Functional definition of the tobacco protoporphyrinogen IX oxidase substrate-binding site

Ilka U. HEINEMANN*, Nina DIEKMANN*, Ava MASOUMI*, Michael KOCH†, Albrecht MESSERSCHMIDT‡, Martina JAHN* and Dieter JAHN*¹

*Institute of Microbiology, Technical University Braunschweig, Spielmannstr. 7, 38106 Braunschweig, Germany, †Division of Structural Biology, The Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Headington, Oxford OX3 7BN, U.K., and ‡Department of Proteomics and Signal Transduction, Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany

PPO (protoporphyrinogen IX oxidase) catalyses the flavin-dependent six-electron oxidation of protogen (protoporphyrinogen IX) to form proto (protoporphyrin IX), a crucial step in haem and chlorophyll biosynthesis. The apparent K_m value for wild-type tobacco PPO2 (mitochondrial PPO) was 1.17 μM , with a $V_{\max}$ of 4.27 $\mu\text{M} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and a catalytic activity k_{cat} of 6.0 s^{-1} . Amino acid residues that appear important for substrate binding in a crystal structure-based model of the substrate docked in the active site were interrogated by site-directed mutagenesis. PPO2 variant F392H did not reveal detectable enzyme activity indicating an important role of Phe³⁹² in substrate ring A stacking. Mutations of Leu³⁵⁶, Leu³⁷² and Arg⁹⁸ increased k_{cat} values up to 100-fold, indicating that the native residues are not essential for establishing

an orientation of the substrate conducive to catalysis. Increased K_m values of these PPO2 variants from 2- to 100-fold suggest that these residues are involved in, but not essential to, substrate binding via rings B and C. Moreover, one prominent structural constellation of human PPO causing the disease variegate porphyria (N67W/S374D) was successfully transferred into the tobacco PPO2 background. Therefore tobacco PPO2 represents a useful model system for the understanding of the structure–function relationship underlying detrimental human enzyme defects.

Key words: enzyme kinetics, haem biosynthesis, protoporphyrin, protoporphyrinogen IX oxidase, stopped-flow fluorescence, variegate porphyria.

INTRODUCTION

PPO (protoporphyrinogen IX oxidase; EC 1.3.3.4) catalyses the oxygen-dependent aromatization of protogen (protoporphyrinogen IX) to proto (protoporphyrin IX) [1] (Scheme 1). It is the last common step of tetrapyrrole biosynthesis for the formation of haems and chlorophylls. Initially, the enzyme was characterized from yeast [2] and mammalian liver [3,4]. It catalyses the six-electron oxidation of protogen using a flavin cofactor and molecular oxygen as terminal electron acceptor. PPO is described as a soluble monomer in *Bacillus subtilis* [5], as a membrane-associated monomer in bovine [6] or as a homodimer in *Mycococcus xanthus* and humans [7–9]. In eukaryotes, PPO is associated with the outer surface of the inner mitochondrial membrane [10], while the plant enzyme is additionally located in chloroplasts [11,12].

In humans, partial PPO deficiency causes a disease known as VP (variegate porphyria; OMIMTM accession number 176200) that is inherited as an autosomal dominant trait displaying incomplete penetrance [13]. The biochemical abnormalities found in VP patients include overproduction and increased excretion of porphyrins and porphyrin precursors. VP manifests clinically with photosensitivity and acute attacks, which includes various neuropsychiatric symptoms [14]. In South Africa, VP is common due to a founder effect, where mainly one amino acid exchange from Arg⁵⁹ to tryptophan causes the disease [15–18]. However, there are various additional mutations known to cause VP [19].

In plants, two isoforms of PPO, namely PPO1 (plastidic PPO) and PPO2 (mitochondrial PPO), have been found [11,20,21]. They share an amino acid sequence identity of less than 30%. Both

enzymes operate as homodimers and use FAD as a cofactor. Recently, the crystal structure of tobacco (*Nicotiana tabacum*) PPO2 with the bound inhibitor INH [4-bromo-3-(5'-carboxy-4'-chloro-2'-fluorophenyl)-1-methyl-5-trifluoromethyl-pyrazol] and non-covalently bound FAD has been solved [22]. This crystal structure is the only currently available structural information for eukaryotic PPO at the atomic level. The structure of tobacco PPO2 revealed a loosely associated dimer, each monomer consisting of a membrane-, an FAD- and a substrate-binding domain respectively. The inhibitor INH was found to be located in the active site domain and consists of a phenyl- and pyrazol-ring system. Even though the FAD is not covalently bound to the enzyme it was found in close proximity to the co-crystallized inhibitor in the active site cavity. A potential reaction mechanism was proposed based on the fact that the substrate has only limited space to rotate in the active site. The six-electron oxidation of protogen to proto is supposedly catalysed via three steps. Three times the FAD cofactor is reduced by two electrons from the tetrapyrrole ring and reoxidized by molecular oxygen, which in turn is reduced to H₂O₂. The reduction starts at the C-20 atom of the tetrapyrrole via enamine-imine tautomerization and H rearrangements [23].

Using a model based on the solved crystal structure of tobacco PPO2, we have generated 14 variant proteins carrying amino acid residue exchanges in the corresponding positions of the active site domain. The mutant variant proteins were kinetically characterized by steady-state and stopped-flow fluorescence spectrometry. Furthermore, the amino acid residues responsible for the most common cause of VP in humans were replaced and analysed on the basis of the crystal structure of PPO2 from tobacco.

Abbreviations used: INH, 4-bromo-3-(5'-carboxy-4'-chloro-2'-fluorophenyl)-1-methyl-5-trifluoromethyl-pyrazol; Ni-IDA, Ni²⁺-iminodiacetic acid; PPO, protoporphyrinogen IX oxidase; PPO2, mitochondrial PPO; proto, protoporphyrin IX; protogen, protoporphyrinogen IX; VP, variegate porphyria.

¹ To whom correspondence should be addressed (email d.jahn@tu-bs.de).

Scheme 1 Reaction scheme of PPO

Protopogen is oxidized to proto by molecular oxygen with the generation of hydrogen peroxide. Hydrogen atoms eliminated during the reaction are written in bold letters.

EXPERIMENTAL

Materials

Proto was purchased from Sigma–Aldrich (Hamburg, Germany). Tween 20 was obtained from Carl Roth GmbH (Karlsruhe, Germany). The remaining chemicals were purchased from Sigma–Aldrich unless specified otherwise. Mutagenesis experiments were performed using the QuikChange® site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). Oligonucleotides were purchased from Biomers.net GmbH (Ulm, Germany). S-Tag Rapid Assay kit was from Novagen (Darmstadt, Germany). Cells were disrupted using a sonotrode from Bandelin (Berlin, Germany). Protino® Ni-IDA (Ni²⁺-iminodiacetic acid) resin was from Machery–Nagel (Düren, Germany).

Site-directed mutagenesis of tobacco PPO2 gene

The *N. tabacum* PPO2 gene expression vector pET32a-PPO2(M1-K224Q) was described previously [22]. The plasmid was used as a template for site-directed mutagenesis. Primers to generate N67R, N67W, R98A, R98E, R98K, L356N, L356V, L372N, L372V, S374D, F392E and F392H respectively were as follows.

N67R: 5'-GGGATGAAGGGGCACGTACTATGACTG-3' and 5'-CAGTCATAGTACTTGCCCTTCATCCC-3'; N67W: 5'-GGGATGAAGGGGCATGGACTATGACTG-3' and 5'-CAGTCATAGTCCATGCCCTTCATCCC-3'; R98A: 5'-CCACTTTCACAAAACAAGGCCTACATTGCCAGAAATGG-3' and 5'-CCATTCTGGCAATGTAGGCCTTGTTTTGTGAAAGTGG-3'; R98E: 5'-CCACTTTCACAAAACAAGGAGTACATTGCCAGAAATGG-3' and 5'-ATTCTGGCAATGTACTCCTTGTTTTGTGAAAGTGG-3'; R98K: 5'-CCACTTTCACAAAACAAGAGTACATTGCCAGAAATGG-3' and 5'-CCATTCTGGCAATGACTTTGTGTGAAAGTGG-3'; L356N: 5'-GGTGGGGTTAATGTACCTTCCAAGGAGC-3' and 5'-GCTCCTTGGAAGTACATTACCCAAAGCCC-3'; L356V: 5'-GGGCTTTGGGGTTGTGTACCTTCCAAGGAGC-3' and 5'-GCTCCTTGGAAGGTACAACAACCCCAAGCCC-3'; L372N: 5'-GACACTAGGCCAACAACCTCTCTTCTATGATGTTCC-3' and 5'-GGAAACATCATAGAAGAGAAGTTGGTGCCTAGTGTC-3'; L372V: 5'-GACACTAGGCACCGTCTTCTCTTCTATGATGTTTCC-3' and 5'-GGAAACATCATAGAAGAGAAGACGGTGCCTAGTGTC-3'; S374D: 5'-GACACTAGGCACCCTCTTCGATTCTATGATGTTTCC-3' and 5'-GGAAACATCATAGAATCGAAGAGGGTGCCTAGTGTC-3'; F392E: 5'-CTCTATACTACTGAGTTGGTGAAGCC-3' and 5'-GGCTTCCACCAACCTCAG-

TAGTATAGAG-3'; F392H: 5'-CTCTATACTACTCATGTTGGTGGGAAGCC-3' and 5'-GGCTTCCACCAACATGAGTAGTAGAG-3'. Individual mutations were combined to generate the double-mutation-carrying variants N67R/S374D and N67W/S374D respectively. For all introduced mutations, overall PPO2 gene integrity was verified by DNA sequence determination.

Recombinant production and purification of *N. tabacum* PPO2 and its variants

Recombinant proteins were produced using the *Escherichia coli* strain BL21(DE3)RIL (Stratagene) containing the original or mutated pET32a-PPO2 plasmids. Cells were grown in LB (Luria–Bertani) medium at 37 °C under vigorous aeration. When the cultures reached a D_{578} of 0.7, protein production was induced by the addition of 250 μ M isopropyl-1-thio- β -D-galactopyranoside. Cells were further cultivated overnight at 25 °C and 150 rev./min, harvested, washed with buffer A (10 mM Tris/HCl, pH 8.0, 300 mM NaCl, 10 mM MgCl₂ and 0.05 % Triton X-100) and resuspended in a minimal volume of buffer A. Cells were broken by sonication (Bandelin HD 2070, 0.5 s sound, 0.5 s paused, MS73 tip, 70 % amplitude) and the cell-free extract was cleared by centrifugation at 66 000 rev./min for 45 min using a Ti70 rotor (Beckman). Protein integrity was verified via Western-blot analysis. Recombinant PPO2 was purified to apparent homogeneity by Protino® Ni-IDA resin (Machery–Nagel) as outlined in the manufacturer's instructions. PPO concentrations were determined via the S-Tag Rapid Assay kit. Protein purification and integrity were analysed by MS and SDS/PAGE as outlined previously [24]. The overall protein folding was analysed by CD spectroscopy using a Jasco J810 CD spectropolarimeter as described previously [25,26]. The flavin content of each mutant enzyme was determined spectroscopically as outlined previously and is given in Table 1 [9,27].

PPO assays

The substrate protogen was produced as described previously [28,29]. PPO2 activity was monitored using a continuous assay via the detection of proto fluorescence. Stopped-flow kinetic experiments were performed under aerobic conditions using a Jasco 810 stopped-flow spectrometer and data obtained were analysed with Biokine Acquire Kinetics (Jasco, Überlingen, Germany). Averages of three to five individual transients were analysed. Protogen was dissolved in 50 mM Mes buffer (pH 6.0), 1 % (v/v) Triton X-100 and 20 mM EDTA. Protogen solution and

Table 1 Kinetic parameters of wild-type and mutant PPO2

The determination of kinetic constants was performed as described in the Experimental section. The Michaelis–Menten constant K_m , the k_{cat} and the k_{cat}/K_m values for the *N. tabacum* PPO2 were determined from substrate velocity plots by measuring the constant velocity formation of proto from protogen over the substrate range 1–25 μ M. Values were determined by computerized Lineweaver–Burk iterative curve fitting (SigmaPlot 8.0 Enzyme Kinetics v1.1). The FAD content of wild-type and mutant PPO2 were determined spectroscopically. The FAD content was calculated using standard FAD (Sigma). The standard error of the results shown was between 5 and 10%. n.d., Not detectable.

Protein variant	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m (μ M $^{-1}$ s^{-1})	FAD content (mol/mol of subunit)
Wild-type	1.17	6	5.1	0.63
Substrate binding ring A				
F392H	n.d.	n.d.	n.d.	0.58
F392E	11.20	11	1.0	0.60
Substrate binding ring B				
L356N	11.0	38	3.4	0.55
L356V	7.30	300	41.1	0.62
L372N	16.40	7	0.4	0.60
L372V	103	597	5.8	0.53
Substrate binding ring C				
R98K	2.6	37	14.4	0.50
R98E	12.50	34	2.7	0.51
R98A	8.30	365	44	0.57
VP				
S374D	10.9	208	18.5	0.55
N67R	97	97	1.0	0.61
N67W	n.d.	n.d.	n.d.	0.48
N67R/S374D ('human PPO')	1.4	111	79.4	0.65
N67W/S374D ('human VP')	21	12	0.57	0.38

PPO2 preparations were mixed in a 6:1 ratio in a total volume of 300 μ l and fluorescence resulting from proto formation was continuously recorded via excitation at 409 nm and emission

measurement above 450 nm. Approximately 8000 measurement points per assay were recorded. Proto concentrations were calculated using an appropriate calibration curve. The percentage of substrate consumed was less than 5%.

Determination of kinetic constants

The Michaelis–Menten constant (K_m), the maximal velocity V_{max} and the catalytic constant k_{cat} were determined from substrate velocity plots by measuring the constant velocity formation of proto from protogen over a substrate range 1–25 μ M and a time course of up to 2 h at 20 °C. The initial reaction rates were linear and values were determined by computerized Lineweaver–Burk iterative curve fitting using SigmaPlot 8.0 Enzyme Kinetics v1.1. For the calculation of the catalytic efficiency k_{cat} , the maximal velocity V_{max} was divided by the corresponding enzyme concentration.

RESULTS AND DISCUSSION

Active site architecture of *N. tabacum* PPO2

The crystal structure of tobacco PPO2 revealed a close insight into the three-dimensional structure of the enzyme [22]. Modelling of the protogen into the active site of tobacco PPO2 is based on the first model proposed by Koch et al. [22]. Arg⁹⁸, Phe³⁹², Leu³⁷² and Leu³⁵⁶ co-ordinated the inhibitor INH which was co-crystallized within the active site of PPO2. These four amino acid residues were found highly conserved throughout the PPO family. Ring A is co-ordinated via aromatic stacking with Phe³⁹² (Figure 1A) [22]. The side chains of Leu³⁵⁶ and Leu³⁷² interact hydrophobically with ring B. The side chain of Arg⁹⁸ forms a salt bridge to the propionate carboxy group of ring C. Additionally ring C might be stabilized by flipping and subsequent

Figure 1 Active site cavity of PPO2

(A) Active site of tobacco PPO2. Depicted are the investigated residues Arg⁹⁸, Leu³⁵⁶, Leu³⁷² and Phe³⁹². Phe³⁹² is shown to co-ordinate ring A of the protogen. Leu³⁵⁶ and Leu³⁷² are stacking protogen ring B from the 'back' and the 'front', and Arg⁹⁸ is supposed to co-ordinate the propionate carboxylate group of the protogen ring C. (B) VP mutants of tobacco PPO2. Asn⁶⁷ and Ser³⁷⁴ which play an important role in human VP, are depicted.

stacking of the conserved residue Phe³⁵³. The dome conformation of the protogen is probably stabilized by the carbonyl oxygen of the highly conserved Gly¹⁷⁵.

Experimental determination of amino acid residues important for substrate binding

Based on the proposed model, we functionally investigated the molecular basis of substrate co-ordination and its catalytic implications. We constructed an appropriate set of mutant PPO2 genes, isolated the respective mutant protein variants and determined their kinetic parameters via fluorescence stopped-flow spectrometry in comparison with wild-type PPO2. We used the strategy of introducing conservative and non-conservative amino acid exchanges for residues of interest in order to evaluate their chemical contribution to substrate binding and catalysis. Obtained results are listed in Table 1. The apparent K_m value of purified wild-type PPO2 protein for its substrate was $1.17 \mu\text{M}$ with a k_{cat} value of 6.0 s^{-1} . The determined kinetic parameters were within the range of values measured for PPOs from other organisms, e.g. $K_m = 1.0 \mu\text{M}$ and $k_{\text{cat}} = 0.0031 \text{ s}^{-1}$ for *B. subtilis* [30]; $K_m = 0.1 \mu\text{M}$ for *Saccharomyces cerevisiae* [31]; $K_m = 2.8 \mu\text{M}$ for *Aquifex aeolicus* [32] and $K_m = 0.85 \mu\text{M}$ and either $k_{\text{cat}} = 0.175 \text{ s}^{-1}$ [7] or $k_{\text{cat}} = 5.95 \text{ s}^{-1}$ [9] respectively for *Homo sapiens*. The FAD content of PPO2 was $0.63 \text{ mol of FAD/subunit PPO2}$. All PPO2 mutant enzymes revealed a similar FAD content, the only exception being the double mutant N67W/S374D, which is discussed in detail below (Table 1).

The crystal structure revealed that ring A of the inhibitor INH is kept in position by aromatic stacking with Phe³⁹², which stabilizes the electron transfer over the ring during the reaction. An amino acid exchange to Glu³⁹² in the variant F392E was tolerated within the active site cavity and resulted in inferior binding of the substrate protogen ($K_m = 11.2 \mu\text{M}$). However, once the substrate protogen was bound, its conversion was catalysed sufficiently ($k_{\text{cat}} = 11 \text{ s}^{-1}$). The tetrapyrrole ring is made positive by the hydride abstraction at the N-5 atom of FAD. Thus the negative charge of the introduced glutamate might stabilize the ring and catalysis may proceed equally fast. In this case, only the stabilization of the substrate by aromaticity is lowered and so its binding is weaker. Additionally, the PPO2 variant F392H was found to be virtually inactive. Determination of reliable kinetic parameters was not possible. Histidine at a pH below its pK at 6.1 is positively charged. Here, at a pH of 6.0, this holds true for most histidine molecules of PPO2. The positive charge of the histidine ring at this position leads to repulsion and obstructs substrate binding; consequently, reaction kinetics collapsed (Figure 1A). For detailed kinetic parameters of the mutant proteins see Table 1.

In the model for substrate binding deduced from the PPO2–INH crystal structure, ring B of protogen is sandwiched between the conserved residues Leu³⁵⁶ and Leu³⁷². Both amino acid residues were mutated to either valine or asparagine respectively. In both cases the conservative amino acid exchange to valine enhanced catalysis up to 100-fold ($k_{\text{cat}} = 300$ and 597 s^{-1} respectively). However, in the case of an amino acid exchange of Leu³⁷² to valine, binding of the substrate protogen was 100-fold reduced ($K_m = 103 \mu\text{M}$). In contrast with that, the PPO2 variants L356N and L372N showed an only slightly inferior binding capacity compared with the wild-type and revealed comparable catalytic PPO2 activities (see Table 1). Valine possesses comparable hydrophobic features to leucine. However, the carbon backbone of the side chain is shorter than that of leucine. Most likely the appropriate location of the hydrophobic residue in relation to the co-ordinated substrate ring is essential, explaining the

increasing K_m values of L356V and especially L372V. Once the substrate is bound, catalysis is found to be enhanced for both mutants. Most likely the smaller side chain of valine better accommodates the necessary complex arrangement of the substrate during catalysis. On the other hand, asparagine obviously provides the necessary side-chain length and chemical character to supplement for leucine in both positions (Figure 1A). The next amino acid residue potentially involved in substrate binding to be investigated was Arg⁹⁸. It was deduced from the *N. tabacum* PPO2 crystal structure that Arg⁹⁸ provides the counter charge for the carboxylate group of the inhibitor which most likely mimics the propionate group of ring C of protogen. The PPO2 variants R98K, R98E and R98A were chosen to evaluate the contribution of this positively charged amino acid. While the conservative exchange R98K improved enzyme activity ($k_{\text{cat}} = 37 \text{ s}^{-1}$), the non-conservative exchange R98E resulted in inferior substrate binding ($K_m = 12.5 \mu\text{M}$). Most likely the introduced negative charge prohibited the ionic interaction necessary for the stabilization of ring C. In agreement with this assumption, the replacement of Arg⁹⁸ with the non-polar amino acid alanine resulted in an enzyme variant (R98A) with an 8-fold increased K_m value (Figure 1A). Interestingly, all three variants in position 98 showed an increased k_{cat} value, especially variant R98A ($k_{\text{cat}} = 309 \text{ s}^{-1}$), indicating improved turnover in the absence of ionic interaction between enzyme and substrate. Nevertheless, the overall response of PPO2 towards the amino acid exchanges introduced in position 98 is nicely reflected by the k_{cat} and K_m values.

In summary, by measuring enzyme activity of mutant PPO2s we were able to verify that the well-conserved residues Arg⁹⁸, Phe³⁹², Leu³⁵⁶ and Leu³⁷² are functionally involved in substrate co-ordination within the active site.

VP

The dominantly inherited genetic disorder VP in humans results from impaired PPO activity as a consequence of an amino acid residue exchange. In more than 94 % of the VP cases in South Africa, Arg⁵⁹ is mutated to tryptophan [16]. The residue corresponding to human Arg⁵⁹ in the tobacco PPO2 is Asn⁶⁷. It is positioned on a longer loop between a β -strand and an α -helix exactly between the FAD-binding and substrate-binding sites (Figure 1B). The residue is conserved in all known mitochondrial and plastidic plant PPO amino acid sequences. We started to mimic the situation of the human enzyme on the basis of the related crystal structure of PPO2 from tobacco by mutating Asn⁶⁷ to arginine. The obtained mutant N67R was found to be reasonably active. Even though the mutation resulted in inferior substrate binding, the catalytic efficiency was acceptable. This observation is in agreement with the findings of Maneli et al. [9], who underlined the importance of a hydrophilic, favourably positively charged amino acid at position Arg⁵⁹ in the human enzyme. Replacing Asn⁶⁷ with tryptophan abolished enzyme activity. The bulky nature of an aromatic tryptophan in position Asn⁶⁷ results in impaired enzyme activity. Thus the enzymatic defect responsible for most VP cases in humans can be transferred on to tobacco PPO2. The spatial structure of PPO2 from tobacco can be used as a model for human PPO. However, in the tobacco PPO2 crystal structure, the residue opposite Asn⁶⁷ is Ser³⁷⁴ (Figure 1B). The corresponding residue in human PPO is Asp³⁴⁹, conserved in all known animal PPOs. Asp³⁴⁹ is likely to form a salt bridge with Arg⁵⁹, which would sterically fit in this area of the protein [22]. In order to adapt tobacco PPO2 further towards the human counterpart, we first mutated Ser³⁷⁴ to aspartate. The S374D single mutant showed reduced substrate binding capacity with a significantly increased k_{cat} value ($k_{\text{cat}} = 208 \text{ s}^{-1}$).

Subsequently, we mutated Asn⁶⁷ of S374D to arginine to reconstruct the assumed salt bridge Asp³⁷⁴/Arg⁶⁷ in the human enzyme for our tobacco VP model. The resulting N67R/S374D double mutant showed an almost wild-type level K_m value ($K_m = 1.4$). We were able to obtain a PPO2 variant with wild-type-like substrate recognition and even improved catalytic properties ($k_{cat} = 111 \text{ s}^{-1}$) by a reconstruction of the salt bridge in the tobacco enzyme. These results argue for the existence of a salt bridge linking Arg⁵⁹ and Asp³⁴⁹ in the human enzyme as well. The double mutation N67W/S374D, mimicking VP in the human enzyme, significantly reduced enzyme activity. The K_m value was increased by a factor of 20, while the k_{cat}/K_m value decreased 10-fold. Obviously, the reduced FAD content of 0.38 mol of FAD/subunit for the PPO2 variant N67W/S473D, representing approx. 60% of the wild-type enzyme content, does not account for the observed decrease in enzyme activity. Similar observations were made previously for the human R59W mutant enzyme [9]. Most importantly, in contrast with most other generated mutant enzymes, this PPO2 variant showed an increased K_m with a parallel decreased k_{cat} compared with the double mutant N67R/S374D. Both amino acids neighbouring Asp³⁴⁹ (Ser³⁷⁴ in PPO2) in the human enzyme have previously been characterized. A mutation of Trp³⁴⁸ of the human enzyme to cysteine resulted in a wild-type-like K_m value and a 10-fold reduced k_{cat} [9]. A mutation of Ser³⁵⁰ to proline abolished enzyme activity [18]. These observations underscore that the structural integrity of this region is important for enzyme activity.

Consequently, the mutant tobacco PPO2 enzymes generated, the single as well as the double mutants, may serve as a model for further investigations towards the structural understanding of the disease-causing situation in the human PPO.

Conclusions

We conclude from our investigation that it is most likely that all four rings of the protogen substrate of PPO2 are being co-ordinated during substrate binding by tobacco PPO2. Ring A is stacked by Phe³⁹², ring B is sandwiched in between Leu³⁵⁶ and Leu³⁷², and the propionate carboxylate of ring C is bound to Arg⁹⁸. Furthermore, ring D might be stacked against the A ring of the iso-alloxazine moiety of the FAD. Most of the generated PPO2 variants were characterized by an increase in K_m and k_{cat} values. One may conclude that tobacco PPO2 was optimized during evolution towards a stringent substrate recognition and discrimination, and not towards catalytic turnover.

The results obtained for the VP-type enzyme variants showed that the enzymatic defect responsible for causing VP in humans can be mimicked with the tobacco PPO2 model.

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