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## Undirected biocatalytic amination of unactivated C( $sp^3$ )–H bonds

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### Abstract

Selective catalytic functionalization of C( $sp^3$ )–H bonds represents a powerful means to access valuable products from ubiquitous starting materials. In a recent *JACS* paper, Arnold and co-workers engineered P450 nitrene transferases to aminate unactivated C( $sp^3$ )–H bonds with excellent site- and stereoselectivities.

By repurposing and evolving natural enzymes to catalyze unnatural reactions, new-to-nature biocatalysis has significantly expanded nature's catalytic repertoire.<sup>1,2</sup> Over the past decade, significant progress has been made in this emerging field of research, leading to a range of synthetically useful biocatalytic processes, including stereoselective carbene- and nitrene transfer,<sup>3</sup> photoenzyme<sup>4,5</sup> and metalloenzyme<sup>6,7</sup>-catalyzed radical transformations, and carbonic anhydrase-catalyzed ketone reduction.<sup>8</sup> Among these unnatural enzymatic activities, carbene and nitrene transferase-catalyzed C( $sp^3$ )–H functionalization reactions are particularly notable, as they allow for the conversion of ubiquitous molecular scaffolds into valuable functionalized products. In nature, P450 monooxygenases are capable of hydroxylating unactivated C( $sp^3$ )–H bonds using a highly reactive Fe=O intermediate with unrivaled site- and enantioselectivities.<sup>9</sup> Previously, despite almost a decade of research,<sup>3</sup> mechanistically related intermolecular C( $sp^3$ )–H amination via unnatural enzymatic Fe-nitrene ("Fe=NH") intermediates has been limited to electronically activated C( $sp^3$ )–H systems, including benzylic, allylic, and propargylic C–H bonds.<sup>10–12</sup>

In a recent *JACS* Article, groundbreaking contributions from the Arnold group demonstrated that site- and stereoselective aminations of simple, unactivated C( $sp^3$ )–H bonds could be accomplished using newly evolved P450 nitrene transferases.<sup>13</sup> Previously, pioneering work from the same group showcased the ability of directed evolution to empower P450 enzymes to catalyze enantioselective intermolecular amination of activated benzylic, allylic, and propargylic substrates.<sup>10–12</sup> In the present work, the Arnold lab further expanded the substrate scope to encompass unactivated C( $sp^3$ )–H bonds for nitrogen insertion processes, including direct C–H amination to furnish unprotected primary amines (Fig 1A).

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At the outset of this research, using methylcyclohexane as the model substrate, a collection of previously engineered heme enzymes was evaluated, leading to the identification of hits with encouraging, albeit low, levels of activities. In this screening effort, the highest activity for C–H primary amination was observed with the *APA6* variant from their previous allylic amination lineage.<sup>10</sup> In the present work, *APA6* was named as *uPA0* (i.e., unactivated primary amination parent). Using hydroxyamine esters as protected nitrene precursors, the highest activity for C–H amidation was observed with the *iAMD5 Y263V* variant, a previously engineered enzyme for related intermolecular amidation.<sup>11</sup> This starting variant was named as *uAMD0* (i.e., unactivated amidation parent).

After identifying hits for C–H amination and amidation, despite the low levels of enzymatic activities (< 5 total turnover number (TTN) for C–H amination and 10 TTN for C–H amidation, respectively), Arnold and coworkers set out to improve enzyme activities by directed evolution. Due to the low initial activities and the presence of multiple possible C–H functionalization isomers, the authors first focused on increasing the enzyme activity and total substrate conversion. Through iterative rounds of site-saturation mutagenesis (SSM) and random mutagenesis via error-prone polymerase chain reaction (epPCR), they were able to quickly improve enzyme activities and arrived at final variants *uPA9* and *uAMD9* exhibiting substantially improved TTNs. With these enzyme mutants, the site- and enantioselectivity of biocatalytic C–H functionalization processes were then carefully determined using chromatographic analysis.

With the final variant *uPA9*, the C–H amination of methylcyclohexane provided *cis*-C3-aminated product with 90 TTN, 86% site selectivity (*ss*), 8:1 diastereomeric ratio (*d.r.*), and 93:7 enantiomeric ratio (*e.r.*). With the *uAMD9* variant, the C–H amidation of methylcyclohexane resulted in *trans*-C2-amidated product with 120 TTN, 91% *ss*, 7:1 *d.r.*, and 85:15 *e.r.*. Further studies showed that ethylcyclohexane also represented a suitable substrate. Using the *uPA9* variant, C3-aminated product formed with 100 TTN, >99% *ss*, 10.5:1 *d.r.*, and 98:2 *e.r.*. With *uAMD9*, C2-amidated product formed with 205 TTN, 78% *ss*, 1:1 *d.r.*, 98:2 *e.r.* (for *cis*-isomer), and 75:25 *e.r.* (Fig 1B). Importantly, preliminary substrate scope evaluation revealed that the evolved enzymes possessed high degrees of promiscuity towards a broad range of hydrocarbon substrates. For example, pentane, hexane, heptane, 3-methylpentane, and several acyclic alkanes were found to be viable substrates. This previously elusive enzyme activity is expected to provide the basis for developing effective amination biocatalysts that can act on various unactivated C(*sp*<sup>3</sup>)-H bonds.

Computational studies using density functional theory (DFT) calculations and molecular dynamics (MD) simulations provided further insights into the reaction mechanism. In accord with previous studies on P450-catalyzed nitrene transfer reactions,<sup>14</sup> a stepwise radical pathway was proposed. In this process, the active Fe-nitrene species was first generated from the ferrous protein catalyst and the nitrene precursor. This Fe nitrene is effectively a diradical species, with significant spin density located at the N atom. Subsequent irreversible hydrogen atom transfer (HAT) between the hydrocarbon and the diradical Fe nitrene leads to a new carbon-centered radical, which then undergoes radical rebound to furnish the amination product and regenerate the ferrous protein catalyst. The authors proposed that the regio- and enantioselectivity is determined by the irreversible HAT event (Fig 1C). The

DFT-computed activation energy of the HAT step was 29.7 kcal/mol, suggesting the kinetic barriers associated with the functionalization of unactivated C(*sp*<sup>3</sup>)-H bonds. Through further MD simulations, the authors suggested that favorable dispersion interactions between the protein scaffold and the substrate/reactive intermediate lowers the activation barrier with the enzyme catalyst.

In summary, the elegant work from the Arnold lab highlights the power of directed evolution in addressing challenging problems in biocatalysis and asymmetric catalysis. For a long time, within the realm of P450 biocatalysis, unactivated C(*sp*<sup>3</sup>)-H bond functionalization could only be accomplished with native P450 monooxygenases. Results demonstrated in the present work showed that evolved P450 nitrene transferases could now allow for the amination of these challenging C-H bonds, thereby complementing the natural enzymatic C-H hydroxylation activities. Notably, at the time of this publication, small-molecule transition-metal catalysts capable of catalyzing the primary amination of unactivated C-H bonds remains elusive. Thus, the present work underscored the ability of enzymes to solve demanding problems that remained out of the reach of classic synthetic systems. Furthermore, the ability of evolved biocatalysts to convert abundant hydrocarbon feedstocks into enantioenriched amines will pave the way for the rapid preparation of pharmaceutically and agrochemically valuable molecular entities.

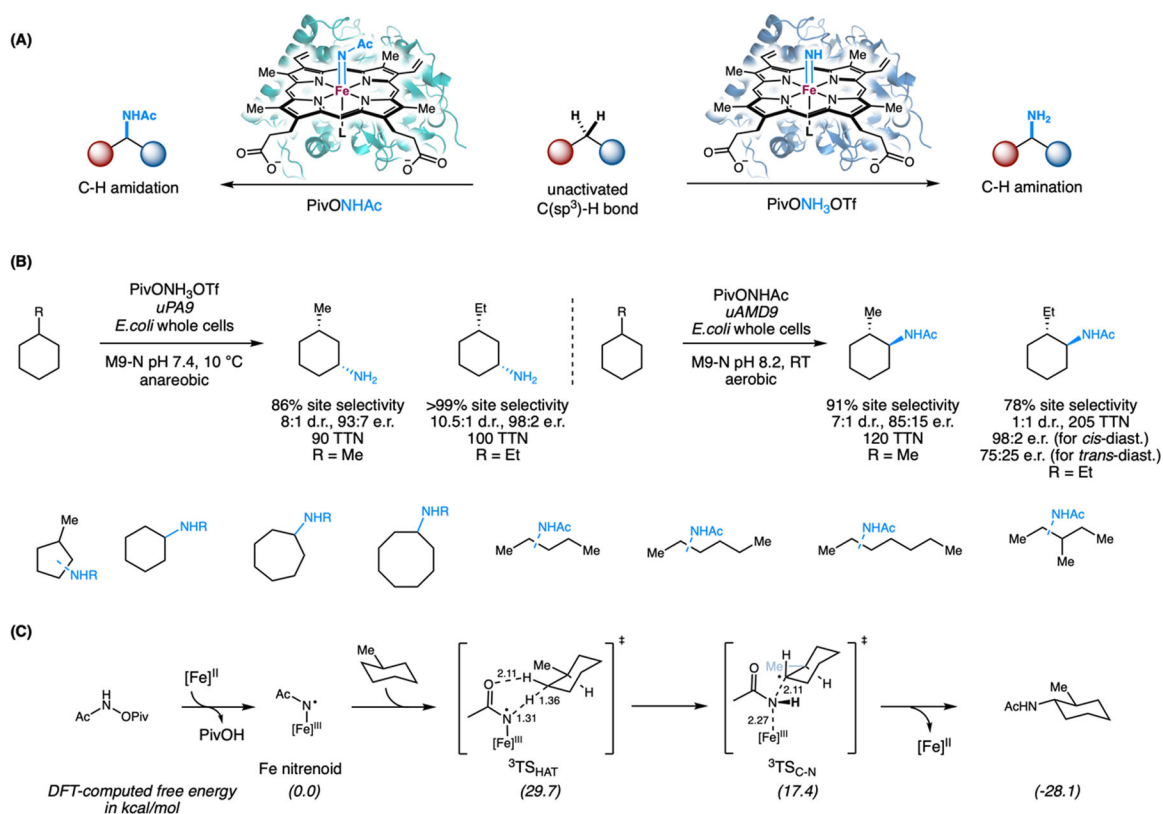
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**Fig 1.** (A) Two different strategies to form direct nitrogen insertion product using unactivated C–H bonds in biological systems; (B) Site-, diastereo-, and enantioselective C–H amination and amidation of hydrocarbon substrates using evolved P450 enzymes; (C) Proposed mechanism and DFT-computed free energy of C–H amidation.