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Novel Hybrid Esterase-Haloacid Dehalogenase Enzyme

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The existence of different catalytic mechanisms (or reaction types) in the same active site is an example of catalytic promiscuity.[1,2] The promiscuity can result from natural evolution of an enzyme, which enhances organism metabolic flexibility and environmental fitness, or from laboratory evolution and can be exploited in numerous synthetic applications.[2] α/β-Hydrolase-fold proteins belong to one of the largest protein superfamilies within the α/β class of folds and exhibit enormous sequence diversity,[3,4] fold plasticity, and activities.[2e,f] As they also exhibit high conservation of tertiary structures and catalytic triads, they have been suggested to have evolved from a common protein ancestor,[5] from which divergent evolution led to the emergence of a large number of promiscuous enzymes.[5b,6]

Serine esterases and haloacid dehalogenases[7] are α/β-type hydrolases that differ in their topological features, the nature/position of the nucleophile, and the geometry of their catalytic scaffolds, while esterases (EC 3.1.1.1) preferentially hydrolyze water-soluble simple esters and contain a Ser/Asp(Glu)/His catalytic triad, haloacid dehalogenases (HAD; EC 3.8.1.2) catalyze the conversion of haloacid compounds into the corresponding alcohols and hydrogen halides by means of an Asp/Asp/His catalytic triad. Although, it has been suggested that dehalogenases are evolutionarily related to esterases,[8] so far no protein, either naturally occurring or laboratory generated, has been reported to possess both activities.

We describe here a multifunctional α/β-hydrolase-fold enzyme,[9] designated REBr, mined from a metagenome library established from the DNA of a microbial community from seawater contaminated with crude oil. The protein showed a novel hydrolytic phenotype, namely the cleavage of both common *p*-nitrophenyl (*p*NP) and short aliphatic esters, and organic haloalkanoates. The existence of these two activities in a single protein is remarkable as they involve distinct catalytic mechanisms (for details see Figure S1 in the Supporting Information).

The *rEBr* gene (933 bp),[9] encodes a protein (310 AA, M_r = 33 852 Da) that exhibits high homology (up to 63% identity, 75% similarity) with a number of α/β-fold hydrolases (see Figure S2). Not only does this protein hydrolyze a series of commercially available common *p*NP and nonactivated short fatty acid esters as propyl propionate and ethyl butyrate, but also haloacids: $[(k_{cat}/K_m)]_{ester}/[(k_{cat}/K_m)]_{haloacid}$ factor of ~4:1 for the best substrates, optimally at 40 °C and pH 8.0–8.5 (Figure S3; Tables 1 and S2). Weak though measurable activity with haloalkanes was detected and no epoxide tested was hydrolyzed. The enzyme cleaved a full set of halides at both terminal and subterminal positions, with catalytic efficiencies increasing in the order bromide (1-fold), fluoride (1.1-fold), chloride (12-fold),

Table 1. Kinetic parameters for the wild-type REBr enzyme.[a]

Substrate	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} M^{-1}]
bromoacetate	0.42 ± 0.04	1614.0 ± 23.3	3.8×10^6
2,3-dibromopropionate	0.52 ± 0.07	1183.0 ± 82.2	2.3×10^6
2-bromopropionate	0.34 ± 0.02	624.0 ± 41.5	1.8×10^6
3-bromopropionate	0.31 ± 0.02	298.0 ± 21.5	9.6×10^5
2-bromobutyrate	12.4 ± 3.10	341 ± 14.3	2.8×10^4
2-bromocaproate	22.5 ± 4.20	261.8 ± 20.0	1.2×10^4
monochloroacetate	1.01 ± 0.24	307.0 ± 18.3	3.0×10^5
monoiodoacetate	3.25 ± 0.32	2.7 ± 0.3	8.0×10^2
monofluoroacetate	0.21 ± 0.04	739.0 ± 26.8	3.5×10^6
p-nitrophenyl butyrate	1.64 ± 0.35	26 500 ± 830	16.2×10^6
ethyl butyrate	90 ± 5.0	11.7 ± 0.6	1.3×10^2

[a] For reaction conditions (pH 8.5, T = 40 °C) see ref. [9]. Activities towards other common esters and haloacids are shown in detail in Table S4.

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and iodide (4800-fold), within the same substrate series. We further estimated the apparent enantioselectivity (E_{app}) of the REBr enzyme by separate measurements of the kinetic parameters for the hydrolysis of two model enantiomers, namely (*S*)- and (*R*)-3-bromo-2-methylpropionate. It should be mentioned that the ratios obtained by these measurements were not true enantiomeric ratios (E_{true}), because the rates of hydrolysis of the enantiomers were measured separately. As shown in Table S2, the enzyme showed a higher preference for the *S*-configured halogen over the *R*-configured substrate ($E_{app} \sim 41$).

The genes of three other α/β-hydrolases[10] homologous to REBr were cloned from bacterial genomes and hyperexpressed, then their products were purified. All enzymes showed slightly lower (ca. sixfold) k_{cat}/K_m values for the cleavage of *p*NP esters than REBr, and none of them hydrolyzed haloacids or haloalkanes. These REBr homologues thus behaved as normal ester hydrolases (Table S3; Figure S3).

Halo-organics inhibited the catalytic activity of REBr towards *p*NP butyrate sevenfold, and *p*NP butyrate strongly inhibited the HAD reaction (75-fold; Figure 1); this demonstrates the promiscuous behavior of the REBr enzyme and its ability to concomitantly hydrolyze both esters and carbon–halogen bonds. This unusual biochemical feature pointed to the possibility of the coexistence of two different catalytic abilities in the same protein.

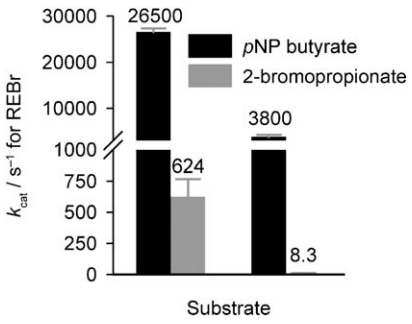

Figure 1. Reciprocal substrate inhibition of REBr-mediated hydrolysis of *p*NP butyrate and 2-bromopropionate. The two left-hand bars show the k_{cat} values for hydrolysis of the individual substrates alone, whereas the right-hand bars show the values obtained with an equimolar mixture of substrates. Reaction conditions: $[E]_o = 0$–12 nM, 3 mM *p*-nitrophenyl butyrate or 2-bromopropionate, 100 mM Tris-sulfate, pH 8.5, $T = 40\,^{\circ}C$. Kinetic parameter (k_{cat}) was calculated as in ref. [9]. Results shown are the average of three independent assays ± the standard deviation.

In order to identify important residues in REBr for substrate binding and catalysis, we developed a 3D model (Figures S4 and 2) to guide site-directed mutagenesis. It revealed a catalytic pocket conserved in esterases (S139/D259/H287), which was confirmed by establishing and analyzing S139G, D259N, and H287Q variants of REBr. The K_m, k_{cat} and k_{cat}/K_m values for *p*NP esters were all significantly reduced (Table S4). A radiochemical assay with the substrate methyl [1-^{14}C]butyrate[9] has confirmed its binding and formation of the acyl-enzyme intermediate of S139 (not shown), which is consistent with its role as a nucleophile. Surprisingly, the D259N and H287Q variants showed an

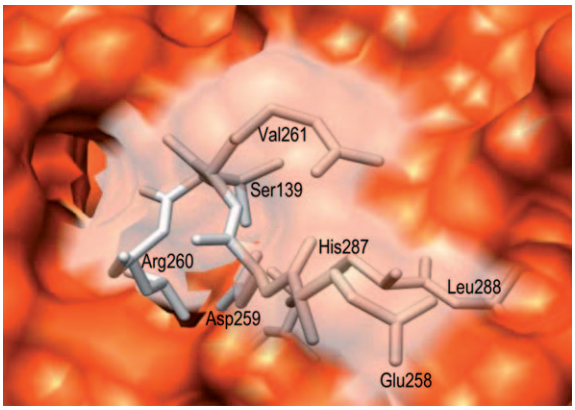

Figure 2. Proposed catalytic and stabilizing residues of the REBr protein. The hydrolases CumD (EC 3.7.1.9; PDB: 2D0D; 20% identity) of *Pseudomonas fluorescens* IPO1 and HasD (EC 3.7.1.8; PDB: 2VF2; 25% identity) from *Mycobacterium tuberculosis* were identified as the best templates and used for homology modeling.

almost complete loss of activity (catalytic efficiencies of the mutants could not be determined because of the apparently high value of K_m), and S139G exhibited significant reduction (ca. 20000-fold) in catalytic efficiency for 2-bromopropionate (Table S4). This last result can be explained by the up to 75-fold greater K_m value coupled with a significant (328-fold) reduction in the k_{cat} value.

In addition, amino acids E258, R260, V261, and L288 were identified from the 3D model as being potential halide-stabilizing residues (Figure 2), therefore E258Q, R260L, V261A, and L288A variants were generated.[9] None of the mutations had a dramatic effect on the esterase activity of REBr (33–99% rel. K_m; 52–116% rel. k_{cat}; 62–198% rel. k_{cat}/K_m; Table S4), but the E258Q, R260L, and V261A substitutions dramatically reduced activity towards halogenated compounds due to an up to 480-fold lower k_{cat} and 117-fold higher K_m towards 2-bromopropionate.

The kinetic constants of 2-bromopropionate binding to REBr were further measured by monitoring intrinsic protein fluorescence by stopped-flow methods.[9] Addition of 2-bromopropionate (which was used as model halo-organic) at different concentrations to REBr resulted in a clear fluorescence enhancement (not shown) similar to that reported for the halohydrin dehalogenase of *Agrobacterium radiobacter*.[11] The kinetics could be fitted to a single exponential equation,[11] thus yielding an observed rate constant (k_{obs}) from which k_2 and k_{-2} values could be calculated together with K_1 and K_d values (Table 2).

The rate of substrate binding (k_2: ~153 s^{-1}) was about twofold higher than the rate of release of alkyl halide from the active site (k_{-2}: ~43–78 s^{-1}); this suggests that binding is not a limiting step for substrate conversion. This observation is consistent with the higher carbon–halogen cleavage rates of REBr (see Table 1), as compared with those of known dehalogenases (k_{cat} up to 41 s^{-1}).[12] Like the parent protein, REBr mutants D259N, H287Q, and E258Q exhibited fluorescence enhancement upon addition of halo-organics at different concentrations, and displayed similar K_d values (Figure 3). However, sub-

Table 2. K_1, k_2, k_{-2} and K_d constants for the REBr enzyme. 2-Bromopropionate in 100 mM Tris-sulfate (pH 8.5) was used as substrate for binding experiments[a]

K_1 [mM]	k_2 [s^{-1}]	k_{-2} [s^{-1}]	K_d [μM]
1.21 ± 0.44	152.5 ± 13.5	77.9 ± 9.6	0.41 ± 0.09

[a] Constants were calculated according to refs. [9] and [11].

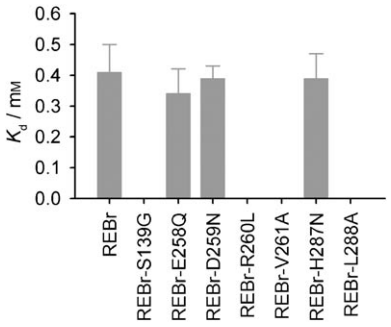

Figure 3. Apparent dissociation constant (K_d) of wild-type REBr and its mutants with 2-bromopropionate. Experiments were carried out in 100 mM Tris-sulfate, pH 8.5, at 40 °C. Results shown are the average of three independent assays ± the standard deviation. Values were calculated according to Equation (5) in refs. [9] and [11].

strate addition to REBr mutants S139G, R260L, V261A, and L288A did not induce any marked fluorescence increase (so kinetic parameters could not be determined); this suggests that the mutated residues in these mutants affected the binding sites of the enzyme.

Time courses of substrate consumption and covalent enzyme–intermediate complex formation were followed in a reaction of 43 μM of [^{14}C]2-bromopropionate and 78 μM of wild-type protein. After filtration of the acid-quenched reaction samples, enzyme, substrate-bound enzyme, and unconsumed substrate were separated by HPLC.[9] Covalent attachment of a radiolabel to the enzyme at position E258 was demonstrated by separating the quenched reaction mixtures of parental and mutant enzyme by SDS-PAGE. There was a coincidence of the ^{14}C label and the parental enzyme band, and an absence of labeled mutant enzyme (not shown), thereby confirming the role of E258 as the nucleophile in carbon–halogen cleavage.

These biochemical analyses on mutant enzymes confirmed residues S139, E258, D259, R260, V261, H287, and L288 in the REBr parental enzyme as multifunctional amino acids participating in substrate binding and cleavage of a number of different halogenated and unhalogenated substrates (Figure 4), with S139, D259, and H287 serving as nucleophile, acid, and base, respectively, for the cleavage of common esters. Furthermore, these amino acid residues, together with E258, R260, V261, and L288, play significant roles in the transformation of haloorganics (see the putative catalytic mechanism in Figure 4). Indeed, E258 could act as the nucleophile, whereas H287 might activate the water molecule needed for carbon–halogen cleavage, with D259 stabilizing the H287 during the reaction. It seems likely that the putative primary halide-stabilizing resi-

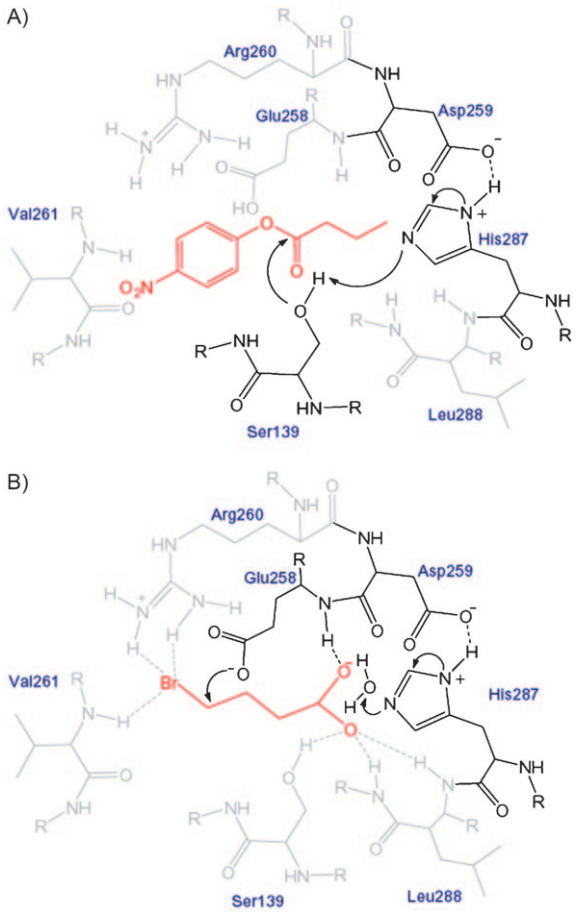

Figure 4. A) Suggested esterase and B) haloacid dehalogenase catalytic mechanisms for the REBr enzyme. Catalytic and binding residues are shown in each model (for details see Figure S6). Substrates are shown in red. Residues marked in gray in panel A are not thought to play a direct role in esterase activity, whereas residues in gray in panel B are suggested to play a role in substrate binding. Although, the model might not represent the exact locations of residues in the protein structure, it summarizes in a graphical view the interactions that should exist around the catalytic core in the REBr protein.

dues are R260 and V261, in combination with S139, which is probably involved in stabilization of the COOH group, and L288. Further determination of the structure of REBr by X-ray crystallography is needed to confirm these notions.

Sequence alignments (Figure S5) to detect whether the amino acids identified here as critical for activity are located in conserved regions of α/β-hydrolases showed that three out of seven—S139, D259 and H287 (REBr numbering)—are absolutely conserved in all proteins. The quality of the alignment in this region is very good, with no gaps and with an almost exact superimposition of secondary-structure predictions. No variants of either REBr, or any of the three other α/β-hydrolase homologues analyzed in this study that bears a mutation in these positions exhibited any catalytic activity. However, the core motif E258·R260·V261·L288 determined in this study to be critical for dehalogenase activity is not present in any other homologue protein. These differences might explain the substrate specificity of the protein under investigation, which, together with previous studies,[6] suggests that active-site ar-

chitecture relatedness is a major determining factor for the evolution of new catalytic activities.

In conclusion, our study has further demonstrated the utility of metagenomics to access novel catalytic activities, and has delivered the first experimental evidence of a functional switch from esterase to dehalogenase activity in a single protein. Our results have several implications. First, they demonstrate the global role of the highly conserved triad Ser/Asp/His in the hydrolysis of both ester– and carbon–halogen bonds. Second, the co-occurrence of both esterase and dehalogenase activities in the REBr protein could provide a significant competitive advantage in organisms thriving in environments containing both classes of substrates.[13,14] In this context, mutations at residues that have a significant role in stabilization, abstraction or orientation of substrates in the active site (also having a role in defining a certain level of physical/chemical freedom) will provide variants from which new specificities can evolve with or without an increase in catalytic promiscuity.

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[9] a) Detailed experimental procedures are available in the Supporting Information. b) Tentative phylogenetic affiliation is shown in Figure S2. c) A graphical representation of optimal pH (~8.5) and temperature (~40 °C) is shown in Figure S3.

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