Directed evolution of cytochrome c for carbon–silicon bond formation: Bringing silicon to life

S. B. Jennifer Kan, Russell D. Lewis, Kai Chen, Frances H. Arnold*

Enzymes that catalyze carbon–silicon bond formation are unknown in nature, despite the natural abundance of both elements. Such enzymes would expand the catalytic repertoire of biology, enabling living systems to access chemical space previously only open to synthetic chemistry. We have discovered that heme proteins catalyze the formation of organosilicon compounds under physiological conditions via carbene insertion into silicon-hydrogen bonds. The reaction proceeds both in vitro and in vivo, accommodating a broad range of substrates with high chemico- and enantioselectivity. Using directed evolution, we enhanced the catalytic function of cytochrome c from *Rhodothermus marinus* to achieve more than 15-fold higher turnover than state-of-the-art synthetic catalysts. This carbon-silicon bond-forming biocatalyst offers an environmentally friendly and highly efficient route to producing enantiopure organosilicon molecules.

**BIOCATALYSIS**

Silicon constitutes almost 30% of the mass of Earth’s crust, yet no life form is known to have the ability to forge carbon–silicon bonds (1). Despite the absence of organosilicon compounds in the biological world, synthetic chemistry has enabled us to appreciate the distinctive and desirable properties that have led to their broad applications in chemistry and material science (2, 3). As a biocompatible carbon isostere, silicon can also be used to optimize and repurpose the pharmaceutical properties of bioactive molecules (4, 5).

The natural supply of silicon may be abundant, but sustainable methods for synthesizing organosilicon compounds are not (6–9). Carbon-silicon bond-forming methods that introduce silicon motifs to organic molecules enantioselectively rely on multistep synthetic campaigns to prepare and optimize chiral reagents or catalysts; precious metals are also sometimes needed to achieve the desired activity (9–19). Synthetic methodologies such as carbene insertion into silanes can be rendered enantioselective using chiral transition metal complexes based on rhodium (11, 12), iridium (13), and copper (14, 15).

These catalysts can provide optically pure products, but not without limitations: They require halogenated solvents and sometimes low temperatures to function optimally and have limited turnovers (<100) (16).

Because of their ability to accelerate chemical transformations with exquisite specificity and selectivity, enzymes are increasingly sought-after complements to, or even replacements for, chemical synthesis methods (17, 18). Biocatalysts that are fully genetically encoded and assembled inside of cells are readily tunable with molecular biology techniques. They can be produced at low cost from renewable resources in microbial systems and perform catalysis under mild conditions. Although nature does not use enzymes to form carbon–silicon bonds, the protein machineries of living systems are often “promiscuous”—that is, capable of catalyzing reactions distinct from their biological functions. Evolution, natural or in the laboratory, can use these promiscuous functions to generate catalytic novelty (19–21). For example, heme proteins can catalyze a variety of non-natural carbene-transfer reactions in aqueous media, including N–H and S–H insertions, which can be greatly enhanced and made exquisitely selective by directed evolution (22–24).

We hypothesized that heme proteins might also catalyze carbene insertion into silicon-hydrogen bonds. Because iron is not known to catalyze this transformation (25), we first examined whether free heme could function as a catalyst in aqueous media. Initial experiments showed that the reaction between phenylidyrmethylsilane and ethyl 2-diazopropanoate (MeEDA) in neutral buffer (pH 7.4) at room temperature gave racemic organosilicon product 3 at very low levels, a total turnover number (TTN) of 4 (Fig. 1A). No product formation was observed in the absence of heme, and the organosilicon product was stable under the reaction conditions.

We next investigated whether heme proteins could catalyze the same carbon–silicon bond-forming reaction. Screening a panel of cytochrome P450 and myoglobin variants, we observed product formation with more turnovers compared to the hemin and hemin with bovine serum albumin (BSA) controls, but with negligible enantioinduction (table S4). Cytochrome c from *Rhodothermus marinus* (*Rma* cyt c), a Gram-negative, thermohalophilic bacterium from submarine hot springs in Iceland (26), catalyzed the reaction with 97% enantiomeric excess (ee), indicating that the reaction took place in an environment where the protein exerted excellent stereocatalysis. Bacterial cytochromes c are well-studied, functionally conserved electron-transfer proteins that are not known to have any catalytic function in living systems (27). Other bacterial and eukaryotic cytochrome c proteins also catalyzed the reaction, but with lower selectivities. We thus chose *Rma* cyt c as the platform for evolving a carbon–silicon bond-forming enzyme.

The crystal structure of wild-type *Rma* cyt c (Protein Data Bank (PDB) ID: 3CP5; (26)) reveals that the heme prosthetic group resides in a hydrophobic pocket, with the iron axially coordinated to a proximal His (H49) and a distal Met (M100), the latter of which is located on a loop (Fig. 1, B and C). The distal Met, common in cytochrome c proteins, is coordinatively labile (28, 29). We hypothesized that M100 must be displaced upon iron-carbeneoid formation, and that mutation of this amino acid could facilitate formation of this adventitious “active site” and yield an improved carbon–silicon bond-forming biocatalyst. Therefore, a variant library made by site-saturation mutagenesis of *M100* was cloned and recombinitely expressed in *Escherichia coli*. After protein expression, the bacterial cells were heat-treated (75°C for 10 min) before screening in the presence of phenylidyrmethylsilane (10 mM),

References (28–33)

6 July 2016; accepted 24 October 2016

10.1126/science.aaf5067

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA.

*Corresponding author. Email: frances@cheme.caltech.edu
Me-EDA (10 mM), and sodium dithionite (Na2S2O4 10 mM) as a reducing agent, at room temperature under anaerobic conditions. The M100D mutation stood out as highly activating: This first-generation mutant provided chiral organosilicon compounds other than Me-EDA could be used for carbon–silicon bond formation (Fig. 1D).

Amino acid residues V75 and M103 reside close (within 7Å) to the iron heme center in wild-type Rma cyt c. Sequential site-saturation mutagenesis at these positions in the M100D mutant led to the discovery of triple-mutant V75T M100D M103E, which catalyzed carbon–silicon bond formation in >1500 turnovers and >99% ee. This level of activity is more than 15 times the total turnovers reported for the best synthetic catalysts for this class of reaction (16). As standalone mutations, both V75T and M103E are activating for wild-type Rma cyt c, and the beneficial effects increase with each combination (table S5). Comparison of the initial reaction rates established that each round of evolution enhanced the rate: Relative to the wild-type protein, the evolved triple mutant catalyzes the reaction more than seven times faster, with turnover frequency (TOF) of 46 min⁻¹ (Fig. 1E).

Assaying the new enzyme against a panel of silicon and diazo reagents, we found that the mutations were broadly activating for enantioselective carbon–silicon bond formation. The reaction substrate scope was surveyed with the use of heat-treated lysates of E. coli-expressing Rma cyt c V75T M100D M103E under saturating conditions for both silane and diazo ester to determine TTN. Whereas many natural enzymes excel at catalyzing reactions on only their native substrates and little else (especially primary metabolic enzymes), the triple mutant catalyzed the formation of 20 silicon-containing products, most of which were obtained cleanly as single enantiomers, demonstrating the broad substrate scope of this reaction with just a single variant of the enzyme (Fig. 2). The reaction accepts both electron-rich and electron-deficient silicon reagents, accommodating a variety of functional groups including ethers, aryl halides, alkyl halides, esters, and amides (5 to 10). Silicon reagents based on naphthalenes or heteroarenes (11 to 13), as well as vinyldialkyl- and trialkylsilanes, could also serve as silicon donors (14, 15, 18). In addition, diazo compounds other than Me-EDA could be used for carbon–silicon bond formation (16, 17) (16).

The evolved Rma cyt c exhibits high specificity for carbon–silicon bond formation. Even in the presence of functional groups that could compete in carbene-transfer reactions, enzymatic carbon–silicon bond formation proceeded with excellent chemoselectivity. For example, styrenyl olefins, electron-rich double bonds, and terminal
**Fig. 2. Scope of Rma cyt c V75T M100D M103E-catalyzed carbon–silicon bond formation.** Standard reaction conditions: lysate of *E. coli* expressing Rma cyt c V75T M100D M103E (OD$_{600}$ = 1.5; heat-treated at 75°C for 10 min), 20 mM silane, 10 mM diazo ester, 10 mM Na$_2$S$_2$O$_4$, 5 vol % MeCN, M9-N buffer (pH 7.4) at room temperature under anaerobic conditions. Reactions performed in triplicate. [a] OD$_{600}$ = 5 lysate. [b] OD$_{600}$ = 0.5 lysate. [c] OD$_{600}$ = 0.5 lysate. [d] 10 mM silane. [e] OD$_{600}$ = 0.15 lysate.

**Fig. 3. Chemoselectivity and in vivo activity of evolved Rma cyt c.** (A) Chemoselectivity for carbene Si–H insertion over N–H insertion increased markedly during directed evolution of Rma cyt c. Standard reaction conditions as described in Fig. 2. Reactions were performed in duplicate using heat-treated lysates of *E. coli* expressing Rma cyt c with protein concentration normalized across variants. Product distribution was quantified after 2 hours of reaction time (before complete conversion, no double insertion product was observed under these conditions). (B) In vivo synthesis of organosilicon compound 22.
alkynes that are prime reaction handles for synthetic derivatization are preserved under the reaction conditions, with no competing cyclopropanation or cyclopropane opening activity observed. As a result, organosilicon products 12 and 13 and 18 to 20 were afforded with 210 to 5010 turnovers and excellent stereoselectivities (98 to >99% ee). Preferential carbon–silicon bond formation could also be achieved with substrates bearing free alcohols and primary amines, yielding silicon-containing phenol 21 (910 TTN, >99% ee) and aniline 22 (8210 TTN, >99% ee). This capability removes the need for functional-group protection and/or manipulation, offering a streamlined alternative to transition-metal catalysis for incorporating silicon into small molecules. Indeed, when the same reactants were subjected to rhodium catalysis [1 mol % Rh(2)(OAc)(4)], the same reactants were subjected to rhodium catalysis [1 mol % (Rh(2)(OAc)(4)), O–H and N–H insertions were the predominant reaction pathways, and copper catalysis [10 mol % Cu(OTf)(2)] gave complex mixtures of products (table S7). Tolerance of these highly versatile functionalities in enzymatic carbon–silicon bond-forming reactions provides opportunities for their downstream processing through metabolic engineering, bioorthogonal chemistry, and other synthetic endeavors.

We next asked whether all Rma cyt c variants would catalyze carbon–silicon bond formation selectively over insertion of the carbene into an N–H bond in the same substrate. We re-examined the evolutionary lineage and tested all four generations of Rma cyt c (wild-type, M100D, V75T M100D, and V75T M100D M103E) with Me-EDA and 4-(dimethylsilyl)aniline (M100D, V75T M100D, and V75T M100D M103E) in glucose-supplemented M9-N buffer were given silane 23 (0.1 mmol) and Me-EDA (0.12 mmol) as neat reagents. The enzyme in this whole-cell system catalyzed carbon–silicon bond formation with 3410 turnovers, yielding organosilicon product 22 in 70% isolated yield (>95% yield based on recovered silane 23) and 98% ee (Fig. 3B). These in vitro and in vivo examples of carbon–silicon bond formation using an enzyme and Earth-abundant iron affirm the notion that nature's protein repertoire is highly evolvable and poised for adaptation: With only a few mutations, existing proteins can be repurposed to efficiently forge chemical bonds not found in biology and grant access to areas of chemical space that living systems have not explored.
Directed evolution of cytochrome c for carbon–silicon bond formation: Bringing silicon to life

S. B. Jennifer Kan, Russell D. Lewis, Kai Chen and Frances H. Arnold

Science 354 (6315), 1048-1051.
DOI: 10.1126/science.aah6219

Bringing carbon-silicon bonds to life

Organic compounds containing silicon are important for a number of applications, from polymers to semiconductors. The catalysts used for creating carbon-silicon bonds, however, often require expensive trace metals or have limited lifetimes. Borrowing from the ability of some metallo-enzymes to catalyze other rare carbene insertion reactions, Kan et al. used heme proteins to form carbon-silicon bonds across a range of conditions and substrates (see the Perspective by Klare and Oestreich). Directed evolution experiments using cytochrome c from Rhodothermus marinus improved the reaction to be 15 times more efficient than industrial catalysts. Science, this issue p. 1048; see also p. 970