Considerations Regarding Activity Determinants of Fungal Polyphenol Oxidases Based on Mutational and Structural Studies

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ABSTRACT Polyphenol oxidases (PPOs) are an industrially relevant family of enzymes, being involved in the postharvest browning of fruits and vegetables, as well as in human melanogenesis. Their involvement lies in their ability to oxidize phenolic or polyphenolic compounds, which subsequently form pigments. The PPO family includes tyrosinases and catechol oxidases, which, in spite of their high structural similarity, exhibit different catalytic activities. Long-standing research efforts have not yet managed to decipher the structural determinants responsible for this differentiation, as every new theory is disproved by a more recent study. In the present work, we combined biochemical along with structural data in order to better understand the function of a previously characterized PPO from *Thermothelomyces thermophila* (TtPPO). The crystal structure of a TtPPO variant, determined at 1.55 Å resolution, represents the second known structure of an ascomycete PPO. Kinetic data for structure-guided mutants prove the implication of “gate” residue L306, residue H_{B1}+1 (G292), and H_{B2}+1 (Y296) in TtPPO function against various substrates. Our findings demonstrate the role of L306 in the accommodation of bulky substrates and show that residue H_{B1}+1 is unlikely to determine monophenolase activity, as was suggested from previous studies.

IMPORTANCE PPOs are enzymes of biotechnological interest. They have been extensively studied both biochemically and structurally, with a special focus on the plant-derived counterparts. Even so, explicit description of the molecular determinants of their substrate specificity is still pending. For ascomycete PPOs, only one crystal structure has been determined so far, thus limiting our knowledge on this tree branch of the family. In the present study, we report the second crystal structure of an ascomycete PPO. Combined with site-directed mutagenesis and biochemical studies, we depict the amino acids in the vicinity of the active site that affect enzyme activity and perform a detailed analysis on a variety of substrates. Our findings improve current understanding of structure-function relations of microbial PPOs, which is a prerequisite for the engineering of biocatalysts of desired properties.

KEYWORDS polyphenol oxidase, X-ray structure, site-directed mutagenesis, tyrosinase, kinetic analysis, *Thermothelomyces thermophila*, protein engineering

Polyphenol oxidases (PPOs) are a family of enzymes comprising tyrosinases (TYR; EC 1.14.18.1), catechol oxidases (CO; EC 1.10.3.1), and aureusidin synthases (AUS; EC 1.21.3.6). Genes expressing them can be found in all domains of life, from humans, animals, and insects to plants, fungi, bacteria, and archaea. Their role in nature is basically

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the synthesis of pigments for protective or other purposes, depending on the organism. This takes place by the oxidation of phenolic compounds into quinones, which then undergo nonenzymatic reactions toward the formation of pigments (1, 2). Organisms from different kingdoms use different precursors for their pigments; hence, they express enzymes with affinity to these specific compounds. For example, animals synthesize their pigments from nitrogen-bearing phenolic compounds, such as tyrosine, dopamine, and catecholamines, while in plants and fungi the most common melanin precursors are catechol, 1,8-dihydroxynaphthalene, and some phenolic acids (e.g., gallic, caffeic, and protocatechuic acids). Bacteria, on the other hand, seem to be able to utilize both types of precursors to form their melanins (3).

In this manner, PPOs are classified, depending on their substrate specificity, as those that hydroxylate tyrosine or other similar monophenolic compounds (TYR-monophenolase activity) or those that can oxidize catecholic derivatives (TYR/CO/AUS-diphenolase activity). This classification seems to be somewhat outdated, though, since recent studies have shown that previously classified COs possess monophenolase activity for their natural substrates, like in the case of AUS from *Coreopsis grandiflora* (CgAUS) (4). Similarly, the PPO from *Aspergillus oryzae* was initially characterized as a CO (AoCO4) because it cannot oxidize tyrosine. However, it seems to be able to hydroxylate small monophenols, as well as diphenols in the *ortho*-position, prior to their further oxidation to quinones (5). These recent findings are indicative of the need to reconsider the classification and nomenclature of members of this enzyme family.

PPOs contain one type-III copper site, which reversibly binds dioxygen and consists of two copper ions (CuA and CuB), each coordinated by three histidine residues (H_{A1} to H_{A3} and H_{B1} to H_{B3} , respectively). Type-III copper sites can be found in at least four states: *oxy*-, *met*-, *deoxy*-, and *hydroperoxide*-forms. In the *oxy*-form, the two Cu^{II} ions bind a dioxygen molecule, while in the *met*-form, which is considered the resting state, the two tetragonal coppers are bridged by a hydroxide or water molecule (6). Reaction of an *o*-diphenol with the *met*-form results in the reduced *deoxy*-form (Cu^I -dicopper), which can quickly turn into the active *oxy*-form by binding dioxygen. The *hydroperoxide*-form can be formed either by oxygen binding to the reduced site or by peroxide activation for substrate hydroxylation (7, 8). Monophenols, on the other hand, require the *oxy*-PPO form to start the catalytic cycle (6).

Even though the active sites of all type-III proteins show high similarity, their substrate scope differs. In the past decade, a lot of effort has been made toward the elucidation of the reaction mechanism of PPOs and the molecular determinants of their ability to hydroxylate monophenols. However, these determinants still remain unclear and every theory is overthrown by a newly discovered enzyme or crystal structure. One of the first theories for the lack of monophenolase activity in COs was the presence of a bulky residue (otherwise known as a “gate” residue) atop CuA (most commonly Phe in plant COs), which was thought to sterically hinder monophenols from binding to CuA, where the hydroxylation step was thought to take place (9). This theory was disproved when the first crystal structure of a plant TYR was elucidated as having the bulky Phe at this position while still retaining its monophenolase activity (10). Additionally, the work of Goldfeder et al. showed that both substrate types bind between the two metal ions, slightly oriented toward CuA (11), while for AoCO4, which possesses hydroxylase activity, it was shown that, upon substrate binding, a conformational change of the “gate” residue takes place (5). Another key residue important for the binding of common tyrosinase substrates (like tyramine) was thought to be the one adjacent to the second CuB coordinating histidine ($H_{B2}+1$), with Arg favoring substrate binding (8).

It can be seen that deprotonation of a monophenol is considered to be the crucial initial step in order to begin the monophenolase catalytic cycle, and the base implicated in this step has long been under investigation. The possession of hydroxylase activity has been related to the presence of a water molecule that is coordinated and activated by an Asn at position $H_{B1}+1$ and a highly conserved Glu positioned several

residues before $H_{B1}+1$ (11). This water molecule is said to deprotonate the incoming monophenol, which is the first step toward its hydroxylation (12). In the case of a PPO from *Vitis vinifera* (VvPPO), the mutation of Gly at position $H_{B1}+1$ to Asn resulted in increased enzyme activity on tyramine and tyrosol (13). Examples of enzymes that do not have an Asn at this position but can still hydroxylate monophenols (not necessarily tyrosine and tyramine) are CgAUS (Thr), two *Agaricus bisporus* PPOs (Asp), two apple TYRs (Ala and Gly), and AoCO4 (Gly) (8). In fact, a mechanism for the hydroxylation by AoCO4 has been proposed, where the enzyme's copper site is in *hydroperoxide*-form and monooxygenation occurs by electrophilic aromatic substitution without the need for deprotonation (5). Recently, docking studies revealed that the preference toward diphenolic substrates exhibited by PPOs bearing a Gly in position $H_{B1}+1$ is explained by a "laying down" orientation of the substrate, which is stereochemically prohibited in PPOs bearing a bulkier Asn residue (14).

In addition to the above, many recent studies point out the His residues that coordinate the active site copper ions as potential proton-accepting bases. Matoba et al. were the first to unveil that monophenol deprotonation takes place via the active-site-bound peroxide. They also highlighted the mobility of both copper ions and the release of H_{A2} and H_{A3} from CuA during each catalytic cycle (15, 16). Crystallographic work on *A. oryzae* TYR also pointed out the migration of both copper ions and the detachment of H_{A3} , potentially acting as a base to accept a proton from the bound tyrosine substrate (17). The increased flexibility of copper-coordinating His residues (H_{A2} , H_{B1} , and H_{B2}) and their role as deprotonating bases was further corroborated by measuring the increase in monophenolase activity of various CgAUS mutants (18).

Our group recently discovered a novel fungal PPO from the ascomycete fungus *Thermothelomyces thermophila* (TtPPO) that is homologous to AoCO4 (45% sequence identity for 82% coverage). Even though TtPPO could not oxidize tyrosine, it presented high activity on other monophenolic substrates, such as guaiacol, hydroquinone, and 1,8-dihydroxynaphthalene. Additionally, it was able to oxidize and remove various chlorophenols. Site-directed mutagenesis was performed on the proposed key activity determinants and the effect on chlorophenol removal was studied (19). In the present work, we evaluate the effect of these mutations on the kinetic characteristics of TtPPO variants and discuss the results using the crystal structure of one of these variants (TtPPO-GN), determined at 1.55 Å resolution. The crystal structure of TtPPO is to our knowledge the second structure of an ascomycete PPO belonging to the short tyrosinase family and thus provides further evidence regarding the structure-function relations of these promiscuous enzymes.

RESULTS AND DISCUSSION

PPOs are enzymes of high biotechnological interest, since they are used in the fields of biocatalysis, biosensors, and bioremediation, and their inhibition has been widely studied due to their implication in postharvest browning of fruits and mushrooms, as well as in human melanogenesis (20–26). Despite several PPOs from various sources (mostly plants) having been biochemically and structurally characterized, the molecular determinants of the monophenolase/diphenolase activity ratio have been a long-lasting debate over the past decade. In the present work, we attempted to gain a deeper understanding of these enzymes' catalytic behavior by combining mutational, biochemical, and structural studies. To this end, we focused on a novel PPO (TtPPO) that had been previously reported as a potential bioremediation agent (19).

Selection of mutation points was performed based on proposed activity determinants, as discussed above. These point mutations have been shown to increase the monophenolase activity of PPOs in some cases (13, 27, 28). The corresponding residues in TtPPO are Leu306 (gate residue), Gly292 (residue $H_{B1}+1$), and Tyr296 (residue $H_{B2}+1$) (Fig. 1), and the produced variants involved the following mutations: G292N, L306A, Y296V, G292N/L306A, and G292N/Y296V.

FIG 1 Structure-based sequence alignment of *TtPPOm*, *AoCO4* (PDB ID: 4J3R), *BmTyr* (PDB ID: 3NM8), and *ScTyr* (PDB ID: 1WX2). Copper-coordinating histidine residues are indicated by blue triangles (CuA). The residues that have been mutated are denoted by yellow triangles. The residues that were not included in the structural model are denoted as insertions. Identical and similar residues are printed in white on a red background and in red on a white background, respectively. The figure was prepared using ESPript (<http://esprict.ibcp.fr>) (51).

TABLE 1 Kinetic constants of wild-type (WT) *Tt*PPO versus its mutants on phenolic substrates

Substrate	Parameter ^a	Mean value (SD) ^b for:					
		WT	G292N	L306A	Y296V	G292N/L306A	G292N/Y296V
Catechol	k_{cat}	8.6 (0.6)	5.4 (0.2)	26.2 (3.0)	4.1 (0.1)	15.4 (0.5)	4.4 (0.6)
	K_m	32.5 (4.3)	8.1 (0.9)	7.5 (1.3)	33.9 (2.3)	5.0 (0.4)	12.56 (3.0)
	k_{cat}/K_m	0.3 (0.0)	0.7 (0.1)	3.5 (0.7)	0.1 (0.0)	3.1 (0.3)	0.35 (0.1)
4-Chlorocatechol	k_{cat}	324.8 (8.6)	17.6 (0.4)	33.9 (0.9)	32.2 (0.9)	26.2 (0.3)	82.2 (3.7)
	K_m	1.8 (0.1)	3.5 (0.2)	0.4 (0.0)	2.2 (0.2)	0.5 (0.0)	4.3 (0.3)
	k_{cat}/K_m	180.4 (11.9)	5.0 (0.3)	81.6 (6.0)	14.6 (1.2)	48.3 (2.0)	18.9 (1.7)
L-DOPA	k_{cat}	4.3 (0.5)	7.5 (1.0)	4.0 (0.2)	2.1 (0.1)	235.1 (81.1)	1.0 (0.1)
	K_m	2.4 (0.7)	6.2 (1.4)	4.6 (0.5)	1.0 (0.1)	37.7 (14.7)	0.6 (0.1)
	k_{cat}/K_m	1.8 (0.5)	1.2 (0.3)	0.9 (0.1)	2.1 (0.3)	6.2 (3.3)	1.6 (0.4)
Catechin	k_{cat}	1619.1 (549.2)	2.9 (0.3)	415.0 (35.1)	547.3 (84.6)	337.9 (11.2)	33.3 (1.7)
	K_m	33.7 (14.0)	4.3 (1.0)	2.8 (0.5)	9.7 (2.4)	4.3 (0.3)	10.3 (0.8)
	k_{cat}/K_m	48.1 (25.8)	0.7 (0.2)	147.2 (30.6)	56.2 (16.6)	78.9 (6.1)	3.2 (0.3)
Guaiacol	k_{cat}	39.3 (2.2)	6.6 (0.3)	- ^c	12.8 (0.9)	0.5 (0.0)	1.5 (0.1)
	K_m	4.6 (0.6)	12.4 (1.5)	-	13.3 (1.9)	6.9 (1.7)	27.3 (4.5)
	k_{cat}/K_m	8.6 (1.3)	0.5 (0.1)	-	1.0 (0.2)	0.07 (0.0)	0.06 (0.0)
Hydroquinone	k_{cat}	67.5 (3.9)	61.3 (2.9)	66.5 (1.9)	12.3 (0.5)	40.3 (3.9)	34.1 (3.4)
	K_m	59.1 (6.0)	49.3 (4.3)	21.6 (1.6)	22.8 (2.5)	75.3 (4.7)	103.0 (14.4)
	k_{cat}/K_m	1.1 (0.1)	1.2 (0.1)	3.1 (0.2)	0.5 (0.1)	0.5 (0.1)	0.3 (0.1)

^a k_{cat} is expressed in min^{-1} , K_m in mM, and k_{cat}/K_m in $\text{min}^{-1} \text{mM}^{-1}$.^bValues in parentheses are the standard deviation (SD) of the mean value of 3 measurements.^cThe symbol “-” indicates not calculated.

Substrate specificity of *Tt*PPO variants. As previously reported (19), *Tt*PPO shows activity on some monophenolic substrates, such as guaiacol, hydroquinone, vanillin, 1,8-dihydroxynaphthalene, cresols, and tyrosol. However, since it cannot oxidize L-tyrosine, it cannot be categorized to the tyrosinase family based on the standing nomenclature. Hence, we could say that *Tt*PPO is a CO with limited monophenolase activity, especially on small monophenols.

The monophenolase/diphenolase ratio for all mutants, measured either on phenol/catechol or on 4-chlorophenol/4-chlorocatechol, was reduced or unaltered compared to that of the wild type (WT) (data not shown). Even though these mutations did not succeed in substantially increasing the enzyme's activity on monophenolic substrates, they appear to affect the substrate specificity of the enzyme. Kinetic analysis of the substrates most efficiently oxidized by *Tt*PPO and its variants clearly shows that the mutated residues play an important role in binding of the substrates (Table 1).

For catechin, which is a bulky polyphenol, all variants showed improved affinity compared to the WT (3 to 12 times decreased K_m); however, the turnover number (k_{cat}) decreased 3 to 558 times as well. More specifically, mutant G292N was almost inactivated, due to an extreme decrease of k_{cat} combined with a moderate increase of affinity (7.8 times). In contrast, L306A showed a 3-fold increased efficiency (k_{cat}/K_m) due to a considerable increase of affinity (12 times) and a more moderate decrease in k_{cat} (4 times). Mutant Y296V and the WT exhibited similar efficiencies due to a proportional decrease of both k_{cat} and K_m . Concerning double variants, both bearing the devastating G292N mutation, G292N/L306A only reduced its efficiency 2-fold compared to the single variant L306A, while G292N/Y296V seemed to be more affected by the G292 mutation, reducing its efficiency by 18-fold compared to the single (Y296V) variant. Based on the above, it can be seen that the size of the gatekeeper residue is critical for the accommodation of bulky substrates.

L-Dihydroxyphenylalanine (L-DOPA) was the only catecholamine tested, bearing a long substitution at the *p*-position of the ring. All variants showed catalytic efficiency similar to that of the WT, except for variant L306A, which had a lower k_{cat}/K_m , due to reduced affinity for the substrate. The double mutant G292N/L306A, on the other hand, showed over 3 times increased catalytic efficiency. Even though its affinity for the substrate decreased 15-fold, this variant showed a remarkable increase in its turnover number, which was not witnessed for the two single variants, suggesting that

FIG 2 Specific activity of TtPPO (black) and its variants G292N (red), L306A (green), Y296V (yellow), G292N/L306A (blue), and G292N/Y296V (magenta) measured at 40°C. Error bars represent the standard deviation of triplicates.

the combinational mutation of these two positions is rather beneficial for the efficiency on L-DOPA.

For the WT as well as for all the variants, efficiency on 4-chlorocatechol was higher (7 to 600 times) than on unsubstituted catechol. However, all mutants showed decreased catalytic efficiency on 4-chlorocatechol compared to the WT (2 to 36 times), mostly due to significant reduction of k_{cat} (4 to 18 times). Concerning catechol, on the other hand, the single mutants G292N and L306A showed catalytic efficiency increases of 2- and 12-fold, respectively, while Y296V's efficiency was decreased 3-fold. Double variants showed k_{cat}/K_m values in between those of the single variants. Similarly to the previous observations on catechin, the kinetic constants of the double variant G292N/L306A were very similar to those of the L306A mutant, indicating that an alteration in the nature of the gate residue affects more significantly the catalytic behavior of TtPPO.

The two tested phenolic derivatives were guaiacol, which is an *o*-methoxyphenol, and hydroquinone, which is a *p*-hydroxyphenol. All mutations appeared to be devastating for the activity on guaiacol, with the single mutants G292N and Y296V retaining almost the same k_{cat} , but with a concurrent decrease in affinity. Kinetic constants could not be calculated for the L306A mutant because activity was barely detectable, implying that this residue may play an important role in binding of the substrate. In contrast, this mutation proved to be rather beneficial for the catalytic efficiency (3-fold increase) on hydroquinone. Mutant G292N showed kinetic characteristics very similar to those of the WT. While the other two single variants showed decreased K_m (almost 3-fold), the affinity of the double variants for this substrate was decreased, with a concurrent decrease in k_{cat} . Mutation Y296V seems to have the most pronounced negative effect on TtPPO activity and affinity for hydroquinone.

Specific activity measurements with vanillin, which is a *p*-substituted guaiacol, showed that, as with guaiacol, all variants lose most of their activity. Variants containing mutation L306A showed superior activity on epinephrine, pyrogallol, and caffeic acid, while variant G292N demonstrated that only on the latter substrate. Interestingly, even though the WT enzyme and variant G292N did not show any activity on gallic acid, the rest of the variants did (Fig. 2). All variants were also tested in overnight reactions for phenolic substrates, on which they showed lower activity, with the exception of L-tyrosine and *p*-hydroxyphenylacetic acid. In contrast to the WT, all variants were active on these compounds, especially the single variants L306A and Y296V. Interestingly, the WT enzyme had some activity on D-tyrosine, which increased 5-fold

FIG 3 (A) Ribbon representation of overall TtPPO structural model. (B) B-factor analysis of TtPPO-GN.

with mutation G292N. Most variants showed decreased activity for the small phenols that the WT enzyme could oxidize, such as *o*- and *p*-cresol, *p*-hydroxybenzoic acid, and tyrosol. The exception was the L306A mutant, which showed enhanced activity on tyrosol (almost 4 times) and *p*-cresol (almost 2 times), and mutant Y296V on *p*-cresol (1.5 times). Other results worth mentioning are the enhanced activity of all variants against resorcinol, with the most pronounced (5-fold) increase being observed for variant G292N (Fig. S1 at <https://doi.org/10.5281/zenodo.4612056>).

What can actually be deduced from the substrate specificity experiments in the present study is that no specific pattern was detected for each of the variants, even though the targeted amino acids clearly influence enzyme specificity. Variants containing the mutation L306A presented enhanced activity on many substrates, including monophenols, in most cases due to increased affinity (lower K_m) (Table 1). This could be due to the smaller Ala side chain and thus a more “spacious” active site; however, crystallographic studies of TtPPO WT and variants in various complex substrates are needed to corroborate this assumption. The implication of this residue in substrate binding has been shown in the case of dandelion PPOs, where it was proposed that a Phe at this position contributes to substrate binding via π stacking interactions, also involving H_{B2} (29). In order to get a better understanding of the possible enzyme-substrate interactions, the determination of TtPPO crystal structure was pursued.

Crystal structure of TtPPO-GN (variant G292N). The TtPPO-GN crystal structure was determined at 1.55 Å resolution. According to DSSP calculations (30, 31), the TtPPO-GN structure involves 9 α -helices, in which ~34% of the observed amino acids (130 out of 381) participate. These α -helices are equally distributed in two amino acid sections: $\alpha 1$ to $\alpha 4$ are located between amino acids 22 and 140, while $\alpha 5$ to $\alpha 9$ are located between amino acids 251 and 332 (Fig. 1). As already observed for the AoCO4 structure, a four-helix bundle ($\alpha 3$, $\alpha 4$, $\alpha 8$, and $\alpha 9$) containing the copper-coordinating His forms the catalytic site of the protein (Fig. 3A). Interestingly, just after $\alpha 3$ and $\alpha 8$ and right before $\alpha 4$ and $\alpha 9$ helices, only a small loop of a few amino acids (5 and 15, respectively) interferes, dividing the former helices into two partially linked helices ($\alpha 3$ - $\alpha 4$ and $\alpha 8$ - $\alpha 9$). The area between helices $\alpha 1$ and $\alpha 2$ (amino acids [aa] 49 to 70) mainly consists of a bulged loop separated in three sections by a strand (aa 61 to 63) and a 3/10 helix (aa 66 to 68) (Fig. 1).

Fourteen N-terminal (Glu1 to Val14), as well as 14 C-terminal (Gln406 to Asp419), residues were not visible in the electron density map, while Gly50, Glu51, Ala52,

FIG 4 (A) Copper site in the crystal structure of *TtPPO-GN*. An $F_o - F_c$ omit map for the water molecule between the copper ions is shown as gray mesh at the 5σ contour level. (B) View of the serine molecule, located on top of the dicopper center with its $2F_o - F_c$ electron density map (gray mesh) contoured at 1σ . Hydrogen bonds are denoted as gray dashed lines and the corresponding atomic distances are marked in Å.

Gly192, Val193, Ser194, Gly195, Ala338, Asp339, and Gly340 were also excluded from the structure due to insufficient density. Based on molecular weight (MW) calculations, the crystallized species should be full-length *TtPPO-GN*, corresponding to the higher-MW band observed in SDS-PAGE electrophoresis (Fig. S2A). Missing residues 192 to 195 belong to an elongated loop, located between helices $\alpha 4$ and $\alpha 5$ and shielding the copper-containing active site (Fig. 1, Fig. 3B). The relevant loop in homologous enzymes has been previously shown to allow substrate access to catalytic copper ions via its rearrangement (5). The mobility of this loop is also observed in the *TtPPO-GN* structure, as evidenced by the relatively higher B-factors of its residues (Fig. 3B), further confirming its suggested implication in the entrance of substrates to the active site.

In accordance with the predicted glycosylation sites identified in a previous study (19), the crystal structure of full-length *TtPPO* revealed three *N*-glycosylation sites: Asn197 (one *N*-acetylglucosamine [NAG]), Asn216 (NAG), and Asn259 (NAG-NAG) (Fig. 3A). On the basis of the electron density, an additional residue (β -D-mannose [BMA]) is probably present on the Asn259 glycosylation chain; however, map quality in this specific area did not allow for successful modeling of this additional sugar. The overall structure is stabilized by two disulfide bonds: (i) Cys55-Cys400, which connects and stabilizes the N and C termini of the polypeptide chain, and (ii) Cys83-Cys142, which connects the $\alpha 2$ helix with an area just outside the $\alpha 4$ helix in the active site.

Dicopper site analysis. The two copper ions in the catalytic center of *TtPPO-GN* are coordinated by three histidine residues each: His110/ H_{A1} ($\alpha 3$ helix), His118/ H_{A2} (loop before $\alpha 4$), and His127/ H_{A3} ($\alpha 4$) coordinate CuA, while His291/ H_{B1} ($\alpha 8$), His295/ H_{B2} ($\alpha 8$), and His319/ H_{B3} ($\alpha 9$) coordinate CuB, with His118 being the only residue not participating in an α -helix motif. Additional density between the two copper ions was modeled as a bridging water molecule (H_2O 870). The distance of this water molecule is 1.9 Å from both CuA and CuB, while the distance between the two copper ions is 3.71 Å (Fig. 4A).

There is an ongoing discussion concerning the exact nature of this additional electron density, evident at several structures of coupled binuclear copper (CBC) enzymes, which is directly related to the oxidation state of their active site. In the *oxy*-form, the two Cu^{2+} ions are bridged by a dioxygen molecule, with a Cu-Cu distance of approximately 3.6 Å; in the *deoxy*-form, both copper ions are reduced to the Cu^+ state, with their distance increasing to 4.6 Å. Under aerobic conditions, the *deoxy*-form quickly falls to the *oxy*-form by dioxygen binding. Concerning the *met*-form, it has been suggested

that it consists of two Cu^{2+} ions at a distance of 3.4 Å bridged by one endogenous ligand (such as hydroxide) (7). In *TtPPO*-GN, based on the distance between the two copper ions and the observed electron density, one could speculate that the active site of the enzyme is in the *met*-form. However, lack of spectroscopic data, as well as the overall resolution of the crystallographic structure, hinders identification of the existing state.

Before data collection, *TtPPO*-GN crystals were soaked in mother liquor supplemented with various substrates; however, no relevant electron density was observed in their active site. Instead, all crystals had an additional density on top of the dicopper center and close to Tyr296, which was modeled as serine, an ingredient of the “amino acid mix” in the crystallization solution. This additional amino acid seems to be firmly stabilized in this area via hydrogen bonds with $\text{H}_{\text{B}2}$ (3.10 Å) and the carbonyl oxygen of Gly303 (2.90 Å) (Fig. 4B). Tyr296 has been highlighted as critical for successful substrate orientation in the active site (11); thus, serine’s location and tight binding may explain the unsuccessful detection of tested substrates at the active site. A close inspection of the *TtPPO*-GN active site with the bound serine molecule also reveals the stereochemical constraints induced by the “bulky” Leu306, positioned on top of CuA, when it comes to substrate access to the catalytic dicopper center (Fig. 4B). It is worth mentioning the recent work by Biundo et al. (32), who showed that some plant and mushroom PPOs could present proteolytic activity, cleaving a particular esterase’s extended loop between residues Ser and Ile, which is not a typical protease cleavage site. Even though the mechanism of this PPO activity is unclear, it points out the affinity of their active site for serine residues.

Comparison with other PPO structures. A search for structural homologues using the Dali server (33) revealed that *TtPPO*-GN is most similar to AoCO4 (PDB code [5OR3](#)), with a root mean square deviation (RMSD) of 1.6 Å, a Z-score of 51.9, and 43% sequence identity. This similarity is also reflected in the substrate specificity exhibited by the two PPOs, since both are active on catechol, caffeic acid, catechin, phenol, tyrosol, *p*-cresol, and guaiacol (19, 34). The second and third closest structural homologues are of almost equal similarity to *TtPPO*-GN: *Bacillus megaterium* tyrosinase (*BmTyr*, PDB code: [6QXD](#), 31% sequence identity, an RMSD of 2.1 Å, and a Z-score of 27.7), followed by *Streptomyces castaneoglobisporus* tyrosinase (*ScTyr*; PDB code: [2ZMZ](#), 31% sequence identity, an RMSD of 2.1 Å, and a Z-score of 26.4).

The overall structure of *TtPPO*-GN is very close to that of AoCO4, with the main differences observed in loop regions located quite distant from the active site (Fig. 5A). *TtPPO*-GN displays a longer conformation in the loop region just after helix $\alpha 9$ (residues 335 to 346) (Fig. 1). Another difference is observed in part of the flexible loop in the vicinity of the copper-coordinating site, comprising residues 224 to 236 in *TtPPO*-GN. The relevant region in AoCO4 has an insertion of several amino acids (residues 216 to 224) and is more tilted toward the active site (Fig. 5A, highlighted in a blue frame). The two small β -strands in AoCO4 that are located between helices $\alpha 4$ and $\alpha 5$ are not observed in the *TtPPO*-GN structure. Furthermore, there are three disulfide bridges in the AoCO4 structure, two of which are also present in *TtPPO*-GN. Even though the third disulfide bond, which is absent in *TtPPO*-GN, has been suggested to stabilize two long loops in AoCO4, the two enzymes have similar thermostabilities, as shown by previously published biochemical data (19).

Superimposition of the *TtPPO*-GN structure with AoCO4 in complex with resorcinol (PDB code [6GSG](#)) showed that the bound serine in the former is positioned slightly above the ligand observed in the active site of its closest structural homologue (Fig. 5B). Resorcinol in the AoCO4 structure is coordinated by the side chain of Ser302 and the carbonyl oxygen of Gly296. Both residues are conserved in *TtPPO*-GN (Ser309 and Gly303), suggesting a possible implication in the binding of the substrate in its active site. In addition to that, the superimposed structures clearly show that the bulkier “gate” residue in *TtPPO*-GN (Leu instead of Val in AoCO4) results in a more congested active site, with potential implications for substrate specificity. This is in accordance

FIG 5 (A) *TtPPO*-GN structure (in green) superimposed onto *AoCO4* (PDB ID 6GSG, beige). The loop region comprising residues 224 to 236 of *TtPPO* is highlighted in a blue frame. (B) Superposition of the copper sites of *TtPPO*-GN and *AoCO4* (with resorcinol bound, PDB ID 6GSG). The residues implicated in copper and substrate coordination are shown in stick representation.

with the biochemical data, where mutation of Leu306 to alanine resulted in increased affinity (lower K_m) for the majority of the examined substrates.

As mentioned above, monophenolase activity in PPOs has been tightly correlated with the occurrence of an Asn residue located just after H_{B1} (His291 in *TtPPO*). This Asn and a conserved Glu residue positioned several residues before H_{B1} coordinate a water molecule, which is suggested to assist in deprotonation of monophenolic substrates, thus launching a tyrosinase catalytic cycle (11). The Glu residue is conserved in native *TtPPO*; however, there is a Gly after H_{B1} . This led us to estimate the effects of the G292N mutation on both the structure and function of the enzyme. By comparing our structure with *BmTYR* (PDB code 3NM8), it can be seen that the Glu residue (Glu280 in *TtPPO*-GN) points toward a different direction, and its side chain does not contribute to the coordination of a water molecule in common with Asn292 (Fig. 6). H_2O786 , however, is coordinated by the side chain amide group of Asn292 and the main chain carbonyl of Glu280, but is unlikely to play a role in deprotonating water, due to its distant location from the suggested substrate-binding site. On the other hand, the side chain carbonyl of Asn292 is at 3.7 Å distance from nitrogen ND1 of H_{B1} (His291) (Fig. 6, left panel), and could thus form a hydrogen bond with the latter through a minimal rearrangement upon substrate binding, rendering H_{B1} a potential base for the deprotonation of incoming monophenols, as has been recently suggested (18). This is, however, not in accordance with the biochemical data, which did not show any increase in the monophenolase activity of this variant. In contrast, the double variant G292N/L306A had twice the activity on phenol compared to the WT enzyme, but because its activity on catechol increased 10-fold, the ratio of monophenolase to diphenolase activity decreased. Based on the above, it could be assumed that substrate deprotonation originates from the flexible His residues (H_{A2} and H_{A3}) that coordinate CuA and that are released upon substrate binding, thus acting as bases, as recently shown for *A. oryzae* and *S. castaneoglobisporus* tyrosinases (15–17).

In conclusion, the present work aspired to shed light on the structural determinants of PPO function, which has been a long-lasting enigma in the field, especially regarding the variability in monophenolase/diphenolase activity exhibited by these enzymes. The biochemical characterization of *TtPPO* mutants, in combination with the crystal structure of variant *TtPPO*-GN, resulted in the identification of amino acids in the vicinity of the copper-binding site that influence the catalytic activity of the enzyme. It is

FIG 6 Active site of *TtPPO*-GN (left) and *BmTyr* (PDB ID 4P6R; right). $H_{B1}+1$ (Asn), the conserved Glu residue, and the coordinated water molecule are highlighted. The distance between the side chains of Asn292 and H_{B1} (His291) of *TtPPO*-GN is shown with a dashed yellow line.

clear that the “gate” residue Leu306, positioned on top of CuA, as well as residues $H_{B1}+1$ (Asn292) and $H_{B2}+1$ (Tyr296) influence the catalytic activity of *TtPPO*. The most-pronounced changes were observed for the former, since its mutation to a smaller amino acid (Leu to Ala) resulted in an increase in the activity of the enzyme against most of the tested substrates. Additionally, mutation of residue $H_{B1}+1$ (G292N) resulted in a variant with considerably increased activity on resorcinol, *D*-tyrosine, and *p*-hydroxyphenylacetic acid. A comparative examination of *TtPPO*-GN structure, along with biochemical data, showed that residue $H_{B1}+1$ is unlikely to contribute to substrate deprotonation as suggested from previous studies, thus pointing to a recently suggested theory where substrate deprotonation is mediated by one of the copper-coordinating His residues. The determination of *TtPPO* structure in complex with its preferred substrates will allow the engineering of a biocatalyst with improved properties, given its potential use as a bioremediation agent.

MATERIALS AND METHODS

Expression and purification of recombinant enzymes. *Pichia pastoris* recombinant strains expressing *TtPPO* and its variants were cultivated in 500 ml of buffered complex methanol medium (BMMY) supplemented with 25 μ M CuSO_4 . Induction took place for 4 days at 23°C and 200 rpm by the addition of 0.5% (vol/vol) methanol per day, based on previous optimization (19). Culture broth was concentrated and dialyzed against 50 mM Tris-HCl–300 mM NaCl (pH 8) buffer and loaded on an immobilized metal cobalt affinity chromatography (IMAC) column as described previously (35).

Variant G292N (*TtPPO*-GN), which was used for crystallographic studies, was expressed in 2-liter cultures as mentioned above. The culture broth was concentrated and the recombinant protein was deglycosylated in the crude protein solution by the addition of 30 μ g ml^{-1} EndoH (endo- β -*N*-acetylglucosaminidase, EC 3.2.1.96) from *Streptomyces plicatus*. After 1.5 h of incubation at 37°C, the crude protein was dialyzed and purified using IMAC as above. Fractions containing the purified *TtPPO*-GN were pooled, concentrated, applied onto a HiPrep 16/60 Sephacryl S-100 HR column (GE Healthcare, Chicago, IL, USA), equilibrated, and eluted with 20 mM Tris-HCl–150 mM NaCl (pH 8) buffer. Fractions of 2 ml were collected at a flow rate of 1 ml min^{-1} , and the ones with pure *TtPPO*-GN were pooled, dialyzed against 20 mM Tris-HCl (pH 8) buffer, and concentrated to 20 mg ml^{-1} . The homogeneity of the protein was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Biochemical characterization of *TtPPO* variants. A typical enzymatic assay was performed as described previously (19). Briefly, reaction mixtures containing 230 μ l of 5 mM substrate in 0.1 M sodium phosphate buffer (pH 7) were mixed with 20 μ l of enzyme and incubated in a SpectraMax 250 microplate reader (Molecular Devices, USA) set at 40°C. The increase in absorbance at each product's λ_{max} was

TABLE 2 Diffraction data and refinement statistics for *TtPPO*-GN

Parameter	Value
Data collection	
Beamline	P14 (EMBL, Hamburg, Germany)
Wavelength (Å)	0.98012
Space group	$P 2_1 2_1 2$
Cell dimensions (a, b, c) (Å)	$a = 86.40, b = 97.18, c = 46.99$
Resolution range (Å)	43.20–1.55 (1.72–1.55) ^a
Ellipsoidal resolution (Å) (direction)	1.44 (a*) 1.93 (b*) 2.24 (c*)
No. of molecules per asymmetric unit	1
No. of unique reflections	30,047 (1,502) ^a
Completeness (%) (spherical)	52.0 (10.1) ^a
Completeness (%) (ellipsoidal)	86.3 (61.3) ^a
R_{merge} (%) ^b	15.5 (179) ^a
R_{pim} (%) ^c	4.4 (51.6) ^a
Mean $I/\sigma(I)$	12.4 (1.8) ^a
$CC_{1/2}$ ^d	0.999 (0.605) ^a
Multiplicity	13.3 (12.6) ^a
Wilson B-value (Å ²)	17.7
Refinement statistics	
$R_{\text{work}}/R_{\text{free}}$ (%)	15.74/20.66
RMSD, bond length (Å)	0.015
RMSD, bond angle (°)	1.83
No. of protein atoms	3,016
No. of solvent atoms	359
Avg. B-values (Å ²)	
All proteins	21.70
Water molecules	30.34
Copper ions	21.23
Ramachandran statistics (%) ^e	
Favored	96.78
Outliers	0
Allowed	3.22
PDB ID	6Z1S

^aValue in parentheses refers to the highest-resolution shell.^b $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.^c $R_{\text{pim}} = \sum_{hkl} (n - 1)^{-1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.^d $CC_{1/2}$ is the correlation coefficient between two random half data sets.^eCalculated using MolProbity (<http://molprobity.biochem.duke.edu/>).

recorded for 20 min. One unit (U) of enzymatic activity was determined as $1 \Delta A_{\lambda_{\text{max}}} \text{ min}^{-1}$. The substrate range of the purified enzymes and their specific activities were determined as described previously (19).

Kinetic studies of the purified enzymes were performed by assaying various concentrations of 4-chlorocatechol (0 to 5 mM), catechol (0 to 60 mM), catechin (0 to 10 mM), L-DOPA (0 to 7 mM), vanillin (0 to 5 mM), guaiacol (0 to 15 mM), and hydroquinone (0 to 70 mM). Kinetic constants were estimated using a nonlinear regression model in GraphPad Prism 5 from GraphPad Software, Inc. (USA).

The purified enzyme was quantified through absorbance at 280 nm (A_{280}) measurements (36) using a molar extinction coefficient of $75,540 \text{ M}^{-1} \text{ cm}^{-1}$ calculated with the ProtParam tool from ExPASy (37).

Crystallization, data collection, and structure determination of *TtPPO*-GN. Similarly to the native enzyme (19), SDS-PAGE analysis showed that purified *TtPPO*-GN appears as two bands, which after deglycosylation with EndoH appeared to be approximately 48 kDa and 39 kDa. Based on our previous analysis, the higher-MW band must correspond to full-length *TtPPO*-GN, while the lower-MW band is assumed to be an N-terminally cleaved enzyme variant. Neither IMAC nor gel filtration purification steps were successful in separating these two bands; they were thus concentrated to 10 mg ml^{-1} in 20 mM Tris-HCl (pH 8.0) and submitted to crystallization experiments at the High Throughput Crystallization Laboratory (HTX Lab) of EMBL Grenoble via the sitting drop vapor diffusion technique (38). Crystals (Fig. S2B) appeared after 1 month in a single condition of the Morpheus screen (Molecular Dimensions; condition H12: 0.1 M amino acid mix, 0.1 M Tris Bicine [pH 8.5], 37.5% [vol/vol] precipitant mix 4) and were prepared for X-ray data collection experiments using the CrystalDirect technology (39). Data collection was carried out at 100K on beamline P14 of EMBL Hamburg, Germany. The crystals diffracted

anisotropically, and the X-ray data were processed using the software STARANISO (40) as implemented in the autoPROC pipeline (41). TtPPO crystals belong to space group $P2_12_12_1$, with unit-cell dimensions $a = 86.40$, $b = 97.18$, and $c = 46.99$ Å, and one molecule per asymmetric unit. The structure of TtPPO-GN was resolved via molecular replacement at 1.55 Å resolution using Phaser (42) as implemented in the CCP4 suite (43). The search model was derived from the crystal structure of *A. oryzae* catechol oxidase (AoCO4), which shares 45% sequence identity for 82% coverage with TtPPO (PDB entry 4J3R) (44). The final model was constructed by alternating cycles of refinement and model building in REFMAC (45) and Coot (46). Refinement converged to R_{work} and R_{free} values of 15.74% and 20.66%, respectively. The validation of the final refined model was performed with PDB-REDO (47), MolProbity (48), and pdb-care (49) Web servers, respectively. Atom coordinates and structure factors have been deposited in the PDB (accession code 6Z1S). Data collection and refinement statistics are summarized in Table 2.

Structure figures were prepared in PyMol (The PyMOL Molecular Graphics System, version 2.3.2; Schrödinger, LLC). Structure-based sequence alignment was performed in UCSF Chimera (50) using the structures of TtPPO-GN, AoCO4 (PDB ID: 4J3R), BmTyr (PDB ID: 3NM8), and ScTyr (PDB ID: 1WX2). The alignment output was visualized using ESPript (<http://esprict.ibcp.fr>).

Data availability. Data regarding structure determination are available at <https://www.rcsb.org> under the ID 6Z1S.

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