

Origin and Control of Chemoselectivity in Cytochrome *c*-Catalyzed Carbene Transfer into Si–H and N–H bonds

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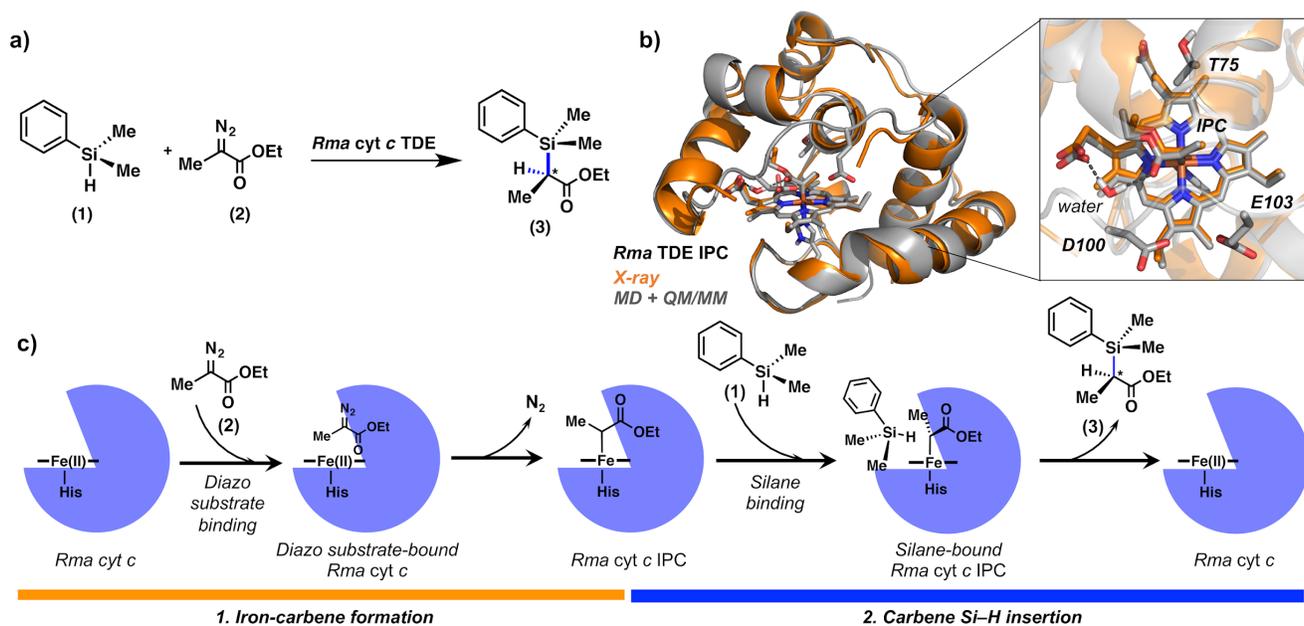
ABSTRACT

A cytochrome *c* heme protein was recently engineered to catalyze the formation of carbon–silicon bonds via carbene insertion into Si–H bonds, a reaction that was not previously known to be catalyzed by a protein. High chemoselectivity towards C–Si bond formation over competing C–N bond formation was achieved, although this trait was not screened for during directed evolution. Using computational and experimental tools, we now establish that activity and chemoselectivity are modulated by conformational dynamics of a protein loop that covers the substrate access to the iron-carbene active species. Mutagenesis of residues computationally predicted to control the loop conformation altered the protein’s chemoselectivity from preferred silylation to preferred amination of a substrate containing both N–H and Si–H functionalities. We demonstrate that information on protein structure and conformational dynamics, combined with knowledge of mechanism, leads to understanding of how non-natural and selective chemical transformations can be introduced into the biological world.

INTRODUCTION

The Arnold lab has expanded the catalytic repertoire of enzymes towards new-to-nature chemistries, and recently created a carbon–silicon bond-forming enzyme¹ by directed evolution of *Rhodothermus marinus* cytochrome c (*Rma* cyt c), whose native function is electron transport.² The laboratory-evolved protein (*Rma* cyt c V75T M100D M103E variant, *Rma* TDE) is capable of catalyzing carbene insertions into Si–H bonds to form new carbon–silicon bonds (**Scheme 1**). *Rma* cyt c has also been evolved to efficiently catalyze carbene insertions into B–H bonds in a selective manner,^{3, 4} highlighting the versatility and evolvability of this protein scaffold towards unnatural carbene transfer reactions. Although the catalytic activities of these novel enzymes are established in previous work,^{1, 3-5} how and why this particular protein scaffold leads to new biocatalysts that efficiently and selectively catalyze these new-to-nature reactions remains unknown. Here, we investigate the reaction mechanism, and use our mechanistic studies to help explain how certain mutations alter and significantly enhance enzyme reactivity and selectivity.

We employed experimental and computational tools to investigate the mechanism of *Rma* TDE-catalyzed carbene Si–H insertion (**Scheme 1a**); our results are similar to those reported by Fasan and Zhang for a similar myoglobin-catalyzed carbene Si–H insertion.⁶ We then investigate how the reaction is affected by the *Rma* TDE protein scaffold and mutations introduced by directed evolution. We propose that the conformational dynamics of a single loop near the heme play a key role in determining the activity and chemoselectivity of the enzyme. Finally, we use our findings to guide enzyme engineering to switch silylation-amination chemoselectivity, and demonstrate that this altered selectivity is mediated by new and distinct conformational dynamics of the loop.



Scheme 1. a) Stereoselective carbon–silicon bond formation catalyzed by *Rma* cytochrome *c* V75T M100D M103E (*Rma* TDE). *Rma* TDE was evolved to catalyze carbene insertions into Si–H bonds using silane **1** and ethyl 2-diazopropanoate (Me-EDA, **2**) as substrates. b) Overlay of the X-ray crystal structure of carbene-bound *Rma* TDE (1.29 Å; PDB ID: 6CUN; in orange) and the computationally modelled (MD + QM/MM-optimized, in gray) structure from our previous work.⁷ Iron porphyrin carbene (IPC), mutated residues, and computationally characterized bridging water molecule are shown in stick format. c) Schematic description of the proposed mechanism for *Rma* cytochrome *c*-catalyzed carbon–silicon bond formation via 1) iron-carbene formation followed by 2) carbene Si–H insertion.

RESULTS AND DISCUSSION

1. Characterization of the reaction mechanism of *Rma* cyt *c*-catalyzed Si–H carbene insertions.

Heme protein-catalyzed carbene insertion reactions are proposed to occur through the formation of a reactive iron porphyrin carbene (IPC) intermediate, followed by transfer of the carbene to a second substrate such as an olefin or an amine N–H bond (**scheme 1**).^{7–10} Previous studies of iron porphyrin-catalyzed carbene transfer reactions have suggested that three distinct IPC electronic states could be involved in these reactions: the closed-shell singlet (CSS), open-shell singlet (OSS), and triplet (T) states.^{8–13} The protein structure, particularly the amino acid ligand that coordinates the iron porphyrin, has been shown to influence the relative stabilities of various electronic states.⁷

To characterize the mechanism of carbene insertion into Si–H bonds catalyzed by *Rma* cytochrome *c* M100D V75T M103E (*Rma* TDE), we performed Density Functional Theory (DFT) calculations using a simple truncated model (imidazole-ligated iron porphyrin) and hybrid quantum mechanics/molecular mechanics (QM/MM) calculations (see SI Methods, section IIb). In our previous work, MD simulations revealed the presence of a bridging water molecule that establishes persistent H-bond interactions with the heme carboxylate group and the carbonyl group of the carbene once the IPC is formed (**Scheme 1b**).⁷ This interaction helps maintain the carbene in a single conformation and controls the stereoselectivity of the carbene transfer reaction. In order to further characterize the impact of this water molecule on the reaction mechanism, we explicitly considered it in the QM/MM calculations which account for the effect of the protein environment surrounding the histidine-ligated iron porphyrin. We considered the closed-shell singlet (CSS), open-shell singlet (OSS), and triplet (T) electronic states along the reaction pathway (see SI Methods, section IIa).

The first step of the reaction (**Scheme 1c**) involves iron-carbene formation from the ethyl 2-diazopropanoate substrate (Me-EDA, **2**) and loss of N₂. Both DFT and QM/MM calculations showed that the closed-shell singlet transition state (TS) is lowest in energy, while the open-shell singlet TS and triplet TS are higher in energy (**Figure 1a** and **Figure S4a**). This differs from earlier studies showing the open-shell singlet is favored with a thiolate ligand.¹¹ In the second reaction step, C–Si bond formation, DFT and QM/MM calculations showed that the closed-shell singlet transition state is lowest in energy. Intrinsic reaction coordinate (IRC) calculations corresponding to the lowest-energy QM/MM transition state (**TDE-TS2-CSS**) show that the enzymatic Si–H insertion is concerted but highly asynchronous, consisting of partial hydride-transfer from the silane to the carbene carbon, followed by C–Si bond formation (**Figure 1c**). In contrast, the higher in energy triplet transition state (**TDE-TS2-T**) favors a radical stepwise pathway (**Figures S4-S6**). These results are consistent with those recently reported by Fasan and Zhang on myoglobin (Mb)-catalyzed carbene insertions into Si–H bonds, where they used truncated model DFT calculations and radical trap experiments to conclude that the Mb-catalyzed carbene insertion into Si–H bonds takes place through a concerted mechanism involving a closed-shell singlet transition state.⁶

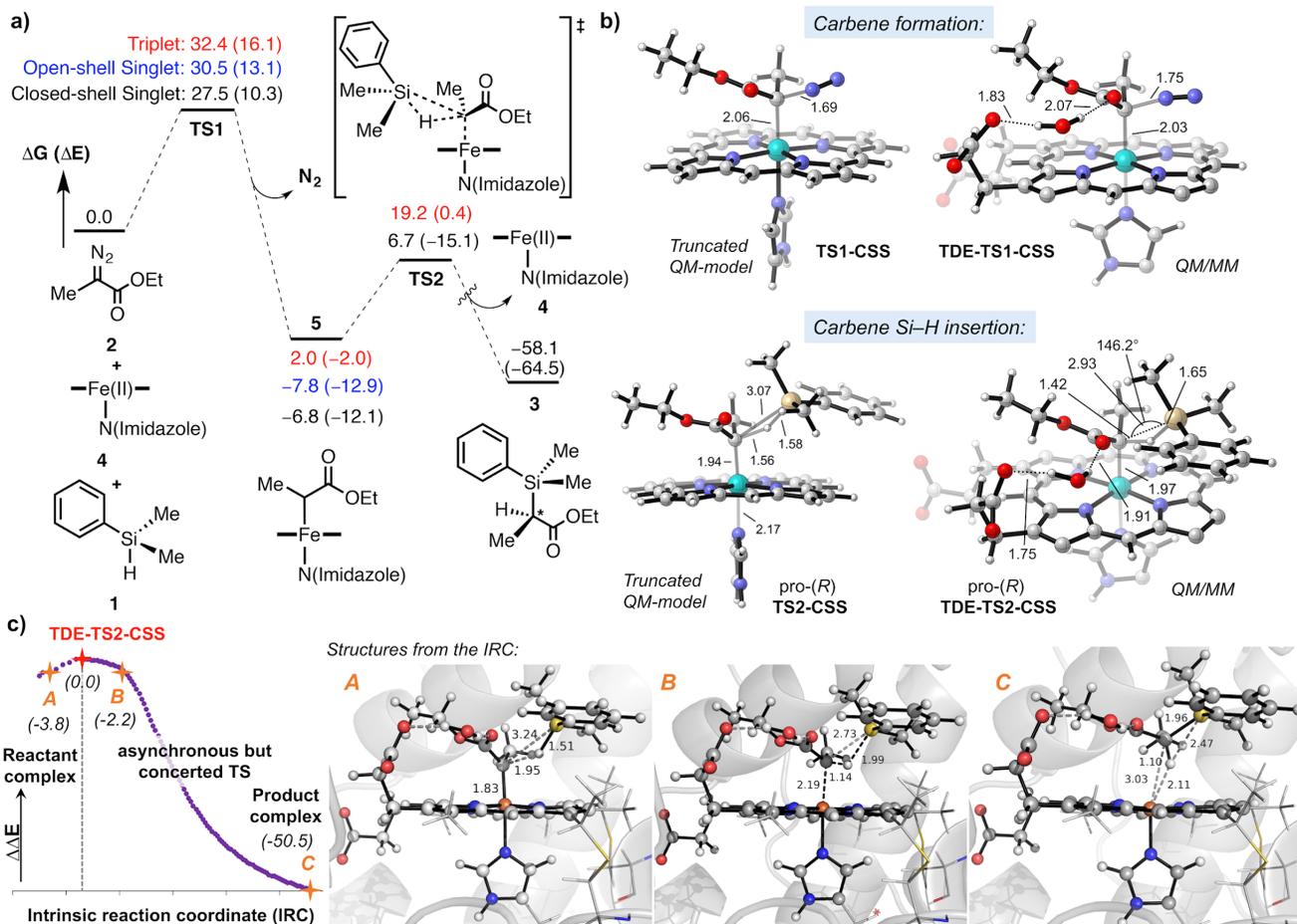


Figure 1. a) Computed reaction pathway for carbene Si-H insertion reaction between PhMe₂SiH **1** and Me-EDA **2** catalyzed by a model imidazole-ligated iron porphyrin (truncated DFT model). Results with three iron electronic states are given. The calculated intrinsic electronic and Gibbs free energies are given in kcal·mol⁻¹. Gibbs free energies do not consider the formation of substrate-substrate or enzyme-substrate reactant complexes, thus neglecting the stabilizing interactions that overcome the unfavorable -TAS term of a bimolecular reaction (usually about 12 kcal/mol in the free energies). b) Optimized geometries for the lowest in energy TS1-CSS and TS2-CSS obtained from truncated QM and QM/MM models (only atoms included in the QM region are shown for clarity). c) Intrinsic reaction coordinate (IRC) calculation for the lowest energy Si-H carbene insertion transition state (TDE-TS2-CSS) calculated using QM/MM (atoms included in the QM region are shown in ball and stick format). Relative electronic energies are given in kcal·mol⁻¹. Key distances and angles are shown in Å and degrees.

Our earlier Hammett analysis¹ showed that electron-donating substitutions on the aryl group of the silane increase the initial rate of the reaction, which indicates that the Si–H insertion step is rate-determining in *Rma* TDE-catalyzed reaction. To further interrogate the reaction mechanism and rate-determining step (RDS) experimentally, the initial-rate primary kinetic isotope effect (KIE) of the *Rma* TDE-catalyzed reaction was determined using dimethyl(phenyl)silane (PhMe₂Si-H) and dimethyl(phenyl)silane-*d* (PhMe₂Si-D). The KIE value obtained for *Rma* TDE was 2.1 ± 0.2 , demonstrating that Si–H bond cleavage is rate-determining in this particular biocatalytic system (see **Figure S1**). The experimental initial-rate KIE for *Rma* TDE is comparable to that computed for **TDE-TS2-CSS** (1.74, see **Table S1**). Similarly, a KIE value of 1.19 was recently reported for Mb(H64V,V68A)-catalyzed reaction between ethyl α -diazoacetate (EDA) and silane **1**.⁶ The larger KIE measured for the *Rma* TDE-catalyzed reaction might indicate that it corresponds to a later TS on the concerted hydride transfer and C–Si bond-forming reaction coordinate, as compared to the Mb(H64V,V68A)-catalyzed reaction. This is supported by the **TDE-TS2-CSS** QM/MM optimized geometry, where the C–Si and C–H bonds being formed (2.93 Å and 1.42 Å, respectively, **Figure 1b**) are shorter (i.e. more product-like) than those in the optimized enzyme-free QM model **TS2-CSS** structure (3.07 Å and 1.56 Å, respectively, **Figure 1b**) and the QM model TS reported by Fasan and Zhang (3.1 Å and 1.7 Å, respectively).⁶ The occurrence of a TS in *Rma* TDE in which the hydride is *more transferred* to the carbene is attributed to the presence of the water molecule that is H-bonded to the carbene carbonyl group. This hydrogen bond interaction, characteristic of the *Rma* TDE enzyme, increases the electron withdrawing character of the ester group conjugated to the carbene C atom, which in turn enhances the electrophilicity of the carbene. Consequently, the hydride transfer occurring during the asynchronous but concerted process is favored (**Figure 1c**).

In addition to the initial-rate KIE value, the end-point KIE value was also measured for *Rma* TDE and determined to be 1.34 ± 0.08 (see **Figure S1**). This is similar to the end-point KIE values reported for Rh-, Ir-, and Cu-catalyzed reactions (1.29 and 1.5 for Rh, 1.6 for Ir, 1.08 for Cu), where the C–Si bond formation step is believed to be both rate-limiting and concerted,¹⁴⁻¹⁶. Overall, our experimental results suggest that C–Si bond formation step is rate-limiting in the *Rma* TDE-catalyzed carbene Si–H insertion reaction and support our computational conclusion that the reaction follows a concerted, asynchronous mechanism.

2. Understanding how directed evolution and silane binding affect the catalytic efficiency of *Rma* TDE-catalyzed C–Si bond formation.

Mutations V75T, M100D and M103E, introduced to wild-type *Rma* *cyt c* during directed evolution, improve the total turnover number of this enzyme for C–Si bond formation.¹ Previously, we showed that these mutations played a role in 1) generating space near the catalytic iron-heme cofactor and therefore creating a new enzyme active site in *Rma* TDE and 2) facilitating the access of silane substrate **1** to the active site of carbene-bound *Rma* TDE by modulating the conformation and dynamics of a loop that covers the access to the heme pocket (“front loop” residues 98–103) (**Figure 2a-b**).⁷ We hypothesized that the protein front loop conformation and dynamics might also modulate silane binding in a catalytically competent pose and thus contribute to the rate-determining step of the reaction (**Scheme 1**).

Using umbrella sampling MD (US-MD) simulations (see SI Methods, section IIc), we compared the silane binding to carbene-bound wild-type *Rma* *cyt c* (WT IPC) and carbene-bound *Rma* TDE (TDE IPC) in a reactive conformation (**Figure S7**). In both cases, the protein front loop must change conformation to accommodate the incoming silane substrate, and the silane substrate is only loosely bound by both protein complexes. The silane substrate is highly solvent exposed and not completely surrounded by the protein residues (**Figure 2c**). Nevertheless, the silane binding affinity in the TDE IPC reactive intermediate estimated from US-MD simulations is ca. 2 kcal·mol⁻¹ more favorable than the silane-bound WT IPC. MD simulations show that the front loop is more flexible in TDE IPC than in WT IPC (**Figure 2b** and **Figure S7**), which allows the front loop to adopt conformations that better favor silane binding (**Figure S8**, residues 98–103). This increased flexibility in the front loop may be due to the hydrophilicity of the D100 and E103 side chains in TDE IPC, which are solvent exposed and are pointing outwards the active site in contrast to M100 and M103, facilitating the silane approach to the reactive carbene (see **Figures 2a-b**).

From kinetics experiments, we have determined that *Rma* TDE has a 7-fold increase in initial rate of carbene Si-H insertion relative to the wild-type protein.¹ According to transition state theory (TST), a 7-fold increase in rate represents an energy difference of ca. 1.2 kcal·mol⁻¹ between the RDS barriers for wild-type *Rma* *cyt c* and *Rma* TDE. This experimentally estimated energy difference ($\Delta\Delta G^{\ddagger}_{\text{RDS}}$) of 1.2 kcal·mol⁻¹ agrees with the computationally estimated energy difference for silane binding in a catalytically competent pose (**Figure S7**). Taken together, these results describe how

silane binding in a reactive conformation differs between *Rma* cyt *c* and *Rma* TDE, and indicate that changes in silane binding were key to improving the catalytic activity of *Rma* cyt *c* toward carbene Si–H insertions.

US-MD simulations also showed that the preferred conformation of the carbene in the enzyme active site, which we characterized in our previous work,⁷ is not altered as the silane approaches the enzyme. This observation, together with the concerted nature of the Si–H carbene insertion TS we characterized, support our previous hypothesis of how the cytochrome *c* protein controls stereoselectivity: the stereochemical outcome of C–Si bond formation is solely determined by how the silane substrate approaches the IPC intermediate in the enzyme active site (**Figures 2a** and **S7**).

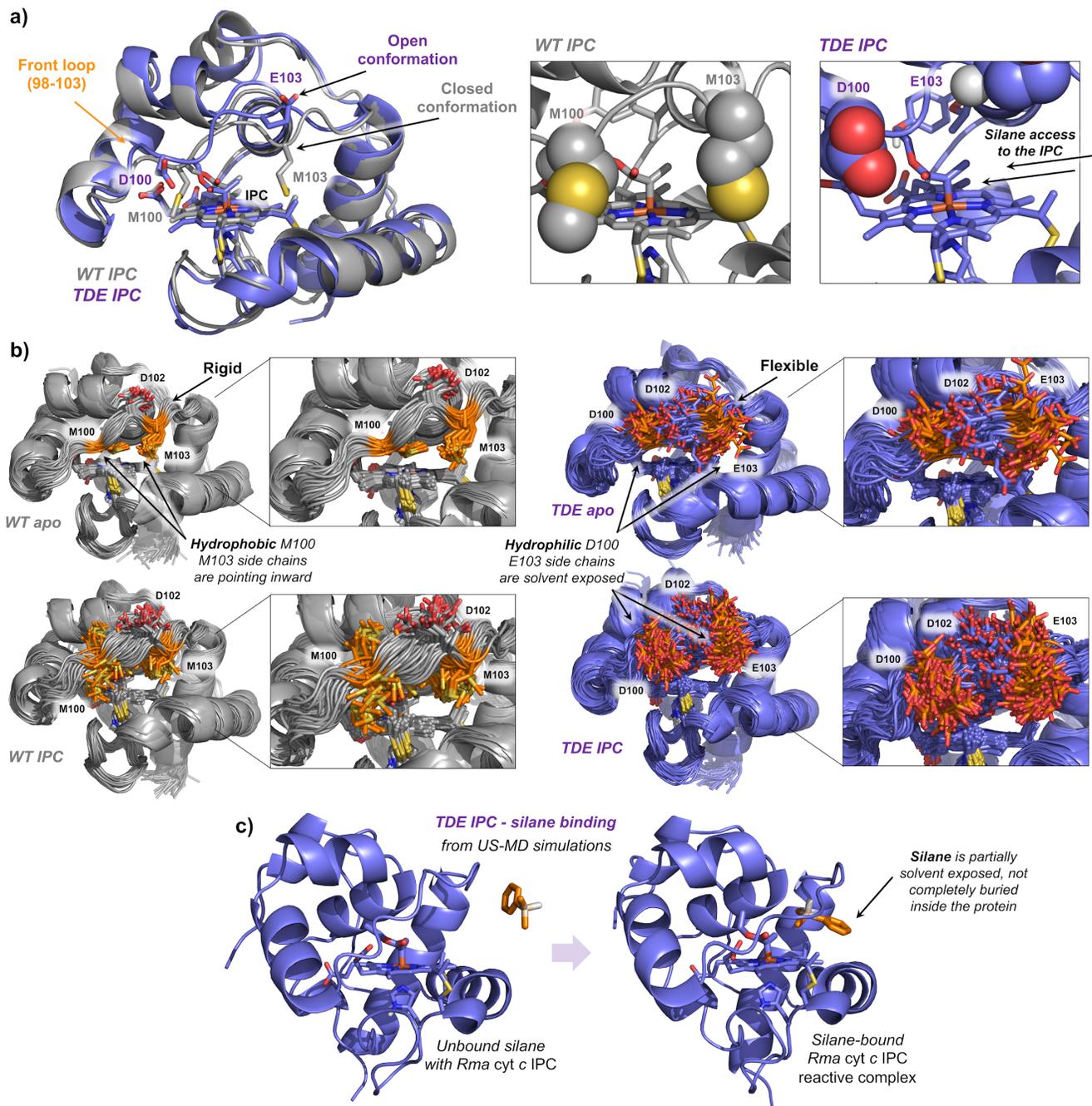


Figure 2. **a)** Representative snapshots obtained from MD simulations of IPC bound in wild-type *Rma cyt c* (gray), and the *Rma* TDE variant (purple). Front loop M100/D100 and M103/E103 residues are represented in space filling format. Hydrophobic methionines (M100 and M103) close the substrate channel in wild-type *Rma cyt c*, but mutations M100D and M103E introduce hydrophilic residues that are exposed to the solvent in *Rma* TDE along the MD simulations, allowing an easier access of the silane substrate to interact with the IPC. V75T creates new key H-bond with Y71 that induce a more hydrophobic environment for the IPC intermediate. **b)** Overlay of 50 snapshots (every 10 ns) obtained from 500 ns MD simulations for wild-type *Rma cyt c* and *Rma* TDE variants in its apo and IPC-bound states. Residues mutated in the front loop (original M100 and M103 in WT, and D100 and E103 in TDE variant) are shown in orange. Insets highlight the flexibility acquired by the front loop due to newly introduced mutations (going from “WT apo” to “TDE apo”) and the formation of the IPC (going

from “WT apo” and “TDE apo” to “WT IPC” and “TDE IPC”, respectively). Corresponding RMSF analysis are reported in **Figure S8. c**) Representative snapshots of silane binding to *Rma* TDE IPC in a catalytically relevant pose obtained from umbrella sampling MD simulations.

3. How *Rma* TDE controls chemoselectivity: C–Si vs. C–N bond formation.

In addition to catalyzing the formation of C–Si bonds, carbene transfer biocatalysts can catalyze formation of other C–X bonds (X= C, N, O, S, and B).^{3, 4, 17-25} In synthetic chemistry, metal carbenes are known to be reactive toward a broad array of functional groups, most notably unsaturated bonds (e.g. olefins and alkynes) and nucleophiles, as well as a variety of X–H bonds.²⁶⁻³² However, such reactivity also makes chemoselectivity difficult to control.^{21, 33, 34} Because chemoselective carbene transfer catalysts would be useful for chemical synthesis, we sought to elucidate the principles that govern carbene transfer chemoselectivity. Previously, we discovered that *Rma* TDE differentiates reactions at Si–H and N–H bonds with 97% selectivity for silylation, even though selection pressure for chemoselectivity was not applied during the directed evolution of this enzyme (where silane **1** was used for activity screening).¹ This preference for Si–H functionalization differs from the intrinsic reactivities of a theoretical protein-free reaction (see below), and is the opposite of what is observed in related carbene transfer competition experiments conducted with a myoglobin enzyme.²¹ Interestingly, wild-type *Rma* cyt *c* functionalizes N–H and Si–H bonds with approximately equal preference, which means that the mutations in *Rma* TDE somehow enhance chemoselectivity for Si–C bond formation.¹

The mechanisms of imidazole-ligated iron porphyrin-catalyzed carbene transfers to N–H and Si–H bonds were compared for the reaction of *p*-dimethylsilylaniline (**Figures 3a–b**). N–H functionalization can occur through a concerted N–H insertion mechanism akin to that discussed earlier for Si–H insertion (**Figure 3c**), or through nucleophilic attack by the nitrogen lone pair followed by subsequent hydrogen shift from the ylide (**Figure 3b**).^{11, 19} Using DFT calculations to model the reaction between imidazole-ligated IPC and *para*-dimethylsilylaniline **6**, the preferred amination pathway was determined to be *N*-nucleophilic attack followed by ylide rearrangement, in agreement with Shaik’s previous mechanistic proposal.¹¹ This transition state for nucleophilic amination is lower in energy than that for concerted Si–H insertion ($\Delta\Delta G^\ddagger = 3.5 \text{ kcal}\cdot\text{mol}^{-1}$, **Figures 3a–c**); thus, in the absence of the protein scaffold, amination is preferred over Si–H carbene insertion. In addition, silylation and amination have geometrically distinct transition states, as the required substrate approaches to the carbene carbon are different in these reactions (**Figures 3a–b**).

We investigated how chemoselectivity might be controlled in the enzyme active site of *Rma* cyt *c*. Using carbene-bound wild-type *Rma* cyt *c* and *Rma* TDE as starting structures, we docked *p*-dimethylsilylaniline **6** into both enzyme active sites, with conformation and orientation of **6** resembling how this substrate attacks the carbene in the DFT-optimized *N*-nucleophilic attack TS (**Figure 3b**). The docked structures then served as starting points for MD simulations, in which the N(substrate **6**) – C(carbene) distance was kept constrained during the entire simulation (see SI Methods, section IIc). These constrained MD simulations showed that in wild-type *Rma* cyt *c*, **6** preferentially explores a near attack conformation that resembles the *N*-nucleophilic attack TS, in which the measured \angle C(carbene)-N(substrate **6**)-C(substrate **6**) angle is 121.5° (**Figures 3d** and **3f**). This is in contrast to the *Rma* TDE system, where **6** prefers a binding pose that resembles the near attack conformation for the energetically disfavored DFT optimized N–H hydrogen abstraction TS ($\Delta G^\ddagger = 41.7 \text{ kcal}\cdot\text{mol}^{-1}$, **Figure 3c**) with an enlarged \angle C(carbene)-N(substrate **6**)-C(substrate **6**) angle (**Figures 3e–f**), rather than the corresponding *N*-nucleophilic attack TS. This change in the preferred near attack conformation of the substrate is due to the new conformation explored by the protein front loop in *Rma* TDE, which is induced by mutations as previously described (**Figure 3b** and **Figure 2a**). Taken together, DFT calculations and MD simulations suggest that *Rma* TDE prevents amination by disfavoring the *N*-nucleophilic attack transition state through front loop conformation and dynamics that, at the same time, favor silylation.

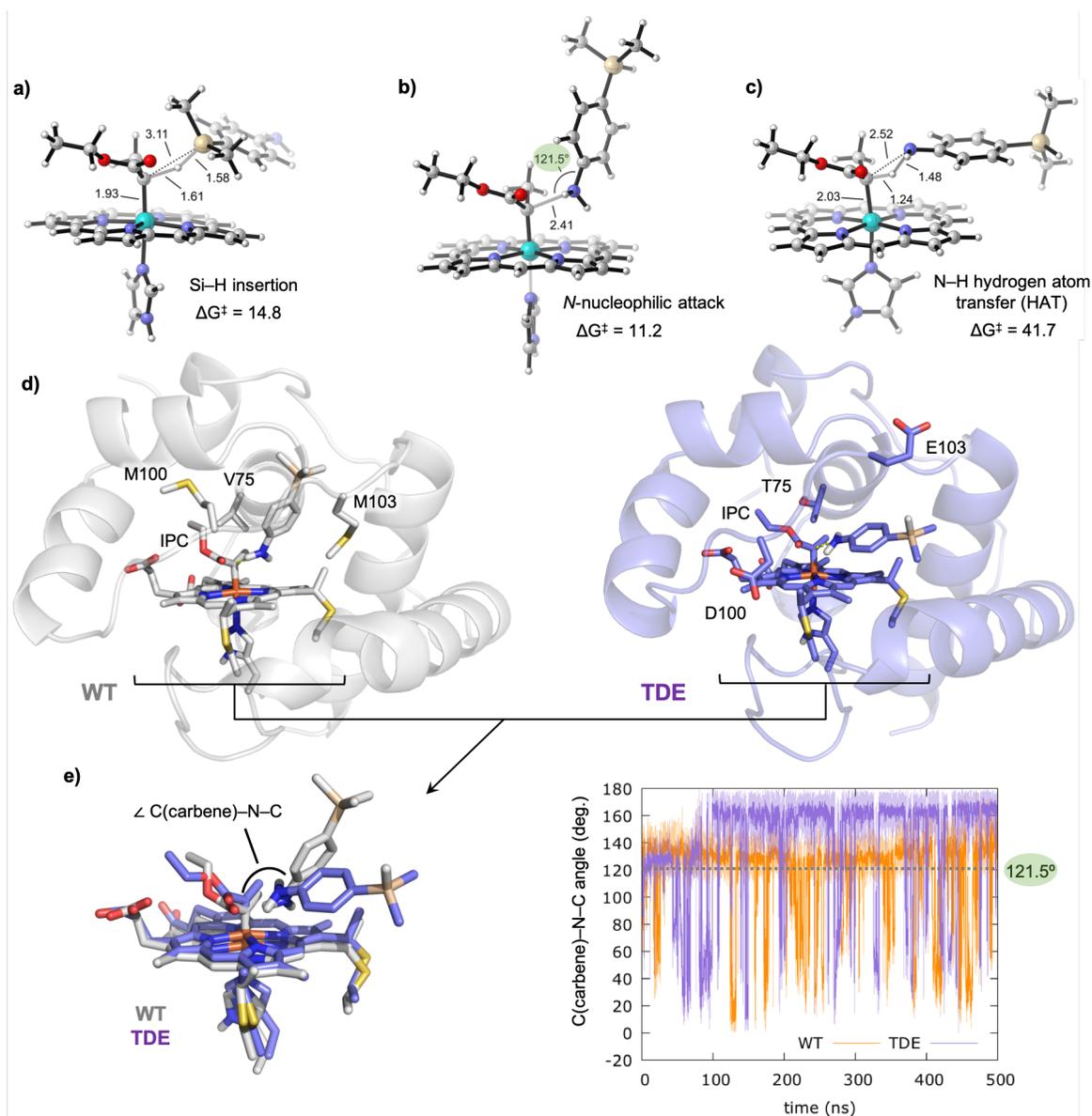


Figure 3. DFT optimized transition states using *p*-dimethylsilylaniline **6** as substrate: **a)** Si–H carbene insertion; **b)** *N*-nucleophilic attack to the electrophilic IPC carbon atom; and **c)** direct N–H carbene insertion. Gibbs free energies are estimated from lowest in energy OSS IPC 5, and are given in kcal·mol⁻¹. Key distances and angles are given in Å and degrees, respectively. Different orientations of the substrate towards the carbene are required. Analysis of the substrate binding pose and near attack conformations through constrained MD simulations (N(silane) – C(carbene) distance constrained along 500 ns MD trajectories). Representative snapshot of constrained MD simulations (constrained N – C distance to 2.5 Å, see SI Methods section IIc for details) in the **d)** wild-type and TDE IPC *Rma* enzyme active sites. **e)** C(carbene)-N(silane)-C(silane) attack angles explored along the constrained MD simulations in wild-type (orange graph) and TDE (purple graph) IPCs. The ideal C(carbene)-N(silane)-C(silane) attack angle value for an effective *N*-nucleophilic attack is 121.5°, determined from DFT optimized TS.

4. From mechanistic understanding to new protein function: switching the chemoselectivity of *Rma* TDE from silylation to amination.

The development of novel carbene transfer enzymes has largely been driven by repurposing existing heme proteins, and evolving these proteins for improved activity for a specific reaction.^{1, 3, 4, 17-21, 23, 24, 35} Fundamentally, the process of repurposing and evolving enzymes for novel reactivity is an alteration of an enzyme's selectivity, taking advantage of their intrinsic catalytic promiscuity.³⁶ Understanding the factors that determine enzyme selectivity may therefore facilitate the development of new carbene transfer biocatalysts for chemical synthesis. In order to further tailor these enzymes for new catalytic applications, we sought to engineer and characterize an enzyme variant with inverted chemoselectivity, favoring amination over silylation.

We investigated the engineering of *Rma* TDE to favor the amination product of *p*-dimethylsilylaniline (**7**) instead of the competing silylation product (**8**), which *Rma* TDE prefers. We hypothesized that this altered selectivity could be achieved by modulating the conformational dynamics of the front loop. To test this, we introduced selectivity-altering mutations to three selected positions to modify the front loop conformation, guided by MD simulations. Taking *Rma* TDE as parent protein, the amino acid residues chosen for site-saturation mutagenesis were E103, M99, and N80 (**Figure 4**). E103 (on the front loop) was selected because it was previously shown to affect the chemoselectivity.¹ Residues M99 (on the front loop) and N80 (on the D helix of *Rma* cyt c) were selected because our MD simulations suggested that these residues might play an important role in determining front loop conformation by establishing different hydrogen bonding interactions between their main and side chains (**Figure S9** and **Figure S10**).

We generated site-saturation mutagenesis libraries at each of the three sites and screened the variants for chemoselectivity, as quantified by the product ratios of carbene transfer to an amine substrate (*p*-isopropylaniline) and a silane substrate (dimethylphenylsilane, **1**) in a single competition reaction. Variants displaying improved selectivity for the amination reaction were then evaluated for their chemoselectivity on *p*-dimethylsilylaniline (**6**). The most amination-selective variants from each library were determined to be E103I, M99P and N80F, which produced the amination product as 27–34% of the total product, as compared to 3% for the parent *Rma* TDE (**Figure 4a**). When we combined these three mutations in a single protein (*Rma* TD N80F M99P E103I, abbreviated as *Rma* TDFPI), the resulting enzyme produces the amination product as 90% of the total product, suggesting that the front loop now occupies a new and distinct

conformation that selectively favors amination over silylation. *Rma* TDFPI also exhibited ca. 1.9-fold increased turnover with respect to the parent *Rma* TDE variant, mainly attributed to E103I mutation (**Figure 4b**).

A computational model for carbene-bound *Rma* TDFPI was then generated and used to perform MD simulations to understand the effects of the new mutations. Our simulations revealed a significant change in front loop conformation in *Rma* TDFPI compared to *Rma* TDE (**Figure 4b**). Notably, the M99P and E103I mutations work synergistically to disfavor the silylation transition state by changing the conformational preference of the front loop (M99P) and blocking the preferred binding pose required for the silane to approach the IPC (E103I) (**Figure 4c**). The new active site found in *Rma* TDFPI has a substrate access tunnel between the D helix and the protein front loop that guides substrate **6** to approach the carbene-bound heme from the top, a trajectory that favors a near attack, amination conformation via an *N*-nucleophilic attack transition state (**Figure 4c**). This trajectory is also favored by the N80F mutation: due to steric repulsion between the phenylalanine side chain and the protein front loop, the front loop is now distanced from the D helix where N80F resides. Finally, by docking substrate **6** into carbene-bound *Rma* TDFPI with an amination-like conformation and constraining the N(substrate **6**) – C(carbene) distance (2.5 Å), constrained MD simulations were used to analyze the catalytically competent binding poses of substrate **6** in *Rma* TDFPI. These simulations showed that the binding pose of substrate **6** is modulated by the new loop conformation and hydrophobic interactions between E103I and the aromatic ring of **6**, which stabilizes the substrate in a near attack conformation that leads to the *N*-nucleophilic attack TS (**Figure 4c** and **Figure S11**). These results demonstrate that the conformational dynamics of the front loop play a fundamental role in determining the reactivity profile of *Rma* cyt *c*-derived carbene transfer catalysts, and that these dynamics are easily altered through mutagenesis, generating enzymes with altered selectivity.

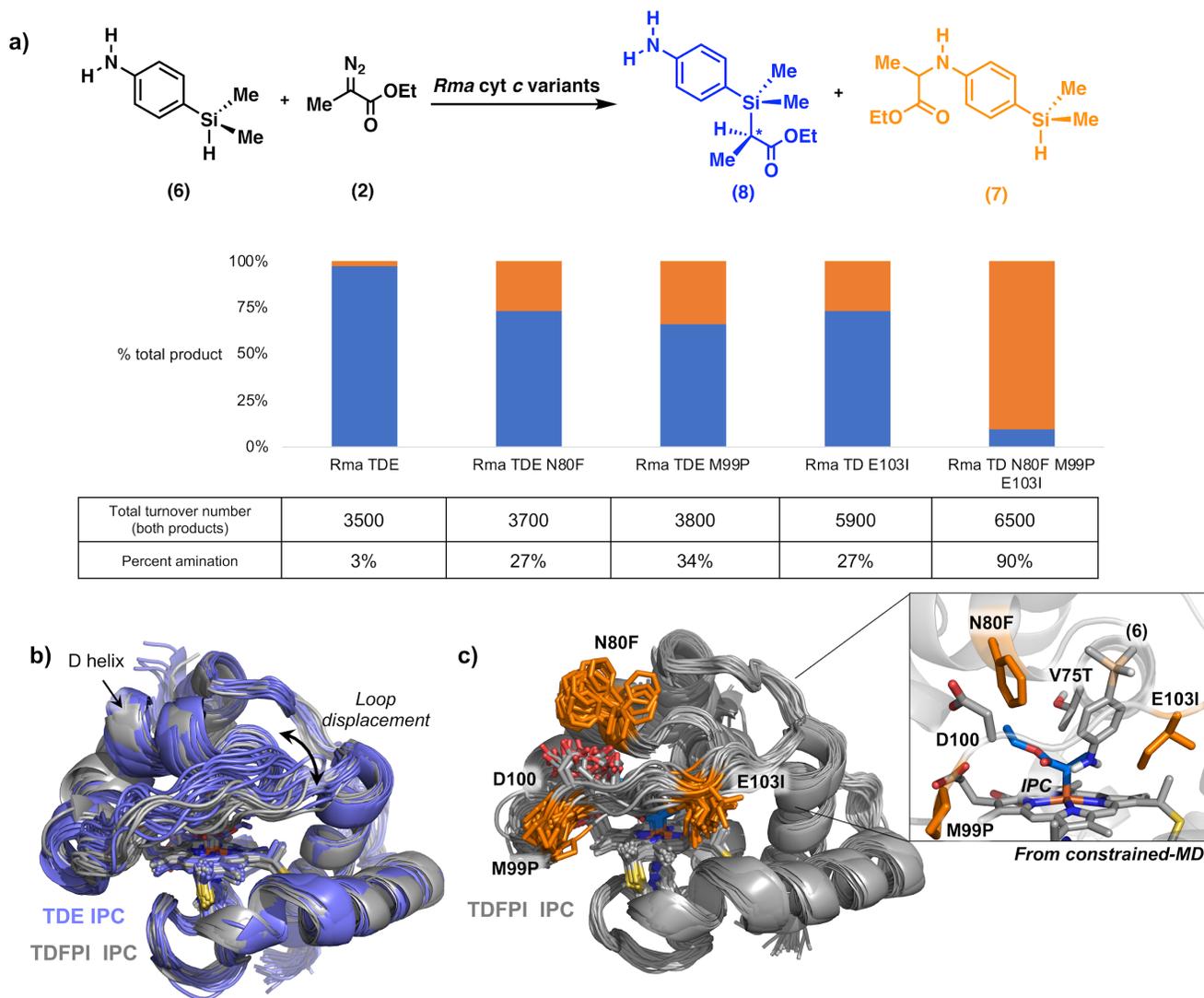


Figure 4. **a)** Site saturation mutagenesis of *Rma* TDE for chemoselective amination over Si–H carbene insertion using *p*-dimethylsilylaniline **6** and diazo compound **2**. Reactions were performed with heat-treated cell lysates with 1 μ M *Rma* cyt *c* protein, 10 mM **6**, 10 mM **2**, and 10 mM sodium dithionite in M9–N buffer. Chemoselectivity was measured as the percent of amination product over total product formed, and was determined by gas chromatography. Total turnover number (TTN) is defined as the combined concentration of **7** and **8**, over protein concentration. See SI for full description of the experimental procedures. **b)** Overlay of different snapshots obtained from 500 ns MD trajectories of *Rma* TDE (purple) and *Rma* TDFPI (gray) IPC-bound complexes. The most important structural changes correspond to the preferred “top-open” conformation adopted by the front loop (residues 98-103) in *Rma* TDFPI as compared to the parent *Rma* TDE variant. **c)** Overlay of snapshots obtained from 500 ns MD trajectory for *Rma* TDFPI IPC-bound complex. The mutations N80F, M99P, and E103I are highlighted in orange. Inset: Snapshot from constrained-MD simulation of *Rma* TDFPI IPC-bound complex with substrate **6**, showing how substrate **6** prefers to adopt a binding pose that favors amination via *N*-nucleophilic attack.

CONCLUSIONS

The catalytic versatility and selectivity observed in engineered carbene transfer enzymes has led to growing interest in developing these systems for chemical catalysis. Understanding the catalytic mechanism of these engineered enzymes and how the protein scaffold influences reaction outcomes offers fundamental insights into how non-natural enzymes function and how we might design new ones. Here, we have characterized, through computation and experiment, how laboratory-evolved *Rma* *cyt c* variants catalyze the formation of carbon–silicon bonds. We determined that *Rma* TDE catalyzes carbene insertion into Si–H bonds through a concerted, nonradical mechanism, and that Si–C bond formation corresponds to the rate-limiting step. We also found that specific H-bond interactions in the *Rma* TDE active site increase the electrophilicity of the carbene as compared to other enzymatic systems. Binding organosilane to the enzyme in a catalytically competent pose requires unfavorable displacement of the protein front loop, rendering C–Si bond formation the rate-determining step of the overall reaction. Directed evolution transformed *Rma* TDE into a faster C–Si bond-forming enzyme by making silane binding in a catalytic conformation more favorable in *Rma* TDE than in the wild-type protein; this in turn reduces the rate-determining energy barrier of carbene Si–H insertion. The mutations in *Rma* TDE also confer chemoselectivity to the enzyme by stabilizing a substrate binding pose that favors silylation over amination, through modulation of the protein front loop conformational dynamics. Finally, taking advantage of the mechanistic details uncovered in this study and guided by computational modelling, we introduced mutations that ultimately switched the enzyme chemoselectivity from silylation to amination by favoring a new conformation of the protein front loop. These results demonstrate how protein conformational dynamics and knowledge of reaction mechanism can guide the evolution of new enzymatic reactions, offering a framework for future mechanism-guided engineering of useful and novel carbene transfer enzymes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Detailed description of experimental and computational methods and procedures, spectroscopic data for all new compounds, supplementary figures and tables, and cartesian coordinates of DFT optimized structures.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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