

Different Structural Origins of the Enantioselectivity of Haloalkane Dehalogenases toward Linear β -Haloalkanes: Open–Solvated versus Occluded–Desolvated Active Sites

Veronika Liskova⁺, Veronika Stepankova⁺, David Bednar, Jan Brezovsky, Zbynek Prokop, Radka Chaloupkova, and Jiri Damborsky*

Abstract: The enzymatic enantiodiscrimination of linear β -haloalkanes is difficult because the simple structures of the substrates prevent directional interactions. Herein we describe two distinct molecular mechanisms for the enantiodiscrimination of the β -haloalkane 2-bromopentane by haloalkane dehalogenases. Highly enantioselective DbjA has an open, solvent-accessible active site, whereas the engineered enzyme DhaA31 has an occluded and less solvated cavity but shows similar enantioselectivity. The enantioselectivity of DhaA31 arises from steric hindrance imposed by two specific substitutions rather than hydration as in DbjA.

Enzymatic catalysis is a powerful tool for preparing optically pure chemicals.^[1–4] However, enzyme engineering is often required to develop biocatalysts that are enantioselective with non-natural substrates. Computational methods can increase the effectiveness of engineering efforts,^[5–10] but even when such methods are applied, an understanding of the factors that govern the interactions of the active site with enantiomers is essential for rational engineering.

Haloalkane dehalogenases (HLDs; EC 3.8.1.5) catalyze the hydrolytic cleavage of carbon–halogen bonds by an S_N2 mechanism (see Figure 1 in the Supporting Information).^[11,12] They are useful for the enantiodiscrimination of β -bromoalkanes, α -bromoesters, and α -bromoamides,^[13–16] thus yielding a variety of enantiomerically pure intermediates that are useful for the synthesis of drugs and other bioactive compounds.^[14,17,18] HLDs are good model systems for studying the principles of enantioselectivity because individual family members show different enantioselectivity and their 3D structure has been determined at atomic resolution. There

has been particular interest in DbjA from *Bradyrhizobium japonicum*^[19] and DhaA from *Rhodococcus rhodochrous*,^[20] which belong to the same subfamily but show different enantioselectivity toward β -bromoalkanes.^[13,21] Whereas DbjA is highly enantioselective ($E = 174$) toward racemic 2-bromopentane (2-BP), DhaA exhibits low enantioselectivity ($E = 18$).^[13] Analysis of the crystal structures revealed that the DbjA active site is more open and solvent-accessible than that of DhaA. On the assumption that the active-site geometry is crucial for substrate recognition, it was proposed that the enantioselectivity of DbjA could be transferred to DhaA by transplanting the active site of the former enzyme into the latter. To this end, eight point mutations and an 11 amino acid insertion were introduced into DhaA to create the mutant DhaA12, whose active site and access tunnel are almost identical to those of DbjA at the atomic level (Figure 1). Although the transplantation yielded a correctly folded and functional enzyme, its enantioselectivity was low. This low enantioselectivity was attributed to differences in dynamics and hydration, which are very difficult to engineer rationally.^[21]

Whereas the systematic reengineering of DhaA did not increase enantioselectivity, another DhaA variant created by semirational engineering aimed at enhancing activity with toxic 1,2,3-trichloropropane, DhaA31,^[22] exhibits excellent enantioselectivity toward 2-BP ($E = 179$), which is remarkable because DhaA31 is less structurally similar to DbjA than DhaA (Figure 1). DhaA31 bears five point mutations (I135F, C176Y, V245F, L246I, and Y273F) that insert large aromatic side chains into its access tunnels, thus narrowing them relative to DhaA and occluding the active site. Its level of active-site hydration, which is considered important for the enantioselectivity of DbjA,^[21] is far lower than in that enzyme, thus suggesting a different structural basis for enantioselectivity toward 2-BP. Herein we present a systematic study on the molecular basis of enantioselectivity in DbjA, DhaA, and DhaA31 involving thermodynamic analysis, steady- and pre-steady-state kinetics, site-directed mutagenesis, and molecular modeling.

The enthalpic and entropic contributions to enantioselectivity can be determined by studying the temperature dependence of the enantioselectivity.^[23] Such experiments with DhaA31 and DhaA revealed that mutations in the former enzyme significantly increased the enthalpic contribution (Table 1; see also Figure 2 in the Supporting Information). The preferential conversion of the *R* enantiomer by DhaA31 is thus due to a high differential activation enthalpy, which

[*] V. Liskova,^[†] Dr. V. Stepankova,^[†] D. Bednar, Dr. J. Brezovsky, Dr. Z. Prokop, Dr. R. Chaloupkova, Prof. J. Damborsky
Loschmidt Laboratories, Department of Experimental Biology and RECETOX, Faculty of Science, Masaryk University
Kamenice 5, 625 00 Brno (Czech Republic)
and
International Clinical Research Center, St. Anne's University Hospital
Pekarska 53, 656 91 Brno (Czech Republic)
E-mail: jjiri@chemi.muni.cz
Dr. V. Stepankova^[†]
Enantis s.r.o.
Kamenice 34, 625 00 Brno (Czech Republic)

[†] These authors contributed equally.

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under:
<http://dx.doi.org/10.1002/anie.201611193>.

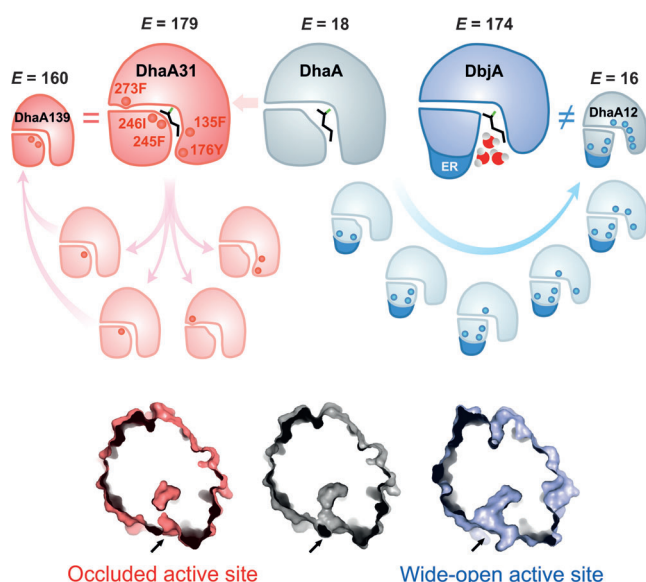


Figure 1. Structural bases of HLD enantioselectivity toward linear β -haloalkanes. Top right: Transplantation of the 11 amino acid extra region (ER) and 8 point mutations from highly enantioselective DbjA (which has an open, solvent-accessible active site) into weakly enantioselective DhaA yields weakly enantioselective DhaA12. The enantioselectivity of DbjA results from water-mediated interactions of the 2-bromopentane (2-BP) alkyl chain with the hydrophobic wall of the active site,^[13] which are difficult to manipulate rationally. Top left: The introduction of five point mutations into DhaA yields strongly enantioselective DhaA31, whose active site is occluded and not solvent-accessible. DhaA139 bearing only two of these mutations retains high enantioselectivity with a much more occluded and less open active site than that of similarly enantioselective DbjA. The enantioselectivity of DhaA31 and DhaA139 is due to interactions of the hydrophobic substrate with the occluded active site, which force (*R*)-2-BP into a reactive configuration. Bottom: Cross-sections of the crystal structures of DhaA31 (red; PDB ID: 3RK4), DhaA (gray; PDB ID: 4HZG), and DbjA (blue; PDB ID: 3A2M), highlighting the differences in the solvent-accessibility of their active sites. The molecular surface of each enzyme is displayed, and black arrows indicate the entrances to the active sites. DhaA exhibits 51% sequence identity with DbjA and a root-mean-square deviation (RMSD) of C α atoms 0.54 Å; whereas DhaA and DhaA31 share 98% sequence identity with RMSD of C α atoms 0.09 Å.

Table 1: Enantioselectivity values (*E*) and thermodynamic components for the kinetic resolution of racemic 2-BP.

Enzyme	$E^{293\text{K}}$	$E^{313\text{K}}$	$\Delta_{R-S}\Delta H^\ddagger$ [kJ mol ⁻¹]	$T\Delta_{R-S}\Delta S^\ddagger^{+293\text{K}}$ [kJ mol ⁻¹]	$\Delta_{R-S}\Delta G^\ddagger^{+293\text{K}}$ [kJ mol ⁻¹] ^[a]
DhaA	18	13	-15.4	-8.1	-7.2
DhaA31	179	17	-91.6	-78.6	-13.0
DbjA ^[b]	174	28	-69.5	-56.8	-11.7

[a] $\Delta_{R-S}\Delta G^\ddagger^{+293\text{K}}$ stands for differential free energy of activation between the *R* and *S* enantiomers of 2-BP ($\Delta_{R-S}\Delta G^\ddagger^{+293\text{K}} = \Delta_{R-S}\Delta H^\ddagger - T\Delta_{R-S}\Delta S^\ddagger^{+293\text{K}}$) and its enthalpic ($\Delta_{R-S}\Delta H^\ddagger$) and entropic ($T\Delta_{R-S}\Delta S^\ddagger^{+293\text{K}}$) terms. [b] The values for DbjA were reported previously by Prokop et al.^[13]

may be related to differences in the orientation of the enantiomers in the transition state. A similar explanation for the enantioselective conversion of 2-BP was proposed for DbjA. The entropic component (which is around 83% as

large as the enthalpic component) also contributes significantly to the enantioselectivity of DbjA.^[13] The large entropic component is attributed to the high hydration of the binding site.^[21] Water promotes hydrophobic interactions between a wall in the active site and the alkyl chains of the enantiomers in such a way that (*R*)-2-BP binds exclusively in a reactive mode but (*S*)-2-BP adopts both reactive and nonreactive binding modes.^[13] The entropic contribution for weakly enantioselective, less hydrated DhaA is much smaller.^[24] If the DhaA and DhaA31 active sites have similar levels of hydration, they should have similar entropic contributions. However, the entropic component for DhaA31 is large (86% of the size of the enthalpic component), like that of DbjA, but unlike that of DhaA. The differential activation entropy is a complex term covering differences between the conformations of enantiomers inside the protein, losses in conformational degrees of freedom of the substrate, and differential solvation effects.^[25] The contribution of entropy to the enantiodiscrimination of 2-BP by DhaA31 is unlikely to be connected with differences in hydration, given the low accessibility of the active site to water, but it is rather due to differences in the spatial freedom of (*R*)- and (*S*)-2-BP inside the active site (Figure 1). The greater differential activation entropy of DhaA31 as compared to DhaA indicates a more rigid transition state for the preferred enantiomer and greater dependence of the active-site flexibility on the temperature.^[25]

Steady-state kinetic parameters were measured for the hydrolysis of the separated enantiomers of 2-BP.^[13] The kinetic data were fitted by using a competitive steady-state model (Table 2). The contribution of substrate inhibition was included only for the *R* enantiomer; substrate inhibition was not observed with the *S* enantiomer. Steady-state kinetics revealed that DhaA31 exhibits similar k_{cat} values for both enantiomers, but that its K_m value for the *R* enantiomer is 155-fold lower than for the *S* enantiomer. A similar trend has been reported for DbjA,^[13] whereas weakly enantioselective DhaA has very similar K_m values for both enantiomers, thus implying that both DhaA31 and DbjA are enantioselective primarily owing to differences in their K_m values for the two enantiomers (Table 2). The K_m value is the ratio of the maximum rate of decomposition of the enzyme–substrate complex (which corresponds to the S_N2 reaction in HLDs)^[11] to the apparent rate of substrate binding.^[26]

We performed pre-steady-state rapid-quench burst and stopped-flow fluorescence analyses of DhaA31 and DbjA with the separated enantiomers to determine whether their enantioselectivity toward 2-BP was governed by substrate binding or the subsequent S_N2 reaction. The kinetic mechanisms of DhaA31 and DbjA are similar. Global kinetic analysis (see Figure 3 in the Supporting Information) indicated that hydrolysis of the alkyl–enzyme intermediate (k_3 and k_7) is rate-limiting for all reaction pathways (see Table 1 and Figures 4–5 in the Supporting Information). However, neither the rate-limiting step nor substrate binding contributes substantially to the enantiodiscrimination of 2-BP by DhaA31 and DbjA. Instead, enantiodiscrimination arises primarily from carbon–halogen bond cleavage (k_2 and k_6). The ratios of the rates of this process for (*R*)- versus (*S*)-2-BP

Table 2: Steady-state kinetic parameters for the hydrolysis of separated (*R*)- and (*S*)-2-BP at 20 °C and pH 8.6. Each value is represented as the mean \pm standard error (lower limit; upper limit).^[a]

Enzyme	<i>(R)</i> -2-BP			<i>(S)</i> -2-BP	
	k_{cat} [s ⁻¹]	K_{m} [mM]	K_{si} [mM]	k_{cat} [s ⁻¹]	K_{m} [mM]
DhaA	0.338 \pm 0.002 (0.336; 0.498)	0.0110 \pm 0.0002 (0.0063; 0.0149)	4.01 \pm 0.20 (0.80; 7.70)	0.044 \pm 0.001 (0.044; 0.044)	0.0159 \pm 0.0008 (0.0107; 0.0201)
DhaA31	0.036 \pm 0.001 (0.035; 0.041)	0.00011 \pm 0.00003 ($<$ 0.00001; ^[b] 0.00084)	5.10 \pm 0.02 (2.09; 5.13)	0.036 \pm 0.001 (0.036; 0.036)	0.017 \pm 0.002 (0.014; 0.018)
DbjA	0.269 \pm 0.001 (0.267; 0.298)	0.0100 \pm 0.0001 (0.0036; 0.0173)	1.42 \pm 0.01 (1.10; 1.45)	0.55 \pm 0.02 (0.35; 1.04)	1.28 \pm 0.06 (0.66; 2.92)

[a] Data were analyzed by using a competitive steady-state model with substrate inhibition for the *R* enantiomer. [b] The lower limit could not be precisely calculated; confidence contour analysis provided the value 1.13×10^{-7} .

were 72 for DhaA31 and 15 for DbjA, thus implying that both enzymes preferentially convert the *R* enantiomer because it undergoes a faster S_N2 reaction. Together with the thermodynamic data and previous findings on the kinetic mechanism of HLDs,^[11,27] the results of global kinetic analysis suggest that the positioning of the *R* and *S* enantiomers in the active site of DhaA31 dictates the ability of the enzyme–substrate complex to reach the S_N2 transition state, in keeping with previous findings for DbjA.^[13]

Molecular docking was used to study the positioning of (*R*)- and (*S*)-2-BP in the DhaA31 active site and revealed reactive and nonreactive binding modes for each enantiomer (Figure 2). (*R*)-2-BP adopts a reactive binding mode when its

alkyl chain is in close contact with Asn41, whereas close contact with Trp107 corresponds to a nonreactive mode. The opposite is true for (*S*)-2-BP.

Quantum-mechanical/molecular-mechanical adiabatic mapping along the reaction coordinate revealed large differences in activation energy between the two binding modes. The frequencies of potentially reactive positions for each enzyme–enantiomer pair were determined by computing the proportion of time that the enzyme–substrate complex spent in a near-attack conformation (NAC) over two molecular dynamics simulations with a duration of 60 ns. In the case of DhaA31, the NAC proportions were (5.5 \pm 1.1)% for (*R*)-2-BP and (0.6 \pm 0.9)% for (*S*)-2-BP. The corresponding values for weakly enantioselective DhaA were (0.6 \pm 0.6) and (0.4 \pm 0.6)%: The values did not differ significantly for this enzyme (see Table 2 in the Supporting Information). Similar results to those for DhaA were obtained for DbjA, in which both enantiomers bound primarily at the same wall of the active site but adopted mirror-image orientations with displaced chiral centers. The *R* enantiomer was sampled exclusively in a reactive binding mode, but the *S* enantiomer adopted both reactive and nonreactive binding modes.^[13] The free access of water molecules to the DbjA active site seems to be important for its binding of hydrophobic linear haloalkanes and their enantiodiscrimination. Molecular dynamics simulations on the complexes of DhaA31 with (*R*)- and (*S*)-2-BP revealed that the main driver for its enantioselectivity is not hydration, as in DbjA,^[13,21] but complementary interactions between the occluded active site and the reactive binding mode of (*R*)-2-BP, thus increasing its frequency of occurrence threefold relative to DhaA (see Table 3 in the Supporting Information). Both enantiomers bind weakly to DhaA, thus leading to rapid release from its less occluded active site.

We used site-directed mutagenesis to construct DhaA variants with different combinations of the five DhaA31 mutations to identify those essential for high enantioselectivity (Figure 3; see Table 4 in the Supporting Information). A variant bearing C176Y and Y273F in the main tunnel was previously studied by Bosma et al.^[28] Although both mutations are believed to reduce the size of the active site and affect substrate binding, the enantioselectivity of this variant (*E* = 25) is similar to that of wild-type DhaA. Therefore, three new variants carrying one of the other DhaA31 mutations

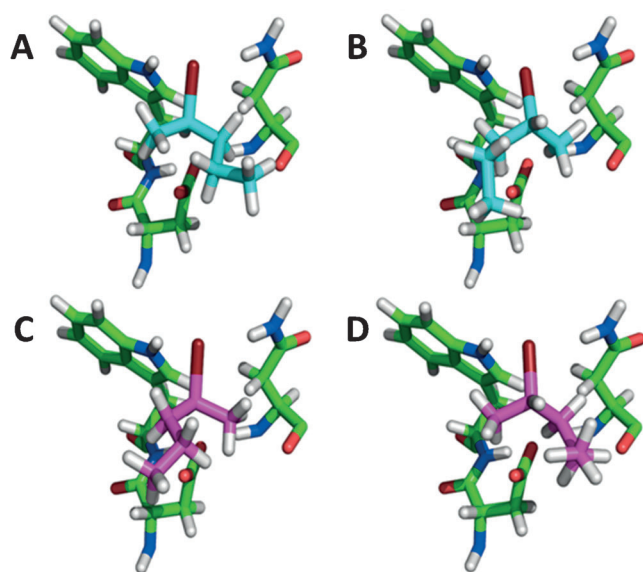


Figure 2. Binding modes of 2-BP enantiomers, as determined by molecular docking. (*R*)- and (*S*)-2-BP are shown in azure and violet, respectively; the catalytic nucleophile (Asp) and two halide-stabilizing residues (Trp and Asn) of DhaA/DhaA31 are shown in green. A) Reactive binding mode with the alkyl chain of the *R* enantiomer in close contact with Asn41; B) nonreactive binding mode with the alkyl chain of the *R* enantiomer in close contact with Trp107; C) reactive binding mode with the alkyl chain of the *S* enantiomer in close contact with Trp107; D) nonreactive binding mode with the alkyl chain of the *S* enantiomer in close contact with Asn41.

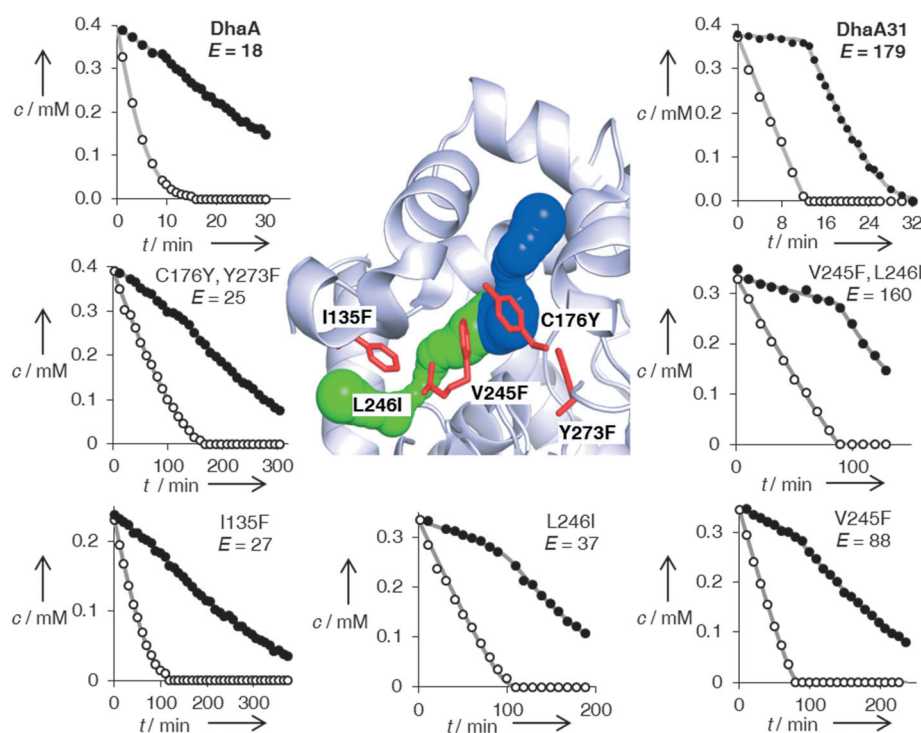


Figure 3. Kinetic resolution of 2-BP by DhaA, DhaA31, and DhaA variants with selected DhaA31 point mutations. The central image shows the side chains of mutated residues (in red) in the main tunnel (shown in blue) and the slot tunnel (in green). C176Y and Y273F affect the main tunnel, residue 245 is located at the interface of the tunnels and the active site, and residues 135 and 246 are in the slot tunnel. The tunnels were analyzed by using the software CAVER 3.01.^[29] ● (S)-2-BP; ○ (R)-2-BP.

(I135F, V245F, and L246I) were constructed. V245F and L246I increased enantioselectivity five- and two-fold, respectively, but I137F had no effect. Kinetic-resolution profiling suggested that both V245F and L246I reduced the K_m value for the *R* enantiomer. A combination of the two beneficial mutations yielded DhaA139, whose enantioselectivity ($E = 160$) matches that of DhaA31. There is evidence from molecular modeling that the new bulky residues create steric hindrance in the active site, thus promoting favorable interactions between (*R*)-2-BP and the active site. Consequently, the *S* enantiomer only binds in a reactive configuration after the *R* enantiomer has been wholly consumed (Figure 3).

In summary, we have shown that the origins of enantioselectivity in engineered DhaA31 and wild-type DbjA^[13] are very different, even though both enzymes achieve similar enantiodiscrimination with the substrate 2-BP. In DbjA, the preferred *R* enantiomer adopts a reactive binding mode more frequently than the *S* enantiomer owing to water-promoted interactions of the alkyl chain with the hydrophobic wall of the active site. In DhaA31, two point mutations create an occluded active site that complements (*R*)-2-BP and non-preferred (*S*)-2-BP binds only after complete conversion of (*R*)-2-BP. Our results show that the enantioselectivity of an enzyme depends on its structure as well as its hydration and dynamics, so one cannot assume that all members of an enzyme family share the same structural basis of enantioselectivity. We also created a highly enantioselective HLD from

a nonselective enzyme through just two point mutations. Whereas previous unsuccessful attempts to engineer an enantioselective DhaA involved transplanting the wide-open and solvent-accessible DbjA active site,^[21] the enantioselectivity-increasing mutations in DhaA31 occluded access to its active site. A future challenge will be to engineer an enantioselective enzyme by modulating the hydration of the active site, but will require new computational methods for quantitative site-specific analysis of protein hydration.

Acknowledgements

We thank Prof. Petr Klan (Masaryk University, Brno) for help with the synthesis of enantiomerically pure substrates. Financial support from The Czech Science Foundation (16-06096S, 17-24321S), the Ministry of Education, Youth, and Sports of the Czech Republic (LH14027, LO1214, LM2015051, LQ1605), and Masaryk University (MUNI/M/1888/2014) is gratefully acknowl-

edged. Access to the METACentrum and CERIT-SC super-computing facilities is highly appreciated (LM2015042 and LM2015085).

Conflict of interest

Zbynek Prokop and Jiri Damborsky are founders and Veronika Stepankova is CEO of Enantis.

Keywords: enantioselectivity · enzyme catalysis · enzymes · molecular modeling · protein engineering

- [1] M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Keßeler, R. Stürmer, T. Zelinski, *Angew. Chem. Int. Ed.* **2004**, *43*, 788–824; *Angew. Chem.* **2004**, *116*, 806–843.
- [2] C. G. Acevedo-Rocha, R. Agudo, M. T. Reetz, *J. Biotechnol.* **2014**, *191*, 1–8.
- [3] U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185–194.
- [4] J. M. Choi, S. S. Han, H. S. Kim, *Biotechnol. Adv.* **2015**, *33*, 1443–1454.
- [5] H. J. Wijma, S. J. Marrink, D. B. Janssen, *J. Chem. Inf. Model.* **2014**, *54*, 2079–2092.
- [6] J. B. Siegel, A. Zanghellini, H. M. Lovick, G. Kiss, A. R. Lambert, J. L. St. Clair, J. L. Gallaher, D. Hilvert, M. H. Gelb,

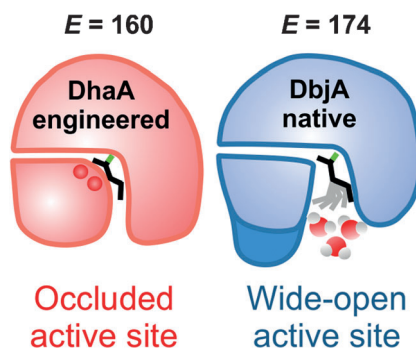
Communications



Enzyme Catalysis

V. Liskova, V. Stepankova, D. Bednar,
J. Brezovsky, Z. Prokop, R. Chaloupkova,
J. Damborsky* ————— ■■■■-■■■■

Different Structural Origins of the
Enantioselectivity of Haloalkane
Dehalogenases toward Linear β -
Haloalkanes: Open–Solvated versus
Occluded–Desolvated Active Sites



Two recipes for success: Two distinct mechanisms are described for the enantiodiscrimination of 2-bromopentane by haloalkane dehalogenases. Highly enantioselective DbjA has a very open, solvent-accessible active site. The engineered enzyme DhaA31 has a more occluded and less solvated cavity (see picture) but shows similar enantioselectivity as a result of steric hindrance imposed by two specific residues, rather than hydration as in DbjA.