Haloalkane dehalogenases (HLDs) are α/β-hydrolases that convert halogenated compounds to their corresponding alcohols. The overall kinetic mechanism proceeds via four steps: (i) binding of halogenated substrate, (ii) bimolecular nucleophilic substitution (S_N2) leading to the cleavage of a carbon-halogen bond and the formation of an alkyl-enzyme intermediate, (iii) nucleophilic addition of a water molecule resulting in the hydrolysis of the intermediate to the corresponding alcohol and (iv) release of the reaction products – an alcohol, a halide ion and a proton. Although, the overall reaction has been reported as irreversible, several kinetic evidences from previous studies suggest the reversibility of the first S_N2 chemical step. To study this phenomenon, we have engineered HLDs to stop the catalytic cycle at the stage of the alkyl-enzyme intermediate. The ability of the intermediate to exchange halides was confirmed by a stopped-flow fluorescence binding analysis. Finally, the transhalogenation reaction was confirmed with several HLDs and 2,3-dichloropropene in the presence of a high concentration of iodide. The formation of the transhalogenation product 3-iodo-2-chloropropene catalysed by five mutant HLDs was identified by gas chromatography coupled with mass spectrometry. Hereby we demonstrated the reversibility of the cleavage of the carbon-halogen bond by HLDs resulting in a transhalogenation. After optimization, the transhalogenation reaction can possibly find its use in biocatalytic applications. Enabling this reaction by strategically engineering the enzyme to stop at an intermediate in the catalytic cycle that is synthetically more useful than the product of the natural pathway is a novel concept.

Keywords: enzyme catalysis; halide exchange; haloalkane dehalogenase; nucleophilic substitution; redirection of reaction; transhalogenation

The field of protein engineering benefits from directed evolution when the structural information about the target enzyme is limited and the structure-function relationships are not sufficiently understood. A recent trend tends to prioritize a semi-rational design to minimize the library size and the screening effort. This greatly accelerates the discovery of novel biocatalysts for biomedical and pharmaceutical applications. However, to achieve a good rational design, a detailed understanding of the structural basis related to enzyme function is critical. Model enzymes are of great value for the investigation of molecular mechanisms of enzyme catalysis as well as the computational analysis of chemical concepts. One class of enzymes, which is recognized as an interesting model system, are haloalkane dehalogenases (HLDs; EC 3.8.1.5).[1] HLDs catalyse the cleavage of a carbon-halogen bond of organohalogen compounds by nucleophilic substitution, followed by the hydrolysis of the alkyl-enzyme intermediates producing the corresponding alcohols (Scheme 1A).

The kinetic mechanism of three representative HLDs has been studied in detail, DhlA from *Xanthobacter autotrophicus* GJ10,[3] DhaA from *Rhodococcus rhodochrous* NCIMB 13064[4] and LinB from *Sphingobium japonicum* UT26.[5] For LinB, kinetic signs for the reversibility of the first chemical step (S_N2) could be observed. To study this phenomenon, we have investigated the formation of the product of the reversed reaction by performing the conversion of halogenated substrates in the presence of a high



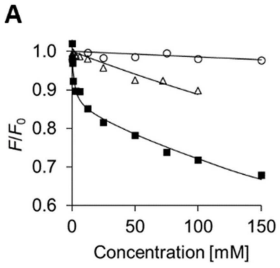
Scheme 1. Catalytic mechanisms of HLDs and their engineered variants. (**A**) The reaction mechanism of HLDs (adapted from Verschueren *et al.*).[2] (**B**) The kinetic mechanism of transhalogenation ($[X^-_2] \gg [X^-_1]$) by engineered HLDs. Substitution of the catalytic histidine prevents the hydrolytic step (red cross). Halogenated substrate R–X, enzyme-substrate complex E.R–X, complex of alkyl-enzyme intermediate with bound halide ion E–R.X, enzyme-product complex E.R-OH and halide ion X^- (X represents Cl, Br or I).

concentration of different halides. This design is based on the ability of the alkyl-enzyme intermediate to exchange the halide anion released from the substrate by a different ion, which is added to the reaction mixture in a large excess. In the case that the alkyl-enzyme intermediate undergoes the reversed reaction, the corresponding transhalogenated compound should be detectable in the reaction mixture (Scheme 1B).

The wild type HLDs DbeA from *Bradyrhizobium elkani* USDA94[6] and DbjA from *Bradyrhizobium japonicum* USDA110[7] were selected for the reversibility tests since the large opening of their active sites makes the exchange of halides for the enzyme in the state of the alkyl-enzyme intermediate most likely. Gas chromatography coupled with mass spectrometry (GC-MS) was used to monitor the conversion of 1-bromobutane with DbeA and DbjA in the presence of a large excess (1 M) of chloride ions. Both wild type enzymes catalysed the formation of the corresponding alcohol, however, the transhalogenation product 1-chlorobutane was not detected (Supplementary Figure S1). This result is consistent with a previous kinetic analysis indicating that hydrolysis is significantly faster in comparison to the possible halide release from the alkyl-enzyme intermediate or the rate of the reversed reaction.[5]

To maximize the chance of the reversed reaction at the stage of the alkyl-enzyme intermediate, we have constructed the variant DbjA H280F which lacks the catalytic histidine. In such an HLD mutant,[8] the hydrolytic step is prevented and the alkyl-enzyme intermediate is trapped, which provides enough time for a halide exchange and a subsequent transhalogenation (Scheme 1B). Since the reaction of this mutant with 1-bromobutane in the presence of chloride still did not produce the transhalogenation product, the halide exchange appeared to be the limiting step.

The binding of chloride, bromide and iodide was studied in detail with DbjA H280F by using the stopped-flow fluorescence analysis. All the obtained fluorescence values showed that the equilibrium was reached rapidly, confirming that there is no kinetic limitation for the halide interaction with the enzyme (Supplementary Figure S2). Both chloride and bromide showed a nonspecific fluorescence quenching at increasing concentrations, reflecting the role of these anions as general fluorescence quenchers. The fluorescence profile with iodide showed a strong additional quenching activity, suggesting a specific binding to DbjA H280F (Figure 1).



B

	K_Q M^{-1}	K_a mM^{-1}
chloride	0.15 ± 0.09	-
bromide	1.3 ± 0.2	-
iodide	2.0 ± 0.2	0.6 ± 0.4

Figure 1. Halide binding to the engineered enzyme DbjA H280F. (**A**) Dependence of amplitude of rapid equilibrium fluorescence quenching recorded upon mixing of DbjA H280F with chloride (open circle), bromide (open triangle) and iodide (black square). (**B**) The apparent association equilibrium constants of the non-specific quenching (K_Q) and the specific binding (K_a) of halides were calculated by using extended Stern-Volmer equation.

The specific binding of iodides was confirmed also for the alkyl-enzyme intermediates after the reaction of DbjA H280F with 1,2-dibromoethane and 1-chlorobutane (Supplementary Figure S3). The equilibrium dissociation constants for the specific binding of iodide ($K_d = 1/K_a$) calculated from the fluorescence analysis were 2 ± 1, 200 ± 100 and 40 ± 20 mM for free DbjA H280F, and alkyl-enzyme intermediate after the reaction with 1,2-dibromoethane and 1-chlorobutane, respectively.

After we confirmed the specific binding of the iodide to DbjA H280F in both free and intermediate states, the transhalogenation of 1-chlorobutane, 1,2-dibromoethane, bis(2-chlorethyl)ether and 2,3-dichloropropene to their corresponding iodinated analogues was tested (Figure 2, Supplementary Table S1).

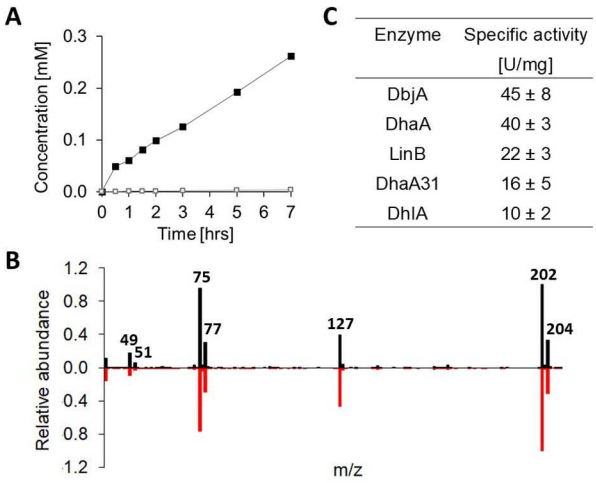


Figure 2. Transhalogenation reaction catalysed by engineered HLDs. (A) Formation of the transhalogenation product 3-iodo-2-chloropropene from 2,3-dichloropropene and iodide catalysed by the variant DbjA H280F. (■) with DbjA H280F (□) without DbjA H280F. **(B)** MS spectrum of the product of the transhalogenation reaction (black) and 3-iodo-2-chloropropene standard (red). The match of both MS spectra is 98%. (**C**) Specific activities of different HLDs with mutation in the catalytic histidine for the conversion of 2,3-dichloropropene to 3-iodo-2-chloropropene.

The formation of the iodinated analogue was observed only in the reaction of DbjA H280F with 2,3-dichloropropene (Figure 2A). To identify the transhalogenated product, we applied GC-MS (Figure 2B) followed by a fragmentation analysis and an analysis of isotopic clusters with the assistance of the Mass Frontier 1.0 software (HighChem, Slovak Republic). The heaviest ion at the mass-to-charge ratio (m/z) 202 is corresponding to the molecular weight of 3-iodo-2-chloropropene (C_3H_4ICl). The isotopic cluster at m/z 202–204 with an abundance of 100/31 (M/(M+2)) matches the isotopic ratio 100/32 predicted by Mass Frontier for the presence of one chlorine and one iodine atom in the molecule. The m/z 127 represents iodine with the characteristic monoisotopic abundance. The m/z 75 (C_3H_4Cl) represents the rest of the molecule after the cleavage of iodine. The m/z 49 represents the fragment CH_2Cl produced from m/z 75 by the cleavage of C_2H_2. Both clusters m/z 75–77 and m/z 49–51 showed an isotopic abundance characteristic for the presence of one chlorine in the molecule. Additionally, the MS spectrum of the transhalogenated product was compared to an MS spectrum obtained for a commercially synthesized chemical standard of 3-iodo-2-chloropropene with 98% match (CF Plus Chemicals, Czech Republic).

Finally, the conversion of 2,3-dichloropropene to 3-iodo-2-chloropropene was tested with a set of selected HLDs carrying the mutation in the catalytic histidine (DbjA H280F, DhaA H272F, LinB H272F, DhaA31 H272F and DhlA H289F).[9] All the tested dehalogenases carrying the mutation in the catalytic histidine were able to catalyse the transhalogenation of 2,3-dichloropropene in the presence of iodides, even though the overall reactions were slow. The most efficient formation of 3-iodo-2-chloropropene was observed with the engineered variants of DbjA and DhaA, followed by LinB and DhaA31. The lowest activity was shown with the DhlA variant (Figure 2C), very likely due to its closed active site cavity. This view is supported by the difference in the rates of DhaA and DhaA31, the latter mutant possessing a more occluded active site cavity.[10]

In summary, the collected data provides a direct experimental evidence for the reversibility of the first chemical step (S_N2) of the catalytic mechanism of HLDs. These observations could pave the way for a potential new chemistry and reactions to synthetically more useful products catalysed by HLDs by strategically stopping the mechanistic cycle at the alkyl-enzyme intermediate. However, additional research and further optimization would be needed to make the enzymatic transhalogenation more efficient.

Experimental Section

Construction of DbjA H280F and DhlA H289F, protein expression and purification. The recombinant genes *dbjA H280F* and *dhlA H289F* were obtained by site-directed mutagenisis. Expression of the HLDs was achieved using *E. coli* BL21(DE3) containing the constructed expression vectors. LB medium with ampicillin (1 L) was inoculated with 5 mL of a freshly grown preculture. The bacteria were grown at 37 °C, until the optical density measured at 600 nm reached approximately 0.6. Then the cultures were cooled to 20 °C, 0.5 mM IPTG was added, and growth continued at 20 °C. After overnight growth, cells were harvested using centrifugation for 10 min at 8000 rpm. A washing step was carried out using approximately 125 mL of washing buffer. Cells were harvested by centrifugation (10 min at 8000 rpm). Cell pellets were resuspended in 40 mL of purification buffer A and then frozen at −80 °C. Cell disruption was achieved by sonication (4× 8 min, 0.3 cycle, 85 amplitude). The crude extract was centrifuged (20–60 min at 14000 rpm) to remove the cell debris. The cell-free extracts were filtered through a Rotilabo-Spritzenfilter (0.45 µm) to remove remaining cell debris. Samples were purified using metal affinity chromatography with a small adaptation of stepwise increased imidazole concentration for washing (10 mL of 4%, 10 mL of 6%, and 15 mL of 10%). Finally, imidazole was removed by a double-dialysis step in 50 mM phosphate buffer, pH 7.5. Protein samples were filtered through a sterile 0.22 µm Rotilabo-Spritzenfilter for storage. The concentration of the prepared enzyme solutions was determined in triplicate using Bradford reagent, and the purity was checked by SDS-PAGE.

Conversion of 1-bromobutane by DbeA wt, DbjA wt and DbjA H280F in presence of chloride. Dehalogenation reac-

tions were performed at 37 °C in 25 ml Reacti Flasks closed by Mininert Valves. The reaction mixtures were prepared by mixing of 15 ml of 100 mM glycine buffer containing 0.8 M NaCl (pH 8.6) and 1-bromobutane as a substrate to a final concentration of 9.3 mM. The reactions were initiated by addition of 300 µl enzyme in a concentration of 0.78 mg/ml (DbeA wt), 0.69 mg/ml (DbjA), or 1.10 mg/ml (DbjA H280F). Buffer (300 µl) instead of enzyme was added to the reaction mixture as a control. The reactions were monitored by taking 1 ml samples at 5, 10, 15, 20, 25, 30, 40, 50 and 60 minutes from the reaction mixtures. The reaction was stopped by addition of 0.5 ml diethylether containing 1,2-dichloroethane as an internal standard to the reaction mixture. After extraction, samples were analysed using a gas chromatograph (Agilent 7890, USA) equipped with the capillary column DB-FFAP (30 m × 0.25 mm × 0.25 µm, Phenomenex) and connected with a mass spectrometer (Agilent 5975 C, USA). The 5 µl of sample were injected into a Split-Splitless inlet at 250 °C, with split ratio of 1:20. The temperature programme was isothermal at 40 °C for 1 min, followed by an increase to 140 °C at 20 °C/min. The flow of carrier gas (He) was 1.2 ml/min. The MS was operated at SCAN mode (30 to 240 amu). The temperatures of the ion source and GC-MS interface were 230 °C and 250° C, respectively.

Stopped-flow fluorescence analysis. The binding of halides to the free DbjA H280F or its enzyme-alkyl intermediates was performed with the stopped-flow instrument SFM-300 (Bio-Logic, France) combined with MPS-70. Fluorescence emission from tryptophan residues was observed through a 320 nm cut-off filter upon excitation at 295 nm. The reactions were performed at 37 °C in a glycine buffer pH 8.6. The enzyme-alkyl intermediate was prepared by mixing the enzyme with a solution of 1 mM 1-chlorbutane or 5 mM 1,2-dibromoethane in 50 mM glycine buffer pH 8.6 and a subsequent incubation at room temperature for 10 minutes. Substrate that was not converted by the enzyme was removed by evaporation. Final concentration of the enzyme in the solution was 0.25 mg/ml. The chloride and bromide data were fitted to Stern-Volmer quenching equation:

$$F/F_0 = \frac{1}{1 + K_Q.[X^-]}$$

where, F/F_0 is the relative fluorescence, and K_Q is the quenching constant (which is the apparent association equilibrium constant of the non-specific quenching interaction between halide (X^-) and enzyme. For the iodide the extended Stern-Volmer was used:

$$F/F_0 = \frac{1}{1 + K_Q.[X^-]} \cdot \frac{1 + f.K_a.[X^-]}{1 + K_a.[X^-]}$$

where, f is the fluorescence level of the halide-bound enzyme complex, and K_a is the association equilibrium constant of the specific binding of the halide to the active site. Data fitting was performed by using the software Origin 6.1 (OriginLab, USA).

Reaction of selected substrates with engineered HLD variants in the presence of iodide. Transhalogenation reactions were performed at 37 °C in 25 ml Reacti Flasks closed by Mininert Valves. The reaction mixtures were prepared by mixing 5 ml of 100 mM glycine buffer containing 200 mM KI (pH 8.6) and 3–12 mM substrate. The reactions were initiated by addition of 5 ml enzyme in a concentration of 5 mg/ml. Buffer instead of enzyme was added for the controls. The reactions were monitored by taking 0.8 ml samples from the reaction mixtures. The reaction was stopped by gentle vortexing of the reaction mixture with 1.6 ml diethylether containing 1,2-dichloroethane as an internal standard. After extraction, the samples were analysed using a gas chromatograph (Agilent 7890, USA) equipped with the capillary column ZB-5 (30 m × 0.25 mm × 0.25 µm, Phenomenex) and connected with a mass spectrometer (Agilent 5975C, USA). For analysis, 1 µl of the sample was injected into a Split-Splitless inlet at 250 °C, with a split ratio of 1:20. The temperature programme was isothermal at 40 °C for 1 min, followed by an increase to 250 °C at 20 °C/min. The flow of carrier gas (He) was 1.2 ml/min. The MS was operated at SCAN mode (15 to 250 amu). The temperature of the ion source and GC-MS interface was 230 °C and 250 °C, respectively.

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