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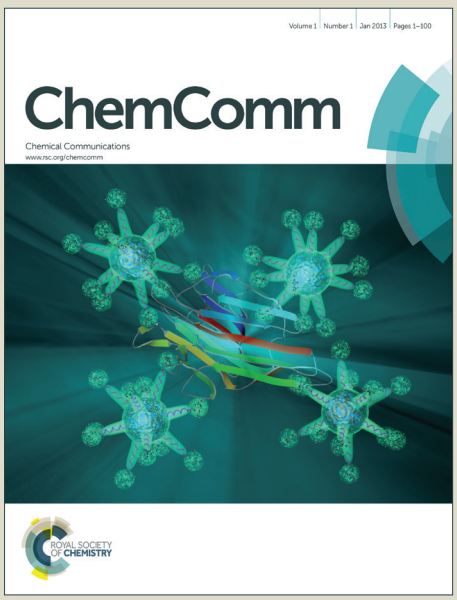

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Myoglobin-catalyzed intermolecular carbene N-H insertion with arylamine substrates

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Gopeekrishnan Sreenilayam and Rudi Fasan*

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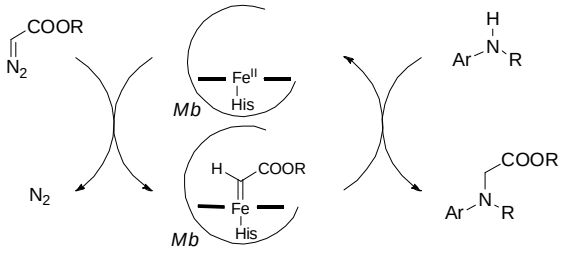
Engineered variants of the heme-containing protein myoglobin can efficiently catalyze the insertion of α-diazo esters into the N—H bond of arylamines, featuring a combination of high chemoselectivity, elevated turnover numbers, and broad substrate scope.

The widespread occurrence of amine functionalities in synthetic building blocks and biologically active natural and man-made molecules make catalytic methods for formation C—N bonds of fundamental synthetic relevance. A potentially very powerful approach to the construction of such bonds is through the insertion of metallo-carbenoid species, generated via metal-catalyzed decomposition of readily available diazo reagents, into the N—H bond of an aryl or alkyl amine.[1] This strategy has provided a concise route to the synthesis of α-amino acid derivatives, α-amino ketones, and nitrogen-containing heterocycles.[2] In the context of this reaction, a variety of transition metal catalysts have been investigated over the past years, including Cu-, Rh-, Ru-, Ag-, and Fe-complexes.[3] Since this transformation has no counterpart among those catalyzed by naturally occurring enzymes, the development of biocatalysts capable of supporting these transformations has direct relevance toward expanding the toolbox of environmentally friendly and sustainable processes for the synthesis of organic molecules.

Recent studies from the Arnold group and our own laboratory demonstrated that a number of heme-containing enzymes and proteins exhibit carbene transfer reactivity. Arnold and coworkers showed that engineered variants of the bacterial cytochrome $P450_{BM3}$[4] as well as other P450s[5] can catalyze the cyclopropanation of styrenes in the presence of α-diazo esters as carbene donors. More recently, the same group reported that these enzymes can also promote carbene N—H insertion reactions with aniline and derivatives thereof as the substrates, supporting up to 480 turnovers.[6] Concurrent studies in our laboratory have recently led to the development of engineered myoglobin (Mb) variants capable of mediating the cyclopropanation of aryl-substituted alkenes with very high catalytic efficiency along with excellent selectivity.[7] The remarkable reactivity toward cyclopropanation prompted us to investigate the catalytic potential and scope of these Mb-based catalysts in the context of N—H insertion (**Scheme 1**). Here, we report that engineered myoglobins can efficiently catalyze this transformation across a variety of arylamine substrates. Furthermore, we show how active site mutagenesis can provide a viable means to optimize the activity of these catalysts toward a specific amine substrate or α-diazo ester reagent.

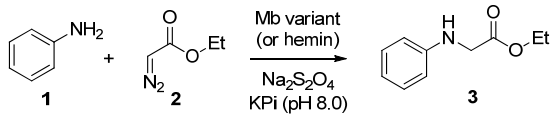


Scheme 1. Putative mechanism for the myoglobin-catalyzed carbene N—H insertion reactions with arylamines.

In initial studies, we tested the activity of wild-type sperm whale Mb toward catalyzing the conversion of aniline (**1**) to ethyl 2-(phenylamino)acetate (**3**) in the presence of ethyl α-diazoacetate (EDA, **2**) (**Table 1**). Under anaerobic conditions and in the presence of dithionite as a reductant, formation of the desired product **3** was observed, thus demonstrating that this hemoprotein is able to mediate carbenoid N—H insertion. Negligible formation of **3** was

noted in the absence of reductant or in the presence of oxygen, indicating that ferrous Mb is responsible for the observed reactivity and that molecular oxygen interferes with it, most likely through competing with the diazo reagent for binding to the heme iron. No product formation upon complexation of the ferrous Mb to carbon monoxide provided further evidence for the direct involvement of the heme cofactor in catalysis. In previous studies, we established that the Mb variant Mb(H64V,V68A) possesses greatly enhanced carbene and nitrene transfer activity in the context of olefin cyclopropanation[7] and arylsulfonyl azide cyclization[8], respectively. Upon testing, Mb(H64V,V68A) was found to exhibit significantly higher N—H insertion reactivity than wild-type Mb (>500 vs. 210 TON, **Table 1**), motivating our choice of this variant for further studies.

Table 1. Catalytic activity of hemin, wild-type sperm whale myoglobin (Mb), and the Mb(H64V,V68A) variant in the N—H insertion reaction with aniline and EDA.



1 2 3

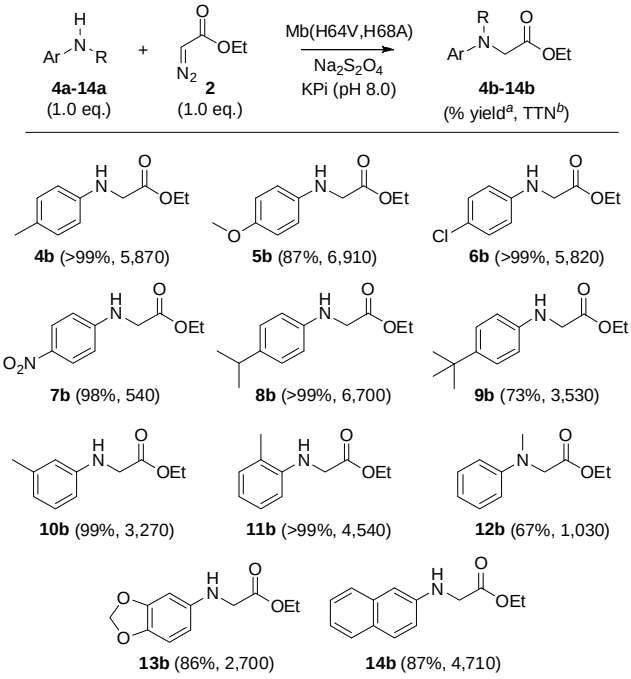
Catalyst[a]	[cat] (mM)	[aniline] (mM)	[EDA] (mM)	Conversion[b]	TON
Hemin	0.01	10	5	18%	180
Mb	0.01	10	5	21%	210
Mb(H64V,V68A)	0.01	10	5	23%	230
Mb(H64V,V68A)	0.02	10	5	45%	223
Mb(H64V,V68A)	0.02	10	10	>99%	499
Mb(H64V,V68A)	0.02	10	10	40%[c]	200[c]
Mb(H64V,V68A)	0.02	40	40	51%	1,010
Mb(H64V,V68A)	0.02	160	160	37%	2,970
Mb(H64V,V68A)	0.001	10	10	61%	6,150

[a] 400 μL scale reactions conducted for 12 hours at room temperature and under anaerobic conditions. [b] Relative to aniline and as determined based on GC conversion using calibration curves with isolated **3**. Error is within 15%. [c] Using 20 μM $Na_2S_2O_4$.

Following reaction optimization, we established that quantitative conversion of aniline to **3** could be obtained at millimolar substrate concentration (0.01 M) using Mb(H64V,V68A) at 0.2 mol% and an equimolar ratio of the amine and diazo reagent (**Table 1**). As a comparison, 10- to 25-fold higher catalyst loadings have been reported in association with similar transformations and yields using transition metal complexes.[2b,3h] Relatively high turnover numbers (200 TON) were obtained also in the presence of stoichiometric amounts of dithionite relative to the Mb catalyst (**Table 1**), indicating that an excess of reductant is beneficial but not essential for the transformation. Importantly, Mb(H64V,V68A) was found to remain active in the presence of the amine substrate and EDA at a concentration as high as 0.16 M, which corresponds to ~15 g aniline/L (**Table 1**). This finding is noteworthy considering that aniline is known to coordinate the heme iron in heme-containing enzymes and thus potentially inhibit their function.[9] Furthermore, no formation of EDA dimerization byproducts (i.e., ethyl fumarate and maleate) or of the double addition product (Ph-N($CH_2COOEt)_2$) was noted in the Mb(H64V,V68A) reactions (**Figure S1** in ESI†), even under the aforementioned high-substrate-loading conditions. This result is in contrast with the mixture of single and double addition products generated from similar reactions in the presence of iron-porphyrins[10] or free hemin[6] as catalysts. At the highest substrate concentration tested (0.16 M), Mb(H64V,V68A) catalyzes nearly ~3,000 turnovers. With 10 mM aniline and a catalyst loading of 0.001 mol%, over 6,000 total turnovers (TTN) were supported by this Mb variant. This value is an order of magnitude higher than that recently reported engineered $P450_{BM3}$ variants[6] and ranks among the highest TTNs reported for catalytic N—H insertion reactions with acceptor-only diazo compounds.[10] The Mb(H64V,V68A)-catalyzed reaction is also remarkably fast, proceeding at an initial rate of 740 turnovers min^{-1} over the first 10 min, as determined by time course experiments.

In order to investigate the substrate scope of the Mb-based catalyst, a range of substituted anilines (**4a**-**12a**) and other arylamines (**13a**, **14a**) were subjected to Mb(H64V,V68A)-catalyzed N—H functionalization in the presence of EDA. As summarized in **Scheme 2**, all of the aniline derivatives, including *para*- (**4a**-**9a**), *meta*- (**10a**), and *ortho*-substituted anilines (**11a**), could be converted to the desired N—H insertion product in very good to excellent yields (67-99%). In terms of substituents on the aniline ring, both electron-donating (**5a**) and electron-withdrawing (**6b**, **7b**) groups were well tolerated by the Mb catalyst. Formation of the N-methyl derivative **12b** from N-methylaniline in 67% yield indicated that secondary aryl amines are also converted by the Mb(H64V,V68A) variant, although with somewhat lower efficiency than for aniline. Addition of a second equivalent of EDA did not increase the yield of **12b**. Substrates **13a** and **14a** were then tested to explore the scope of the reaction across other aromatic amines. Also in this case, high conversions for the Mb(H64V,V68A)-catalyzed reaction were achieved, further supporting the broad substrate scope of this catalyst. Reactions with the amine substrates described above were also carried out in the presence of

Scheme 2. Yields and total turnover numbers (TTN) for Mb(H64V,V68A)-catalyzed carbene N—H insertion with various aryl amines. Reaction conditions: 10 mM amine, 10 mM EDA, 10 mM $Na_2S_2O_4$ with (a) 20 μM (0.2 mol%) and (b) 1 μM (0.01 mol%) hemoprotein.



low catalyst loading (0.01 mol%). Under these conditions, between1,000-6,900 total turnovers were measured, highlighting the reactivity and robustness of the Mb catalyst for N—H carbene insertion across various aromatic amines. Finally, successful isolation of ~15 mg of **3** in 80% yield from a larger scale reaction provided a proof-of-principle demonstration of the scalability of the myoglobin-mediated transformation.

An attractive feature of this class of Mb-derived carbene transfer catalysts is that they are genetically encoded and their activity can be in principle modulated by mutagenesis. Despite the broad substrate scope and high activity of Mb(H64V,V68A), comparatively lower yields were observed with N-methyl-aniline as the N—H component (**Scheme 2**). In the interest of determining whether improved activities on these substrates could be obtained by protein engineering, we screened a panel of previously prepared Mb variants containing 1-2 amino acid mutations within the distal cavity of the protein.[7] From the screening, both Mb(H64V) and Mb(H64V,L29A) were found to be considerably more efficient than Mb(H64V,V68A) toward the conversion of N-methyl-aniline, supporting about nearly four-fold higher turnovers on this substrate (3,910 vs. 1,030, **Figure 1**, top panel). Using Mb(H64V,L29A) at 0.2 mol%, **12b** could be thus obtained in higher yields (75%). To examine the performance of the Mb variants in the presence of a different α-diazoester reagent, reactions with aniline were repeated with *tert*-butyl diazoacetate (*t*BDA) as the carbene source. Whereas this reaction is efficiently catalyzed by Mb(H64V,V68A) (3,620 TTN, **Figure 1**, bottom panel), even higher TTN values for formation of **35** were observed for Mb(H64V) and Mb(H64V,L29A) (5,730 and 7,540 TTN, respectively). Collectively, these experiments illustrate how the substrate reactivity of these Mb catalysts toward different amine substrates and diazo reagents can be effectively modulated via modification of the hemoprotein active site.

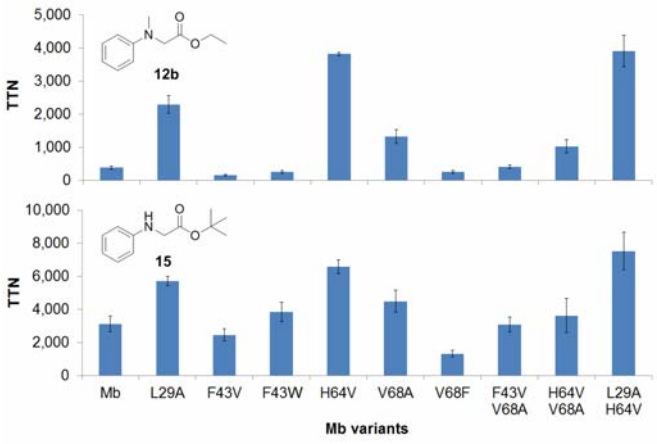


Figure 1. Total turnovers supported by the different Mb variants for formation of N—H insertion products **12b** (N-methyl aniline + EDA) and **15** (aniline + *t*BDA).

An interesting aspect emerging from the studies above concerns the activity-enhancing effect of the H64V substitution, a trend shared across other types of Mb-mediated group transfer reactions we investigated previously[7-8]. Since His64 'blocks' the distal face of the heme to the solvent (**Figure S2** in ESI†),[11] the observed activity enhancement could stem from an increased accessibility of the heme center to the reagents as a result of this mutation. Conversely, the L29A substitution exhibited a distinctive beneficial effect for N—H insertion activity (**Figure 1**), while the same mutation had essentially no impact on Mb-catalyzed olefin cyclopropanation[7], despite a common reactive intermediate (i.e., heme-bound carbenoid) is likely involved in these reactions. As this mutation expands the volume above the heme iron center (Fe—Leu29(C_β) distance ~8Å, **Figure S2** in ESI†), it could play a role in better accommodating the amine substrate prior or after attack by the carbenoid species. Notably, the beneficial effect of this substitution for carbene N—H insertion reactivity appeared to be rather general, as indicated by the high TTNs measured with Mb(L29A) with other aniline derivatives in addition to those considered above (e.g., **5b**: 4,990 turnovers; **6b**: 6,790 turnovers).

In conclusion, this work provides a first demonstration of the ability of engineered Mb variants to efficiently catalyze carbenoid N—H insertion reactions with aryl amine substrates. The excellent chemoselectivity, high numbers of catalytic turnovers, and broad substrate scope across variously substituted anilines and aryl amines make these Mb-derived catalysts a synthetically valuable and competitive alternative to transition metal-based systems typically employed in these transformations. These studies pave the way to exploration of the scope of these Mb-derived catalysts in the context of other classes of amine substrates as well as other carbene transfer reactions.

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Notes and references

[a] Department of Chemistry, University of Rochester, 120 Trustee Rd, Rochester, New York 14627, USA. E-mail: fasan@chem.rochester.edu

† Electronic Supplementary Information (ESI) available: Experimental details, synthetic procedures, characterization data for reaction products. See DOI: 10.1039/c000000x/

1 a) Doyle, M. P.; McKervey, M. A.; Ye, T. *Modern catalytic methods for organic synthesis with diazo compounds: from cyclopropanes to ylides*; Wiley-CH: New York, 1998; b) Moody, C. J., *Angew. Chem. Int. Ed.*, 2007, **46**, 9148; c) Gillingham, D.; Fei, N., *Chem. Soc. Rev.*, 2013, **42**, 4918.

2 a) Bolm, C.; Kasyan, A.; Drauz, K.; Gunther, K.; Raabe, G., *Angew. Chem. Int. Ed.*, 2000, **39**, 2288; b) Morilla, M. E.; Diaz-Requejo, M. M.; Belderrain, T. R.; Nicasio, M. C.; Trofimenko, S.; Perez, P. J., *Chem. Commun.*, 2002, 2998; c) Davies, J. R.; Kane, P. D.; Moody, C. J., *Tetrahedron*, 2004, **60**, 3967; d) Matsushita, H.; Lee, S. H.; Yoshida, K.; Clapham, B.; Koch, G.; Zimmermann, J.; Janda, K. D., *Org. Lett.*, 2004, **6**, 4627; e) Burtoloso, A. C. B.; Correia, C. R. D., *Tetrahedron Lett.*, 2004, **45**, 3355; f) Davies, J. R.; Kane, P. D.; Moody, C. J., *J. Org. Chem.*, 2005, **70**, 7305.

3 a) Yates, P., *J. Am. Chem. Soc.*, 1952, **74**, 5376; b) Saegusa, T.; Ito, Y.; Kobayash.S; Hirota, K.; Shimizu, T., *Tetrahedron Lett.*, 1966, 6131; c) Nicoud, J. F.; Kagan, H. B., *Tetrahedron Lett.*, 1971, 2065; d) Moyer, M. P.; Feldman, P. L.; Rapoport, H., *J. Org. Chem.*, 1985, **50**, 5223; e) Garcia, C. F.; McKervey, M. A.; Ye, T., *Chem. Commun.*, 1996, 1465; f)

Bachmann, S.; Fielenbach, D.; Jorgensen, K. A., *Org. Biomol. Chem.*, 2004, **2**, 3044; g) Aviv, I.; Gross, Z., *Chem. Commun.*, 2006, 4477; h) Liu, B.; Zhu, S. F.; Zhang, W.; Chen, C.; Zhou, Q. L., *J. Am. Chem. Soc.*, 2007, **129**, 5834; i) Lee, E. C.; Fu, G. C., *J. Am. Chem. Soc.*, 2007, **129**, 12066; j) Deng, Q. H.; Xu, H. W.; Yuen, A. W.; Xu, Z. J.; Che, C. M., *Organic Letters*, 2008, **10**, 1529; k) Hou, Z.; Wang, J.; He, P.; Qin, B.; Liu, X.; Lin, L.; Feng, X., *Angew. Chem. Int. Ed.*, 2010, **49**, 4763; l) Mbuvi, H. M.; Klobukowski, E. R.; Roberts, G. M.; Woo, L. K., *J. Porph. Phthal.*, 2010, **14**, 284.

4 a) Coelho, P. S.; Brustad, E. M.; Kannan, A.; Arnold, F. H., *Science*, 2013, **339**, 307; b) Wang, Z. J.; Renata, H.; Peck, N. E.; Farwell, C. C.; Coelho, P. S.; Arnold, F. H., *Angew. Chem. Int. Ed.*, 2014, **53**, 6810.

5 Heel, T.; McIntosh, J. A.; Dodani, S. C.; Meyerowitz, J. T.; Arnold, F. H., *Chembiochem*, 2014, in press (doi: 10.1002/cbic.201402286).

6 Wang, Z. J.; Peck, N. E.; Renata, H.; Arnold, F. H., *Chem. Sci*., 2014, **5**, 598.

7 Bordeaux, M.; Tyagi, V.; Fasan, R., *Angew. Chem. Int. Ed.*, 2014, in press (DOI: 10.1002/anie.201409928).

8 Bordeaux, M.; Singh, R.; Fasan, R., *Bioorg. Med. Chem.*, 2014, **22**, 5697.

9 a) Rein, H.; Maricic, A.; Janig, G. R.; Vuk-Pavlovic, S.; Benko, B.; Ristau, O.; Ruckpaul, K., *Biochim. Biophys. Acta*, 1976, **446**, 325; b) Locuson, C. W.; Hutzler, J. M.; Tracy, T. S., *Drug Metab. Dispos.*, 2007, **35**, 614.

10 Aviv, I.; Gross, Z., *Synlett*, 2006, 951.

11 Vojtechovsky, J.; Chu, K.; Berendzen, J.; Sweet, R. M.; Schlichting, I., *Biophys. J.*, 1999, **77**, 2153.

