Enantioselectivity of Haloalkane Dehalogenases and its Modulation by Surface Loop Engineering**

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Dedicated to Dr. Alfred Bader on the occasion of his 85th birthday

Enzymes are widely used for the synthesis of pharmaceuticals, agrochemicals, and food additives because they can catalyze enantioselective transformations.[1] Understanding the molecular basis of enzyme–substrate interactions that contribute to enantioselectivity is important for constructing selective enzymes by protein engineering.[2] Up to now, emphasis has been on reactions such as lipase- or esterase-based kinetic resolutions,[2,3] as well as lyase-, aminotransferase- and ketoreductase-mediated conversions.[1a,4] An emerging group of enzymes that is explored for enantioselectivity is dehalogenases. Haloalkane dehalogenases can convert a broad range of halogenated aliphatic substrates to their corresponding alcohols by an S_{2}2 mechanism (Scheme 1),[5] and because of the simplicity of the reaction represent a good model system to study the structural basis of reactivity[6] and enantioselectivity.

![Scheme 1. Reaction mechanism of haloalkane dehalogenases with α-bromoesters and β-bromoalkanes. Enz-COO⁻ : active site Asp.

However, only a weak enantioselectivity (enantiomeric ratio, E value < 9)[7] has been reported with haloesters and 1,2- and 1,3-dihaloalkanes for the haloalkane dehalogenases from Xanthobacter autotrophicus (DhlA)[8] and Rhodococcus rhodochrous NCIMB13064 (DhaA). To further understand the enantioselectivity of these enzymes, we explored several dehalogenases for which the X-ray structure is available. This includes DhaA, LinB from Sphingobium japonicum UT26,[10] and DbjA from Bradyrhizobium japonicum USDA110.[11] Kinetic resolution of an expanded set of racemic substrates was analyzed with recombinant proteins, and it revealed that DhaA, LinB, and DbjA possess excellent enantioselectivity for α-bromoesters (Table 1). Furthermore, DbjA showed high enantioselectivity with two β-bromoalkanes.

The steady-state kinetics of DbjA determined with (R)- and (S)-2-bromopentane showed a large difference in Michaelis constants $K_{m}$ (24 and 570 μM, respectively) and similar catalytic constants $k_{cat}$ (0.36 and 0.27 s⁻¹), which indicates that enantioselectivity in this case is mainly the result of substrate binding. The high enantioselectivity of DbjA allowed use of the enzyme for kinetic resolution of 2-bromopentane on a preparative scale. Incubation of racemic substrate (7 g) in a 4:1 mixture of Tris buffer (24 L, 50 mM, pH 8.2) and dimethyl sulfoxide with DbjA enzyme (240 mg as substrate (7 g) in a 4:1 mixture of Tris buffer (24 L, 50 mM, pH 8.2) and dimethyl sulfoxide with DbjA enzyme (240 mg as

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results of thermodynamic and mutagenesis analysis indicate that the enantioselective reactions with 2-bromopentane and methyl 2-bromobutyrate are controlled by different molecular bases.

Next, we tried to link these molecular bases to three-dimensional structures of wild-type and mutant DbjA by molecular modeling. Both enantiomers of 2-bromopentane bind along the same wall of the active-site pocket, and adopt a mirror-image orientation with displaced chiral centers (Figure 2c). This binding is characterized by hydrophobic interactions between the alkyl chain of the substrate and the hydrophobic wall, and by two hydrogen bonds between the bromine atom and the side chains of the halide-stabilizing residues Trp104 and Asn38. During the molecular dynamics simulation, the $R$ enantiomer was sampled exclusively in a reactive binding mode, while ($S$)-2-bromopentane adopted a mixture of reactive and nonreactive binding modes. His139 appeared to modulate the distribution of reactive configurations among these binding modes by its interaction with the substrate molecule (Figure 2c, Supporting Information Figure 1). The simulations showed that the inclined conformation of His139 in DbjA increases reactivity with the $R$ enantiomer and increases reactivity with the $S$ enantiomer (Supporting Information Figure 3 and Table 3), which results in reduced enantioselectivity of this mutant with 2-bromopentane. The effect of the mutation is opposite for the two enantiomers because of the different location of their chiral centers (Figure 2c).

In contrast to what was observed with 2-bromopentane, the enantiomers of methyl 2-bromobutyrate appeared to bind in different orientations with their chiral centers aligned and the two substituting alkyl groups pointing towards different sides of the active site (Figure 2c). These orientations are stabilized by three hydrogen bonds: two between bromine and the side chains of halide-stabilizing residues, and one between the substrate carbonyl group and the side chain of Asn38 or Trp104 for the $R$ and $S$ enantiomer, respectively (Figure 2c, Supporting Information Figure 1). Hydrophobic interactions with the wall of the active-site pocket are less important for methyl 2-bromobutyrate than for 2-bromopentane. The binding free energies calculated for these binding modes favor binding of the $R$ enantiomer over the $S$ enantiomer, irrespective of the protein variant, because of better conformity of the $R$ enantiomer with the active site. The discrimination against the
Senantiomer is further reinforced in the chemical step of the reaction (Supporting Information Table 3). The inclined conformation of His139 in DbjA decreases reactivity with both enantiomers since their chiral centers are spatially aligned. The magnitude of this effect is larger for the $S$ enantiomer, thus resulting in increased enantioselectivity with methyl 2-bromobutyrate (Supporting Information Figure 2 and Table 3).

Our analysis of DbjA enantioselectivity demonstrates that different molecular bases underlie the enantioselective conversion of methyl 2-bromobutyrate and 2-bromopentane (Figure 2c). Furthermore, the enantioselectivity of DbjA can be modulated by mutation at the surface loop region. Assuming that the inclined conformation of His139 in DbjA significantly reduces the volume of the active-site pocket, substitution of His139 by Ala should restore the original enzyme enantioselectivity. Indeed, the enantioselectivity of the DbjA+His139Ala mutant was reconstituted for both substrates (Figure 2a,b). The effects of the mutations were stronger for 2-bromopentane than for methyl 2-bromobutyrate because of different binding orientations and the distinct nature of the interactions involved in their enantiodiscrimination.

In conclusion, we have shown that haloalkane dehalogenases: 1) can kinetically discriminate between enantiomers of two distinct groups of substrates, $\alpha$-bromoesters and $\beta$-bromoalkanes; 2) have enantioselectivity based on distinct molecular interactions, which can be modified separately by engineering of a surface loop; and 3) can adopt an inverse temperature dependence of enantioselectivity for $\beta$-bromoalkanes, but not $\alpha$-bromoesters, by mutating this surface loop and a flanking residue. Our study contributes towards understanding of the molecular basis and thermodynamics of the enantioselectivity of enzymes,[18] and opens up new possibilities for constructing enantioselective biocatalysts by protein engineering.

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[2] a) R. J. Kazlauskas, *Trends Biotechnol.* **1994**, 12, 464–472; b) W. V. Tuomi, R. J. Kazlauskas, *J. Org. Chem.* **1999**, 64, 2638–2647; c) D. Rotticci, J. C. Rotticci-Mulder, S. Denman, T. Norin, K. Hult, S enantiomer is further reinforced in the chemical step of the reaction (Supporting Information Table 3). The inclined conformation of His139 in DbjA decreases reactivity with both enantiomers since their chiral centers are spatially aligned. The magnitude of this effect is larger for the $S$ enantiomer, thus resulting in increased enantioselectivity with methyl 2-bromobutyrate (Supporting Information Figure 2 and Table 3). Our analysis of DbjA enantioselectivity demonstrates that different molecular bases underlie the enantioselective conversion of methyl 2-bromobutyrate and 2-bromopentane (Figure 2c). Furthermore, the enantioselectivity of DbjA can be modulated by mutation at the surface loop region. Assuming that the inclined conformation of His139 in DbjA significantly reduces the volume of the active-site pocket, substitution of His139 by Ala should restore the original enzyme enantioselectivity. Indeed, the enantioselectivity of the DbjA+His139Ala mutant was reconstituted for both substrates (Figure 2a,b). The effects of the mutations were stronger for 2-bromopentane than for methyl 2-bromobutyrate because of different binding orientations and the distinct nature of the interactions involved in their enantiodiscrimination.

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Enantioselectivity of Haloalkane Dehalogenases and its Modulation by Surface Loop Engineering

In the loop: Engineering of the surface loop in haloalkane dehalogenases affects their enantiodiscrimination behavior. The temperature dependence of the enantioselectivity ($\ln E$ versus $1/T$) of $\beta$-bromoalkanes by haloalkane dehalogenases is reversed (red data points) by deletion of the surface loop; the selectivity switches back when an additional single-point mutation is made. This behavior is not observed for $\alpha$-bromoesters.