Oxidases

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Tyrosinase versus Catechol Oxidase: One Asparagine Makes the Difference

*Even Solem, Felix Tuczek, and Heinz Decker**

Abstract: *Tyrosinases mediate the ortho-hydroxylation and two-electron oxidation of monophenols to ortho-quinones. Catechol oxidases only catalyze the oxidation of diphenols. Although it is of significant interest, the origin of the functional discrimination between tyrosinases and catechol oxidases has been unclear. Recently, it has been postulated that a glutamate and an asparagine bind and activate a conserved water molecule towards deprotonation of monophenols. Here we demonstrate for the first time that a polyphenoloxidase, which exhibits only diphenolase activity, can be transformed to a tyrosinase by mutation to introduce an asparagine. The asparagine and a conserved glutamate are necessary to properly orient the conserved water in order to abstract a proton from the monophenol. These results provide direct evidence for the crucial importance of a proton shuttle for tyrosinase activity of type 3 copper proteins, allowing a consistent understanding of their different chemical reactivities.*

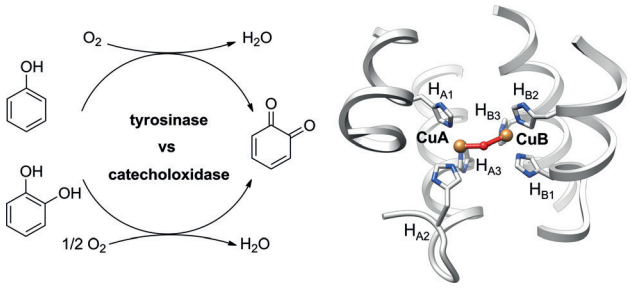

Figure 1. Left: Reactivities of tyrosinase (top) and catechol oxidase (bottom). Right: Type 3 copper center of *Vv*PPOg from wine grapes[19] in its met form with copper ions A and B, and the six histidines provided by the four α-helices.

Melanin is ubiquitous. The high-molecular-weight pigment is involved in the coloring of skin and hairs,[1–3] protection against UV and ionizing radiation,[4–6] immunological defense,[7,8] and other important biological functions.[9–13] It is formed by the polymerization of dopaquinone, which is generated by monooxygenation of tyrosine, in turn mediated by the enzyme tyrosinase (TY). Alternatively, dopaquinone can also be produced from L-DOPA with the help of a related enzyme, catechol oxidase (CO). In vivo catechol oxidases also mediate the two-electron oxidation of other diphenols to *ortho*-quinones (Figure 1 left).

TYs and COs are commonly referred to as phenoloxidases (PPOs).[14] These enzymes have similar active sites in which two copper ions, CuA and CuB, are coordinated by six histidines (Figure 1 right). Dioxygen is reversibly bound as peroxide in a side-on bridging coordination.[15,16]

Besides the natural substrate tyrosine TYs also hydroxylate other monophenols in *ortho*-position to diphenols, followed by oxidation to *ortho*-quinones (monophenolase activity). COs, in contrast, can only perform the latter reaction (diphenolase activity).[15] Although being of significant biological, medical, and economic importance, the molecular basis of the functional discrimination between tyrosinase and catechol oxidase is still unclear.[4,15,17,18]

Recently, the Fishman group presented crystal structures of the tyrosinase from *Bacillus megaterium* (Tyr*Bm*) with and without bound mono- or diphenolic substrates such as tyrosine, *p*-tyrosol, and L-DOPA.[17,18] They suggested that a highly conserved glutamate and an asparagine bind and activate a water molecule towards deprotonation of monophenols, which is required for tyrosinase activity. Here we provide the first direct experimental proof of this hypothesis.

We were able to determine the coding sequence for a specific latent polyphenoloxidase (L-*Vv*PPOcs-3) based on total RNA extracted from wine leaves (*V. vinifera* L. cv. Cabernet Sauvignon), followed by first strand cDNA (complementary DNA) synthesis, cloning, and sequencing. Lately, the Dirks-Hofmeister/Moerschbacher group established an expression system for plant PPOs in *E. coli*.[20] Based on these studies we were able to produce L-*Vv*PPOcs-3 heterologously after some modification. The expression construct he expression construct was designed without the coding sequence for the N-terminal transit peptide. Additionally, the recombinant enzymes were fused to an N-terminal Strep II affinity tag (WSHPQFEK) encoded by the specific expression vector (pET51b). The protein was purified to about 95 % by applying Strep-Tactin affinity chromatography (Figure S1).

L-*Vv*PPOcs-3 has a mass of about 60 kDa and is organized in three domains. The N-terminal domain carries the active site which is shielded by the C-terminal domain. A short flexible linker peptide connects the N- and C-terminal domains.

Around histidines H_{B1} and H_{B2} that coordinate CuB (cf. Figure 1), L-*Vv*PPOcs-3 shows the sequence -I-E236-N-V-

[*] E. Solem, Prof. Dr. H. Decker
Institute of Molecular Biophysics, Johannes Gutenberg University
Jakob Welder Weg 26, 55128 Mainz (Germany)
E-mail: hdecker@uni-mainz.de
Prof. Dr. F. Tuczek
Institute of Inorganic Chemistry
Christian Albrechts University Kiel
Max Eyth Straße 2, 24118 Kiel (Germany)

Supporting information (including experimental procedures, supplemental results and discussion, supplemental references, tables, and figures) for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201508534.


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                                 HB1  HB2
VvPPOg                  : LEHAPHNIVHK: 244
L-VvPPOcs-3             : IENVPHGPVHI: 245
G241N                   : IENVPHNPVHI: 245
G241N/P242I             : IENVPHNIVHI: 245
P239del/G241N/P242I     : IENV-HNIVHI: 244
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Figure 2. Sequence alignment of *Vitis* PPOs and the muteins. The alignment is based on the structure of Grenache PPO (*Vv*PPOg; PDB-ID 2P3X).[19] The wild-type L-*Vv*PPOcs-3 (Cabernet Sauvignon) and its muteins G241N, G241N/P242I and P239del/G241N/P242I are shown.

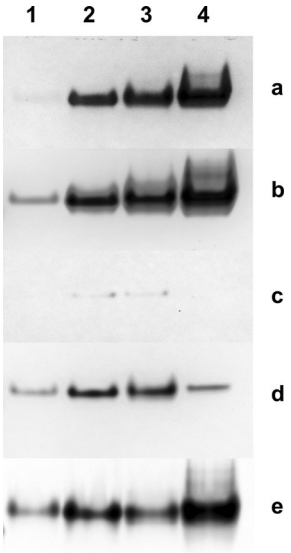


Figure 3. In-gel activity assay using L-*Vv*PPOcs-3 with monophenols (tyramine and *p*-tyrosol) and a diphenol (4-methylcatechol). Catalytic activities after two different reaction times and in the presence of 1.5 mM SDS as activator: a) *p*-tyrosol (10 min); b) *p*-tyrosol (120 min); c) tyramine (10 min); d) tyramine (120 min); e) 4-MC (5 min). The enzyme and the muteins are given as follows.: 1) L-*Vv*PPOcs-3wt, 2) G241N mutein, 3) G241N P242I mutein, 4) P239del G241N P242I mutein. An alkaline PAGE (10%) was applied using a 0.1 M maleate-Tris buffer, pH 5.5; the sensitivity of the assay was increased by addition of 6 mM MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate) for monophenols and 10 mM MBTH for the diphenol. The absorption was measured at 505 nm. The concentrations of L-*Vv*PPOcs-3 (wt and the muteins) were 15 µg. The concentrations for the substrate were 6 mM tyramine, 6 mM *p*-tyrosol, and 10 mM 4-MC.

P-H_{B1}240-G-P-V-H_{B2}-I- which includes a strictly conserved glutamate (Figure 2).

As evident from an in-gel activity assay (Figure 3), wild-type L-*Vv*PPOcs-3 catalyzes the oxidation of 4-methylcatechol (4-MC) but it catalyzes the oxygenation of monophenols such as tyramine and *p*-tyrosol only to a negligible degree. In contrast to the structure of *Bacillus megaterium* tyrosinase (Tyr*Bm*) no asparagine is found in this sequence part of L-*Vv*PPOcs-3. This residue should be located next to the CuB-coordinating H_{B1}240. In L-*Vv*PPOcs-3, however, there is a glycine (G241) at this position, resulting in a loss of monophenolase activity. A glycine can also be found at the corresponding position in the *To*PPO-1 and *To*PPO-2 from dandelion (*Taraxacum officinale*), for which only diphenolase activity has been observed so far.[20]

We discovered that L-*Vv*PPOcs-3 can be converted to a monophenolase by site-directed mutagenesis of G241N. In the presence of SDS (sodium dodecyl sulfate) as activator[21–23] the monophenols tyramine and *p*-tyrosol are efficiently converted to the corresponding *ortho*-quinones (Figure 2 and Supporting Information). Thus, apart from the conserved E236 the presence of asparagine as a direct neighbor of H_{B1} on its downstream side is required for monophenolase activity, in full agreement with all PPOs that act as tyrosinases. If, on the other hand, the asparagine is missing, only diphenolase activity is observed.

The sequence part of L-*Vv*PPOcs-3 around H_{B1} also contains two prolines which are of special interest since they are known to disturb secondary structures. In fungal tyrosinases (e.g., *Agaricus bisporus*) the glutamate and asparagine are provided by one α-helix which also carries two of the three CuB-coordinating histidines (H_{B1} and H_{B2}; Figure 4a).[24] In plant PPOs, this part of the sequence contains

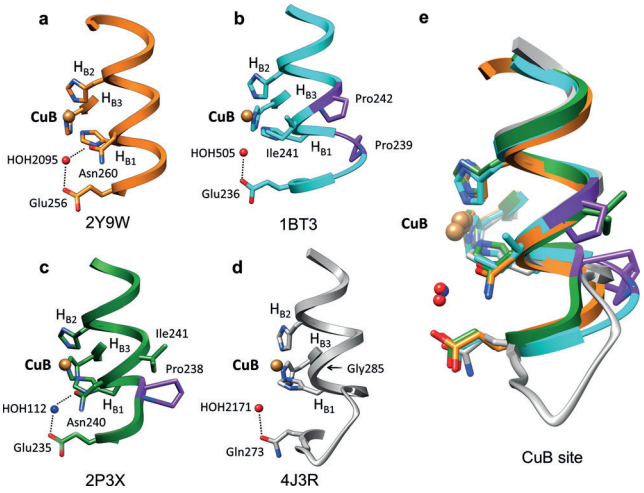


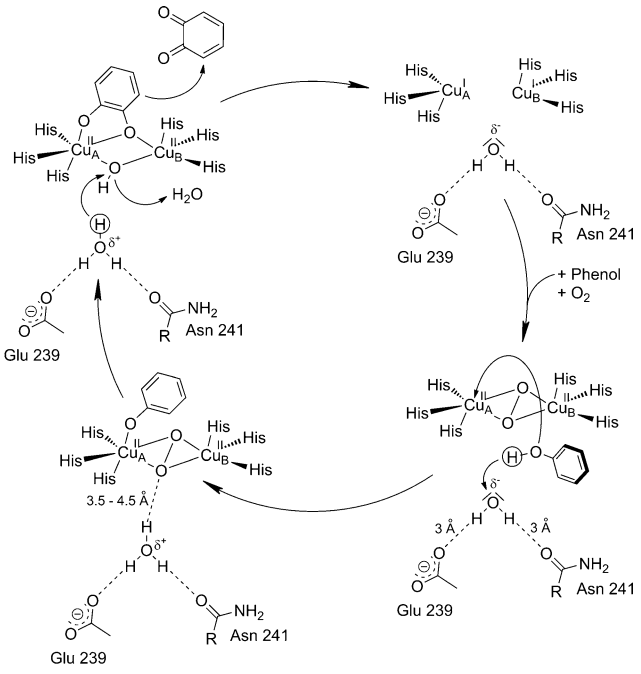
Figure 4. Parts of the α-helices providing the CuB-coordinating histidines H_{B1} and H_{B2} from different PPO structures: a) Champignon *A. bisporus Ab*Tyr, PDB-ID 2Y9W (orange); b) sweet potato *I. batatas Ib*PPO, PDB-ID 1BT3 (cyan); c) wine *V. vinifera Vv*PPOg, PDB-ID 2P3X (green); d) mould fungus *A. oryzae Ao*CO-4, PDB-ID 4J3R (gray, here a Gln substitutes Glu); e) superposition of a–d. The pyrrolidine rings of prolines in direct neighborhood to H_{B1} are highlighted in purple; the copper atoms of the CuB site are colored light brown. As demonstrated in the superposition (e), the conserved glutamates and asparagines (if present) take the same topological position, independent of the presence of prolines. A water molecule (HOH112) shown in blue is incorporated in the *Vv*PPOg structure on the basis of a re-refinement of its published structure[19] (2P3X*; see Figures S3 and S4).

two prolines, one being strictly conserved and the other one more variable. The latter one (P242 in sweet potato PPO,[25] *I. batatas*, 1BT3) is located between H_{B1} and H_{B2} (Figure 4b) similar to the situation in L-*Vv*PPOcs-3. Replacing this proline in L-*Vv*PPOcs-3 by an isoleucine, in addition to the mutation G241N, further increases the efficiency of the monooxygenation of *p*-tyrosol and tyramine in L-*Vv*PPOcs-3; in addition, the catalytic oxidation of 4-methylcatechol (4-MC) is enhanced (Figure 3). The resulting sequence corre-

sponds to that of *Vv*PPOg[19] (Figure 4c) and a closely related wine *Vv*PPOr (Riesling) for which tyrosinase activity has also been established.[26]

The other proline (P239 in L-*Vv*PPOcs-3) flanking H_{B1} at the upstream side is strictly conserved among plant PPOs, but does not exist in PPOs from other organisms (Figure S2). Apparently the presence of this proline is compatible with monophenolase activity as was also shown for PPOs from wine grapes[26] and walnut leaves,[27] as long as the glutamate and the asparagine residues are present. In L-*Vv*PPOcs-3, deletion of this proline P239del in addition to the mutations G241N and P242I results in an even higher turnover rate of *p*-tyrosol (Figure 3). With respect to the monooxygenation of tyramine, however, the catalytic efficiency is drastically reduced.

Thus, the presence of an asparagine (besides the conserved glutamate) is necessary to induce monophenolase activity. The conserved prolines modulate this activity and/or lead to a substrate specificity. If only two prolines are present but no asparagine, only diphenolase activity is observed (Figure 4b).[25] On the other hand, if asparagine and glutamate are present but both prolines are absent, then monooxygenase activity is very strong. This applies to PPOs from other organisms such as bacteria and fungi (Figure 4a).[24] If asparagine is missing in these enzymes, tyrosinase activity is lost again (Figure 4d).[28] Independent of the presence of prolines, however, the conserved glutamate and asparagine (if present) as well as the conserved water molecule always take the same topological position (Figure 4e). Importantly, this also applies to wine *Vv*PPOg (PDB-ID 2P3X), where the presence of a conserved water molecule is revealed by a re-refinement of the published structure[19] (PDB-ID 2P3X*; Figures S3 and S4).



Scheme 1. Deprotonation of a phenolic substrate in the tyrosinase cycle.

By hydrogen bonding to the two amino acids glutamate and asparagine, the conserved water molecule is fixed at a distance of about 3 Å from these residues (Scheme 1). Moreover, it is oriented in such a way that its negatively charged side attracts the proton of the hydroxyl group of monophenols. When approaching the dicopper(II) peroxo site, the substrate passes the conserved water molecule which becomes protonated, forming a hydronium ion. The resulting phenolate shifts to the free coordination site at CuA[29] where it is bound at a distance of about 2 Å (Figure 5). Alternatively,

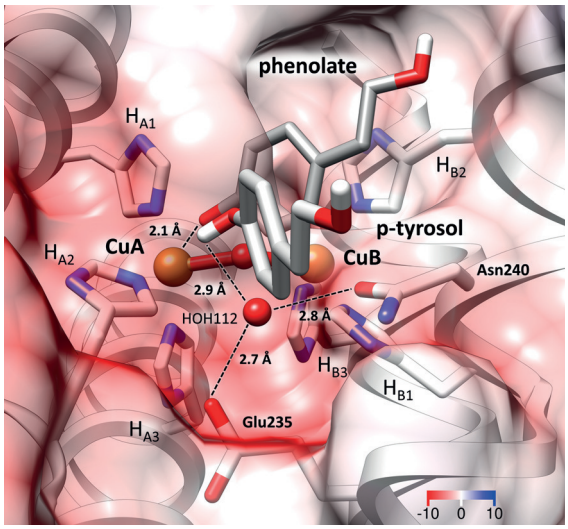


Figure 5. Deprotonation and approach of *p*-tyrosol to the active site in the refined *Vv*PPOg structure (PDB-ID 2P3X*, see the Supporting Information). The incoming tyrosol coordinates as phenolate to CuA at a distance of 2.1 Å. The proton of the phenolic substrate is transferred over a minimum distance of 2.9 Å to the conserved water molecule bound by Glu235 and Asn240. The position of the *p*-tyrosol was adopted from the PPO structure of *B. megaterium* co-crystallized with *p*-tyrosol[17] (PDB-ID 4P6T). The electrostatic charge distribution of the surface was calculated with the Coulombic Surface Coloring option implemented in the UCSF Chimera software (version 10.1).

the proton is released when the free phenolic substrate binds to CuA and is transfered to the conserved water molecule. Afterwards, electrophilic attack of the peroxide occurs in *ortho*-position of the phenolate,[15,17,29] leading to an asymmetrically bound catecholate. The other oxygen of peroxide forms a bridging hydroxide. Then the coordinated catecholate is oxidized in a two-electron process and released as quinone. This process is facilitated by back transfer of the proton over a distance of about 3.5–4.5 Å from the hydronium ion to the hydroxide ligand, forming water. The two copper centers are thereby reduced to Cu^{I} and able to react with O_2 again.

In latent PPOs the C-terminal domain covers the entrance of the catalytic site.[15,23] In the presence of SDS, however, di- and even very weak monophenolase activity is observed in wild-type L- *Vv*PPOcs-3 (see above). Recent MD simulations based on the crystal structure of the structurally related hemocyanin from *Octopus dofleini* (PDB-ID 1JS8) confirmed the SDS-mediated displacement of the C-terminal domain, providing an access to the catalytic site.[30]

The interaction of the substrate with the active site is potentially further influenced by a gate residue,[15,17–19,25] which in plant PPOs can be phenylalanine, tyrosine, or leucine. In contrast to an earlier hypothesis,[25,29] recent experiments indicate that the steric bulk of these residues is not correlated with the enzymatic activities of these systems.[18] Moreover, a molecular dynamics simulation of *Vv*PPOr from Riesling[26] based on the structure of *Vv*PPOg from Grenache[19] reveals that the aromatic ring of phenylalanine potentially functioning as a gate residue in fact rotates to provide free access of the substrate to CuA.[30] The functional discrimination between tyrosinases and catechol oxidases thus can exclusively be traced back to the presence of an asparagine which together with a glutamate binds a conserved water. As a result, the conserved water molecule becomes basic enough to bind the proton released by phenolic substrates upon coordination to the active site. Apparently, interaction with a single residue (e.g., glutamate) does not lower the pK_B of the conserved water sufficiently to play this role. In this case only diphenolase activity is possible which does not rely on a deprotonation pathway.

These conclusions are fully consistent with the results from small-molecule copper chemistry.[31] In contrast to catecholoxidase mimics, tyrosinase models require the addition of base.[32] Neutral (i.e., protonated) phenols inevitably react with Cu-O_2 intermediates to generate phenoxyl radicals, leading to unphysiological reaction products.[15] Whereas originally an excess of triethylamine had to be employed to deprotonate phenolic substrates,[33,34] application of a stoichiometric amount (or a slight excess) of diamine ligand to achieve catalytic activity in these model systems has been shown only recently.[35] Like the activated water molecule in the enzyme, this ligand could act as an "internal base" to enable catalytic monooxygenation of phenols.[15,36]

In summary, we have demonstrated the dependence of tyrosinase activity on an asparagine which along with a glutamate binds and activates a conserved water molecule towards deprotonation of monophenolic substrates. This result, which is supported by the first crystal structure of a plant tyrosinase (from walnut leaves) published recently,[37] provides a consistent understanding of the reactivities of type 3 copper systems on a molecular level as well as important structural insights for modifying or inhibiting these enzymes and applying them in biotechnology.[12]

Acknowledgements

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Keywords: catechol oxidase · monophenolase activity · polyphenoloxidase · type 3 copper proteins · tyrosinase

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