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Research Article

Promiscuous Dehalogenase Activity of the Epoxide Hydrolase CorEH from *Corynebacterium* sp. C12

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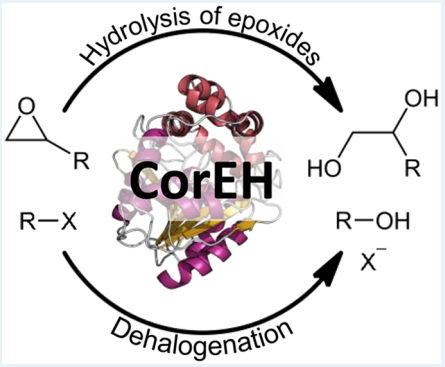
ABSTRACT: Haloalkane dehalogenases and epoxide hydrolases are phylogenetically related and structurally homologous enzymes that use nucleophilic aspartate residues for an S_N2 attack on their substrates. Despite their mechanistic similarities, no enzymes are known that exhibit both epoxide hydrolase and dehalogenase activity. We screened a subset of epoxide hydrolases, closely related to dehalogenases, for dehalogenase activity and found that the epoxide hydrolase CorEH from *Corynebacterium* sp. C12 exhibits promiscuous dehalogenase activity. Compared to the hydrolysis of epoxides like cyclohexene oxide (1.41 μmol min^{-1} mg^{-1}), the dehalogenation of haloalkanes like 1-bromobutane (0.25 nmol min^{-1} mg^{-1}) is about 5000-fold lower. In addition to the activity with 1-bromobutane, dehalogenase activity was detected with other substrates like 1-bromohexane, 1,2-dibromoethane, 1-iodobutane, and 1-iodohexane. This study shows that dual epoxide hydrolase and dehalogenase activity can be present in one naturally occurring protein scaffold.



KEYWORDS: *catalytic promiscuity, Corynebacterium, epoxide hydrolase, haloalkane dehalogenase, dual activity*

INTRODUCTION

The α/β-hydrolase-fold family is one of the largest structural superfamilies, including many enzymes that catalyze a broad range of chemically diverse reactions.[1,2] The α/β-hydrolase enzymes share a common fold where the core is formed by a mostly parallel 6−8 stranded β-sheet connected via 4−6 α-helices. They possess a catalytic triad consisting of a catalytic nucleophile, a histidine, and an acidic residue (Figure 1).[2] The α/β-hydrolase-fold enzymes are catalytically diverse and span several distinct enzyme classes including acyltransferases, oxidoreductases, lyases, and isomerases.[3]

The most commonly employed Ser-His-Asp catalytic triad facilitates the formation of an acyl-enzyme intermediate, an ester derivative of the catalytic serine that can be hydrolyzed by a water molecule activated by the His-Asp pair. While this mechanism works very well for the hydrolysis of esters and amides, it is not suitable for the hydrolysis of epoxides and organohalogen compounds, which would result in the formation of nonhydrolyzable ether intermediates. Instead, epoxide hydrolases (EH, E.C. 3.3.2.10), haloacetate dehalogenases (HacD, E.C. 3.8.1.3), and haloalkane dehalogenases (HLD, E.C. 3.8.1.5) all employ the same Asp-His-Asp/Glu catalytic triad. S_N2 attack on epoxides or halocarbons by the nucleophilic aspartate results in the formation of an alkyl-enzyme intermediate, an ester derivative of the catalytic aspartate. The alkyl-enzyme intermediate is then hydrolyzed by a water molecule activated by the His-Asp/Glu pair[4,5] (Figure 1). The charge relay acid can be located at two different positions in the sequence (Figure S1). In most epoxide hydrolases and dehalogenases of HLD subfamilies I and III, the charge relay acid is found at the second position, close to the histidine base. In dehalogenases of HLD subfamily II and some epoxide hydrolases, the charge relay acid is located at the first position, close to the catalytic nucleophile.

In addition to the catalytic triad, both epoxide hydrolases and dehalogenases have other supporting catalytic residues that are highly conserved within their group. The ring-opening catalyzed by epoxide hydrolases is usually assisted by a tyrosine-tyrosine pair,[10] but some epoxide hydrolases use a histidine-tyrosine[11] pair instead. Haloalkane dehalogenases make use of one halide-stabilizing tryptophan, usually paired with either another tryptophan residue[8] (subfamily HLD-I) or asparagine (subfamilies HLD-II and HLD-III).[4,9] One dehalogenase, DatA from *Agrobacterium tumefaciens*, has been reported to have a special halide-stabilizing pair, consisting of an asparagine-tyrosine pair.[12,13] Some dehalogenases, DmxA,[14] DsaA,[15] DmrB,[16] and DsvA[17] have been reported to have only one halide-stabilizing residue.

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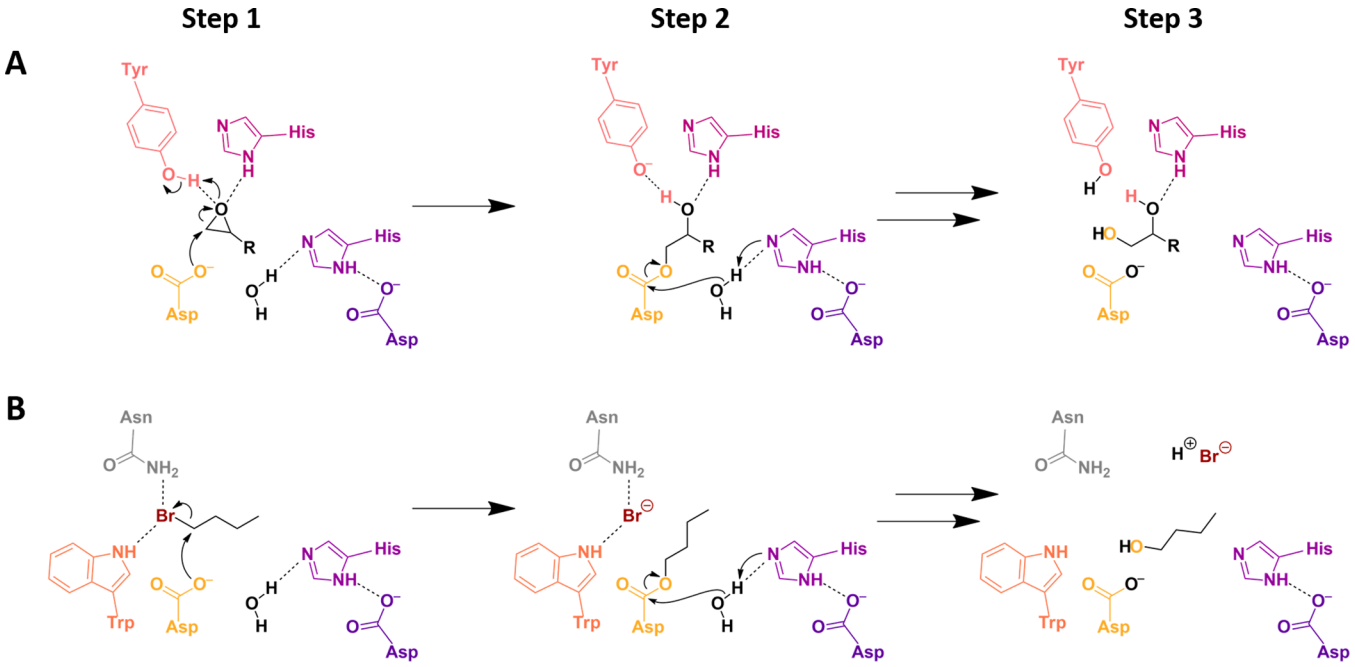

Figure 1. Catalytic mechanisms for epoxide hydrolases[6,7] and haloalkane dehalogenases.[8,9] Both reactions start (step 1) with a nucleophilic attack on the epoxide or halocarbon by the nucleophilic aspartate (yellow), which results in the formation of an alkyl-enzyme intermediate. The alkyl-enzyme intermediate is hydrolyzed (step 2) by a water molecule activated by the histidine-aspartate pair (purple). Some enzymes utilize a histidine-glutamate pair instead. This leads to the release of a vicinal diol after epoxide hydrolysis or the release of an alcohol in the case of dehalogenation (step 3). Possible further intermediates and transition states between step 2 and step 3 were omitted for clarity. Epoxide ring-opening (A) is assisted by a tyrosine-tyrosine or histidine-tyrosine pair (pink), while the halide is stabilized (B) by at least one tryptophan (orange) and usually one other halide-stabilizing residue like asparagine or another tryptophan (gray).

Within protein superfamilies the primary function of one family member is often found as promiscuous activity in other family members and the same promiscuous activity is usually shared by more than one family member.[18,19] Rather than rare exceptions, promiscuous activities are frequently observed, and it is commonly believed that catalytic promiscuity plays an important role in the emergence of novel functions by providing a starting point for evolution by natural selection.[18] The versatility of the catalytic triad employed by α/β-hydrolases allows for the catalysis of several distinct chemical reactions, explaining why catalytic promiscuity is often observed.[3] It is believed that the structurally homologous and phylogenetically closely related epoxide hydrolases, haloacetate dehalogenases, and haloalkane dehalogenases evolved from a common ancestor with an Asp-His-Asp/Glu catalytic triad and both epoxide hydrolase and dehalogenase activities.[20] Due to the structural and mechanistic similarities within the α/β-hydrolase-fold superfamily, it is also possible to engineer enzymes with one catalytic activity to catalyze another reaction.[21−24] As an example, it was possible to convert an esterase from *Pseudomonas fluorescens* into an epoxide hydrolase.[23] Although epoxide hydrolases and dehalogenases are more similar in the exerted catalytic mechanism than esterases and epoxide hydrolases, interconversion of these two activities by protein engineering has not been reported. No enzyme that is able to catalyze both reactions, epoxide hydrolysis and the dehalogenation of haloalkanes, has been described yet.

As it is generally believed that enzyme promiscuity plays an important role in enzyme evolution and the diversification of enzymes, it is highly likely that a promiscuous epoxide hydrolase-dehalogenase enzyme exists. In this study, we describe the discovery of an epoxide hydrolase with promiscuous dehalogenase activity. Promising candidates were selected using phylogenetic analysis and screened utilizing sensitive halide detection assays. We explain the mechanisms for both reactions by structure−function analysis and docking studies and show that both activities can be present in one naturally occurring protein scaffold. Our findings support the hypothesis that a common ancestor with both haloalkane dehalogenase and epoxide hydrolase activities could have existed.

RESULTS AND DISCUSSION

Selection and Screening of Epoxide Hydrolases. Several epoxide hydrolases were selected for screening for dehalogenase activity based on phylogenetic analysis of a set of characterized α/β-hydrolases. These were EliEH2 from *Erythrobacter litoralis*, PpuEH from *Pseudomonas putida*, NpuEH2 from *Nostoc punctiforme*, SceEH from *Saccharomyces cerevisiae*, ScoEH from *Streptomyces coelicolor*, AciCIF from *Acinetobacter* sp., PaeCIF from *Pseudomonas aeruginosa*, and CorEH from *Corynebacterium* sp. C12. These sequences were selected based on their relatively close relation to the dehalogenase sequences in the data set and should therefore be more likely to possess promiscuous dehalogenase activity (Figure 2).

CorEH Catalyzes Epoxide Hydrolysis and Dehalogenation. Following initial screening experiments with the selected epoxide hydrolase sequences employing a recently developed halide oxidation (HOX) assay,[25] CorEH was selected for further analysis (Figure S2). The HOX assay for the detection of halides is much more sensitive than other halide assays, like the commonly employed Iwasaki assay,[26]

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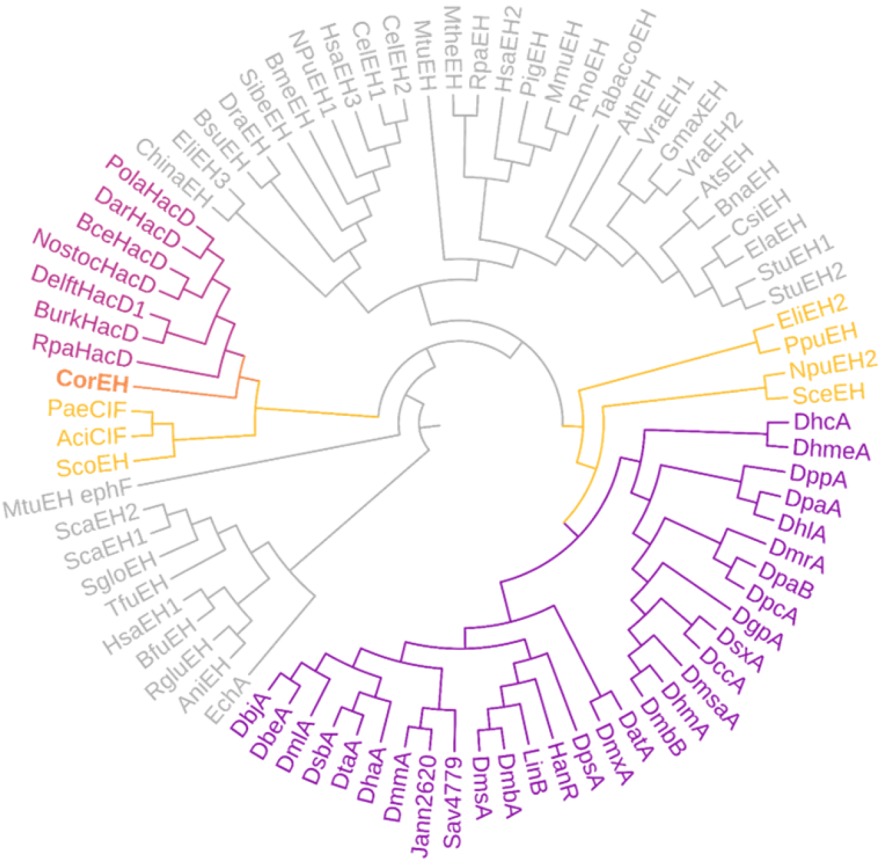

Figure 2. Phylogenetic tree based on characterized epoxide hydrolases and dehalogenases. The branches closest to the root (gray) consist only of epoxide hydrolases, while haloalkane dehalogenases (purple) and haloacetate dehalogenases (pink) are found further from the root. Branches containing only epoxide hydrolases that are more similar to dehalogenases are highlighted in yellow. The epoxide hydrolase CorEH is highlighted in orange. As branch length is not important for the purpose of this figure, it was ignored for clarity.

enabling the detection of very low dehalogenase activities. In all further screenings, the HOX assay was used to detect halide formation with bromoalkanes as substrates, while iodoalkanes were screened using an iodide-specific variation of the HOX assay.[27]

CorEH was first described as the enzyme responsible for epoxide hydrolysis by *Corynebacterium* sp. C12 in 1998.[28] The highest activity was observed with C_6 and C_7 carbocyclic epoxides, while C_5 and C_8 carbocyclic epoxides were hydrolyzed with less than 20% of the activity against cyclohexene oxide. CorEH also hydrolyzed linear 1,2-epoxyalkanes, preferring substrates with long alkyl chains, suggesting that hydrophobic interaction might be important for substrate binding. CorEH was confirmed to hydrolyze several other epoxides in 2006,[5] highlighting the diverse substrate scope of this promiscuous epoxide hydrolase. In this study, we explore the promiscuous dehalogenase activity of the epoxide hydrolase CorEH using activity assays and structural analysis.

Using the HOX assay, wild-type CorEH was reproducibly shown to exhibit significant dehalogenase activity with 1-bromobutane, with a specific activity of approximately 0.25 nmol min^{-1} mg^{-1}. This was confirmed by gas chromatography−mass spectrometry (GC-MS, Figure 3A,C,D). To exclude unspecific reactions of substrates with the protein, two catalytic triad variants of CorEH, D123N (catalytic acid) and H264F (catalytic base), were also tested for dehalogenase activity. These variants alter the charge-relay acid and the histidine base, respectively, and consequently the alkyl-enzyme intermediate formed during the reactions cannot be hydrolyzed by an activated water molecule.[29,30] The loss of activity for these variants confirms that CorEH is actively converting 1-bromobutane to 1-butanol and that the reaction is an enzymatically catalyzed reaction rather than a non-enzymatic conversion.

The dehalogenase activity of wild-type CorEH with 1-bromobutane is about 4000-fold lower than the average activity of several natural dehalogenases (1 μmol min^{-1} mg^{-1}).[4] In addition to the activity with 1-bromobutane, dehalogenase activity was detected with other substrates like 1-bromohexane, 1,2-dibromoethane, 1-iodobutane, and 1-iodohexane (Figure 3B). The promiscuous dehalogenase activity of CorEH with 1-bromobutane (0.25 nmol min^{-1} mg^{-1}) is also approximately 5000-fold lower compared to the highest reported primary activity of CorEH with cyclohexene oxide (1.41 μmol min^{-1} mg^{-1})[28] but only 1000-fold lower when compared to the activity with other epoxides like 1,2-epoxyoctane (0.22 μmol min^{-1} mg^{-1}, Figure S3).

Structure−Function Analysis. To gain insight into the differences and similarities between CorEH, dehalogenases, and other epoxide hydrolases, we determined the crystal structure of CorEH to 2.2 Å resolution (Figure 4A). As a member of the α/β-hydrolase-fold superfamily, CorEH has the typical β-sheet surrounded by α-helices and is covered by a cap-domain.

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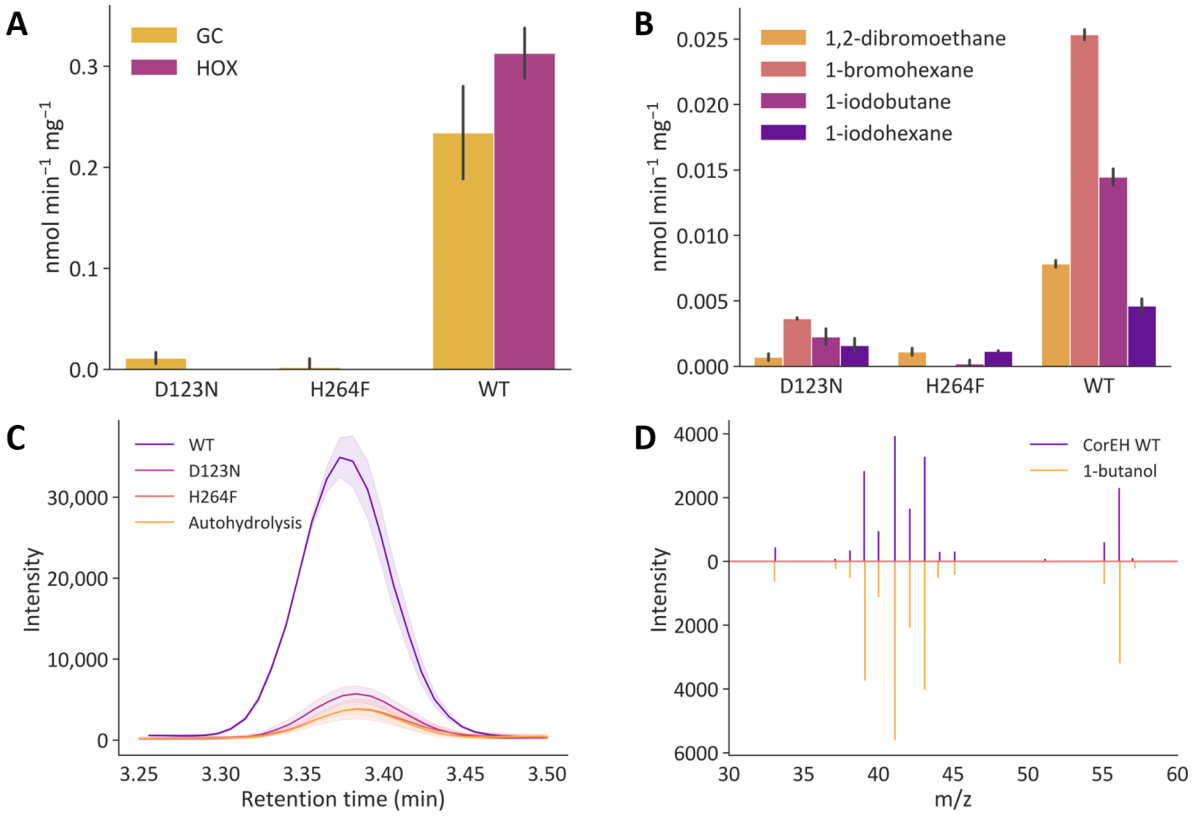

Figure 3. Dehalogenase activity of CorEH and the catalytic triad variants D132N and H264F. (A) Specific activity of CorEH for 1-bromobutane. Activity was determined using the halide oxidation (HOX) assay, for bromide formation, and gas chromatography (GC), for the production of butanol. (B) Specific activities of CorEH toward several other halogenated compounds, showing a preference for 1-bromohexane. (C) Overlay of gas chromatograms for the peak identified as butanol, clearly showing the difference between the active CorEH and autohydrolysis. The differences between the catalytic triad variants D123N and H264F and autohydrolysis for both the production of butanol and the formation of bromide are small, and the peak of H264F is almost the same as autohydrolysis. (D) Mass spectra of the peak in panel C compared to a butanol standard (500 μM). In order to easily compare the two spectra, the standard spectrum is inversed. All reactions were performed in triplicate and standard deviations are shown (A–C). Experiments were repeated multiple times, resulting in similar values.

The active site of CorEH (Figure 4B,C) includes the catalytic triad typical for epoxide hydrolases and dehalogenases. It consists of the catalytic nucleophile D99, the charge-relay acid D123 and the histidine base H264. Similar to other closely related epoxide hydrolases, the epoxide ring-opening in CorEH is assisted by a noncanonical His/Tyr-pair[11] made up of H145 and Y209 (Figure S4). Unlike most other epoxide hydrolases and dehalogenases of subfamilies HLD-I and HLD-III, the charge-relay acid of CorEH is located at the first position close to the catalytic nucleophile.

Natural haloalkane dehalogenases have three potential positions for their halide-stabilizing residues. Some dehalogenases have only a single primary halide-stabilizing residue,[17] but most dehalogenases utilize a pair to stabilize the halide. The main halide-stabilizing residue is a tryptophan located directly C-terminal to the catalytic nucleophile in the sequence and is very conserved among haloalkane dehalogenases and epoxide hydrolases. The tryptophan flanking the nucleophile is found in almost all haloalkane dehalogenases and in most epoxide hydrolases (70%). Aside from halide-stabilization, this tryptophan also assists to position the catalytic nucleophile in the correct orientation for nucleophilic attack.[32] Some epoxide hydrolases have a leucine, isoleucine, phenylalanine, or tyrosine residue at this position instead. CorEH possesses a potentially halide-stabilizing tryptophan, W100, at the primary position but lacks other canonical stabilizing residues. This could explain the low promiscuous dehalogenase activity of CorEH.

In dehalogenase subfamilies HLD-II and HLD-III, the primary halide stabilizing residue is paired with an asparagine located in the HGxP-motif, where x is any residue. Instead of an asparagine, the HGxP-motif of CorEH includes a tryptophan residue (W34), but a Trp/Trp-pair at these two positions is not found in known dehalogenases. Either tryptophan residue might play a role in the dehalogenase activity of CorEH, as they are not present as a pair in any of the other epoxide hydrolases studied in the initial screenings (Figure S4). In the subfamily HLD-I, the stabilization-pair is completed by a tryptophan in the variable cap-domain, but the sequence in this region does not align with epoxide hydrolases.

Despite the presence of a tryptophan at the primary halide stabilizing position in about 70% of known α/β-hydrolase-fold epoxide hydrolases, only one other enzyme, Ylehd from *Yarrowia lipolytica*, has been reported to exhibit both epoxide hydrolase and dehalogenase activities. Supposedly, Ylehd catalyzes the hydrolysis of epoxides at pH 8, while catalyzing the dehalogenation of several haloalkanes at pH 4. Unfortunately, that publication lacked proper controls for autohydrolysis. We were unable to reproduce the dehalogenase activity claimed by Bendigiri et al.[33] (Figure S5).

Docking Studies with Typical Substrates. Docking calculations were performed to study the binding of substrates

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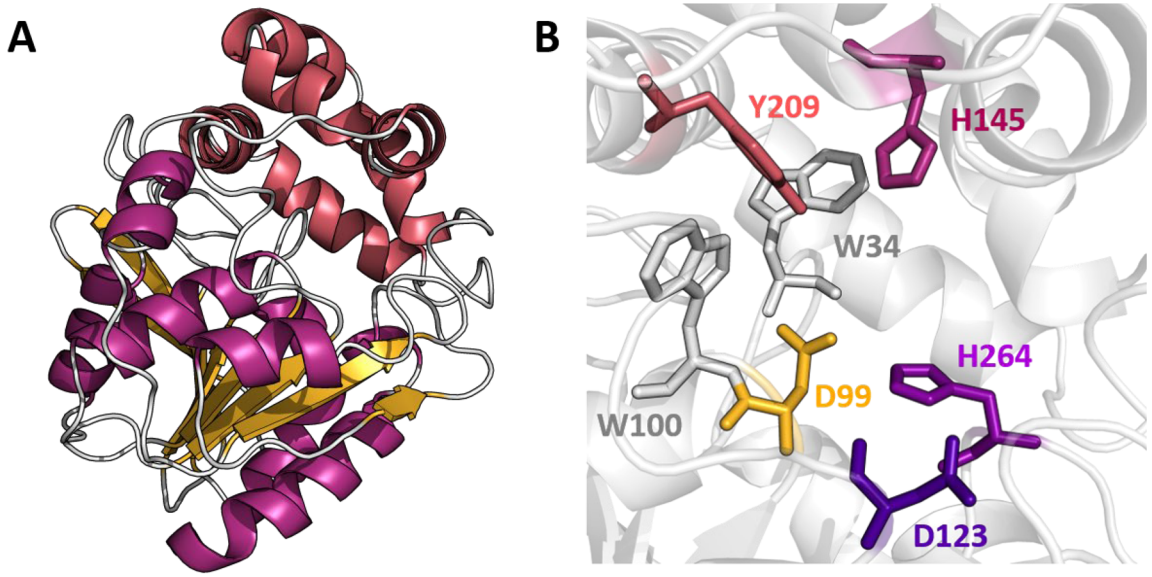

C

Enzyme	HS_2	CN/HS_1	CRA_1	RO_1	HS_3	RO_2	CRA_2	B
CorEH	HGWP	GHDWGG	ILDMI	INHIFF	-----	QWYAT	AWGGS	CGHFV
BsuEH	HGFP	GHDWGG	AINIP	SSYIAY	-----	NWYRA	MED--	ASHWI
LinB	HGNP	VHDWGS	YMEAI	------	QAF--	-WPRQ	EPGAL	GAHFI
DhlA	HGEP	VQDWGG	IMNA-	------	TAW--	-FPKM	MKDKL	AGHFV
DatA	HGNP	LQDYGA	FFEPV	------	AKL--	-GPRN	KPGFL	GIHFV
DmxA	HGQP	CHDWGS	FMEAM	------	QTL--	-FPQN	EPGFL	GLHYI

Figure 4. Crystal structure of CorEH and comparison of the active site to dehalogenases and epoxide hydrolases. The crystal structure of CorEH is available in the Protein Data Bank under the PDB ID 7AC0. (A) A single monomer of CorEH highlighting the highly conserved main domain consisting of a β-sheet (yellow) surrounded by α-helices (hot pink) and the variable cap domain (pink) typical for members of the α/β-hydrolase fold superfamily. (B) Close-up of the active site of CorEH showing the catalytic triad with the nucleophile D99 (yellow), the charge-relay acid D123 (dark purple), and the catalytic histidine H264 (purple). H145 and Y209 form the noncanonical epoxide ring-opening pair and two tryptophan residues (W100 and W34) are found at the primary and secondary halide-stabilizing positions, respectively. (C) Alignment of the active site of CorEH with the epoxide hydrolase from *Bacillus subtilis* (BsuEH[5]) and the dehalogenases from *Sphingomonas paucimobilis* (LinB[31]), *Xanthobacter autotrophicus* GJ10 (DhlA[8]), *Agrobacterium tumefaciens* (DatA[13]), and *Marinobacter* sp. (DmxA[14]) and catalytic residues are highlighted. The catalytic triad is shown with the catalytic nucleophile (CN, yellow), the charge-relay acid (CRA, dark purple) at position 1 or 2 (CRA_1 and CRA_2 respectively), and the histidine base (B, purple). The epoxide ring-opening pair (RO_1 and RO_2, hot pink and pink respectively) and halide-stabilizing residues with the primary residue (HS_1, orange) and secondary residues (HS_2 or HS_3, orange) are also visualized.

typical for both epoxide hydrolysis and dehalogenation in CorEH. The formation of reactive Michaelis−Menten complexes indicated by favorable binding energies was compared with experimental results for CorEH, the closely related epoxide hydrolase PaeCIF, and the haloalkane dehalogenase DhlA (Table S1). Plausible binding poses were identified for CorEH with both epoxides and bromoalkanes (Figure 5). The distance of the oxygen atom of the nucleophile (D99) to the attacked carbon atom is 3.4 Å for 1,2-epoxyhexane and 4.0 Å for 1-bromobutane. The epoxide ring of 1,2-epoxyhexane is stabilized by hydrogen bonds with the stabilizing residues Y209 and H145, while the bromine atom of 1-bromobutane can be stabilized by W100 and Y209. The orientation of the side chain of W34 in the crystal structure makes it unlikely that this residue is able to form hydrogen bonds with the substrate to stabilize the halogen atom. The iodinated substrates could bind with favorable energies, but no stabilization of the leaving halogen was observed.

Mutational Studies of CorEH Variants. To investigate the potential involvement of the two tryptophan residues in halide stabilization, we performed mutational studies with the CorEH variants W34N, W34F, and W100A for dehalogenase and epoxide hydrolase activity (Figures S6 and S7).

All three variants lost most of both dehalogenase and epoxide hydrolase activities when compared to the wild-type activity. Variant W34F still shows some of the wild-type activity, retaining approximately 24% of the activity toward 1,2-epoxyoctane and 45% toward 1-bromobutane. The variant W34N loses almost all activity toward 1,2-epoxyoctane (5% of the wild-type activity) and most activity toward 1-bromobutane (10% of the wild-type activity). Both variants target the tryptophan at the potential secondary halide stabilizing position, substituting a large nonpolar residue by a polar residue (W34N) or another large nonpolar residue (W34F).

Misawa et al. already proposed that hydrophobic interactions might be important for substrate binding,[28] and it is possible that the change in hydrophobicity causes the observed loss of activity. Like W34N, the variant W100A loses most of the wild-type activity, exhibiting only 13% of the wild-type activity toward 1,2-epoxyoctane and 8% of the wild-type activity toward 1-bromobutane. W100A also introduces a large change in hydrophobicity in the active site, exchanging the largest hydrophobic amino acid tryptophan with the smallest

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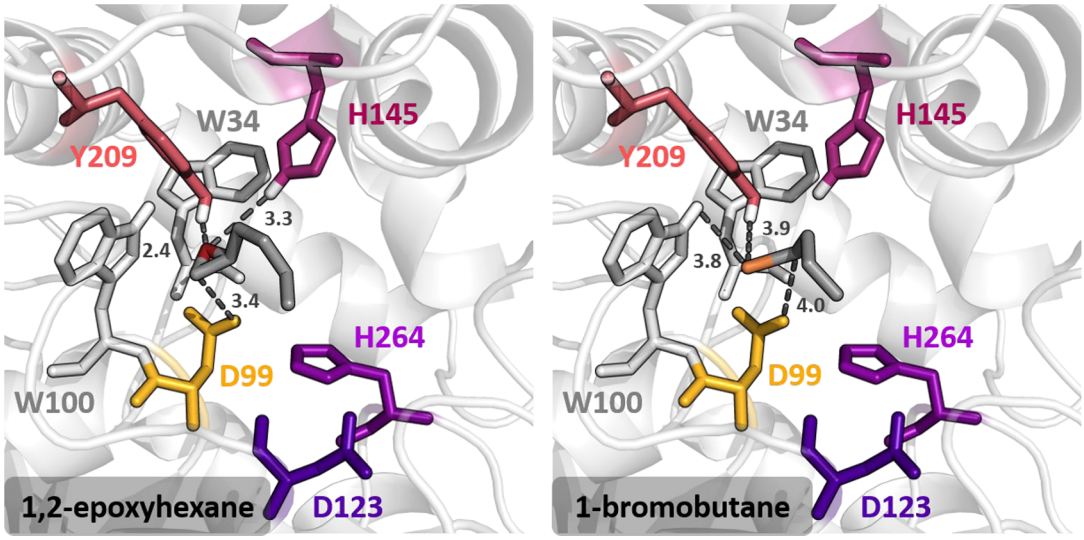

Figure 5. Active site of CorEH with the predicted binding mode for the epoxide substrate 1,2-epoxyhexane and the haloalkane 1-bromobutane. The catalytic triad is shown with the nucleophile D99 (yellow), the charge-relay acid D123 (dark purple), and the catalytic histidine H264 (purple). H145 and Y209 form the noncanonical epoxide ring-opening pair, and two tryptophan residues (W100 and W34) are found at the primary and secondary halide-stabilizing positions, respectively. The distances between the catalytic nucleophile and the attacked carbon atom, between the stabilizing residues and the oxygen atom of the epoxide ring, and between stabilizing residues and the bromide atom of the haloalkane are visualized with dashes and reported in Å.

hydrophobic amino acid alanine. The loss of both epoxide hydrolase and dehalogenase activities for variant W100A can also be explained by the secondary function of the tryptophan;[32] removal of W100 might lead to the incorrect positioning of the catalytic nucleophile for the nucleophilic attack involved in both epoxide hydrolysis and dehalogenation. Both W34 and W100 seem to be important for substrate binding to the active site, but it is currently unclear whether they are directly involved in halide-stabilization.

CONCLUSIONS

We have shown that CorEH is an epoxide hydrolase that can also catalyze the dehalogenation of haloalkanes, particularly bromoalkanes such as 1-bromobutane and 1-bromohexane. Our structure−function studies suggests that the dehalogenase activity of CorEH probably stems from the presence of at least one halide stabilizing residue. Although this could not be confirmed experimentally via mutagenesis, computational modeling of Michaelis−Menten complexes utilizing the crystal structure of CorEH, determined to 2.2 Å in this study, supports this hypothesis. Removal of the tryptophan at the primary halide stabilizing position resulted in the loss of both activities, likely due to the loss of its secondary function to properly position the catalytic nucleophile.[32] Substitution of the uncommon tryptophan in the HGxP-motif with phenylalanine does not completely remove the dehalogenase activity. Nevertheless, it causes a significant drop in both haloalkane dehalogenase and epoxide hydrolase activities, indicating that this residue is important for catalysis. Enzyme promiscuity plays an important role in enzyme evolution and the diversification of enzymes. Our findings show that dual epoxide hydrolase and dehalogenase activity can occur in one natural protein scaffold, supporting the hypothesis that a common ancestor with both haloalkane dehalogenase and epoxide hydrolase activities could have existed. It is highly likely that more enzymes with this dual activity exist in nature, waiting to be discovered in further investigations.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.1c00851.

Active site of epoxide hydrolases and dehalogenases, screening of epoxide hydrolases and CorEH variants for dehalogenase activity and hydrolysis of epoxides, alignment active sites epoxide hydrolases, failure to verify the claim that Ylehd has dehalogenase activity, and experimental procedures (PDF)

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Author Contributions

U.T.B. conceived and directed the project. E.D.S. designed and performed most experiments supported by C.S.P.B. L.B. performed the crystallization experiments, and G.J.P. measured the crystals and solved the crystal structure. M.L. directed/supervised the crystallization experiments. J.M., D.B., and J.D. carried out computational analysis. E.D.S. drafted the manuscript to which all authors contributed. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ DEDICATION

Dedicated to Prof. Klaus Buchholz on the occasion of his 80th birthday.

■ ABBREVIATIONS

EH, epoxide hydrolase; HLD, haloalkane dehalogenase; HacD, haloacetate dehalogenase; HOX, halide oxidation

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