

Article

Computational Study on the Mechanism and Origin of Enantioselectivity in Non-Heme Iron Enzyme-Catalyzed Alkene Trifluoromethyl Azidation

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Abstract

Non-heme iron enzyme-catalyzed enantioselective alkene trifluoromethyl azidation represents a powerful strategy for constructing valuable chiral organofluorine compounds, yet the mechanistic origins of enantioselectivity controlled by the key residues in the enzyme's chiral environment remain elusive. Herein, we integrate density functional theory (DFT) calculations, classical molecular dynamics (MD) simulations, and quantum mechanical/molecular mechanical (QM/MM) calculations to elucidate the mechanism and the origin of enantiocontrol in this transformation. Our computational study reveals that radical addition from the CF₃ radical to the 4-methoxystyrene substrate constitutes the enantioselectivity-determining step. The enantioselectivity arises from energetic differences driven by H···H repulsions and C-H···O and N-H···F interactions between the substrate and the binding pocket defined by key residues I335V, F188Q, and R324 near the active site, which stabilize the transition state leading to the major enantiomer. These findings provide mechanistic insights into non-heme iron enzyme-catalyzed asymmetric azido-trifluoromethylation of alkenes and establish a structural framework for rational biocatalyst design.

Keywords: non-heme iron enzyme; trifluoromethyl azidation; QM/MM calculations; reaction mechanism; enantioselectivity



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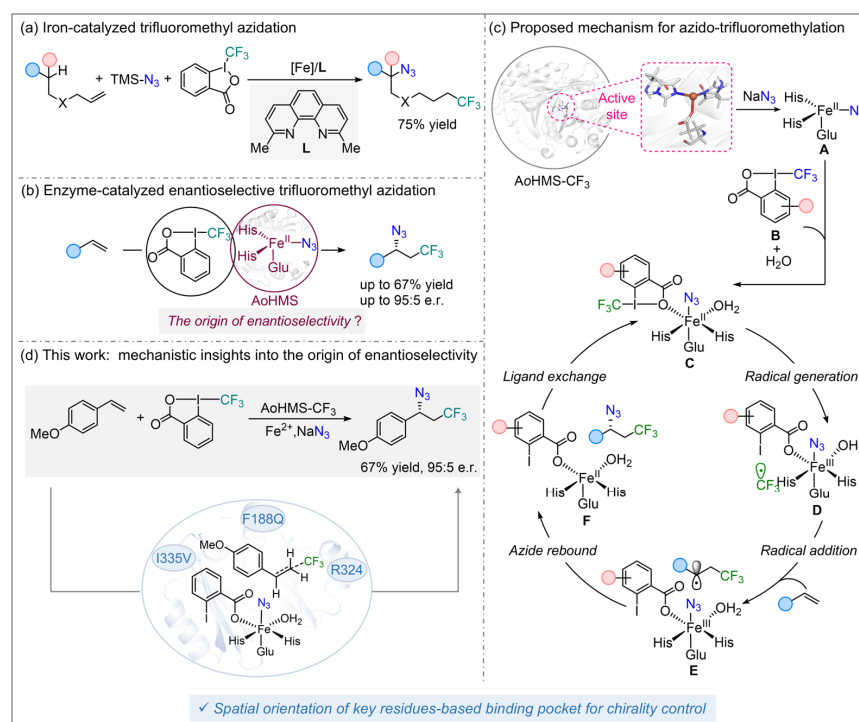
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1. Introduction

Fluorinated compounds have attracted considerable attention due to their unusual pharmacokinetic properties and high metabolic stability in medicinal chemistry [1–9]. The development of efficient methods for the introduction of trifluoromethyl (CF₃) groups into organic molecules is of great importance and yet highly challenging in organic synthesis. The first synthesis of a trifluoromethylated aromatic compound was achieved by Swarts via the reaction of benzotrichloride with antimony trifluoride [10]. Other methods have been developed to efficiently introduce the CF₃ group into organic molecules [11–19]. Notably, alkenes are highly regarded due to their ready availability and efficient conversion into high-value products in fluoroalkylation [20–26]. Among them, metal-catalyzed trifluoromethylation of alkenes represents one of the most straightforward and efficient strategies

for preparing valuable functional molecules containing C(sp²)-CF₃ bonds [27–30]. In this regard, iron-catalyzed difunctionalization of alkenes has enabled azido-trifluoromethylation with excellent regiocontrol [30] (Scheme 1a). Despite these advances, the direct and highly enantioselective incorporation of trifluoromethyl groups remains a formidable challenge. Biocatalysis offers a promising strategy to address this challenge [31–38], as enzymes are renowned for their exceptional ability to precisely control reaction enantioselectivity—a capability that can be further refined using protein engineering techniques such as directed evolution [39–43]. Recently, Huang’s group established a breakthrough non-heme iron-dependent enzymatic platform for enantioselective trifluoromethylation reactions, employing hypervalent iodine(III) reagent 1-trifluoromethyl-1,2-benziodoxol-3-(1H)-one (Togni reagent II) as CF₃ radical precursors and sodium azide as the external azide source [38] (Scheme 1b). They found that utilizing the wild-type hydroxymandelate synthase from *Amycolatopsis orientalis* (AoHMS) only afforded a 4% yield of the product, whereas the AoHMS variant AoHMS-CF₃ achieved up to 67% yield and 95:5 e.r. The originally proposed mechanism for this reaction is detailed in Scheme 1c. Coordination of hypervalent iodine(III) reagent B and H₂O with the active-site Fe(II) center of enzyme A generates the six-coordinate Fe(II) species C. Subsequent activation of B oxidizes A to form Fe(III) species D, concomitantly generating a CF₃ radical. This radical then undergoes radical addition with the alkene substrate to afford radical intermediate E, which undergoes azide rebound with the Fe(III)-N₃ intermediate to form the chiral trifluoromethyl azide-coordinated intermediate F. Finally, ligand exchange releases the product and regenerates the active catalyst C.



Scheme 1. The development of alkene trifluoromethyl azidation. (a) Iron-catalyzed trifluoromethyl azidation; (b) Enzyme-catalyzed enantioselective trifluoromethyl azidation; (c) Proposed mechanism for azido-trifluoromethylation; (d) This work: mechanistic insights into the origin of enantioselectivity.

Although the mechanisms of non-heme iron enzyme-catalyzed reaction have been extensively studied [44–46], the enantioselectivity-determining step and the mechanistic origins of enantioselectivity controlled by the key residues of the AoHMS-CF₃ in this trifluoromethyl azidation remain poorly understood. Therefore, we decided to investigate the

detailed reaction mechanism using density functional theory (DFT) calculations, molecular dynamics (MD) simulations and quantum mechanical/molecular mechanical (QM/MM) calculations. The computational results reveal that steric hindrance and weak interactions between the substrate and the binding pocket defined by key residues I335V, F188Q, and R324 are the key factors governing chirality control (Scheme 1d).

2. Results and Discussion

2.1. Reaction Mechanism

We first performed DFT calculations employing the model reaction illustrated in Figure 1a to investigate the plausible reaction mechanism. In this computational setup, 4-methoxystyrene **1a** and Togni reagent II **2a** served as the model substrates, while the catalyst utilized was a simplified analog (cat) of the AoHMS enzyme active site, wherein two histidine residues were replaced by imidazole and a glutamate residue was substituted with acetate. The corresponding energy profile for the azido-trifluoromethylation of **1a** with **2a** in the absence of the enzymatic environment is depicted in Figure 1b. Possible spin states were considered, including singlet (both closed- and open-shell), triplet, and quintet states. Herein, we focus on the quintet potential energy surface, while other spin states are detailed in the Supporting Information (see Figure S1 for details). Starting from $^5\text{INT1}$, I–CF₃ bond cleavage occurs via transition state $^5\text{TS1}$ to generate the CF₃ radical-complexed intermediate $^5\text{INT2}$, with an energy barrier of 12.1 kcal/mol. This is followed by complexation with substrate **1a** to form intermediate $^5\text{INT3}$, which then undergoes radical addition via transition state $^5\text{TS2}$ to give the trifluoromethyl-substituted benzyl radical-complexed $^5\text{INT4}$. Subsequently, C–N bond formation via azide rebound was examined at both the N³ and N¹ sites (transition states $^5\text{TS3}$ and $^5\text{TS4}$, respectively). The N¹-site pathway ($^5\text{TS4}$) lies 1.3 kcal/mol higher in energy than the N³-site pathway ($^5\text{TS3}$), and both lead to the product **3a**-complexed intermediate $^5\text{INT5}$.

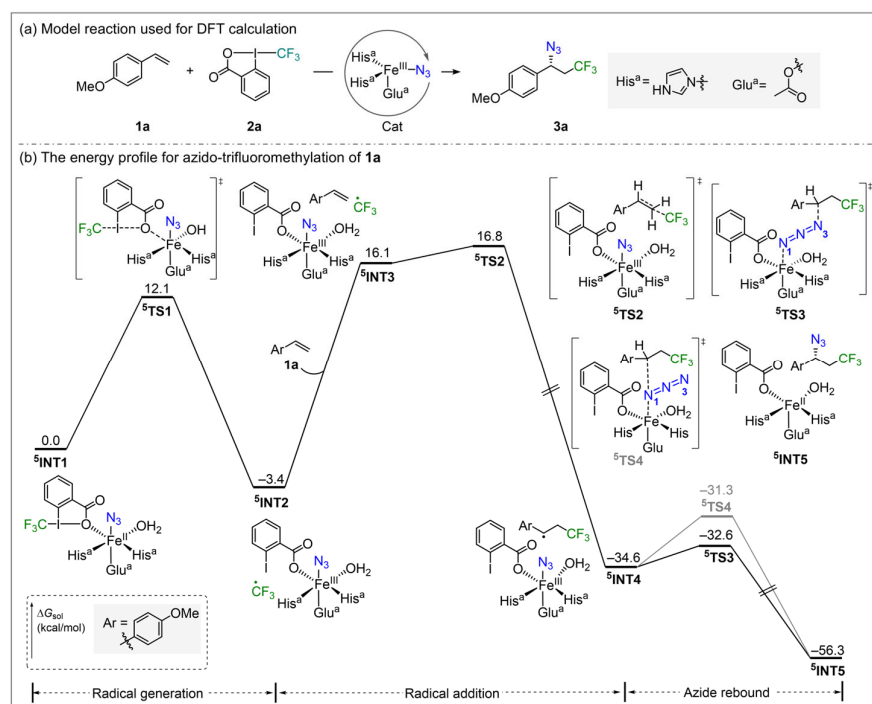


Figure 1. DFT calculations. (a) Model reaction used for DFT calculation; (b) The energy profile for azido-trifluoromethylation of **1a**.

Our calculations indicate that azide rebound proceeds rapidly, with a barrier of only 2.0 kcal/mol—a result that is in excellent agreement with Huang’s work [38]. By contrast, the radical addition is assumed to be the rate-determining step based on the model calculations, exhibiting a substantially higher barrier of 20.2 kcal/mol. The DFT-computed geometries of the CF_3 radical formation transition state $^5\text{TS1}$, radical addition transition state $^5\text{TS2}$, and azide rebound transition states $^5\text{TS3}$ and $^5\text{TS4}$ are shown in Figure 2a. To further investigate the radical character of this transformation, NPA spin density distribution analyses were performed on key intermediates. In $^5\text{INT2}$, the four unpaired electrons are essentially localized on the Fe metal center (spin density: 3.661), with negligible spin density on the N_3 and CF_3 groups. Upon I- CF_3 bond cleavage, the resulting $^5\text{INT2}$ features one unpaired electron on CF_3 (0.979), while the spin density on Fe decreases to 2.988; the N_3 group remains essentially spin-free (−0.056). Following radical addition to afford $^5\text{INT4}$, the spin distribution changes markedly, with unpaired electron density now distributed over Fe (3.949), N_3 (0.393), and the benzylic C1 atom of substrate **1a** (−0.646). These findings collectively support a radical reaction mechanism that is fully consistent with experimental mechanistic studies [38].

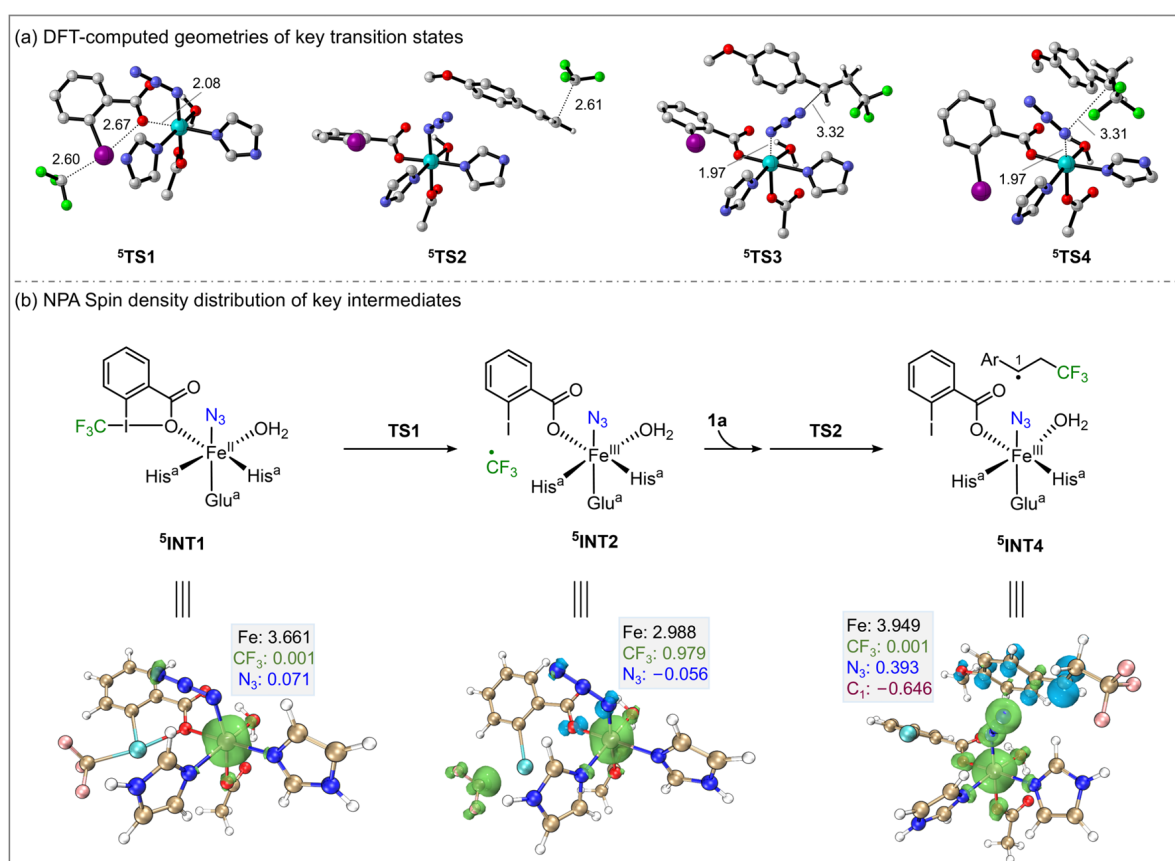


Figure 2. DFT-computed geometries of key transition states and NPA spin density distribution of key intermediates. (a) DFT-computed geometries of key transition states; (b) NPA Spin density distribution of key intermediates. The distances are shown in Å.

2.2. The Origin of Enantioselectivity

DFT model calculations indicate that CF_3 radical addition acts as the rate-determining step and is followed by rapid azide rebound. Interestingly, QM/MM calculations on the potential energy surface of the full enzyme (see Figure S2 for details) reveal that the energy barriers for CF_3 radical formation and radical addition are 8.9 and 8.2 kcal/mol, respectively. These comparable barriers suggest that either process could serve as the rate-determining

step, in contrast to the DFT model predictions, thus demonstrating that the enzyme environment does modulate the kinetic barrier of the reaction. Despite this discrepancy, given the experimentally observed rapid azide rebound, the radical addition is an irreversible process, making this step the enantioselectivity-determining step. Consequently, we focused our subsequent analysis on this addition step to elucidate how the *AoHMS*-CF₃ governs the observed enantiopreference. In the enzyme environment, substrate **1a** and the CF₃ radical can access two major conformations, which are preorganized through radical addition transition states (*R*)-**TS2** and (*S*)-**TS2** to afford (*R*)-**INT4** and (*S*)-**INT4**, respectively (Figure 3).

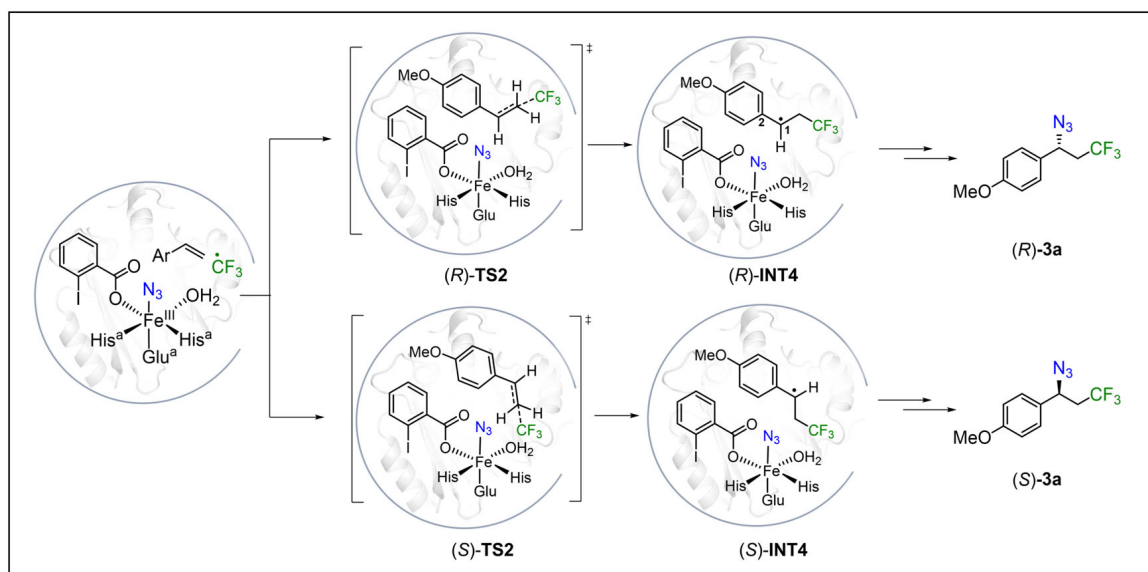


Figure 3. Key radical addition step in the enzyme environment.

To account for the dynamic nature of the protein scaffold, several representative snapshots were extracted from the MD trajectory (see Scheme S3 for details). The corresponding radical addition transition states were calculated using QM/MM methods. Here, we obtained six independent snapshots (Conf 1–6, Figure 4). The corresponding calculated activation free energy ($\Delta G^\ddagger$) differences between the transition states (*R*)-**TS2** and (*S*)-**TS2** are 1.5, 0.8, 1.9, 13.4, 2.5, and 0.9 kcal/mol, respectively. Notably, the $\Delta G^\ddagger$ values exhibit a remarkable sensitivity to the conformational snapshot extracted from the MD trajectory. Among the six representative snapshots, the energy gap for the *R*- vs. *S*-enantiomer varies from 0.8 kcal/mol to 13.4 kcal/mol. This large fluctuation underscores the critical role of substrate–active-site conformational dynamics in modulating the transition-state stability. Despite these fluctuations, the quintet (*R*)-⁵**TS2** consistently lies lower in energy than (*S*)-⁵**TS2** across all snapshots, consistent with the experimental formation of (*R*)-**3a** as the major product. To provide a more robust energetic basis for mechanistic conclusions, we employed a Boltzmann-weighted ensemble average over these transition states. The resulting averaged barrier ($\Delta G^\ddagger_{\text{average}} = 1.4$ kcal/mol) provides a statistically meaningful description that reconciles the structural diversity observed in the MD simulations. We therefore emphasize that the stereoselectivity of *AoHMS*-CF₃ cannot be rationalized by a single static snapshot, but rather emerges from the dynamic conformational ensemble of the enzyme–substrate complex.

To gain deeper insight into the origin of the observed enantioselectivity, we systematically analyzed the steric effects and interactions between the substrate and surrounding residues at all of these transition states. Our results show that the spatial orientation of the binding pocket, defined by key residues F188Q, I335V, and R324, dictates the observed

enantioselectivity. To this end, we selected the most populated conformation Conf5 from the second MD simulation and the moderately populated Conf1 from the first MD simulation as representative conformations, and performed a detailed comparative analysis of the energy barrier differences between the R- and S-pathways. Other results are shown in Figures S3–S6.

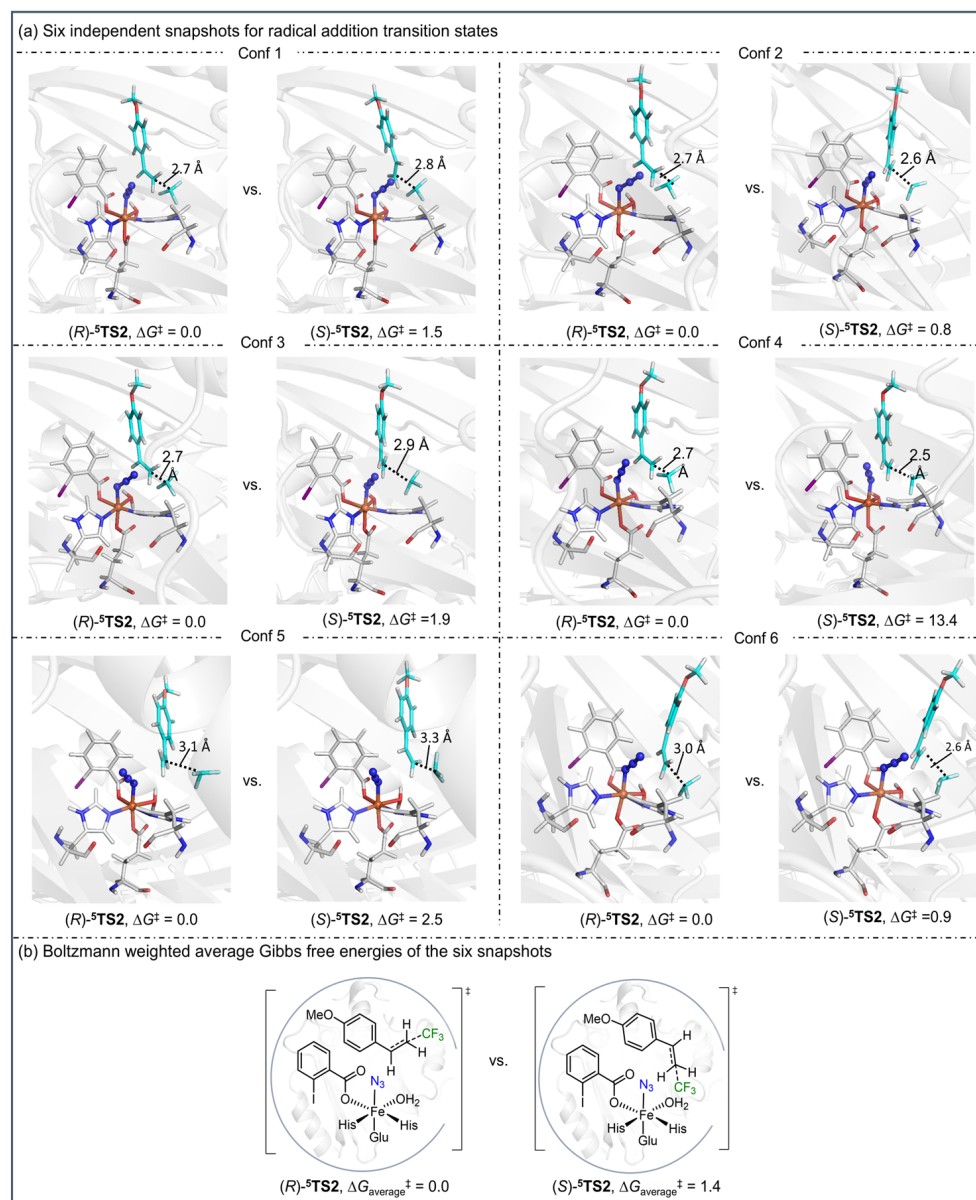


Figure 4. QM/MM calculations. (a) Six independent snapshots for radical addition transition states; (b) Boltzmann weighted average Gibbs free energies of the 6 snapshots.

As shown in Figure 5a, in Conf1 transition state (S)-⁵TS2, residue I335V undergoes a H···H repulsion (2.4 Å) with the substrate, whereas residues F188Q and R324 form comparatively weak C-H···O (3.8 Å) and N-H···F (3.2 Å) interactions, respectively. By contrast, in (R)-⁵TS2, no significant H···H repulsion (2.9 Å) is present between I335V and the substrate. More importantly, F188Q and R324 engage in substantially stronger C-H···O (3.1 Å) and N-H···F (2.8 Å) interactions with the substrate, respectively. IGMH analysis was performed to visualize the steric repulsion and noncovalent interactions between these key residues and the substrate in transition states (see Figure S7 for details). Similar interactions are also observed in the two transition states of Conf5 (Figure 5b) and in

other conformations (see Figures S3–S6 for details), indicating the important role of weak interactions between the substrate and residues in governing enantioselectivity.

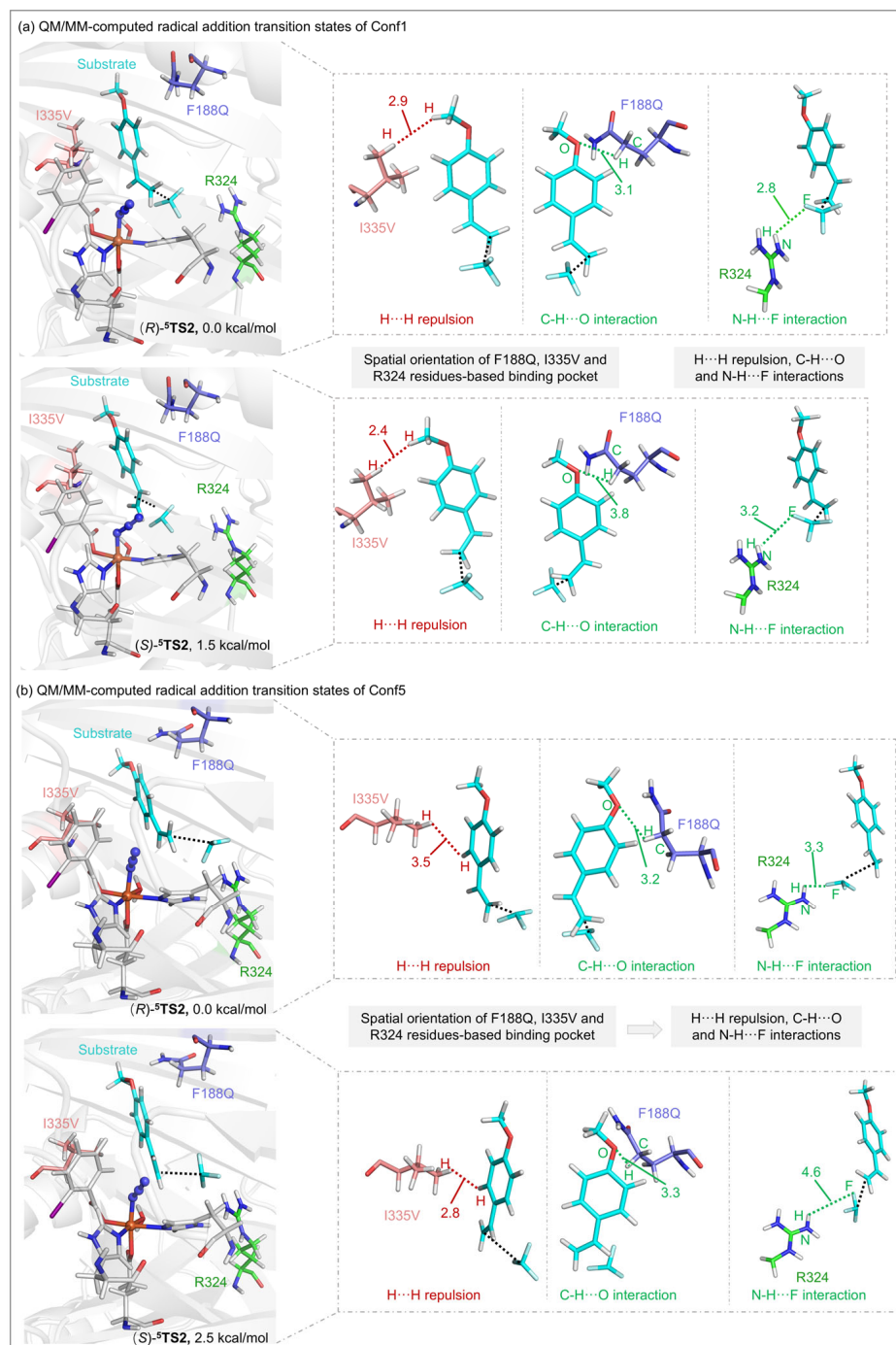


Figure 5. The origin of enantioselectivity. (a) QM/MM-computed radical addition transition states; of Conf1; (b) QM/MM-computed radical addition transition states of Conf5. The energies and distances are shown in kcal/mol and Å, respectively.

Furthermore, monitoring of the H...H, C-H...O, and N-H...F distances between the substrate and residues I335V, F188Q, and R324 over time reveals that these interactions are maintained throughout the dynamic simulation (Figure 6). This further highlights the critical role of H...H, C-H...O, and N-H...F interactions in chirality control.

Taken together, the steric hindrance and noncovalent interactions exerted by the residue-based binding pocket within the enzymatic environment rationalize the energy

barrier difference between these two radical addition pathways, ultimately leading to the observed enantioselectivity. To probe the effect of the I335V substitution on pocket properties and reaction selectivity, we introduced the I335L mutation into AoHMS-CF₃ and performed MD simulations followed by QM/MM calculations on a representative structure. The I335L mutation significantly increased the pocket volume, and the computed barrier for the (*R*)-⁵TS3 was 3.1 kcal/mol lower than that for the (*S*)-⁵TS3, suggesting that this substitution enhances enantioselectivity (see Figure S8 for details).

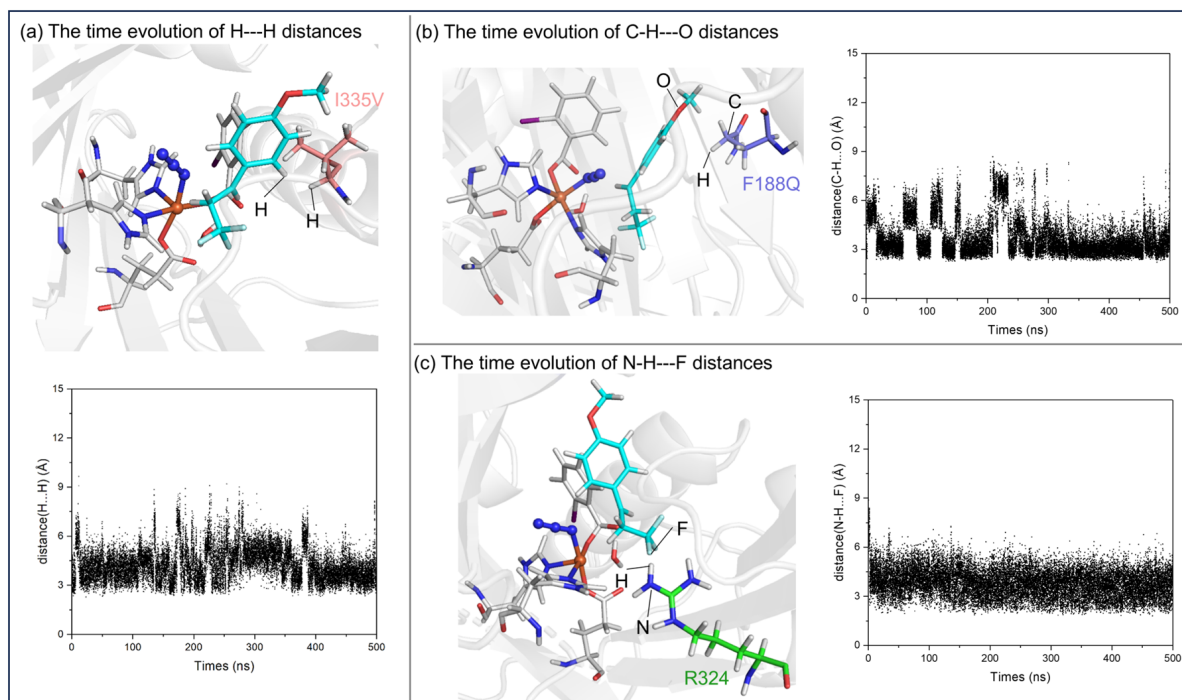


Figure 6. The time evolution of key bond distances between the substrate and key residues. (a) The time evolution of H...H distances; (b) The time evolution of CH...O distances; (c) The time evolution of NH...F distances.

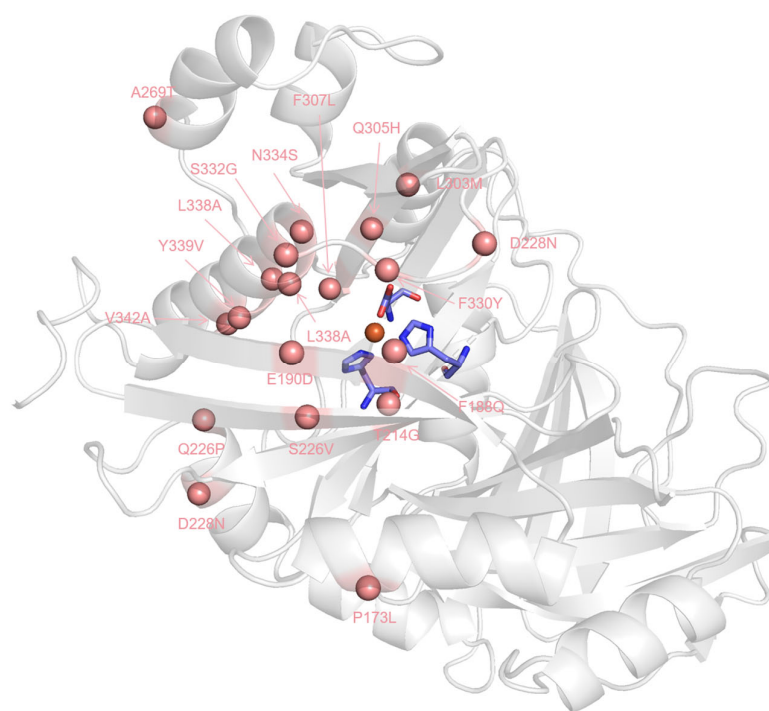
3. Materials and Methods

3.1. DFT Calculations

All DFT calculations were carried out with Gaussian 16 [47]. Geometry optimizations were performed with the (U)B3LYP-D3 method [48–50]. The LANL2DZ [51] basis set with ECP was used for Fe and I, and the 6-31G(d) [52,53] basis set was used for other atoms. To ensure that the correct unrestricted wavefunctions were obtained, a stability test was carried out using Gaussian keyword *stable = opt*. Frequency analysis was conducted at the same level of theory to verify that the stationary points are minima or saddle points. Single-point energies were computed at the (U)B3LYP-D3/def2-TZVP level [54] of the theory on gas phase optimized geometries in diethylether with the application of the SMD solvation model [55]. To validate the accuracy of the calculation results, we recalculated single-point energies of key transition states using the M06-2X and ω B97X-D functional with the same basis set. We obtained similar results, which further support the reliability of our calculations (see Figure S9 for details). The 3D diagrams of computed species were generated using CYLView [56]; detailed DFT calculations computational procedures are provided in the Supplementary Information (SI).

3.2. MD Simulations

Classical molecular dynamics (MD) simulations were carried out using the pmemd module of the GPU-accelerated Amber 20 package [57]. The initial geometry of AoHMS-CF₃ was derived from the X-ray crystal structure of the AoHMS enzyme (PDB ID: 2R5V), in which 19 amino acid residues were mutated by PyMOL 2.4.0 software [58] (Scheme 2). The substrate was docked into AoHMS-CF₃ using AutoDock vina 1.1.2 [59] (see Scheme S1 for details), and the resulting conformation served as the starting point for constrained MD simulations. Hydrogen atoms were added to standard residues using the H++ web server [60]. Parameters for the Fe³⁺ ion and its coordinating residues were obtained using the Metal Center Parameter Builder (MCPB.py) [61]. The atomic partial charges of the benzyl radical substrate were derived using the RESP charge-fitting method [62,63] based on electrostatic potential, with the corresponding electrostatic potential data calculated at the B3LYP/6-31G(d) level using Gaussian 16. Parameters for both the hypervalent iodine reagent and the benzyl radical substrate were generated using the GAFF force field [64], standard amino acid residues were treated with the Amber ff19SB force field [65], and solvent water molecules were described using the TIP3P model [66]. Following initial equilibration, three 500 ns MD simulations were performed using an isothermal-isobaric ensemble (NPT). Three independent 500 ns molecular dynamics simulations were completed (see Scheme S2 for details). The representative structures were characterized by K-means clustering analysis (see Scheme S3 for details). The Cpptraj [67] module from Ambertools utilities was used to process and analyze the trajectories. Protein structures were visualized using PyMOL; detailed MD simulation computational procedures are provided in the Supplementary Information (SI).



Scheme 2. Crystal structure of WT AoHMS (PDB: 2R5V). The salmon-colored spheres represent the mutation sites in the final variant (AoHMS-CF₃).

3.3. QM/MM Calculations

The ONIOM algorithm [68,69] integrated in Gaussian 16 software was employed for QM/MM calculations. Water molecules and counterions within 5 Å of the enzyme were retained. Residues greater than 6 Å away from the QM region were kept fixed

during geometry optimization. For the QM region, geometry optimization and vibrational frequency calculations were performed at the B3LYP-D3/6-31G(d)-LANL2DZ (Fe, I) level, and the resulting frequencies were used to obtain the free-energy correction values (G_{corr}) for the ONIOM energies. Single-point energies were computed at the B3LYP-D3/def2-TZVP level. For the MM region, the same force field parameters from the classical MD simulations discussed above were used. The mechanical embedding scheme was used in geometry optimization, and the resulting ONIOM energy was denoted as E_1 . Single-point energy calculations were carried out using the electronic-embedding scheme to better describe the electrostatic interactions between the QM and MM regions, and the resulting energy was denoted as E_2 . The sum of E_2 and G_{corr} values was defined as the free energy and used to assess the relative stabilities of different species. The initial input files for the ONIOM calculations were generated using molUP [70].

Boltzmann-weighted average Gibbs free energies ($\Delta G_{\text{average}}^\ddagger$) of the 6 snapshots from the MD trajectory were calculated using the following formula, where n is the number of structures ($n = 6$), k_B is the Boltzmann constant, and T is the temperature ($T = 298.15$ K):

$$\Delta G_{\text{average}}^\ddagger = -k_B T \ln \left(\frac{1}{n} \sum_{i=1}^n \exp \left(\frac{-\Delta G_i^\ddagger}{k_B T} \right) \right)$$

4. Conclusions

In summary, we have presented a computational study using DFT calculations, MD simulations, and QM/MM calculations to elucidate the mechanism and the origin of enantioselectivity in non-heme iron enzyme-catalyzed alkene trifluoromethyl azidation. The computations show that the catalytic cycle consists of three major steps, including radical generation, radical addition, and azide rebound. The radical addition from the CF_3 radical to the 4-methoxystyrene substrate represents the enantioselectivity-determining step of the reaction. QM/MM analysis of the key enantioselectivity-determining transition states reveals that the observed high enantioselectivity stems from differential $\text{H}\cdots\text{H}$ repulsion and $\text{C-H}\cdots\text{O}$ and $\text{N-H}\cdots\text{F}$ interactions between the enzyme's key residues (F188Q, I335V and R324) in the binding chiral pocket and the substrate. This work provides fundamental atomistic insights into how a non-heme iron enzyme exerts enantiocontrol through finely tuned spatial environments in its active site. The elucidated mechanism and the identified role of critical binding pocket residues establish a clear structural framework for the rational design and optimization of biocatalysts for asymmetric radical trifluoromethylation and related transformations.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/catal16080695/s1>, Scheme S1: Initial model of the docked complex for molecular dynamics simulation; Scheme S2: The RMSD plot over 500ns in holo-MD simulation; Scheme S3: K-means clustering analysis of the 500 ns MD trajectory; Figure S1: The other possible spin states of the energy profile for the azido-trifluoromethylation of 1a with 2a; Figure S2: QM/MM computed full-enzyme potential energy surface; Figure S3: Analysis of the interactions between the substrate and key residues in (R)-configuration transition states; Figure S4: Analysis of the interactions between the substrate and key residues in (R)-configuration transition states; Figure S5: Analysis of the interactions between the substrate and key residues in (S)-configuration transition states; Figure S6: Analysis of the interactions between the substrate and key residues in (S)-configuration transition states; Figure S7: IGMH analysis of interactions between key residues and the substrate; Figure S8: Virtual mutant screening; Figure S9: Benchmark of single-point energy methods. Table S1: Zero-point correction (ZPE), thermal correction to enthalpy (TCH), thermal correction to Gibbs free energy (TCG), energies (Esol), and Gibbs free energies (Gsol) (in Hartree) of the structures calculated at (U)B3LYP-D3/def2-TZVP-SMD(diethylether) //(U)B3LYP-D3/6-31G(d)-LANL2DZ level of theory.

Table S2: Zero-point correction (ZPE), thermal correction to enthalpy (TCH), thermal correction to Gibbs free energy (TCG), E1 and E2 (in Hartree) of the structures calculated at ONIOM-EE(B3LYP-D3/def2-TZVP)//ONIOM-ME(B3LYP-D3/6-31G(d)-LANL2DZ) level of theory [71–74].

Author Contributions: H.W.: data curation; formal analysis; visualization; writing—original draft; funding acquisition. L.L.: data curation; formal analysis; visualization; investigation. Y.-B.S.: supervision; writing—review and editing. Y.-F.Y.: conceptualization; supervision; writing—review and editing; funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data that support the findings of this study are available in the Supplementary Information of this article.

Conflicts of Interest: The authors declare no conflicts of interest.

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