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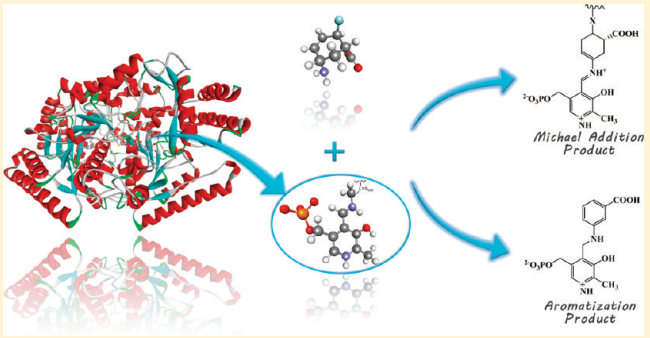
Theoretical Study on HF Elimination and Aromatization Mechanisms: A Case of Pyridoxal 5′ Phosphate-Dependent Enzyme

Hatice Gökcan and F. Aylin Sungur Konuklar*

Informatics Institute, Computational Science and Engineering Programme, Istanbul Technical University, Ayazağa Campus 34469, Maslak, Istanbul, Turkey

Supporting Information

ABSTRACT: Pyridoxal 5-phosphate (PLP), the phosphorylated and the oxidized form of vitamin B6 is an organic cofactor. PLP forms a Schiff base with the ϵ-amino group of a lysine residue of PLP-dependent enzymes. γ-Aminobutyric acid (GABA) aminotransferase is a PLP-dependent enzyme that degrades GABA to succinic semialdehyde, while reduction of GABA concentration in the brain causes convolution besides several neurological diseases. The fluorine-containing substrate analogues for the inactivation of the GABA-AT are synthesized extensively in cases where the inactivation mechanisms involve HF elimination. Although two proposed mechanisms are present for the HF elimination, the details of the base-induced HF elimination are not well identified. In this density functional theory (DFT) study, fluorine-containing substrate analogue, 5-amino-2-fluorocyclohex-3-enecarboxylic acid, is particularly chosen in order to explain the details of the HF elimination reactions. On the other hand, the experimental studies revealed that aromatization competes with Michael addition mechanism in the presence of 5-amino-2-fluorocyclohex-3-enecarboxylic acid. The results allowed us to draw a conclusion for the nature of HF elimination, besides the elucidation of the mechanism preference for the inactivation mechanism. Furthermore, the solvent phase calculations carried out in this study ensure that the proton transfer steps should be assisted either by a water molecule or a base for lower activation energy barriers.



1. INTRODUCTION

Pyridoxal 5-phosphate (PLP)-dependent enzymes play a prominent role in many biological activities. The studies on PLP-dependent enzymes mostly concentrate on their inactivation mechanisms and development of inhibitors.[1−4] Particularly, fluorine-containing substrate analogues have attracted much attention in recent years as potential mechanism-based inactivators. In medicinal chemistry, the fluorine is explored as hydrogen mimic because of it being the first halogen atom next to hydrogen that can be bound carbon covalently.[5] HF substitution in substrate analogues of PLP-dependent enzymes usually occurs at the β position of amines. This enhances the possibility of covalent binding to the enzyme active site, thus facilitating the inactivation.

GABA aminotransferase (GABA-AT, E.C. 2.6.1.19), a member of subfamily II of α-family of PLP-dependent enzymes, degrades γ-aminobutyric acid (GABA) to succinic semialdehyde.[6,7] Reduction of GABA concentration in the brain causes convolution besides several neurological diseases such as epilepsy, Parkinson's disease, Huntington's chorea, and Alzheimer's disease where an increase in the concentration of GABA in the brain has an anticonvulsant effect.[8−11] Because of the inability of GABA to cross the blood brain barrier, substrate analogues are used to inactivate the GABA-AT.[12−17] The natural product gabaculine is a highly potent irreversible inactivator of GABA-AT and belongs to a class of highly specific irreversible enzyme inhibitors.[18,19] However, gabaculine was found toxic for therapeutic applications because of the possibility of its high reactivity with some other enzymes.[6,19] Silverman and coworkers have reported some fluorine-containing conformationally restricted analogues of GABA as potential mechanism-based inactivators.[3,15] 5-Amino-2-fluorocyclohex-3-enecarboxylic acid is a composite structure that has the form of cyclohexene analogue of (1*R*,4*S*)-(+)-4-amino-2-cyclopentene-1-carboxylic acid but with an added fluorine atom, which enables in the irreversible inhibition of GABA-AT.[8] This compound could go through enzyme-catalyzed HF elimination that inactivates GABA aminotransferase either by Michael addition that leads to a covalently modified active site residue or an aromatization mechanism that produces a modified coenzyme (Scheme 1). The presence of double bond and fluorine in the structure is important for empowering the inhibition process. The mechanism of base-induced HF elimination is not well identified yet. The two proposed mechanisms are concerted E2 or stepwise E1cb pathways and are recently studied on systems with a pyridyl ring by means of computational tools.[21−24]

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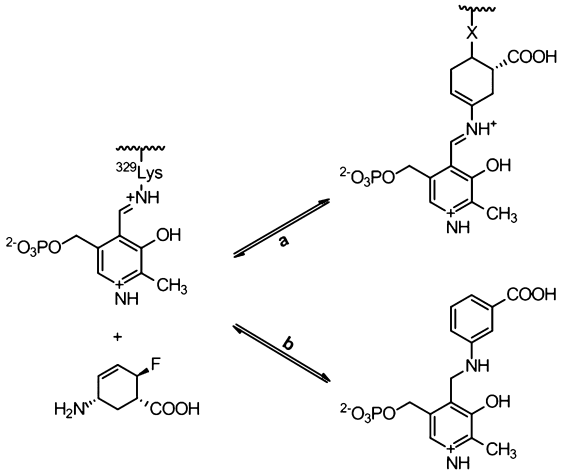
Scheme 1. Experimentally Proposed Products of Inactivation of GABA-AT with 5-Amino-2-fluorocyclohex-3-enecarboxylic Acid[a]



[a](a) via Michael addition mechanism, (b) via aromatization mechanism.

The previous computational studies related to PLP-dependent enzymes were concentrated mostly on the formation of the external aldimine.[5,18,19,25−27] The initial step of the external aldimine formation from an internal aldimine where the PLP is covalently bound to ε-amino group of active site lysine is common for all PLP-dependent enzymes. According to those studies, PLP binds to lysine residue with an imine bond and produces an internal aldimine.[18,28] Moreover, a new imine bond is formed because of the reaction of the substrate with the Schiff base that produces external aldimine from Michaelis complex.[18,19,29] This reaction is called transimination or transaldimization and is symmetrical.[18] It is also known that in most PLP-dependent enzymes the nitrogen of pyridine ring is protonated and the interactions in enzymatic residues are stabilized via the electron-sink effect.[25,30−33] After the formation of external aldimine, the mechanism shows a wide range of diversity based on the activity type of the enzyme. The effects of acid−base catalysis and the solvent effects on proton transfer have been investigated in many studies.[21,25−27,34−36] It is known that the water molecule is important to get reliable reaction barriers.[34−36] However, the assistance of water in modeling 1,3-proton transfers may not always lower the barrier height.[34]

To obtain a deeper understanding of mechanisms taking place in the presence of fluorine, a density functional theory (DFT) study of the external aldimine formation and the HF elimination reactions has been carried out. Although a variety of GABA analogues are present in literature, 5-amino-2-fluorocyclohex-3-enecarboxylic acid is particularly chosen as a substrate analogue in order to explain the details of the HF elimination reactions. Furthermore, experimental studies revealed that aromatization competes with Michael addition mechanism in the presence of 5-amino-2-fluorocyclohex-3-enecarboxylic acid (Scheme 1).[37−45] The results enabled us to explain the details of the HF elimination reaction taking place in the presence of a PLP and the aromatization reaction taking place during the inactivation process.

2. COMPUTATIONAL DETAILS

The study is conducted using both the Gaussian'03 (G03) and Gaussian'09 (G09) program packages.[46,47] The G03 program package is used for gas phase geometry optimizations and frequency calculations, with the B3LYP[48] method and the 6-31+G(d,p) basis set. Frequency calculations were carried out at stationary points so as to derive correction for thermal energies. For transition states, a single imaginary frequency was obtained, and the corresponding normal mode was inspected to check the correct transition state had been located. Furthermore, intrinsic reaction coordinate (IRC) calculations were performed for the characterization of the transition states.

The presence of charged species along the reaction path makes the optimization calculation in aqueous medium necessary. The solvation effect of water has been considered by B3LYP/6-31+G(d,p) optimization calculations using the polarizable continuum model (PCM) of the Tomasi group[49] as implemented in G09. The single point MP2 calculations on the optimized structures were also performed with the 6-31+G(d,p) basis set. The single point MP2 energies are given in the Supporting Information. Moreover, natural bond orbital (NBO) analysis was performed to figure out the importance of stereoelectronic effects on the stability of the structures along the reaction path.[50] The energies that are depicted in the paper are the free energies unless they are indicated differently. The coordinates of all stationary points that are obtained from gas phase calculations are given in the Supporting Information.

3. RESULTS AND DISCUSSION

To mimic the external aldimine formation and HF elimination mechanisms on PLP-dependent enzymes, a model system has been used. The model system studied is based on the experimental work in which the inactivation mechanism of PLP-dependent enzyme GABA-AT is studied in the presence of substrate analogue 5-amino-2-fluorocyclohex-3-enecarboxylic acid. In our work, Lysine329 (Lys329) residue attached to PLP has been represented with a methylamine. This method of representation in enzymes is common and widely accepted in literature.[25,27,34,35,51] In PLP-dependent enzymes belonging to the transaminases family, the enzymatic residues that have carboxylate groups stabilize the protonated pyridine nitrogen.[25] In GABA-AT, the Asp298 is the candidate for such interaction with the protonated nitrogen. Therefore, the nitrogen of pyridine ring is protonated,[21,22,52] while the phosphate group on PLP is replaced with a methyl group.[5,21,26,35]

The inactivation mechanism is initiated with the external aldimine formation, which is common to all PLP-dependent enzymes (Scheme 2, mechanism 1). It is followed by the HF elimination, which also occurs in experimental studies (Scheme 2, mechanism 2). Once the HF is eliminated from the enzyme substrate complex, the inactivation mechanism either continues with Michael addition (Scheme 2, mechanism 3) or aromatization paths (Scheme 2, mechanism 4). Michael addition simply involves the nucleophilic attack of the neutral amine to the carbon−carbon double bond on the defluorinated cyclohexene ring, which leads to the formation of covalently modified active site residue (Scheme 2, mechanism 3). Two different types of aromatization mechanisms can be proposed on the basis of the literature, which differs only in the sequence of the proton abstraction steps (Scheme 2, mechanism 4) where a modified coenzyme is produced at the end.[8,29,53] The mechanism of aromatization reaction is also modeled for the first time to elucidate the inactivation pathway of PLP-dependent enzyme GABA-AT.

3.1. External Aldimine Formation. External aldimine intermediate formation starts with the geminal diamine formation through the nucleophilic attack of the amine group of the substrate to the PLP−methylamine complex (**00TS01**, Figure 1). The distance between the atoms C8 and N7 (2.133 Å) is comparable with a previous study on external aldimine

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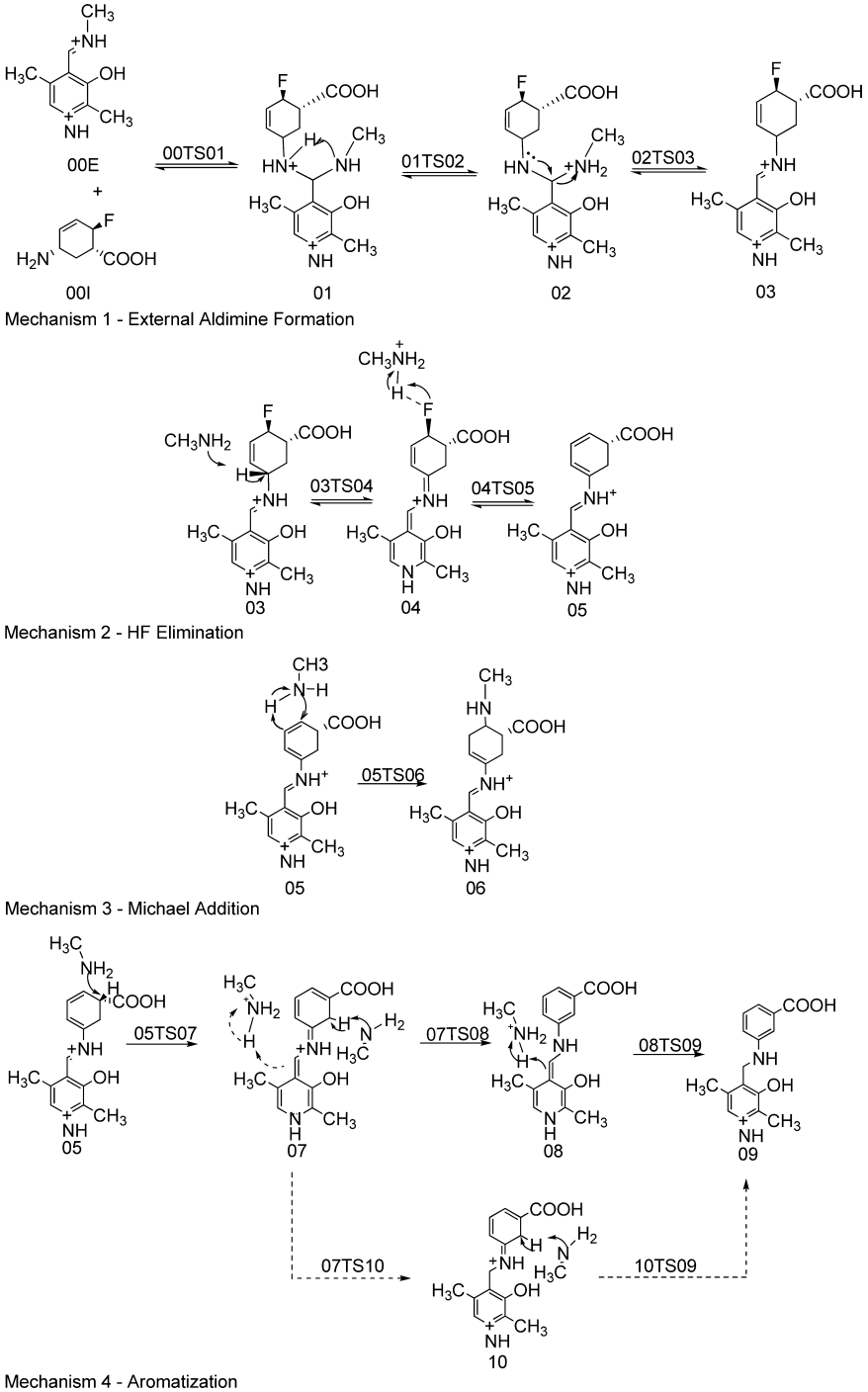
Scheme 2. Schematic Representation of Inactivation Mechanisms of GABA-AT with 5-Amino-2-fluorocyclohex-3-enecarboxylic Acid[a]



[a]Mechanism 1: External aldimine formation. Mechanism 2: HF elimination from PLP-5-amino-2-fluorocyclohex-3-enecarboxylic acid complex. Mechanism 3: Inactivation via Michael addition mechanism. Mechanism 4: Inactivation via aromatization mechanism. The dashed arrows correspond to an alternative aromatization mechanism.

formation with γ-vinyl GABA (1.970 Å).[27] In gas phase calculations, a prereaction complex (**00EI**, Figure 1) is obtained, which is 13.2 kcal/mol lower in energy than the infinitely apart reactants (Figure 2), while the NBO charges on atoms C8 and N7 are 0.21 and −0.89, respectively, which is appropriate for the formation of a transition state. The free energy barrier for the formation of geminal diamine is found to be 2.8 kcal/mol. The MP2 single point energy calculations at 6-31+G(d,p) level of theory, which is given in the Supporting Information, are in qualitative harmony with the DFT calculations. However, when the solvent effect is taken into consideration, the activation barrier is increased by 11.2 kcal/mol. In addition to the increase in activation energy in solvent, the intermediate structure **00EI** is disappearing and the transition state **00TS01** occurs directly from separated reactants. The loss of complex in the presence of

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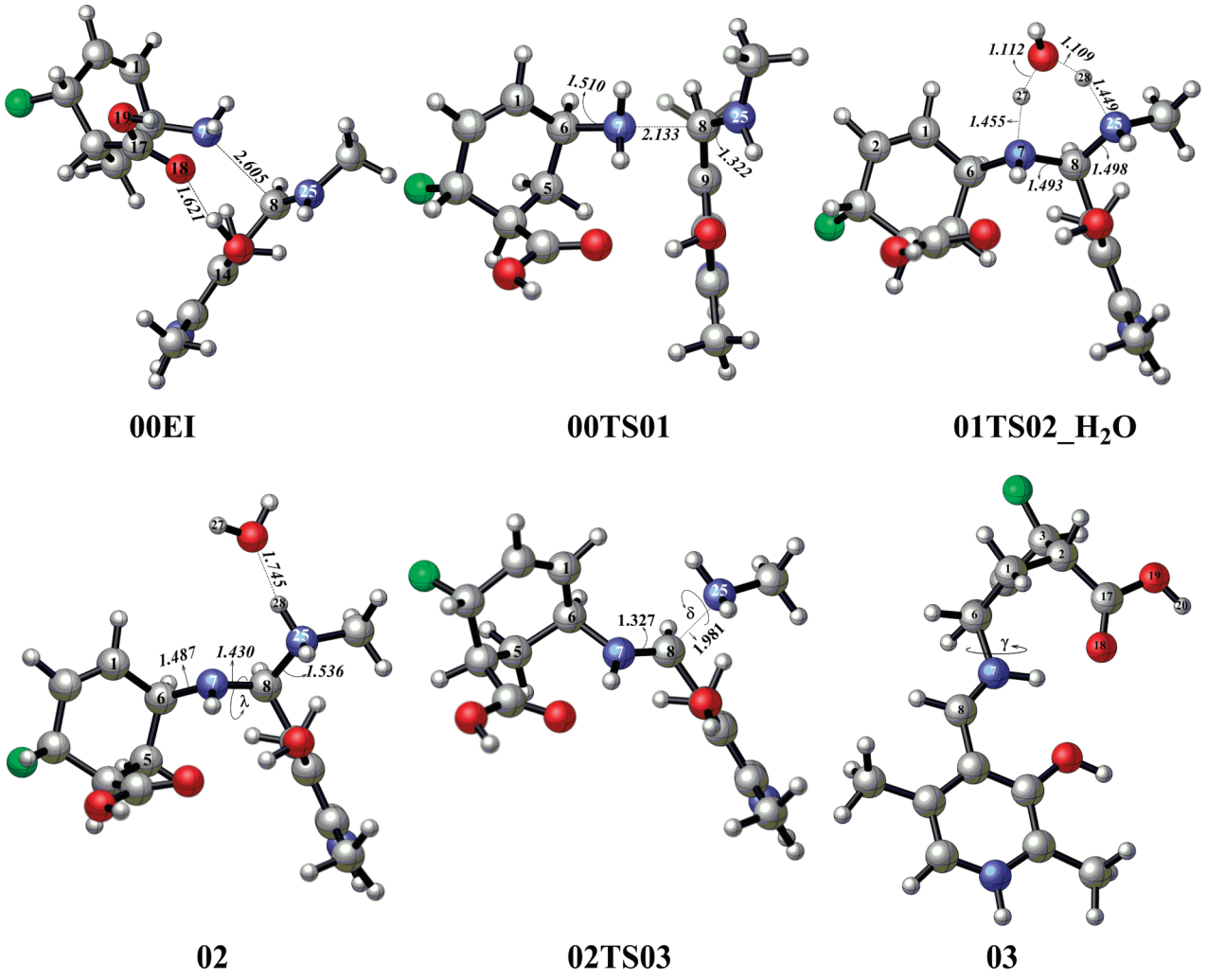

Figure 1. Three-dimensional geometries of the transition state and intermediate structures of external aldimine formation.

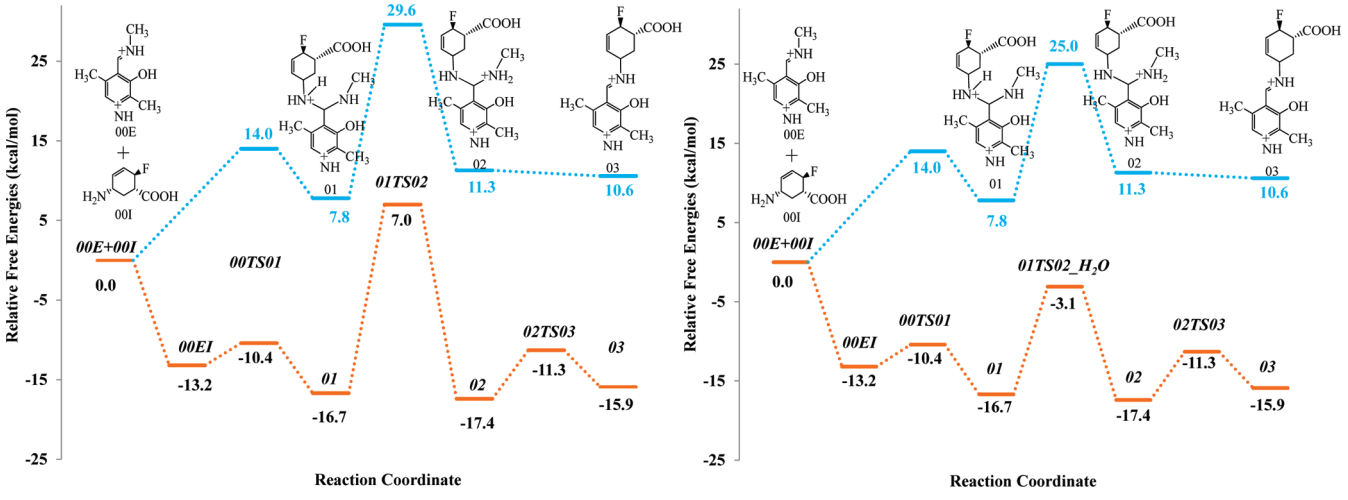

Figure 2. Energy profile for external aldimine formation without assistance (left) and with water assistance (right). Solvent optimization results are represented by blue, while the gas phase is represented by orange. Relative free energies are given as kcal/mol.

a solvent effect is mostly seen in computational calculations.[35,54,55]

The reaction continues with the 1,3 proton transfer between amino groups of a geminal diamine. The four-membered transition state (**01TS02**) is characterized with one imaginary frequency of 1603.92 cm^{-1} having a barrier of 23.7 kcal/mol. The H27 atom is located in between N7 and N25 atoms with distances 1.358 and 1.302 Å, respectively, where similar distances and the same activation energy barrier have been reported in a computational mechanistic study on the PLP-dependent enzyme

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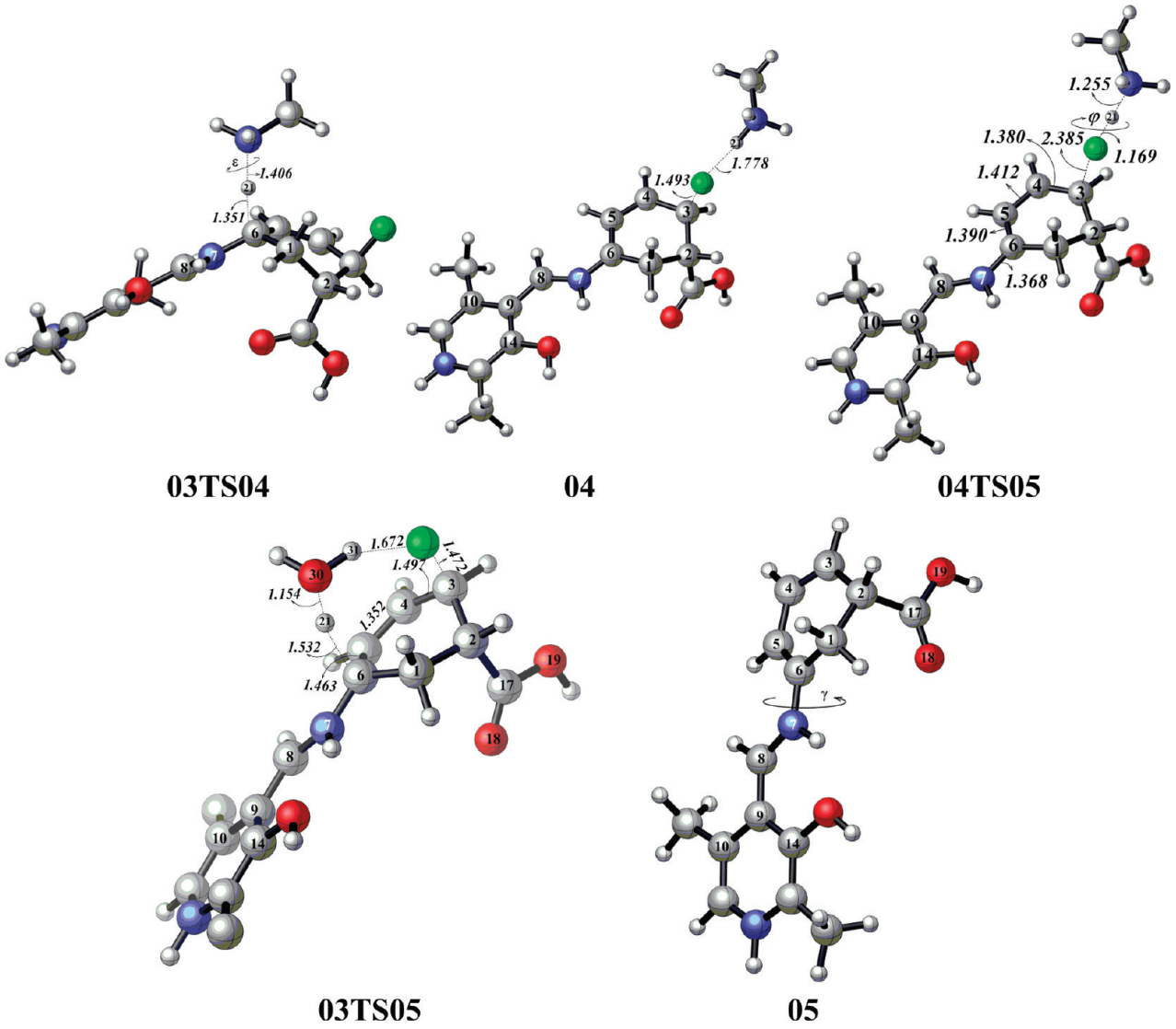

Figure 3. Three-dimensional geometries of the transition state and intermediate structures of HF elimination path.

ornithine decarboxylase (ODC) for a transamination reaction.[21] Moreover, the obtained value is also in harmony with a previous study with γ-vinyl GABA where the gas phase barrier for the 1,3 proton transfer step is calculated to be 24.2 kcal/mol.[27] When the geometry optimization is performed in the presence of water as a solvent, the activation energy barrier is obtained as 21.8 kcal/mol for 1,3 proton transfer step, which is only 2.1 kcal/mol lower than the gas phase calculation.

The 1,3 proton transfer between amino groups of geminal diamine is also studied with an assistance of a single water molecule. The six-membered transition state (**01TS02_H_2O**, Figure 1) is obtained with a relative free energy barrier of 13.6 kcal/mol. The energy barrier of the same step was calculated 12.6 kcal/mol in the case of other PLP-dependent enzymes.[21] Moreover, the calculated gas phase barrier is compatible with a previous work with γ-vinyl GABA, where it was calculated to be 14.5 kcal/mol.[27] The distances between the transferring protons (H27 and H28) and the corresponding amino group are found to be almost equal (∼1.450 Å). Besides, the H27 and H28 is placed in such a position that they are equally distant to the oxygen of water (∼1.110 Å). The transition state yielded the product **02** (Figure 1), where N25 is still bounded to C8. The oxygen atom of water molecule interacts with H28 by a distance of 1.745 Å, while the dihedral λ is found to be 149.3° in structure **02**. The solvent optimization calculations result in the same transition state with 3.6 kcal/mol increase in relative free energy barrier (17.2 kcal/mol). Although the large energy barrier might be lowered by addition of more water molecules, the assistance of an acid or a base in solution might further lower the barrier in enzymatic reactions.[35]

After the 1,3 proton transfer step, a relative free energy barrier of 6.1 kcal/mol is observed for the bond breakage between N25 and C8 (**02TS03**, Figure 1). The geometry parameters of the intermediate structure **03** obtained from IRC calculation is comparable to the one obtained in a previous study (Figure 1).[21] The distances between N7−C8 and C8−N25 are found to be 1.327 and 1.981 Å, respectively. The transition state structure could not be located in the solvent phase; the possibility of a diffusion-controlled reaction should not be omitted.

External aldimine formation is found to be exergonic according to the gas phase calculation, while the reaction turns out to be endergonic when solvent effect with the optimized geometries are taken into account (Figure 2). The gas phase optimization and single point MP2 calculations yields a

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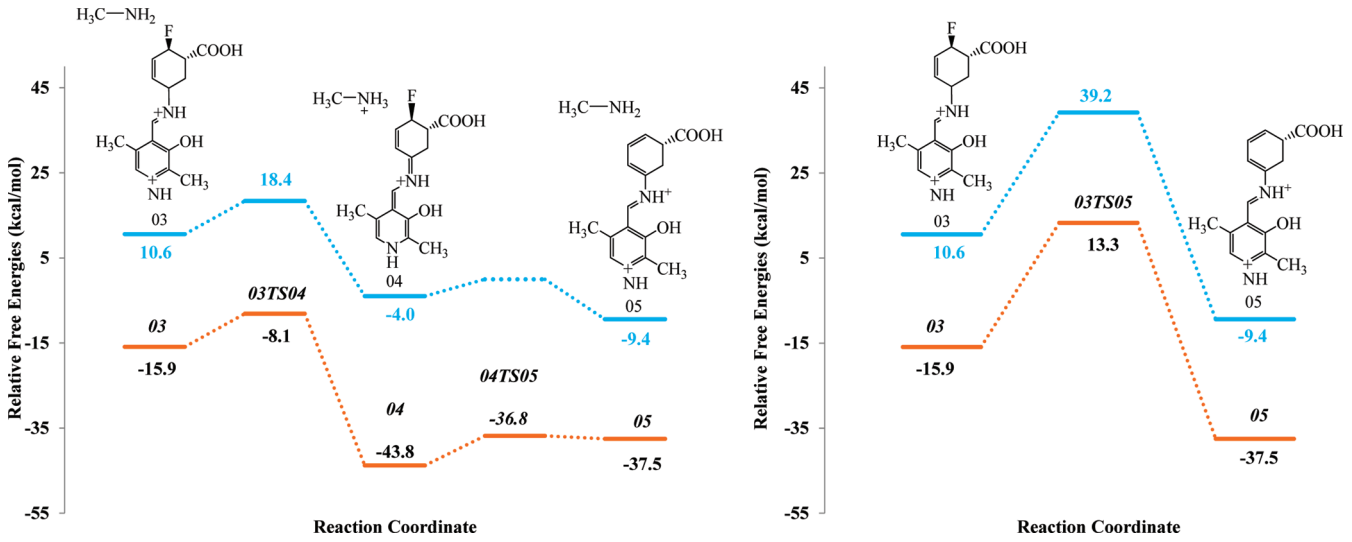

Figure 4. Energy profile for HF elimination via methylamine-assisted E1cb mechanism (left) and water-assisted E2 mechanism (right). Solvent optimization results are represented by blue, while the gas phase is represented by orange. Relative Gibbs free energies are given as kcal/mol.

prereaction complex, while the complex seems to disappear in the solvent optimization calculations. According to the IEFPCM calculations, the reaction starts with **00TS01** with an energy barrier of 14 kcal/mol. It is well-known that the use of water for shuttling the proton in [1,3] proton transfer reactions lowers the activation energy barrier.[21,27,34,35] The activation barrier for **01TS02_H_2O** is found to be 17.2 kcal/mol in solvent calculations, which is almost 5 kcal/mol less than the barrier of the corresponding nonassisted transition state (**01TS02**) (Figure 2). It is noteworthy to state that the transition state for the bond cleavage reaction (**02TS03**) is located in gas phase calculations, while it is predicted to occur almost barrierless according to the solvent phase calculations.

3.2. Fluoride Ion Elimination in the Form of HF. The external aldimine formation is followed by the HF elimination step. The reaction is a typical example of base-induced β-elimination reaction with the formation of carbon−carbon double bond. In an experimental study performed by Silverman et al., the HF elimination step is proposed to occur with a stepwise fashion where the abstraction of the γ proton at C6 position by one of the basic amino acid residues of the enzyme is followed by the elimination of the fluorine ion.[8] In literature, the computational studies on the HF elimination calculations have carried out both for water-assisted concerted E2 elimination mechanism and for the methylamine-assisted stepwise path, which is denoted as E1cb elimination mechanism.[20,23,24,56] Both the experimental and theoretical studies have provided some clues regarding the nature of the mechanism.[23,24] In the E2 mechanism the rate determining step is expected to be the bond breaking of the carbon−leaving group bond. In E1cb mechanism the bond lengthening of carbon−leaving group bond is observed because of the possible hyperconjugation.[57,58] It should also be noticed that the presence of protonated nitrogen on the PLP provides an extra stabilization to the carbanion intermediate formed during the elimination reaction.

In this study, the HF elimination via the E1cb mechanism is studied with the assistance of methylamine on the basis of the experimental labeling studies in which the Lys329 is reported as a candidate base for assistance in elimination.[20,56] The first step for HF elimination is the transfer of γ proton from C6 to methylamine. The transition state (**03TS04**, Figure 3) is modeled with a free energy barrier of 7.8 kcal/mol (Figure 4). The solvent optimizations are also carried out, and the calculations revealed a transition state having the same geometrical parameters with the same activation energy barrier with the gas phase results. The transition state in gas phase is characterized with one imaginary frequency of −1128.72 cm^{-1} and yields the structure **04** (Figure 3, Figure 4).

The subsequent transition state is the last step of the HF elimination via proton transfer to the fluorine atom from the $CH_3NH_3^+$ moiety (**04TS05**, Figure 3). The gas phase calculations generated a transition structure having an activation barrier of 7.0 kcal/mol and the dihedral angle φ is 162.9°, which is equal to −168.8° in structure **04**. Alunni et al. have reported that after the formation of the carbanionic intermediate, which corresponds to a shallow minimum according to the looseness of C−F bond, the fluorine elimination would occur fast because of the low energy barrier and high exothermicity.[23] In solvent calculations, in conjunction with the previous literature results, the fluorine elimination occurs fast, and the transition structure could not be obtained, which should have a low activation energy barrier and high exothermicity.

In the water-assisted concerted transition state structure (**03TS05**, Figure 3), the proton is transferred to a water molecule, the single bond between fluorine atom and C3 is broken, and the lone pairs of F atom interact with the O30−H31 antibonding orbital via O30−H31−F angle of 153.3°. During the HF elimination via E2 elimination mechanism, which follows a concerted path, C3−C4 and C5−C6 single bonds (1.507 and 1.515 Å, respectively) turn into be double bonds (1.368 and 1.380 Å, respectively), while the C4−C5 double bond (1.338 Å) lengthens to a single bond (1.427 Å). Moreover, the λ dihedral angle alters to −10.4° in structure **05** (Figure 3), from −105.5° in structure **03** (Figure 1). The relative free energy barrier for this transition state (**03TS05**, Figure 3) is calculated as 29.2 kcal/mol. The solvent optimization calculation resulted with the same six member transition state with an activation energy barrier of 28.6 kcal/mol.

The overall HF elimination reaction is found to be exergonic. The detailed energy profile for HF elimination mechanism is depicted in Figure 4. Alunni et al. reported that when the fluoride is the leaving group, an E1cb mechanism is more prone to occur

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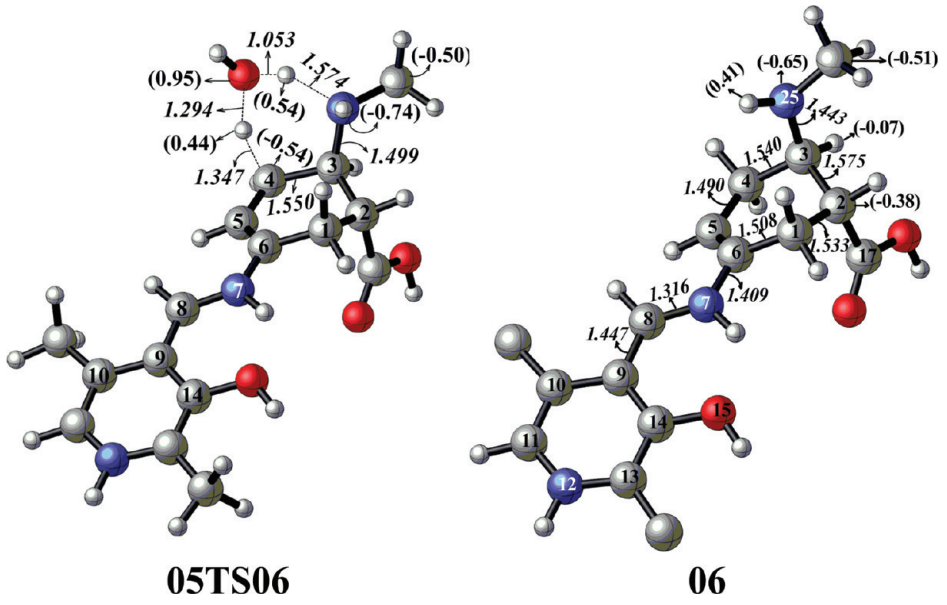

Figure 5. Three-dimensional geometries of Michael addition path. NBO charges are written in parentheses.

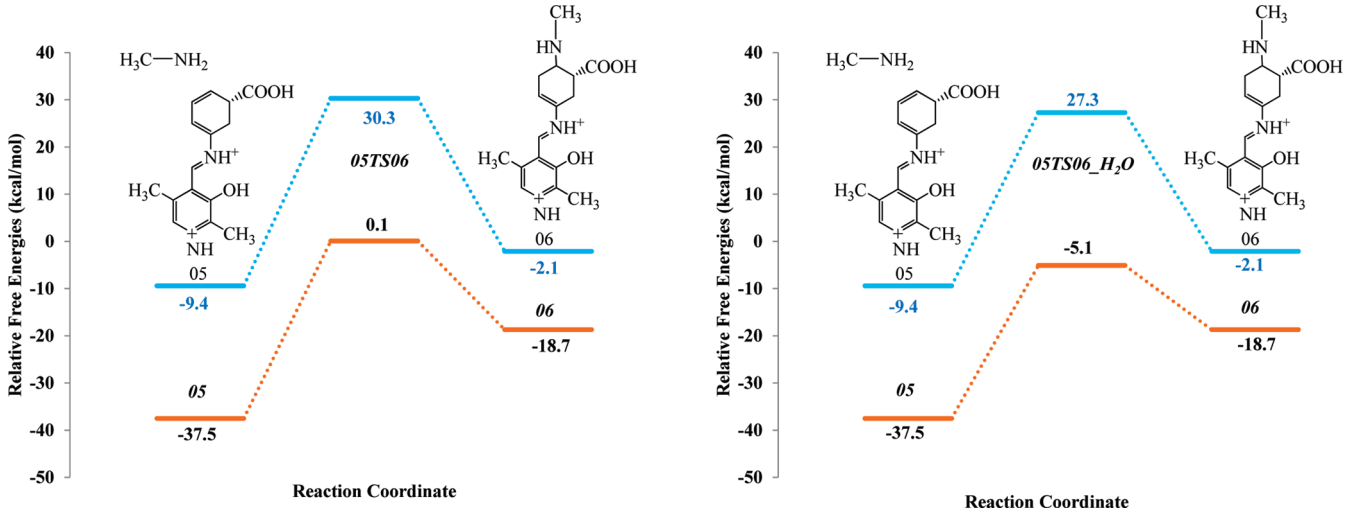

Figure 6. The energy profiles for the nonassisted (left) and the water-assisted Michael addition mechanisms (right). Solvent optimization results are represented by blue, while the gas phase calculations are represented by orange. The relative free energies are given as kcal/mol.

than E2 mechanism because of the electron withdrawing power of fluorine, which promotes β-anion stability.[23,24] It is found that the E1cb mechanism with methylamine assistance (**03TS04**) is more plausible than an E2 mechanism, which is assisted by a water molecule (**03TS05**). Moreover, Clift et al. reported that the R conformer of 3-F-GABA would follow the E1cb mechanism.[20] It is noteworthy to say that the intermediate structure **03** adopts R conformer, and it is found to be that when (*R*)-**03** is the reactant, the E1cb mechanism is favorable over E2 mechanism.

3.3. Michael Addition. The Michael addition reaction in the enzyme GABA-AT consists of the addition of the side chain amine of Lys329 to a carbon−carbon double bond on the substrate. The experimental results proposed a stepwise mechanism for the Michael addition reaction where nucleophilic attack of the neutral amine to the double bond is followed by the proton transfer.[8] However, previous theoretical findings have shown that the reaction proceeds through a concerted mechanism where both the nucleophilic attack and the proton transfer steps occur simultaneously.[8] In addition, it has been shown that Michael addition can occur with the assistance of explicit water molecules, which has a capability of lowering the energy barriers.[27,34,52,59,60]

A transition state (**05TS06**) with N25−C3 distance equal to 1.518 Å is modeled for the concerted Michael addition reaction. The proton transfer step occurs simultaneously with distances 1.460 and 1.340 Å between N25−H27 and H27−C4, respectively. The Michael adduct (**06**, Figure 5) is formed with an energy barrier of 37.6 kcal/mol in gas phase. The overall process is highly exergonic according to gas phase calculations. The solvent optimization calculations yield the same transition state geometry with a slight increase in the activation energy barrier (∼2 kcal/mol). Moreover, the calculated energy barrier with solvent optimization is comparable with the single point solvent effect calculation in the previous study with λ-vinyl GABA that was reported as 39.6 kcal/mol.[27] The energy profile for the Michael addition mechanism is depicted in Figure 6. The

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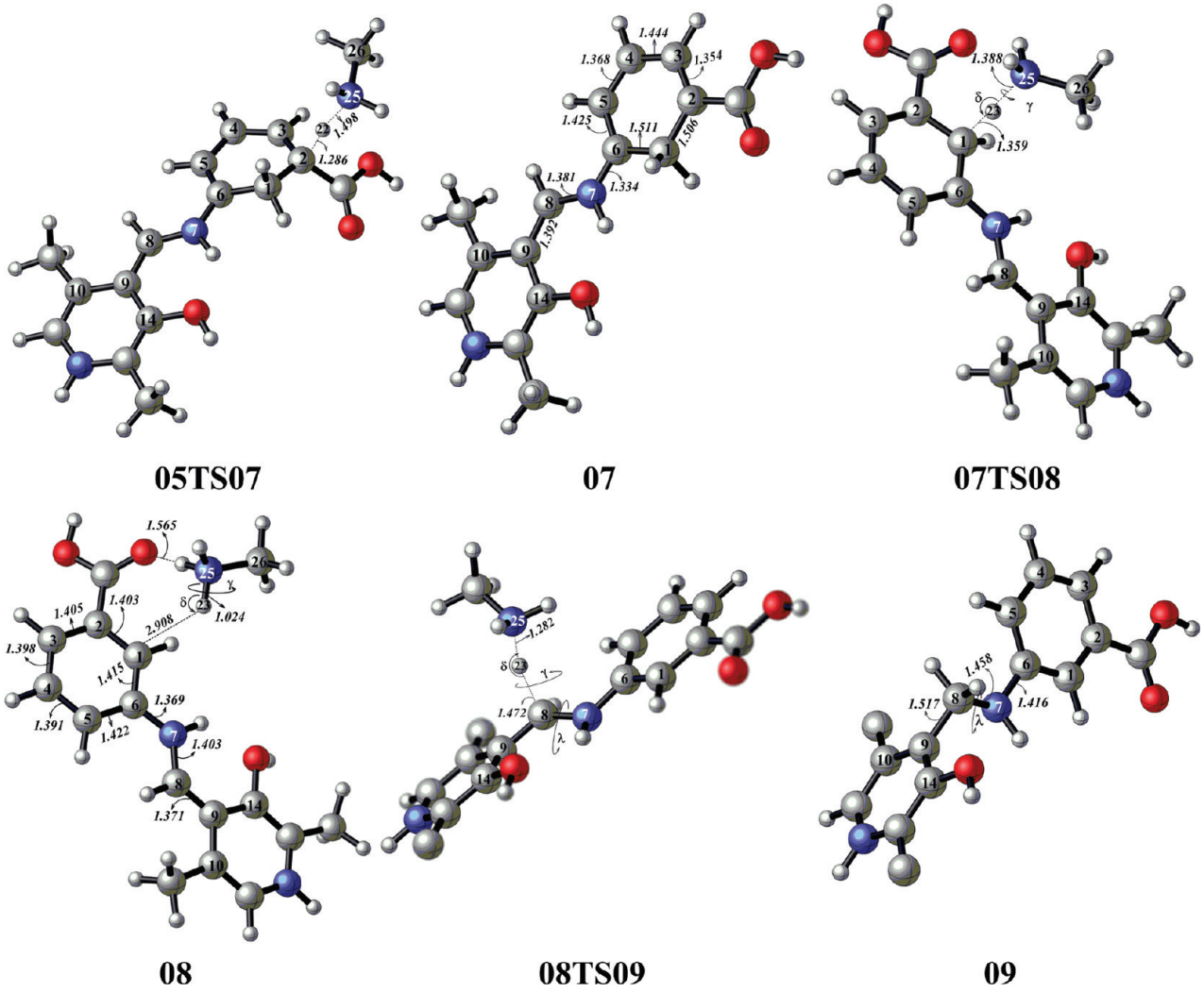

Figure 7. Three-dimensional geometries of the transition state and intermediate structures of aromatization mechanism.

reaction becomes less exergonic with the addition of the solvent effect.

The concerted Michael addition may also be modeled with the assistance of a water molecule.[27,59] The presence of a water molecule leads to a six membered transition state structure (**05TS06_H_2O**, Figure 5), thus providing a decrease in the ring strain relative to the four membered structure. The gas phase energy barrier is found to be 32.4 kcal/mol, while it was found 40.7 kcal/mol in λ-vinyl GABA case.[27] The product obtained is identical the one obtained in the nonassisted process (**06**, Figure 5). Furthermore, in MP2 gas phase calculations, the activation barrier for the transition state structure (**05TS06_H_2O**) is calculated 26.2 kcal/mol, which is comparable with a previous work where it was reported as 23.4 kcal/mol.[59] However, the solvent optimization calculations on the water-assisted path lead to the formation of the same product with a $\Delta G^{\#}$ value of 36.7 kcal/mol.

In the Michael addition mechanism, the presence of electron-withdrawing group next to the double bond between C3 and C4 atoms triggers the push−pull effects. The removal of electron density from the central double bond enables the pyramidalization of the related atoms and facilitates the proton transfer.

3.4. Aromatization Mechanisms. The elimination of the fluoride ion leads to the formation of structure **05** (Figure 3), which is the protonated form of an intermediate present in the inactivation of the enzyme by the gabaculine.[29] The aromatization mechanism involves two deprotonation steps sequentially, which are followed by a protonation step.[18,29,53,61,62] In an enzyme, the deprotonation should occur with the attack of the side chain of an amino acid containing primary amine. To figure out the convenient amino acids for deprotonation and protonation steps, the PDB structure of γ-vinyl GABA bonded GABA-AT (1OHW) is inspected. Because of the planarity of structure **05**, where γ-vinyl GABA is almost perpendicular to the molecular plane of the PLP ring, the susceptible amino acids for deprotonation and protonation reactions around the active site are Lys329[29,62] and Arg445.

The aromatization mechanism starts with the proton elimination from α carbon (C2) of the substrate ring. The methylamine is the proton acceptor where the proton (H22) is transferred to N25 with a C2−H22 distance of 1.286 Å and H22−N25 distance of 1.498 Å (**05TS07,** Figure 7). Severe structural changes are observed during the proton transfer; the double bond between C3−C4 (1.368 Å) turns out to be a single bond (1.444 Å), while the double bond between C5−C6 shifts to C6−N7, and C5−C6 bond distance lengthens from 1.380 to 1.425 Å, leading to the formation of intermediate **07** (Figure 7). The proton transfer step occurs by overcoming a 13.6 kcal/mol high barrier. Additionally, the MP2 single point calculations result in a comparable activation energy barrier (15.8 kcal/mol).

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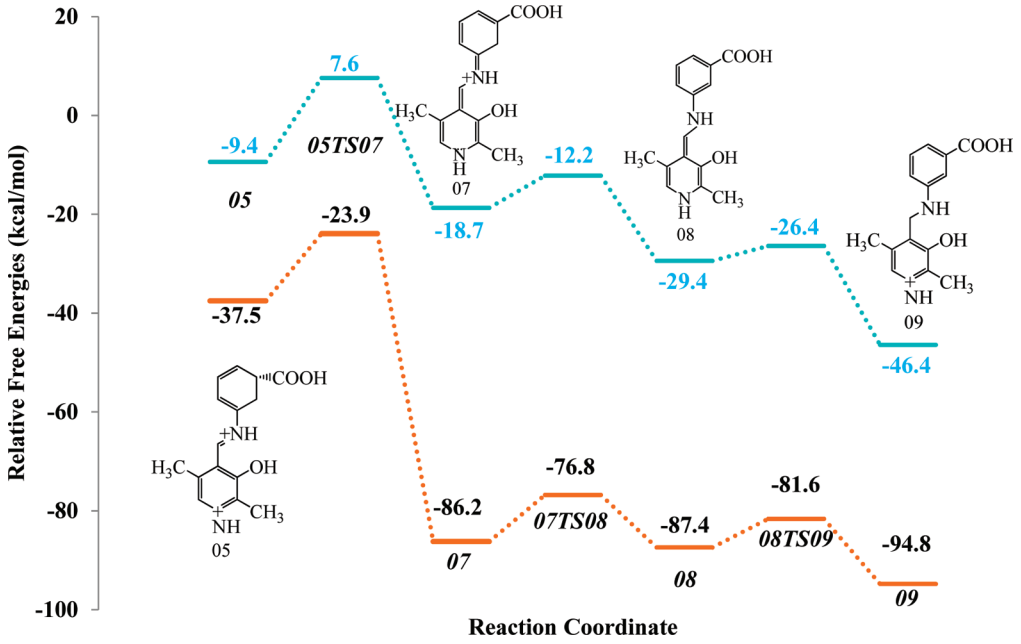

Figure 8. Energy profile of mechanism 3a. Solvent optimization results are represented by blue, while the gas phase is represented by orange. Relative free energies are given as kcal/mol.

On the other hand, the solvent optimization calculations revealed that the relative free energy barrier for the transition structure **05TS07** is 17.0 kcal/mol. The PLP and the substrate rings are coplanar in the intermediate structure **07**, providing an energetic stability to the structure with respect to the structure **05**. In addition, the presence of an intramolecular long-range interaction stemming from the nature of the hydroxylamine increases the energetic stability.

The aromatization mechanism may proceed either through the deprotonation of the C1 on the substrate ring or the protonation of the C8 from the double bond next to the imine nitrogen. In the study, the methylamine is used as the proton acceptor again for the deprotonation step (**07TS08**, Figure 7). The gas phase calculations have revealed an energy barrier of 9.4 kcal/mol, while it decreases to 6.5 kcal/mol when solvent optimization is taken into account (Figure 8). The transferred proton is located in between C2 and N25 with 1.359 and 1.388 Å distances, respectively. In addition, the dihedral γ and angle δ values are $-101.30°$ and 168.70°, respectively, which determines the position of methylamine in Cartesian space. The accuracy of this step is validated with the IRC calculations, where the transition state produces protonated methylamine and structure **08** (Figure 7) with a relative energy of −29.4 kcal/mol in solvent (Figure 8). The coplanarity of the two rings is still seen in the structure **08**, allowing the delocalization of electrons throughout the rings.

The last step consists of a proton transfer from the protonated methylamine to the carbon−carbon double bond next to the amine. The proton transfer from protonated methylamine to C8 is achieved in such a way that the hydrogen atom is located with a distance 1.282 and 1.472 Å away from the N12 and the C8 atoms, respectively. The angle δ is found to be 163.70°, where the dihedrals γ and λ are equal to 144.10° and −173.70°, respectively. In gas phase calculations, the transition state (**08TS09**, Figure 7) is determined with one imaginary frequency having a value of $-1143.34\ cm^{-1}$ and has a 5.8 kcal/mol free energy barrier (Figure 8). In structure **09** (Figure 7), the aromaticity on the PLP ring is recovered with the protonation of the C8. The coplanarity of the two rings is disturbed; moreover, two rings are located perpendicular to each other. The solvent optimization calculations yield the same geometries, while the relative free energy barrier for the transition state **08TS09** is calculated as 3.0 kcal/mol. Furthermore, the single point MP2 energy calculations pointed out a barrier of 2.5 kcal/mol.

The product of an alternative aromatization mechanism is equivalent to the structure **09**. However, the path is sequentially different than the one explained above (Scheme 2). In the alternative path, prior to the deprotonation of the C1 on the substrate ring, the protonation occurs at C8. The protonation is achieved with the protonated methylamine in which the gas phase energy barrier is found to be around 53.0 kcal/mol. The formation of the product structure **09** is not possible via an alternative path because of the presence of higher energy barrier.

4. CONCLUSION

The external aldimine formation is a common step for PLP-dependent enzymes. The rate determining step is found to be 1,3 proton transfer both in the gas phase and solvent optimizations for the external aldimine path. The assistance of water molecules in the proton transfer step is crucial for lowering the energy barrier without changing the nature of the rate determining step. The fluorine elimination step either follows the concerted water-assisted E2 mechanism or base-induced stepwise E1cb mechanism. The results are consistent with stepwise experimental suggestion where the stepwise E1cb mechanism is more prone to occur with the assistance of methylamine, which is used to mimic lysine side chain.

The assistance of a water molecule in the Michael addition mechanism is found to be inevitable, which helps to decrease activation energy at least 5 kcal/mol. The alternative mechanism of inactivation is aromatization, which has lower activation barriers relative to the Michael addition mechanism. The product obtained after aromatization is a modified coenzyme that is lower in energy relative to the Michael addition product. The formation

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of the modified coenzyme via an aromatization path is both kinetically and thermodynamically favored.

The proton transfer steps that are assisted either by a water molecule or a base have lower activation barriers with respect to the corresponding nonassisted ones. Nevertheless, the gas phase calculations on charged systems yields less reliable energy barriers. Therefore, geometry optimizations and frequency calculations should be also carried out in aqueous phase.

■ ASSOCIATED CONTENT

Supporting Information

The coordinates of all stationary points that are obtained from gas phase calculations and energy profiles of the single point MP2 calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: konuklar@itu.edu.tr. Fax: +90 212 2857073.

Notes

The authors declare no competing financial interest.

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