Biochemical and structural consequences of a glycine deletion in the \( \alpha-8 \) helix of protoporphyrinogen oxidase

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A rare Gly210 deletion in protoporphyrinogen oxidase (PPO) was recently discovered in herbicide-resistant Amaranthus tuberculatus. According to the published X-ray structure of Nicotiana tabacum PPO, Gly210 is adjacent to, not in, the PPO active site, so it is a matter of interest to determine why its deletion imparts resistance to herbicides. In our kinetic experiments, this deletion did not affect the affinity of protoporphyrinogen IX nor the FAD content, but decreased the catalytic efficiency of the enzyme. The suboptimal \( k_{cat} \) was compensated by a significant increase in the \( k_{s} \) for inhibitors and a switch in their interactions from competitive to mixed-type inhibition. In our protein modeling studies on herbicide-susceptible PPO and resistant PPO, we show that Gly210 plays a key role in the \( \alpha-8 \) helix-capping motif at the C-terminus of the \( \alpha-8 \) helix which helps to stabilize the helix. In molecular dynamics simulations, the deletion had significant architecture consequences, destabilizing the \( \alpha-8 \) helix-capping region and unraveling the last turn of the helix, leading to enlargement of the active site cavity by \( \sim 50 \% \). This seemingly innocuous deletion of Gly210 of the mitochondrial PPO imparts herbicide resistance to this dual-targeted protein without severely affecting its normal physiological function, which may explain why this unusual mutation was the favored evolutionary path for achieving resistance to PPO inhibitors.

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1. Introduction

Protoporphyrinogen oxidase (PPO, also Protox in some literature), a key enzyme in the synthesis of tetrapyrroles, catalyzes the oxidation of protoporphyrinogen IX (Protogen) to protoporphyrin IX (Proto). Plant PPO1 is compartmentalized in the thylakoid and in the envelope membranes of chloroplasts, whereas the mitochondrial isoform PPO2 is localized on the outer surface of the inner mitochondrial membrane [1,2]. In the chloroplast, the porphyrin pathway leads to both chlorophyll and heme, whereas it leads exclusively to heme in the mitochondrin [3].

The three-dimensional (3D) structure of plant mitochondrial PPO revealed that this homodimer folds into a compact structure that includes an FAD-binding, a substrate binding, and a membrane-binding domain [4]. The PPO FAD-binding domain has structural homologies to other flavoenzymes [5], and it is known to be near the binding site of xenobiotic inhibitors [6,7]. A number of herbicide classes (e.g., diphenyl ethers, oxadiazoles, phenylphthalimides and triazolinones) act as competitive inhibitors of PPO [3,8,9]. This enzyme still remains one of the most important target sites for herbicide development. Numerous synthesis efforts are generating new lead compounds [10,11], and a new commercial product has recently been released [12].

Inhibition of this enzymatic step results in unregulated cytosolic accumulation of Proto [13–18], which is responsible for the photodynamic herbicidal action of PPO-inhibiting herbicides [19]. Crystal structures of PPO complexed with various inhibitors have revealed that their binding within the catalytic domain is located near the C-terminus capping region of the \( \alpha-8 \) helix [47]. Gly210 plays an important role in the \( \alpha-8 \) helix cap.

Interestingly, biotypes of the weed Amaranthus tuberculatus (Moq.) Sauer (water hemp) have evolved resistance to inhibitors of protoporphyrinogen oxidase via the selection of an unusual amino acid deletion [20]. This mutation occurred within the mitochondrial isoform, PPO2. However, in A. tuberculatus, as has also been reported in some other species, the gene encoding the mitochondrial isoform
contains an amino-terminal extension that leads to dual-targeting of the enzyme to both chloroplasts and mitochondria [20].

Helix-capping motifs are specific patterns of hydrogen bonding and hydrophobic interactions found at or near the ends of helices in both proteins and peptides [21]. The interactions play an important role in stabilizing the helices. For the C-terminal of an α-helix, the nomenclature for the helix and flanking residues is [21]:

\[ ... \text{C}3\text{C}2\text{C}1\text{Ccap}\text{C}1’\text{C}2’\text{...} \]

in which \( \text{C}1, \text{C}2, \text{etc.}, \) are residues which belong to the helix, \( \text{Ccap} \) is the boundary residue of the helix and the \( \text{C}’ \) residues can participate in the capping. C-terminal capping has been classified into two motifs, Schellman motif and \( \text{C}’ \) motif, depending upon the hydrogen bonding pattern. The \( \text{C}’ \)-cap of α-8 helix of \( \text{PPO} \) matches well the \( \text{C}’ \) motif, which typically has a \( 5 \rightarrow 1 \) hydrogen bond between \( \text{N}^\circ \text{H} \text{C}1 \) and \( \text{C}’ \) of \( \text{C}3 \); a hydrophobic stabilization between \( \text{C}3 \) or \( \text{C}4 \) and one of the \( \text{C}’ \) residues, \( \text{C}’\text{C}2’ \text{C}4’ \); and a glycine as the \( \text{C}’1 \) residue [21-23]. In \( \text{PPO} \), the corresponding residues are \( \text{Gly}210 \text{C}1 \), which hydrogen bonds to \( \text{Ala}206, \) the \( \text{C}3 \) moiety, and there is a key hydrophobic interaction between \( \text{Val}205 \) in the \( \text{C}4 \) position and \( \text{Pro}213 \) in the \( \text{C}4’ \) position.

Recently published molecular modeling studies designed to understand the mechanism of drug resistance found that \( \text{Gly}210 \) deletion significantly affected the surrounding hydrogen bonding network and led to conformational changes in the active site of the mutant enzyme [24].

In this work, our experimental studies showed that the \( \text{Gly}210 \) deletion did not affect the \( K_m \) of the substrate nor the FAD content, but that the catalytic efficiency was reduced for the resistant enzyme. This was compensated by an increase in the \( K_m \) for the inhibitors and an altered interaction with the substrate binding domain. We report molecular dynamics simulations in which the glycine deletion in \( \text{A. tuberculatus} \) \( \text{PPO}2 \) destabilized the helix capping region and caused the last turn of the α-8 helix to unravel. Considering the benefits of a \( \text{Gly}210 \) deletion mutation over single-point amino acid mutations provides some insight as to the evolutionary path favored under the selection pressure exerted by \( \text{PPO} \) inhibitors.

2. Methods

2.1. Cloning of \( \text{S-} \) and \( \text{R-PPO} \)

Two 1.6 kb fragments encoding S- and \( \text{R-PPO} \) were amplified independently from \( \text{A. tuberculatus} \) cDNAs [20] using Tripler Master Taq Polymerase and primers with restriction enzyme adaptors: Start-\( \text{PPX2-EcoRI-F} \) (5′-\text{GGATTCCTACGCGGTCTTCTCATC-3′}) and Stop-\( \text{PPX2-HindIII-R} \) (5′-\text{ATCAAGCTTTTACGCGGTCTTCTCATC-3′}). One fragment was digested with EcoRI and HindIII, cloned in-frame into the pRSET- B vector (Invitrogen, Life Technologies Corp., Carlsbad, CA) forming the sensitive pRSET-S-\( \text{PPX2} \) and resistant pRSET-R-\( \text{PPX2} \) clones, and sequenced to ensure their encoded proteins were identical except for the Gly210 deletion. Transformsants were grown on Luria-Bertani (LB) media containing 50 µg mL\(^{-1}\) ampicillin and 25 µg mL\(^{-1}\) kanamycin and grown at 37 °C for 18 h [25,26].

2.2. Expression and purification of \( \text{S-} \) and \( \text{R-PPO} \)

Clones in pRSET vector were transformed into BL21(DE3)-pLysS strain of \( \text{E. coli} \). Cells were grown in 250 mL of LB with 100 µg mL\(^{-1}\) of carbenicillin, shaking overnight at 37 °C. Cultures were diluted in 1 L of LB with antibiotic and grown at 37 °C shaking for 2 h, induced with 1 mM IPTG and grown at 25 °C shaking for 5 more hours. The cells were harvested by centrifugation at 1600 \( \times g \), washed with 0.05% NaCl, and stored at −80 °C.

Cells were lysed using a French press (Spectronics Instrument) at 140 MPa in 50 mM sodium phosphate pH 7.5, 1 M NaCl, 5 mM imidazole, 5% glycerol, and 1 µg mL\(^{-1}\) leupeptin. Following lysis, 0.5 U of benzonase (Novagen, EMD Chemicals, Inc., Gibbstown, NJ) and PMSF (final concentration of 1 mM) were added. Cell debris was removed by centrifugation at 3000 \( \times g \). His-tagged \( \text{PPO} \) proteins were purified on a nickel activated Hitrap Chelating HP column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) equilibrated with 20 mM sodium phosphate pH 8.0, 50 mM NaCl, 5 mM imidazole, 5 mM MgCl\(_2\), 0.1 mM EDTA, and 17% glycerol. 

\( \text{PPO} \) eluted with 250 mM imidazole. The active protein was desalted on a PD-10 column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) equilibrated with a 20 mM sodium phosphate buffer, pH 7.5, 5 mM MgCl\(_2\), 1 mM EDTA and 17% glycerol. Each liter of culture provided approximately 10 mg of pure \( \text{PPO} \), which was stored at −20 °C until being used in assays.

2.3. \( \text{PPO} \) assay

Proto was purchased from Sigma-Aldrich (Milwaukee, WI). \( \text{Proto} \) was prepared according to Jacobs and Jacobs [27]. Assays were conducted in 100 mM sodium phosphate pH 7.4 with 0.1 mM EDTA, 0.1% Tween 20, 5 µM \text{FAD}, and 500 µM \text{imidazole}. Dose–response curves with the \( \text{PPO} \) inhibitors acifluorfen, lactofen, and MC-15608 were obtained in the presence of 150 µM \( \text{Proto} \). Enzymatic activity was monitored on a RF-5301 PC spectrofluorophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) with excitation and emission wavelengths set at 395 and 626 nm, respectively. The excitation and emission bandwidths were set at 1.5 and 30 nm, respectively. All assays were triplicated and the experiments were repeated. Dose–response curves were analyzed with the add-on package for dose–response curves, drc [28], for R version 2.10.0 [29]. Means and standard deviations were obtained using the raw data, and \( I_{50} \) values were one of the parameters in the regression curves. The regression curves were imported into SigmaPlot version 10 (Systat Software Inc., San Jose, CA).

2.4. Biochemical characterization

Apparent \( K_m \) for \( \text{Proto} \) was determined with either 13 or 130 µg of purified S- and \( \text{R-PPO} \), respectively, in the presence of various amount of \( \text{Proto} \). Inhibitory constants, \( K_m \), were determined by adding the diphenyl ether inhibitor (lactofen, acifluorfen or MC15608) at a concentration approximating its \( I_{50} \) as determined by the dose–response curves. The kinetic analysis was performed with the enzyme kinetic module of SigmaPlot version 10. The FAD content was then analyzed by measuring absorption at the maximum wavelength (450 nm) and expressed as absorption mg\(^{-1}\) protein mL\(^{-1}\) as described by Maneli et al. [30], using a UV3101PC spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD).

2.5. Homology modeling

The sequences of PPO genes from \( \text{A. tuberculatus} \) encoding for wild-type (S-PPO) and herbicide-resistant mutant lacking \( \text{Gly}210 \) (R-PPO) were obtained from PUBMED, accession numbers ABD52329 and ABD52330, respectively [20]. The 3-dimensional (3D) model of S-PPO was prepared starting from the coordinates of the crystal structure of \( \text{Nicotiana tabacum} \) (tobacco) PPO2 complexed with a phenyl-pyrazole inhibitor (1sez.pdb), since no experimental 3D structure is available for \( \text{A. tuberculatus} \) PPO [4]. The PPO sequences of the two species were aligned using CLUSTALW with a Gonnet matrix, a gap open penalty of 10 and a gap extension penalty of 0.2 [31]. The comparative model of S-PPO was built using MODELLER 9.1 [32]. Five different models were constructed and the best model was selected based on the Discrete Optimized Protein Energy (DOPE) score of the models [33]. The model was validated using PROCHECK analysis (PROCHECK4.2) and Profile-3D analysis (Accelrys). The coordinates for FAD were transferred from the crystal structure template into the final model. An in silico model
of S-PPO having Gly210 mutated to Ala210 was also generated starting from the S-PPO homology model.

2.6. Molecular dynamics simulation

The S-PPO holoenzyme model was subjected to minimization and molecular dynamics (MD) simulation using AMBER 9 [34]. We developed the force field parameters for FAD using antechamber. The complexes were solvated in a water box extending 10 Å away from any protein atom. Steepest-descent minimization was carried out for 250 steps followed by conjugate-gradient minimization for 4750 steps. The minimized complex was then heated from 0 to 300 K in six 10 ps steps, each time increasing the system temperature by 30 K, with harmonic constraints of 5 kcal/mol/Å². After heating (50 ps), the density was allowed to equilibrate for a further 40 ps using weak constraints of 1 kcal/mol/Å² on the complex followed by 500 ps of constant volume equilibration at 300 K. All MD simulations used SHAKE on hydrogen atoms, a 2 fs time step and Langevin dynamics for temperature control. Finally a 10 ns production simulation was carried out for the S-PPO model with constant volume equilibration at 300 K with no constraints, recording the coordinates every 10 ps. The reliability of the MD simulation was demonstrated by the stability of the whole complex during the simulation. The trajectories from the MD simulation were analyzed using the ptraj module of AMBER 9. The model stability was checked after each step, monitoring the root mean square deviation (RMSD) of the backbone as well as the temperature, density and energy (total, kinetic and potential) of the system. The overall atom root mean square fluctuation (RMSF) during MD simulation was calculated. Plots of RMSD and RMSF vs. time for the S-PPO and R-PPO simulations are given in the Supporting Information (Figs. S1 and S2, respectively). The distance between the N5 atom of FAD and the carbonyl oxygen of Gly207 was measured using ptraj.

Once the S-PPO model was found to be stable, a snapshot was selected from t = 3 ns of the production run to be used as the starting point for the R-PPO model. Gly210 was deleted and the system was minimized, followed by equilibration and a 10 ns production run and analysis, using the identical procedure to that used for the S-PPO model.

2.7. Secondary structure analysis

The secondary structure of the whole protein was monitored during the MD simulations using the ptraj module in Amber9. The DSSP method proposed by Kabsch and Sander was used for the prediction of secondary structure information for residues [35]. The Kabsch and Sander algorithm identifies secondary structure by hydrogen bond patterns.

2.8. Active site analysis

For each of S-PPO and R-PPO, an average structure was calculated from the MD trajectory using ptraj module of Amber9. However, such an average structure represents a hypothetical conformation of the protein, so we next determined which snapshot from the MD trajectory was closest to the average protein conformation, using the one with lowest backbone RMSD. This snapshot was extracted from each trajectory and utilized for active site volume analysis.

The extracted protein coordinates were subjected to minimization, using the same procedure as mentioned earlier, to relieve any strains and short contacts within the protein. The optimized protein was then subjected to SiteMap analysis. SiteMap scans the surface of a protein to find possible binding sites which are then further analyzed [36,37]. The volume and size of the active site were measured using SiteMap. The models were visualized and images were prepared using PyMOL [38].

3. Results

3.1. Biochemical consequences of glycine deletion on substrate kinetics

Heterologous expression of the S-PPO and R-PPO from A. tuberculatus yielded functional enzymes, but the activity of R-PPO was about 10 times lower than that of S-PPO (Table 1). The Michaelis–Menten constant of S-PPO for Protogen was similar to that of R-PPO (Km ≈ 1 µM) (Table 1), which is within the range of 1 and 5 µM reported with etiopropazine preparations of other plant species [17,39–41]. On the other hand, the Vmax of S-PPO is several folds greater than those reported with crude enzyme preparations [17,39–41]. Since PPO represents a small fraction of the total protein in etiopropazine preparations, the apparent Vmax values calculated under these conditions were most likely underestimated. While the Gly210 deletion did not affect the affinity of Protogen for PPO, it had a deleterious effect on its ability to catalyze the reaction. The Vmax and turnover number (kcat) of R-PPO were approximately 10 times lower than those of S-PPO (Table 1).

The FAD-binding domain has structural homologies to other flavoenzymes [5]. Certain PPO mutations affect the binding of flavin adenine dinucleotide (FAD). In particular, an Arg59 to Trp mutation proximal to the FAD-binding domain reduced FAD-binding and resulted in an inactive mammalian PPO [30]. On the other hand, amino acid mutations affecting the architecture of the Protogen binding domain do not necessarily affect FAD-binding [6]. This seems to be the case with the Gly210 deletion. The S- and R-holoenzymes had similar levels of FAD (0.27 ± 0.01 and 0.28 ± 0.01 OD500 FAD⁻¹ protein ml⁻¹, respectively).

3.2. Biochemical consequences of glycine deletion on inhibitor kinetics

R-PPO was 100 to 500 times less sensitive than the wild-type A. tuberculatus S-PPO to the diphenyl ether inhibitors [Fig. 1 and Table 1], which is in agreement with the resistance pattern observed in vivo [42–44]. As reported numerous times in other plant species, the diphenyl ether herbicides are competitive inhibitors of PPO [45–51]. We also observed competitive inhibition for A. tuberculatus S-PPO (Fig. 2A). Biochemical characterization of the binding of the PPO inhibitors to R-PPO, however, showed a dramatic difference. These compounds no
longer competed directly with the binding of Protogen in R-PPO, but
instead behaved as mixed-type inhibitors (Fig. 2 Ba n dSupplementary
Fig. S3). In all cases, the regression curves intersect above the 1/[S] axis,
which suggests that the Gly210 deletion has altered the architecture of
the A. tuberculatus PPO substrate binding domain, thereby enabling the
R-PPO—inhibitor complexes to still bind Protogen, but with lower
affinities than the enzyme alone. The $K_i$ values were greater in R-PPO
than in the wild-type (Table 1).

3.3. Structural features of the Protogen binding site

The mitochondrial PPO2 from N. tabacum shares more than 65%
sequence similarity with S-PPO (Supplementary Fig. S4). Hence, the
crystal structure of N. tabacum PPO complexed with a phenyl-pyrazole
inhibitor (1sez.pdb)[4] was selected as the template for building the 3D
model of S-PPO. No major discrepancies were observed in the geometry,
solvent accessible surface area, side chain conformational probabilities,
or backbone and side chain conformations of the model. A Ramachan-
dran plot of the model indicated that, as expected, most $\phi$ angles were
negative and $\psi$ angles were positive (Supplementary Fig. S5). Analysis of
the Ramachandran plot showed 98% of the non-glycine residues to be in
the 'most favored or allowed' regions. Our model showed slightly better
PROCHECK analysis compared to the model reported by Hao et al. (e.g.,
89.5% residues in most favored region compared to 87.1% for Hao et al.)
[24]. The few outliers were located in loops towards the outside of the
protein and distant from the substrate binding domain.

The boundary of the substrate binding domain consists of the
isoalloxazine ring of FAD acting as a roof of the active site, with its
re face opposite to Gly207. Arg128 (equivalent to Arg98 in
N. tabacum PPO) is well conserved across species and has been proposed to participate in
ionic interactions with the propionic acid side-chain on ring C of the
substrate[7], but apparently is not involved in orientating the substrate
with respect to the FAD cofactor[6]. The carbonyl oxygen of the
conserved Gly207 protrudes from the $\alpha$-8 helix into the center of the
cavity, and serves as a putative center for the bound tetrapyrrole
macrocyle [7]. Mechanistically, the position of Gly207 relative to
the opposing FAD ring is critical for catalysis as it limits the distance
between the substrate and the cofactor.

Gly207 is located towards the end of the $\alpha$-8 helix of PPO. In most $\alpha$
helices, C-terminus capping is provided by backbone partners from an
adjacent peptide turn [22] and the backbone dihedral angle, $\psi$, of the
residue at C’ is required to have a positive value, hence glycines are
commonly involved in helix capping [21]. In S-PPO, the $\alpha$-8 helix is
capped by C Gly210, forming a stable $\alpha$-motif[22]. Hydrogen bonding
interactions between Gly210 (C’) N–H and Ala206 (C3) result in 5–1
hydrogen bonding (Fig. 3A). Hydrophobic interaction between Pro213
(C4') and Val205 (C4) further supports the presence of the αL motif[23]. Before molecular dynamics (MD) simulation, for S-PPO Gly210 had a positive ψ (88.2°), whereas Gly211 had a negative ψ (−87.5°). After MD, the Gly210 and Gly211 ψ angles changed (112.6° and −110.8°, respectively), but retained the same sign and approximate magnitude. Also the key nonbonded interactions were well maintained (compare Fig. 3A and C).

3.4. Architectural consequences of the Gly210 deletion

Deletion of Gly210 leading to R-PPO removed key hydrogen bonding interactions within the αL motif and fundamentally altered the helix capping (compare Fig. 3C and 3D). Two glycine residues (Gly210 and Gly211) are present at the end of the α-8 helix in S-PPO. In R-PPO, the remaining glycine, Gly211, could in principle serve as a capping residue for the helix, but only after some significant conformational changes.

From the analysis of secondary structure and intramolecular contacts, we were able to detect significant conformational changes caused by deletion of the Gly210. In the R-PPO model, the last turn of the α-8 helix was found to be unstable after 6 ns of MD simulation. In the MD simulations, the secondary structure of the α-8 helix varied considerably in the R-PPO model compared to the S-PPO model, especially near the C-terminus of the helix. As can be seen from Fig. 4, for the S-PPO model Phe204, Val205 and Ala206 retained their helical conformation throughout the MD simulation, while the % helical content for Gly207, Thr208 and Cys209 decreased stepwise in that order but still was >50%. By contrast, in the R-PPO model, the % helical content for Phe204 was ~90% and subsequent residues had stepwise decreased % helical content with Gly207 and Thr208 ~50% and Cys209 having none. Variations in secondary structure of amino acids as a function of time are depicted in Fig. 5. Gly207 and Thr208 maintained non-helical secondary structure throughout the trajectory, unlike for the S-PPO model, in which the α-helical nature of these amino acids was maintained for >60% of the trajectory. Similarly, Val205 and Ala206 lost their α-helical nature (their ψ angles became positive) after 6 ns of MD simulation. Thus the last turn of the α-8 helix unraveled, even though the stabilizing hydrophobic interaction between Val205 and Pro213 was maintained in the simulations.

The unraveling of the last turn of the α-8 helix in the R-PPO model increased the volume of the active site by ~50% (Fig. 6 and Supplementary Fig. S6) (comparing R-PPO with S-PPO, both after MD simulation).
The volumes of the S- and R-PPO active sites, as calculated using the SiteMap module of the Schrödinger suite [36,37], were ∼551 Å³ and ∼848 Å³, respectively. The larger volume observed in R-PPO is due to the opening up of the cavity to include a pocket near the active site (Supplementary Fig. S6). The wider cavity of the R-PPO model can hence accommodate both the substrate and inhibitor simultaneously. The Gly210 deletion also affected the distance between the carbonyl O of Gly207 and N5 of the FAD cofactor, $d_{ON}$. We found that $d_{ON}$ showed oscillating behavior in R-PPO, sometimes increased and sometimes similar to $d_{ON}$ in S-PPO (Fig. 7), which could be predicted to reduce the catalytic efficiency of this enzyme, because this longer distance would impede the sequential hydride abstraction at C20 of Protogen by N5 of the isoalloxazine ring of FAD [4]. Analysis of the distribution of $d_{ON}$ from the MD trajectories demonstrated that there is an average increase of ∼1.5 Å from S-PPO to R-PPO. The distance distribution of 1000 snapshots from each trajectory is displayed in Fig. 7B. For S-PPO, a significant number of snapshots had $6 < d_{ON} < 8$ Å, while for R-PPO, $d_{ON}$ was between 7 and 9 Å in most of the snapshots. Thus overall there was an increase in the distance between the catalytic center FAD (N5) and carbonyl O of Gly207.

3.5. Potential advantages of a glycine deletion over an amino acid substitution

The occurrence of a codon deletion is thought to be fairly uncommon, relative to the natural frequency at which amino acid substitutions occur [52]. Therefore, an in silico simulation was used to evaluate the consequences of amino acid substitution at position 210. Ala was initially selected because it differs from Gly by a single methyl group and is known to be involved in helix capping [21,53]. This amino acid substitution resulted in placement of the β-carbon of Ala into the hydrophobic pocket of the mitochondrial PPO lined by Phe467, Val360, Pro361, Leu362, Gly422, Gly423 and Phe420 (Fig. 8). This simple

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**Fig. 5.** Variations in the secondary structure of amino acids in α-8 helix. Amino acids 204–206 retained their α-helical nature (shown as blue) throughout the trajectory for S-PPO; while in the R-PPO MD simulation, 204–206 started to lose their α-helical nature after ∼6 ns.

**Fig. 6.** Protein fold of the S-PPO (A) and R-PPO (B) models after molecular dynamics. As can be seen, the number of turns in the α-8 helix of the two models differs. Overlay of the S-PPO (cyan) and R-PPO models (red) is shown for comparison (C). Conserved Gly residue is shown in stick mode. The bound cofactor FAD (green) is shown in stick mode in all the images for reference.
amino acid stabilizes the substrate binding domain of PPO because in the wild-type this mutation was also reported in plants resistant to PPO inhibitors [20]. A Val311 to methionine point mutation was found in Bacillus subtilis, GenBank accession number PPO, but this alteration did not lead to a significant increase in resistance to PPO inhibitors [54].

4. Discussion

The seemingly innocuous deletion of Gly210 has a dramatic effect on biochemical functioning of PPO. Our MD simulations also predict that the deletion significantly changes the architecture of the substrate binding domain of PPO because in the wild-type this amino acid stabilizes the α-8 helix by its role in α8 motif C-terminal helix capping [22]. In the MD simulations, deletion of Gly210 led to unraveling of the last C-terminal helical turn which greatly increased the volume of the cavity. These conformational changes agree with the biochemical differences observed between the wild-type and herbicide-resistant forms of PPO. Specifically, the enlargement of the substrate binding domain would not affect the binding of Protogen (Km remains fairly constant), but the displacement of Gly207 would have a deleterious effect on the catalytic efficiency (lower turnover number).

The carbonyl oxygen of Gly207 protruding from the α-8 helix into the center of the cavity is thought to serve as a center for the tetrapyrrole macrocycle [7], positioning the substrate at the correct distance and orientation from FAD. The optimum distance for hydride abstraction by an isoalloxazine ring from its substrate is 2.67 Å [55]. While no PPO complexed with either Protogen or Proto has been successfully crystallized, docking of Proto within the crystal structure of *N. tabacum *PPO estimated the distance between C20 of Protogen and N5 of the isoalloxazine ring of FAD to be ~2.2 Å [4], which would be suitable for the sequential hydride abstraction occurring at C20 of Protogen.

Hao et al. [24] reported a homology model in which they found that R-PPO had a very similar structure to S-PPO. However, in their approach after preparing the R-PPO model starting from the S-PPO three-dimensional structure, they did not allow the R-PPO model to relax to a less-strained conformation before analysis. In subsequent MD simulations they observed significant adjustments to the R-PPO protein conformation, but reverted to their unrelaxed R-PPO model for docking, which, not surprisingly, resulted in similar binding modes of the inhibitors into S-PPO and R-PPO. It would be worthwhile to attempt docking into the fully relaxed R-PPO structure. Hao et al. assumed that the herbicides were competitive PPO inhibitors in both the wild-type and resistant proteins but did not perform any kinetic characterization of the compounds’ binding to PPO, relying instead on the published plant growth inhibition data of these herbicides. However, our biochemical analysis revealed that the Gly210 deletion mutation changes the binding of acifluorfen and lactofen from competitive to mixed-type inhibition, which suggests that the inhibitor and Proto IX can occupy the R-PPO binding pocket simultaneously in the resistant protein.

In our modeling of R-PPO, deletion of Gly210 increased the distance between the Gly207 and the FAD ring in most of the trajectory snapshots, by an average of ~1.5 Å. Since the rate of the FAD catalyzed reaction is limited by the hydride transfer step, such a change in the hydrogen-transfer reaction coordinate in R-PPO would be expected to reduce the enzyme’s catalytic efficiency, and indeed such a reduction is observed. In plants not exposed to inhibitors, this reduced efficiency may be partially compensated by the native chloroplastic PPO, which is still present. In the presence of inhibitors, the significant increase in the Km has enabled plants expressing the R-PPO in the chloroplast to survive.

With respect to the inhibitors, studies with isolated etioplasts have demonstrated that all known PPO inhibitors compete with Protogen at or near the catalytic site on the enzyme [45–51]. Accordingly, the crystal structure of *Myxococcus xanthus *PPO complexed with acifluorfen shows that a diphenyl ether herbicide fits tightly within the Protogen binding cavity, with the nitrophenyl ring extending toward the back of the hydrophobic pocket and the trifluoromethyl group on the other phenyl ring positioned between the isoalloxazine ring of FAD and the bottom surface of the pocket [7]. The enlargement of the substrate binding pocket resulting from the reorganization of the α-8 helix which we observed in the MD simulations could enable the binding of both Protogen and the inhibitor simultaneously, which would explain the mixed-type inhibition kinetics observed in the mutant PPO.

Evolved resistance to PPO inhibitors has followed a convoluted path; specifically, it involved the selection of an amino acid deletion on the mitochondrion PPO isoform and the presence of a dual-targeting transit peptide to redirect this mutant protein to the chloroplast [20]. The reason for selecting a combination of two relatively uncommon genetic alterations rather than a simple amino acid substitution does not impart resistance to diphenyl herbicides since Ala is known to occupy position 210 in the mitochondrial PPO of several herbicide-sensitive plant species (e.g., GenBank accession number AF273767). Substitutions to amino acids larger than Gly or Ala would cause severe steric clashes in this region of PPO, a plausible explanation of why single-point mutations at position 210 have not appeared in plants resistant to PPO inhibitors [20]. A Val311 to methionine point mutation was also reported in *Bacillus subtilis* PPO, but this alteration did not lead to a significant increase in resistance to PPO inhibitors [54].
acid substitution is intriguing [52]. Indeed, low level herbicide-resistant Arabidopsis thaliana plants have been generated via single-point mutagenesis of PPO1 (chloroplast isoforms) by others [56]. Mutation of A. thaliana Tyr426 resulted in a highly functional resistant PPO, and was selected to develop a double mutation (Tyr426Met + Ser305Leu) selectable marker. Although inferior to the Tyr426 residue, substitution of Ala220 (which corresponds to Gly210 in the mitochondrial isoform of A. tuberculatus) with Val, Thr, Leu, Cys, or Ile also provided low level herbicide resistance. A mutation at the corresponding residue of N. tabacum PPO1 also was obtained from cell culture selection [57].

Nonetheless, no amino acid substitutions at position 210 of the mitochondrial PPO, such as those identified in chloroplastic PPOs [56,57], have been reported as a mechanism of resistance in plants exposed to PPO inhibitors. In silico simulation suggests that these substitutions would strain the backbone dihedral angle of the terminal residue of the α-8 helix, and so the level of resistance achieved by such a mutation may carry too high a fitness cost or provide little or no resistance.

The deletion of Gly210 may provide a superior level of resistance to PPO inhibitors than that obtained via a single amino acid substitution. Thus, it is remarkable that the Gly210 codon in the mitochondrial PPO isoforms of A. tuberculatus happens to be located within a short nucleotide repeat, a genetic environment that fosters its deletion [20,52]. No corresponding repeat region is found in the sequence of the chloroplastic isoform, which is an evolutionarily distinct isozyme sharing only 25% amino acid identity with the mitochondrial isoforms, nor is this unique genetic environment distinct isozyme sharing only 25% amino acid identity with the mitochondrial isoforms. The requirement for the deletion of an amino acid specifically involved in helix capping, rather than a substitution, and the presence of the transit peptide leading to the dual-targeting of the gene product may partially explain why resistance to PPO-inhibiting herbicides has been slow to evolve.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbapap.2010.04.004.

References


Fig. 8. Wall-eyed stereoview of the active site of PPO after Gly210Ala mutation. The γ-carbon of the mutated amino acid (Ala in this case, shown in ball and stick mode) is located within the hydrophobic pocket lined by Phe467, Val360, Pro361, Leu362, Gly422, Gly423 and Phe420. Mutation of Gly210 to any amino acid other than Ala would cause steric clashes with the hydrophobic side chains of these amino acids.