Theoretical study of the reaction mechanism of phenolic acid decarboxylase

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Introduction

Phenolic acid decarboxylases (PADs) are cofactor-free enzymes that catalyze the non-oxidative decarboxylation of phenolic acids to their corresponding p-vinyl derivatives (Scheme 1) [1,2]. PAD-type enzymes can be classified based on their substrate specificity, such as p-coumaric acid decarboxylase (PDC) and ferulic acid decarboxylase (FAD). Phenolic acids are important compounds in the cell wall of plants [3], linking lignin to hemicellulose and cellulose. They can be released from these polymers by hemicellulases [4] and cinnamic acid esterases [5]. A number of organisms have evolved an ability to transform these potentially toxic phenolic acids [6,7] by some metabolism pathways [8], including PAD-catalyzed reactions [9]. As lignin is an

Abbreviations
BsPAD, phenolic acid decarboxylase from Bacillus subtilis; DFT, density functional theory; EsFAD, ferulic acid decarboxylase from Enterobacter sp.; FAD, ferulic acid decarboxylase; HCHL, hydroxycinnamoyl-CoA hydratase-lyase; LpPDC, p-coumaric acid decarboxylase from Lactobacillus plantarum; PAD, phenolic acid decarboxylase; PCA, p-coumaric acid; PDC, p-coumaric acid decarboxylase; ScFAD, ferulic acid decarboxylase from Saccharomyces cerevisiae; VAO, vanillyl alcohol oxidase; ZPE, zero-point energy.
abundant and renewable resource, PAD-type enzymes can be used as biocatalysts to produce styrene derivatives as precursors for the polymer industry [10] and to generate flavor and fragrance compounds for the food-processing industry [11]. PAD-type enzymes have recently also been reported to catalyze the hydration [12] and carboxylation [13] of hydroxystyrenes, increasing further their biocatalytic utility.

PAD-type enzymes can catalyze the decarboxylation of several phenolic acids, but are inactive toward substrate analogues without a hydroxy group at the para position of the aromatic ring [14,15]. This substrate specificity indicates that a p-quinone methide intermediate pathway (Scheme 2) is involved in the decarboxylation process, rather than a 1,2-addition pathway [16]. Based on this, the following reaction mechanism has been proposed [14]. The reaction starts with the deprotonation of the phenolic p-hydroxy group, followed by a proton transfer from a general acid at the active site to the nucleophilic C2 carbon of the substrate, resulting in the formation of a quinone methide intermediate. Subsequently, the quinone intermediate promotes the electron flow from the carboxylate, causing the C–C bond cleavage and the generation of the p-vinyl phenol and CO2 products. This kind of quinone intermediate has also been suggested in the reactions of other enzymes with p-hydroxylated aromatic compounds as substrates, such as hydroxycinnamoyl-CoA hydratase-lyase (HCHL) [17] and vanillyl alcohol oxidase (VAO) [18].

In recent years, several crystal structures of PAD-type enzymes from different organisms have been solved [19–25]. The crystal structures of two wild-type enzymes were first solved for PDC from Lactobacillus plantarum (LpPDC) [19] and PAD from Bacillus subtilis (BsPAD) [20]. Subsequently, another structure of wild-type LpPDC and structures of three mutants of LpPDC (Tyr20Phe, Glu71Ser and Arg48Gln) were obtained [22]. Comparison of these four structures of LpPDC indicated that the wild-type LpPDC and the Tyr20Phe mutant are in an ‘open’ conformation, while the Glu71Ser and the Arg48Gln mutants are in a ‘closed’ conformation [22]. Recently, the structure of the Tyr19Ala mutant of BsPAD was solved in complex with the substrate p-coumaric acid (PCA) [23]. In addition, the structures of FAD from Enterobacter sp. (EsFAD) [13,24] and Saccharomyces cerevisiae (ScFAD) [25] have also been determined.

The structures of LpPDC and BsPAD show high overall similarity [22,23]. The active site is located at the internal cavity in a conserved β-barrel and changes from the ‘open’ to the ‘closed’ conformation by the movements of several loops, induced by substrate binding, especially the β1–β2 loop. The binding mode of the substrate in the crystal structure of BsPAD (shown in Fig. 1) [23] is consistent with that in the proposed model based on molecular docking simulations of LpPDC [22]. In the crystal structure of BsPAD, two tyrosine residues (Tyr11 and Tyr13) interact with the substrate carboxylate group, and an arginine residue (Arg41) forms a hydrogen bond with the phenol moiety. Glu64 is suggested to act as a general base to accept a proton from the p-hydroxyl group [23]. In this orientation, Tyr19 is the only group that can protonate the C2 = C3 double bond of the substrate. The importance of these residues has been demonstrated by mutational analysis [23]. Namely, the mutation of Tyr11 or Tyr13 to phenylalanine caused an almost complete loss of activity, and the Tyr11Ala/Tyr13Ala, Glu64Ala and Arg41Ala variants were inactive. The Tyr19Ala mutant retained 4% activity, indicating that this residue is not the proton source for the reaction, or at least not the only one. In HCHL and VAO, two tyrosine residues equivalent to Tyr11 and Tyr13 are also observed in the active sites [17,26]. However, the crystal structures of these enzymes show that the substrate is flipped 180° compared to the orientation of the substrate in BsPAD. That is, the two tyrosines interact with the p-hydroxyl group of the substrate. In the proposed mechanisms for hydration and carboxylation of hydroxystyrenes catalyzed by

![Scheme 1](image1.png)

**Scheme 1.** Reaction catalyzed by PAD-type enzymes.

![Scheme 2](image2.png)

**Scheme 2.** Proposed mechanism of PAD-type enzymes involving quinone methide intermediate [14].
PAD-type enzymes, a substrate orientation similar to that in HCHL and VAO has been implicated [12,13].

In the present study, the reaction mechanism of PAD from *B. subtilis* was investigated by means of density functional theory (DFT) calculations. A very large model of the active site is designed on the basis of the crystal structure of the substrate-bound form of the enzyme [23]. Reaction pathways with different substrate orientations, general acids and protonation states of Glu64 and the substrate are considered. Energy profiles are presented and the roles of various active site residues are analyzed. The methodology used here has been successfully employed to study a large number of enzymatic reaction mechanisms [27–31].

Decarboxylases are increasingly used in organic synthesis, and mechanistic studies have attracted great interest [32–35]. The information obtained here on the structures and energetics of the *Bs* PAD-catalyzed reaction may help to further understand PAD-type enzymes and to extend their biocatalytic applicability.

**Computational methodology**

**Technical details**

All the calculations presented here were carried out using the Gaussian 09 program [36] with the B3LYP hybrid density functional method [37,38]. Geometries were optimized with the 6-31G(d,p) basis set. To obtain more accurate energies, single-point calculations on the optimized structures were performed with the larger basis set 6-311 + G(2d,2p). The SMD solvation model was used to consider the effects of the rest of the enzyme that was not included in the model [39]. Single-point energies at the same level of theory as the geometry optimization were calculated, and the dielectric constant of the enzyme environment was chosen to be ε = 4. Frequency calculations were performed with the 6-31G(d,p) basis set to obtain zero-point energies (ZPEs). Dispersion corrections were obtained using the DFT-D3(BJ) method [40,41]. The energies presented in this paper are thus the large basis set energies corrected for ZPEs, solvation and dispersion effects.

As will be discussed below, a number of atoms were fixed to their crystallographic positions during the geometry optimizations to prevent unrealistic movements of the residues. This procedure introduces a number of small imaginary frequencies, in this case less than 20, typically below 50i cm⁻¹. In general, entropy has only a small effect on the chemical steps of enzymatic reactions [42,43]. However, the entropy effects cannot be ignored when the release or binding of a molecule is involved in the reaction. According to previous studies on similar enzymatic reactions [44,45], the entropy gain from the release of a small gas molecule can be estimated to be equal to the translational entropy for the free molecule. The entropy of the release of CO₂ is calculated to be 11.1 kcal mol⁻¹ at room temperature and this value is added to the energy of the C–C bond cleavage step.

**Active site model**

In the current study, a model of the PAD active site was constructed on the basis of the crystal structure of the Tyr19Ala mutant of *Bs*PAD in complex with the substrate PCA (PDB 4ALB, Fig. 1) [23]. To restore the enzyme to its wild-type, the mutated Ala19 residue was modified back to the native tyrosine. As shown in Fig. 2, the active site model consists of the PCA substrate, crystallographic water molecules Wat1 and Wat2, the residues suggested to act as general acids or bases in the reaction (Tyr19 and Glu64), the residues potentially forming hydrogen bonds directly or via Wat1 with the carboxylate group and phenolic hydroxyl group of the substrate (Tyr11, Tyr13, Arg41, Thr66, Thr68 and Thr98), and the residues Tyr31, Trp62 and Gln102 that can form hydrogen bonds to Glu64. In addition, a number of residues that also contribute to make up the active site (Ile33, Val38, Val70, Leu72, Ile85 and Phe87) were included. The water molecules Wat1 and Wat2 included in the active site model have also been observed in the same positions in other crystal structures of PAD-type enzymes [19–22,24]. Truncations of all the amino acids were made at the α-carbons, and hydrogen atoms were
added manually. In order to avoid unrealistic movements of the groups during the geometry optimizations, the truncated α-carbons and also a number of β-carbons were kept fixed to their crystallographic positions, as marked with asterisks in Fig. 3.

As discussed above, the substrates of HCHL and VAO are proposed to bind in a different orientation [17, 26] compared to that in BsPAD and LpPDC. One can envisage a similar binding mode also in BsPAD, however, such that the two tyrosine residues (Tyr11 and Tyr13) interact with the p-hydroxyl group instead of the carboxylate group of the substrate. Therefore, two binding modes (termed Mode A and Mode B) with different substrate orientations are investigated in the current study (see Fig. 3). Mode A is the one assumed on the basis of the binding modes in HCHL and VAO, while Mode B is the one that was suggested by the crystal structure of BsPAD (PDB 4ALB) [23]. For each binding mode, different protonation states of the substrate p-hydroxyl group and the residue Glu64 are considered, and also different proton sources for the protonation of the substrate are examined.

It should be noted here that the crystal structure of BsPAD shows that a water molecule (Wat3 in Fig. 1) is located in the vicinity of where the mutated Tyr19 should be. An equivalent water molecule has also been observed in the other structures of PAD-type enzymes [19–22, 24]. Since this water molecule can contribute to the stabilization of the Tyr19 when it acts as the general acid in the reaction mechanism (see discussion below), we have added it to the model in all the scenarios where Tyr19 is examined as the proton source, as shown for example in Fig. 3B. The total sizes of the active site models used in this study range between 306 and 309 atoms.
Results and discussion

Reaction mechanism

Reaction pathways with different substrate orientations, proton sources and protonation states have been examined in this study. First, we describe the one that was found to have the most plausible energy barriers (see Scheme 3). The other alternatives that were calculated to have higher energies will be discussed briefly in the following sections.

In the most favorable pathway, the substrate turned out to be bound according to Mode A, i.e. similarly to what has been observed in the crystal structures of HCHL [17] and VAO [26]. It has been proposed that the substrate p-hydroxyl group should be deprotonated to facilitate the formation of quinone methide intermediate [22–24]. However, in the substrate orientation according to Mode A, there is no general base in the vicinity of the p-hydroxyl group to accept the proton. From the optimized structure shown in Fig. 3A one can see that, upon binding, the substrate p-hydroxyl group is hydrogen-bonded to two tyrosine residues (Tyr11 and Tyr13). These interactions make the hydroxyl group much more acidic, and we therefore suggest that the substrate p-hydroxyl group loses its proton upon binding to the active site [46]. Two equivalent tyrosine residues in the active sites of HCHL and VAO have also been suggested to play a similar role in the reactions of those enzymes [17,26]. Interestingly, in the proposed mechanisms for the PAD-catalyzed hydration and carboxylation of hydroxystyrene, the substrate (which corresponds to the product of the present study) was also suggested to bind to the active site in an orientation corresponding to Mode A [12,13].

Initially, we optimized a structure of the enzyme-substrate complex in which the Glu64 residue is in the protonated form. However, we could also locate a structure in which this proton moves to the carboxylate group of the substrate. The latter structure (called React-A, see Fig. 3A) has lower energy and will therefore constitute the zero point of the relative energy scale of the reaction. In React-A, the distance between

Scheme 3. Reaction mechanism of BsPAD suggested on the basis of the present calculations.

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the proton on the substrate carboxyl and the oxygen of Glu64 carboxylate is 1.68 Å. The substrate carboxyl group further forms a hydrogen bond to Wat1, which in turn is hydrogen-bonded to Thr66 and Thr98. Three hydrogen bonds are found between Glu64 and the surrounding groups Tyr31, Trp62 and Wat2. Additionally, Arg41 forms hydrogen bonds with Tyr31 (see Fig. 3A).

In order for the reaction to proceed, the proton on the substrate carboxyl has to be transferred to Glu64 first (Int1, Fig. 4). This structure is calculated to be 5.6 kcal mol\(^{-1}\) higher in energy than React-A. In Int1, the distance between the transferred proton and the oxygen atom of the substrate is 1.47 Å. Differently from React-A, Tyr31 and Arg41 now form hydrogen bonds with the substrate carboxylate group.

The following step of the reaction is the protonation of the substrate C2 = C3 double bond. This is achieved by the protonated Glu64 as the general acid. The calculated energy barrier for this step is 16.0 kcal mol\(^{-1}\) relative to React-A. In the optimized structure of the corresponding transition state TS1, the proton on Glu64 transfers to the C2 atom with H–C and O–H distances of 1.30 Å and 1.36 Å, respectively (see Fig. 4). The hydrogen bond between the substrate carboxylate and Tyr31 is kept, and now Arg41 also forms hydrogen bonds with the same carboxylate group. This indicates that these two residues contribute to the stabilization of the negative charge of the substrate carboxylate group in this step.

Downhill from TS1, the quinone methide intermediate (Int2) is formed, which is calculated to be 6.9 kcal mol\(^{-1}\) higher in energy than React-A. The hydrogen bond pattern changes between TS1 and Int2. In TS1, the substrate carboxylate group is stabilized by Tyr31, Arg41 and Wat1, while in Int2 Tyr31 has rotated to form a hydrogen bond with the negatively charged Glu64, and Arg41 forms hydrogen bonds with both Tyr31 and Glu64. Notably, a new hydrogen bond is formed between Tyr19 and the substrate carboxylate group (Fig. 4).

The next step is the collapse of the quinone methide intermediate Int2 to generate the vinyl phenol and to release CO\(_2\). The energy barrier for this C–C bond cleavage step is calculated to be 9.0 kcal mol\(^{-1}\) relative to Int2, i.e. 15.9 kcal mol\(^{-1}\) higher than React-A. The reaction energy of this step is –5.3 kcal mol\(^{-1}\) relative to Int2, including the entropy contribution discussed in the Technical Details section. At TS2, the C2–C3 bond length is 2.28 Å, increased from 1.60 Å in Int2. The hydrogen bond between Tyr19 and the substrate carboxylate group is still maintained at TS2, but is slightly elongated.

The calculated energy profile for the entire reaction is given in Fig. 5. As seen, the energies of the transition states for the substrate protonation (TS1) and the C–C bond cleavage (TS2) are very close (16.0 and

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**Fig. 4.** Optimized structures of stationary points along the proposed reaction pathway. Atoms fixed during geometry optimization are marked with asterisks. Distances are given in ångströms. For clarity, only the substrate and selected residues are shown here.
15.9 kcal·mol\(^{-1}\), respectively). Therefore, it is not possible on the basis of the current calculations to determine which of the steps is the rate-limiting one.

It is interesting to note that, in the optimized structure of the quinone methide intermediate Int2, Tyr19 is found to form a hydrogen bond to the substrate carboxylate group, which is maintained in the structure of the C–C bond cleavage transition state (TS2). To investigate the influence of this interaction on the energetics of the reaction, we have optimized a different structure of Int2 (called Int2\(^\prime\)), in which this hydrogen bond is not formed (see Data S2 for the optimized structure). In this case, the calculated energy is 3.2 kcal·mol\(^{-1}\) higher than Int2. Very importantly, the barrier for the C–C bond cleavage starting from Int2\(^\prime\) is 5.0 kcal·mol\(^{-1}\) higher than TS2 (see Data S2 for the optimized structure). These results thus show that Tyr19 is important for the catalysis.

### Alternative reaction pathways with Mode A

As mentioned above, several alternative pathways with different protonation states and proton sources have also been considered for Mode A. All of these scenarios turned out to have higher energy barriers. We will briefly discuss these options here.

In the mechanism suggested on the basis of the calculations (Scheme 3), the substrate p-hydroxyl group is in the deprotonated form and Glu64 acts as the general acid. In the scenario where this hydroxyl group is in the neutral form, the protonation of the substrate double bond cannot generate a quinone methide intermediate. As expected, the calculated energy of the intermediate with protonated C2 atom is very high, 32.2 kcal·mol\(^{-1}\) higher than the corresponding reactant (see Data S3 for the optimized structures), which shows that this scenario can be ruled out and that the hydroxyl group of the substrate has to be deprotonated prior to C2 protonation.

As discussed above, it has been suggested that Tyr19 is the general acid, protonating the substrate double bond [23]. This option has also been examined in the current study. Starting from the enzyme–substrate complex with the same protonation state as React-A, the protonation of the substrate double bond by Tyr19 can generate a quinone methide intermediate (see Data S3 for the optimized structures). However, the energy of this intermediate is calculated to be 22.7 kcal·mol\(^{-1}\) relative to the reactant, which is 16 kcal·mol\(^{-1}\) higher than the corresponding intermediate in the suggested mechanism of Scheme 3.

For the scenario in which both the substrate p-hydroxyl group and Glu64 are deprotonated and Tyr19 is the general acid, we optimized the structures of intermediates and transition states along the reaction (see Data S3). The calculated barrier for the substrate protonation is 17.7 kcal·mol\(^{-1}\) relative to the reactant, which is reasonable. However, the energies of the following stationary points are calculated to be much higher. In particular, the barrier for the C–C bond cleavage is 34.3 kcal·mol\(^{-1}\), which is 18 kcal·mol\(^{-1}\) higher than that of the suggested mechanism of Scheme 3. From these results, one can dismiss the Tyr19 residue as being the general acid in Mode A.

Finally, it should be noted that the substrate can also bind to the active site according to Mode A but with the other face of the substrate receiving the proton from Glu64. In this orientation, the interaction between the substrate and the surrounding groups is slightly different from that in Mode A. The overall energy barrier of this scenario is 21.2 kcal·mol\(^{-1}\), which is 5 kcal·mol\(^{-1}\) higher than the suggested mechanism. Optimized structures and the energy profile for this scenario are also given in the Data S2.

### Reaction pathways with Mode B

For the binding mode suggested by the crystal structure (Mode B) [23], the only possible proton source for the substrate double bond is Tyr19. Two scenarios with different protonation states of the substrate p-hydroxyl group have been investigated here.

As shown in the optimized structure of the active site model for Mode B (React-B, Fig. 3B), the substrate carboxylate group forms hydrogen bonds with Tyr11 and Tyr13, and the p-hydroxyl group forms hydrogen bonds with Wat2 and Glu64. In the mechanism proposed on the basis of the crystal structure, Glu64 is suggested to accept the proton of the substrate p-hydroxyl group in the formation of the quinone methide intermediate [23]. Using this model, we attempted to optimize the structure of the quinone methide intermediate by moving the proton from Tyr19 to the C2 position of the substrate and the
substrate $p$-hydroxyl proton to Glu64. However, in optimization, the proton on the Glu64 carboxyl always moves back to the substrate $p$-hydroxyl group (see Data S4 for the optimized structures). The resulting intermediate is calculated to be 36.2 kcal-mol$^{-1}$ higher in energy than React-B, showing that this pathway is not plausible.

Another scenario for Mode B has also been examined in which both the substrate $p$-hydroxyl group and Glu64 are deprotonated. The calculated energy profile for this scenario is similar to that in Mode A with same protonation states and proton source (see Data S4 for optimized structures and energy profiles). The barriers for the substrate protonation and the C–C bond cleavage are 18.0 kcal-mol$^{-1}$ and 30.2 kcal-mol$^{-1}$ respectively relative to the reactant, and this scenario can thus be ruled out too.

Here, it is interesting to compare the calculated energies for the enzyme–substrate complexes with different substrate orientations. The energy of React-B is 9.4 kcal-mol$^{-1}$ higher than the structure corresponding to React-A with the extra water molecule hydrogen bonding to Tyr19. Similarly, in the case where both the substrate $p$-hydroxyl group and Glu64 are deprotonated, the reactant with Mode B is also calculated to be higher in energy by 8.3 kcal-mol$^{-1}$ compared to that with Mode A. Thus, substrate binding according to Mode A is more favorable, and the energy barriers for the scenarios with Mode B become even higher compared to the reactants with Mode A.

Comparison with experimental results

No experimental rate constant ($k_{cat}$) has been measured for $Bs$PAD. However, $k_{cat}$ values for PCA decarboxylation catalyzed by several PAD-type enzymes from other organisms have been reported to be between 113 s$^{-1}$ and 6000 s$^{-1}$ [10,47,48], corresponding to energy barriers of about 12–15 kcal-mol$^{-1}$. Thus, the calculated overall barrier of 16 kcal-mol$^{-1}$ obtained here (Fig. 5) is in good agreement with these observations.

It is also interesting to compare the obtained mechanism with the results of mutational analysis. $Bs$PAD was found to be inactive when Glu64 was mutated to alanine [23]. This is consistent with the proposal put forward here that this residue is responsible for the substrate protonation (Scheme 3). Very interestingly, a glutamate residue has been suggested to function as the general acid–base group in the reaction mechanism of HCHL [17].

In the previous mechanistic proposal for $Bs$PAD, Tyr19 was suggested to be the proton source, which was based on the fact that the Tyr19Ala variant retained only 4% activity [23]. In the current mechanism, Tyr19 forms a hydrogen bond to the carboxylate group of the substrate in the decarboxylation step, which is suggested by the calculations to lower the barrier for this step (see above). This result thus reconciles the calculated mechanism with the mutational experiments for the Tyr19Ala variant.

Mutation of the Tyr11 or Tyr13 residues to phenylalanine resulted in an almost complete loss of activity, and the double mutant Tyr11Ala/Tyr13Ala was inactive in $Bs$PAD [23]. This indicates that these two tyrosines are very important for the reaction. In the calculated mechanism, the suggested role of these tyrosines is to induce the deprotonation of the substrate $p$-hydroxyl group upon binding to the active site, which is critical for the subsequent formation of the quinone methide intermediate. These hydrogen bonds between the substrate and Tyr11/Tyr13 can also stabilize other intermediates of the reaction, thereby lowering the barriers. In fact, a similar role has been implicated for the equivalent tyrosines in both HCHL and VAO [17,26].

The Arg41Ala variant of $Bs$PAD showed a complete loss of activity [23]. As seen from the optimized structures in Fig. 4, Arg41 forms hydrogen bonds to the carboxylate group of the substrate in Int1 and TS1, and to Glu64 and Tyr31 in Int2, TS2 and Prod. This indicates its role to be in the binding of the substrate and the stabilization of intermediates along the reaction pathway. In the proposed mechanisms for the PAD-catalyzed hydration and carboxylation of hydroxystyrene, this arginine residue is suggested to form hydrogen bonds with the bicarbonate molecule involved in the reaction, indicating that it is also important in these alternative reactions [12,13].

Finally, it is worth noting that similar mutations of active site residues corresponding to the ones discussed above have also been performed in $Lp$PAD and $Es$FAD, yielding very similar results as in $Bs$PAD [22,24].

Conclusions

In the present paper, we have investigated the reaction mechanism of PAD using DFT calculations. Reaction pathways with different substrate orientations, different proton sources for the substrate protonation and different protonation states of the substrate and the active site Glu64 residue have been considered. The energetically most favorable mechanism is shown in Scheme 3 and the associated energy profile is given in Fig. 5. Cartesian coordinates of the optimized structures are provided in Data S5. The calculations give general support to the proposed mechanism in which a quinone methide
intermediate is formed by the protonation of the substrate double bond, followed by C–C bond cleavage to generate the p-vinyl phenol and CO₂ products. However, significant changes compared to the literature proposal [22,23] are suggested here on the basis of the calculations.

From the obtained energies and energy barriers, it is concluded that the substrate adopts a different orientation in the active site compared to the previous proposal [22,23]. Namely, the p-hydroxyl group of the substrate, rather than the carboxylate group, interacts with the Tyr11 and Tyr13 residues, which results in the deprotonation of this hydroxyl group upon binding to the active site. This substrate orientation is analogous to that in other enzymes with p-hydroxylated aromatic compounds as substrates, such as HCHL and VAO, in which the hydroxyl group has also been observed to bind to two conserved tyrosines at the active site. One might therefore speculate that it can be a general feature for substrate recognition and activation in this kind of enzyme.

As discussed above, PAD-type enzymes have recently been reported to catalyze the non-natural reactions of hydration and carboxylation of hydroxystyrene derivatives [12,13]. Also in the proposed mechanisms of these two reactions the p-hydroxyl group of the substrate interacts with the two tyrosine residues, and the present calculations are thus consistent with these proposals.

In the previous mechanistic proposal for PAD the Tyr19 residue was suggested to act as the general acid in the reaction, protonating the C–C double bond of the substrate [22,23]. The present calculations show that this mechanism is associated with high energy barriers. It is instead suggested that the Glu64 residue has this role in the reaction, which results in much more plausible barriers. The Tyr19 residue is suggested to be engaged in hydrogen bonding to the substrate carboxylate group in the quinone methide intermediate, which is demonstrated to lower the barrier for the C–C bond cleavage step.

The overall energy barrier for the whole reaction is calculated to be in good agreement with kinetics data and the details of the mechanism are consistent with the available results of mutagenesis experiments. We believe that the new mechanistic insights provided here, most importantly into the substrate orientation and the nature of the general acid in the reaction, can be generalized to other members of the PAD family. This information might also be useful in the analysis of promiscuous reactions of the PAD-type enzyme and ultimately in the design of new enzyme variants for other biocatalytic applications.

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Author contributions

XS, ML and FH designed the project. XS and ML performed the calculations. XS, ML and FH analyzed the results and wrote the paper.

References

1 Priefert H, Rabenhorst J & Steinbüchel A (2001) Biotechnological production of vanillin. Appl Microbiol Biotechnol 56, 296–314.
2 Rodríguez H, Curiel JA, Landete JM, de las Rivas B, de López Felipe F, Gómez-Cordovés C, Mancheño JM & Muñoz R (2009) Food phenolics and lactic acid bacteria. Int J Food Microbiol 132, 79–90.
3 Iiyama K, Lam TBT & Stone BA (1994) Covalent cross-links in the cell wall. Plant Physiol 104, 315–320.
4 de Vries RP, Poulsen CH, Madrid S & Visser J (1998) aguA, the gene encoding an extracellular α-glucuronidase from Aspergillus tubingensis, is specifically induced on xylose and not on glucuronic acid. J Bacteriol 180, 243–249.
5 Christov LP & Prior BA (1993) Esterases of xylan-degrading microorganisms: production, properties, and significance. Enzyme Microb Tech 15, 460–475.
6 Zaldivar J & Ingram LO (1999) Effect of organic acids on the growth and fermentation of ethanologenic Escherichia coli LY01. Biotechnol Bioeng 66, 203–210.
7 Campos FM, Couto JA & Hogg TA (2003) Influence of phenolic acids on growth and inactivation of Oenococcus oeni and Lactobacillus hilgardii. J Appl Microbiol 94, 167–174.
8 Rosazza JPN, Huang Z, Dostal L, Volm T & Rousseau B (1995) Review: Biocatalytic transformations of ferulic acid: an abundant aromatic natural product. J Ind Microbiol 15, 457–471.
9 Tran NP, Gury J, Dartois V, Nguyen TKC, Seraut H, Barthelmels L, Gervais P & Cavin JF (2008) Phenolic acid-mediated regulation of the padC gene, encoding the phenolic acid decarboxylase of Bacillus subtilis. J Bacteriol 190, 3213–3224.
10 Jung DH, Choi W, Choi KY, Jung E, Yun H, Kazlauskas RJ & Kim BG (2013) Bioconversion of p-Coumaric acid to hydroxystyrene using phenolic acid decarboxylase from B. amyloliquefaciens in biphasic reaction system. Appl Microbiol Biotechnol 97, 1501–1511.
11 Vanbeneden N, Gils F, Delvaux F & Delvaux FR (2008) Formation of 4-vinyl and 4-ethyl derivatives from hydroxycinnamic acids: occurrence of volatile phenolic flavour compounds in beer and distribution of Pad1-activity among brewing yeasts. Food Chem 107, 221–230.

12 Wuensch C, Gross J, Steinkellner G, Gruber K, Glueck SM & Faber K (2013) Asymmetric enzymatic hydration of hydroxystyrene derivatives. Angew Chem Int Ed 52, 2293–2297.

13 Wuensch C, Pavkov-Keller T, Steinkellner G, Gross J, Fuchs M, Hromic A, Lyskowski A, Fauland K, Gruber K, Glueck SM et al. (2015) Regioselective enzymatic β-carboxylation of para-hydroxystyrene derivatives catalyzed by phenolic acid decarboxylases. Adv Synth Catal 357, 1909–1918.

14 Hashidoko Y & Tahara S (1998) Stereochemically specific proton transfer in decarboxylation of 4-hydroxycinnamic acids by 4-hydroxycinnaminate decarboxylase from Klebsiella oxytoca. Arch Biochem Biophys 359, 225–230.

15 Edlin DAN, Narbad A, Gasson MJ, Dickinson JR & Lloyd D (1998) Purification and characterization of hydroxycinnamate decarboxylase from Brettanomyces anomalus. Enzyme Microb Technol 22, 232–239.

16 Gramatica P, Ranzi BM & Manitto P (1981) Decarboxylation of cinnamic acids by Saccharomyces cerevisiae. Bioorg Chem 10, 14–21.

17 Bennett J, Bertin L, Mouton B, Fairlamb JJ, Brzozowski AM, Walton NJ & Grogan G (2008) A ternary complex of hydroxycinnamoyl-CoA hydratase-lyase (HCHL) with acetyl-CoA and vanillin gives insights into substrate specificity and mechanism. Biochem J 414, 281–289.

18 Fraaije MW & van Berkel WJ (1997) Catalytic mechanism of the oxidative demethylation of 4-(methoxymethyl) phenol by vanillyl-alcohol oxidase. J Biol Chem 272, 18111–18116.

19 Joint Center for Structural Genomics. Crystal structure of p-coumaric acid decarboxylase (NP_786857.1) from Lactobacillus plantarum at 1.70 Å resolution. PDB entry: 2P8G. Release year: 2006.

20 Joint Center for Structural Genomics. Crystal structure of phenolic acid decarboxylase (2635953) from Bacillus subtilis at 1.36 Å resolution. PDB entry: 2P8G. Release year: 2007.

21 Matte A, Grosse S, Bergeron H, Abokite K & Lau PC (2010) Structural analysis of Bacillus pumilus phenolic acid decarboxylase, a lipocalin-fold enzyme. Acta Crystallogr F: Struct Biol Cryst Commun 66, 1407–1414.

22 Rodriguez H, Angulo I, de las Rivas B, Campillo N, Páez JA, Muñoz R & Mancheño JM (2010) p-Coumaric acid decarboxylase from Lactobacillus plantarum: structural Insights into the active site and decarboxylation catalytic mechanism. Proteins 78, 1662–1676.

23 Frank A, Eborall W, Hyde R, Hart S, Turkenburg JP & Grogan G (2012) Mutational analysis of phenolic acid decarboxylase from Bacillus subtilis (BsPAD), which converts bio-derived phenolic acids to styrene derivatives. Catal Sci Technol 2, 1568–1574.

24 Gu W, Yang J, Lou Z, Liang L, Sun Y, Huang J, Li X, Cao Y, Meng Z & Zhang KQ (2011) Structural basis of enzymatic activity for the ferulic acid decarboxylase (FADase) from Enterobacter sp. P6–4. PLoS One 6, e16262.

25 Bhuiya MW, Lee SG, Jez JM & Yu O (2015) Structure and mechanism of ferulic acid decarboxylase (FDC1) from Saccharomyces cerevisiae. Appl Environ Microbiol 81, 4216–4223.

26 van den Heuvel RH, van den Berg WA, Rovida S & van Berkel WJ (2004) Laboratory-evolved vanillyl-alcohol oxidase produces natural vanillin. J Biol Chem 279, 33492–33500.

27 Siegbahn PEM & Himo F (2009) Recent developments of the quantum chemical cluster approach for modeling enzyme reactions. J Biol Inorg Chem 14, 643–651.

28 Blomberg MRA & Siegbahn PEM (2010) Quantum chemical studies of proton-coupled electron transfer in metalloenzymes. Chem Rev 110, 7040–7061.

29 Hopmann KH & Himo F (2010) Quantum chemical modeling of enzymatic reactions-applications to epoxide-transforming enzymes. In Comprehensive Natural Products Chemistry II Chemistry and Biology (Mander LN & Liu H-W, eds), pp. 719–747. Elsevier, Oxford Volume 8, Enzymes and Enzymatic Mechanisms.

30 Siegbahn PEM & Himo F (2011) The quantum chemical cluster approach for modeling enzyme reactions. Wiley Interdiscip Rev: Comput Mol Sci 1, 323–336.

31 Blomberg MRA, Borowski T, Himo F, Liao R-Z & Siegbahn PEM (2014) Quantum chemical studies of mechanisms for metalloenzymes. Chem Rev 114, 3601–3658.

32 Kourist R, Guterl JK, Miyamoto K & Sieber V (2014) Enzymatic decarboxylation—an emerging reaction for chemicals production from renewable resources. ChemCatChem 6, 689–701.

33 Nestl BM, Hammer SC, Nebel BA & Hauer B (2014) New generation of biocatalysts for organic synthesis. Angew Chem Int Ed 53, 3070–3095.

34 Jordan F & Patel H (2013) Catalysis in enzymatic decarboxylations: comparison of selected cofactor-dependent and cofactor-independent examples. ACS Catal 3, 1601–1617.
35 Li T, Huo L, Pulley C & Liu A (2012) Decarboxylation mechanisms in biological system. *Bioorg Chem* **43**, 2–14.
36 Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Scalmani G, Barone V, Mennucci B, Petersson GA *et al.* (2013) Gaussian 09, Revision D.01; Gaussian, Inc., Wallingford, CT.
37 Becke AD (1993) Density functional thermochemistry. III. The role of exact exchange. *J Chem Phys* **98**, 5648–5652.
38 Lee C, Yang W & Parr RG (1988) Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density. *Phys Rev B* **37**, 785–789.
39 Marenich AV, Cramer CJ & Truhlar DG (2009) Universal solvation model based on solute electron density and on a continuum model of the solvent defined by the bulk dielectric constant and atomic surface tensions. *J Phys Chem B* **113**, 6378–6396.
40 Grimme S, Antony J, Ehrlich S & Krieg HA (2010) Consistent and accurate Ab initio parametrization of density functional dispersion correction (DFT-D) for the 94 elements H–Pu. *J Chem Phys* **132**, 154104.
41 Grimme S, Ehrlich S & Goerigk L (2011) Effect of the damping function in dispersion corrected density functional theory. *J Comput Chem* **32**, 1456–1465.
42 Hu P & Zhang Y (2006) Catalytic mechanism and product specificity of the histone lysine methyltransferase SET7/9: An Ab initio QM/MM-FE study with multiple initial structures. *J Am Chem Soc* **128**, 1272–1278.
43 Senn HM, Thiel S & Thiel W (2005) Enzymatic hydroxylation in p-hydroxybenzoate hydroxylase: a case study for QM/MM molecular dynamics. *J Chem Theory Comput* **1**, 494–505.
44 Blomberg MRA & Siegbahn PEM (2012) Mechanism for N₂O generation in bacterial Nitric oxide reductase: a quantum chemical study. *Biochemistry* **51**, 5173–5186.
45 Lind MES & Himo F (2014) Theoretical study of reaction mechanism and stereoselectivity of arylmalonate decarboxylase. *ACS Catal* **4**, 4153–4160.
46 Model calculations using phenol with two hydrogen bonding water molecules indicate that the pKa of phenol decreases by ca 6 and 3 units using ε = 4 and ε = 78, respectively. See Data S1 for details.
47 Godoy L, Martínez C, Carrasco N & Ganga MA (2008) Purification and characterization of a p-coumarate decarboxylase and a vinylphenol reductase from *Brettanomyces bruxellensis*. *Int J Food Microbiol* **127**, 6–11.
48 Huang HK, Tokashiki M, Maeno S, Onaga S, Taira T & Ito S (2012) Purification and properties of phenolic acid decarboxylase from *Candida guilliermondii*. *J Ind Microbiol Biotechnol* **39**, 55–62.

**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s website:

**Data S1.** Model calculations using phenol with two hydrogen bonding water molecules.

**Data S2.** Optimized structures of Int2 and TS2’.

**Data S3.** Additional results concerning alternative pathways with Mode A.

**Data S4.** Additional results concerning alternative pathways with Mode B.

**Data S5.** Cartesian coordinates of all stationary points of the suggested mechanism.