

Iron Heme Enzyme-Catalyzed Cyclopropanations with Diazirines as Carbene Precursors: Computational Explorations of Diazirine Activation and Cyclopropanation Mechanism

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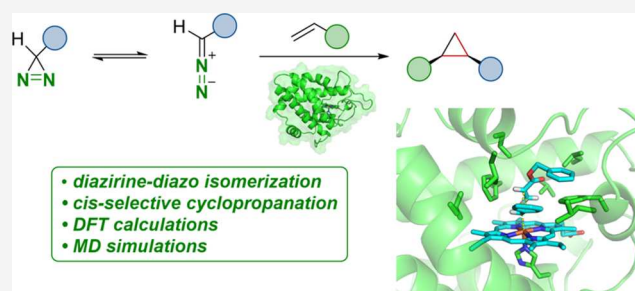


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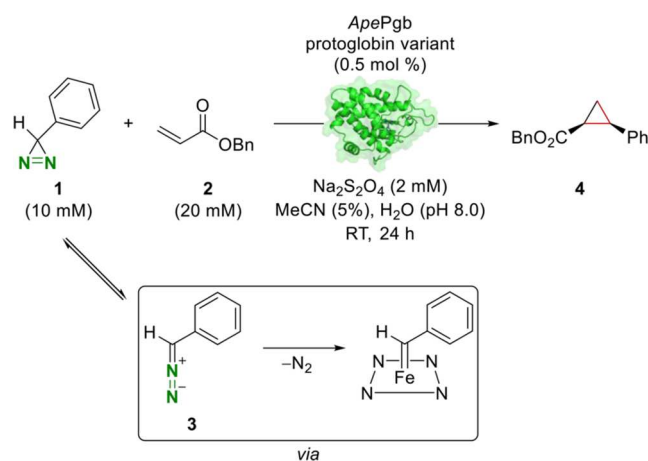
ABSTRACT: The mechanism of cyclopropanations with diazirines as air-stable and user-friendly alternatives to commonly employed diazo compounds within iron heme enzyme-catalyzed carbene transfer reactions has been studied by means of density functional theory (DFT) calculations of model systems, quantum mechanics/molecular mechanics (QM/MM) calculations, and molecular dynamics (MD) simulations of the iron carbene and the cyclopropanation transition state in the enzyme active site. The reaction is initiated by a direct diazirine-diazo isomerization occurring in the active site of the enzyme. In contrast, an isomerization mechanism proceeding via the formation of a free carbene intermediate *in lieu* of a direct, one-step isomerization process was observed for model systems. Subsequent reaction with benzyl acrylate takes place through stepwise C–C bond formation via a diradical intermediate, delivering the cyclopropane product. The origin of the observed diastereo- and enantioselectivity in the enzyme was investigated through MD simulations, which indicate a preferred formation of the *cis*-cyclopropane by steric control.



INTRODUCTION

During the last 2 decades, biocatalysis has attracted expanding attention in academia as well as in the chemical and pharmaceutical industries.¹ In particular, the widespread use of directed evolution has enabled the development of highly efficient and selective enzyme catalysts for a variety of new-to-nature transformations, with important applications in, *inter alia*, molecular synthesis and drug discovery.² Bioengineered iron heme enzymes have been shown to facilitate a range of useful non-natural carbene and nitrene transfer reactions.³ Cyclopropanations and X–H (X = C, N, Si, S, etc.) insertions via iron carbene intermediates were accomplished with iron heme enzyme catalysis, delivering the desired products with outstanding efficiencies and excellent levels of enantioselectivity.⁴ However, these transformations almost exclusively relied on the use of diazo compounds as carbene precursors, which require the presence of stabilizing electron-withdrawing substituents to avoid potentially hazardous thermal decomposition processes.⁵ Recently, bench-stable but challenging-to-activate diazirines have been employed as alternative carbene sources in a variety of carbene transfer reactions, including *cis*-selective cyclopropanations with acrylates (Scheme 1).⁶ While experimental mechanistic investigations via trapping experiments provided evidence for a diazirine-diazo isomerization taking place in the presence of the enzyme catalyst and facilitated by the iron heme complex prior to the formation of

Scheme 1. Diazirines for *cis*-Selective Cyclopropanation with Enzyme Catalysis⁶

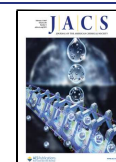


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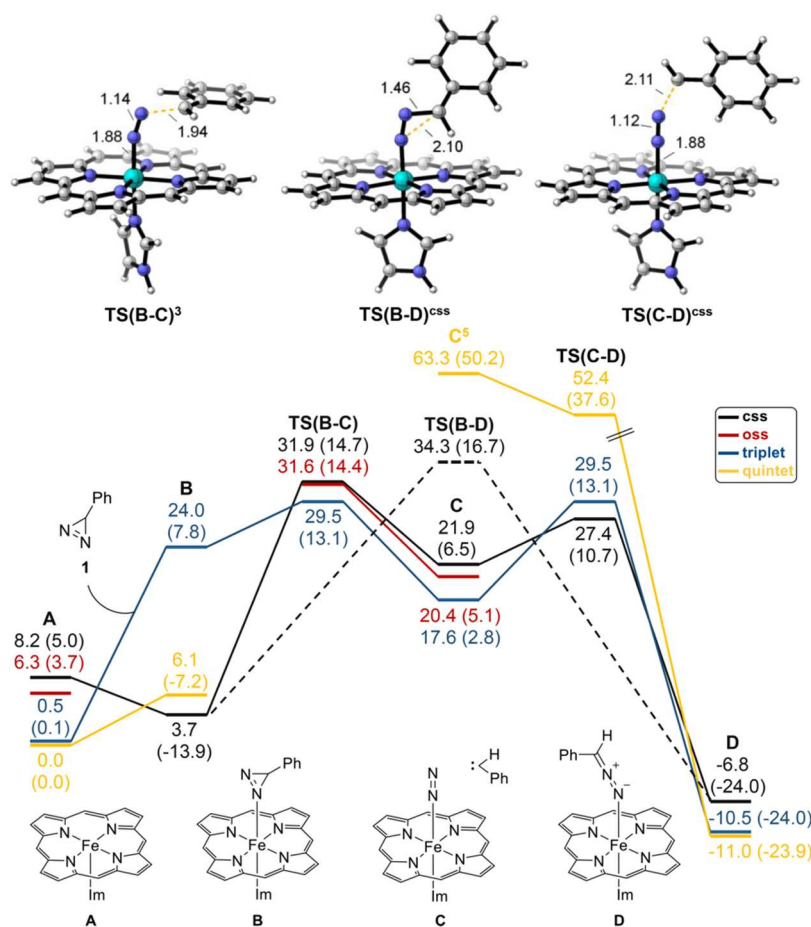


Figure 1. Calculated Gibbs free energy diagram (in kcal mol⁻¹) for the isomerization of diazirine **1** to diazo species **3**. Values in parentheses correspond to relative enthalpies (in kcal mol⁻¹). Distances are given in Å. Superscripts correspond to the spin state. css = closed-shell singlet; oss = open-shell singlet.

the key iron carbene intermediate, a detailed understanding of the complete reaction mechanism is thus far lacking.

We became interested in delineating the mechanism of the diazirine-diazo isomerization as well as the subsequent cyclopropanation reaction by means of density functional theory (DFT) and quantum mechanics/molecular mechanics (QM/MM) calculations. Furthermore, molecular dynamics (MD) simulations in the active site of the enzyme were performed to explore the key iron carbene intermediate as well as the enantio- and diastereoselectivity-controlling factors.

RESULTS AND DISCUSSION

We initiated our investigation by studying the formation of phenyldiazomethane (**3**) via isomerization of 3-phenyl-3H-diazirine (**1**) at the B3LYP-D3(BJ)/def2-TZVP+CCP-M(Et₂O)/B3LYP-D3(BJ)/6-31G(d),LANL2DZ(Fe) level of theory.⁷ In the calculations, an iron(II) porphyrin complex bearing an imidazole ligand in the axial position was employed as a model for the histidine-coordinated iron(II) heme active site in the enzyme, which is formed in the presence of Na₂S₂O₄ as reductant.^{8,9} The implicit CPCM solvation model with Et₂O as the solvent was employed to simulate the hydrophobic environment found inside the active site of the enzyme.^{9a,d,e,h} In our calculations, a quintet ground state¹⁰ was found for the intermediate **A**, which is in line with previously reported experimental observations.¹¹ Starting from the resting state intermediate **A**, coordination of diazirine **1** via one of the

nitrogen lone pairs results in the formation of intermediate **B** in a closed-shell singlet (css) spin state via spin-crossover (Figure 1). The subsequent ring-opening of the diazirine motif via transition state **TS(B-D)** has an activation free energy of 34.3 kcal mol⁻¹ on the closed-shell singlet surface to lead to intermediate **D**. The isomerization via **TS(B-D)** takes place on the css surface and does not represent a stable stationary point in other spin states. However, further investigations revealed an alternative, energetically favorable two-step isomerization process, likely involving a spin-crossover from the closed-shell singlet to the triplet spin state. In the first step, carbene intermediate **C** is formed via **TS(B-C)** with a Gibbs free energy of activation of 29.5 kcal mol⁻¹ on the triplet surface and an enthalpic energy barrier of 27.0 kcal mol⁻¹, thus representing the turnover-limiting step. Typically, decreased entropies and enthalpies of activation are observed in the active site of the enzyme, thus resulting in lowered Gibbs free energies of activation.¹² Furthermore, a number of residues surrounding the active site, such as F73, R90, and F93, offer the potential for stabilizing stacking interactions with the phenyl substituent of diazirine **1**, thus possibly leading to a further decrease in the Gibbs free energy of activation and allowing for the reaction to occur at room temperature (Figure S10 in the SI). In **C**, the free carbene remains in relatively close proximity to the coordinated dinitrogen ($d_{N_2-C} = 3.2$ Å, Figure S4 in the SI) and does not dissociate, thus subsequently allowing for a fast and energetically feasible C–N bond

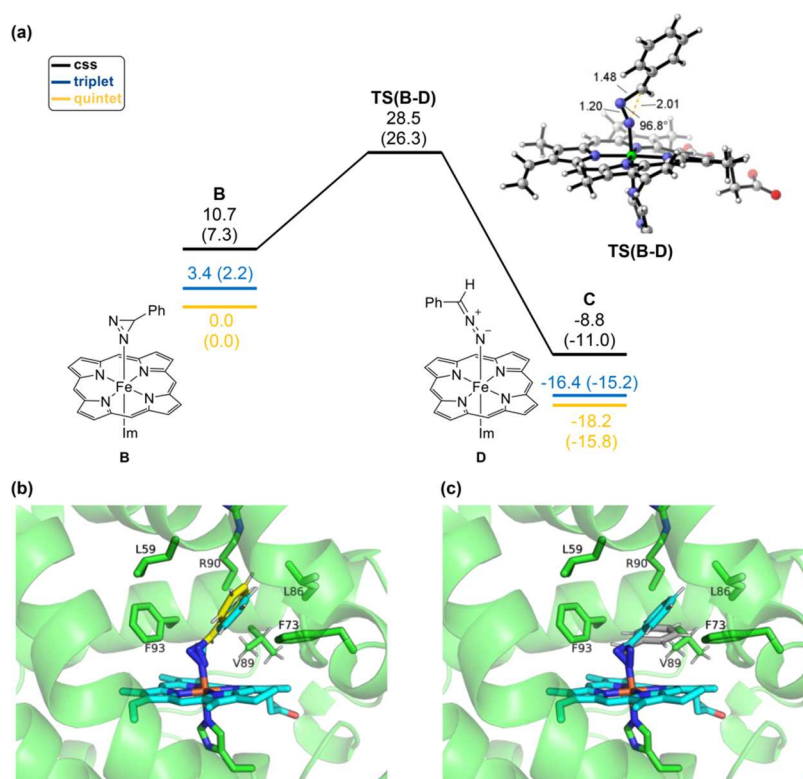


Figure 2. QM/MM calculation of the isomerization step. (a) Calculated Gibbs free energy diagram (in kcal mol⁻¹). Values in parentheses correspond to relative enthalpies (in kcal mol⁻¹). Distances are given in Å, and peptide residues are omitted for clarity; (b) aligned structure of TS(B-D) (yellow) to B¹ (cyan); (c) aligned structure of DFT-optimized B¹ (gray) to B¹ (cyan). Nonrelevant hydrogens are omitted for clarity in panels (b) and (c).

formation via TS(C-D), resulting in the formation of diazo-coordinated complex D and reasonably outcompeting a potential dissociation of phenylcarbene. Presumably, the binding of the diazine to form complex B in the enzyme is thermally favorable since the higher Gibbs free energy of B with respect to A can be attributed to an unfavorable entropy (8–12 kcal mol⁻¹ $-T\Delta S$ of a bimolecular reaction). This assumption is further substantiated by the significantly lower relative enthalpies of -13.9 and -7.2 kcal mol⁻¹ relative to A⁵ on the css and quintet spin surface, respectively, thereby highlighting the unfavorable entropy for coordination of diazine. Furthermore, the substoichiometric concentration of the reductant Na₂S₂O₄ renders an involvement of Na₂S₂O₄ or a mechanism proceeding via an iron(III) species unlikely for the catalytic diazine activation. The observation of a two-step mechanism being operative *in lieu* of a direct diazine-diazo isomerization pathway is in good agreement with previously reported studies on thermal, metal-free diazine isomerizations.¹³

We also investigated the isomerization process using the QM/MM method, where the whole enzyme is modeled (Figure 2a). In our calculation, substrate, heme, and coordinated histidine are described using DFT, and all of the rest are treated by a MM force field (see the SI for details). Our QM/MM calculations reveal that only the direct isomerization transition state TS(B-D) can be located in the enzyme active site, which means the two-step transition state TS(B-C) and the formation of the corresponding carbene intermediate is disfavored by the enzyme environment (Figures S7 and S8). The barrier from B¹ to TS(B-D) is also lowered dramatically, from 30.6 to 17.8 kcal mol⁻¹. In the

structures, we observed C–H/ π and π – π interactions between the diazine and surrounding nonpolar side chains (Figure S7). Our computations also reveal that the enzyme preorganizes the conformation of B¹, causing it to closely resemble the transition state TS(B-D) (Figure 2b). By aligning the DFT-optimized intermediate B¹ in the enzyme site, we found a strong steric repulsion between it and nearby α helix residues (V89) (Figure 2c). This reveals the enzyme's role in controlling the substrate's binding conformation through the evolution of a structurally rigid scaffold. We believe such substrate prearrangement plays an important role in the catalysis of the enzyme, resulting in the lowering of the barrier and inhibition of formation of the free carbene intermediate.

The mechanism of *cis*-selective cyclopropanation was investigated next (Figure 3). Initially, intermediate D undergoes a shift in coordination from the terminal nitrogen to the C–N double bond, resulting in the formation of the more stable complex E (Figure S3 in the SI). Subsequently, the key carbene intermediate F is generated by facile C–N bond cleavage via TS(E-F) with an activation free energy of 21.9 kcal mol⁻¹. The reaction of F with benzyl acrylate (2) takes place in a concerted fashion (TS(F-P)) in a closed-shell singlet spin state, generating the *cis*-configured cyclopropane P and regenerating initial complex A in a single step. In contrast, on the open-shell singlet and triplet surfaces, a facile, stepwise process involving the formation of diradical intermediate G was observed. Intermediate G is generated by C–C bond formation between the carbene carbon C1 and the terminal alkene carbon C_{term} in TS(F-G), which is preferred over the concerted cyclopropanation by 2.7 kcal mol⁻¹. The observation of a preferred stepwise pathway is further in line with

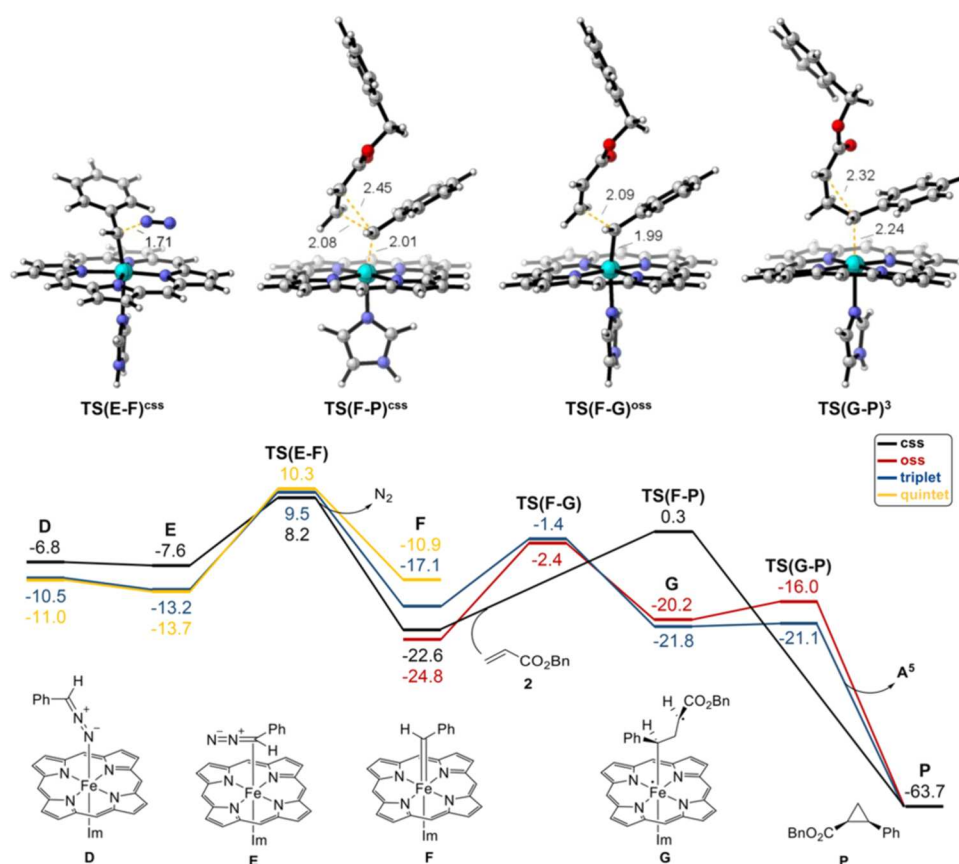


Figure 3. Calculated Gibbs free energy diagram (relative to A^5 , in kcal mol^{-1}) for the cyclopropanation with acrylate **2**. Distances are given in Å. Superscripts correspond to the spin state. css = closed-shell singlet; oss = open-shell singlet.

previously reported studies on related enzyme-catalyzed reactions.^{9b,14} An alternative C–C bond formation taking place with the internal alkene carbon was found to be unfeasible due to significant steric interactions between the ester substituent and the porphyrin ring. Subsequently, the cyclopropane is formed by simultaneous C–C bond formation and Fe–C bond cleavage in TS(G–P) with a very low energy barrier of only $0.7 \text{ kcal mol}^{-1}$.

In comparison, the formation of the thermodynamically more stable *trans*-configured cyclopropane **P'** occurs via transition state TS(F–G') with a $1.9 \text{ kcal mol}^{-1}$ lower activation free energy than TS(F–G) (Figure S2 in the SI). Despite the energetic preference for the generation of the *trans*-cyclopropane, the difference in activation free energy is relatively small, thus enabling the enzyme to preferentially catalyze the formation of the *cis*-configured product by destabilizing one transition state and stabilizing the other.

With the results from the DFT calculations in hand, we turned to molecular dynamics (MD) simulations to gain insights into the structure and dynamics of the active site of the best-performing protoglobins enzyme variant, ApePgb GLAVRSQL.¹⁵ Employing a monomer of the functional homodimeric enzyme, MD simulations on the carbene intermediate revealed that the side chain of the R63 residue located on a flexible open-loop motif preferentially orients away from the active site and toward the solvent (Figure 4). The preferred orientation of R63 toward the solvent is fully consistent with previously reported cryogenic electron microscopy (cryo-EM) structures of the strongly related enzyme variant ApePgb GLVRSQL, which lacks the additional

V60A, G61V, and F175L mutations present in the fully optimized variant.^{6,16} During the directed evolution campaign, the introduction of the V63R, V60A, and G61V mutations into the parent ApePgb enzyme resulted in a considerable increase in cyclopropanation activity, which was attributed to the disruption of a rigid helix region, forming the above-mentioned open loop and thus allowing for easier access to the active site. In addition, a I149L mutation lead to an approximately 2-fold increase in activity, which can be reasonably explained by the further reduced steric congestion in the active site. In contrast, other beneficial mutations, that is, C45G and C102S, are located at distal positions with respect to the active site, while L175 is located directly at the dimer interface and likely influences the activity by modulating the stability of the functional homodimeric enzyme (Figure S9). Furthermore, due to the small size of the carbene, a significant rotation around the Fe–C1 bond was observed in the MD simulations, with the N1–Fe–C1–C2 dihedral fluctuating between 0° and 180° and preferentially adopting a dihedral angle of approximately 75° and 145° . At a dihedral angle of $\sim 0^\circ$, a short distance between the phenyl substituent and the F93 side chain was observed, suggesting the presence of stabilizing π – π stacking interactions, which were previously observed in cryo-EM measurements of a strongly related carbene intermediate.¹⁶ In contrast, at dihedral angles of 75° and 145° , destabilizing steric interactions between the phenyl substituent and the surrounding hydrophobic residues, in particular, the F73 side chain, are minimized. However, due to the observed large fluctuations and in contrast to the cryo-EM structure, no single preferred conformation is adopted by the carbene,

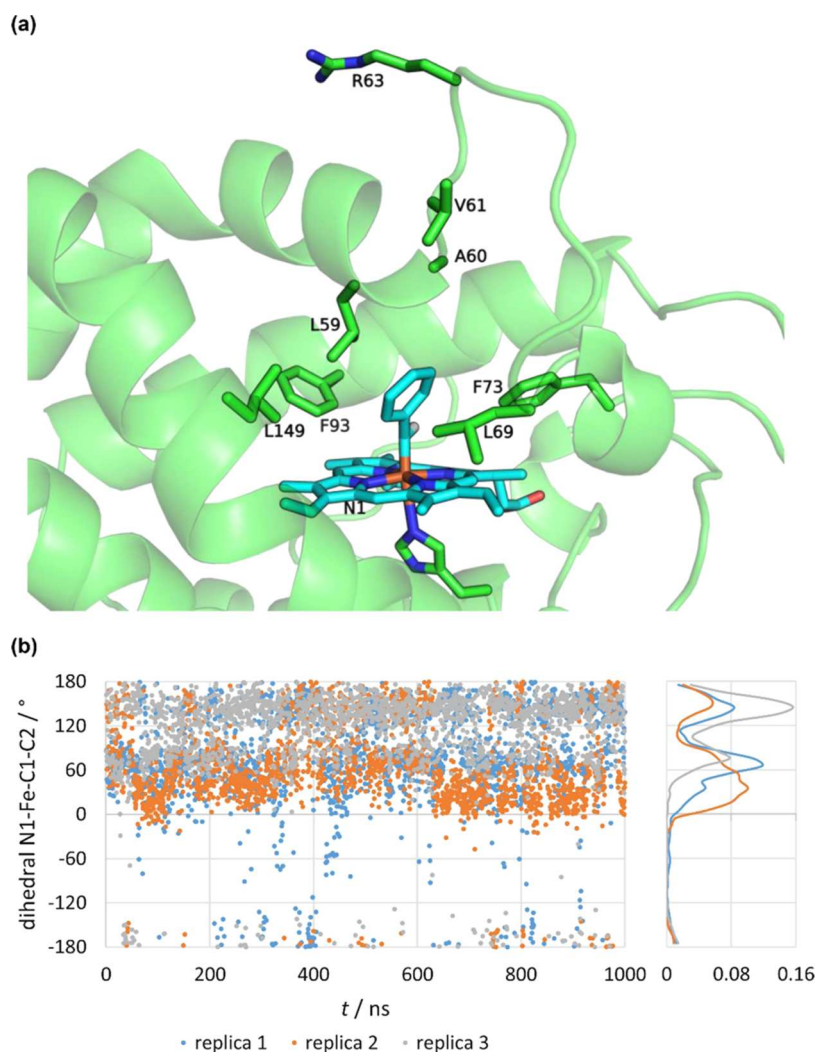


Figure 4. MD simulations on the key iron carbene intermediate. (a) Representative structure of the most populated cluster; (b) plot of the dihedral angle over time (left) and probability density plot (right) for three independent replicas. Nonrelevant hydrogens are omitted for clarity.

thereby indicating that subsequent cyclopropanation can occur from a number of carbene conformations. While a reaction of the carbene with either active site amino acid side chains or the porphyrin can not be fully excluded as a side reaction, it can likely be disregarded due to the hydrophobic nature of all amino acid residues in close proximity.¹⁷

To understand the enantioselectivity- and diastereoselectivity-controlling factors in the enzyme, the transition states leading to all four possible isomers were investigated by MD simulations. In the simulations, the Fe–C1, C1–C_{term}, and C1–C_{int} distances were restrained to mimic the DFT-optimized transition state geometries. For the (1*S*,2*R*)-isomer, the alkene was found to be preferentially oriented almost perfectly parallel to the Fe–C1 bond, which is in good agreement with the optimal conformation obtained in the DFT calculations (Figure 5a).¹⁸ Furthermore, the benzyl ester substituent is positioned in a sterically accessible area within the active site, thereby minimizing destabilizing interactions. In contrast, the simulations on the TS-like structure leading to the (1*R*,2*S*)-configured cyclopropane **4** revealed a significant deviation from the optimal parallel arrangement (Figure 5b). The alkene was found to rotate by approximately 60° to avoid unfavorable steric interactions between the benzyl ester substituent and the large F93, L56, and L59 residues, thus

destabilizing the C–C bond forming transition state and rendering formation of (1*R*,2*S*)-**4** less feasible.

Structures resulting in the formation of the *trans*-configured products, i.e., (1*R*,2*R*)-**4** and (1*S*,2*S*)-**4**, either displayed a significantly broadened distribution of the crucial dihedral angle ranging from 120° to 180° (Figure 5c) or a very unfavorable perpendicular orientation of the alkene with respect to the Fe–C1 bond (Figure 5d), thus rationalizing the experimentally observed high diastereoselectivity.

Based on our simulations, the L56, L59, F73, and F93 residues are crucial for achieving high selectivities in the cyclopropanation reaction. However, the significant steric bulk of these residues likely also prevents efficient cyclopropanations with sterically more demanding olefins from taking place. This observation is in line with a previously reported lack of product formation, when more bulky olefins, i.e., 4-methoxy styrene and allyl benzene, are employed as substrates.⁶ Consequently, targeting these sites in future directed evolution campaigns could enable the transformation of a variety of thus far unreactive substrates.

CONCLUSIONS

In conclusion, we have reported the first detailed investigations on the mechanism of iron heme enzyme-catalyzed cyclo-

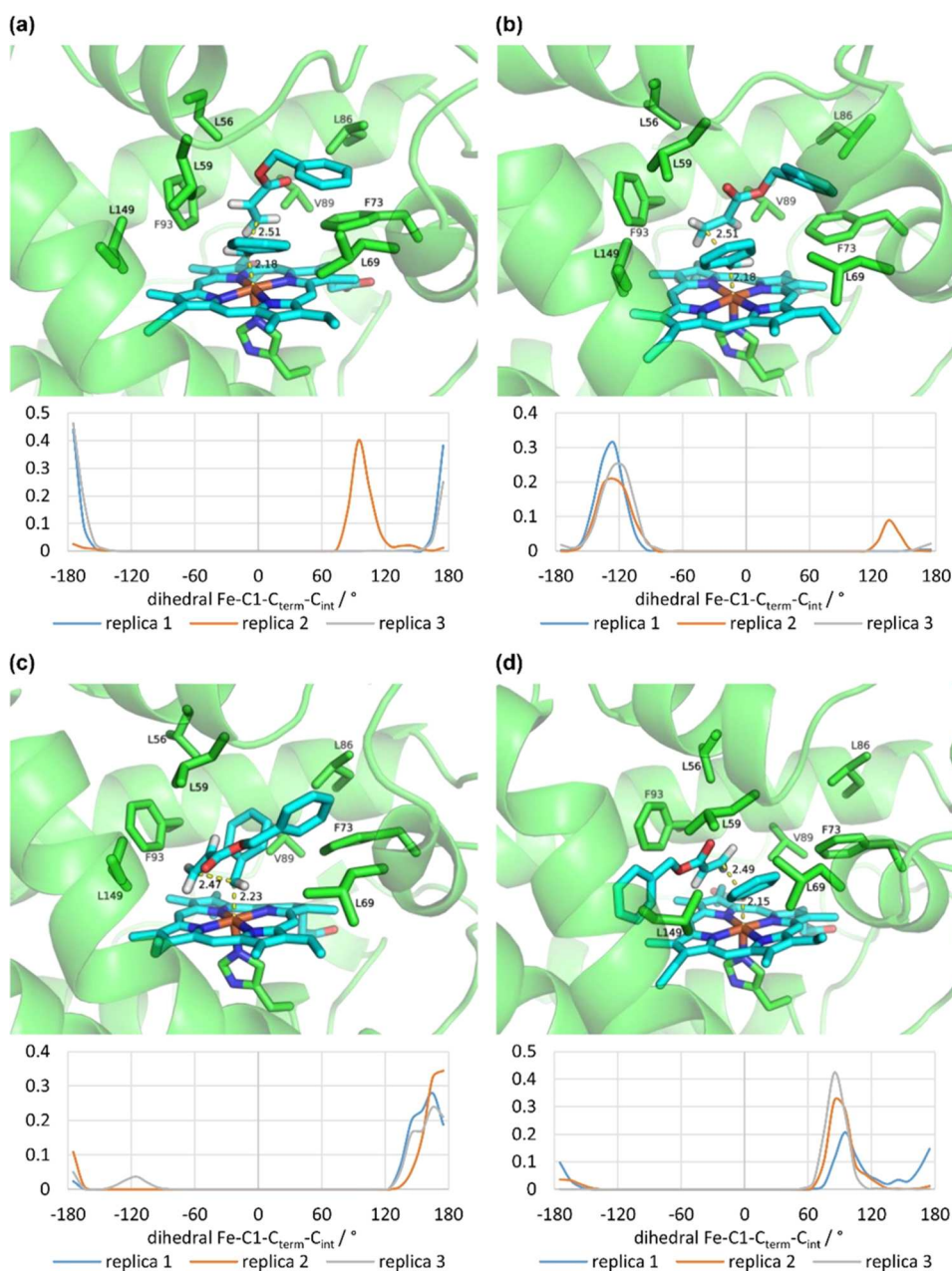


Figure 5. MD simulations on C–C bond forming transition state mimics leading to (a) the major product (1R,2S)-4, (b) (1S,2R)-4, (c) (1R,2R)-4, and (d) (1S,2S)-4. Distances are given in Å. Nonrelevant hydrogens are omitted for clarity.

propanations with stable and user-friendly diazirines as carbene precursors. Our studies revealed a two-step isomerization pathway involving a free carbene intermediate to be operative for structurally simple model systems, while a direct, one-step isomerization mechanism is preferred in the active site of the enzyme. In addition, the cyclopropanation was found to proceed via two energetically feasible distinct C–C bond formations within a diradical mechanism instead of an often proposed concerted process. The origin of diastereo- and enantioselectivity was rationalized through MD simulations of key intermediates and transition state mimics in the enzyme active site.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.3c06030>.

Computational methods, energies, Cartesian coordinates of calculated structures, and molecular dynamics data (PDF)

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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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Notes

The authors declare no competing financial interest.

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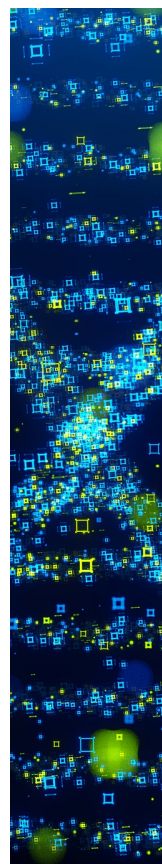
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