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Cytochrome P450-Catalyzed Insertion of Carbenoids into N-H Bonds

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Abstract

Expanding nature's catalytic repertoire to include reactions important in synthetic chemistry will open new opportunities for 'green' chemistry and biosynthesis. We demonstrate enzyme-catalyzed insertion of carbenoids into N-H bonds. This type of bond disconnection, which has no counterpart in nature, can be mediated by variants of the cytochrome P450 from *Bacillus megaterium*. The N-H insertion reaction takes place in water, provides the desired products in 26-83% yield, forms the single addition product exclusively, and does not require slow addition of the diazo component

Introduction

The insertion of carbenoids into amine N-H bonds is a powerful method of forming C-N bonds.[1] This type of reaction has been used for synthesis of many bioactive molecules such as unnatural amino acids, alkaloids, and N-heterocyclic compounds.[2] For instance, N-H insertion by rhodium catalysts was used in the synthesis of thienamycin, which is one of the most potent antibiotics ever identified and can only be accessed in significant quantities via organic synthesis.[3] Carbene insertion reactions typically utilize diazo compounds, which readily generate reactive carbenoid intermediates with loss of dinitrogen in the presence of a catalyst.[4] In recent years, a variety of catalysts employing transition metal complexes,[5] ureas[6] or artificial metalloenzymes[7] have been developed. However, no enzyme or antibody has ever been reported to exhibit this mode of activation.

Recently we showed that variants of the cytochrome P450 from *Bacillus megaterium* (P450-BM3) are excellent catalysts for intermolecular cyclopropanation of styrenes using synthetic diazo compounds in water.[8] We hypothesize that this reaction proceeds through an iron-carbenoid intermediate that forms at the hemin prosthetic group when the protein is treated with ethyl diazoacetate (EDA) under anaerobic conditions. The yields and selectivities exhibited by the P450-BM3 variants are comparable to the best cyclopropanation catalysts based on rhodium.[8] Given that metal-carbenoids react with other electron-rich partners like amines,[9] we hypothesized that P450-BM3 may also be a competent catalyst for carbenoid N-H insertion (Figure 1). N-H insertion with metal porphyrins has been reported in organic solvents, but these reactions often form a mixture of the single and double N-H insertion products when primary amines are used.[10] An enzyme, on the other hand, should be able to sterically select for one product compatible with the binding pocket.

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Although we have postulated that P450-BM3 forms iron-carbenoids in the presence of diazo compounds,[8] this carbenoid has not been characterized or observed directly. Thus, the ability of engineered P450s to catalyze classical carbenoid insertions provides additional evidence that an iron-carbenoid intermediate is formed at the heme prosthetic group.

Results and Discussion

We combined aniline with EDA in the presence of a reductant ($Na_2S_2O_4$) under argon atmosphere and tested the mixture with seven P450-BM3 variants previously identified as competent catalysts for cyclopropanation.[8a] In choosing this set of P450s, we hypothesized that cyclopropanation activity would correlate with ability to generate the iron-carbenoid intermediate that is also necessary for N-H insertion. Whereas wildtype BM3 (WT) provided only trace amounts of the desired product (**1**, Table 1, entry 1), a few variants gave **1** in good yields after 12 h at room temperature. In particular, variant H2-5-F10, which is derived from a thermostable P450-BM3 lineage[11] and differs from WT by 15 mutations, formed **1** in 47% yield and 473 turnovers (TTN) using 0.1% protein relative to EDA (entry 6).

The Cys at position 400 in P450-BM3 coordinates axially to the iron-heme. Interestingly, variants possessing a mutation of Cys at position 400 to serine (P411-CIS and P411-T268A, entries 7 and 8) were less active than their cysteine analogues. This contrasts with the much greater activity of the Ser-ligated P411 enzymes observed in styrene cyclopropanation.[8b] The Cys to Ser substitution does not result in substantial geometric changes at the active site.[8b] It is possible that a less electron-rich ligand in the axial position of the heme prosthetic group lowers the activity of the catalyst in N-H insertion reactions.

When CO was bubbled gently through the protein solution before the addition of EDA and aniline, no product formation was observed (Table 1, entry 7), presumably due to complexation of CO to the iron center. Performing the reaction under aerobic conditions significantly reduced the yield of **1**, suggesting that O_2 also inhibits the transformation (Table 1, entry 10). Additionally, variants BM3-CIS, H2-4-D4, and H2-5-F10 (entries 3, 4, and 6, see supporting information Table S.2 for amino acid sequences) differ by only 1-2 active site amino acids from variant H2-A-10 (entry 5), yet exhibit a range of activity (24-47% yield). This demonstrates that changes in the protein sequence, and presumably the geometry around the active site, can modulate N-H insertion activity.

We have previously observed that reductant is not consumed stoichiometrically in carbene transfer reactions with P450-BM3 and is only needed to reduce the Fe(III) resting state to the Fe(II) active catalyst.[7a] Accordingly, decreasing the amount of $Na_2S_2O_4$ from 10 mM to 2.5 mM did not significantly affect the efficiency of the N-H insertion reaction, but omitting $Na_2S_2O_4$ from the reaction mixture altogether resulted in no product formation (Supporting Information, Table S.1). Furthermore, only 2% conversion was observed when we switched from $Na_2S_2O_4$ to a biological reductant like NADPH. This is likely due to inefficient reduction of Fe(III) to the active Fe(II) catalyst for carbene transfer, as the redox potential of NAD^+/NADH ($E^{\circ\prime} = -320$ mV; all potentials versus standard hydrogen electrode) is less negative than that of low spin Fe(III)/Fe(II) in P450-BM3 ($E^{\circ\prime} = -430$ mV). Because P450s exist in both the ferric and ferrous forms in intact *Escherichia coli* cells,[12] we posited that catalysis could also be achieved using intact cells. Addition of EDA and aniline to cells expressing H2-5-F10 provided **1** in 38% yield.

Under the current optimized reaction conditions, complete conversion to the N-H insertion product was never observed. We reasoned that incomplete conversion could be due to depletion of EDA in competing side reactions, inhibition of the enzyme by **1**, or deactivation of the enzyme over time. When an equimolar ratio of EDA and aniline was used, unreacted

aniline could be detected by GC at the end of reaction but dimeric side products like diethyl fumarate or maleate were not observed. When we combined EDA and aniline in a 1:2 ratio, then added a second equivalent of EDA after 2 h, no further **1** was produced. This indicates that the moderate yield of **1** is not due to consumption of the limiting reagent, EDA, by competing side reactions. To test for product inhibition, 1.0 equivalents of ethyl 2-(p-tolylamino) acetate (**4**), the N-H insertion product from the reaction of EDA with toluidine, was added to a mixture of enzyme, reductant, aniline and EDA. The yield of **1** remained unchanged. These experiments suggest that deactivation of the P450 catalyst may be responsible for the moderate conversion to **1**. Efforts to elucidate the mechanism of this deactivation are currently on going.

We examined a variety of substrates for N-H insertion using P450 variant H2-5-F10 and found that this catalyst is fairly general and can catalyze N-H insertion with both primary and secondary anilines (Figure 2). Substitution is well-tolerated on the aniline partner on both the *ortho* and *para* positions. Reaction of aniline and EDA was also performed in milligram scale without any organic cosolvent to provide **1** in 65% isolated yield. On the other hand, alkyl amines like morpholine, cyclohexyl amine and benzyl amine were poor substrates for this enzyme, which gave <3% conversion to the insertion products (Supporting Information, Table S.3).

Diazo amides can be used as the carbene precursor for this reaction, and product **8** can be formed in moderate yields. Products such as **8** are valuable because they are precursors to important synthetic building blocks such as amino amides and 1,2-diamines. Only one transition metal-based method has ever been described for N-H insertion with diazo amides; TTNs of 16-32 were reported.[13] In comparison, P450 variant H2-5-F10 catalyzes the insertion reaction with more than 150 TTN.

As P450-BM3s are competent catalysts for both N-H insertion and cyclopropanation, we performed competition experiments with styrene and aniline to determine the relative rates of these two reactions. When 1 equivalent of EDA was combined with 1 equivalent of aniline and 1 equivalent of styrene, the N-H insertion and cyclopropanation products were formed in 63% and 9% yield, respectively (Figure 3a). This suggests that N-H insertion reaction with H2-5-F10 is significantly faster than the cyclopropanation reaction (Supporting Information, Table S.4). When EDA is combined with substrate **9** containing both amine and olefin functionalities, the single N-H insertion product **10** is formed in 32% yield, with only trace amounts of the single cyclopropanation product (Figure 3b). This chemoselectivity for N-H insertion is consistent with our observation that N-H insertion is faster than cyclopropanation.

The enzyme is also selective. No double insertion products were ever observed in the enzyme-catalyzed N-H insertion reactions, as determined by GC-MS and ^{1}H NMR of the products in milligram-scale reactions. In contrast, when the isolated hemin prosthetic group alone was used as catalyst, the single and double insertion products were produced in 2.5:1 to 3.5:1 ratios (Figure 4). This product distribution is consistent with what has been observed with other metalloporphyrins such as iron tetraphenylporphyrin (FeTPP).[10] The ability of P450 variants to make the single insertion product exclusively agrees with our hypothesis that the protein binding pocket can control reaction selectivity and constitutes a major advantage of protein-mediated catalysis over catalysis by isolated metalloporphyrins.

Conclusions

Enzymes offer an advantage over transition metal catalysts because they operate under mild conditions in aqueous solvent, are highly active, and can be highly selective for one product

when multiple are possible.[14] By screening variants of P450-BM3 for activity on unnatural substrates, we have uncovered a new mode of enzyme-catalyzed amine synthesis and report one of the first example of N-H insertion by metal carbenoids catalyzed in water. Variants of P450-BM3 reacted readily with diazo compounds and amines to give α-amino esters and amides. These reactions proceeded to good yields without the need for slow addition of the diazo reagent. In contrast to N-H insertion mediated by other metalloporphyrins, no double insertion products were observed in the P450-catalyzed reaction, even when equal molar ratios of amine and diazo compounds were used. Our results support the hypothesis that an iron-carbenoid is formed when P450-BM3 reacts with diazo compounds and suggest that many other insertion reactions will also be catalyzed by P450s.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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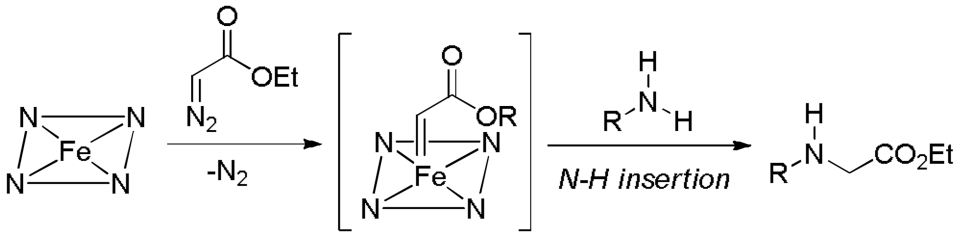
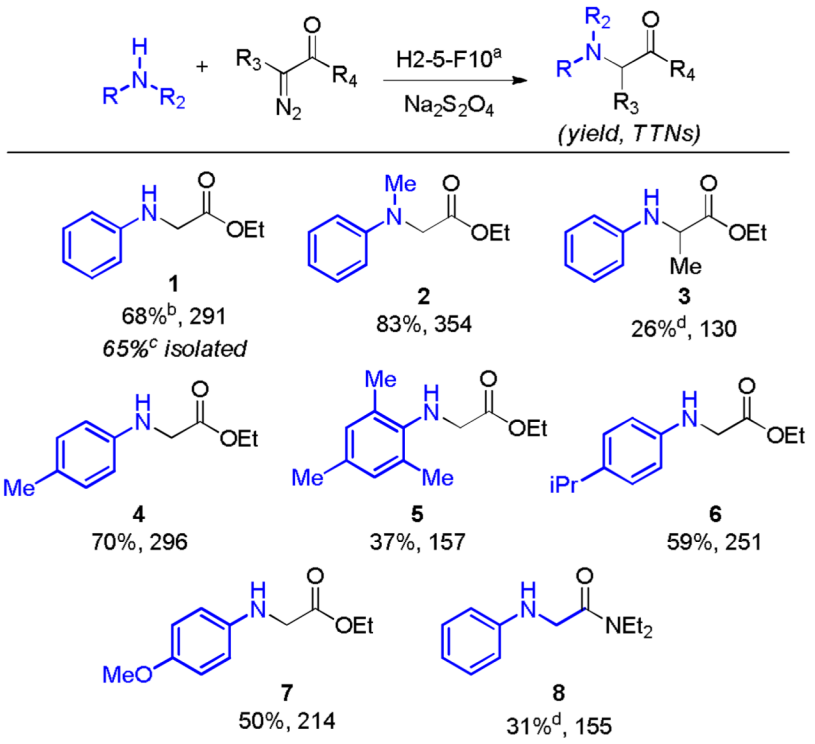


Figure 1.
Proposed carbenoid N-H insertion reaction by cytochrome P450.



[a]Reactions were carried out with protein (20 μM), EDA (8.5 mM), aniline (20 mM) and $Na_2S_2O_4$ (10 mM) in phosphate buffer (pH 8) and allowed to shake at room temperature for 12 h. Yields are reported in parentheses and TTN are presented in Table S.1 in supporting information. [b]Yields were determined by GC calibrated for each product. [c]Isolated yield based on milligram scale reaction: 25 mM EDA, 25 mM aniline, 25 μM protein, and 25 mM $Na_2S_2O_4$. [d]Reactions carried out with protein (20 μM), EDA (10.0 mM), aniline (20 mM) and $Na_2S_2O_4$ (10 mM).

Figure 2.
Substrate scope for cytochrome P450-catalyzed N-H insertion.

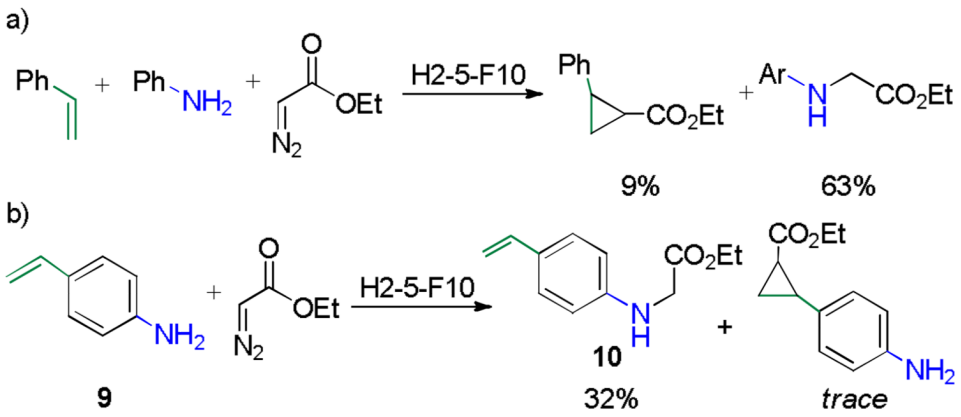


Figure 3.

(a) Competition reaction of styrene and aniline for cyclopropanation and N-H insertion, respectively, with EDA. (b) Reaction of bifunctional substrate **9** with EDA.

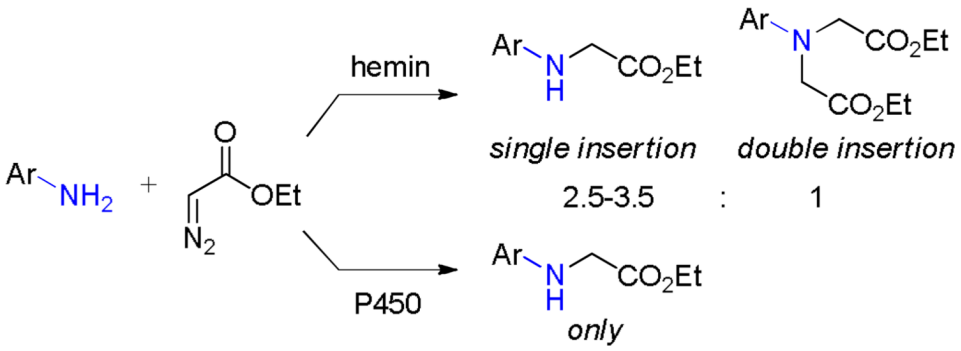
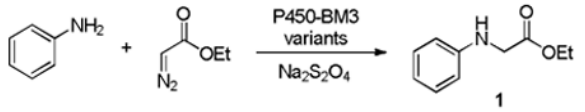


Figure 4.
Product ratio of N-H insertion catalyzed by hemin versus P450 variant H2-5-F10.

Table 1

N-H insertion using variants of cytochrome P450-BM3.



1

Entry	Catalyst[a,b]	Conditions	% Yield[c]	TTN
1	WT	Anaerobic[d]	1.7	17
2	T268A	Anaerobic	16	160
3	BM3-CIS	Anaerobic	43	433
4	H2-4-D4	Anaerobic	34	340
5	H2-A-10	Anaerobic	24	238
6	H2-5-F10	Anaerobic	47	473
7	P411-CIS	Anaerobic	14	136
8	P411-T268A	Anaerobic	9.5	95
9	H2-5-F10 + CO	Anaerobic	0	0
10	H2-5-F10	Aerobic	2	20
11	H2-5-F10	Anaerobic	68[d]	291

[a] Reactions were carried out with protein (10 μM), EDA (8.5 mM), aniline (20 mM) and $Na_2S_2O_4$ (10 mM) in phosphate buffer (pH 8) with shaking at room temperature for 12 h.

[b] See supplemental information for specific amino acid substitutions from WT for each mutant.

[c] Yields were determined by GC calibrated for **1**.

[d] Anaerobic reactions performed under Ar atmosphere. [d] Reaction performed with 20 μM protein.