Directed Evolution of a Fe(II)- and α-Ketoglutarate-Dependent Dioxygenase for Site-Selective Azidation of Unactivated Aliphatic C-H Bonds

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Abstract

Fe(II)- and α-ketoglutarate-dependent halogenases and oxygenases can catalyze site-selective functionalization of C-H bonds via a variety of C-X bond forming reactions. Achieving high chemoselectivity for functionalization using non-native functional groups remains rare, however, particularly for non-native substrates. The current study shows that directed evolution can be used to engineer variants of an engineered dioxygenase, SadX, that address this challenge. Site-selective azidation of succinylated amino acids and a succinylated amine was achieved using variants with improved azidation yield and selectivity on a probe substrate as a result of mutations throughout the SadX structure. The installed azide group was reduced to a primary amine, and the succinyl group required for azidation was enzymatically cleaved to provide the corresponding amine. These results provide a promising starting point for evolving additional SadX variants with activity on structurally distinct substrates and for enabling enzymatic C-H functionalization with other non-native functional groups.

Introduction

Selective incorporation of nitrogen-containing functional groups into organic structures constitutes a major goal of synthetic chemistry due to the prevalence of amines in biologically active compounds and other materials.[1] C-H functionalization via C-N bond formation is particularly attractive since it could minimize functional group transformations required for nitrogen installation and enable late-stage functionalization.[2] A variety of metal-catalyzed amination methods compatible with different substrate classes, including those containing suitable directing groups, pendant aminating agents (for intramolecular reactions), and acidic or tertiary sp^3 C-H bonds, have therefore been developed.[3–7] Most of these methods involve reaction of the organic substrate with pre-activated nitrogen sources, including hypervalent iodine reagents, haloamines, and nitrene precursors.[8–10] While sp^3 C-H amination is rare in nature,[11] engineered heme-[12–15] and non-heme Fe(II)-dependent enzymes[16,17] can catalyze intramolecular cyclization of sulfonyl azides via nitrene insertion into pendant C-H bonds. Cytochrome P450 variants have also been engineered to catalyze intermolecular amination of benzylic, allylic, and propargylic C-H bonds using sulfonyl azides[18] or *N*-hydroxylamine esters.[19–22] Interestingly, a *N*-acetoxy intermediate was proposed as nitrene precursor for intramolecular aziridination catalyzed by the P450 BezE in the biosynthesis of benzastatin.[23] On the other hand, introducing simple masked ammonia equivalents like azides, which can be readily converted to primary amines, heterocycles, and other nitrogen-containing functional groups, into unactivated sp^3 C-H bonds remains a challenging task.[24,25]

In considering how enzymes could be used to address this synthetic challenge, we were drawn to a report showing that the Fe(II)- and α-ketoglutarate-dependent halogenase (FeDH) SyrB2[26] can catalyze site-selective azidation of unactivated sp^3 C-H bonds using sodium azide in the presence of dioxygen as a terminal oxidant.[27] This remarkable transformation is believed to proceed via a radical rebound mechanism analogous to that proposed for Fe(II) and α-ketoglutarate-dependent oxygenases (FeDOs, Figure 1, outer cycle).[28] Unlike the FeDOs, however, which bind their Fe(II) cofactor via a His/His/Asp facial triad, SyrB2 and other FeDHs possess a Gly or Ala residue in place of the Asp residue, which allows for halide (X^- = Cl^-, Br^-) binding to the iron center.[29] Selective rebound of $X^\bullet$ over $HO^\bullet$ leads to halogenation by FeDHs (Figure 1, inner cycle).[30,31] Binding of several anions was spectroscopically characterized in SyrB2, but rebound was only observed for chloride, nitrate, and azide.[27] Mutating Ala to Gly to create more space for the azide ligand led to an estimated azidation yield of approximately 13%, but SyrB2 has only been shown to possess activity on carrier protein-linked substrates,[32,33] so it is unclear whether it could be engineered for functionalization of small molecule substrates.

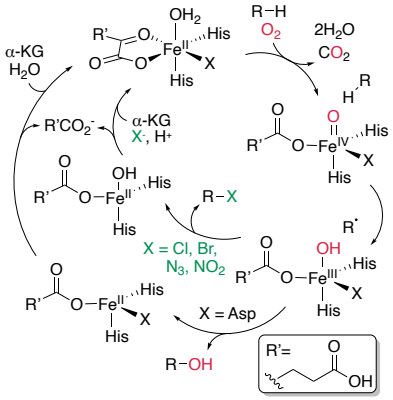


Figure 1. Catalytic cycle showing proposed mechanisms for FeDO (outer cycle) and FeDH (inner cycle) activity.[27,28]

Recently, several FeDHs with activity on small molecule substrates have been identified,[34] and azidase activity has been reported for some of these. SwHalB,[35] one of several BesD-like FeDHs with activity on amino acids, and an engineered variant of WelO5*[36] were reported to possess azidase activity, but no conversion or chemoselectivity data were provided for the azidation reactions. SaDAH catalyzes azidation of a complex alkaloid,[37] but trace amounts of chloride in the reaction buffer were sufficient to provide similar yields of chlorination and azidation (in addition to hydroxylation) by this enzyme. An alternative approach to biocatalytic azidation was recently achieved by using a non-heme iron pyruvate dioxygenase to catalyze non-native radical relay reactions.[38] In this system, a pre-oxidized *N*-fluoroamide substrate is believed to react with the azide-bound Fe(II) center of the enzyme to form an amidyl radical and a Fe(III)(N_3)(F) intermediate in analogy to a previously reported fluorination reaction involving simple Fe(II) salts[39]. Substrate-controlled 1,5-HAT of the former species leads to a carbon centered radical, and more favorable rebound of azide relative to fluoride sidesteps the chemoselectivity challenge noted above to deliver azide products.

These efforts highlight that while non-heme iron enzymes are promising candidates for non-native catalysis, selective azidation of small molecule substrates with catalyst-controlled site selectivity

at unactivated C-H bonds has not yet been reported. Indeed, achieving high chemoselectivity for any non-native C-H functionalization reactions (i.e. halogenation, azidation, etc.) of non-native substrates via rebound pathways using WT and engineered FeDHs remains rare,[36,40–42] demonstrating how precise tuning of these enzymes is required to achieve selective rebound of X• over HO• (Figure 1). Herein, we report that directed evolution can be used to engineer variants of the FeDO SadA[43] that catalyze site-selective azidation of succinylated amino acids and a succinylated amine. The installed azide group can be reduced to generate a primary amine, and the succinyl group required for azidation can be enzymatically cleaved to provide the free amine. Computational modeling shows that the improved function of the engineered variants results from mutations throughout the SadA structure, suggesting that subtle changes throughout the enzyme structure are required to enable selective C-H functionalization.

Results and Discussion

SadA is one of only two FeDOs[40,43] in which a facial triad mutation (D157G) results in halogenase activity (the only one reported when we started our evolution efforts), suggesting that it has a relatively high propensity for rebound of non-native X-type ligands. The substrate tolerance of the WT enzyme, which hydroxylates a variety of succinylated amino acids,[44–46] also suggests that non-native activity could extend to other substrates bearing a suitably placed carboxylic acid substituent. Recently, we reported that expression of this enzyme is improved by fusing it to maltose binding protein (MBP) to generate MBP-SadA D157G (hereafter SadX), and we evaluated SadX activity on *N*-succinyl leucine (**1a**) in the presence of different anions.[47] SadX exhibits relatively broad scope with respect to X• rebound (X• = Cl•, Br•, N_3•, NCO•), suggesting that it could serve as a useful platform for non-native FeDO catalysis.

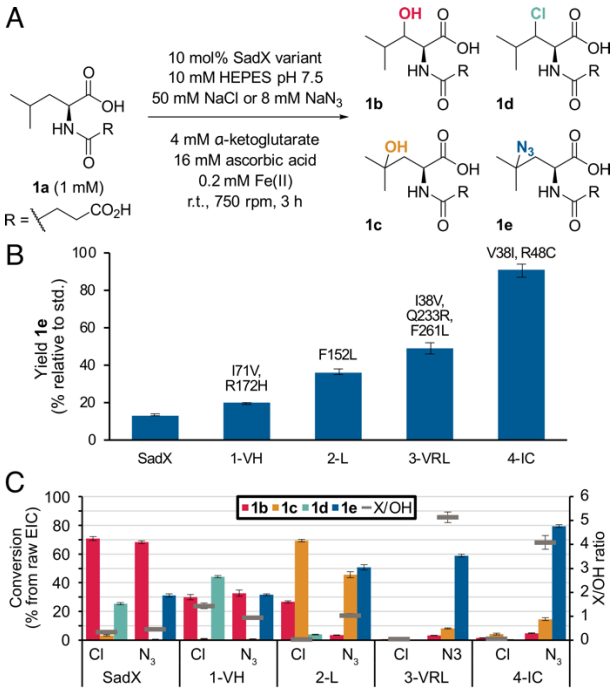


Figure 2. Biocatalysis using engineered SadX variants. A) Reaction conditions for evaluating SadX variant yields using purified enzyme. B) Azidase lineage showing improvement in azidation yields as determined by triplicate LC/MS measurements relative to *N*-acetyl-L-valine internal

standard. C) Complete product distributions for enzymes in the azidase lineage showing changes in site and chemoselectivity as determined by raw extracted ion chromatogram peak intensities from triplicate LC/MS measurements. See Figure S1 for complete data.

The improved expression of SadX allowed for LC/MS analysis of non-native X$^{\bullet}$ rebound in crude *E. coli* cell lysate containing the desired X^{-} salt and excess ascorbate[48] (Figure 2A). Our initial evolution efforts focused on improving the chlorinase activity of SadX on **1a**. A library of 900 SadX variants containing an average of 3-4 amino acid mutations/gene was generated using error prone PCR. Improved conversion of **1a** to **1d** was observed for variant 1-VH (SadX I71V R172H), which provides **1d** as the major product and gives a 3-fold increase in selectivity for chlorination over hydroxylation. Examining 1-VH reactivity in the presence of NaN_3 revealed that a 20% yield of azidation product **1e** could be obtained (Figure 2B) with a chemoselectivity improvement similar to that observed for chlorination (Figure 2C). We then established that even SadX gives comparable yield and selectivity toward azidation and chlorination.[47] Saturation mutagenesis of active site residues in SadX that were reported to improve SadA-catalyzed oxygenation of *N*-succinyl-3,4-dimethoxyphenylalanine (G79 and F261) was therefore pursued, but improved azidation yields were not observed.[45]

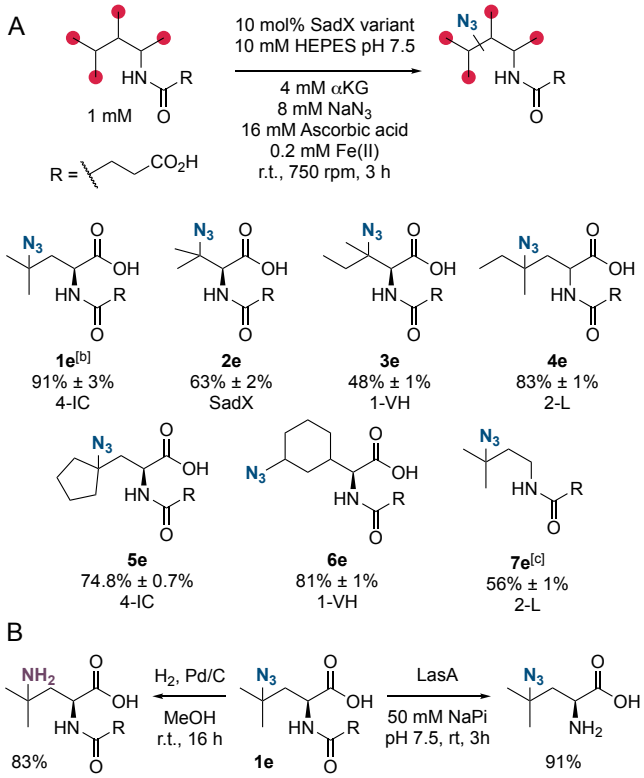
Given that 1-VH provided similar yield and selectivity for chlorination and azidation, we opted to continue evolving the latter reaction (Figure 2B). The lack of improvement observed using site saturation libraries led us to again use error prone PCR for library creation. A second library of 900 variants of 1-VH was constructed, and evaluation of azidase yield on **1a** led to the finding that variant 2-L (1-VH F152L) provided 1.8-fold increased conversion to **1e**. Product **1c** was detected as the major hydroxylated product, and the chemoselectivity **1e/(1b+1c)** was nearly identical to the **1e/1b** ratio from the parent variant 1-VH. Similar analysis of a library of 900 variants of 2-L revealed that variant 3-VRL (2-L I38V Q233R F261L) gave a modest increase in conversion to **1e** but an increase of more than 4-fold in **1e/(1b+1c)** when compared to 2-L. A final error-prone PCR library of 3-VRL variants was constructed with an average of 1–2 amino acid mutations per sequence. Screening for improved azidation on **1a** revealed several additional variants. Single and double mutants of 3-VRL incorporating these new mutations were cloned and evaluated. The final result was variant 4-IC (3-VRL V38I R48C), which provided a 91% assay yield of **1e** (6.7-fold increase with respect to SadX) and a **1e/(1b+1c)** ratio of 4.4 (7.5-fold increase with respect to SadX).

Analysis of product distributions for SadX variants in the azidase lineage revealed subtle relationships between the site and chemoselectivity of the azidation, chlorination, and hydroxylation reactions catalyzed by these enzymes (Figure 2C). As previously reported,[47] SadX prefers hydroxylation to chlorination by over two-fold, and a similar preference for hydroxylation over azidation is observed. Notably, however, hydroxylation and chlorination occur at the β-position of the substrate, while azidation occurs at the γ-position. This difference indicates that the Fe(IV)(oxo)(N_3) intermediate responsible for C-H abstraction (Figure 1) can access either the β or γ C-H bond of the substrate but that the relative orientation of the resulting substrate radical and the Fe(III)(OH)(N_3) intermediate could control rebound selectivity for HO$^{\bullet}$ or $N_3^{\bullet}$, as was proposed to rationalize HO vs Cl rebound in SyrB2.[32] Variant 1-VH, which was obtained from a screen aimed at improving chlorination, shows increased chemoselectivity and a large decrease in hydroxylation. The reduced oxygenase activity of 1-VH translates to the azidation reaction, so

even though its azidation activity was not improved over SadX, its chemoselectivity was. In the second round of evolution, however, improved azidation was accompanied by a change in the site selectivity of hydroxylation, and a large decrease in chlorinase activity. This finding seems to indicate that the active site is better optimized for functionalization of the γ-position since reaction of the β-position is greatly diminished. The final two rounds of evolution led to markedly improved γ-azidation activity and specificity, perhaps by further improving substrate positioning to minimize the potential for HO$^{\bullet}$ rebound.[32]

The substrate scope of enzymes along the azidase lineage was next evaluated on several *N*-succinylated amino acids (Figure 3A). Collectively, enzymes along the lineage catalyze azidation of β- and γ-branched substrates. Good-to-high yields (56-91%) of azidated products are obtained for all substrates except for *N*-succinyl isoleucine (48%). While azidation of tertiary C-H bonds is typically observed regardless of whether they are at the amino acid β or γ position, azidation of a secondary C-H bond of succinyl-protected cyclohexyl alanine to give **6e** shows that the enzyme can override this general preference. The need for multiple enzymes to achieve high selectivity on different substrates is consistent with previous studies highlighting how precise substrate positioning is required for selective halogenation by FeDHs.[30,32] While no single enzyme is suitable for all substrates examined, the diversity of active site structures along the azidase lineage was sufficient to accommodate the moderate structural variation of the substrates examined. To demonstrate the synthetic utility of our evolved azidases, we established that the azide could be readily reduced to the corresponding amine (Figure 3B). This product could be isolated via ion exchange chromatography followed by semi-preparative HPLC. We also showed that LasA,[44] which desuccinylates **1a**, also works on **1e** to provide the free azidated amino acid.

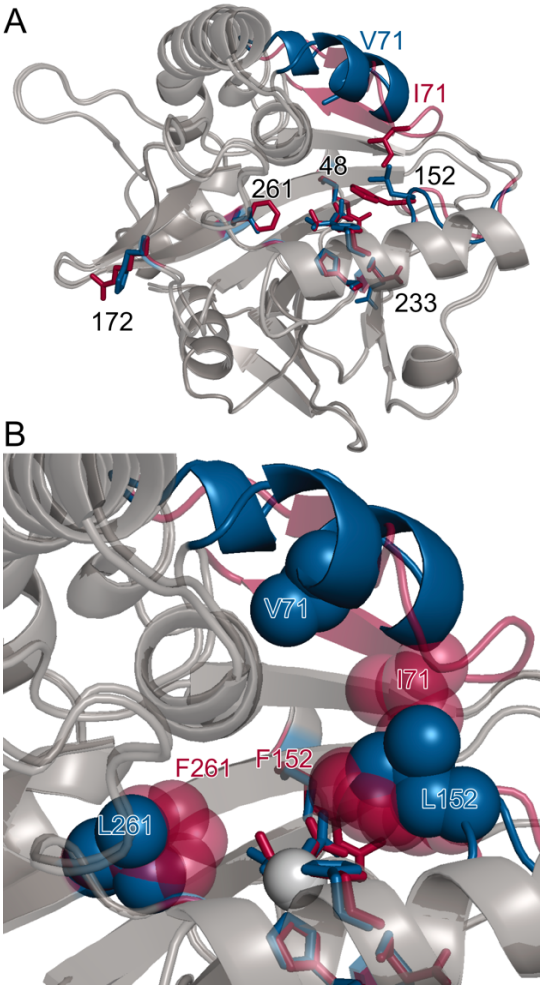
Figure 3. A) Representative substrate scope of evolved azidases[a] and B) reduction and desuccinylation of **1e**.



[a]Yields determined by comparison of EIC peak areas (area azidation product/areas substrate and products). [b]Yield determined by LC/MS quantitation relative to *N*-acetyl-L-valine internal standard. [c]Standard reaction conditions with 8 mM αKG.

Finally, computational modeling was used to gain initial insight into the locations of the six mutations introduced into SadX to generate 4-IC and how those mutations might influence catalysis (Figure 4). The crystal structure of SadA (PDB ID 3W21)[46] is missing loops that span S59-S75 and G147-A153. Because I71V and F152L in variant 4-IC fall in these ranges, models of SadA were generated using SWISS-MODEL,[49] Rosetta,[50] and AlphaFold[51]. The SWISS-MODEL and Rosetta structures both had poorly packed S59-S75 and G147-A153 loops and showed F152 projecting out of the active site (Figure S2). The AlphaFold structure, on the other hand, predicts that the S59-S75 loop forms a β-hairpin-like structure that caps the active site and that F152 sits directly above the Fe-coordinating H155 residue in the active site (Figure 4A). F261L and F152L, the latter of which led to the change in hydroxylation selectivity from the β-position to the γ-position of **1a** and improved azidation at the γ-position, are both predicted to increase the active site volume proximal to the Fe(II) center (Figure 4B). The four remaining mutations, R48C, I71V, R172H, and Q233R, are all >12 Å from the Fe(II) center. I71V is predicted to significantly alter the structure of the S59-S75 loop, but no major structural changes are predicted as a result of the other mutations. This finding suggests that subtle changes in substrate binding lead to the high chemoselectivity of SadX for X$^{\bullet}$ over HO$^{\bullet}$ rebound, but more sophisticated modeling approaches[30] will be required to explore the origins of these changes.

Figure 4. Overlaid models of SadX (red) and 4IC (blue) generated using Alphafold showing A) the locations of mutations in the respective structures and B) changes to the active site structure as a result of F152L, F261L, and I71V.



Conclusion

FeDHs and FeDO facial triad variants have the potential to enable site-selective functionalization of C-H bonds via a variety of C-X bond forming reactions. This capability stems from their unique mechanism, which involves C-H abstraction by an Fe(IV)(X)-oxo intermediate and rebound of X$^{\bullet}$ over HO$^{\bullet}$ from the resulting Fe(III)(OH)(X) intermediate (Figure 1). Because rebound chemoselectivity is largely controlled by substrate orientation relative to the X and OH ligands within the active site,[30,31] achieving high chemoselectivity for rebound of non-native X groups using WT and engineered FeDH/Os remains rare, particularly for non-native substrates[36,40,41]. Indeed, while azidated products have been detected using several such enzymes,[35–37] highly selective azidation of small molecule substrates with catalyst-controlled site selectivity at unactivated C-H bonds has not yet been reported. The current study shows that directed evolution can be used to engineer variants of SadX that address this challenge. Site-selective azidation of succinylated amino acids and a succinylated amine was achieved using variants with improved azidase activity and selectivity on a probe substrate (**1a**) as a result of mutations throughout the SadX structure. The installed azide group was reduced to a primary amine, and the succinyl group required for azidation was enzymatically cleaved to provide the corresponding amine. These results provide a promising starting point for evolving additional SadX variants with activity on

substrates increasingly distinct from the native succinylated amino acid substrates of the FeDO SadA and for enabling enzymatic C-H functionalization with other non-native functional groups.

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Data Availability Statement
All data reported in this manuscript (i.e. Figures 2 and 3) are provided in the Supplementary Information file on the Angew. Chem. Int. Ed. website.

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