Theoretical studies on the pyridoxal-5'-phosphate dependent enzyme dopa decarboxylase: Effect of Thr 246 residue on the co-factor-enzyme binding and reaction mechanism

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Decarboxylation of amino acid is a key step for biosynthesis of several important cellular metabolites in the biological systems. This process is catalyzed by amino acid decarboxylases and most of them use pyridoxal-5'-phosphate (PLP) as a co-factor. PLP is bound to the active site of the enzyme by various interactions with the neighboring amino acid residues. In the present investigation, density functional theory (DFT) and real-time dynamics studies on both ligand-free and ligand-bound dopa decarboxylases (DDC) have been carried out in order to elucidate the factors responsible for facile decarboxylation and also for proper binding of PLP in the active site of the enzyme. It has been found that in the crystal structure Asp271 interacts with the pyridine nitrogen atom of PLP through H-bonding in both native and substrate-bound DDC. On the contrary, Thr246 is in close proximity to the oxygen of 3-OH of PLP pyridine ring only in the substrate-bound DDC. In the ligand-free enzyme, the distance between the oxygen atom of 3-OH group of PLP pyridine ring and oxygen atom of Thr246 hydroxyl group is not favorable for hydrogen bonding. Thus, present study reveals that hydrogen bonding with O3 of PLP with a hydrogen bond donor residue provided by the enzyme plays an important role in the decarboxylation process.

Keywords: Enzyme, Molecular dynamics, Quantum mechanics, Density functional theory, Pyridoxal-5'-phosphate, Carbodopa, Decarboxylase, Aldimine

Pyridoxal-5'-phosphate (PLP) is derived from vitamin B₆ and serves as a versatile enzyme co-factor that facilitates many biochemical transformations, including racemization, decarboxylation, deamination and transamination reactions of amino acids¹⁻⁵. The first obligatory chemical step in all PLP-dependent enzymatic reactions is the formation of a Schiff base between co-enzyme aldehyde and substrate amino group (Scheme I)⁶⁻⁸. This common intermediate is termed as “external aldimine”. Proper functioning of PLP depends on its ability to stabilize carbanions, formed adjacent to the Schiff base in external aldimine intermediate through delocalization into the extended π-bonded system, i.e. Schiff base and pyridine ring.

According to the proposition of Dunathan, the common fundamental mechanism that PLP-dependent enzymes use to control the reaction specificity is specific binding of external aldimine intermediate such that the bond to be broken is oriented parallel to
the π-orbitals of the π-bonded system (Scheme II)\(^9\). This orientation maximizes orbital overlap between the nascent p-orbital on C\(_\alpha\) and the π-system in the transition state, thereby minimizing transition state energy. The structure of PLP in which both pyridine nitrogen and aldimine nitrogen atoms are protonated is generally assumed to be the active form of co-enzyme in enzyme-catalyzed reactions (Scheme III). This assumption is primarily based on the studies from Bruice’s laboratory\(^10,11\).

Toney et al\(^12\) reported a computational study of non-enzymatic and enzymatic pyridoxal phosphate-catalyzed decarboxylation of 2-aminoisobutyrate (AIB). They studied four prototropic isomers of a model aldimine between AIB and 5'-deoxypyridoxal with an acetate group interacting with the pyridine nitrogen in both gas phase and water model of decarboxylation pathways. They found that unprotonated Schiff base contribution is larger than that of pyridine ring even when it is protonated.

An active site model of dialkylglycine decarboxylase has also been constructed and validated which has revealed the possibility of a proton transfer from LYS272 to PLP C4' simultaneously with C\(_\alpha\)-C bond cleavage. NMR, absorption, and fluorescence studies of model compounds for the internal and external aldmines have shown that there is a keto-enol equilibrium, corresponding to an intra-molecular proton transfer\(^13-20\) (Scheme III). NMR studies of protonation and hydrogen bond states of PLP and PLP model Schiff bases in different environments starting from aqueous solution, organic solid state to polar organic solution and finally to the enzyme environments have been reported by Limbach et al.\(^21\)

Lin et al\(^22\) reported a quantum mechanics/molecular mechanics (QM/MM) study on the substrate bound PLP-dependent dopa decarboxylase (DDC) to elucidate the factors that contribute to tautomeric equilibrium of intra-molecular proton transfer in external PLP-L-dopa Schiff base. They found that presence of a carboxylate anion on \(\alpha\)-carbon of Schiff base stabilizes zwitterions and shifts the equilibrium in favor of oxoenamine tautomer. Protonation of PLP pyridine nitrogen further drives equilibrium toward oxoenamine direction. On the contrary, solvent effects favor hydroxyimine configuration. They reported the hydroxyimine form of PLP(H\(^+\))-L-dopa Schiff base as major isomer in DDC and explained by hydrogen bonding interactions of Asp271 and Lys303 with pyridine nitrogen of PLP and imino nitrogen of Schiff base, respectively. The role of Thr246 as H-bond donor, as well as H-bond acceptor in oxoenamine and hydroxyimine tautomer, respectively is also predicted. Recently, Lin et al\(^23\) reported MD simulation studies using a combined QM/MM potential to investigate the internal proton transfer equilibrium of external aldmine species in L-dopa decarboxylase and carbanion stabilization by the enzyme co-factor in active site of alanine racemase.

In this paper, we report our thorough QM and MD simulation studies to investigate the critical role of hydrogen bond formation between O3 of PLP and Thr246 hydroxyl hydrogen on decarboxylation.

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**Scheme II**—Stereoelectronic requirements of the C\(_\alpha\)-C bond for the facile decarboxylation

**Scheme III**—Tautomeric equilibrium of an external PLP aldimine in PLP-dependent enzyme
mechanism. Earlier, Lin et al. have reported that Thr246 can act as H-bond donor when external aldimine exists in ketoenamino form, thus we have selected this structure as a model for our QM calculation. Effect of electrostatic potential (ESP) on the co-factor and enzyme binding process has also been studied.

**Methodology**

**Collection of crystal structure**
Crystal structures of several amino acid decarboxylases were searched and downloaded from Protein Data Bank. Crystal structure analysis were performed using VMD software.

**Computational methods**
All initial geometries were optimized by DFT method using B3LYP hybrid functional and 6-31G* basis set. Quantum mechanical calculations were performed using GAMESS software and final geometries were analyzed and drawn using Molden software.

Two crystal structures were selected for present MD calculation—1JS6.pdb, a ligand-free DDC enzyme and 1JS3.pdb, a carbidopa-bound enzyme. MD simulations were carried out using NAMD program and CHARMM topology and parameter files were used for this purpose. X-PLOR topology file for PLP was modified to the CHARMM format. Topology file for substrate carbidopa was generated with the help of tyrosine topology which is already present in the CHARMM topology file. Before starting MD, all bad contacts were removed by minimizing the energy of the system. In addition to minimization, the system was also heated and brought into equilibrium before starting MD simulation (30 ps). Simulations were performed using a canonical NVT ensemble. A constant temperature for the simulated system was implemented by rescaling the velocities periodically, so that the entire system was set to the desired temperature.

Solvent around the proteins was described with explicit water molecules consisting of TIP3P water molecules and resultant structure was a protein surrounded with water molecules. The range of the truncated solvent buffer was 10 Å from the surface of the protein, so that total amount of water molecules varied from 4278 to 5494, depending on the area and shape of molecular surface. A cut-off distance of 8 Å was used to carry out long-range electrostatics with Particle Mesh Ewald (PME) and for van der Waals forces. Simulations were conducted in periodic boundary conditions (PBC). Simulations were carried out for 2 ns, including 2000000 cycles. The magnitude of each time step was 1 fs. Every 1000th step was saved to the trajectory, resulting in a set of 2000 coordinates for the analysis of the several H-bonding distances. Analysis of the final result was done with the help of VMD software and GNUPLOT was used to plot different results.

**Calculation of electrostatic potential (ESP)**
Calculation of ESP of the enzyme macromolecule was performed using Adaptive Poisson-Boltzmann Solver (APBS) program. The PQR file necessary for this calculation contains van der Waals radius and charge of each atom of macromolecule. For our present work, these PQR files were generated by PDB2PQR server proved by National Biomedical Computation Resource and supported by NIH grant GMO69720-01 to NAB and NPACI Alpha Project program. ESP of PLP was calculated using wave function based program provided by Molden software. Color mapping of electrostatic potential on solvent accessible surface was performed using VMD software.

**Results and Discussion**

**Crystal structural analysis of different PLP-dependent decarboxylases at their active site for locating the binding residues**

Based on amino acid sequences PLP-dependent decarboxylases can be divided into four groups of independent evolutionary origin. Crystal structures of all PLP-dependent decarboxylases available in the Protein Data Bank were analyzed in detail and are reported in Table 1. DDC which belongs to group-II is reported in two crystalline forms. Active sites of both ligand-free and carbidopa bound crystal structures (Figs 1A & B) showed a Thr246 residue at a close proximity to O3 of PLP. DDC is a PLP-dependent enzyme, which catalyzes irreversible decarboxylation reaction of aromatic-L-amino acid substrates, such as dopa, phenylalanine and tryptophan. It plays an important role in the synthesis of key neurotransmitters dopamine and serotonin via decarboxylation of L-3, 4-dihydroxy phenylalanine (L-Dopa, an anti-Perkinson drug) and L-5-hydroxy tryptophan respectively.

Crystal structure of ligand-free DDC and its complex with anti-Perkinson drug carbidopa has been recently reported. It is a tightly associated
α2-dimer and the active site, as revealed from crystal structure is located near the monomer-monomer interface, but is composed mainly of residues from one monomer only. In the internal aldime form of ligand-free DDC (PDB entry: 1JS6.pdb), PLP binds to Lys303 through a Schiff base linkage. The salt bridge between carboxylate group of Asp271 and protonated pyridine nitrogen atom of PLP provides a strong electron sink capable of stabilizing the obligatory carbanionic intermediates. Phosphate group of PLP is further attached to the protein through an extended hydrogen bonding network. Careful observation of crystal structure also revealed a Thr246 residue in the proximity of O3 of PLP-pyridine ring. Distance between this O3 atom and that of Thr246 hydroxyl oxygen was 5.48 Å (Fig. 1A) which was not a favorable distance for H-bond formation. On the other hand, this distance in carbidopa bound enzyme (PDB entry: 1JS3.pdb) was only 2.93 Å (Fig. 1B), which was quite appropriate for hydrogen bonding. This observation was quite interesting and led to the idea that hydrogen bonded Thr246 residue with O3 of PLP had a determining role in the decarboxylation process.

Glutamic acid decarboxylase (GAD), a widely distributed enzyme in all eukaryotes and many prokaryotes, catalyzes the conversion of glutamate to γ-aminobutyric acid (GABA)34. In vertebrates, GABA is an important neurotransmitter and two isoforms of the enzyme, viz., GAD65 and GAD67 are responsible for its (GABA) synthesis34,35. The active sites of the crystal structures of both of GAD65 and GAD67 also

<table>
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<tr>
<th>Entry No.</th>
<th>Class</th>
<th>Enzyme</th>
<th>PDB Entry</th>
<th>Residue near pyridine N of PLP (distance in Å between pyridine N &amp; residue O)</th>
<th>Residue near 3 oxygen (distance in Å between 3 O &amp; residue oxygen)</th>
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<tr>
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<td>Thr339 (3.27 Å)</td>
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<td>Ser246 &amp; H2O (2.68 Å), Thr212 (5.26 Å)</td>
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Fig. 1—Active site of the crystal structures [(A): Ligand-free enzyme (PDB entry: 1JS6.pdb); and (B): Ligand-bound enzyme (PDB entry: 1JS3.pdb) showing the distances between O3 of PLP and Thr246, N1 of PLP and Asp271]
show Thr348 and Thr339, whose hydroxyl groups coordinate to PLP O3 atom. However, the bacterial GAD shows another additional residue Ser246 that interacts with the O3 atom through a water molecule. Comparison of interacting residues in all crystal structures as listed in Table 1 revealed that a Thr moiety might be considered as a conserved species for binding with O3 of PLP in Group-II decarboxylases.

Both the crystal structures of Group-III showed the Trp unit as the binding residue for the oxygen atom (Table 1). The hydrogen atom in the NH group of Trp was quite polar in nature and could bind through hydrogen bond to the O3 atom of the co-factor. All the enzymes of group-IV showed an Arg residue at the vicinity of O3 (Table 1). In many cases, a well-ordered water molecule was identified through which the Arg residue could interact with PLP O3 atom.

All these observation suggested that various enzymes in their respective groups were quite selective in binding the O3 atom of PLP and within a specified group, a conserved amino acid residue was used for this purpose. In the next section, our quantum chemical investigation will reveal how two binding sites assisted the progress of decarboxylation by modifying the potential energy surface (PES).

Quantum mechanical study

Quantum chemical study was performed to investigate the effect of hydrogen bonding between O3 of PLP and a hydrogen bond donor residue at the proximity of this atom on the activation energy of decarboxylation step. For this study, 3-hydroxy-pyridine-4-aldehyde was considered as an approximated structure of PLP. The aldimine of this approximated system with L-alanine was employed to study the decarboxylation process. All the structures (Scheme IV) were optimized by DFT method using B3LYP hybrid functional and 6-31G* basis set.

The protonation at pyridine nitrogen of PLP is reported to decrease the energy of activation of decarboxylation process\(^\text{10,11}\). We should be talking about two tautomeric forms and their resonance canonical forms (Scheme III). In the oxoenamino or ketoenamino form, there is a negative charge on O3 and it is expected that protonation at this oxygen of substrate-bound PLP will make the co-factor much more electron demanding. As a result, stability of C\(_\alpha\)-COO\(^-\) \(\sigma\)-bond of substrate will also be different in comparison to non-protonated counterpart by interacting with the pi-system of pyridine ring of PLP.

The effect of protonation on the stability of co-factor substrate complex was studied by attaching protons at O3 and at pyridine nitrogen and optimizing the geometries in a conformation favorable for decarboxylation. Geometries of substrate-bound PLP with C\(_\alpha\)-COO\(^-\) \(\sigma\)-bond aligned in a perpendicular plane to the pyridine ring were optimized after attaching protons at O3 and at pyridine nitrogen. Results shown in Table 2 revealed that in almost all cases, the system underwent to its optimized state after increasing the length of C\(_\alpha\)-COO\(^-\) \(\sigma\)-bond, indicating the decarboxylation process becoming more facile on the protonated species.

To understand the effect of H-bonding with O3 of PLP on energy profile of decarboxylation process we constructed some models of external aldimine with binding residues and investigated the PES of the corresponding reaction. At the beginning, the approximated co-factor mentioned earlier was taken into consideration to study the change in energy with increasing length of C\(_\alpha\)-COO\(^-\) \(\sigma\)-bond (dissociation of CO\(_2\)). The curve (bond length vs. PE) showed no maxima and thus the reaction was expected to proceed without any transition state (Fig. 3, curve A). Then a formate ion was placed at a fixed distance to act as a counter ion for the protonated pyridine nitrogen of PLP. The initial optimized geometry is described in the Fig. 2A. The variation of PE with the bond length of C\(_\alpha\)-COO\(^-\) is shown in the Fig. 3, curve B,
which followed an energy maximum, stabilized by 4.5 Kcal/mole with respect to the non-coordinated co-factor at the same point and this could be an energy profile for decarboxylation reaction. Fig. 2A’ represents the optimized geometry at the energy maximum of the curve-B.

One methanol molecule at a fixed distance was employed to approximate a threonine residue for coordinating with O3 of PLP. Figure 2B & B’ represent the optimized geometries of minimum and maximum points on the PES (Fig. 3, curve C) respectively, in which methanol molecule was coordinated to O3 of PLP ring. In this case, pyridine nitrogen atom of PLP was left unprotonated to quantify the effect of protonation only on O3 of PLP. Curve-C of Fig. 3 represents the progress of decarboxylation process with the assistance of only protonated O3. In contrast to curve-B, it showed a broad maxima and the system at energy maximum was lowered by 1.6 Kcal/mole with respect to the system represented by curve-A. This clearly indicated that coordination at the O3 of PLP by an external hydrogen bond donor contributed stability to some extent for the TS of the decarboxylation process, though not much as shown by the coordination at pyridine nitrogen (Fig. 3, curve B). Finally, the combined effect of both, the coordinated pyridine nitrogen atom and O3 of PLP was studied. This effect

### Table 2—Results obtained from the quantum mechanical calculation on various substrate-mimics of PLP-enzyme complexes

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<tr>
<th>Trial structure</th>
<th>Substituents</th>
<th>Result during optimization</th>
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<td>R1 = R2 = l.p</td>
<td>Does not cleave Cα-COO’ bond</td>
<td>Optimized to stable geometry</td>
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<tr>
<td>R1 = l.p; R2 = H</td>
<td>Cleaves Cα-COO’ bond</td>
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l.p.= lone pair.

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Fig. 2—(A) Optimized geometry of model PLP with formic acid (A’) Transition structure for decarboxylation process in assistance with formic acid (B) Optimized geometry of model PLP with methanol (B’) Transition structure for decarboxylation process in assistance with methanol (C) Optimized geometry of model PLP with formic acid and methanol (C’) Transition structure for decarboxylation process in assistance with formic acid and methanol
can be visualized in the PES represented by curve-D of Fig. 3, which showed an energy maximum stabilized by 1.1 and 5.2 Kcal/mole with respect to the systems represented by curve-B and A respectively (Fig. 3). The optimized geometries of the energy minimum and maximum on the PES of PLP-substrate complex (Fig. 3, curve-D) in which both pyridine nitrogen and O3 atom were protonated are shown in Figs 2C & C,’ respectively.

Thus, the present computational study revealed that for facile decarboxylation process protonation at both electron-rich sites (pyridine nitrogen and O3 of PLP) was a necessary condition.

**Molecular dynamics study**

From the outcome of the above DFT study, it was obvious that formation of hydrogen bond between O3 of PLP and a hydrogen bond donor amino acid residue of the decarboxylases was quite significant in the facile decarboxylation process. This finding was further reinforced from the results of MD simulations. Ligand-free DDC (PDB entry: 1JS6.pdb) and carbidopa-bound DDC (PDB entry: 1JS3.pdb) were selected for present investigation. After completion of 2 ns simulations, the root mean square deviation (RMSD) values were plotted against time and presented in Fig. 4. RMSD is a numerical measure of the difference between two structures. From the given data, it was clear that RMSD values for ligand-free DDC was relatively higher than that of carbidopa-bound enzyme (1.41 Å and 1.22 Å, respectively). This indicated that substrate-bound DDC was conformationally much more rigid than ligand-free enzyme. Possibly, this was due to difference in number of hydrogen bonding networks at the active site of two decarboxylases.

As the crystal structure of DDC is a tightly associated dimer of two chains (chains A and B), to elucidate the effect of Thr246 on PLP binding and catalytic activity, the distances between O3 and hydroxyl oxygen of Thr (OG1) for both chains were plotted against time steps (Figs 5A & B). The time averaged distances calculated for ligand-free and substrate-bound DDCs as reported in Table 3 indicated
that this distance was higher for ligand-free enzyme than that of substrate-bound enzyme. This result supported the observation obtained from native crystal structure already mentioned in the previous section.

The distances between O3 of PLP and hydrogen atom of Thr246 hydroxyl group were also plotted against time steps for both monomeric structures (Figs 6A & B & Table 3). Here also the distance was much longer in the case of native enzyme in comparison to carbidopa-bound enzyme. Figure 7 represents snapshots of the active site clefts of ligand-free and ligand-bound DDCs during the time of simulation. From Fig. 7 it was clear that the distances between O3 of PLP and Thr246 hydroxyl hydrogen was much shorter in the ligand-bound DDC in comparison to ligand-free enzyme.

All these observations obtained from the present MD simulations established that Thr246 residue was in close proximity of PLP only in the substrate-bound enzyme, so that it could act as hydrogen bond donor. This hydrogen bond not only maintained the tight binding of co-factor with the enzyme in the active site cleft, but was also responsible for withdrawing electron density from PLP and consequently facilitated the decarboxylation process. DFT calculation reported in the previous section also supported the role of O3 of PLP on the PES of
decarboxylation process when hydrogen bonded to amino acid residue having H-bond donor group. The present dynamics study also indicated that when PLP was bound to Lys residue in the internal aldimine, this hydrogen bonding was not important for binding PLP at the active site cleft.

**Effect of electrostatic potential**

ESP of PLP and that of active site clefts for ligand-free and carbidopa-bound DDCs were calculated. Solvent accessible surfaces were color mapped with electrostatic potential (Figs 8A to F). It was found that
negative electrostatic potential field was generated around the phosphate group and O3 atom and positive potential field was around pyridine nitrogen atom of PLP (Fig. 8B). Careful observation of ESP surfaces of active site clefts of both DDCs (Figs 8C to F) revealed that in these regions PLP pyridine nitrogen atom was surrounded by negative potential field generated by the enzyme itself. On the contrary, O3 and also the phosphate group of PLP were covered by strong positive potential field originated from the other part of the enzyme. ESP surface generated around PLP and that of the active site clefts of the PLP-dependent decarboxylases were complementary to each other. So, there was strong reason to consider the electrostatic effect as an important contributor to the co-factor-enzyme binding process.

Conclusion

Present computational study reveals that protonation at O3 of PLP is also a necessary condition for the facile decarboxylation process. Thr246 residue has an important role in binding PLP to the substrate. It also plays a crucial role in the decarboxylation process by activating PLP which is due to the electron pulling of Thr246 from pyridine ring through hydrogen bond formation with O3 of PLP. The electrostatic interaction between PLP and decarboxylases plays a crucial role to maintain the proper conformational orientation of PLP in the active site cleft during the decarboxylation process.

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