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Reaction Mechanism of Histone Demethylation in α KG-dependent Non-Heme Iron Enzymes

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ABSTRACT

Histone demethylases (KDMs) catalyse histone lysine demethylation, an important epigenetic process that controls gene expression in eukaryotes and represent important cancer drug targets for cancer treatment. Demethylation of histone is comprised of sequential reaction steps including oxygen activation, decarboxylation and demethylation. The initial oxygen binding and activation steps have been studied. However, the information on the complete catalytic reaction cycle is limited, which has impeded the structure-based design of inhibitors targeting KDMs. Here we report the mechanism of the complete reaction steps catalyzed by a representative non-heme iron α KG-dependent KDM, PHF8 using QM/MM approaches. The atomic-level understanding on the complete reaction mechanism of PHF8 would shed light on the structure-based design of selective inhibitors targeting KDMs to intervene cancer epigenetics.

1. INTRODUCTION

Histone demethylases control the methylation states of histone proteins and play an important role in stabilizing the structure and function of chromatin in an eukaryotic cell ^{1,2}. Histone lysine demethylases (KDMs) enzymes have emerged as attractive cancer drug targets in the pharmaceutical industry since overexpression or mutations of KDMs is related to various forms of cancer by interfering cancer epigenetics ³. There are two families of KDMs, namely FAD (Flavin Adenine Dinucleotide)-dependent amino oxidase lysine demethylase 1 (KDM1) and α KG (α -ketoglutarate)-dependent Jumonji C (JmjC)-domain containing histone demethylases (JHDMs) ⁴. JHDMs are the largest group of KDMs with a non-heme iron in the active site, which can act on tri-, di- and mono-methylated lysine residues in histone proteins to remove the methyl mask from the methylated histones ⁵.

Although KDMs are emerging cancer drug targets, so far few inhibitors have been identified to target these enzymes, particularly the JHDMs. Understanding the complete reaction process is urgently needed for developing selective inhibitors towards α KG-dependent KDMs.

Theoretical studies on the demethylation by a few non-heme enzymes have been reported. The demethylation in an DNA repair enzyme AlkB was studied using QM/MM method starting from the catalytically active Fe(IV)=O intermediate ^{6,7}. Recently, a DFT study on the mechanism of demethylation starting from the Fe(IV) oxo species was reported for a JHDM enzyme, JMJD2A ⁸. However, so far little has been reported on the detailed reaction mechanism of the entire demethylation catalytic cycle including oxygen activation, decarboxylation and demethylation catalysed by KDMs ⁹. Here we report the detailed mechanism of the reactions catalyzed by PHF8 ¹⁰, an α KG-dependent KDM using QM/MM calculations. This research would provide the structural basis for the design of selective KDMs inhibitors.

2. METHODS

2.1 System setup

The crystal structure of the PHF8 (PDB: 3KV4) in complex with substrate histone (H3) and cofactor analogue N-oxalylglycine¹⁰ was used as the initial structure. The missing linker region residues 65-79 were built using Modeller software¹¹. The α KG cofactor was built by replacing the nitrogen of N-oxalylglycine with a carbon atom. The charges of α KG were calculated according to the Merz–Singh–Kollman scheme^{12,13} using Gaussian 09 package¹⁴ at HF/6-31G* level using restrained electrostatic potential (RESP) method¹⁵. The parameters for the di- and trimethylated lysine residues were obtained from the previous literature¹⁶.

The protonation states of the side chains of the titratable amino acids were assessed using H++ server at pH 7¹⁷. The amber parameters for the penta-coordinated (5C) Fe(II) iron active site (ground state: high spin S=2)^{18,19,20,21} and the coordinating ligands were prepared using the Metal Centre Parameter Builder (MCPB) using MCPB.py²². The missing hydrogen atoms and counter ions were added to the structure of the PHF8 enzyme using tleap. The enzyme was solvated into a truncated octahedral box with TIP3P²³ water molecules such that no protein atom was within 10 Å to any edge of the box.

2.2 MD simulations

FF14SB²⁴ force field was used and the zinc ion and its coordinating ligands in the PHD domain were described using zinc AMBER force field (ZAFF)²⁵. The periodic boundary conditions were employed in all the simulations. Long-range electrostatic interactions were calculated using the particle mesh Ewald (PME) method²⁶ with a cut-off of 8 Å for the direct space

Coulomb and vdW forces. All MD simulations were performed using the GPU version of the PMEMD ²⁷ engine integrated with Amber 14 ²⁸.

The energy minimization of the enzyme complex was performed using steepest descent and conjugate gradient algorithms. The minimized enzyme complex was then subjected to controlled heating from 0 to 300K at constant volume using Langevin thermostat with a collision frequency of 1 ps⁻¹ using a canonical ensemble (NVT) for 400 ps. The solute molecules were restrained using harmonic potential of 10 kcal mol⁻¹ Å² during the heating process. The SHAKE algorithm was used to constrain the bonds of all hydrogen atoms. Then the system was equilibrated at 300K in an NPT ensemble for 1 ns without restrains on the solute molecules and the Berendsen barostat was used to maintain the pressure at 1 bar. The SHAKE algorithm was used to constrain bonds involving hydrogen ²⁹. A production MD run with explicit solvent was performed for continuous 50 ns (Supplementary information, Figure S1) in a NPT ensemble with a target pressure of 1 bar and a pressure coupling constant of 2 ps. The trajectories were analysed using CPPTRAJ and VMD ³⁰.

The system is composed of 465 amino acid residues, 3 Na⁺ ions and 7,520 water molecules, in total 75,143 atoms are included in the MD simulations.

2.3 QM/MM calculations

The choice of the starting structure is crucial in the QM/MM studies ³¹. QM/MM calculations were conducted based on selected snapshots from MD simulations. The selected snapshots obtained from cluster analysis on the equilibrated MD trajectory were subjected to molecular mechanics (MM) minimization using the steepest descend (2,000) followed by conjugate gradient (2,000) algorithms using Amber14 ²⁸. The minimized snapshots were then prepared for

QM/MM setup using TAO package ³² and the calculations were performed using ONIOM ^{33, 34, 35} implemented in Gaussian09 package ¹⁴. The system was divided into two layers. The high layer was treated with BP86 or B3LYP density functionals and the low layer was treated using molecular mechanics with the parameters obtained from Amber force field ³⁶. The interactions between the high and the low layer were treated using electronic embedding scheme ³⁷.

In QM/MM setup, the JmjC domain was kept while the PHD domain and linker region were removed. 279 amino acids residues, 3 Na⁺ ions and 1,323 water molecules were kept in the QM/MM calculations with 8,025 atoms in total. The QM region for penta-coordinated iron centre (the resting state of non-heme iron) consisted of H319, H247, D249, N333, α KG and dimethylated lysine residue (Figure 1C). The sixth coordination site was saturated with a molecular oxygen (Figure 1D). The QM region for the decarboxylation and the demethylation steps were composed of 69 and 66 atoms with the total charge of the system being 0 and -2, respectively. The residues within 12Å of non-heme iron including water molecules were allowed to move freely and the rest of the system was frozen during geometry optimization. The link atoms approach was used to saturate the dangling bond in the QM/MM calculation.

The potential energy surface (PES) of the reaction catalyzed by PHF8 was studied using adiabatic mapping approach which describes the energy as a function of the chosen reaction coordinates. The QM/MM optimized structures were taken as the starting points for PES scan along the chosen reaction coordinates with a step size of 0.05 to 0.1Å. The reaction coordinates for the decarboxylation step was chosen as the C1-C2 bond of the α KG cofactor and O_p-O_d bond of the peroxy group to be broken, respectively. The reaction coordinate for the demethylation step was the distance between the hydrogen atom of the second methyl group (me2) of histone K9 and the oxygen of the Fe(IV)=O species. The minima and the transition states (TS) obtained

from the PES were fully optimized and validated by frequency calculations. The TSs were characterized by presence of one imaginary frequency and the minima structures had no imaginary frequency. The non-bonded van der Waals parameters for the iron were obtained from the previous literature ³⁸. All the calculations were performed on S=2 (unless otherwise stated) with DFT using UBP86 functional with 10% Hartree-Fock exchange, an experimentally calibrated functional able to reproduce the geometry and electronic spectra of {FeNO}7 complex ^{39,40}. The Pople 6-311g* basis set was used for Fe and the atoms that are directly coordinated to the non-heme iron. The rest of the atoms were treated with 6-31G* basis set ^{39,40}. Furthermore, single point correction to the electronic energy was performed using the Grimme's DFT dispersion corrections with Becke-Johnson damping (GD3BJ). The zero-point energy (ZPE), thermal and entropic energy contributions were computed for all the TSs and their related minima structures at 298.15K and 1.0 bar by performing frequency calculations using the Gaussian09 package. The stability of the DFT wavefunction was checked using opt=stable keyword for all the optimized structures of the TSs and the minima obtained from the QM/MM study. To be comparable with the literature ^{7,8,41,42}, we also performed benchmark calculations using UB3LYP ^{43,44,45,46} functional with LanL2DZ ⁴⁷ basis set on Fe and split-valance 6-31G* set on the rest of the atoms (Table S1).

3 RESULTS AND DISCUSSION

3.1 Dioxygen activation and oxidation reaction

3.1.1 Resting state of non-heme Fe

PHF8 consists of a plant homeodomain (PHD) and a JmjC domain connected through a linker region. The crystal structure of PHF8 (PDB code: 3KV4) ¹⁰ shows the histone peptide is bound

through trimethylated lysine residue (K4) and dimethylated lysine (K9) to the PHD and JmjC domain, respectively (Figure 1A). The non-heme ferrous ion (Fe(II)) located in the conserved β -barrel fold of the JmjC domain is coordinated with a His-His-Asp catalytic triad in a monodentate fashion and the α KG substrate binds to the metal centre in a bidentate fashion^{18,48}. The iron centre is prone to bind with an molecular oxygen to give a high-valent Fe(IV)=O oxo reactive intermediate, which removes the methyl group from the dimethylated lysine residues of the histone substrate^{9,19,49}.

MD simulations were run and the representative structures obtained from the cluster analysis of the MD trajectory were selected as the starting structures for scanning the reactions pathway of PHF8 (Table S3). The resting state of R1 is quintet, with the d_{xy} orbital doubly occupied and a single α -electron in each of the other four d orbitals. From our MD simulation of PHF8, the water molecule was observed in the 6C site (Figure S2), in agreement with the most X-ray structures of non-heme Iron enzymes, where a water molecule is observed at the 6C site. In the presence of the cofactor and the substrate, the water molecule would leave the 6C site and therefore allow the oxygen to bind to the iron centre. Loss of water ligand would result in the conversion from 6C to 5C Fe coordination, creating an open empty site for O₂ attack⁴⁹. Therefore, a penta-coordinated (5C) square pyramidal geometry of the non-heme active site was used as the resting state R1(Figure 2A). This is consistent with other non-heme iron containing enzymes where the binding of both cofactor α KG and substrate in the active site results in the loss of water ligand and tetragonal pyramidal configuration^{48,50,51}. The spin density on the iron ion (3.73) in the resting state approximately corresponds to +2 oxidation state⁵² and the pyramidal configuration allows the triplet oxygen molecule to bind to the vacant coordination

site of iron (Figure 2A). These results are in agreement with the CD/MCD spectroscopy experiments for the 2OG dependent non-heme enzymes^{53,54}.

The non-heme iron in the active site of PHF8 is oxidized from Fe(II) in the resting state R1 to Fe(III) oxidation state in the superoxide radical IM2 (the spin density on the iron center changes from 3.73 to 4.13) (Figure 2B). The coordinate bond formed with α KG in the intermediate IM2 is slightly shorter than those in R1, which is attributed to the more stable hexa-coordination of Fe in IM2 than the penta-coordination in R1. The change of electron density in the formation of the Fe(III)-OO[•] superoxide radical is shown in Figure S3.

3.1.2 Fe(III)-OO[•] Superoxide Radical Intermediate

The binding of the molecule oxygen to R1 results in the formation of an intermediate IM2 with distorted octahedral (6C) geometry of the iron center. The IM2 intermediate may have several possible spin states (triplet, quintet or septet). A study of the QM cluster model study on the O₂ activation in 4-hydroxyphenylpyruvate dioxygenase (HPPD) showed the formation of a peroxo-bridged Fe(IV)-OO species with triplet ground state (S=1) which then undergoes spin crossing from triplet to quintet surface to produce the experimentally observed quintet ground state (S=2) Fe(IV)=O intermediate⁴⁰. Bridged intermediate was also reported in other non-heme enzymes such as α -KAO⁶.

Since protein environment may have significant impact on the results for the KDM enzymes, we conducted QM/MM study on Fe(III)-OO[•] under both triplet state S=1 and quintet state (S=2) using UBP86 with 10% HF. Interestingly, the QM/MM calculations with S=2 results in the Fe(III)-OO[•] superoxide radical intermediate IM2, whereas the optimization with S=1 results in a Fe(IV)-OO²⁻ peroxy species (Figure S4). The energy of the Fe(III)-OO[•] intermediate (S=2) was

almost isoenergetic with the Fe(IV)-OO²⁻ species (S=1) (Table S1). QM/MM potential surface scans were conducted using the C1-C2 bond in α KG as the reaction coordinate. Notably, a transition state was located on the quintet surface leading to a peroxo-bridged Fe(III) intermediate IM3, whereas no transition state was located on the triplet surface (Figure S5), indicating the quintet state is the preferred ground state for the superoxide radical intermediate.

We also performed QM/MM calculations with UB3LYP, a commonly adopted DFT functional employed in the study of non heme iron containing enzymes^{43,44,45,46}. The QM/MM energy of the quintet state (S=2) was 7.2 kcal/mol lower than that of triplet state (S=1) (Table S1, Figure S6).

The electronic structure of the quintet IM2 is best described as high spin S=5/2 Fe(III) antiferromagnetically coupled to superoxide radical anion (S=1/2). The bond lengths of the Fe-O and O_d-O_p bond in IM2 is 2.11Å and 1.28Å, respectively (Figure 2B). These results are in good agreement with the QM/MM optimized structure of Fe(III) superoxide radical species in Histone Demethylase JMJD2A, where the calculated Fe-O_p bond distance is 2.13Å and the O_d-O_p distance is 1.28Å using UB3LYP functional with LANL2DZ basis set on Fe and double- ζ valence 6-31G(d) basis set on rest of atoms⁵⁸. The electronic structure of IM2 obtained in our study is also in accordance with a recent QM study on a 2-OG dependent non-heme iron oxygenase (α -KAO), where the reported bond lengths of the Fe-O and O_d-O_p bond were 2.11Å and 1.29Å, respectively by using UB3LYP-D3/cc-pVTZ method⁶.

In the QM/MM optimized structure of the Fe(III) superoxide radical anion IM2, the NH group of dimethylated lysine residue of the histone substrate makes a hydrogen bond with the non-coordinating oxygen O1 of α KG. As a result, the reactive methyl group (me2) moves close to

vicinity of non-heme iron active site. The non-coordinating carboxylate group of the α KG is stabilized by the ionic interaction with K264 as well as the hydrogen bonding interactions with the two surrounding residues Y257 and T244 (Figure 3). In addition, the second sphere residue N333 also helps to maintain the configuration of the enzyme active site by making a stable hydrogen bond with the carboxylic group of D249, which is coordinated to the non-heme iron (Figure 3). These favorable interactions deposit the substrate histone and α KG in a catalytically active pose, enabling the subsequent reactions to happen.

3.1.3 Orbital interaction in formation of IM3

Oxygen is in its triplet state with a α -spin electron in each of its π^* orbital. The orbital interaction between d_z^2 with one of the oxygen atoms and d_{xz} with the other π^* orbital results in a strong Fe-O sigma bond (β 149) and a second weaker π bond (β 153). The molecular orbitals of the QM/MM optimized quintet state of Fe(III)-OO $^\bullet$ superoxide radical intermediate are shown in Figure 4. In TS2, an electron pair of the distal O_d atom is transferred to the keto group of α KG in a normal nucleophilic attack. The unpaired alpha spin orbital (α 157) on that oxygen makes a π -type interaction with the C-CO₂ sigma bond (Figure S9). Such π -bond is also formed in the corresponding unoccupied beta spin orbital (β 154). The resultant lower bond order of the C-CO₂ is evident in the structure, which shows the CO₂ moiety is almost linear. In IM3, the bond to the CO₂ moiety is a single electron bond (α 150). The corresponding beta orbital is beta 149. The d_{xy} orbital is doubly occupied (α 151, β 150) (Figure 4, Figure S7).

3.2 Decarboxylation

3.2.1 Formation of peroxo-bridged intermediate from Fe(III)-OO $^\bullet$

The distal oxygen O_d of the superoxide radical anion then undergoes a nucleophilic attack on the C2 carbon atom of αKG to give a peroxo-bridged intermediate IM3 (Figure 2D, Figure 5A). Our calculations show that in IM2 the absolute value of the spin density on the O_d (0.47) is higher than that on O_p (0.29) (Figure 2B), indicating that the distal oxygen has more radical character and therefore is more liable for the subsequent nucleophilic attack. This reaction proceeds through a transition state TS2 (Figure 2C) with an activation energy barrier of 12.2 kcal/mol (Figure 2H). In TS2, the NH group of the dimethylated lysine makes a hydrogen bond with the O1 oxygen of αKG, stabilizing the CO₂ to be released. The C1-C2 bond distance of αKG is elongated to 2.03Å in the TS2 and the O_p - O_d bond distance increased from 1.28Å to 1.40Å. The distal oxygen O_d formed a covalent bond with the C2 of αKG. The Fe-O_p bond distance is 1.98Å and the spin density of 4.04 indicates the oxidation state of iron is approximately +3⁵².

The effect of the conformational sampling on the QM/MM activation barrier for the decarboxylation reaction was considered by optimizing the TS2 transition state starting from five different QM/MM snapshots obtained from the MD trajectory. The computed Boltzmann weighted average of the electronic energy barrier (relative electronic energy without zero-point energy (ZPE) and entropic effect) was 11.0 kcal/mol based on the PES scans starting from the four different snapshots (Figure S8) and the related QM/MM optimized structures are shown in Figure S9.

The peroxo-bridged Fe(III) intermediate IM3 is formed with a relative energy of -26.2 kcal/mol (Figure 2H), indicating this step of reaction is exergonic. The exergonic nature of the IM3 is in accordance with the QM studies performed for other non-heme iron α-keto acid dependent oxygenases⁶. The Fe-O_p distance in IM3 intermediate is 2.06Å and spin population of iron becomes 3.75, indicating the oxidation state of iron being +3 (Figure 2D). The C1-C2 bond

distance has further increased to 3.53Å and the NH bond of dimethylated lysine remains H-bonded with the oxygen of CO₂ to be released.

3.2.2 Formation of Fe(IV)=O from peroxo-bridged intermediate

Heterolytic cleavage of the O_p-O_d bond in IM3 gives a Fe(IV)=O oxo intermediate IM4 (Figure 2F). It goes through the transition state TS3 (Figure 2E) with a small activation barrier of 3.5 kcal/mol (Figure 2H). The O_p-O_d bond distance is 1.77Å and Fe-O_p bond distance is 1.88Å in the TS3. The spin density of 3.97 on the iron in the TS3 approximately indicates that it has changed its oxidation state from Fe (II) to Fe (III). In the final product IM4, the bond length of Fe-O_p is reduced to 1.62Å, indicating a double bond is formed between O_p and Fe (Figure 2F). Based on the spin density on the Fe, the oxidation state of the iron center is +4 in Fe(IV)=O oxo intermediate, in agreement with the previous computational and experimental studies of other non-heme iron enzymes^{7,41,55,57,59,60}.

Previous QM/MM studies on halogenase SyrB reported the mechanism study on the O₂ activation steps and suggested the oxo rotation in the reaction process⁶¹. Superposition of the three intermediates IM2, IM3 and IM4 of PHF8 shows the extent of oxo rotation in relation to the methyl group of the histone lysine (K9) to be demethylated (Figure 2G). Notably, the oxo rotation is a continuous process in the decarboxylation starting from the Fe(III)-OO[•] intermediate IM2 and consummating at the Fe(IV)=O intermediate IM4. The distance between the hydrogen atom ^{me2}H (H1) of the dimethylated histone substrate and the O_p oxygen decreased gradually from the intermediate IM2 to IM3 and IM4. The reduction in the distance is attributed to the extra space created by releasing the CO₂ to give the Fe(IV)=O intermediate, which is

critical for positioning the enzyme for the subsequent hydrogen abstraction and the demethylation reaction.

The change in the free energy of the reaction ΔG_r from IM2 to IM4 is associated with a value of -41.1 kcal/mol, indicating the overall decarboxylation reaction process is significantly exergonic (Figure 2H), which is consistent with previous studies on non-heme iron α KG dependent enzymes^{6,40}. The IM4 exhibits a 5C trigonal bipyramid geometry, consistent with the results obtained from the nuclear resonance vibrational spectroscopy data on the non-heme iron α KG dependent enzyme where Fe(IV)=O intermediate was found to penta-coordinated⁵⁹. The penta-coordinated Fe(IV)=O intermediate was also reported in previous QM/MM study on the hydrogen atom abstraction catalyzed by the non-heme iron containing α KG dependent enzyme AlkB⁸. A recent QM/MM study on the hydroxylation reaction in Taurine/ α Ketoglutarate Dioxygenase was also reported using a penta-coordinated Fe(IV)=O intermediate⁶². Furthermore, it was suggested that the penta-coordinated Fe(IV)=O intermediate adopts quintet spin state and the hexa-coordinated Fe(IV)=O intermediate by triplet spin state⁶³, underpinning with the quintet spin state adopted in the present research.

The dominant spin states of iron in non-heme enzymes remains a long controversy^{40,43,44, 64-66}, although quintet state has recently been suggested to be the ground state for the resting state and the Fe (IV)=O intermediate in other non-heme enzymes^{45,46,55,56,57}. Since the ground state may change along the sequential reaction steps, here we calculated the different spin states (namely, triplet, quintet and septet spin states) of all the species involved in the decarboxylation process using QM/MM optimization. It is shown that the quintet state is consistently the ground state for all the species in the reaction process (Table S2). Furthermore, the S=2 reaction path

demonstrates to be the most preferable pathway among the three spin states considered (Figure S10).

In summary, the demethylation of the histone substrate catalyzed by α KG-dependent non-heme iron KDM enzymes proceeds with sequential reaction steps including oxygen binding and activation, decarboxylation and demethylation. In the initial steps, one dioxygen molecule is bound to the non-heme iron R1 and the bound oxygen then abstracts one electron from iron, being reduced to superoxide radical anion IM2, which then undergoes nucleophilic attack on the α KG co-substrate. Following the nucleophilic attack, a bridged intermediate IM3 is formed, which then releases a CO₂ molecule to yield the Fe(IV)-oxo intermediate IM4, consummating the decarboxylation reaction. The rate-limiting step in the decarboxylation reaction is the nucleophilic attack of the oxo group in the superoxide radical intermediate IM2 to yield the peroxo-bridged IM3 intermediate (Figure 2H, Table S3).

3.3 Demethylation of dimethylated lysine in histone

3.3.1 Hydrogen abstraction

The scope of Fe(IV)=O ferryl intermediate to abstract a hydrogen atom from the DNA base in a non-heme iron containing α KG-dependent enzyme AlkB enzyme was investigated, and it was found that the reaction went through a lower activation barrier of 23.4 kcal/mol than the hydrogen-atom abstraction for the Fe(III)-OO[•] superoxide radical ⁸. A DFT study on the dimethylated lysine indicated the dimethylated lysine adopted either a H-in (NH of the histone close to the Fe=O group) or a H-out (a methyl group of the histone close to the Fe=O group) conformation relative to the Fe(IV)=O ferryl species ⁹. In the H-in conformation, an iminium

intermediate was formed, whereas in the H-out conformation, the reaction proceeded by formation of a hydroxymethylaminium intermediate.

The demethylation reaction of the histone substrate catalyzed by α KG-dependent non-heme KDMs is triggered by hydrogen abstraction from the dimethylated lysine residue of histone by the IM4 intermediate Fe(IV)=O , the main oxidant ferryl species generated from the aforementioned oxygen activation and decarboxylation reactions (Figure 5B). In PHF8, only the H-out conformation of the dimethylated histone can be observed (Figure 2F), *i.e.* the second methyl group of the dimethylated lysine me2 points towards the Fe(IV)=O group and the NH group points towards the non-coordinating oxygen of succinate group. The hydrogen-atom abstraction from the me2 of the dimethylated lysine residue results in a hydroxylated IM5 intermediate (Figure 6B). The transition state TS4 is characterized by C-H and O-H bond distance of 1.45Å and 1.16Å, respectively (Figure 5B, Figure 6A). These results are in agreement with the DFT-optimized saddle point associated with the H-abstraction in the conversion of dimethylated substrates by a JHDM enzyme, JMJD2A ⁹ and with the QM/MM calculations of the AlkB enzyme ⁸. It is worth noting that in TS4 the NH of dimethylated lysine approaches the non-coordinating oxygen of succinate to form a hydrogen bond with distance of 2.35Å (Figure 6A). The new H-bond interaction effectively restrains the histone in the catalytic site and helps to position it appropriately to enable the hydrogen abstraction reaction to happen.

3.3.2 Formation of carbinolamine

The transfer of the hydroxyl group from the intermediate IM5 to the carbon cation radical on me2 of the histone substrate proceeds via a transition state TS5 (Figure 6C), giving a hemiaminal intermediate IM6 (Figure 6D). The CH_2OH group of the hemiaminal intermediate IM6 then

dissociates to release formaldehyde and monomethylated histone. The succinate molecule dissociates from the active site, allowing for the coordination of the water molecules and subsequently α KG to bind to Fe (II) in the active site to complete the catalytic cycle of the enzyme^{22,49}.

QM/MM PES scan disclosed that hydrogen abstraction occurs via an activation barrier of 18.74 kcal/mol. The energy of intermediate IM5 formed is 2.7 kcal/mol higher than that of IM4, indicating the hydrogen abstraction step is slightly endergonic. The subsequent hydroxyl transfer is associated with an activation barrier of 6.8 kcal/mol. According to the overall reaction profile in the demethylation process (Figure 7, Table S3), we demonstrate that the rate-limiting step for the demethylation process is the hydrogen abstraction from IM4 to yield the IM5 intermediate. This is in good accordance with the experimental measured kinetic data ($k_{\text{cat}} = 5.4 \pm 0.3 \text{ hr}^{-1}$) for the PHF8 enzyme in the catalytic conversion of the dimethylated histone peptide¹⁰, which is corresponding to an activation barrier of 20.1 kcal/mol.

4 CONCLUSIONS

KDM enzymes play an important role in cancer epigenetics and therefore have emerged as promising drug targets in the development of cancer therapies. Despite of their significance in cancer epigenetics, few selective inhibitors have been reported due to the lack of understanding in the reactions these enzymes catalyse. The elucidation of the substrate binding mode and characterization of the electronic structures of the intermediates and the transitions states on the pathway of reactions catalysed by the KDMs would provide us a valuable insight for design of novel selective inhibitors regulating the cancer epigenetics.

In this study, we elucidated the mechanism of the complete reactions catalysed by PHF8, a typical α KG-dependent KDM enzyme. The reaction catalysed by the PHF8 enzymes proceeds with the oxygen binding and followed by activation and decarboxylation. The non-heme iron centre is oxidized from the penta-coordinated resting state Fe(II) sequentially through the Fe(III)-OO[•] superoxide radical, Fe(III)-OO peroxo-bridged species and then to the Fe(IV)=O oxo intermediate. The QM/MM calculations disclosed that the rotation of the oxo group from the Fe(III) to Fe(IV) oxidation states aligns it in a perpendicular orientation relative to the second methyl group (me2) of the dimethylated lysine (K9) of histone peptide, enabling the subsequent reactions to happen. The rate-limiting step of the overall reactions is the hydrogen atom abstraction by Fe(IV)=O oxo intermediate from the second methyl group of the dimethylated histone lysine residue.

The present study reports the complete reaction cycle catalyzed by a non-heme enzyme PHF8, which would provide the structural basis for the binding modes of the substrate, intermediates and transition states involved in the sequential steps of reaction catalyzed by α KG-dependent KDM enzymes and shed light on structure-based design of novel selective inhibitors as potential cancer therapies.

Figure 1. The X-ray structure of PHF8 (PDB: 3KV4) complex with histone and α KG¹⁰. The PHD domain and JmjC domain are drawn by ribbon representation in purple and cyan, respectively. The linker region connecting PHD and JmjC domain substrate peptide are shown in green and histone shown in brown. The trimethylated lysine and dimethylated lysine residues are

shown by stick representation and are labelled as K4 and K9. The α KG substrate is shown by stick and ball representation in red. The divalent zinc and iron metal ions are shown in sphere representation in blue and pink, respectively. (B) The QM/MM reaction setup of PHF8. The solvated residues of the JmjC domain and histone peptide substrate are shown in ribbon representation in blue and red, respectively. (C) The 5C active site of PHF8, (D) The 6C active site of PHF8 formed with triplet oxygen molecule approaching the iron centre.

(D)

(F)

(G)

(H)

Figure 2. The QM/MM optimized critical points for the decarboxylation reaction in PHF8. (A)-(H) The spin densities are shown in a tabular format in the gold color next to the optimized structures. The important distances are shown in Å. (A) 5-coordinated Fe(II) complex R1, (B) 6-coordinated octahedral Fe(III) superoxo radical anion intermediate IM2, (C) transition state TS2 connecting intermediates IM2 and IM3, (D) Bridged peroxo intermediate Fe(III)-OO IM3, (E) transition state TS3 connecting intermediate IM3 and the Fe(IV)=O oxo intermediate IM4, (F) Fe(IV)=O oxo intermediate IM4, (G) The oxo rotation occurring during the decarboxylation of the PHF8 enzyme. The color schemes for IM2, IM3 and IM4 are dark blue, light blue and red, respectively. (H) The QM/MM reaction energy profile for the decarboxylation reaction in PHF8.

Figure 3. The interactions of the second sphere residues in the QM/MM optimized structure of Fe(III)-OO[•] superoxide radical anion intermediate. The distances are in Å.

Figure 4. The β -spin molecular orbitals for the QM/MM optimized structures at $S=2$ spin state. (A) Fe(III)-OO $\cdot^-$ superoxide radical intermediate IM2, (B) TS2, (C) IM3. The MOs were analyzed using the Multiwfn⁶⁷. The isosurfaces of the orbitals were drawn using GPview⁶⁸.

(A)

(B)

Figure 5. 2D scheme of the reaction mechanism of PHF8. (A) Formation of Fe(IV)-oxo intermediate via oxygen activation and decarboxylation reactions, (B) yielding of demethylated histone via demethylation reaction.

Figure 6. The QM/MM optimized critical points for the demethylation reaction in PHF8. (A) transition state associated with the proton transfer from ^{me2}C of Histone lysine to $Fe(IV)=O$ oxo intermediate (B) hydroxylated intermediate, (C) transition state associated with hydroxyl transfer, (D) hemiaminal intermediate. The important distances are shown in Å. The ^{me2}C is the carbon atom of the me_2 and ^{me2}H is the hydrogen atom of me_2 of the dimethylated lysine.

Figure 7. The QM/MM reaction profile for the demethylation reaction catalyzed by PHF8.

ASSOCIATED CONTENT

Supplementary Information

The supplementary Information is attached separately in PDF format and is available free of charge on the ACS Publication website.

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Author Contributions

WS and MH conducted the experiments. MH supervised the project. WS, MH, DQ and TM wrote the paper. All authors read and approved the manuscript.

Notes

The authors declare no competing financial interest.

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