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Structural studies of a fungal polyphenol oxidase with application to bioremediation of contaminated water†

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Abstract: Polyphenol oxidases (PPOs) are a group of Cu-containing enzymes exhibiting two activities, catechol oxidase and tyrosinase. Their precise mechanism of action and the structural elements that determine the distinction between the two activities are yet to be fully understood. In nature, PPOs catalyse the oxidation of several phenols to *o*-quinones, considerably affecting the colour and nutritional properties of numerous agricultural products. On the other hand, PPOs have been widely employed as biocatalysts in food, pharmaceutical, and cosmetic industries. *Tt*PPO is a PPO from the thermophilic fungus *Thermothelomyces thermophila* (*Tt*PPO), capable of degrading of chlorophenols (CPs), contagious by-products of various pesticides. The present work aims to clarify the structural determinants of *Tt*PPO function, by performing protein-ligand docking experiments via *YASARA* software. The docking results are compared with biochemical data, and the role of specific amino acids in *Tt*PPO function is investigated. The identification of the amino acids involved in binding of the different substrates to the active site of the enzyme would allow the structure-based design of a more efficient biocatalyst for wastewater treatment.

Keywords: polyphenol oxidase; tyrosinase; phenol derivative; docking; structure-function relations

1. Introduction

Polyphenol oxidases (PPOs) are a group of metalloenzymes containing a type-III copper centre and are distributed among bacteria, fungi, archaea, plants, insects and animals [1]–[5]. Type III copper center consists of two copper ions (CuA and CuB) coordinated by three histidine residues each [6]. PPOs exhibit two different enzymatic activities: a. Tyrosinase (L-tyrosine, L-DOPA:oxygen oxidoreductase; EC 1.14.18.1), which catalyzes the ortho-hydroxylation of monophenols and the subsequent two electron oxidation of the o-diphenols to the corresponding o-quinones, and b. Catechol oxidase (1,2-benzene-diol:oxygen oxidoreductase; EC 1.10.3.1), which only catalyzes the latter diphenolase reaction [7]. The quinones produced as a result of PPO activity lead to the production of complex polymers known as melanins, which cause enzymatic browning in fruits and vegetables, considerably reducing their market value. [8]. One of the most important although least studied application of PPOs is as sensitive detectors of phenol derivatives in polluted waters [9] as well as efficient tools for the biodegradation of these substances [10].

In spite of various crystallographic and molecular docking studies, the structural determinants that affect the distinction between the two enzymatic activities and the substrate specificity exhibited by PPOs are still not fully understood. For instance, molecular studies on two walnut (*Juglans regia*)

tyrosinase isoenzymes (*Jr*PPO1 and *Jr*PPO2) showed that the activity towards monophenolic substrates was dependent on the occurrence of an asparagine residue located after one of the CuB coordinating histidines [11]. However, there are many PPOs that do not have an Asn residue in this position but still display monophenolase activity [7].

The present work is focused on *Tt*PPO, a PPO from the thermophilic fungus *Thermothelomyces thermophila* that is capable of degrading chlorophenols (CPs), contagious by-products of pesticides [12]. Various *Tt*PPO mutants, designed based on a structural model and according to existing literature, were previously produced and biochemically characterized. In order to shed light on the structure-function relations of *Tt*PPO, we determined the tertiary structure of *Tt*PPO mutant (G292N) by X-ray crystallography (PDB code 6Z1S), however, subsequent efforts to determine *Tt*PPO structure in complex with various substrate analogues have not been yet successful. In the present work, we used YASARA software to dock 3,4-Dihydroxy-L-phenylalanine (L-DOPA) on native *Tt*PPO and 2 mutants that exhibited substantial differentiations in their catalytic efficiency. Based on the docking results, we discuss the implication of specific aminoacids on substrate specificity of *Tt*PPO.

2. Methods

Enzyme-substrate docking experiments were performed using *YASARA* software [13]. The crystal structure of *Tt*PPO (mutant G292N, PDB entry 6Z1S) was prepared for molecular docking as a two-step process. Initially, the pdb file was introduced to *YASARA* and water molecules, ligands, ions as well as oligosaccharides from glycosylation site were excluded from the structure. Next, all missing hydrogen atoms were added and residues having several conformations were identified (only one of the multiple conformations was kept). The structure of L-DOPA (in pdb format) was obtained from Ligand Expo [14], and formatted into *YASARA*-object files (*.yob) for later use. *YASARA* offers two docking methods, either AutoDock LGA [15-16] or VINA [17]. Binding poses were searched in a simulation cell, extended at 11Å around the two copper ions of the active site. AutoDock VINA was selected as docking method while the exhaustiveness was set to 25 and the same number of poses were calculated for each target and substrate. Poses that significantly deviated from the expected binding pose were "flagged as 'unreasonable' poses. All visualizations were created using PyMOL 2.3.2 (The PyMOL Molecular Graphics System, Version 2.3.2, Schrödinger, LLC).

3. Results and Discussion

The original purpose of the present work was to analyze the structural determinants that affected the specificity of *Tt*PPO, a recently purified and characterized oxidase [12]. Interestingly, *Tt*PPO had the ability to degrade various chlorophenols, rendering it a biocatalyst of biotechnological interest. In order to be able to improve *Tt*PPO efficiency as a bioremediation agent, via protein engineering, a detailed knowledge of substrate binding in its active site is a prerequisite. We thus performed docking experiments, using various phenolic substances, including chlorophenols. However, all docking attempts using chorophenols resulted in their positioning outside the expected substrate binding pocket. L-DOPA, on the contrary, seemed to be located in a catalytically favorable position. This finding, in combination with the fact that mutation of specific aminoacids affected substantially *Tt*PPO efficiency against this particular substrate led us to use these docking results in order to identify aminoacids in *Tt*PPO active site, actively implicated in its oxidative efficiency.

More specifically, kinetic studies of *Tt*PPO and its mutants, using a variety of substrates, showed that the double mutation G292N/L306A resulted in a variant with significantly increased activity on L-DOPA (data not shown). The latter was therefore chosen as docking target, and the relevant results are presented in Table 1.

Table 1. Molecular docking results of L-DOPA on native *Tt*PPO and mutants **G292N, G292N/L306A**.

	Best Bind.energy [kcal/mol]	Dissoc. constant [μM]
Native	4.937	240.508
G292N	5.097	183.590
G292N/L306A	5.614	76.715

The predicted binding poses are quite close to the crystallographically determined location of ligands in PPO active site, with the bound ligand being positioned between the two active site copper ions [1]. As shown in Figure 1A, L-DOPA is stabilized in native *Tt*PPO by forming hydrogen bonds with residues Ser309, His291 and Tyr119. It also seems to form a π-π stacking interaction with His295. When glycine 292 is mutated to asparagine (Fig. 1B), the aliphatic part of the ligand is shifted towards the opposite direction, forming additional hydrogen bonds with the backbone carbonyls of G304, L306 and N292, as well as with the side chain hydroxyl of Tyr296. In addition, the molecule shifts closer to the active site copper ions. When additional mutation L306A is introduced (Fig. 1C), resulting in a less stereochemically congested active site, the aliphatic side chains shifts back to its original position. In this case, L-DOPA seems to be stabilized in the active site by a higher number of hydrogen bonds, when compared to the native enzyme, involving amino acids Ser309, Tyr119, Asn292 and Tyr296.

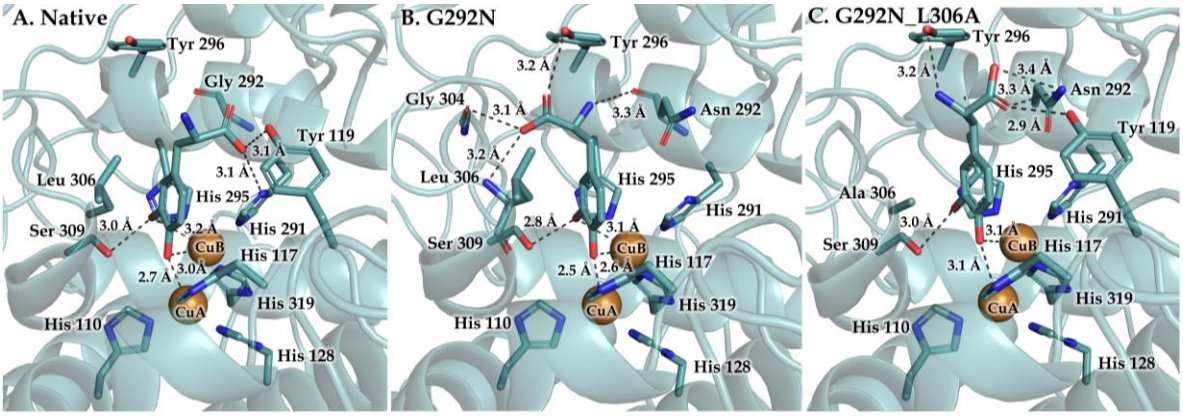

Figure 1. Docking poses of L-DOPA in (A) Native *Tt*PPO, (B) variants G292N and variant G292N_L306A (C).

4. Conclusions

Previous studies concerning the structure-function relations of PPOs, with a special focus on the amino acids affecting the ratio of monophenolase/diphenolase activity, have revealed the implication of (a) the "gate" residue [18], positioned on top of CuA (in our case L306) and (B) the residue adjacent to the first CuB coordinating histidine residue (H_{B1+1}, in our case N292) [19]. Docking of L-DOPA in *Tt*PPO active site corroborates the involvement of these residues in the positioning of the substrate. These findings, combined with biochemical experiments, set the basis for structure-based design of *Tt*PPO mutants with desired activity.

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Conflicts of Interest: The authors declare no conflict of interest.

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