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Exploring the Biocatalytic Potential of Fe/α-Ketoglutarate-Dependent Halogenases

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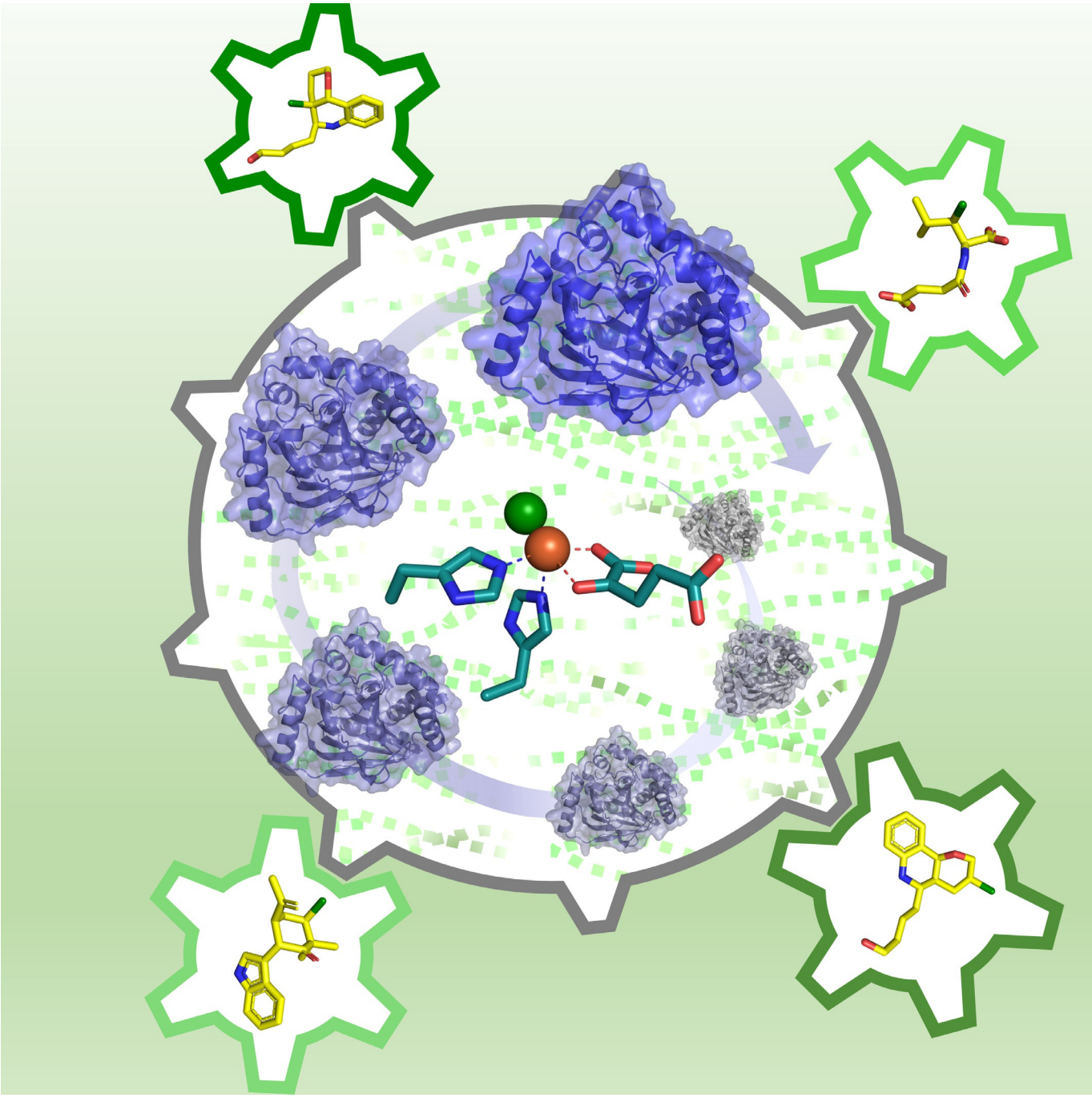

Abstract: Freestanding Fe/α-ketoglutarate-dependent halogenases are oxidoreductases that catalyze the installation of halogen atoms into unactivated sp^3-hybridized carbon centers with high stereo- and regioselectivity. Since their discovery in 2014, a small number of indole alkaloid and amino acid halogenases have been identified and characterized. First enzyme engineering examples suggest that the accessible substrate range of these enzymes may be expanded through the use of rational enzyme design and directed evolution. Structural investigations of non-heme iron halogenases acting on freestanding as well as tethered substrates are beginning to inform about the principles of the underlying halogenation mechanism.

Introduction

The number of employed biocatalytic steps in industrial processes is continuously increasing and today enzymes are routinely used to catalyze a variety of chemical transformations, prominent examples including the synthesis of chiral alcohols and amines.[1]

The manufacturing processes benefit from the high regio- and stereoselectivity of the—typically engineered—biocatalysts, enabling the selective chemical synthesis while reducing associated protection, deprotection, and purification steps.[2] As the discipline of biocatalysis is maturing, scientists turn to improve the technological readiness of intrinsically more complex enzyme classes and begin to tap into novel reactivities that go well beyond the current state-of-the-art. Especially C–H activating enzymes such as halogenases are interesting targets as they are valuable tools for the production of high-value compounds such as pharmaceuticals and agrochemicals.[3]

The substitution of a hydrogen with a halogen atom alters a molecule's properties and affects its pharmacokinetics as well as its biological activity. Unsurprisingly, around 20% of small molecule drugs and 30% of agrochemicals are halogenated.[4] In addition, organic halides are useful synthetic handles for functional group transformations and—if installed on aromatic moieties—for the formation of C–C bonds.[5] However, although halogenated compounds find wide application in academia and industry, the chemical synthesis of organic halides remains challenging and traditionally require alcohols, alkenes, and acid containing compounds as precursors. Even modern approaches only offer few methods for the functionalization of unactivated $C(sp^3)$–H bonds, which typically place specific demands on substrate structure and may suffer from lack of stereoselectivity.[6]

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Enzymes as Valuable Biocatalysts for Halogenation

Turning to nature, the more than 4700 halogenated compounds, which have been discovered to date,[7] underline the importance of halogenation in biosynthetic pathways. Notably, in approximately half of these compounds the carbon atom to which the halogen is bound is sp^3 hybridized[5] emphasizing that stereocontrolled halogenation must be a critical strategy in natural product synthesis.

Today's known halogenating enzymes are classified according to their catalytic mechanism. α-Ketoglutarate-dependent halogenases (αKGHs), which are the focus of this Concept paper, harness a radical mechanism, while the other known halogenases either follow an electrophilic or a nucleophilic mechanism. Even though the catalytic cycle of the latter two enzyme families has been extensively reviewed elsewhere,[3b,6c,8] a brief overview of nature's alternative halogenation approaches is helpful to contextualize the unique synthetic advantages attainable through the halogenation strategy employed by αKGHs.

Heme-iron-dependent[9] and vanadium-containing halogenases,[10] which are referred to as haloperoxidases, oxidize a halide anion with the help of hydrogen peroxide and form hypohalous acid (HOX) as the halogenation agent.[11] Necessarily, these halogenases follow an electrophilic halogenation mechanism and act on electron-rich substrates, like aryl- or alkene moieties. In addition, the HOX is released by the enzyme into the reaction media resulting in the unselective halogenation of the substrate,[11] thus rendering these haloperoxidases unsuitable for selective chemical transformations. In contrast to haloperoxidases, flavin-dependent halogenases (FDHs), which also generate Cl^+,[12] Br^+,[13] or I^+ equivalents,[14] are substrate specific and enable regioselective halogenation. The reactive HOX is formed *via* oxidation of a halide anion by a C4a-hydroperoxyflavin species. However, instead of being released, the HOX is channeled through a 10 Å-long tunnel to the substrate binding site,[15] where it interacts with an active site lysine presumably either by reaction or *via* hydrogen bonding,[16] enabling selective halogenation of the deeply buried electron-rich substrates.

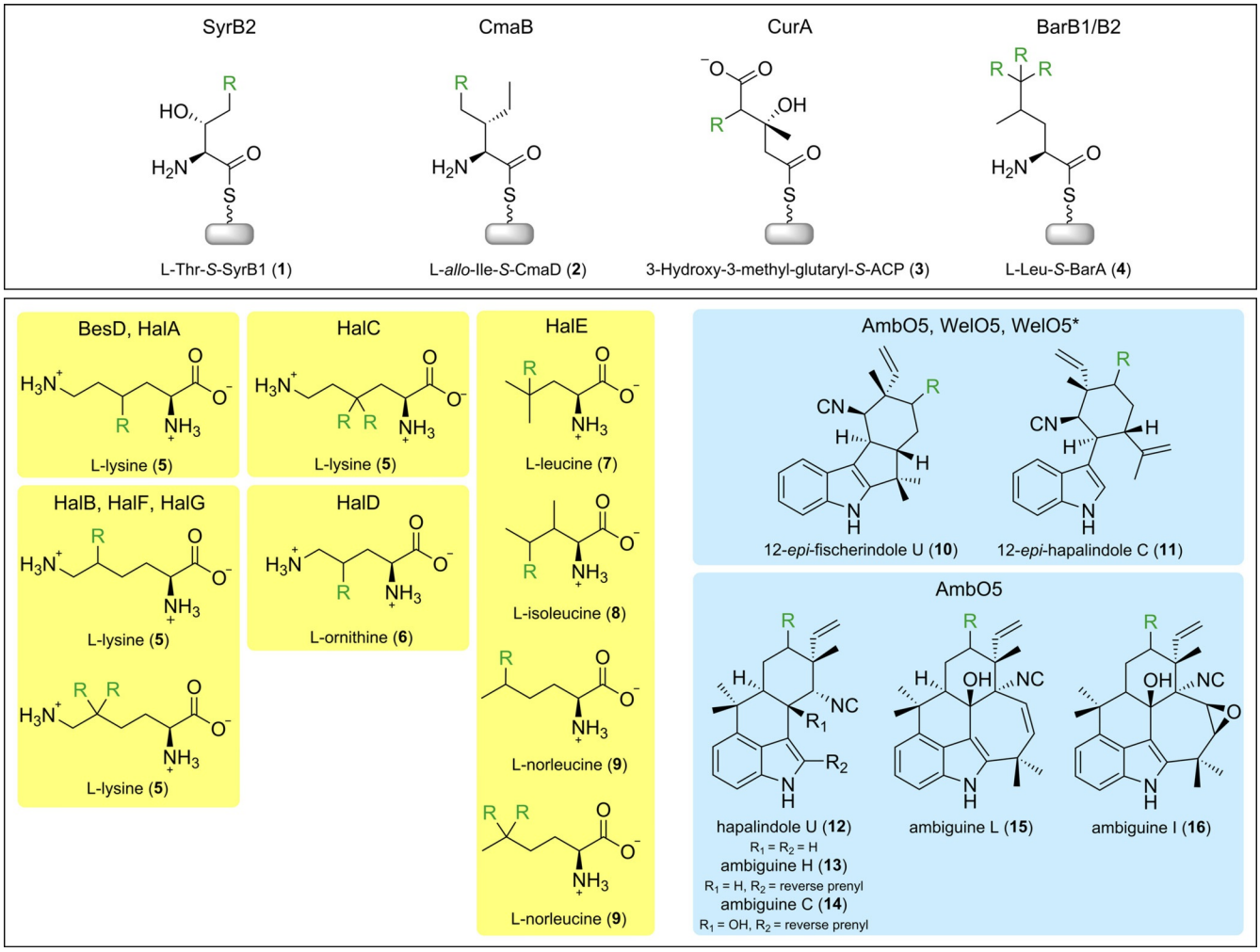
S-Adenosyl-L-methionine (SAM)-dependent halogenases, the only known enzyme family capable of installing fluoride into a substrate molecule, operate through a nucleophilic mechanism. Here, the halide ion attacks the C-5′ carbon atom of SAM in a S_N2 substitution reaction in which L-methionine acts as leaving group.[17] This reaction was described for the fluorinase from *Streptomyces cattleya*,[18] resulting in the corresponding 5′-fluorodeoxy adenosine (5′-FDA), with additional fluorinases catalyzing the same reaction identified in the following

years.[19] Besides the fluorinases, a chlorinase with a corresponding overall mechanism was identified.[20]

Fe/αKG-Dependent Enzymes Enable Selective C(sp^3)-Halogenation

The superfamily of α-ketoglutarate-dependent oxygenases (αKGDs) is diverse with members discovered in bacteria, fungi, plants, and vertebrates. Their biological function includes collagen biosynthesis, plant and animal development, transcriptional regulation, nucleic acid modification/repair, fatty acid metabolism, and secondary metabolite biosynthesis.[21] The transformations catalyzed by αKGDs reflect this diversity and include reactions such as hydroxylation, demethylation, desaturation, epoxidation, epimerization, cyclization, and halogenation.[22] αKGD coordinate Fe^{II} in the active site by two conserved histidine residues, a carboxylate residue (in halogenases: glycine or alanine) and the cofactor α-ketoglutarate, stabilized in a double-stranded β-helix (DSBH) fold.[23] The radical reaction mechanism of αKGDs involves the formation of a high-valent Fe^{IV}=O intermediate, which is formed at the expense of αKG and molecular oxygen. This ferryl intermediate abstracts a hydrogen from an unactivated C(sp^3)–H bond in the primary substrate yielding Fe^{III}–OH and the substrate radical, which subsequently reacts according to the employed enzyme's reaction specificity (*vide infra*).[24] Differing from the well-studied hydroxylases, halogenases do not contain the 2-His-1-carboxylate facial triad. Their active site is characterized by the so-called HXG motif, in which the carboxylate residue is replaced by a glycine or alanine allowing space for the coordination of a halide ion. Rebound of the coordinated halide to the substrate radical finally affords the halogenated product (Scheme 2, below).[25]

To date, a number of αKGHs have been described: The carrier protein-dependent halogenases BarB1 and BarB2,[26] SyrB2,[27] CytC3,[28] CmaB,[29] HctB,[30] CurA,[31] and the freestanding halogenases WelO5,[32] WelO5*,[33] *Wi*-WelO15,[34] AmbO5,[35] as well as the recently identified BesD family (Scheme 1).[36]



Scheme 1. Substrates (R = H) and products (R = Cl) of Fe/αKG-dependent halogenases with mono-, di- or trihalogenation activities. Only substrates names are shown. Carrier-protein-dependent halogenases (top panel) require covalently tethered substrates. Freestanding halogenases (bottom panel) act on free compounds. Enzymes belonging to the BesD/HalA-G (yellow) and AmbO5/WelO5 (blue) families halogenate amino acids and indole alkaloids, respectively. AmbO5 is also active towards other indole alkaloids not shown here. In all given examples, halogenation occurs regio- and stereoselectively, but resulting stereochemistries are not depicted. In some cases, bromination (R = Br) or azidation (R = N_3) has been demonstrated.

Table 1. Overview of the kinetic parameters of selected native and engineered Fe/αKG-dependent halogenases.[a]

Origin	Enzyme	Substrate	TTN	k_{cat} [min^{-1}]	K_M [mM]	k_{cat}/K_M [min^{-1} mM^{-1}]	Ref.
Carrier protein-dependent							
P. syringae pv. syringae B301D	SyrB2	**1**	7	29	3.1	9	[27]
P. syringae pv. tomato DC3000	CmaB	**2**	16	–	–	–	[29]
Freestanding							
Amino acid halogenases							
P. fluorescens	HalA	**5**	–	14.7	0.28	53	[36]
P. kilonensis	HalD	**6**	–	10.1	0.03	330	[36]
B. ambifaria	SadA D157G variant	**19**	3	–	–	–	[43]
Indole alkaloid halogenases							
F. ambigua UTEX 1903	AmbO5	**10**	–	1.7	–	–	[35]
		11	–	0.71	–	–	[35]
		12	–	0.21	–	–	[35]
		13, 14, 15, 16	–	0.67–0.7	–	–	[35]
H. welwitschii UTEX B1830	WelO5	**10**	75	1.8	–	–	[32, 35]
		11	–	0.73	–	–	[35]
H. welwitschii IC-52–3	WelO5*	**10, 11**	–	1.8–1.9[b]	–	–	[33]
	WelO5* CB2 variant	**17**	33	3.0	0.67	4.5	[37]
W. intricata HT-29–1	WelO15 *Wi*-5 variant	**18**	–	2.8	0.026	108	[34]
	WelO15 *Wi*-11 variant	**18**	–	11.1	0.137	81	[34]

[a] –: Not available. [b] Reported as k_{obs}.

The initially discovered carrier protein-dependent halogenases exhibit high specificity for the chlorination of their native substrates, like L-threonine (**1**) for SyrB2[27] and L-alloisoleucine (**2**) for CmaB,[29] both tethered to their respective carrier protein. The low activity of these enzymes (e.g., 7 TTN (total turnover number) for SyrB2,[27] see Table 1) and the mandatory covalent tethering of the substrate to the carrier protein has frustrated early attempts to use αKGHs for the biocatalytic C–H functionalization of amino acid type substrates. However, the recent discovery of freestanding αKGHs has rekindled interest in the area of enzymatic C(sp^3)-halogenation and has led to the identification[32,36] and tailoring[34,37] of αKGHs for academic and industrial applications.

Identification of Freestanding αKGHs by Pathway Elucidation

Liu and co-workers discovered the first freestanding Fe/αKG-dependent halogenases AmbO5 and WelO5 by pathway elucidation leading to indole alkaloids in the cyanobacteria *Fischerella ambigua* UTEX 1903 and *Hapalosiphon weltwitschii* UTEX B1830, respectively.[38] The authors found strong indication that chlorination occurred at the final steps of the biosynthetic routes because chlorinated and dechlorinated analogs were present at the same time, which led them to infer and verify the presence of a late-stage halogenase. Acquisition of amino acid sequence data permitted the cloning of the genes encoding the first freestanding αKGHs. Their subsequent heterologous expression in *E. coli* allowed for the first time detailed studies to be performed on the biochemical characteristics, catalytic properties and possible applications of the enzymes.

Whereas αKGH AmbO5 is able to halogenate the C13 atom of different fischerindole, hapalindole and ambiguine alkaloids (Scheme 1),[35] WelO5 has a more limited substrate scope and preferentially chlorinates the C13 of 12-*epi*-fischerindole U (**10**). Notably, WelO5 was the first freestanding αKGH for which a crystal structure was solved.[25] Analyzing the structural features of the enzyme, Liu and co-workers suggested that a distinctive phenomenon in WelO5 is the conformational change undergone by the external C-terminal α-helix spanning residues 220–235, which shifts 9 Å towards the active site upon substrate binding. As this α-helix is a highly variable region in AmbO5 and WelO5, it is considered to determine substrate tolerance and specificity. Substitution of 18 residues in this region or construction of a WelO5-AmbO5 chimera resulted in WelO5 variants with a substrate scope as broad as that of AmbO5.[35]

The discovery of WelO5 served as the basis for the identification and characterization of a further hapalindole alkaloid halogenase, WelO5* from *Hapalosiphon weltwitschii* IC-52-3, which shares 95% protein sequence identity with WelO5 - equivalent to a difference of 15 amino acids.[33] The fact that eleven out of these 15 residues reside on the C-terminal α-helix region most likely explains its enhanced specificity towards **10** compared to WelO5.[33] An additional WelO5 homolog investigated is *Wi*-WelO15 from *Westiella intricata* HT-29-1.[34] This halogenase shares 99% protein sequence identity with WelO5.

In 2019, the group of Chang published the discovery and characterization of a freestanding amino acid αKGH named BesD. The enzyme was identified while studying a gene cluster responsible for the biosynthesis of terminal alkyne amino acids in soil bacteria.[40] BesD, an amino acid halogenase involved in the biosynthesis of β-ethynylserine in *Streptomyces cattleya*,[40] is able to chlorinate L-lysine (5) at the γ-carbon (Scheme 1). Notably, BesD shares low protein sequence identity with other freestanding halogenases such as AmbO5 (25%) and WelO5 (11%) but is much closer related to Fe/α-ketoglutarate-dependent oxygenases. Based on the crystal structure of BesD in complex with 5, which was later solved by the same group,[36] this halogenase also seems to rely on a covering lid to fix the amino acid substrate in the active site. In addition to the HXG

motif, the residues His134 and Asn219 were determined to be important for chemoselectivity control through second-sphere interactions with the substrate and the oxo-ligand, respectively. Using BLAST and manual motif identification, potential αKGH sequences were selected and then narrowed down by removal of redundant and shorter sequences, sequence similarity network analysis by clustering and evaluation of their genomic contexts (amino acid metabolism). Twenty-one candidates originating mostly, though not only, from *Streptomyces* and *Pseudomonas* were grouped into eight clusters (HalA-H) and functionally screened towards a mix of different amino acids. Nineteen candidates from clusters HalA-G could catalyze the chlorination, bromination or azidation of **5**, **6**, **7**, **8** or **9** with different regioselectivities (Scheme 1), while two gene products belonging to cluster HalH did not exhibit amino acid halogenase activity.[36]

The discoveries of AmbO5, WelO5, and BesD have opened opportunities for further exploration of genes encoding new freestanding Fe/αKG-dependent halogenases. However, although important contributions, subsequent studies using known halogenase genes as templates will likely lead to candidates with similar substrate scopes. Thus, a possible strategy to source genes encoding novel Fe/αKG-dependent halogenase families is through biosynthetic pathway elucidation leading to C(sp^3)-halogenated natural products different from hapalindole or amino acid chemical cores. Methods such as genome mining for natural product discovery or phylogeny-guided mining for identification of new biosynthetic gene clusters from microbial genomes or metagenomes have extensively been reviewed and could be applied for this purpose.[41] The process of metabolic gene cluster discovery has become faster owing to the development of powerful bioinformatic tools and the continuously dropping costs of next-generation sequencing technologies.[42] Last but not least, new halogenases can be obtained by engineering Fe/αKG-dependent hydroxylases given the high structural similarity between the two enzyme families.[43]

Fe/αKG-Dependent Halogenases as Targets for Protein Engineering

To date, halogenase engineering has mainly focused on FDHs[44] or haloperoxidases[45] to halogenate non-natural substrates for their use as final products[46] or as intermediates to more valuable aryl-, alkoxy-, or amino compounds.[47] Due to their electrophilic reaction mechanism FDHs and haloperoxidases are generally limited to the conversion of aromatic compounds, though there are some exceptions, such as the synthesis of chloramphenicol[48] or antifungal dichlorinated diaporthins.[49] Interested to complement this existing biocatalytic halogenation toolbox with enzymes capable of asymmetric halogenation of unactivated C(sp^3)-centers, first groups have targeted to engineer Fe/αKG-dependent enzymes for the transformation of non-natural substrates.

Creating Halogenases from Hydroxylases

Until the discovery of WelO5 in 2014,[38b] the repertoire of Fe/αKG-dependent halogenases consisted exclusively of carrier protein-dependent enzymes. Therefore, many groups have tried to convert standalone hydroxylases of the Fe/αKG enzyme family into halogenases, with the expectation that these could also perform halogenations without the need of a tethered substrate. The employed strategy focused on remodeling the metal-coordination motif by replacing Asp or Glu of the HXD/E hydroxylase motif by Gly or Ala in an attempt to mimic the HXG motif of halogenases. This single-site substitution seemed logical as the Asp/Glu carboxylate ligand was known to take the space the halide should occupy for iron coordination.[50]

Unfortunately, this approach failed when applied to well-characterized Fe/αKG-dependent hydroxylases like taurine dioxygenase (TauD) and prolyl-4-hydroxylase (P4H).[51] Not only did the resulting variants not behave as halogenases but also lost their hydroxylase activity. Explanations were inefficient iron binding, as determined in TauD variant D101A or insufficient chloride ion binding in the active site, though this was not investigated. In case of P4H, the affinities of the constructed variants D414A/G for iron were never determined, but inefficient iron binding would be expected as wild type P4H has already low affinity for iron.[52] The authors also suggested that hydroxylation did not occur due to the lack of a proper hydrogen bonding network conferred by second-sphere residues. Indeed, in the structures of carrier protein-dependent halogenases CytC3 and SyrB2 residues that do not make direct contact with the active site iron were revealed to be important to bind α-ketoglutarate and chloride.[50,53]

Reversing the mode of the previous investigations, Drennan and co-workers attempted to turn halogenase SyrB2 into a hydroxylase by replacing Ala118 with Asp or Glu. However, halogenase activity was annulled and hydroxylation was not detected, even though the enzyme retained its ability to bind iron.[50] Unlike in the SyrB2 example, Boal and co-workers succeeded to convert WelO5 into an exclusive hydroxylase by replacing Gly with Asp. Crystal structure of variant G166D showed coordination of iron by Asp as found in hydroxylases.[25]

Besides the requirements of space and electrostatic interactions for halide accommodation and coordination, studies on the reactivity of SyrB2 showed that for halogenation to occur the substrate needs to reside distal to the oxygen of the Fe^{IV}=O intermediate and therefore spatially distinct from substrates targeted for hydroxylation.[43,54] Substrate positioning far from the oxo moiety and closer to the halide has been proposed to be an essential feature favoring the Fe^{III}–Cl rebound (halogenation) over the Fe^{III}–OH rebound (hydroxylation) in carrier protein-dependent halogenases (Figure 1 and Scheme 2). This hypothesis was further confirmed by a substrate-extension approach: L-norvaline, a compound which contains an additional methylene group compared to L-threonine, was hydroxylated by SyrB2 because its longer side chain brought it closer to the oxo group.[54a]

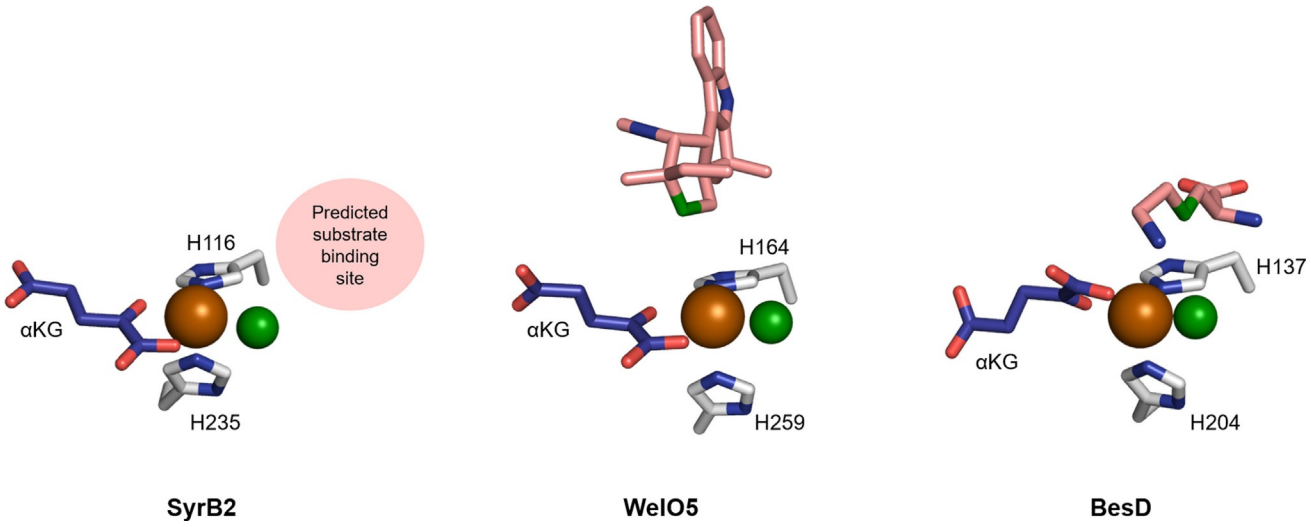
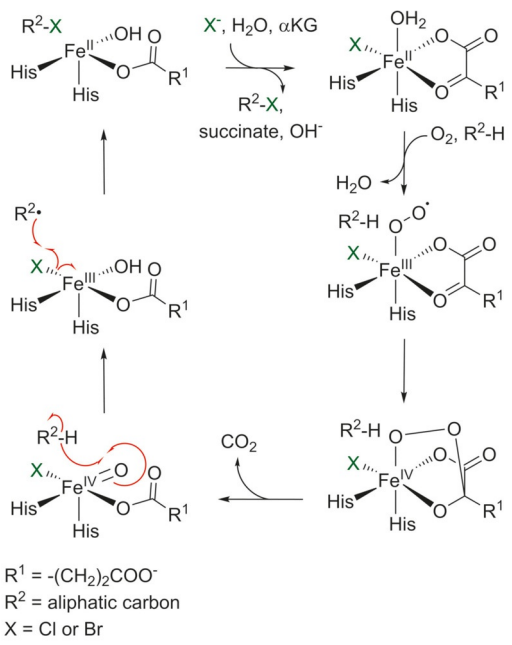


Figure 1. Substrate positioning in SyrB2 (PDB ID: 2FCT), WelO5 (PDB ID: 5IQT), and BesD (PDB ID: 6NIE). Given the requirement of a tethered substrate, there is no available substrate-bound crystal structure for SyrB2. In SyrB2, the substrate-binding site has been proposed to be close to the chloride ligand. In the structure of WelO5 bound with **10**, the substrate is localized in a different quadrant than in SyrB2 and on the same side as the putative oxygen-binding site.[25] In BesD, αKG, His204 and chloride coordinate iron in a different geometry and the targeted carbon of **5** appears to be on the opposite side of the putative oxygen-binding site.[36] The images were generated with PyMOL. Substrates are depicted in pink and the target carbon for halogenation is shown in green.



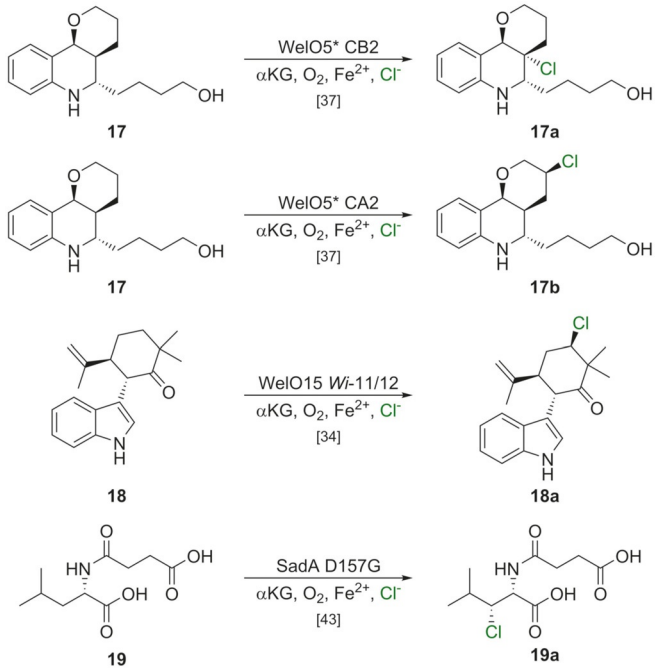
Scheme 2. Proposed reaction mechanism for Fe/α-ketoglutarate-dependent halogenase WelO5. Mechanism adapted from Mitchell et al.[25] and Galonić et al.[39]

Notably, the WelO5 structure displays a different substrate positioning than the orientation identified in SyrB2 and the co-crystallized 12-*epi*-fischerindole U (**10**) was found surprisingly far from the halide ligand.[25] However, Boal and co-workers suggested that a rearrangement of the active site occurs during the catalytic cycle favored by an unusual α-ketoglutarate conformation and second sphere hydrogen bonding interactions.[25] In BesD, the vacant site for oxygen binding is distal to the substrate, but similar to the proposed WelO5 mechanism a substrate conformational change and a shift of the oxo ligand before, during or after αKG decarboxylation has been proposed to occur during catalysis (Figure 1 and Scheme 2).[36]

Taking into account the previous structural work, the Boal group utilized the structure of WelO5 in complex with its natural substrate (**10**) to identify a similar hydroxylase, SadA from *Burkholderia ambifaria* (PDB ID: 3W21),[55] and tailored it to halogenate one of its natural substrates, *N*-succinyl-L-leucine (**19**).[43] Although SadA and WelO5 share only 19% sequence identity, their active sites possessed high structural homology. As a first step, the Asp in the HXD motif of hydroxylase SadA was replaced by a glycine. In contrast to the preceding investigations, variant SadA D157G did halogenate the C3 position of **19** in the presence of NaCl or NaBr with high regioselectivity but low halogenation/hydroxylation ratio. As mutation S189A in WelO5 was previously shown to promote hydroxylation, replacing the residue in the equivalent position (G179 in SadA) to a serine was expected to suppress hydroxyl transfer. Unfortunately, SadA D157G/G179S was less active than D157G and chemoselectivity remained unchanged. This suggested that the oxo intermediate was stabilized in a different way in wild type SadA than in WelO5. This remodeling study may certainly serve as a proof of concept that single-site substitution in the HXD motif can be successful provided there is significant local structural similarity between the subject and the query. However, in absence of more conclusive structural data the (subtle) differences determining hydroxylase versus halogenase function seem to prevent the elucidation of more general engineering rules at the moment.

Engineering Substrate Specificity, Activity and Reaction Control

In the first example of engineering freestanding halogenases for the acceptance of non-indole alkaloid type substrates, Buller and co-workers tailored WelO5* for the regio- and stereoselective chlorination of a martinelline-derived fragment (**17**)[37] with reported anti-cancer activity (Scheme 3).[56] By



Scheme 3. Examples of non-natural substrates converted by freestanding halogenase variants.

screening a set of free-standing αKGHs (WelO5, AmbO5, WelO5*, and the engineered SadA D157G[43]), WelO5* was the only enzyme identified to halogenate the target substrate. However, initial activity and chemoselectivity of WelO5* were low, with the major detected product being the hydroxylated side product (1% conversion towards the chlorinated product **17b** compared to 40% towards the hydroxylated product). In order to increase the activity and chemoselectivity, WelO5* was engineered using a structure-guided evolution approach. Substrate docking studies led to the identification of nine residues (N74, F77, V81, A82, I84, A88, V90, R153 and I161), which were selected as targets for single-site saturation mutagenesis. Libraries of residues A82, A88 and R153 comprised variants with a 3- to 5-fold increase in halogenation activity, while libraries on residues V81 and I161 contained mutants with 10- to 20-fold increase in activity compared to the wild type. In the screening process, an alternative chlorinated product was observed, highlighting the existence of regioselectivity switches in the active site. Based on their different regioselectivities, best variants from the first evolution round, CA1 and CB1, were separately subjected to iterative saturation mutagenesis.

Second round variant CA2 (V181L/I161M) showed a further 10-fold improvement in chlorination activity and shifted hydroxylation regioselectivity compared to CA1. A simultaneous 2-site randomization of CB1 afforded second round variant CB2 (V181R/I161S), which almost completely converted **17** to **17a**, reducing hydroxylation to less than 5%. *In vitro*, CB2 displayed more than 290-fold higher TTN and a 400-fold higher apparent turnover towards **17** compared to the wild type enzyme. CB2 was also shown to brominate **17**, though it had lower preference for bromide than chloride.[37]

In a further study to expand the substrate-scope of freestanding αKGHs, Höbenreich and co-workers recently investigated the activities and selectivities of *Hw*-WelO15 from *Hapalosiphon weltwitschii* IC-52-3 (identical to WelO5*) and *Wi*-WelO15 variants towards five non-natural hapalindole derivatives.[34] Through comparison of substrate acceptance by the enzymes, the authors observed that the hapalindole vinyl group was not relevant for substrate recognition whereas the presence of the isonitrile group was important as substrates lacking it were hardly converted. The crystal structure of a *Wi*-WelO15 variant, named *Wi*-0 (V6I/D284N), was utilized as a starting point for four rounds of structure-guided directed evolution including the identification of hotspots through active site docking of the hapalindole derivative (**18**). The final purified variants *Wi*-11 (N47R/V81T/A82M/A88V/V90P/S93L/S103A) and *Wi*-12 (N47R/A82L/V90P/S93D) were identified as the best in terms of conversion (up to 91%) and chemoselectivity (96%) for the conversion of 0.5 mM of **18**. Variants from earlier evolution rounds were more efficient towards the other derivatives which contained a keto group and indole substitutions. In all cases, chlorination was >99% regio- and stereoselective for C13 and the (*R*)-configuration. Particularly, chemoselectivity was impacted by substitution I84H and caused *Hw*-WelO15 wild type (WelO5*) and *Wi*-WelO5 variants to shift their preference towards 30% hydroxylation of **18**, giving further evidence that chemoselectivity is controlled by substrate positioning.[34]

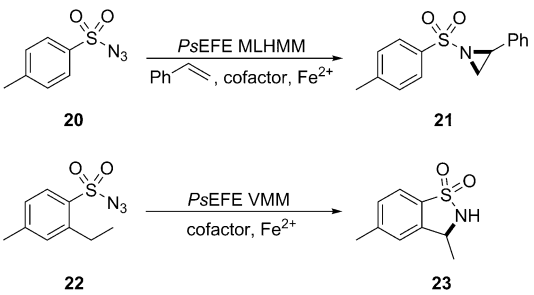
These initial engineering studies on WelO5 homologs suggest that the reported narrow substrate scope of freestanding αKGHs hinge on few hotspot residues in the active site and thus can be expanded in a straightforward manner using rational design and directed evolution. Furthermore, the achieved optimization of chemoselectivity, turnover number and apparent k_{cat} bode well for the expansion of the application scope of halogenases as these improvements demonstrate that the modulation of the enzyme's performance does not necessarily rely on complex synergistic arrays of functional residues that may limit evolvability.

Catalytic Promiscuity

Although not an engineering work on a αKGH *per se*, an enzyme belonging to the family of αKGDs has recently been shown to catalyze non-natural nitrene transfer reactions.[57]

This study highlights that selected Fe/αKG-dependent dioxygenases, in a similar fashion as heme-iron enzymes, are capable of reaction promiscuity under anaerobic conditions.[58] An ethylene-forming dioxygenase from *Pseudomonas savastanoi*

(*Ps*EFE)—known to naturally hydroxylate L-arginine—catalyzed the intermolecular aziridination of *p*-toluenesulfonyl azide (**20**) and styrene to form **21** as well as the intramolecular nitrene C–H bond insertion of 2-ethylbenzenesulfonyl azide (**22**) to form sultam (**23**) (Scheme 4). As nitrene transfer would proceed without formation of the reactive iron-oxo intermediate, *Ps*EFE was hypothesized to not require αKG as co-substrate. To prove this, the authors used αKG analogs such as acetate and *N*-oxalylglycine (NOG), which yielded 7- to 8-fold higher aziridination activity towards **20** compared to reactions carried out with αKG. After two rounds of site-saturation mutagenesis and recombination on five active site residues, a quintuple-mutant, referred to as *Ps*EFE MLHMM, showed a TTN of 120, 15-fold higher than that of the wild type, as well as a significant increase in its (*R*)-selectivity. When the library was screened against **22**, the triple-mutant *Ps*EFE VMM stood out with a TTN of 730 and total chemoselectivity for insertion over reduction. Even though these remarkable results were achieved with an atypical member of the αKGD family, this study demonstrates the native capability of Fe/αKG-dependent enzymes to catalyze new chemistries.



Scheme 4. Non-natural nitrene transfer reactions catalyzed by *Ps*EFE variants. Either α-ketoglutarate, *N*-oxalylglycine or acetate was used as cofactor. Only target products are displayed.

Summary and Outlook

Asymmetric C(sp^3)-halogenation is a highly desirable transformation for the organic chemist's toolbox. It is especially valuable in the context of late-stage functionalization as introducing a halogen early within a synthesis scheme may become a liability to key transformations later on.[5] However, only few synthetic methods exist which allow the selective modification of complex compounds. Turning to nature, the use of Fe/α-ketoglutarate-dependent halogenases may offer a viable strategy toward the asymmetric halogenation of unactivated C–H bonds. First members of this family acting on freestanding substrates have been discovered and pioneering protein engineering studies suggest that the substrate scope of freestanding αKGHs can be more easily broadened than anticipated.[34,37] Nevertheless, the young field has to tackle a range of remaining challenges to bring Fe/α-ketoglutarate-dependent halogenases to their full potential. Most important will be to increase the low turnover number of the short-lived αKGHs, which likely suffer from autoxidation. To date, this problem has been primarily addressed by the addition of antioxidants or reducing agents in the reaction mixture whereas studies to engineer enzyme stability have not yet been conducted. Drawing parallels to pivotal studies carried out, for example, by the groups of Arnold,[59] Flitsch[60] and Turner[61] to improve turnover numbers of P450s, which also follow a radical mechanism, hopes are high that αKGH performance may be improved in a similar way.

Apart from broadening the substrate scope of existing halogenases by enzyme engineering, the biocatalytic halogenase toolbox would benefit from an influx of new wild type enzymes accepting a wider range of chemical core structures. Unfortunately, the identification of novel freestanding Fe/α-ketoglutarate-dependent halogenases has proven challenging, with only a handful of new enzymes identified since their initial discovery in 2014. In the context of the current explosion of available genome information, this low success rate is surprising—however, it can be rationalized when considering the overall low sequence identity of the identified halogenases (e.g. indole alkaloid versus amino acid halogenases). Consequently, learning to reprogram the growing number of diverse Fe/α-ketoglutarate-dependent hydroxylases[62] to halogenases still represents an attractive avenue forward, even in the light of the limited achievements enzyme engineers have obtained to date. Importantly, the establishment of engineering rules will depend on a more in-depth understanding of which factors determine a hydroxylation versus halogenation reaction outcome. Towards this goal, structural studies of substrate-bound WelO5* and *Wi*-WelO15 variants on the evolutionary trajectory toward their tailored progeny may shed some light on this aspect of the enzyme's structure–function relationship as the amount of hydroxylated product produced by the halogenases significantly drops in the course of evolution.[34,37]

Today, Fe/α-ketoglutarate-dependent halogenases may already be applied for the synthesis of compounds that can be used in structure–activity relationship (SAR) studies.[63] Looking forward, the introduction of αKGHs into biosynthetic pathways, analogously to studies carried out with flavin-dependent halogenases,[47a,64] may be an attractive tool to increase the diversity of accessible products and to synthesize bioactive molecules or valuable intermediates. Combined with additional enzyme engineering examples and further mechanistic studies to provide a clearer picture of the catalytic cycle, such applications will turn Fe/α-ketoglutarate-dependent halogenases into versatile tools for the selective halogenation of traditionally difficult-to-derivatize target substrates.

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Conflict of interest

The authors declare no conflict of interest.

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