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Catalytic Roles of Coenzyme Pyridoxal-5'-phosphate (PLP) in PLP-dependent Enzymes: Reaction Pathway for Methionine- γ -lyase-catalyzed L-methionine Depletion

Zhe Li^{a,b}, Yunsong Zhao^a, Huifang Zhou^a, Hai-Bin Luo^{a,*}, Chang-Guo Zhan^{b,*}

^aSchool of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou 510006, P.R. China

^bDepartment of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 South Limestone Street, Lexington, KY, 40536

Abstract

Pyridoxal-5'-phosphate (PLP), the active form of vitamin B₆, is an important and versatile coenzyme involved in a variety of enzymatic reactions, accounting for about 4% of all classified activities. However, the detailed catalytic reaction pathways for PLP-dependent enzymes remain to be explored. Methionine- γ -lyase (MGL), a promising alternative anti-tumor agent to conventional chemotherapies whose catalytic mechanism is highly desired for guiding further development of re-engineered enzymes, was used as a representative PLP-dependent enzyme, and the catalytic mechanism for L-Met elimination by MGL was explored at the first-principles quantum mechanical/molecular mechanical (QM/MM) level with umbrella sampling. The QM/MM calculations revealed that the enzymatic reaction pathway consists of 4 stages for a total of 19 reaction steps with five intermediates captured in available crystal structures. Furthermore, the more comprehensive role of PLP was revealed. Besides the commonly known role of “electron sink”, coenzyme PLP can also assist proton transfer and temporarily store the excess proton generated in some intermediate states by using its hydroxyl group and phosphate group. Thus, PLP is participated in most of the 19 steps. This study not only provided a theoretical basis for further development and re-engineering MGL as a potential anti-tumor agent, but also revealed the comprehensive role of PLP which could be used to explore the mechanisms of other PLP-dependent enzymes.

***Correspondence to:** Chang-Guo Zhan, Ph.D., Director, Molecular Modeling and Biopharmaceutical Center (MMBC), Director, Chemoinformatics and Drug Design Core of CPRI, Endowed College of Pharmacy Professor in Pharmaceutical Sciences, Professor, Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 South Limestone Street, Lexington, KY 40536, Phone: 859-323-3943, FAX: 859-257-7585, zhan@uky.edu; luohb77@mail.sysu.edu.cn (H.-B. Luo).

Supporting Information Available

Supporting information including the following contents: RMSD graph of MGL being equilibrated by MM-based MD simulation; Reaction coordinate selection for each step; Comparison between the possible pathways for the deprotonation of L-Met; Proton transfer assisting the cleavage of C^{PLP}-N^{Lys} bond; The average values of electron densities and their fluctuations for steps 6 and 8; Tyr114 is not possible to deprotonate Tyr114; Exploration of the possible pathways for the protonation of MeS⁻; Change of free energy along reaction coordinate; The effect of different basis set to the calculation result; The experimental thermodynamic data for Methionine- γ -lyase-catalyzed L-methionine depletion; The comparison between the calculated and crystal structures; and Coordinates of the QM region of the optimized geometries obtained during the QM/MM calculation. This material is available free of charge via the Internet at <http://pubs.acs.org>.

DECLARATION OF INTERESTS

The authors declare no competing financial interest.

Graphical Abstract

Keywords

Pyridoxal-5'-phosphate; Methionine- γ -lyase; L-methionine depletion; Catalytic mechanism; QM/MM calculation

Introduction

Pyridoxal-5'-phosphate (PLP, the main metabolically active form of vitamin B6) is a versatile cofactor/coenzyme for a variety of enzymes that are involved in the metabolism of amino compounds – mostly amino acids and biosynthesis of biomolecules such as neurotransmitters serotonin, dopamine, epinephrine, norepinephrine, gamma-aminobutyric acid (GABA), and histamine. PLP is so important that it is involved in more than 140 PLP-dependent enzyme activities, accounting for ~4% of all classified activities.^{1–3} The PLP-dependent enzyme activities can be classified into different types of reactions, including transamination, decarboxylation, racemization, elimination, substitution, and retro-aldol or retro-Claisen cleavages.³ The versatility comes from the electrophilicity of PLP: the PLP covalently binds the substrates by forming external aldimines in the beginning of the reactions, and then different types of carbanionic reaction intermediates can be stabilized by PLP through resonance effect.^{3–5} This function of PLP is also known as “electron sink”. Although being such an important coenzyme and many mechanisms were proposed based on experimental results and crystal structures, the detailed reaction pathway of PLP is still not clear. Besides the “electron sink” effect, since almost all kinds of PLP-dependent reactions generate cation and anion intermediates, acid and base groups assisting proton transfer are just as important. In this study, the detailed catalytic reaction pathway of a representative PLP-dependent enzyme, L-Methionine- γ -lyase (MGL), was explored by using the state-of-the-art first-principles quantum mechanical/molecular mechanical (QM/MM) approach with umbrella sampling.^{6–8} The role of PLP as an “electron sink” and other important functional groups involved in the proton transfer were clearly revealed.

MGL belongs to the γ -family of PLP-dependent enzymes that catalyze the α , γ -elimination of L-methionine (L-Met) and its derivatives such as L-homocysteine, L-ethionine, and L-seleno-methionine to α -keto acids, ammonia, and thiols.^{3, 9} Extensive studies have revealed

that many malignant human cell lines and primary tumors have substantial requirements for L-Met,^{10–13} whereas normal cells are relatively resistant to methionine depletion. Tumor cells are unable to survive with L-Met concentration decreasing from ~30 μ M (the normal serum concentration),¹⁴ to a threshold around 5 μ M.^{15–17} Conventional chemotherapeutic agents, that are cytotoxic by means of interfering with cell division, can damage normal cells which divide rapidly and exhibit substantial adverse effects including myelosuppression, mucositis, and alopecia,¹⁸ especially for patients with advanced cancers. As an alternative strategy to conventional chemotherapy, MGL induced L-Met depletion exhibited a broad spectrum of anti-tumor activities^{13–18} and has been studied as a potential treatment for cancer.¹¹ Extensive studies have demonstrated that the growth of various tumors such as glioblastoma,¹⁹ Lewis lung carcinoma,²⁰ and human colon cancer lines²¹ can be successfully inhibited by MGL.

MGL is present only in bacteria and fungi.²² Although L-Met depletion has long been studied and was proven to have many advantageous over conventional chemotherapies,^{23–25} The use of MGL was set back by its poor stability in serum and unwanted immunogenicity in humans.^{26–27} To overcome these challenges, one promising alternative is to use re-engineered structurally homologous PLP-dependent eukaryote enzymes, *e.g.* human cystathionine- γ -lyase (hCGL), instead of MGL from bacterial sources.²⁸ Stone, *et al.* reported that the re-engineered PEGylated hCGL enzyme showed promising results since the immunogenicity and the instability of this enzyme have been greatly decreased.^{28–29} However, the re-engineering hCGL has both lower activity and lower binding affinity against L-Met than the most studied *Pseudomonas putida* sourced MGL.^{28–29} Hence, structurally homologous enzyme mutants with low immunogenicity, high stability, and high activity against L-Met is highly desired for further development of the anti-tumor enzyme.

Understanding of the catalytic mechanism of MGL could lead to important insights for the rational design of homologous eukaryote enzymes (especially human sourced enzymes) with high catalytic activity and stability. Besides, the MGL-catalyzed α , γ -elimination reaction is also one of the most complicated PLP-dependent catalytic reactions, and thus the study can also provide a more comprehensive understanding of the role of PLP. According to our first-principles QM/MM calculations, this enzymatic reaction pathway consists of 4 stages for a total of 19 reaction steps. Proton transfer is very important during the α , γ -elimination reaction process and is involved in 15 out of the 19 reaction steps. As expected, the “electron sink” effect, which is the commonly known function of PLP, is quite significant. However, to our surprise, the PLP can not only act as an “electron sink”, but also assist proton transfer and temporarily store the excess proton generated in some intermediate states by using its hydroxyl and phosphate groups that further assist the reaction. The detailed catalytic reaction pathway provides important insights into the key amino-acid residues for the catalytic reaction and a solid basis for further development of re-engineered MGL enzymes. The new mechanistic insights could also help to explore the role of PLP in other PLP-dependent enzymes.

Methods

Preparation of the simulation system

Pseudomonas putida sourced MGL (pMGL) exhibits higher catalytic activity, lower K_M , and relatively higher k_{cat} values compared to the enzymes from other sources and, thus, was selected for therapeutic development.³⁰ Hence, in this study, the simulation system of MGL was prepared based on the crystal structure of the most studied pMGL (PDB code: 2O7C).³¹ The proper function of MGL depends on the so-called catalytic dimer in which the active site contains the residues from two neighboring subunits, and the structure of the catalytic dimer was kept when preparing the simulation structure. The coenzyme PLP was covalently linked to Lys211 by forming an internal Schiff base. The protonation state of PLP was determined based on the NMR experimental results³² and the surrounding hydrogen-bond network. The binding mode of substrate L-Met was determined based on the crystal structure of C116H mutated MGL-Met complex (PDB code: 3VK3).³³

Molecular dynamics (MD) simulations were first applied to equilibrate the constructed MGL-Met complex by using the AMBER 16 package.^{34–36} First, the partial atomic charges of the Lys211-PLP Schiff base residue and the substrate L-MET were calculated by using the Gaussian 03 program³⁷ at the HF/6–31G* level, and the antechamber program³⁸ was used to fit the restricted electrostatic potential (RESP) and assign generalized amber force field (GAFF) parameters to Lys-PLP and L-MET. The AMBER99SB force field was applied to the protein. The protonation states of all the amino-acid residues in the protein were determined by the Propka program,^{39–42} and the hydrogen-bond networks were further carefully checked. The prepared protein-coenzyme-substrate complex was solvated in to a truncated octahedral box of TIP3P water molecules⁴³ with the surface of the box at least 8 Å away from any atom in the complex, and 15 Na⁺ ions were added to neutralize the system. The resulting simulation system contains 70268 atoms. The system was first energy-minimized by four steps of energy minimization, and each step contained 2500 cycles of the steepest descent energy minimization and 2500 cycles of conjugated gradient energy minimization. Then, the system was heated from 0 K to 300 K in 50-ps MD run in an NVT (constant volume and temperature) ensemble and further equilibrated for 100-ps MD run in an NPT (constant pressure and temperature) ensemble at a pressure of 1 atm. Finally, 8 ns of the production MD run was performed in an NPT ensemble with the temperature set to 300 K and pressure set to 1 atm. The SHAKE algorithm^{42, 43} was applied to constrain all the bonds containing H atoms, and the simulation time step was set to 2 fs. The cutoffs for the van der Waals (vdW) and short ranged electrostatic interactions were both set to 10 Å. Along with the periodic boundary condition (PBC), the particle mesh Ewald (PME) method⁴⁴ was used to calculate the long-range electrostatic interactions. The MD trajectories became stable after 2 ns, and the resulting snapshot after 4 ns was used for the subsequent QM/MM calculations.

Born-Oppenheimer First-principles QM/MM Molecular Dynamic Simulations

The initial structure for the QM/MM calculations was built based on the snapshot after 4 ns of classical MM-based MD simulations. The solvent molecules beyond 30 Å from the center of the L-MET molecule were removed and the resulting QM/MM system contained a total

of 22500 atoms. As shown in Figure 1, in this system, substrate L-Met, Tyr114, Lys211, coenzyme PLP, and a water molecule were partitioned to the QM subsystem and the rest atoms were partitioned to the MM subsystem. The QM subsystem was treated with the B3LYP functional using the 6–31G* basis set. The QM/MM boundary was treated by using the pseudobond approach.^{45–48} All other atoms in the MM subsystem were treated with the same force field used in the classical MD simulations. This QM/MM strategy has been successfully employed to the study of different kinds of enzymes.^{6–8} The spherical boundary condition was applied so that atoms more than 25 Å away from the center of the sphere were fixed. The 18 and 12 Å cutoffs were employed for electrostatic and van der Waals interactions, respectively. There was no cutoff for electrostatic interactions between the QM and MM subsystems.

The prepared system was firstly optimized and ~5-ps QM/MM MD simulations were carried out to equilibrate the system. Then, the QM/MM geometry optimizations were used to map out a minimum energy path with the reaction coordinate deriving method.⁴⁷ As the reaction consists of nearly ~20 steps and most of the steps involve proton transfer, the possible reaction pathways for proton transfer were carefully explored and the one with the lowest energy barrier was considered as the most possible pathway for this catalytic reaction.

Along the minimum-energy path, ~200 windows were created for each step of the reaction. The MM subsystem for each window was further equilibrated by 500-ps free energy perturbation calculations with the QM subsystems frozen. The frozen QM subsystem is the “perturbation” in the FEP method, and this calculation is also performed at the QM/MM level. The aim of this step is only to get an equilibrated structure for the remaining studies. Thus, we did not use the energy difference generated by the FEP method because the further QM/MM MD simulations performed with umbrella sampling is a higher-level calculation in theory and is likely to give a more accurate result. Based on the resulting structures, 25-ps QM/MM MD simulations with umbrella sampling were performed for each reaction window. The simulation time step was set to 1 fs. Harmonic biasing potentials were applied for each reaction window with force constants of 40 to 80 kcal/(mol·Å²). The Berendsen thermostat method⁴⁹ was used to control the system temperature at 300 K, and the Beeman algorithm⁵⁰ was used to integrate the Newton equation of motion. The configurations from the last 20 ps of the trajectory of each window were used for further unbiased potential of mean force (PMF) calculation by using the weighted histogram analysis method (WHAM).^{51–52} All the first-principles QM/MM calculations were performed with the modified Q-Chem⁵³ and Tinker⁵⁴ programs.^{45–48} During the whole catalytic reaction, there are three products generated. After each product is released, before proceeding to the next step, the product from the system, and replaced the original position with water molecules. Based on the newly prepared structure, 500 ps MD simulations was performed for the MM subsystem with the QM subsystem frozen to equilibrate the system, and the QM/MM MD simulations with umbrella sampling were performed based on the method described above. After all the calculations have been done, we combined the PMF results of all the steps together in order to obtain an overall understanding of the kinetic and thermodynamic properties.

Results and Discussions

Being firstly equilibrated by the MM-based MD simulations, as shown in the RMSD graph given in supporting information (SI section S1), the trajectory of the catalytic dimer of MGL became stable after 2 ns, and the snapshot after 4 ns was used for further QM/MM calculations. Based on the minimum-energy pathway mapped out by the reaction coordinate deriving method and further QM/MM MD simulations with the umbrella sampling, the detailed reaction pathway starting from the resting state followed by catalytic steps that degrade L-Met and finally returning to the resting state was revealed. In general, MGL-catalyzed L-Met degradation contains 19 reaction steps in a total of 4 stages, including 1) transamination reaction of PLP, 2) E1cB elimination of MeSH, 3) tautomerization and hydrolysis, and 4) return to the resting state. Different possible reaction pathways were explored, and especially, since many steps contain proton transfer, the possible proton donors/acceptors for each step were carefully studied. The free energy profiles and structural details for each reaction stage were described below.

Stage 1. Transamination reaction of PLP

The QM/MM calculations starting from the MM-based MD equilibrated enzyme-substrate (ES) complex were performed at the B3LYP/6–31G*: AMBER level. As shown in Figure 1, atoms in blue were treated by the QM method at the B3LYP/6–31G* level, atoms in black were treated by the MM method, and atoms in red were treated as pseudo atoms using the pseudobond approach. The QM region includes a water molecule, substrate L-Met, Tyr114, Lys211, and coenzyme PLP.

The computational results revealed that the first stage of the reaction (transamination reaction of PLP) consists of 5 reaction steps, and the structures and free energy details are shown Figure 2. Before the reaction begins, MGL exists in its resting state and coenzyme PLP is covalently linked to Lys211 and forms a Schiff base. Since the catalytic reaction happens at moderate pH, substrate L-Met exists in a zwitterion state (containing both a negatively charged carboxylate and a positively charged α -ammonium group). In the first step, the positively charged protonated α -amino group in L-Met is deprotonated in order to further nucleophilically attack C_{PLP} of the Schiff base. Based on the structure, either a water molecule or O_{hyd} in PLP could act as a proton acceptor, resulting in three possible deprotonation pathways. Energy changes for different pathways were studied (details are given in SI section S3), and QM/MM calculations revealed that O_{hyd} can act as a better proton acceptor than a water molecule. As a result, in the first step, L-Met is deprotonated with the proton transferred to O_{hyd} of PLP, generating an OH group with an energy barrier of 12.5 kcal/mol.

In the second step, the electron pair from the N_{Met} atom of the α -amino group in L-Met can readily attack C_{PLP} of the Schiff base formed by PLP and Lys211. To study the energy barrier of this step, we simply defined the reaction coordinate for this step to be the distance between N_{Met} and C_{PLP}. At the QM/MM level, this step can happen almost spontaneously. After this step, the system becomes a tetrahedral geminal diamine intermediate. In the current state, as discussed in SI section S4, the C_{PLP}-N_{Lys} bond is still too strong for cleavage. However, in the following steps 3 and 4, a proton transfers from N_{Met} to N_{Lys},

making the C_{PLP}-N_{Met} bond stronger and N_{Lys} of Lys211 a better leaving group. Although N_{Met} is very close to N_{Lys} in space, direct transfer of proton from N_{Met} to N_{Lys} via a 4-members ring transition state is not favored due to the high energy barrier for the 4-members ring transition state. According to the structure, the hydroxyl group (O_{hyd}) generated during step 1 is the nearest possible acid to both N_{Met} and N_{Lys} that could assist the proton transfer between the two N atoms. With the assistance of the OH group, as the proton transfer begins, a proton first transfers from O_{hyd}H to N_{Lys} in step 3, and then the proton in N_{Met} further transfers back to O_{hyd} in step 4. In the next step, since N_{Lys} becomes a better leaving group after the proton transfer, the bond between N_{Lys} and C_{PLP} can be readily cleaved. The distance between N_{Lys} and C_{PLP} was chosen as the reaction coordinate for this step, and the calculated energy barrier for this step is only 3.6 kcal/mol. Cleaving the N_{Lys}-C_{PLP} bond results in the dissociation between Lys211 and PLP, and a new external Schiff base is generated between L-Met and PLP after this reaction step.

Stage 2. E1cB elimination of MeSH

After steps 1–5, as shown in Figure 3, the reaction system reaches a state in which a new Schiff base between L-Met and PLP is formed, and the pyridine ring of PLP and N_{Met} contains two positive charges. The H atom on C_α position becomes very acidic since the carbanion intermediate is strongly stabilized through delocalization effect. As the amino group of Lys211 is the only nearest possible base, the acidic proton on C_α transfers to the amino group of Lys211 in step 6. In order to show the delocalization effect to the reaction, the electron density distributions on different atoms were evaluated, and the atoms with the most significant change in electron densities are given in Figure 4. As seen from the electron density change for step 6, after the H_α atom transferred from C_α to the N_{Lys} atom, C_α became a carbanion intermediate, but the charge of this C_α atom only decreased slightly by about 0.15. On the other hand, charges on C₁, C₂, C₃, and N_{PLP} also decreased simultaneously by 0.1–0.2, which means that the resulted negative charge was evenly distributed to the PLP ring through delocalization effect, and the carbanion was stabilized by the PLP ring. Due to the stabilization by delocalization, this step is very favorable thermodynamically, and the deprotonation of this C_α atom further expands the delocalized area of the conjugation π -bond. The remaining positive charge increases the acidity of the proton on C_β through delocalization. C_β can be readily deprotonated with the assistance of a proper base. The possible base that can assist the deprotonation of the C_β was carefully studied. Since the OH group of Tyr114 is close to the C_β in space, there is a possibility that Tyr114 serves as a base. There are three different possible ways in which Tyr114 could accept a proton (see SI Section S6). However, all the three possible pathways have very high energy barriers and are not possible for this reaction. The only left amino-acid residue that could possibly serve as a base is Lys211. Although N_{Lys} is currently in an ammonium ion form, we found that the phosphate group is very close to N_{Lys} of Lys211 in space, and a proton in N_{Lys} can rapidly transfer to the phosphate group with a very low energy barrier of 1.3 kcal/mol. After a proton on N_{Lys} is “stored” on the phosphate group in step 7, Lys211 can serve as a base involved in the deprotonation reaction of the C_β. Thus, in step 8, the C_β atom is deprotonated with the proton transferred to Lys211, and the carbanion intermediate is further stabilized by the positive charge on the PLP ring through delocalization effect, which can be seen from the electron density change that the negative charge was transferred

to N_{Met} and C_1 atoms (Figure 4, SI section S5, Table S2, and S3). After C_β is deprotonated, the resulted carbanion intermediate can further cause E1cB elimination reaction. Following the E1cB elimination, the MeS group, which is connected to the adjacent carbon atom C_γ , can be expelled with the assistance of the carbanion intermediate. Thus, in step 9, MeS^- is eliminated with an energy barrier of 12.1 kcal/mol. In step 10, after the MeS^- is generated, the proton on Lys211, Tyr114, or the phosphate group of PLP could possibly protonate it. All the three different pathways are discussed in SI Section S7, and the proton on the phosphate group can spontaneously transfer to the leaving MeS^- group, generating the first product methyl mercaptan. In the final step of this stage, as the phosphate group is just deprotonated in step 9, the proton on the ammonium group of Lys211 transfers to it since the basicity of phosphate group is stronger than the amino group on Lys211 according to the QM/MM calculations. The deprotonated N_{Lys} in Lys 211 can further act as a nucleophilic agent and attack the C_{PLP} atom in further reaction steps.

Stage 3. Tautomerization and hydrolysis

Structures and free energy details of this step are shown in Figure 5. After stage 2, methyl mercaptan as the first product is expelled from C_γ , and C_γ exists in an unsaturated state. In the beginning of stage 3, C_γ is protonated. After comparing different possible proton donors, the proton on the phosphate group of PLP can transfer to this carbon with the lowest energy barrier, and this step can be classified as a tautomerization reaction within the external Schiff base. After tautomerization, the delocalized area of the PLP ring contains two positive charges, and both two positive charges can increase the electrophilic activity of the C_{PLP} atom. Thus, in the next step (step 12), the N atom of the Lys211 side chain can easily attack C_{PLP} with a very low energy barrier of 1.7 kcal/mol and, as a result, a new tetrahedral geminal diamine intermediate is generated.

In the next three steps of this stage, a hydrolysis reaction happens, resulting in the cleavage of the $C_\alpha-N_{Met}$ bond and the generation of the second product α -Ketobutyric acid. The detailed mechanism is quite similar to the traditional acid-catalyzed addition-elimination mechanism. First, in step 13, the hydroxyl group of Tyr114 can act as an acid to activate the reaction. After different reaction coordinates were examined, the proton on the hydroxyl group of Tyr114 can transfer to C_β with the assistance of a water molecule, which greatly increases the electrophilicity of C_α . In step 14, a water molecule can act as a nucleophilic agent that add to the highly electrophilic C_α . Since in the previous step the hydroxyl group of Tyr114 is deprotonated and turned into its conjugate base, a proton in the water molecule could simultaneously transfers to the base form of Tyr114 as the nucleophilic addition reaction happens, which further increases the nucleophilicity of the water molecule and decreases the reaction barrier. In step 15, the proton on O_{Wat} transfers to N_{Met} with the assistance of a water molecule, which increases the stability of the $C_\alpha-O_{Wat}$ bond and decreases the stability of the $C_\alpha-N_{Met}$ bond. The $C_\alpha-N_{Met}$ bond is cleaved simultaneously as the proton is transferred, and the second product α -Ketobutyric acid is generated in this step.

Stage 4. Return to the resting state of the enzyme

After the first 3 stages, the original L-Met has been cleaved into 3 parts (the first product MeSH, the second product α -Ketobutyric acid, and an amino group linking to the Lys211–

PLP conjugate). In the final stage, the amino group will be expelled and the whole enzyme will return to its resting state. In the first two steps of this stage, the proton on O_{hyd} transfers to N_{Met} , and a proton on N_{Lys} then transfers back to O_{hyd} , which increases the leaving ability of the protonated amino group (N_{Met}) and stabilizes the $O_{\text{hyd}}-N_{\text{Lys}}$ bond. After the two steps of proton transfer, N_{Lys} contains a lone pair of electrons that can further attack C_{PLP} and the NH_3 group is expelled from the tetrahedral geminal diamine intermediate. Finally, in the last step, the proton on O_{hyd} transfers to the amine group, and the whole enzyme returns to the resting state. The structures and detailed free energy profiles are given in Figure 6.

Overall feature of the catalytic reaction pathway

In summary, based on the QM/MM calculations, the whole catalytic reaction pathway follows several (addition/proton transfer)-elimination reaction cycles (Figure 7). When the enzyme exists in its resting state, coenzyme PLP forms an internal Schiff base with Lys211. The first stage of this reaction is an addition-elimination reaction: substrate L-Met is added to C_{PLP} , and N_{Lys} on Lys211 is eliminated subsequently. As a result, Lys211 is replaced by L-Met, and the external Schiff base between PLP and L-Met is formed. The second stage follows a deprotonation-elimination pathway: N_{Lys} and the phosphate group act as bases and deprotonate both C_{α} and C_{β} . The resulting deprotonated carbanion intermediate are strongly stabilized by the PLP-Met complex and the stabilized carbanion intermediate further causes the elimination of MeS^- following the E1cB mechanism. Stage 3 is mainly a hydrolysis reaction with the nucleophilic addition-elimination process. In the beginning of stage three, both C_{PLP} and N_{Met} are involved in the big conjugation π -bond. During the first several steps of this stage, N_{Lys} is added back to C_{PLP} , and C_{β} is protonated, which breaks the conjugation π -bond connecting C_{PLP} and PLP ring and increases the electrophilicity of the C_{α} atom. The nucleophilic addition then occurs with a water molecule attacking C_{α} . As a proton on O_{wat} transfers to N_{Met} , N_{Met} is simultaneously eliminated from the C_{α} atom, resulting in the generation of the second product α -Ketobutyric acid. The final stage of the reaction is a proton transfer-elimination process. The only part of the substrate linked to the Lys-PLP complex is an amino group (N_{Met}). The proton on N_{Lys} transfers to the amino group (N_{Met}), which increases the leaving ability of N_{Met} , and the protonated amino group (N_{Met}) is eliminated subsequently, generating an ammonium ion. After this step, the enzyme returns to its resting state, and L-Met is cleaved into three parts during this whole reaction, including methyl mercaptan, α -Ketobutyric, and ammonium ion.

PLP acts as more than just an “electron sink” during the reaction

During the cleavage procedure of L-Met, there are mainly two types of chemical reactions. One type is nucleophilic addition and elimination reaction which breaks down the connecting bond (including the C_{γ} -S bond and the $C_{\alpha}-N_{\text{Met}}$ bond) inside L-Met, and the other type of the reactions is proton transfer which can neutralize the temporarily generated positive or negative charges or weaken the bond of the leaving groups. Since the pyridoxal ring of PLP containing a positive charge can withdraw electrons and stabilize the corresponding intermediate states, PLP can effectively facilitate the addition-elimination reactions during the catalysis. The electron withdraw effect is also essential for other enzymes involved in the metabolism of amino acids which use PLP as their coenzyme, and

thus PLP is commonly known as an “electron sink” in the proposed catalytic mechanisms. However, in this study, we have demonstrated that coenzyme PLP acts as more than just an “electron sink” and is also involved in the proton transfer reactions which can neutralize the generated carbanions and weaken the bond of the leaving groups. As the proton transfer is so important such that it is involved in 15 out of the 19 reaction steps, future studies on the role of PLP in other PLP-dependent enzymes should also account for its additional role in protein transfer.

The important roles of PLP as an “electron sink” in the addition-elimination reactions

The pyridoxal ring contains a positive charge and, by delocalization through conjugated π -bond, can increase the electrophilicity of the reaction center and stabilize the generated carbanion intermediates and, thus, facilitate the addition-elimination reactions. The effect of this pyridoxal ring plays a key role in various reaction steps. In the beginning of stage 1, the transamination reaction, both the pyridoxal ring and N_{Lys} contain a positive charge which greatly increases the electrophilicity of C_{PLP} , and substrate L-Met can be added to C_{PLP} with a very low energy barrier (step 2). In stage 2, after the external Schiff base between L-Met and PLP is generated, C_{α} and C_{β} are sequentially deprotonated, and the resulted carbanions are strongly stabilized by the two positive charges located on the pyridoxal ring (step 6) and N_{Met} on the Schiff base (step 7) through delocalization effect of the π -bond. The stabilized carbanion intermediate further causes the elimination of MeS^- by the E1cb mechanism, and the resulted positive charge further transfers to the pyridoxal ring (step 9). In the final stage, as the bond between N_{Met} and C_{PLP} is cleaved, the positive charge on the pyridoxal ring greatly weakens the $N_{Met}-C_{PLP}$ bond and increases the leaving ability of N_{Met} (step 18). Among these reaction steps mentioned above, the deprotonation reaction of a carbon in step 6 and the cleavage of C-S bond in step 9 are normally quite difficult to happen. However, with the assistance of the “electron sink” which is also shown in Figure 8, the generated negative charge by carbon deprotonation in step 6 can transfer from the carbanion C_{α} to the pyridoxal ring, and then the negative charge can again transfer back to C_{γ} after the cleavage of $C_{\gamma}-S$ bond in step 9. The mobility of negative charges plays important roles in other PLP-dependent enzymes.

The important roles of PLP in proton transfer

Proton transfer is another important type of reaction and involved in 15 out of the 19 reaction steps. Acids and bases are thus quite important in assisting the proton transfer reactions. Since in most PLP-dependent enzymes, including MGL, the cofactor PLP is covalently linked to Lys in the resting state, considering PLP and Lys211 as a whole, groups on PLP and Lys211 involved in most of the proton transfer reaction steps. Important groups involved in the proton transfer include the hydroxyl group (O_{hyd}) and the phosphate group on PLP, the amino group (N_{Lys}) on Lys211, and the hydroxyl group (O_{Tyr}) on Tyr114.

The hydroxyl group (O_{hyd}) on PLP is mainly involved in the proton transfer between N_{Lys} and N_{Met} atoms, which can be considered as a “proton shuttle”. In the beginning of the reaction (step 1), the hydroxyl group in PLP is in its deprotonation state and, thus, can act as a base to deprotonate the amino group (N_{Met}) of substrate L-Met, making L-Met possible to initiate nucleophilic attack on C_{PLP} . In the subsequent steps, after L-Met, PLP, and Lys211

form the tetrahedral geminal diamine intermediate, the hydroxyl group O_{hyd} is the nearest acid to both N_{Met} and N_{Lys} so as to efficiently assist the proton transfer from N_{Met} to N_{Lys} in steps 3 and 4 as a bridging proton donor/acceptor. The proton transfer from N_{Met} to N_{Lys} strengthens the $C_{\text{PLP}}-N_{\text{Met}}$ bond and weakens the $C_{\text{PLP}}-N_{\text{Lys}}$ bond, which results in the replacement of Lys211 by substrate L-Met. In steps 16 and 17, the proton transfers from N_{Lys} to N_{Met} with the assistance of hydroxyl group O_{hyd} , resulting in the elimination of the final product ammonia.

The phosphate group on PLP, together with the amino group N_{Lys} on the side chain of Lys211, can act as a dual proton acceptor/donor which can accept up to two protons or give out up to two protons. The proton can transfer between the amino groups N_{Lys} and the phosphate group O_{pho} almost freely since the calculated free energy change is very small (< 2 kcal/mol) and the calculated energy barrier is very low (< 2 kcal/mol). Thus, the dual proton acceptor/donor can first act as bases to deprotonate C_{α} and C_{β} then as acids to protonate the first product MeS^- and C_{γ} . Since N_{Lys} is close to both C_{α} and C_{β} , in steps 6 to 8, C_{α} is first deprotonated to N_{Lys} , the proton is further transferred to the phosphate group, and N_{Lys} again acts as a base to deprotonate C_{β} . However, when it comes to the protonation of MeS^- and C_{γ} in steps 8 to 10, as the O_{pho} atom is closer to the S and C_{γ} atoms, the proton transfers from the phosphate group to MeS^- , a proton further transfers from N_{Lys} to the phosphate group, and the phosphate again acts as an acid to protonate C_{γ} . The dual proton acceptor/donor formed by the phosphate group on PLP and the amino group (N_{Lys}) on the side chain of Lys211, together with the “proton shuttle” effect of hydroxyl group (O_{hyd}) on PLP, should also be considered in other PLP-dependent enzymes.

Besides the functional groups mentioned above, the hydroxyl group (O_{Tyr}) on Tyr114 is mainly involved in the hydrolysis reaction during steps 13 to 15 which generates the second product α -Ketobutyric acid. This hydroxyl group-protonated C_{β} and the resulted O_{Tyr} anion further assist the nucleophilic attack of C_{α} by a water molecule.

The computational results are consistent with the experimental catalytic rate constant, thermodynamic data, mutagenesis, and crystal structure results

The overall free energy profile of the catalytic reaction is depicted in Figure 9. According to this calculated free energy profile, the protonation of C_{β} by the hydroxyl group (O_{Tyr}) on Tyr114 in step 13, is the rate-determining step, and the highest energy barrier of this reaction is the energy difference between intermediate 10 to the transition state of step 13, which is 17.6 kcal/mol. According to the experimental catalytic rate constant k_{cat} value (33.4 s^{-1})⁵⁶ for the elimination reaction, the highest energy barrier of this reaction should be 16.2 kcal/mol and is very close to the calculated energy barrier of 17.6 kcal/mol. Several experimental results revealed that the mutation of Tyr114 resulted in a significant decrease of the enzyme activity,⁵⁷ which is also consistent with our calculation result that Tyr114 is involved in the rate-determining step.

In order to further justify our results, we tried to find the experimental thermodynamic data of MGL catalyzing L-Met into α -keto acid, ammonia, and thiol, but only the kinetic properties associating with the reaction rate are available. However, we can calculate the thermodynamic data for this reaction by using the standard Gibbs free energy of formation

(ΔG^0) and solvation free energy (ΔG^0_{solv}) for each reactant and product. As discussed in detail in SI Section S10, the change of Gibbs free energy for the whole reaction ΔG_{total} (calculated by ΔG^0 and ΔG^0_{solv}) is -27.37 kcal/mol. The ΔG of -30.4 kcal/mol from the QM/MM calculation is very close to -27.37 kcal/mol. The difference between the two values may also come from the roundup error for the 19 steps calculation and may also come from lacking consideration of the substrate binding and product releasing processes which can be further explored in the future works. Considering that proton transfer plays important roles in the whole reaction, we also used a larger basis set 6-31G** to examine the basis set effects on the calculation results. As discussed in detail in SI section S9, the energetic results associated with the 6-31G** basis set are reasonably close to the corresponding energetic results associated with the 6-31G* basis set. In particular, the change on the overall ΔG of the reaction was only 0.88 kcal/mol, and the highest energy barrier is still associated with the transition state in step 13. So, the change of the basis set does not affect the qualitative conclusions of this study.

During the reaction, 6 structures with the highest energy barriers are the ES complex (12.5 kcal/mol), intermediate 5 (10.6 kcal/mol), intermediate 7 (15.8 kcal/mol), intermediate 8 (12.1 kcal/mol), intermediate 10 (14.7 kcal/mol), and intermediate 12 (17.4 kcal/mol), respectively. Although no crystal structure for the intermediate state is available for the wild type *Pseudomonas putida* sourced MGL that is used in this work, for the *Entamoeba histolytica* sourced MGL, 5 out of these 6 intermediate structures mentioned above were captured in the reported crystal structures,⁵⁵ which are all indicated on Figure 9 by green asterisks. The 5 intermediate structures with PDB ID are the ES complex (3AEN), intermediate 5 (3AEJ), intermediate 7 (3AEM), intermediate 8 (3AEO), and intermediate 10 (3AEL)⁵⁵, respectively. The superposition between crystal structures and the computational structures are given in SI section S11. Although there has been no crystal structure of intermediate 12 captured, the subsequent reaction step (step 13) has been confirmed to be the rate-determining step by both the previous experiments⁵⁷ and our QM/MM calculations. It is reasonable to understand that reaction step 13 is associated with the highest energy barrier.

Overall, the conclusion of this work is justified by available kinetic data, thermodynamic data, mutagenesis results, and crystal structures.

Conclusion

In this work, we studied the detailed catalytic mechanism of a representative PLP-dependent enzyme MGL eliminating L-Met by umbrella sampling at the first-principles QM/MM level. The calculation results revealed that the whole catalytic reaction consists of 4 stages for a total of 19 steps. The 4 stages include (1) transamination reaction of PLP, (2) E1cB elimination of methyl mercaptan, (3) tautomerization and hydrolysis, and (4) return to the resting state of the enzyme. After the catalytic reaction cycle, the enzyme returns to its initial resting state and substrate L-Met is cleaved into three products including the first product methyl mercaptan, the second product α -Ketobutyric acid, and the third product ammonia. According to the QM/MM calculations, Tyr114 is involved in the rate-determining step, which is consistent with the experimental results of site-directed mutagenesis. Five

intermediates with relatively higher energy barriers were captured in reported crystal structures, and the calculated highest energy barrier (17.6 kcal/mol) is reasonably close to the experimental value of 16.2 kcal/mol. Further, in addition to the commonly known role of PLP as an “electron sink”, PLP can also assist proton transfer and temporarily store the excess proton generated in certain intermediate states by using its hydroxyl and phosphate groups together with the amino group of Lys211 side chain. These mechanistic insights will not only provide a theoretical basis for further development and re-engineering of MGL as a potential anti-cancer agent, but also help to explore the comprehensive role of PLP in PLP-dependent enzymes in general.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Partition of the QM/MM system. In the 2D structure, the atoms in the QM region were depicted in blue and the pseudo atoms were depicted in red. In the 3D structure, the QM region was denoted by ball and stick models.

Figure 2.

The first stage of the reaction (transamination of PLP). The 2D, 3D structures, and the free energy curve correspond to each other. Red lines indicate the separations of different steps, and the horizontal axis is the “generalized reaction coordinate” the details of which are given in SI Section S2, which is also the case for Figures 3, 5, 6, and 8.

Figure 3.

The second stage of the reaction (E1cB elimination of MeSH). The 2D, 3D structures, and the free energy curve correspond to each other.

Figure 4.

Changes of electron density distributions for step 6 and step 8 during umbrella sampling.

The fluctuations are not shown on the figure but are given in SI section S5, Table S2, and S3.

Figure 5.

The third stage of the reaction (tautomerization and hydrolysis). The 2D, 3D structures, and the free energy curve correspond to each other.

Figure 6.

The fourth stage of the reaction (returning to the resting state). The 2D, 3D structures, and the free energy curve correspond to each other.

Figure 7.

Summary of the catalytic cycle of MGL with 4 reaction stages. These 4 stages consist of 19 steps in total, and the enzyme returns to its resting state at the end.

Figure 8.

The charge transfer on the N_{PLP} atom of the pyridoxal ring for steps 6 and 9. (A) and (B) are the histogram representations of the charge distribution on N_{PLP} for different reaction windows in the Umbrella Sampling calculation. The legend step6_before indicates the reaction window before step 6 happened, and step6_after indicates the reaction window after step 6 happened. Step9_before and step9_after are named in the same manner. There are 2500 samples (the time interval between each sample is 10 fs) in each window, and Y-axis means the number of samples that the charge of N_{PLP} lies within the certain range indicated by X-axis.

Figure 9.

The PMF curves of different steps are piecing together to give the overall free energy profile for the MGL-catalyzed L-Met elimination reaction. The free energy (in kcal/mol) of each intermediate and transition state is given in red numbers. Error was estimated based on bootstrapping method. It should be noted that the reaction coordinates for different reaction steps were different and, hence, the free energy of the last step of one PMF is not necessarily the same as that of the first step of the next PMF. Assuming that they are, the PMF curves can be pieced together to show the overall free energy of the entire reaction process. As the reaction coordinates were used for different reaction steps, and the horizontal axis is the “generalized reaction coordinate” each of which corresponds to a specific reaction coordinate as discussed in SI section S2. There are two gaps for the overall free energy profile, one between stages 2 and 3 and the other between stages 3 and 4. For each gap, a product was released (after stage 2 or 3) and removed from the system with a water molecule added to occupy the same position. Assuming there is no free energy change upon product release and water uptake, the relative free energies of the system before and after the product release are approximately the same. The detailed data of this figure is given in SI section S8. The intermediate structures marked by green asterisks were captured by the known crystal structures of *Entamoeba histolytica* sourced MGL.⁵⁵