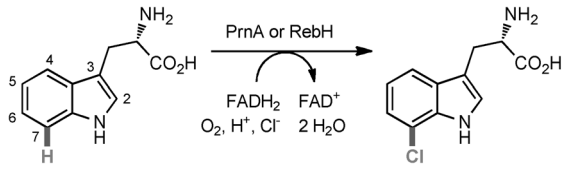
DOI: 10.1002/cbic.201300780

Improving the Stability and Catalyst Lifetime of the Halogenase RebH By Directed Evolution

Catherine B. Poor, Mary C. Andorfer, and Jared C. Lewis*[a]

We previously reported that the halogenase RebH catalyzes selective halogenation of several heterocycles and carbocycles, but product yields were limited by enzyme instability. Here, we use directed evolution to engineer an RebH variant, 3-LR, with a T_{opt} over 5 °C higher than that of wild-type, and 3-LSR, with a T_m 18 °C higher than that of wild-type. These enzymes provided significantly improved conversion (up to fourfold) for halogenation of tryptophan and several non-natural substrates. This initial evolution of RebH not only provides improved enzymes for immediate synthetic applications, but also establishes a robust protocol for further halogenase evolution.

Halogenated organic compounds pervade chemistry and play important roles in industrial, agrochemical, pharmaceutical, and materials products, as well as functioning as essential building blocks and intermediates in organic synthesis.[1–3] Halogenated arenes comprise a particularly important class of compounds, but conventional approaches to arene halogenation by electrophilic aromatic substitution require harsh chemical oxidants and often suffer from poor regioselectivity.[4,5] In nature, selective arene halogenation is catalyzed by flavin-dependent halogenases,[6] which employ halide salts and air as the halogen source and terminal oxidant, respectively (Scheme 1). Several groups have used halogenases, (including



Scheme 1. RebH- or PrnA-catalyzed 7-chlorination of tryptophan.

RebH,[7] PrnA,[8] and point mutants of these enzymes)[9,10] to halogenate tryptophan and related small molecules on an analytical scale. We recently explored the substrate scope and selectivity of RebH and showed that this enzyme can halogenate a range of substituted indoles and naphthalenes on a preparative scale.[11] Although the scope, selectivity, and mild reaction conditions we employed highlight the synthetic utility of enzymatic halogenation, the high enzyme loadings required to achieve synthetically useful product yields hinder the practicability of RebH. During preparative-scale bioconversions in our laboratory, extensive RebH precipitation was observed after several hours of reaction (well after the window in which kinetic data were acquired); this suggests that significant improvements in product yield might be possible by increasing the stability of this enzyme.

Stability is an important property of all enzymes, particularly those exposed to the reaction conditions encountered in industrial processes or for laboratory evolution experiments.[12,13] Improving enzyme thermostability has many benefits, including prolonging catalyst lifetime, increasing enzyme tolerance to stress (such as proteolysis and organic solvents), and enabling reactions to be conducted at higher temperatures, which can increase reaction rates.[14–16] Stable enzymes can also better tolerate mutations introduced to alter other properties, such as substrate scope and specific activity, as random mutations are generally destabilizing.[17] To the best of our knowledge, no halogenase from a thermophilic organism has been characterized. Here, we describe the first use of directed evolution to increase both the thermostability and the optimal operating temperature of RebH.

To improve thermostability without losing catalytic activity, we employed a screen of RebH mutant libraries by incubating at elevated temperatures and examining reaction conversions at room temperature.[18] Error-prone PCR was used to generate a library of RebH variants with an average of two residue mutations. The library was expressed in *Escherichia coli* in 96-well plates; the cells were lysed, and the supernatants were transferred to microtiter plates for heat treatment. Tryptophan halogenation reactions were conducted overnight, and reaction conversions were determined by HPLC analysis.

The first-generation mutant library was constructed from wild-type (WT) RebH as the parent, and 1365 colonies were screened following incubation at 42 °C for 2 h. Mutants showing twice the conversion of WT were identified (confirmed following purification and incubation at 49 °C for 2 h). In addition, the melting temperature (T_m, the midpoint of the thermal unfolding transition curve) of an improved mutant (the single amino acid mutation S2P) was analyzed by circular dichroism (CD) spectroscopy. The S2P mutant had a T_m 2 °C higher than that of WT RebH, thus indicating increased stability. The beneficial mutations identified in improved variants from the first round were combined by using overlap extension PCR, and the best variant (1-PVM: mutations S2P, M71V, and K145M) from this library showed an almost 20-fold improvement in conversion compared to WT (Figure 1 A).

Mutant 1-PVM was used as the parent for a second-generation random mutagenesis library. Of the 1008 colonies

[a] Dr. C. B. Poor, M. C. Andorfer, Prof. J. C. Lewis
Department of Chemistry, University of Chicago
5735 S. Ellis Ave., Chicago, IL 60637 (USA)
E-mail: jaredlewis@uchicago.edu

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201300780.

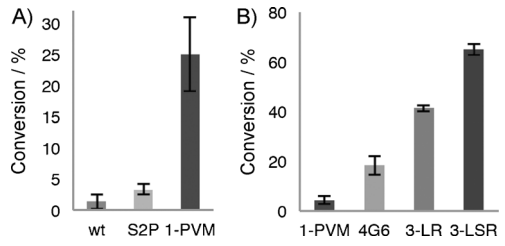


Figure 1. Halogenation conversion after incubation at 49 °C for 2 h. Reactions were performed on tryptophan with A) 2% and B) 0.5% enzyme loading.

screened following incubation at 51 °C for 2 h, variant 4G6 (E423D and E461G) provided a further 2.5-fold increase in conversion. The third-generation random mutagenesis library used 4G6 as the template and comprised 1008 colonies. Each of the three best-performing variants from the third round of screening following incubation at 54 °C for 3 h contained a single amino acid mutation. Following combination, the two best variants were identified as 3-LR (S130L, Q494R) and 3-LSR (S130L, N166S, Q494R; Figure 1 B).

The melting temperatures of the best mutants identified through the rounds of genetic diversification, screening, and recombination were analyzed to probe the relationship between halogenase conversion and thermostability (Figure 2 A).

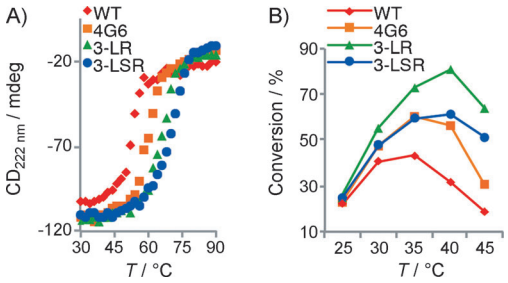
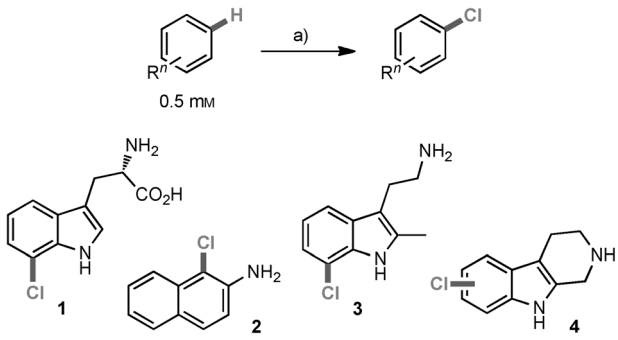


Figure 2. A) Thermal denaturation curves obtained using CD at 222 nm. B) Conversion–temperature profiles of RebH (0.4 mol% RebH).

WT RebH had a melting temperature of 52.4 °C; for the most thermostable variant, 3-LSR, it was 70.0 °C. The 18 °C increase in T_m indicates significant improvement in enzyme stability. To determine whether improved thermostability enables the use of higher reaction temperatures, conversion–temperature profiles of RebH variants were constructed (Figure 2 B). With the accumulation of beneficial mutations, the optimum temperature for halogenation (T_{opt}) of tryptophan based on total conversion to halogenated product (not initial rate) increased by at least 5 °C, from 30–35 °C for WT RebH to 40 °C for 3-LR. Mutant 3-LR produced 100% more 7-chlorotryptophan than WT RebH on an analytical scale when at the respective T_{opt}.

To establish the relevance of these thermostability improvements to preparative-scale biocatalysis, halogenation by 3-LR and 3-LSR of several substrates was examined (Scheme 2, Table 1). Based on HPLC analysis, reaction of tryptophan with



Scheme 2. General scheme for RebH-catalyzed arene halogenation, and substrates used to examine enzyme scope. a) RebH, 0.2 equiv FAD, 40 equiv glucose, cofactor regeneration system, 100 mM NaCl, 25 mM HEPES, 5% *i*PrOH, pH 7.4.

Table 1. Representative yields for preparative 3-L(S)R-catalyzed[a] halogenation reactions and comparisons to WT RebH-catalyzed reactions.

Product	Enzyme ([mol%])	*T* [°C]	*t* [h]	Yield [%][a]	Fold improvement[b]
1	3-LR (0.4)	40	16	69	2.8
2	3-LSR (0.8)	21	30	62	2.3
3	3-LSR (1.0)	40	36	56	4.1
4	3-LSR (2.5)	21	48	67[c]	1.7

[a] Isolated yield of pure product. [b] Ratio of product concentrations relative to internal standard from HPLC analysis of crude reaction mixtures when using 3-L(S)R or WT RebH as specified.[19] [c] 88:12 ratio of 5-/6-halogenation products.

3-LR at 40 °C afforded a 2.8-fold increase in the yield of **1** relative to the reaction with WT RebH at 35 °C (optimal reaction conditions for both enzymes).[19] Furthermore, a 69% isolated yield of **1** was obtained when using only 0.4 mol% 3-LR loading, compared to a 37% yield by the same loading of WT RebH.

Improved conversions (1.7- to 4.1-fold relative to WT) of the non-natural substrates 2-aminonaphthalene, 2-methyltryptamine, and tryptoline (to **2**–**4**, respectively) were also observed with 3-LSR (Scheme 2, Table 1). Reactions with 3-LSR and WT RebH were conducted at 21 °C and 40 °C for identical times and enzyme loadings. In each case, the highest conversion was observed at the same temperature for both 3-LSR and WT RebH: 21 °C for the formation of **2** and **4**, and 40 °C for **3**. Interestingly, the selectivity of tryptoline halogenation (at the 5 vs. the 6 position) increased from 60% with RebH to 88% for 3-LSR (no other changes in selectivity were observed). Bioconversions with 3-LSR continued for 30 h at up to 40 °C, whereas WT RebH began precipitating into inactive aggregates within hours of initiating reactions, thus clearly illustrating the stability of 3-LSR.[11] Therefore, directed evolution can be used to improve halogenase stability, lifetime, and selectivity; 3-L(S)R should prove a good starting point for evolving RebH variants with activity with a range of additional substrates.[20]

To better illustrate the extended lifetimes of these enzymes, halogenation of 2-methyltryptamine (10 mg) with 3-LSR and WT RebH was monitored under optimal conditions for each

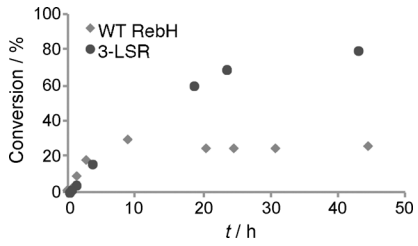


Figure 3. Plots of conversion against time for halogenation of 2-methyltryptoamine by WT and 3-LSR RebH at 40 °C.

enzyme[19] at 40 °C. As noted above, maximum product yields for both enzymes were observed at this temperature; however, the reaction profiles (Figure 3) show that 3-LSR remained active for significantly longer (~threefold), thereby affording an approximately fourfold improvement in yield (Table 1, entry 3). Steady-state kinetic analysis of the enzymes showed that WT RebH has a k_{cat}/K_m similar to that of 3-LSR at 40 °C (Table 2, entries 1 and 2), but a significantly higher k_{cat}/K_m at 21 °C (Table 2, entries 3 and 4). These data are consistent with the notion that stabilized enzymes have decreased conformational flexibility and that whereas this decrease can be beneficial for stability (prolonging lifetime, or enabling reaction at high temperature), it can be detrimental to activity.[21,22] Similarly contrasting stability and kinetic data have been reported for other stabilized enzymes that provided increased product yields,[23,24] but stabilized enzymes with essentially unchanged[25,26] or even increased[27,28] k_{cat}/K_m have also been reported.

Table 2. Kinetic data for halogenation of 2-methyltryptamine by RebH and 3-LSR at 40 °C and 21 °C.[19]

Enzyme	T [°C]	K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} μM^{-1}]
RebH	40	280.1 ± 18.4	0.25 ± 0.0095	8.8×10^{-4}
3-LSR	40	202.5 ± 12.7	0.15 ± 0.0093	7.9×10^{-4}
RebH	21	16.8 ± 3.8	0.060 ± 0.0057	3.6×10^{-3}
3-LSR	21	40.2 ± 3.7	0.013 ± 0.00036	3.3×10^{-4}

A variety of structural features can impart stability to proteins;[29] to gain insight into these features, we solved the crystal structure of 3-LSR and compared it with the WT RebH structure. Phases of 3-LSR were obtained by molecular replacement by using WT RebH (PDB ID: 2OAM) as the search model. The crystal structure was refined to 3.05 Å (final R_{work} = 18%, R_{free} = 24%). WT RebH and 3-LSR are similar overall with a backbone root mean square deviation (RMSD) of 0.32 Å. The differences in the structures are at the eight changed amino acids.

Investigating the location and nature of the mutations in the structure of 3-LSR might provide a molecular basis for the increased thermostability and T_{opt}. Mutation Q494R is on the protein surface (neutral side chain of glutamine, positively charged arginine). Increasing surface charge deters protein aggregation.[30] For the serine-to-proline mutation of S2P (N terminus), proline residues generally increase protein rigidity by decreasing the flexibility of the polypeptide chain. Indeed, the five other RebH crystal structures in the PDB start their models at amino acid 2 or 3;[31,32] in 3-LSR, the electron density map extends to amino acid one, thus indicating increased order at the N terminus. The increased rigidity of the N terminus might also help stabilize the protein by preventing it from acting as a "fraying" point for thermal unfolding.[33] Mutation K145M is near the surface of the protein and in the area of two arginine residues (Figure 4); WT RebH has higher positive charge density around this lysine, and the methionine substitution in 3-LSR might yield stability by reducing the charge density at this position.[34] Moreover, the side chain of methionine has a conformation that enhances packing with neighboring residues; this might enhance thermostability.[35]

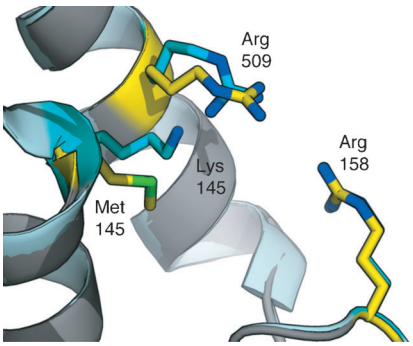


Figure 4. The local environment of the K145M mutation. Overlay of WT RebH (grey backbone carbons, cyan side-chain carbons, blue side-chain nitrogens; PDB ID: 2OAM) and 3-LSR (light blue backbone carbons, yellow side-chain carbons, blue nitrogens, green sulfur; PDB ID: 4LU6).

In our previous work with the tryptophan halogenase RebH, product yield was limited by enzyme instability.[11] Even though RebH has a melting temperature above 50 °C, improvements in stability under our reaction conditions were desired, so a protein engineering approach was pursued. Three rounds of error-prone PCR, recombination, and screening resulted in variants 3-LR (seven mutations) with a T_{opt} more than 5 °C higher than that of WT, and 3-LSR (eight mutations) with a T_m 18 °C higher than that of WT. That different mutants had the highest T_m and T_{opt} values indicates that thermostability and halogenase conversion are not strictly coupled. A common belief accounting for this divergence is that increased rigidity helps stability but hinders activity.[21,22] Ultimately, however, 3-LSR did provide 100% improvement in halogenase conversion at its T_{opt}, and this improvement held for several non-natural substrates. This initial demonstration of RebH evolution not only provides improved enzymes for immediate synthetic applications, but also establishes a robust protocol for further halogenase optimization.

Acknowledgements

This work was supported by an NIH Pathways to Independence Award (5R00GM087551-03) and a Searle Scholar Award (11-SSP-

202) to J.C.L. and an NSF predoctoral fellowship to M.C.A. We thank Prof. Phoebe Rice for the use of crystallography software, Dr. Elena Solomaha for assistance with CD spectroscopy, and James Payne for optimization of protein expression and lysis conditions in 96-well plate format. This work is based on research conducted at the APS on the NE-CAT beamlines, which are supported by a grant from the NIGMS (P41 GM103403) from the NIH. Use of the APS, an Office of Science User Facility operated for the U.S. DOE Office of Science by Argonne National Laboratory, was supported by the U.S. DOE under Contract No. DE-AC02-06CH11357. We thank all beamline staff at NE-CAT for their assistance.

Keywords: biocatalysis · directed evolution · halogenases · RebH · thermostability

[1] J. Fauvarque, *Pure Appl. Chem.* **1996**, *68*, 1713 – 1720.
[2] P. Jeschke, *Pest Manage. Sci.* **2010**, *66*, 10 – 27.
[3] M. Z. Hernandes, S. M. T. Cavalcanti, D. R. M. Moreira, W. F. de Azevedo, Jr., A. C. L. Leite, *Curr. Drug Targets* **2010**, *11*, 303 – 314.
[4] K. Smith, G. A. El-Hiti, *Curr. Org. Synth.* **2004**, *1*, 253 – 274.
[5] A. Podgoršek, M. Zupan, J. Iskra, *Angew. Chem. Int. Ed.* **2009**, *48*, 8424 – 8450; *Angew. Chem.* **2009**, *121*, 8576 – 8603.
[6] K.-H. van Pée, E. P. Patallo, *Appl. Microbiol. Biotechnol.* **2006**, *70*, 631 – 641.
[7] E. Yeh, S. Garneau, C. T. Walsh, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 3960 – 3965.
[8] M. Hölzer, W. Burd, H.-U. Reißig, K.-H. van Peé, *Adv. Synth. Catal.* **2001**, *343*, 591 – 595.
[9] W. S. Glenn, E. Nims, S. E. O'Connor, *J. Am. Chem. Soc.* **2011**, *133*, 19346 – 19349.
[10] A. Lang, S. Polnick, T. Nicke, P. William, E. P. Patallo, J. H. Naismith, K.-H. van Pée, *Angew. Chem. Int. Ed.* **2011**, *50*, 2951 – 2953; *Angew. Chem.* **2011**, *123*, 3007 – 3010.
[11] J. T. Payne, M. C. Andorfer, J. C. Lewis, *Angew. Chem. Int. Ed.* **2013**, *52*, 5271 – 5274; *Angew. Chem.* **2013**, *125*, 5379 – 5382.
[12] M. Adamczak, S. H. Krishna, *Food Technol. Biotechnol.* **2004**, *42*, 251 – 264.
[13] J. D. Bloom, S. T. Labthavikul, C. R. Otey, F. H. Arnold, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 5869 – 5874.
[14] I. Wu, F. H. Arnold, *Biotechnol. Bioeng.* **2013**, *110*, 1874 – 1883.
[15] H. H. Liao, *Enzyme Microb. Technol.* **1993**, *15*, 286 – 292.
[16] H. Zhao, F. H. Arnold, *Protein Eng.* **1999**, *12*, 47 – 53.
[17] A. A. Pakula, R. T. Sauer, *Annu. Rev. Genet.* **1989**, *23*, 289 – 310.
[18] P. C. Cirino, R. Georgescu in *Methods in Molecular Biology, Vol. 230: Directed Enzyme Evolution: Screening and Selection Methods* (Eds.: F. H. Arnold, G. Georgiou), Humana, Totowa, **2003**, pp. 117 – 125.
[19] In preparative-scale reactions, we found that 3-LSR provided the highest conversions without agitation, whereas WT RebH provided the highest conversions with agitation. So, these conditions were used for each enzyme, respectively, in preparative scale reactions. Steady-state kinetics for both enzymes were measured for reactions that were shaken to achieve reproducible results.
[20] J. C. Lewis, S. M. Mantovani, Y. Fu, C. D. Snow, R. S. Komor, C.-H. Wong, F. H. Arnold, *ChemBioChem* **2010**, *11*, 2502 – 2505.
[21] P. Závodszky, J. Kardos, Á. Svingor, G. A. Petsko, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 7406 – 7411.
[22] M. Wolf-Watz, V. Thai, K. Henzler-Wildman, G. Hadjipavlou, E. Z. Eisenmesser, D. Kern, *Nat. Struct. Mol. Biol.* **2004**, *11*, 945 – 949.
[23] X.-Q. Pei, Z.-L. Yi, C.-G. Tang, Z.-L. Wu, *Bioresour. Technol.* **2011**, *102*, 3337 – 3342.
[24] A. McDaniel, E. Fuchs, Y. Liu, C. Ford, *Microb. Biotechnol.* **2008**, *1*, 523 – 531.
[25] D. Zhang, F. Zhu, W. Fan, R. Tao, H. Yu, Y. Yang, W. Jiang, S. Yang, *Appl. Microbiol. Biotechnol.* **2011**, *90*, 1361 – 1371.
[26] Y. Wang, E. Fuchs, R. da Silva, A. McDaniel, J. Seibel, C. Ford, *Starch/Staerke* **2006**, *58*, 501 – 508.
[27] R. Kumar, M. Sharma, R. Singh, J. Kaur, *Mol. Cell. Biochem.* **2013**, *373*, 149 – 159.
[28] D. Maté, C. García-Burgos, E. García-Ruiz, A. O. Ballesteros, S. Camarero, M. Alcalde, *Chem. Biol.* **2010**, *17*, 1030 – 1041.
[29] G. A. Petsko, *Methods Enzymol.* **2001**, *334*, 469 – 478.
[30] M. S. Lawrence, K. J. Phillips, D. R. Liu, *J. Am. Chem. Soc.* **2007**, *129*, 10110 – 10112.
[31] E. Yeh, L. C. Blasiak, A. Koglin, C. L. Drennan, C. T. Walsh, *Biochemistry* **2007**, *46*, 1284 – 1292.
[32] E. Bitto, Y. Huang, C. A. Bingman, S. Singh, J. S. Thorson, G. N. Phillips, Jr., *Proteins Struct. Funct. Bioinf.* **2008**, *70*, 289 – 293.
[33] P. R. Blake, J.-B. Park, F. O. Bryant, S. Aono, J. K. Magnuson, E. Eccleston, J. B. Howard, M. F. Summers, M. W. W. Adams, *Biochemistry* **1991**, *30*, 10885 – 10895.
[34] A. Martin, V. Sieber, F. X. Schmid, *J. Mol. Biol.* **2001**, *309*, 717 – 726.
[35] E. Querol, J. A. Perez-Pons, A. Mozo-Villarias, *Protein Eng.* **1996**, *9*, 265 – 271.

Received: December 13, 2013
Published online on May 21, 2014