Roles of active site residues in catalysis, substrate binding, cooperativity and the reaction mechanism of the quinoprotein glycine oxidase

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Running title: Glycine oxidase catalytic mechanism

Keywords: Cysteine tryptophylquinone, enzyme mechanism, allostery, protein structure, GoxA, LodA-like protein, acid-base catalysis, enzyme cooperativity

Data Deposition: The coordinates of the structures presented here have been deposited in the Protein Data Bank: H583C GoxA + glycine pH 7.5 (6VL7), F316A GoxA + glycine pH 5.5 (6VMW), Y766F GoxA + glycine pH 7.5 (6VMF), H767A GoxA + glycine pH 7.5 (6VMV)

ABSTRACT

The quinoprotein glycine oxidase from the marine bacterium Pseudoalteromonas luteoviolacea (PlGoxA) uses a protein-derived cysteine tryptophylquinone (CTQ) cofactor to catalyze conversion of glycine to glyoxylate and ammonia. This homotetrameric enzyme exhibits strong cooperativity towards glycine binding. It is a good model for studying enzyme kinetics and cooperativity, specifically for being able to separate those aspects of protein function through directed mutagenesis. Variant proteins were generated with mutations in four active-site residues, Phe-316, His-583, Tyr-766 and His-767. Structures for glycine-soaked crystals were obtained for each. Different mutations had differential effects on $k_{cat}$ and $K_{0.5}$ for catalysis, $K_{0.5}$ for substrate binding, and the Hill coefficients describing the steady-state kinetics or substrate binding. Phe-316 and Tyr-766 variants retained catalytic activity, albeit with altered kinetics and cooperativity. Substitutions of His-583 revealed that it is essential for glycine binding and the structure of H583C PlGoxA had no active-site glycine present in glycine-soaked crystals. The structure of H767A PlGoxA revealed a previously undetected reaction intermediate, a carbinolamine product-reduced CTQ adduct, and exhibited only negligible activity. The results of these experiments, as well as those with the native enzyme and previous variants enabled
construction of a detailed mechanism for the reductive half-reaction of glycine oxidation. This proposed mechanism includes three discrete reaction intermediates that are covalently bound to CTQ during the reaction, two of which have now been structurally characterized by X-ray crystallography.

**Introduction**

The phenomenon of cooperativity was initially observed and studied through the allosteric binding of oxygen to hemoglobin over a century ago (1-3). A classical model of positive cooperativity is described as a multimeric protein, in which the affinity for ligand binding to an open site on one subunit is increased by conformational changes induced by binding of a ligand to another subunit. Cooperativity as a mathematical concept, also can describe steady-state kinetic behavior when the increase in initial velocity cannot be fit to a hyperbolic curve using the Michaelis-Menten equation. In each case, binding and kinetics, a sigmoidal curve is characteristic of positive cooperativity. If a protein or enzyme has multiple subunits, which each possess a ligand or substrate binding site, the possibility of cooperativity must be considered.

The quinoprotein glycine oxidase from *Pseudoalteromonas luteoviolacea* (PlGoxA) (4) is an ideal enzyme with which to study the interplay between cooperativity of substrate binding and of steady-state kinetic behavior. PlGoxA uses a protein-derived cysteine tryptophylquinone (CTQ) cofactor (5,6) (Figure 1A). The complete glycine oxidation reaction is oxygen dependent, but the several reaction steps in the reductive half-reaction are not (Figure 1B). Addition of glycine to PlGoxA under anaerobic conditions yields a stable product-reduced CTQ Schiff base intermediate with a characteristic absorbance spectrum distinct from that of the resting oxidized protein. For WT PlGoxA, the initial binding of glycine is slow relative to the reaction steps that form the product Schiff base. As such, an anaerobic titration of PlGoxA with glycine yielded spectral changes that were used to quantitate the fraction of protein to which glycine has bound. This yielded a Hill coefficient ($h$ value) of 3.7 for this homotetrameric protein, which describes strong cooperative binding (7). Analysis of the steady-state kinetics of glycine oxidation by PlGoxA also exhibited positive cooperativity, but with a smaller $h$ value of 1.8 (4). This result was interpreted to mean that the substrate binding was only partially rate limiting for the overall reaction, thus obfuscating the binding cooperativity.

PlGoxA is a member of the family of proteins, named after a lysine ε-oxidase from *Marinomonas mediterranea* (MmLodA) (8). LodA-like proteins are CTQ-bearing enzymes, which unlike previously studied tryptophylquinone dehydrogenases, function as oxidases (9). MmLodA has been described as either a homodimer or tetramer (10). Steady-state kinetic studies revealed the enzyme obeyed Michaelis-Menten kinetics with no discernable cooperativity. A second LodA-like protein from the same bacterium is a glycine oxidase, MmGoxA (11), which forms a homodimer. Analysis of the steady-state kinetics of glycine oxidation by MmGoxA did reveal positive cooperativity, with an $h$ value of 1.7 (12). With MmLodA and MmGoxA, it was not possible to monitor substrate binding because no stable product-CTQ adduct with distinct spectroscopic features was detectable.

Recently, it was shown that mutation of Asp-678 to Glu in the active site of PlGoxA eliminated the observed cooperativity of steady-state kinetic behavior, but it did not diminish the observed cooperativity of glycine binding
The explanation for this was that the mutation slowed the rate of one or more reaction steps such that binding of glycine was no longer even partially rate limiting in the overall reaction. As a consequence of this, it was also possible to spectroscopically characterize a substrate-oxidized CTQ Schiff base reaction intermediate that accumulated during the reaction of A678E PlGoxA, which was not observed during the reaction of the WT enzyme (Figure 1B).

The structure of PlGoxA reveals other amino acid residues that potentially play roles in catalysis and cooperativity (Figure 2). Of particular interest are residues Tyr-766 and His-767. The side chains of these residues project from a loop of one subunit into the active site of another subunit of the homotetramer. The crystal structure of the substrate-bound PlGoxA showed that these residues interact with the carboxyl group of the glycine substrate in the covalent CTQ-adduct that is formed by reaction of the glycine amino group with CTQ (4). The inter-subunit projection of these residues suggests a role in cooperativity. It was previously shown that Phe-237 in MmGoxA plays a role in its cooperative kinetic behavior (12). The corresponding residue in PlGoxA is Phe-316, which suggests that it may also play a role in cooperativity of PlGoxA. Residue His-583 of PlGoxA is seen in the crystal structure to interact with the glycine-CTQ adduct in the active site. This suggests a possible role in substrate binding or catalysis or both. In this study, these four amino acid residues were altered by site-directed mutagenesis and the effects of the mutations on glycine binding, glycine oxidation kinetics and protein structure were determined. The results describe how subtle changes in structure cause dramatic effects in substrate binding, reaction kinetics and cooperativity. Furthermore, alteration of the binding and kinetic mechanism by a H767A mutation allowed structural characterization of another previously undetectable reaction intermediate.

Results
Expression of PlGoxA variant proteins
The following variant proteins were expressed in and isolated from E. coli: H583A, H583C, F316Y, F316A, Y766F, and H767A. In each case, the amount of protein that was purified was comparable to that of the WT recombinant protein. When subjected to size exclusion chromatography, each protein eluted with a mass in the range of 350-400 kDa. This size is consistent with each being a homotetramer of four 91 kDa subunits. Each protein exhibited a visible absorbance spectrum that is characteristic of oxidized CTQ and essentially the same as is seen in WT PlGoxA. In contrast to these results, previous mutagenesis studies of MmLodA and MmGoxA, which involved corresponding amino acid residues, compromised CTQ formation and often had lower yields (14,15). Similarly, mutagenesis of active site-residues in the tryptophan tryptophylquinone (TTQ)-dependent methylamine dehydrogenase compromised TTQ formation (16). Thus, PlGoxA is a particularly robust tryptophylquinone protein, which allows for more detailed structure-function studies of active-site residues than has been possible previously with other CTQ and TTQ dependent enzymes. The parameters that were determined in this study that describe glycine binding and steady state kinetics of glycine oxidation are summarized in Table 1.

Crystallization and structure determination
Crystals for F316A, H583C, Y766F and H767A were grown in similar conditions used for the WT (4). The resulting crystals were yellow in color and isomorphous to the WT (Table 2) with very
similar overall structures (rmsd < 0.3 Å across backbone atoms). Upon soaking in cryoprotection solution containing 10 mM glycine, F316A and Y766F turned deep blue in color, consistent with the formation of a product-reduced CTQ Schiff base in the crystal (17). H583C exhibited no color change while H767A became nearly transparent, which would be consistent with reduced CTQ (7). Diffraction quality crystals of H583A and F316Y could not be obtained from the WT crystallization conditions.

**His-583 variants**

The only PlGoxA variant proteins in this study that did not show any reactivity towards glycine were those in which His-583 was mutated. Mutation of His-583 to either Cys or Ala produced the same result, a CTQ-bearing protein with a characteristic absorbance spectrum that could not be reduced by glycine (Figure 3A). Consistent with this observation, the crystal structure of H583C PlGoxA, which was soaked with glycine, had no glycine present in the active site. (Figure 4). Rather, a pair of water molecules or perhaps a single water with alternate positions interact with the quinone oxygen, and His-767 and Tyr-766 from a neighboring protomer (Figure 4B). Ser-681, which interacts with the bound substrate carboxyl group in the WT structure, is oriented away from the active site in H583C, consistent with a lack of bound substrate in this mutant.

It was previously shown that D678A and D678N PlGoxA variant proteins were not reduced by glycine, but did have glycine present in the active sites in the glycine-soaked crystals (13). In the structures of the glycine-reduced CTQ adduct of WT PlGoxA and the glycine-soaked crystals of D678A and D678N PlGoxA, a His-583 ring nitrogen interacts with a carboxyl oxygen of glycine. This interaction is not possible in the H583A and H583C variants. In the latter case, Cys-583 is oriented away from the substrate-binding site and stabilizes two water molecules not observed in the WT structure due to the presence of the His-583 side chain (Figure 4).

Asp-678 is required to deprotonate the glycine NH$_3^+$ group for Schiff base formation, either directly or via a water. Thus, it can be concluded that His-583 is critical for initial binding of glycine in the active site and positioning it relative to CTQ and Asp-678 to allow adduct formation. A reason for mutating His-583 to Cys is that Cys is present in this position in MmLodA. However, in the structure of the MmLodA-lysine adduct (10), Cys does not interact with the lysine. In that enzyme the Schiff base with CTQ is formed by the ε-amino group of the lysine side-chain rather than the backbone amino group of glycine in GoxA. Other residues within the much larger substrate pocket of that enzyme interact with the bound substrate.

**Phe-316 variants**

F316A and F316Y PlGoxA variants were each reactive towards glycine as judged by glycine-induced changes in the absorbance spectrum, consistent with product Schiff base formation. As such, it was possible to perform a titration with glycine to assess the binding parameters (Figure 5A and B). Whereas, WT PlGoxA exhibited an $h$ value of 3.7 ± 0.4, the F316Y and F316A variants exhibited $h$ values of 1.7 ± 0.1 and 1.4 ± 0.1, respectively. Each mutation increased the $K_{0.5}$ for glycine from 187 μM in WT PlGoxA to 333 μM for F316Y PlGoxA and 783 for μM F316A PlGoxA. Thus, removal of the phenyl ring or even a more modest modification of conversion to Tyr each significantly diminish the cooperativity of glycine binding and its affinity. The variants each exhibited steady-state activity (Figure 5C
and D). For F316Y PlGoxA, the $k_{cat}$ of $8.0 \pm 0.2$ s$^{-1}$ is actually a bit greater than that of WT PlGoxA, while the $k_{cat}$ of $3.1 \pm 0.2$ s$^{-1}$ for F316A PlGoxA is about half that of WT PlGoxA. For WT PlGoxA, the $h$ value determined from fit of the kinetic data was 1.8. For the F316Y and F316A variants, it was 1.6 and 1.2, respectively. These values are comparable to the $h$ values determined from the glycine titration. Thus, the F316Y mutation diminished the cooperativity of substrate binding, and increased $K_{0.5}$, but did not reduce $k_{cat}$. The F316A did reduce $k_{cat}$, as well as affect binding and cooperativity.

The crystal structure of F316A PlGoxA soaked in glycine exhibits electron density consistent with the formation of the product Schiff-base (Figure 6). However, the orientation of the carboxyl group of bound substrate is slightly different from that observed for WT, resulting in altered interactions with active site residues (Figure 6C). In the WT protein, Phe-316 is engaged in a face-to-edge pi stacking interaction with His-767 from the neighboring protomer. The absence of this interaction and the steric bulk of the phenyl ring likely account for the subtle perturbations observed in the active site, which result in the decreased cooperativity and $k_{cat}$.

**Tyr-766 variants**

Tyr-766 and His-767 both project from one monomer of the heterotetramer into the active site of another and interact with the glycine carboxyl group in the crystal structure of the product CTQ Schiff base adduct of PlGoxA. Substitution of Tyr-766 with Phe, which eliminates the interaction with this residue, has a profound effect on substrate binding. Y766F PlGoxA did react with glycine, as judged by changes in the absorbance spectrum. Similar to what was observed with the Phe-316 variants, the $h$ value obtained from the glycine titration of Y766F PlGoxA was decreased to $1.8 \pm 0.1$ (Figure 7). However, in this case the $K_{0.5}$ for binding increased significantly to $527 \mu$M. This decreased affinity was also evident from the steady state kinetic analysis. The $K_{0.5}$ for the reaction also significantly increased to $666 \pm 33 \mu$M. As was observed for the F316Y PlGoxA, the Y766F mutation did not diminish $k_{cat}$, rather it increased to $8.5 \pm 0.2$ s$^{-1}$. The crystal structure of glycine-soaked Y766F PlGoxA is nearly identical to that of the WT enzyme, except for the absence of the OH group on residue 766 (Figure 8).

**His-767 variants**

His-767 also interacts with the glycine carboxyl group in the crystal structure of the product-CTQ Schiff base adduct of PlGoxA. On first look, it did not appear that the His767A PlGoxA variant reacted with glycine, as there was no rapid change in the absorbance spectrum. However, after addition of excess glycine a very slow change was observed. In contrast to WT PlGoxA and the other variant proteins in this study, the spectrum did not change to that of the product CTQ Schiff base intermediate. Instead, it appeared to change to that of the reduced CTQ on the minutes time scale (Figure 9). Given these results, it was not possible to perform glycine titration to assess binding parameters, as was done for the other variant proteins. Steady-state kinetic studies appeared to show no reaction. However, if very high concentrations of glycine were used a slow reaction above background was observed. It appeared to increase linearly with second order rate constant of $17 \text{M}^{-1}\text{s}^{-1}$ with a rate of $0.24 \text{s}^{-1}$ at $10 \text{mM}$ glycine.

Soaking of H767A PlGoxA crystals in $10 \text{mM}$ glycine also seemed to show a bleaching of color. Electron density at the active site showed that a glycine-CTQ adduct was indeed present (Figure 10). However, the electron density was best fit
with the carbinolamine intermediate (See Figure 1B) with an oxygen bound to the \(\alpha\)-carbon of glycine. Attempts to model this structure as the product Schiff-base intermediate resulted in positive difference density at the position of the hydroxyl oxygen and the carboxyl group (Figure 10C), supporting the assignment of the carbinolamine intermediate. This species has not been observed with WT PI\(\text{GoxA}\) or any other variant proteins, but it is consistent with the spectral changes observed after glycine addition. Since the \(\alpha\)-carbon has been hydroxylated, there is no longer a carbon-nitrogen double bond that is present in the product-reduced CTQ adduct. Without this extended conjugation from CTQ through the bound glycine, the spectrum of this species should be similar to that of reduced CTQ, as was observed in Figure 9. Comparison to the WT active site shows very little structural perturbation except the closer approach of Asp-678 to form a hydrogen bond with the hydroxyl group (Figure 10D)

**Discussion**

Previous studies of tryptophylquinone-bearing enzymes have shown that mutation of active site residues is not well-tolerated. Mutation of the active site Asp residue that corresponds to Asp-678 in the TTQ-dependent methylamine dehydrogenase (16) and the CTQ-dependent MmLodA (14) and MmGoxA (12) prevented the formation of the quinone cofactor. Mutation of some other active site residues in these enzymes also prevented or reduced the levels of cofactor biosynthesis. In contrast, the mutagenesis of active site residues in PI\(\text{GoxA}\) did not compromise the completion of CTQ biosynthesis. As such, the results of this and previous studies of PI\(\text{GoxA}\) now allows a detailed description of the kinetic reaction mechanism, identification of reaction intermediates and the roles of active site residues in the reaction mechanism. It also describes the basis for the observed differences in the extent of cooperativity of substrate binding and of steady-state kinetics.

An unusual feature of the reaction of WT PI\(\text{GoxA}\) is that addition of glycine to the enzyme under anaerobic conditions yields a stable Schiff base adduct with the product bound to the reduced CTQ (17). Spectral changes associated with formation of this adduct upon anaerobic titration of PI\(\text{GoxA}\) with glycine yielded an \( h \) value of 3.7 indicative of strong cooperative binding (7). Steady-state kinetic analysis yielded a smaller \( h \) value of 1.8 (4), indicating that a reaction step other than binding was partially rate limiting. Mutagenesis of Asp-678 revealed that it is involved in both the deprotonation of glycine to allow formation of the substrate oxidized CTQ adduct and the subsequent deprotonation of that adduct during conversion to the product reduced CTQ adduct. Studies of a D678E variant revealed that steady state kinetics no longer exhibited cooperativity, even though the cooperativity of binding was retained with an \( h \) value of 3.9 (13). Furthermore, it was possible to observe transient formation of the initial substrate-oxidized CTQ Schiff base adduct. This was because the mutation slowed the rate of conversion to the product-reduced CTQ Schiff base to allow its observation, and was sufficiently slow to be rate limiting for the overall reaction to eliminate the cooperativity of the steady-state kinetics.

The crystal structure of the glycine-soaked H767A PI\(\text{GoxA}\) in the current study revealed the structure of another reaction intermediate, a carbinolamine species that is still bound to CTQ. This results from addition of water to the product Schiff base. Whereas carbinolamines are typically unstable in solution, the intermediate observed in H767A PI\(\text{GoxA}\) is quite stable.
in the active site in the crystal structure, indicating that the hydroxyl moiety is not deprotonated to initiate release of the glyoxylate product (Figure 1B). This suggests that loss of the interaction of His-767 with the CTQ-bound glycine and reaction intermediates has perturbed the position of the carbinolamine species relative to that in the WT enzyme, such that it is not in a position for facile deprotonation by Asp-678 or an activated water. Thus, as a consequence of this mutation, addition of water to the otherwise stable product-reduced CTQ Schiff base is enhanced but the deprotonation of the resulting intermediate is deterred. A similar carbinolamine intermediate was identified during the reaction of the TTQ-dependent aromatic amine dehydrogenase. In that study, crystals of the enzyme were soaked with substrate and flash cooled and it was observed in one of the resultant crystal forms (18). Hydrolysis of the glyoxylate product occurs during oxidation of CTQ yielding an iminoquinone, which is subsequently hydrolyzed to yield the quinone and ammonia. A similar iminoquinone has been shown to be the initial product of the oxidative half-reaction in the TTQ-dependent methylamine dehydrogenase (19) and aromatic amine dehydrogenase (18), as well tyrosylquinone-dependent copper amine oxidases (20).

The residues being studied extend aromatic side chains into the active site, partially surrounding the CTQ. His-583 is to one side of the CTQ and the other residues are “in front” of the quinone oxygen to which glycine binds. Phe-316 is angled downwards and the loop residues Tyr-766 and His-767 are angled upwards in this orientation. The sidechain of Asp-678, above the CTQ and about 5 Å away, meets with the glycine adduct through rotation; the planar aromatic residues appear to stay in place and form a stabilizing “pocket” for the glycine to enter and be oriented. Compromising this stabilizing pocket by altering or removing aromatic rings results in a range of effects, from preventing entrance of glycine to relatively minor changes in binding and reaction kinetics. Results of Y766F PLGoxA also suggest that the presence of the aromatic side chain in this position is more important than the OH group for binding and cooperativity than catalysis, as $k_{cat}$ is not diminished. Mutating the Phe-316 residue affects the reaction in a way similar to Y766F in that it is the...
binding kinetics rather than the $k_{cat}$ that are primarily affected.

Clearly, interactions between His-767 and Tyr-766 from one subunit with the active site of a neighboring subunit are critical for cooperative substrate binding and catalysis. Among these are direct hydrogen bond interactions between these residues and bound substrate as well as pi-stacking interactions between Tyr-766 and Phe-316. We had previously identified a hydrated channel connecting the two active sites, into which the loops bearing His-767 and Tyr-766 project (13). This suggested the binding of substrate to one active site may control access to the other. The crystal structures do not show obvious changes that may impact substrate access resulting from the mutations. However, this is likely to be a dynamic process, which may be difficult to capture by X-ray crystallography. PlGoxA provides a good model for studying enzyme kinetics and cooperativity, specifically for being able to at least partially, tease apart those aspects of the protein through directed mutations. It shares active site residues with many other quinoproteins, some that are dimers and some tetramers, with and without positive cooperativity. This situation provides for the ability to compare similar residues, in some cases nearly identically overlaying arrays of active sites, between related proteins that nonetheless differ in reaction specifics. PlGoxA has uniquely robust protein expression amongst LodA-like proteins, forming CTQ in variants with mutations that prevented expression in other systems, thus allowing the functions of those residues to be studied beyond their involvement in CTQ formation. In addition, PlGoxA has, along with the surface-to-surface interactions shared by all multimeric proteins, unique inter-subunit projections directly into a neighboring unit’s active site. The position of these loop residues, Tyr-766 and His-767, suggest roles in both cooperativity, through communicating ligand binding in one subunit to another, as well as regulating the reaction within the active site into which they project.

**Experimental Procedures**

**Design, expression and purification of PlGoxA variant proteins**

Primers used to generate site-directed mutations are shown in Table 3. Mutagenesis was performed with the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). Amplified mutant gene products were transformed into XL-10 ultracompetent *E. coli* cells, and mutations were verified by Sanger sequencing of harvested plasmids by Genewiz (South Plainfield, NJ). Plasmids harboring the mutated genes were then transformed into *E. coli* Rosetta cells, from which the protein variants were expressed and purified as described previously (4). In each case, cells were transformed with a pET15 vector containing the gene to be expressed with a hexahistidine tag at the N-terminus. Cells were grown overnight to an OD$_{600}$ of 0.8, at which point they were induced with 1 mM IPTG for four hours. Cells were harvested by centrifugation and then lysed via sonication in 50 mM potassium phosphate, pH 7.5. The cell lysate was centrifuged and the supernatant applied to a Ni-NTA affinity column. The column was washed with an imidazole gradient in the same buffer to isolate the tagged proteins.

**Analytical techniques**

Protein purity and native molecular weight were determined by 7.5% SDS-PAGE and size exclusion chromatography using a HiPrep 16/60 column packed with Sephacryl S-300 HR collected in an ÄKTA Pure FPLC system (GE Healthcare Life Sciences, Pittsburgh PA, USA). Absorption
spectra were recorded using an HP 8452 Diode Array spectrophotometer controlled with Olis Globalworks software (Olis, Bogart, GA).

**Binding and activity assays**

Glycine binding titrations were monitored via spectrophotometry from the changes in absorbance from 300 to 650 nm at 20°C in 50 mM potassium phosphate buffer, pH 7.5, under anaerobic conditions as previously described (7). Data from the spectroscopic changes that occurred during the titration were fit to eq 1, which describes allosteric binding. The fraction of PlGoxA with glycine bound was determined from the ΔA/ΔAmax at appropriate wavelengths during the titration.

\[
\frac{\Delta A}{\Delta A_{\text{max}}} = \frac{[\text{glycine}^h]}{(K_d^h + [\text{glycine}]^h)}
\]  

Glycine oxidase activity was determined using a previously described (15) coupled-enzyme assay in which the formation of NH₃ released from glycine (eq 2) is monitored through coupling to the oxidation of NADH by glutamate dehydrogenase (eq 3).

\[
\text{Gly}+\text{O}_2+ \text{H}_2\text{O} \rightarrow \text{glyoxylate} + \text{NH}_3 + \text{H}_2\text{O}_2
\]

\[
\text{NH}_3 + 2\text{-oxoglutarate} + \text{NADH} \rightarrow \text{L-glutamate} + \text{H}_2\text{O} + \text{NAD}^+
\]

The standard assay mixture contained 0.5 μM PlGoxA, 5 mM 2-oxoglutarate, 0.25 mM NADH, and 20 U/ml glutamate dehydrogenase. Reactions were performed in 50 mM potassium phosphate, pH 7.5, at 30°C. Initial velocity was determined by monitoring the rate of disappearance of NADH at 340 nm using the ε₃₄₀ of NADH of 6220 M⁻¹cm⁻¹. Data were analyzed by the Michaelis-Menten equation (eq 4) and the Hill equation (eq 5), in which h is the Hill coefficient.

\[
v/[E] = \frac{k_{\text{cat}}[S]}{(K_m+[S])}
\]

\[
v/[E] = \frac{k_{\text{cat}}[S]^h}{(K_{0.5}^h + [S]^h)}
\]

**Crystallization and structure determination**

Crystals of the PlGoxA variant proteins were grown in batch mode under paraffin oil. Protein at 10 mg/ml was combined with mother liquor containing either 20-25% PEG 3350, 0.1 M ammonium sulfate and 0.1 M HEPES pH 7.5 or 20-25% PEG 3350, 0.1 M citric acid pH 5.5. Crystals were transferred to mother liquor containing 10% v/v PEG 400 as a cryoprotectant for approximately 1 min prior to cryocooling in liquid nitrogen. For glycine soak experiments, 10 mM glycine was included in the cryoprotectant solution.

Diffraction data were collected at 100 K on beamline 5.0.2 at the Advanced Light Source at Berkeley National Laboratory, indexed and integrated with XDS (21,22) and scaled using Aimless (23). The structure was solved by molecular replacement using Phaser-MR (24) or by Fourier synthesis, using the native PlGoxA structure (PDB ID: 6BYW). Manual model building was done in Coot (25) with further rounds of refinement performed using the Phenix suite (26). Figures were prepared using Pymol (http://www.pymol.org), which was also used for pairwise structural alignments.

**DATA AVAILABILITY**

The data related to x-ray crystallography are deposited in the Protein Data Bank and assigned accession codes 6VL7, 6VMW, 6VMF and 6VMV. All other data is contained in this manuscript.
Acknowledgements: The authors thank Yu Tang for technical assistance. This research was funded by the National Institute of General Medical Sciences of the National Institutes of Health, under award R35GM130173 (V.L.D.).

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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The abbreviations used are: CTQ, cysteine tryptophylquinone; MmLodA, L-lysine-ε-oxidase from *Marinomonas mediterranea*; MmGoxA, glycine oxidase from *Marinomonas mediterranea*; PlGoxA, glycine oxidase from *Pseudoalteromonas luteoviolacea*; TTQ, tryptophan tryptophylquinone; WT, wild type
### Table 1. Kinetic and binding parameters for PlGoxA and variants

| PlGoxA variant | Steady state kinetics | Glycine binding |
|----------------|-----------------------|------------------|
|                | $k_{cat}$ (s$^{-1}$) | $K_{0.5}$ (μM)  | $h$ value | $K_{0.5}$ (μM) | $h$ value |
| WT$^1$         | 6.0 ± 0.2             | 187 ± 13         | 1.8 ± 0.2 | 103 ± 3        | 3.7 ± 0.4 |
| D678E$^2$      | 1.0 ± 0.02            | 151 ± 17         | 1.0       | 26 ± 0.1       | 3.9 ± 0.1 |
| H583A          | NR$^3$                | NR               |           | NR             |
| H583C          | NR                    | NR               |           | NR             |
| F316Y          | 8.0 ± 0.2             | 333 ± 17         | 1.5 ± 0.1 | 70 ± 1.2       | 1.7 ± 0.03 |
| F316A          | 3.1 ± 0.2             | 783 ± 115        | 1.2 ± 0.1 | 175 ± 9        | 1.4 ± 0.1 |
| Y766F          | 8.5 ± 0.2             | 666 ± 33         | 1.2 ± 0.04 | 527 ± 13     | 1.8 ± 0.1 |
| H767A          | ND$^4$                |                   |           |                |            |

$^1$From references (4,7)
$^2$From reference (13)
$^3$NR is no reaction
$^4$ND, too slow to be accurately determined
Table 2. Data collection, processing and refinement statistics. Numbers in parentheses are for the high-resolution shell.

|                         | H583C GoxA + Gly pH 7.5 | H767A GoxA + Gly pH 7.5 | Y766F GoxA + Gly pH 7.5 | F316A GoxA + Gly pH 5.5 |
|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| **Data collection**     |                         |                         |                         |                         |
| Wavelength (Å)          | 1.00000                 | 1.00000                 | 1.00000                 | 1.00000                 |
| Space group             | P2_1                    | P2_1                    | P2_1                    | P2_1                    |
| Unit cell parameters    |                         |                         |                         |                         |
| a, b, c (Å)             | 109.8, 93.3, 110.0      | 107.9, 93.0, 109.6      | 90.0, 95.1, 90.0        | 90.0, 91.4, 90.0        |
|                         | 188.6                   | 187.6                   | 90.0, 95.1, 90.0        | 90.0, 91.4, 90.0        |
| α, β, γ (°)             | 90.0, 95.1, 90.0        | 90.0, 95.1, 90.0        | 90.0, 91.4, 90.0        | 90.0, 91.4, 90.0        |
| Resolution range (Å)    | 49.2 – 2.14             | 48.2 – 2.20             | 48.4 – 2.24             | 47.2 – 1.99             |
| Number of reflections   | 770,319/207,973         | 665,403/189,420         | 634,586/173,760         | 843,086/240,912         |
| (measured/unique)       |                         |                         |                         |                         |
| R_merge                 | 0.09 (0.61)             | 0.11 (0.51)             | 0.12 (0.64)             | 0.10 (0.56)             |
| I/σI                    | 9.2 (2.1)               | 5.9 (2.1)               | 5.9 (1.9)               | 9.0 (2.2)               |
| Completeness (%)        | 99.4 (100.0)            | 98.9 (99.6)             | 98.0 (99.7)             | 99.1 (98.5)             |
| Redundancy              | 3.7 (3.9)               | 3.5 (3.3)               | 3.7 (3.7)               | 3.5 (3.1)               |
| **Refinement Statistics** |                         |                         |                         |                         |
| Resolution (Å)          | 2.14                    | 2.20                    | 2.24                    | 1.99                    |
| R_work/R_free           | 0.157/0.211             | 0.187/0.239             | 0.202/0.250             | 0.172/0.223             |
| R.m.s. deviations       |                         |                         |                         |                         |
| Bond lengths (Å)        | 0.013                   | 0.012                   | 0.004                   | 0.008                   |
| Bond angles (°)         | 1.174                   | 1.080                   | 0.719                   | 0.941                   |
| **Ramachandran Statistics** |                         |                         |                         |                         |
| Allowed                 | 99.4%                   | 99.5%                   | 99.5%                   | 99.5%                   |
| Outliers                | 0.6%                    | 0.5%                    | 0.5%                    | 0.5%                    |
| Average B-factor (Å²)   | 47.6                    | 51.8                    | 51.8                    | 32.3                    |
Table 3. Primers used to generate site-directed mutations. Changed bases are underlined.

| Mutation | Primers                                                                 |
|----------|-------------------------------------------------------------------------|
| H583C    | Forward: 5’ - GGT GGT TTT TGC CCT GGC GTT – 3’                         |
|          | Reverse: 5’ – AAC GCC AGG GCA AAA ACC ACC – 3’                         |
| H583A    | Forward: 5’ – GGT GGT TTT GCC CCT GGC GTT – 3’                         |
|          | Reverse: 5’ – AAC GCC AGG GGC AAA ACC ACC – 3’                         |
| F316Y    | Forward: 5’ – AAT ACC GAC TAT GCA GAT AAC TCA AAC TGG – 3’             |
|          | Reverse: 5’ – CCA GTT TGA GTT ATC TGC ATA GTC GGT AAT – 3’             |
| F316A    | Forward: 5’ – TTA AAT ACC GAC GCT GCA GAT AAC TCA – 3’                |
|          | Reverse: 5’ – TGA GTT ATC TGC GTC GGT ATT TAA – 3’                    |
| Y766F    | Forward: 5’ – CTT GGG TTT TGT TGC TTT CAT GCC GAA GGG – 3’             |
|          | Reverse: 5’ – CCC TTC GGC ATG AAA GCC AAC AAA CCC AAG – 3’             |
| H767A    | Forward: 5’ – CTT GGG TTT TGT GCC TAT GCT GCC GAA GGG – 3’             |
|          | Reverse: 5’ – CCC TTC GGC AGC ATA GCC AAC AAA CCC AAG – 3’             |
Figure 1. (A) The protein-derived cysteine tryptophylquinone cofactor. The posttranslational modifications that form CTQ are indicated in red. R signifies the point of attachment to the protein. (B) Reaction scheme and structures of intermediates for glycine oxidation by PlGoxA. This is based on the results of previous studies (13,17) and the current study.
Figure 2. The active site of glycine-soaked crystals of WT PlGoxA (PDB ID: 6EER) showing the product-reduced CTQ Schiff-base adduct and active site residues as sticks colored according to element. Carbons from residues of the neighboring protomer are colored yellow.
Figure 3. The absorbance spectra of WT PlGoxA (black) and H583C PlGoxA (red). Spectra are shown of the oxidized enzymes before and after addition of 5 mM glycine.
Figure 4. The active site of glycine-soaked crystals of H583C PlGoxA (PDB ID: 6VL7). (A) Omit Fo-Fc electron density contoured at 4.0σ for a model omitting active site waters. (B) 2Fo-Fc electron density contoured at 1.0σ for a model including waters. (C) Active site residues shown as sticks colored according to element. Carbons from residues of the neighboring protomer are colored yellow. Waters are shown as red spheres and putative hydrogen bonds shown as dotted lines with distances indicated in Å. (D) Overlay of the H583C (cyan and yellow) and WT (gray) PlGoxA active sites. The hydrogen bonds indicated are from the WT structure.
Figure 5. Substrate binding and steady-state kinetic analysis of F316Y PlGoxA (A and C) and F316A PlGoxA (B and D). In A and B, changes in absorbance at 600 nm were monitored during titration with glycine. The lines are fits of the data to eq 1. In C and D steady-state kinetic data were also fit to eq 5. Each data point is the average of 3 replicates. When error bars are not evident, it is because they too small to extend beyond the circle.
Figure 6. The active site of glycine-soaked crystals of F316A PiGoxA (PDB ID: 6VMW). (A) Omit Fo-Fc electron density contoured at 5.0σ for the active site modeled as CTQ. (B) 2Fo-Fc electron density contoured at 1.0σ. (C) Overlay of the F316A (cyan and yellow) and WT (gray) PiGoxA active sites. Active site residues shown as sticks colored according to element. Carbons from residues of the neighboring protomer are colored yellow. Putative hydrogen bonds are shown as dotted lines with distances indicated in Å.
Figure 7. Substrate binding and reaction kinetics of Y766F PlGoxA. A. Changes in absorbance were monitored at 600 nm during titration with glycine. The dashed line is a fit of the data to equation 1. B. Steady-state kinetic analysis of glycine oxidase activity of Y766F PlGoxA. The dotted line is a fit of the data to equation 5.
Figure 8. The active site of glycine-soaked crystals of Y766F PlGoxA (PDB ID: 6VMF). (A) Omit Fo-Fc electron density contoured at 5.0σ for the active site modeled as CTQ. (B) 2Fo-Fc electron density contoured at 1.0σ. (C) Overlay of the Y766F (blue and cyan) and WT (gray) PlGoxA active sites. Active site residues shown as sticks colored according to element. Carbons from residues of the neighboring protomer are colored yellow. Putative hydrogen bonds are shown as dotted lines with distances indicated in Å.
Figure 9. Spectroscopic changes associated with slow reaction of H767A PlGoxA with glycine. Spectra were recorded immediately after addition of 5 mM glycine to 30 µM H767A PlGoxA (black), 53 seconds later (blue), and 317 seconds later (red).
Figure 10. The active site of glycine-soaked crystals of H767A PlGoxA (PDB ID: 6VMV). (A) Omit Fo-Fc electron density contoured at 4.0σ for the active site modeled as CTQ. (B) 2Fo-Fc electron density contoured at 1.0σ and Fo-Fc density contoured at 4.0σ are shown for the active site adduct modeled as a carbinolamine or (C) as the product Schiff-base. (D) Overlay of the H767A PlGoxA (cyan and yellow) and WT PlGoxA (gray) PlGoxA active sites. Putative hydrogen bonds shown as dotted lines with distances indicated in Å. The hydrogen bonds indicated are from the H767A structure. Residues Y766’ and H767’ are of the neighboring protomer.
Roles of active site residues in catalysis, substrate binding, cooperativity and the reaction mechanism of the quinoprotein glycine oxidase
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J. Biol. Chem. published online March 31, 2020

Access the most updated version of this article at doi: 10.1074/jbc.RA120.013198

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