

# Enzymatic Nitrogen Incorporation Using Hydroxylamine

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**ABSTRACT:** Hydroxylamine-derived reagents have enabled versatile nitrene transfer reactions for introducing nitrogen-containing functionalities in small-molecule catalysis as well as biocatalysis. These reagents, however, result in poor atom economy and stoichiometric organic waste. Activating hydroxylamine (NH<sub>2</sub>OH) for nitrene transfer offers a low-cost and sustainable route to amine synthesis, since water is the sole byproduct. Despite its presence in nature, however, hydroxylamine is not known to be used for enzymatic nitrogen incorporation in biosynthesis. Here, we report an engineered heme enzyme that can utilize hydroxylammonium chloride, an inexpensive commodity chemical, for nitrene transfer. Directed evolution of *Pyrobaculum arsenaticum* protoglobin generated efficient enzymes for benzylic C–H primary amination and styrene aminohydroxylation. Mechanistic studies supported a stepwise radical pathway involving rate-limiting hydrogen atom transfer. This unprecedented activity is a useful addition to the ‘nitrene transferase’ repertoire and hints at possible future discovery of natural enzymes that use hydroxylamine for amination chemistry.

Nature’s ability to use simple and abundant precursors for enzymatic functionalization of biomolecules has long inspired reaction discovery. Heme-dependent peroxxygenases activate hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to access Compound I, for example, and effect a rich repertoire of oxidation reactions (Scheme 1a).<sup>1</sup> Whereas the use of H<sub>2</sub>O<sub>2</sub> for C–H hydroxylation and epoxidation,<sup>2</sup> among other reactions,<sup>3,4,5</sup> has been studied extensively with chemo- and biocatalysts, the development of sustainable and efficient strategies for nitrogen incorporation from the analogous amine feedstock hydroxylamine (NH<sub>2</sub>OH) is underexplored.<sup>6</sup> NH<sub>2</sub>OH is a structural and isoelectronic analog of H<sub>2</sub>O<sub>2</sub>, and forming an iron-bound nitrene intermediate from NH<sub>2</sub>OH would be analogous to accessing Compound I from the hydroperoxo intermediate during the peroxxygenase catalytic cycle.<sup>7</sup> These shared features intimate that heme enzymes could possibly process NH<sub>2</sub>OH for nitrogen incorporation (Scheme 1b), enabling amination reactions while generating water as the sole byproduct.

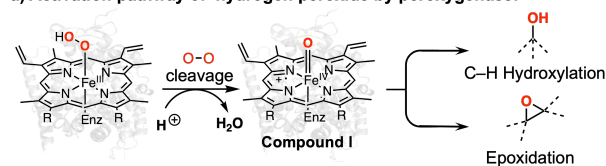
In soil microorganisms, NH<sub>2</sub>OH serves as an intermediate in the nitrogen cycle which is oxidized to nitrates, nitrites and nitrous oxide.<sup>8</sup> Despite its natural incidence and frequent exposure to metalloenzymes,<sup>9,10</sup> however, NH<sub>2</sub>OH is not known to serve as an amine source for nitrogen incorporation. The only natural ‘nitrene transferase’ known to date, cytochrome P450BezE, operates on an *O*-acetylated hydroxylamine precursor (Scheme 1c).<sup>11</sup> Similarly, the analogous amination reactions in synthetic chemistry and biocatalysis that have emerged recently use oxygen-substituted hydroxylamine-derived reagents.<sup>12,13</sup> We previously reported that engineered heme proteins can activate hydroxylamine-derived reagents to form and transfer nitrenes to hydrocarbons.<sup>14</sup> These reagents rely on electron-withdrawing groups on the oxygen to weaken the N–O bond and pull away the bonding pair of electrons in the bond-breaking event *en route* to the nitrene intermediate. While effective in generating myriad C–N bonds, these reagents also generate copious amount of hazardous waste, thus limiting application at scale.

Inspired by peroxxygenases, we sought to investigate the activity of heme proteins toward NH<sub>2</sub>OH for nitrogen incorporation. To do this, a heme protein must overcome two main challenges. First, compared to electrophilic hydroxylamine-derived reagents, the

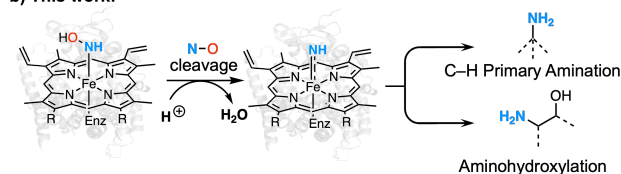
absence of an electron-withdrawing group in NH<sub>2</sub>OH results in an elevated N–O bond dissociation energy.<sup>15,16</sup> Additionally, the heme must selectively bind the nitrogen in NH<sub>2</sub>OH instead of oxygen to form a nitrene intermediate.<sup>6</sup> We hypothesized that an enzyme active site could position NH<sub>2</sub>OH for efficient bond cleavage and facilitate the release of water. The active sites of peroxxygenases contain a highly conserved acid-base catalytic pair that facilitates efficient O–O bond cleavage of H<sub>2</sub>O<sub>2</sub> through a proton relay mechanism.<sup>17</sup> Indeed, introducing this catalytic pair can even switch the activity of oxygen-dependent heme enzymes to that of peroxxygenases.<sup>17,18</sup> We posited that a similar activation mechanism with NH<sub>2</sub>OH could be reprised in a heme protein to create a pathway for amine synthesis with NH<sub>2</sub>OH that parallels the reactivity of peroxxygenases.

## Scheme 1. Background and Summary

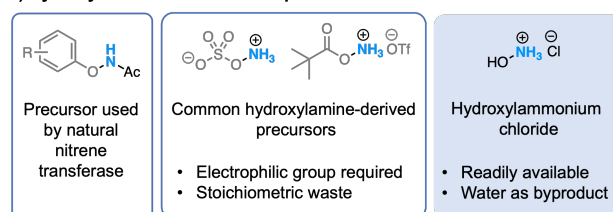
### a) Activation pathway of hydrogen peroxide by peroxxygenase:



### b) This work:



### c) Hydroxylamine-derived nitrene precursors:



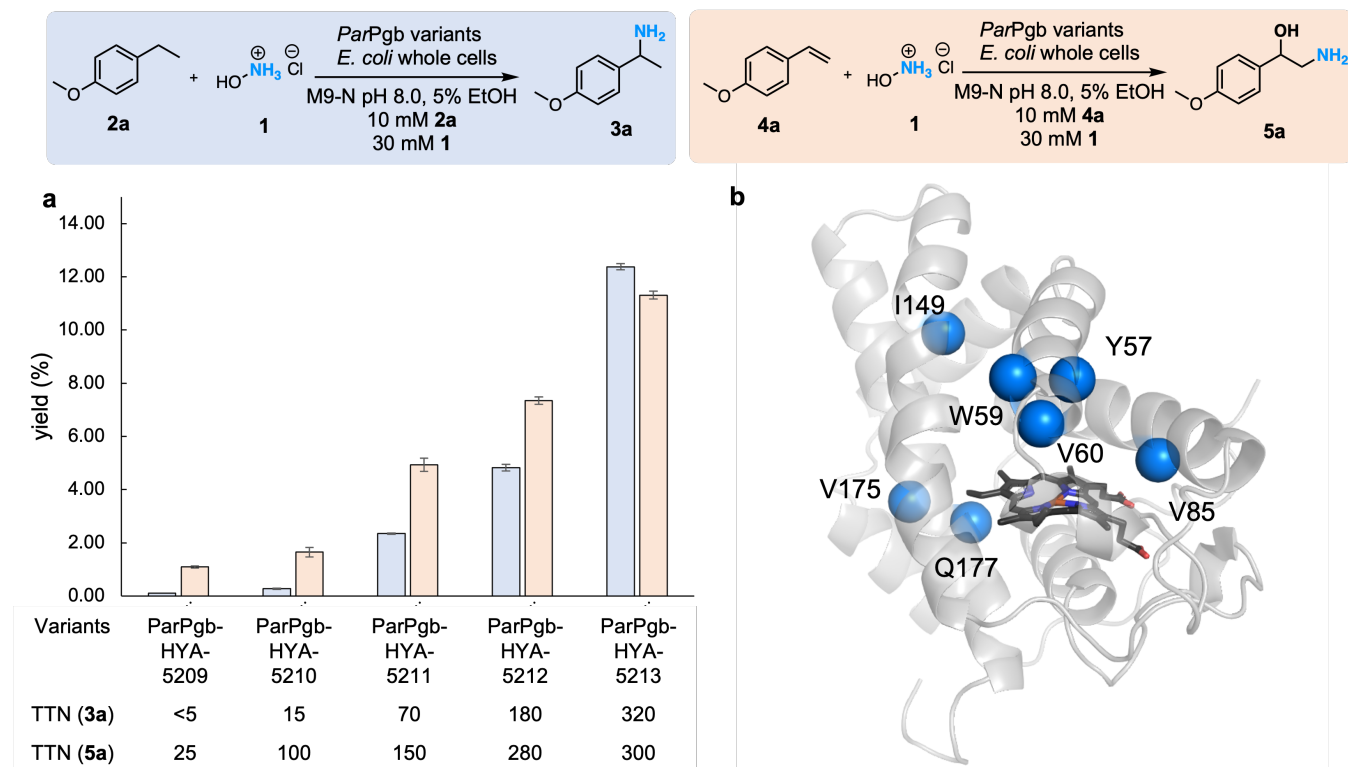
We searched for hints of this catalytic activity using the bench-stable hydrochloride salt of NH<sub>2</sub>OH (**1**) for two different amination reactions enabled by nitrene chemistry, the benzylic C–H primary

amination of 4-ethylanisole **2a** and the aminohydroxylation of 4-vinylanisole **4a**. These two reactions, which share a common reaction pathway via N–O bond cleavage of hydroxylamine precursors, were previously realized using *O*-pivaloylhydroxylammonium triflate (PivONH<sub>3</sub>OTf) as the amine source,<sup>14a,b</sup> and could reveal a general starting point for exploring enzymatic activity with NH<sub>2</sub>OH. A collection of hemoproteins including cytochromes P450, cytochromes *c*, and globins was examined for both transformations. To our surprise, several engineered enzyme variants exhibited trace levels of product formation (see SI, Section 2.). Among the active protein variants, only *Pyrobaculum arsenaticum* protoglobin W59L V60Q, renamed ParPgb-HYA-5209 (ParPgb Hydroxylamine-dependent Aminase) exhibited measurable reactivity for both reactions, furnishing the benzylic C–H primary amination product **3a** with trace activity and aminohydroxylation product **5a** with ~1% yield. Control experiments with free heme and bovine serum albumin showed no product formation. Wild type ParPgb, a small, dimeric, gas-binding hemoprotein whose native function is unknown,<sup>19</sup> also does not catalyze these reactions.

ParPgb-HYA-5209 has two mutations that are presumed to create a more open active site, allowing non-native substrates to enter and react.<sup>20</sup> Previously, we reported protoglobin variants that catalyze carbene transfer for cyclopropanation and X–H bond insertions,<sup>21,22,23</sup> but nitrene transfer activity, especially for C–H functionalization, had not been demonstrated. ParPgb-HYA-5209 is

an appealing starting point for enzyme engineering due to its high thermostability,<sup>24</sup> which allows it to tolerate activating, but often destabilizing, mutations.<sup>25</sup> Additionally, protoglobin-based biocatalysts can be produced readily in *Escherichia coli* at high levels.

Once a promiscuous activity for a new-to-nature reaction is identified in a protein, directed evolution can often enhance that activity.<sup>26,27</sup> We evolved ParPgb-HYA-5209 for benzylic primary amination with **2a** and **1** using a combination of site-saturation mutagenesis (SSM) and random mutagenesis. We identified 20 new, improved variants containing 41 unique mutations (see SI, Section 3.1.). The best of those variants, ParPgb-HYA-5210, was over 4-fold more active, giving 0.27% yield and 15 total turnover numbers (TTN) in whole-cell reactions. We shuffled the leading sequences using staggered extension process (StEP) recombination<sup>28</sup> and screening, for three iterative rounds (Figure 1a). The top variant from recombination, ParPgb-HYA-5213, has seven amino acid substitutions (Y57D, W59L, V60Q, V85I, I149F, V175A and Q177R) relative to the wild-type protein (Figure 1b). Further SSM targeting these seven amino acids gave no improvement. The final variant exhibited a 45-fold improvement in yield compared to ParPgb-HYA-5210, and over 160-fold in yield compared to ParPgb-HYA-5209 (12.4% yield and 320 TTN). Having established a lineage for benzylic C–H primary amination, we also investigated the activity of the engineered enzymes for aminohydroxylation: the activity with styrene **4a** was increased 10-fold, giving 11.3% yield and 300 TTN in whole-cell reactions (Figure 1a.).



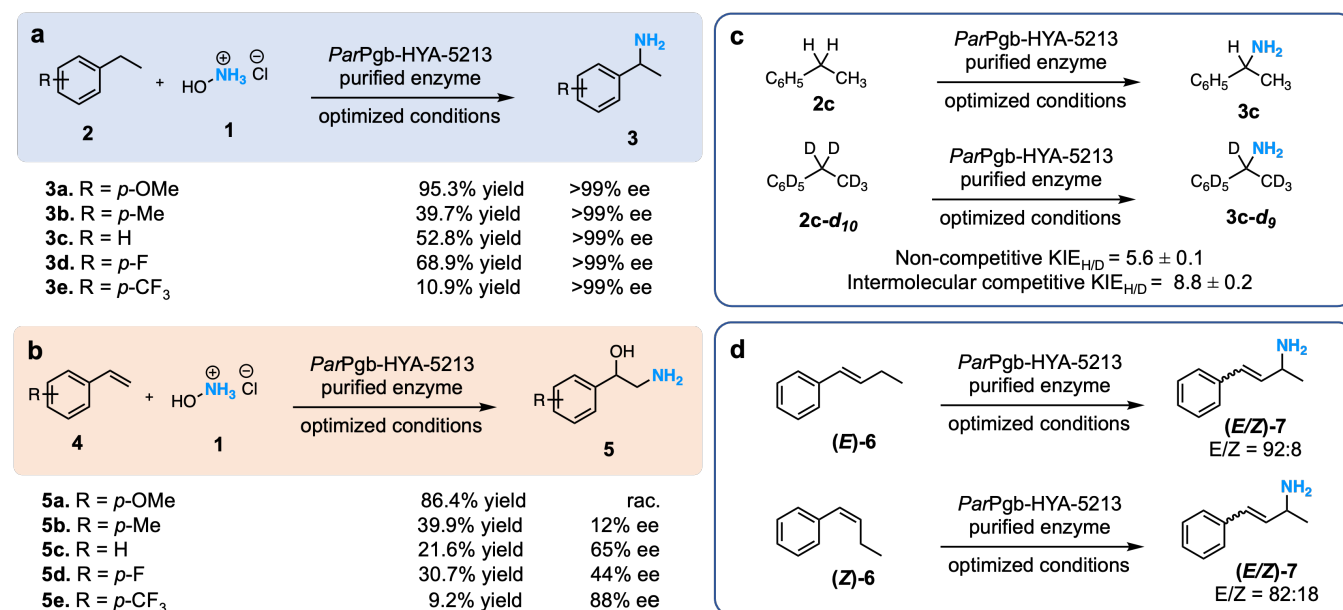
**Figure 1. a)** Evolution trajectory of benzylic C–H primary amination with 4-ethylanisole **2a** (blue) and reactivity of the lineage for aminohydroxylation with 4-vinylanisole **4a** (orange). Experimental details can be found in SI Section 3.3. **b)** The amino acid positions mutated in ParPgb-HYA-5213 are shown (blue spheres) on the homology model of wildtype ParPgb (grey) based on the crystal structure of *Methanosarcina acetivorans* protoglobin (PDB:2VEE).

We next investigated the reaction conditions with purified enzyme. During protein purification, we discovered that the heme occupancy of the protoglobins was only ~25% by comparing the protein and heme concentrations. We reasoned that heme production in *E. coli* does not match the high protoglobin protein expression level.<sup>29</sup> Fortunately, we were able to reconstitute the fully

charged enzymes by supplementing exogenous heme *b* cofactor to the apo protoglobins.<sup>22,30</sup> The amination reactions can proceed aerobically but are more efficient under anaerobic conditions, increasing the yield of **3a** by ~10% (see SI, Section 9.1.). Further reaction optimization using heme-loaded purified enzyme of variant ParPgb-HYA-5213 with sodium dithionite as reducing reagent

under anaerobic conditions greatly improved the yield for benzylic C–H primary amination with **2a**: the reaction with 0.5 mol% enzyme afforded 95.3% analytical yield and 69% isolated yield of primary amine **3a**. The enzymatic product was derivatized to measure enantiomeric excess (ee) (see SI, Section 5.). The reaction gave excellent enantioselectivity of >99% ee, favoring the (*R*)-enantiomer.

To gain insight into how directed evolution improved the reactivity of *ParPgb* using  $\text{NH}_2\text{OH}$ , we compared the kinetic parameters of *ParPgb*-HYA-5213 and its parent *ParPgb*-HYA-5209 for benzylic C–H primary amination using **1** and ethylbenzene **2c** (see SI, Section 7.). The observed turnover number ( $k_{\text{cat}}$ ) of *ParPgb*-HYA-5213 ( $1.5 \text{ s}^{-1}$ ) increased 180-fold compared to *ParPgb*-HYA-5209 ( $0.0087 \text{ s}^{-1}$ ), which is likely the major factor contributing to the increase in overall yield observed along the evolutionary lineage. In addition, the observed  $K_M$  value for  $\text{NH}_2\text{OH}$  decreased from 5.4 mM to 0.30 mM, suggesting the evolution campaign improved productive binding of  $\text{NH}_2\text{OH}$ . Finally, we observed that the rate



**Figure 2.** Substrate scope and mechanistic study. Experimental details can be found in SI Section 4, 5, 8 and 9.2. **a**) Substrate scope of the benzylic C–H substrate **2**. **b**) Substrate scope of the styrenyl substrate **4**. **c**) KIE experiment using **2c** and **2c-d<sub>10</sub>**. **d**) Radical probe experiment using (*E*)- and (*Z*)-**6**.

The mechanism of benzylic C–H primary amination with  $\text{NH}_2\text{OH}$  was investigated using kinetic isotope effects (KIE) experiments and a radical probe. A non-competitive KIE value of 5.6 and intermolecular competitive KIE of 8.8 was obtained, indicating C–H bond cleavage as the rate-determining step in the final variant *ParPgb*-HYA-5213 (Figure 2c). Interestingly, in previous studies of enzymatic benzylic C–H amination with hydroxylamine-derived and azide precursors, the non-competitive KIE values were near unity, indicating nitrene formation was rate-determining.<sup>14c,32</sup> The primary non-competitive KIE value here suggests that nitrene formation with  $\text{NH}_2\text{OH}$  by the final variant is relatively facile. In addition, the relatively large KIE values also suggest a radical-related reaction pathway involving an open-shell nitrene intermediate.<sup>33,34</sup> This agrees with the isomerization of radical probe substrate (*E/Z*)-**6** (Figure 2d), which is consistent with the formation of a carbon-centered radical at the allylic position. Collectively, these results provide evidence in favor of a stepwise radical mechanism for the C–H amination process.<sup>12,35,36</sup>

Given that wild-type *ParPgb* cannot use hydroxylamine for amination, we examined the mutations responsible for this novel

decreases at high concentrations of  $\text{NH}_2\text{OH}$ , reflecting enzyme inactivation and/or substrate inhibition.

We next evaluated the enzymatic activity for benzylic C–H primary amination on various substrates (Figure 2a). Overall, substrates bearing *para* electron-rich and electron-deficient substituents (**3a–e**) are well tolerated, furnishing primary amine products in overall good yield and excellent selectivity. We also examined the substrate scope of aminohydroxylation with styrenes (Figure 2b). Similarly, styrenes bearing both electron-rich and electron-deficient groups on the aryl moiety furnished amino alcohol products in good yields (**5a–e**). In addition to the electronic properties, the sterics of the different substrates will also affect binding in the active site and therefore reactivity. The enantioselectivities of the amino alcohol products vary from highly enantioselective to racemic. We reasoned that the electron-rich aziridine products undergo hydration via a ring-opened carbocation intermediate whereas electron-deficient substrates likely undergo hydration through an  $\text{S}_{\text{N}}2$  pathway.<sup>31</sup>

reactivity. After three rounds of recombination, mutation Y57D, together with mutations W59L and V60Q, were conserved among all the final, most active variants. Furthermore, these residues are directly above the heme cofactor and point towards the heme iron in the generated homology model (see SI, Figure S4). We hypothesized that, much like the key active-site residues in peroxygenases, mutations Y57D and V60Q could serve as an acid-base catalytic pair to activate hydroxylamine through a putative proton relay mechanism, while mutations Y57D and W59L could create a more open active site. We reasoned that introducing these three mutations to other wild type protoglobin homologs could enable them to use  $\text{NH}_2\text{OH}$ . Eleven homologous variants from archaea and bacteria with wild-type protein sequence identities ranging from 51.83% to 82.72% to *ParPgb* were selected for this mutation transfer experiment (Table 1). These three mutations were introduced at their homologous positions by site-directed mutagenesis to yield eleven *Pgb* DLQ variants. All eleven wild-type protoglobins expressed and showed no reactivity towards benzylic C–H primary amination with **2a** and **1**. In contrast, eight out of eleven *Pgb* DLQ variants showed activity, although none were more

active than ParPgb DLQ. The *Pyrobaculum ferrireducens* protoglobin DLQ variant with 82.72% sequence identity to ParPgb gave the highest activity in terms of TTN, comparable to that of ParPgb DLQ. These results suggest the key role of these mutations in activating hydroxylamine. This diverse library of hydroxylamine-dependent protoglobins could serve as a starting point for exploring new-to-nature nitrene transfer reactions.

**Table 1.** Protein expression levels and activities of protoglobin DLQ variants with 4-ethylanisole **2a** and hydroxylammonium chloride **1<sup>a</sup>**

Variant	Sequence identity (%)	Protein expression (mg per L of cells)	TTN for 3a
<i>TpePgb</i> -HYA-5225	51.83	165	< 1
<i>MacPgb</i> -HYA-5217	53.68	35	0
<i>TdaPgb</i> -HYA-5223	54.21	185	1.5
<i>PmePgb</i> -HYA-5220	54.45	10	0
<i>CthPgb</i> -HYA-5216	54.74	180	< 1
<i>ThuPgb</i> -HYA-5224	57.07	180	< 1
<i>AauPgb</i> -HYA-5214	58.12	195	2.8
<i>TamPgb</i> -HYA-5221	58.12	170	2.9
<i>TarPgb</i> -HYA-5222	59.59	190	4.4
<i>ApePgb</i> -HYA-5215	62.83	20	0
<i>PfePgb</i> -HYA-5219	82.72	130	25
<i>ParPgb</i> -HYA-5218	100	220	30

<sup>a</sup>Experimental details can be found in SI Section 6.

In conclusion, we have demonstrated that a hemoprotein can evolve to use a feedstock chemical, hydroxylammonium chloride, for direct incorporation of nitrogen. This unprecedented reactivity enables benzylic C–H primary amination and alkene aminohydroxylation, with water as the sole byproduct. Mechanistic studies revealed a radical reaction pathway with C–H bond cleavage as the rate-determining step. We envision this discovery will enable new sustainable and economical biocatalytic routes to nitrogen-containing molecules. Finally, we anticipate this work will presage the discovery of natural enzymes that utilize NH<sub>2</sub>OH as amine sources.

## ASSOCIATED CONTENT

### Supporting Information

Materials, experimental methods, and compound characterization data, including Tables S1 and Figure S1–9.

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## Notes

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