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Authors

Segraves, Erika N
Chruszcz, Maksymilian
Neidig, Michael L
et al.

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Kinetic, Spectroscopic, and Structural Investigations of the Soybean Lipoyxygenase-1 First Coordination Sphere Mutant, Asn694Gly.

Erika N. SeGRAves[&], Maksymilian Chruszcz[^], Michael L. Neidig[#], Viola Ruddat[&], Jing Zhou[#], Wladek Minor[^], Edward I. Solomon^{#*}, Theodore R. Holman^{&*}.

[&] Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA, 95064, USA.

[^]Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA, 22908, USA.

[#] Department of Chemistry, Stanford University, Stanford, CA, 94305, USA.

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* To whom the correspondence should be sent.
TRH : Phone: (831) 459-5884; Fax: (831) 459-2935;
tholman@chemistry.ucsc.edu
EIS : Phone: (650)723-4694; Fax: (650) 725-0259
edward.solomon@stanford.edu

Running Title: Characterization of N694G sLO-1

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ABBREVIATIONS

hLO, human lipoxygenase; sLO-1, soybean lipoxygenase-1; sLO-3, soybean lipoxygenase-3; WT sLO-1, wild type soybean lipoxygenase; purple lipoxygenase, the HPOD bound Fe^{III} enzyme; Fe^{II}•HPOD, the complex of Fe^{II} and HPOD; N694G, the mutation of Asn694 to glycine; LA, linoleic acid; AA, arachidonic acid; HPOD, 13-(S)-hydroperoxy-9,11-(Z,E)-octadecadienoic acid (the oxidation product of LA); LA-D31, fully deuterated LA; 15-(S)-HPETE, 15-(S)-hydroperoxyeicosatetraenoic acid (the oxidation product of AA); CD, circular dichroism: MCD, magnetic circular dichroism: LMCT, ligand-to-metal charge transfer: K_D , dissociation constant of the enzyme/P complex; k_2 , rate constant of peroxide scission; k_{obsd} , the observed pseudo-first-order rate constant; k_{cat} , the rate constant for product release; k_{cat}/K_m , the rate constant for substrate capture; KIE, primary kinetic isotope effect; k_{cat}^H / k_{cat}^D , the KIE for k_{cat} ; $(k_{cat}^H / K_m) / (k_{cat}^D / K_m)$, KIE for k_{cat}/K_m ;

ABSTRACT: In soybean LO (sLO-1), Asn694 is a weak sixth ligand that is thought to be critical for enzymatic catalysis. In the current investigation N694G sLO-1 was studied to probe its contribution at this sixth ligand position to the kinetic and spectroscopic properties. The k_{cat} value of N694G is ~ 230 times lower than that of WT sLO-1 at 25 °C, which can be partially explained by a lowered reduction potential of the iron as seen by a shift in the visible LMCT band (N694G, $\lambda_{\text{max}} = 410$ nm and WT sLO-1, $\lambda_{\text{max}} = 425$ nm). This conclusion was supported by a faster rate of oxidation of N694G by product than that of WT sLO-1 ($k_2 = 606$ s⁻¹ for N694G and $k_2 = 349$ s⁻¹ for the WT sLO-1). These results suggest a stronger ligand at the active site iron than the native Asn694, which is confirmed to be a water bound to the Fe(II) in the crystal structure. This produces a 6-coordinate CD/MCD spectra for the ferrous N694G and an intermediate rhombic EPR signal for the ferric N694G. The EPR spectrum and its pH dependence suggest that the coordination environment of the ferric N694G contains one hydroxide and one water. Based on both kinetic and structural factors, we propose that the Asn694 water-derived ligand would likely be a hydroxide and the active site, water-derived ligand a water in the ferric state, hence lowering the reaction rate of N694G more than would be expected from the lowered reduction potential alone.

Lipoxygenase (LO) is a mononuclear, non-heme iron dioxygenase that catalyzes the incorporation of molecular oxygen into a 1,4-*cis*, *cis*-pentadiene fatty acid to form hydroperoxide products. In plants, soybean LO isoform-1 (sLO-1) oxygenates linoleic acid (LA) with positional specificity to produce 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13(*S*)-HPOD), which is a precursor to potent signaling molecules in growth regulation (1) and wound response (2). In humans, the lipoxygenase products are members of the eicosanoid family and are implicated in asthma (3), heart disease (4), and various cancers (5).

LO catalyzes the addition of molecular oxygen by substrate activation rather than O₂ activation (6). In the generally accepted mechanism (Scheme 1), the inactive, ferrous enzyme (resting LO) reacts with 13(*S*)-HPOD to generate the catalytically active ferric enzyme (called yellow LO due to a ligand-to-metal charge transfer (LMCT) band ($\lambda_{\text{max}} \sim 425$ nm) (7, 8). The catalytic cycle then begins with the Fe(III)-OH⁻ abstracting the *pro-S* hydrogen atom from the 1,4-*cis*, *cis*-pentadiene fatty acid substrate to form a pentadienyl radical and the Fe(II)-OH₂ intermediate (Scheme 1) (9, 10). A large, temperature-independent kinetic isotope effect (KIE) has been reported for sLO-1 (KIE ~ 80) (11) and human 15-LO (KIE ~ 50) (12, 13), indicating that C-H cleavage is the rate-determining step. The substrate radical reacts with O₂, delivered via an oxygen channel (12, 13), generating a peroxy radical, which is reduced as a result of a hydrogen atom transfer from the Fe(II)-OH₂ species, to produce the hydroperoxide product and the Fe(III)-OH⁻ form of the enzyme. Finally, the purple LO species, named for the peroxide-to-Fe(III) charge transfer band ($\lambda_{\text{max}} =$

580 nm), can be formed by addition of excess HPOD to yellow LO, however it is unclear if this species is generated during catalysis.

The crystal structure of two soybean lipoxygenases, sLO-1 (14) and sLO-3 (15) and one rabbit 15-lipoxygenase (16) (15-rLO) have been reported. Five residues that serve as ligands for the iron-binding site have been shown to be highly conserved across plant and mammalian lipoxygenases, however, a key difference arises in the sixth amino acid ligand of the active site. In most plant lipoxygenases, mammalian 5-LOs, and murine/human platelet 12-LOs, the sixth ligand is Asn (17). In the remaining 12-LOs and reticulocyte 15-LOs, however, the ligand is His (17). Additionally, sequence comparison of the newly reported 15-hLO-2 shows the ligand to be a Ser (18). This difference in coordination has been of continuing interest in our labs because of its relationship with enzyme activity.

WT sLO-1, with an Asn ligation, is the best characterized lipoxygenase, both kinetically and spectroscopically. Utilizing MCD, it was determined that Asn694 was highly flexible and the Fe(II)-Asn bond could vary in length depending on experimental conditions (19). In particular, the ferrous form of the enzyme exists as a 60:40 mixture of 5- and 6- coordinate species, with the Asn varying between bound and un-bound. However, when substrate (linoleic acid) or inhibitor (oleic acid) were added, the coordination environment changed to 100% 6-coordinate, suggesting a structural connection between the active site and the Asn694 ligand (19, 20). This hypothesis of a structural connection between the active site and the iron coordination environment was confirmed by mutating a hydrogen bond network (Gln495, Gln697

and Asn694), which affected the ability of substrate binding to change the iron coordination and subsequently enzyme activity (21, 22).

The importance of the Asn694 in sLO-1 was also confirmed when the Asn was mutated to His (N694H). The ferrous MCD of N694H manifested only a 6-coordinated species, not a mixture of 5- and 6-coordination, due to the strong ligation of the histidine (20). In addition, the ligation of the His reduced the reactivity of sLO-1, due to a decrease in its reduction potential and hence a lowered rate of hydrogen abstraction. It should be noted that this His ligation is found naturally in 15-hLO, whose decreased activity relative to 15-sLO was previously proposed to be due to its lowered reduction potential (23).

In the current study, we further examine the role of Asn694 in sLO-1, by performing kinetic, spectroscopic and structural investigations of the point mutation, Asn694Gly sLO-1 (N694G), which possesses a different coordination environment from WT sLO-1 and manifests a dramatically lowered enzymatic activity.

Materials & Methods

Materials. LA was purchased from Aldrich Chemical Co. and per-deuterated linoleic acid (D-LA) was purified from a mixture of per-deuterated algal fatty acids from Cambridge Isotope Labs, as previously described (20). All commercial fatty acids were re-purified using a Higgins Analytical Haisil (25 cm × 4.6 mm) C-18 column. Solution A was 99.9% MeOH and 0.1% acetic acid, while solution B was 99.9% H₂O and 0.1% acetic acid. An isocratic elution of 85% A:15% B was used to

purify all fatty acids and they were stored at $-80\text{ }^{\circ}\text{C}$ for a maximum of 6 months. All other chemicals were reagent grade or better and were used without further purification.

Mutagenesis, overexpression, and purification of Lipoxygenases. Site-directed mutagenesis, overexpression, and purification of WT and N694G followed a protocol outlined previously (20). In brief, following expression of the protein in BL21-DE3 (*Escherichia coli*), the cells were harvested by centrifugation and their membranes were disrupted by sonication. Cell debris was pelleted and the supernatant was first dialyzed against 20 mM Bis-Tris buffer (pH 6.0) and then applied to a SP-Sephadex high-flow ion exchange column (Pharmacia), which was equilibrated with the same buffer. Eluted fractions containing lipoxygenase activity were pooled, concentrated, dialyzed against 20 mM Bis-Tris buffer (pH 6.0), and applied to a Macro-Prep 25-S ion exchange column (Bio-Rad). After concentration and buffer exchange into 0.1 M Borate (pH 9.2), the isolated enzyme was estimated to be greater than 90% pure (SDS-PAGE). Iron content of all lipoxygenase enzymes were determined on a Finnegan inductively coupled plasma mass spectrometer (ICP-MS) using internal standards of Co(II)-EDTA, and data were compared with standardized iron solutions.

Enzyme activation kinetics. Activation kinetics were carried out as previously described (8, 24). Briefly, kinetic measurements of the enzyme activation were performed under pseudo-first-order conditions (with enzyme as the minor component) using a DX•17MV Applied Photophysics spectrofluorometer equipped with a 150-watt xenon arc lamp light source and 320 nm cutoff filter. The oxidation

of ferrous N694G and WT sLO-1 was observed as a temporal change in fluorescence emission above 320 nm ($\lambda_{\text{ex}} = 280$ nm). The assay samples were $\sim 2 \mu\text{M}$ enzyme (WT or N694G) over a range of $40 \mu\text{M} - 2.5 \text{ mM}$ HPOD in a mixed buffer (25 mM Tris, 25 mM Ches, and 25 mM Caps buffers, 50 mM ionic strength, pH 9.2 at 25 °C). To determine K_D and k_2 , kinetic runs were performed at 7 different concentrations of HPOD; each pseudo-first-order rate constant (k_{obsd}) was the average of five determinations. The stopped-flow apparatus was rinsed with HNO_3 , KOH, and water periodically to prevent kinetic artifacts from enzyme build-up.

Steady-State Kinetic Measurements. Steady-state kinetic values were determined by following the formation of the conjugated product at 234 nm ($\epsilon = 2.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) with a Perkin-Elmer Lambda 40 spectrophotometer. No photo-degradation of the product was observed. All kinetic measurements were standardized to iron content. The assay samples were 2 mL in volume with substrate concentrations ranging from 1-100 μM under constant stirring with a rotating magnetic bar. Stock solution of LA was prepared in 95% ethanol and diluted into buffer so that the total ethanol concentration was less than 1.5%. Fatty acid concentrations were verified by allowing the enzyme reaction to proceed to completion and quantitating the conjugated product at 234 nm. Higher substrate concentrations were avoided due to the formation of micelles, which would alter the free substrate concentration (25). Initial rates (up to the first 15% of the reaction) for each substrate were fitted to the Michaelis-Menten equation using the KaleidaGraph (Synergy) program and errors determined. All subsequent kinetic measurements were handled in a similar fashion.

Kinetic measurements were determined at pH 9.2 in a mixed buffer (25 mM Tris, 25 mM Ches, and 25 mM Caps buffers) at 25° C. The total ionic strength was adjusted to 50 mM by the addition of NaCl. The temperature was controlled using a circulating water bath and jacketed cuvette system.

Kinetic Isotope Effect. The KIE values were determined by comparing steady-state results using pure proteo and pure per-deuterated LA substrate and should be differentiated from competitive KIE values where there is a mixture of proteo and deuterio substrate in the reaction flask. Steady-state KIE values were determined by following the formation of the conjugated product at 234 nm, as discussed above, and were carried out in 0.1 M Borate buffer (pH 9.2) with the temperature-controlled circulating water bath and jacketed cuvette system. For protonated LA, ≈ 120 nM of N694G was used and for the D-LA ≈ 340 nM of N694G was used for each assay (normalized to iron content).

Reaction Rates at Varying O_2 Concentrations. Reaction rates of N694G were determined by measuring oxygen consumption on a Clark oxygen monitor as previously published (26). Reactions were carried out as a function of oxygen concentration in 1 mL solutions, which were constantly stirred and thermostatted to 20 °C. The reaction was initiated by the addition of ≈ 50 nM N696G (normalized to iron content) via a gastight Hamilton syringe to the reaction chamber. The experiments were repeated at a number of different concentrations of oxygen, established by passing mixtures of N_2 and O_2 over stirred solutions in the reaction chamber for 20 minutes. The new oxygen concentration was calibrated against the

value of O₂ dissolved in an air-saturated solution at 20 °C (288 μM O₂). The rate of oxygen consumption was recorded at O₂ concentrations from 5 to 300 μM.

HPLC Analysis of Hydroperoxide Products. Purified LA was reacted with WT sLO-1 and N694G in 0.1 M Borate (pH 9.2) until the reaction was complete (monitored by UV absorbance) and subsequently quenched with HOAc. The hydroperoxides were extracted with MeCl₂, evaporated to dryness, and solubilized in MeOH. The sample was reduced with 100 equivalents of NaBH₄, evaporated, and re-solubilized in acidified water (pH 3.0 with HOAc). The reduced products were extracted with ether, dried with MgSO₄, filtered and evaporated. The methylation was accomplished by reaction with TMS diazomethane in benzene/MeOH for 1 hour in the dark. The reaction solution was evaporated and solubilized in 95% hexane: 5% isopropanol. The oxidized product standards were isolated from the auto-oxidation of LA using RP-HPLC. Each fraction was reduced and methylated as above. Each methyl ester product was resolved via HPLC, equipped with a Chiralcel OD-H column (250mm x 4.6 mm, 5μm; Chiral Technologies), using a mobile phase of hexane-isopropanol (98:2) at a flow rate of 0.5 mL/min. The products were detected at 234 nm and Millenium software (Waters Corporation) was used for integration. Error was determined from replicate HPLC injections (n = 4).

UV-vis Absorption and CD spectroscopies were carried out at 4° C. The UV-vis absorption spectra were recorded on a Perkin Elmer Lambda 40 monochromometer. Spectra were taken in 0.1 M Borate (pH 9.2) at 0.1 M ionic strength (adjusted with NaCl) at 4° C. CD absorption spectra were obtained using an Aviv 60DS

spectropolarimeter in 0.1 M Borate (pH 9.2). CD spectra were baseline corrected by subtracting the buffer and cell background from the raw data. Titrations were performed by the addition of 1 equiv of 13-(*S*)-HPOD to 0.6 mL of lipoxygenase (20 mg/mL).

CD and MCD Spectroscopy. Near-infrared (600-2000 nm) CD and MCD data were recorded on a Jasco J-200D spectropolarimeter with a liquid N₂-cooled InSb detector and equipped with an Oxford Instruments SM4000-7 Tesla (T) superconducting magnet/cryostat capable of fields up to 7T and temperatures down to 1.5 K. The N694G CD sample (~1.8 mM) was exchanged into deuterated CHES buffer (50 mM, pD 9.2) and kept at 5 °C during data collection with a circulating cooling bath attached to the sample holder. Glycerol-*d*₃ was added at 60 % (v/v) to the N694G/Fe(II) solution for preparation of the MCD sample and CD spectra were taken with and without glycerol addition to ensure that the site was unaffected by the glassing agent (Note: no change in the CD spectrum due to glycerol was observed). CD spectra were corrected for buffer and cell baselines by subtraction. Low temperature MCD data were obtained in custom made cells consisting of two Infrasil quartz disks separated by a 0.3 cm thick neoprene O-ring spacer into which the sample was injected and secured between two copper plates. The MCD spectrum was corrected for the natural CD and zero-field baseline effects caused by strain in the glasses by averaging the positive and negative field data at a given temperature (i.e. (7T-(-)7T)/2).

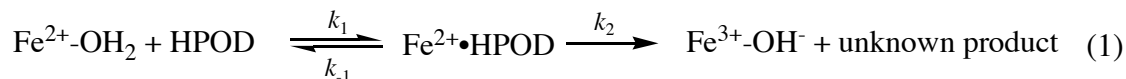
EPR spectroscopy. EPR measurements were performed on a Bruker ESP-500E spectrometer equipped with a dual mode X-band cavity and an Oxford Instruments ESR-900 helium flow cryostat. The program HAM (Dr. M. Hendrich, Carnegie Mellon U.) was used for data manipulation and EPR spectral simulation, as described previously (20). In brief, the signals were fit with an effective $S = 1/2$ model which uses a g -dependent linewidth and properly accounts for field-dependent transition probabilities. The temperature dependence was also fit with this program using the standard Boltzmann equation. The amount of EPR-active Fe(III) in the protein samples was determined relative to a Fe(III)-EDTA standard. A 100 μ M Fe solution was made from a 1000 ppm iron standard (Fischer Scientific), with addition of 10-fold excess of EDTA in 25% glycerol. The EPR spectra were recorded at a modulation frequency of 100 kHz and at microwave frequencies of about 9.63 GHz. Precise microwave frequencies were recorded for individual spectra to insure precise g -alignment. Other EPR parameters are specified in the figure legends.

Crystallization and Data Collection. Crystals of N694G were grown and cryoprotected as previously described (14, 21). The 100K data were collected at the Structural Biology Center 19BM beamline at Argonne National Laboratory, and processed with HKL2000 (27). The N694G crystal was monoclinic with a $P2_1$ space group with the following unit cell parameters: $a=92.88 \text{ \AA}$, $b=92.84 \text{ \AA}$, and $\beta=90.7^\circ$. The structure of native soybean lipoxygenase-1 (PDB code 1F8N) was used as an initial model.

Crystallographic Refinement. Refinement of the mutant structures was performed with the programs REFMAC (28), O (29), and COOT (30). For R_{free} calculations, a subset of 5.1% reflections was used (31). During the refinement, the occupancies of the ferrous ion and its coordinating water molecules were adjusted to 0.7 as for previous LO mutants (21). This resulted in an average displacement factor of the iron-coordinating atoms (27 \AA^2), which is similar to the value of the Fe(II) displacement parameter (29 \AA^2) and closer to the average B factor for all atoms of the structure (Table 1). The programs PROCHECK (32) and MOLPROBITY (33) were used for structure validation. Residues Val312, Asn534, and Ser560 have unusual backbone conformation, as observed for other LO mutants (14). The coordinates and structure factors were deposited in the Protein Data Bank with accession code 1Y4K. Superpositions of molecules or their fragments were done using the program LSQKAB (34) from the CCP4 package (35). Figures were prepared with PyMOL (36).

Results and Analysis

Protein Purification and Kinetic Determinations. N694G and WT sLO-1 were purified with yields of 2-4 mg/L, with metal contents of $50 \pm 10\%$ and $80 \pm 5\%$, respectively. The activation of N694G and WT sLO-1 can be described by equation 1:



where the term K_D will be used as previously defined: $K_D = K_M$, since $((k_{-1} + k_2) / k_1) = K_M$ and $k_{-1} \gg k_2$ (37, 38). N694G and WT sLO-1 showed saturation curves consistent

with formation of the oxidized ferric enzyme (yellow LO, Fe(III)-OH). Using nonlinear regression methods, the dissociation constant K_D and the rate constant k_2 were determined for N694G and WT sLO-1 (Table 2). HPOD binds to N694G with a K_D of $41 \pm 5 \mu\text{M}$, which was comparable to that of WT sLO-1 (K_D of $39 \pm 14 \mu\text{M}$). The rate of oxidation of the iron center for N694G was twice the magnitude as that of the WT sLO-1 ($k_2 = 606 \pm 18 \text{ s}^{-1}$ vs. $k_2 = 349 \pm 31 \text{ s}^{-1}$). The K_D and k_2 values for WT sLO-1 differ slightly from our previously reported values, presumably due to differences in buffer composition (8).

The steady-state parameters, k_{cat} and k_{cat}/K_M (Table 3), were determined at 25 °C and the k_{cat} value of N694G was ~ 230 times lower than that of the WT sLO-1, while the k_{cat}/K_M value was ~ 45 times lower. The KIE data indicate similar mechanistic rate-limiting steps for N694G as for WT sLO-1. The $^{\text{D}}k_{\text{cat}}$ of N694G showed temperature independence (average $^{\text{D}}k_{\text{cat}} = 37 \pm 3$), over the range of 15 - 37 °C (Figure 1). Although the magnitude of the KIE is less than that observed for previous mutants (37, 38), the magnitude of the $^{\text{D}}k_{\text{cat}}$ is much larger than semiclassical theory predicts ($\text{KIE} = 7\text{-}10$) and is consistent with a tunneling mechanism for hydrogen atom abstraction (39). A large, temperature independent $^{\text{D}}k_{\text{cat}}$ is also seen for WT sLO-1, indicating hydrogen atom abstraction is fully rate limiting (40). The analysis of $^{\text{D}}k_{\text{cat}}/K_M$ was hampered by high errors due to extrapolation from the steady-state K_M values; however, the overall trend indicates a slight temperature dependence, with a maximum of 44 ± 19 at 37 °C. The data for N694G is comparable to our previous data for N694H, which manifested a large $^{\text{D}}k_{\text{cat}}/K_M$ of 60 ± 9 at 30 °C (40).

The kinetic parameters at varying O₂ concentrations were analyzed under saturating LA concentrations (5-fold K_M) at 20 °C (Table 3). The $k_{\text{cat(O}_2\text{)}}$ was determined to be $0.71 \pm 0.01 \text{ s}^{-1}$ for N694G, which is in good agreement with the k_{cat} values determined spectrophotometrically (Table 3) and ~320-fold lower than the $k_{\text{cat(O}_2\text{)}}$ for the WT sLO-1 ($230 \pm 15 \text{ s}^{-1}$) (13). There was no decrease in the rate between 5-260 $\mu\text{M O}_2$, which indicates that the $K_{\text{M(O}_2\text{)}}$ is less than 1 $\mu\text{M O}_2$ and therefore the $k_{\text{cat}}/K_{\text{M(O}_2\text{)}}$ value is equal to or greater than $0.7 \mu\text{M}^{-1} \text{ s}^{-1}$. For WT sLO-1, the $k_{\text{cat}}/K_{\text{M(O}_2\text{)}} = 7.5 \pm 0.9 \mu\text{M}^{-1} \text{ s}^{-1}$ ($K_{\text{M(O}_2\text{)}} = 30 \pm 4 \mu\text{M}^{-1} \text{ s}^{-1}$) (13), which is 11-fold higher than that for N694G; however, due to the limitations of the $K_{\text{M(O}_2\text{)}}$ experiment, these values could be closer.

WT sLO-1 is known to oxidize LA with high regio- and stereospecific resolution. Accordingly, the hydroperoxide products of both WT sLO-1 and N694G were analyzed by chiral HPLC after reduction and methylation (12). The dominant product for both enzymes is 13(*S*)-HPOD (83% for WT sLO-1 and 81% N694G), however N694G showed 2% greater promiscuity for the 9(*R*)-HPOD stereoisomer (all error $\leq 0.1\%$, Table 4), which has been previously observed for the Leu546Ala sLO-1 mutant (*vide infra*) (12).

Spectroscopy and Crystallography

(1) Fe(III) Site

Oxidation of N694G was accompanied by an increase at approximately 400 nm (Figure 2b), due to the LMCT band, characteristic of the yellow species (Fe(III)-OH-)

(20). UV-vis CD spectroscopy resolved the LMCT feature from the large protein envelope and revealed a distinct, well-resolved band, with a maximum at 410 nm (Figure 3). This LMCT band is higher in energy than WT sLO-1 (425 nm), which is consistent with a decrease in the reduction potential of the iron, relative to WT sLO (20). The $\Delta\epsilon$ value was corrected for iron content and is comparable in magnitude to that of the WT sLO-1 ($\Delta\epsilon = 1.50 \text{ M}^{-1} \text{ cm}^{-1}$ for N694G and $\Delta\epsilon = 1.45 \text{ M}^{-1} \text{ cm}^{-1}$ for WT sLO-1). Addition of excess HPOD to N694G, produced the purple species (Fe(III)-HPOD), which is due to a peroxide-Fe(III) LMCT (Figure 2c) (41). N694G showed a maximum at 590 nm ($\epsilon = 1750 \text{ M}^{-1} \text{ cm}^{-1}$), while WT sLO-1 showed a maximum at 595 nm ($\epsilon = 1510 \text{ M}^{-1} \text{ cm}^{-1}$) (20). Previously, our labs reported a maximum at 580 nm ($\epsilon = 1550 \text{ M}^{-1} \text{ cm}^{-1}$) for WT sLO-1 under different buffer conditions (24), indicating a buffer dependence of this LMCT.

The EPR spectrum of as-isolated N694G (150 μM iron) in frozen solution at 4 K, exhibited a weak signal at $g \sim 4.3$ (data not shown). The low intensity of this signal represents less than 1% of the total iron and indicates that the enzyme is mostly in its ferrous state. Addition of 1 equiv of 13-(*S*)-HPOD to ferrous N694G oxidized the iron to the catalytically competent yellow, ferric species. The yellow N694G at pH 9.2 (0.1 M Borate, 0.1 M ionic strength) exhibited a complex multicomponent EPR signal with pronounced features at $g \approx 4.3$, 6 and 7 (Figure 4a). In the analysis of the EPR spectrum of yellow N694G (pH 9.2), the $g \approx 6$ signal had axial symmetry ($E/D = 0$), the signal at 7 had intermediate rhombicity ($E/D = 0.05$) and the signal at $g = 4.3$

had rhombic symmetry ($E/D = 0.33$). These features indicate that the yellow N694G form at pH 9.2 contained no less than three species: axial ($E/D = 0$, $g_{x,y,z} = 6, 6, 2$), intermediate rhombicity ($E/D = 0.05$, $g_{x,y,z} = 7.0, 4.0, 1.9$) and rhombic ($E/D = 0.33$, $g_{x,y,z} = 4.3, 4.3, 4.3$). Addition of 3 more equivalents of HPOD generated the purple species at pH 9.2 (0.1 M Borate, 0.1 M ionic strength) with a different EPR spectrum with three, major features at $g \approx 4.3, 5.0$ and 7.0 (Figure 4e). The multicomponent spectrum of the purple N694G (pH 9.2) was comprised of the same three species as the yellow enzyme form, one major signal ($E/D = 0.05$, $g_{x,y,z}$ values of $7.0, 5.0$, and 1.9) and two minor signals with E/D values of 0.33 and 0 , respectively, but in different ratios. The yellow and purple species were also generated at pH 7, to determine any pH dependence. The EPR spectrum of yellow N694G at pH 7.2 (0.1 M Tris, 0.1 M ionic strength) contained the same three species as at pH 9.2, except the relative amount of the axial species at $g = 6$ is decreased (Figure 4c). The spectrum of purple N694G at pH 7.2 was identical to the spectrum at pH 9.2 (data not shown). For all spectra, features with g -values around 2 were obscured by an adventitious Cu(II) signal.

The quantification of the amount of Fe(III) contributing to the axial ($g \approx 6$), intermediate rhombicity ($g \approx 7.0$) and the rhombic ($g \approx 4.3$) signals, for all the samples measured was complicated by the fact that the axial and intermediate rhombicity signals spread over a large field range (≈ 2300 gauss) and overlap. This problem was overcome by EPR spectral simulations. The simulated spectra were simultaneously integrated over the entire field range to obtain the total EPR intensity

from transitions within the $M_s = \pm 1/2$ Kramers doublet for the axial and intermediate rhombicity signals and the middle transition of the rhombic signal. Fe(III)-EDTA was used as a standard for spin quantitation. The double integral was evaluated between 0 and 2500 G, just short of the interfering signal from adventitious Cu(II). The signals for both the yellow and the purple species of N694G were simulated with an effective spin $S' = 1/2$ Hamiltonian (Figures 4b, 4d, 4f), with the parameters given in Table 5. An axial doublet ($g \approx 6$), a doublet with intermediate rhombicity ($g \approx 7.0$), and two rhombic doublets ($g \approx 4.3$) were required for the simulation. The axial simulation parameters were used previously for WT sLO, while the intermediate simulations are distinct from that published for N694H (20). It should be noted that for all of the intermediate rhombicity signal simulations of both the yellow and the purple species, it was difficult to match the variability of the g-strain of each g-component. Therefore, these simulations are approximations to the true signal shape. Two rhombic doublets were required because one would not adequately fit the broad base of the $g \approx 4.3$ signal. These two rhombic components are considered as a single species in the following discussion. The simulation areas were adjusted for the g_{ave} differences of the rhombic and axial signals as described by Aasa and Vangård (42) and linewidth broadening was applied to each g-value.

Quantification of the signal intensity was dependent on the relative populations of the spin manifolds of each of the three EPR active species. In order to account for the spin populations of all the three M_s doublets, the ZFS parameters were approximated

for each species from the temperature dependence of the EPR spectral intensity over the range 3.8 - 40 K under non-saturating conditions. The temperature dependence of the $g \approx 7.0$ and $g \approx 4.3$ signals were evaluated and their signal intensity versus temperature was compared to the previous data for N694H. Based on this comparison, the ZFS of the $g \approx 7.0$ signal for both the yellow and the purple species was determined to have a positive D value of approximately 2 cm^{-1} , while the rhombic $g \approx 4.3$ signal (present in the spectra of both the yellow and purple species) had an |D| of approximately 1 cm^{-1} . These approximate ZFS parameters were used to calculate the Boltzmann population distributions of the three doublets and the spin populations were corrected accordingly for the two signals, $g \approx 7$ (for both the yellow and the purple species) and $g \approx 4.3$. The $g \approx 6$ signal observed had significant spectral overlap with the other two species and therefore its temperature dependence could not be obtained accurately from the EPR spectral data. Its D-value was set to 2 cm^{-1} , based on the fact that in WT sLO-1 two axial signals are observed with D values of approximately 2 cm^{-1} (43).

Utilizing the intensity simulation (g_{ave} corrected), it was determined that the relative concentrations of the three EPR visible species for yellow N694G (pH 9.2) were $28 \pm 3 \%$ for the axial, $64 \pm 6 \%$ for the intermediate rhombicity, and $8 \pm 2 \%$ for the rhombic (Figure 4b). For the yellow species at pH 7.2, the three species remained, but their relative concentrations changed to $18 \pm 2\%$ for the axial, $76 \pm 8\%$ for the intermediate rhombicity and $6 \pm 2\%$ for the rhombic species (Figure 4d). It is

important to note the dominant signal present in yellow N694G at both pH 9.2 and 7.2 was the intermediate rhombicity species ($E/D = 0.05$) at $g_{\text{eff}} = 7$, although it does not appear in the spectrum to be the largest feature. The dominant signal in purple N694G (pH 9.2) is also the intermediate rhombicity species at $g \approx 7.0$ ($E/D = 0.05$), which accounts for 96 ± 10 % of the total signal, while the $g = 6$ and $g = 4.3$ account for 2 ± 1 % each of the total signal (Figure 4f). The $g \approx 7$ signal for purple N694G at pH 7.2 was identical to the signal at pH 9.2 and therefore had the same species ratio.

(2) Fe(II) site

The overall crystal structure of the N694G (PDB code 1Y4K) was very close to the structure of the WT sLO-1 (PDB code 1F8N) - the r.m.s. displacement between C α atoms was 0.41Å. The largest differences between the structures were found in the loops regions, residues 71-77 and 88-91. Leu546 manifests two orientations, which may relate to the product distribution profile. Previously, Knapp et al. (12) demonstrated that mutations of the putative oxygen channel (L546A sLO-1) affected the product distribution. This same change in product distribution is also observed in N694G, which may relate to the conformational change of Leu546 but further study is required. The regions of residues 1-6, 21-30 and 117-121 were flexible and invisible in the electron density map, similar to that of the WT sLO-1 structure.

The removal of the Asn side chain at residue 694 generated a space that is partially occupied by two water molecules, one of which filled the sixth coordination site of the octahedral Fe(II) cation (Asn694 water, water 1003)(Table 6). The Fe(II)···OH₂ distance (2.14 Å) was much shorter than the Fe(II)···O δ 1 Asn694

distance (2.87 Å) in the native protein. The second water molecule (water 1001) was hydrogen bonded to the ligated water (1003) and the backbone carbonyl oxygen of Leu754. In comparison with the WT sLO-1, there were additional small changes in distances between the Fe(II) ion and other coordinating residues, however, they are minimal (Table 6). The three histidine residues are bound in a facial manner and the monodentate carboxylate (Ile839) is hydrogen bonded to the active site water (1002). The active centers in the N694G and WT sLO-1 are presented for comparison (Figure 5).

Near-infrared CD and MCD spectroscopy were employed to study the high-spin ferrous active site in N694G at higher resolution and in solution. The CD and MCD results are presented in Figure 6. The energies and splitting pattern of CD/MCD bands provides information about the geometric and electronic structure of the active site in N694G. In octahedral symmetry, a six-coordinate (6C) ferrous site has a doubly degenerate 5E_g ligand field excited state and a triply degenerate ${}^5T_{2g}$ ligand field ground state split in energy by $10 Dq \sim 10000 \text{ cm}^{-1}$ for biologically relevant nitrogen and oxygen ligands (44, 45). In the low symmetry of a protein active site, these states further split (${}^5E_g \rightarrow d_x^2 - y^2$ and d_z^2) resulting in two ligand field transitions at $\sim 10000 \text{ cm}^{-1}$ and split by $\sim 2000 \text{ cm}^{-1}$ for a distorted 6C ferrous site. Five-coordinate (5C) square pyramidal sites show these transitions at $\sim 10000 \text{ cm}^{-1}$ and $\sim 5000 \text{ cm}^{-1}$ and 5C trigonal bipyramidal sites exhibit one transition at $< 10000 \text{ cm}^{-1}$ and one at $< 5000 \text{ cm}^{-1}$. Distorted four-coordinate sites show only low energy ligand

field transitions in the 4000-7000 cm^{-1} region, due to the much smaller value of $10 Dq$ for tetrahedral complexes ($10 Dq (T_d) = -4/9 10 Dq (O_h)$).

The 278 K CD spectrum of N694G contains two negative ligand field bands at $\sim 8700 \text{ cm}^{-1}$ and $\sim 10700 \text{ cm}^{-1}$ (Figure 6a). CD spectra measured both with and without the glassing agent glycerol present were unchanged. The 5 K, 7T MCD spectrum of N694G (Figure 6b) contains two positive ligand field features at $\sim 9100 \text{ cm}^{-1}$ and $\sim 10700 \text{ cm}^{-1}$. Thus, the CD and MCD spectra for N694G reflect the presence of only one species (in contrast to wt-sLO), which has a distorted 6C ferrous site with an exogenous water ligand binding to the sixth coordination position previously occupied by Asn694.

Discussion

The coordination environment of the iron binding site in lipoxygenase is highly conserved across plant and mammalian species, with the exception of the sixth ligand (46). The sixth ligand of the active site can vary from an Asn in many plant and mammalian lipoxygenases to a His or a Ser in select mammalian enzymes (17, 18). In sLO-1, the sixth ligand, Asn694, is a weak ligand that can adjust its bond distance to the iron depending on the presence of substrate (linoleic acid) or inhibitor (oleic acid), as observed by MCD (20, 22).

We have proposed that the physical connection between substrate binding and the coordination environment of the iron is a highly conserved hydrogen bonding network that connects the substrate cavity to the first coordination sphere of the iron

(Gln495, Gln697 and Asn694) (21). The weak ligation of Asn694 has also been shown to be critical to the activity of sLO-1. Converting the Asn694 ligand to a histidine (N694H) dramatically lowered the enzymatic rate (20, 21). This change in rate was proposed to be due to a decrease in the reduction potential of the active site iron from the stronger His ligation and hence a reduction in the rate of the hydrogen abstraction (20).

In the current investigation, we studied N694G to further probe the role of the sixth ligand position in the kinetic and spectroscopic properties. With regards to catalysis of LA, the k_{cat} value of N694G is ~ 230 times lower than that of WT sLO-1 and ~ 9 lower than that of N694H at 25 °C (20, 47). The $k_{\text{cat}}/K_{\text{M}}$ of N694G value is ~ 45 times lower than that of WT sLO-1 and ~ 6 lower than that of N694H at 25 °C (Table 2) (20, 47). These data are indicative of a greater effect on product release (k_{cat}) than substrate capture ($k_{\text{cat}}/K_{\text{M}}$) for N694G versus WT sLO-1 and are consistent with the previous trend observed for N694H versus the WT sLO-1(20, 21). In addition, the KIE results for both k_{cat} and $k_{\text{cat}}/K_{\text{M}}$ of N694G are consistent with hydrogen atom abstraction being the sole rate-limiting step, indicating that N694G uses the same fundamental mechanism as the WT sLO-1 (20).

One of the primary driving forces for the lipoygenase hydrogen atom abstraction is the reduction potential (E°) of the active site iron (48). Previously, we estimated a lower reduction potential for N694H, relative to WT sLO-1, from a shift in the LMCT band (20). This comparison can also be done with regards to N694G (LMCT $\lambda_{\text{max}} = 410$ nm, $24,390$ cm^{-1}), which is of higher energy than the WT sLO-1 (LMCT $\lambda_{\text{max}} =$

425 nm, 23,530 cm⁻¹). This result signifies a lower E° for N694G than the WT sLO-1 and is a partial contribution to the lowered activity of N694G versus WT sLO-1.

This conclusion is supported by the activation kinetics of N694G. The activation kinetics of lipoxygenase by HPOD measures both the K_D of HPOD binding and the rate at which HPOD oxidizes the ferrous active site iron (k_2). The activation data of N694G demonstrate that the active site of N694G is comparable to that of WT sLO-1 with the K_D values being similar between the two ($K_D = 41 \pm 5 \mu\text{M}$ for N694G and $K_D = 39 \pm 14 \mu\text{M}$ for WT sLO-1). Nevertheless, the rate of iron oxidation for N694G is faster than that of WT sLO-1 ($k_2 = 606 \pm 18 \text{ s}^{-1}$ for N694G and $k_2 = 349 \pm 31 \text{ s}^{-1}$ for the WT sLO-1). These kinetic results, coupled with the blue shifted His-to-Fe(III) LMCT band, indicate a lowered reduction potential for N694G and suggest a stronger ligand at the active site iron than the native Asn694.

This hypothesis is confirmed by the crystal structure, the CD/MCD data for the ferrous N694G and the EPR signal of the ferric N694G. The ferrous structure shows two water molecules in the cavity, occupied by Asn694 in WT sLO-1, with one of the waters bound directly to the iron. The water ligated to the ferrous iron has a short bond distance relative to the Asn in WT sLO-1 (Table 6), indicating a strong ligand interaction and hence a lowered reduction potential (i.e. lowered kinetic activity). Furthermore, the crystallographically defined iron coordination correlates well to CD and MCD studies of N694G, which are consistent with the presence of a purely 6C ferrous site with an exogenous water ligand bound in solution.

The solution EPR spectrum of ferric N694G at pH 9.2 shows a distribution of species with the intermediate rhombic signal being the major component (64%). The intermediate rhombicity species has an E/D value of 0.05, which is similar to the intermediate rhombic signal of N694H ($E/D = 0.08$) (20), but is in contrast to the axial signal ($E/D = 0.01$) of the WT sLO-1 enzyme (49). The axial signal in wt reflects the presence of an open coordination position on Fe(III) (i.e. 5C square pyramidal). These data indicate that the coordination environment of N694G is 6C (not axial) but with one strong unique axis (not completely rhombic), as seen previously with N694H (20). Considering the fact that the Yellow N694G EPR is minimally pH dependent and the yellow WT sLO-1 has a hydroxide bound, it is appropriate to assume that the 6C coordination environment of the ferric N694G contains one hydroxide and one water with the hydroxide corresponding to the strong axis, however it is unclear which coordination position (active site or Asn694) has the water and which has the hydroxide.

The purple species of N694G manifests the same three species as the yellow form; axial ($E/D = 0$), intermediate rhombicity ($E/D = 0.05$) and rhombic ($E/D = 0.33$), however, its appearance is different from the yellow signal due to the increase in the percentage of the intermediate rhombicity species (96%) and the lowered g -strain (Table 5), which sharpens certain features of the signal. If we assume the HPOD is directly bound to the iron, based on the purple LMCT band, then the HPOD has displaced the exogenous, water-derived, ligand, similar to that seen in the HPOD-WT sLO-3 structure (50). The fact that the E/D of the intermediate rhombicity species

does not change upon the anionic, hydroperoxide binding, suggests that a hydroxide has been lost in the coordination environment, leaving HPOD bound to the catalytic site position and a water at position 694 (Figure 7). This is supported by the fact that the purple N694G EPR does not change down to pH 7.2, indicating a bound water. Nevertheless, the fact that the exogenous, Asn694 ligand is a water in the purple N694G, does not indicate that the exogenous, Asn694 ligand is a water in the Yellow form because the decrease in Lewis acidity of the ferric iron upon HPOD binding could raise the pKa of a hydroxide, leading to its protonation

This raises the question of which ligation position, the catalytic site or the 694 site is the hydroxide and which is the water in the ferric form of N694G. A possible answer to this question may be found in analysis of the kinetics of N694G. The activation parameters are twice as fast for N694G than that of the WT sLO-1, while the catalysis of LA hydroperoxidation is over 230 times slower. These results, combined with the higher rhombicity of the yellow N694G EPR signal and a blue-shift in the LMCT band, compared to WT sLO-1, support our previous hypothesis that stronger ligands (than Asn) at position 694 in sLO-1, lower the reduction potential and thus decrease the driving force of the reaction (20). Nevertheless, N694G is over 9-fold slower than N694H even though its LMCT band is at lower energy (410 versus 400 nm, respectively). If the reduction potential were the only factor in the rate of catalysis, one would hypothesize that the rate of catalysis of N694G would be faster than that of N694H, which is not the case. This suggests that there is another factor that affects the rate of catalysis of N694G. One possibility is

that in the ferric state, the water is the active site orientated ligand and the hydroxide is the Asn694 ligand. We and others have proposed that the active site hydroxide is critical to abstracting the hydrogen atom from the substrate (9, 21). If this critical, active site, exogenous ligand is in fact a water in N694G, then the rate of hydrogen atom abstraction would be lowered dramatically. This hypothesis is supported by examination of the hydrogen bond interactions to both water ligands in the ferrous forms of N694G (Figure 8). The active site water is hydrogen bonded to the bound carboxylate (Ile839), which would raise its pKa due to anionic repulsion, favoring protonation. This H-bond is shorter for N694G (2.25 Å) versus WT sLO (2.50 Å), which would raise the pKa of the active site water in N694G even more (Figure 5). In contrast, the Asn694 water, for N694G, is hydrogen bonded to a second water and has a distant hydrogen bond from the NH of Gln697, which could potentially stabilize the charge of a bound hydroxide, upon oxidation of the iron. Based on these structural factors, the Asn694 water ligand would likely become the bound hydroxide in the ferric state, hence accounting for the lowered reaction rate of N694G compared to N694H, even though its reduction potential is not as low. We are currently investigating this possibility.

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Scheme 1. Lipoxygenase mechanism.

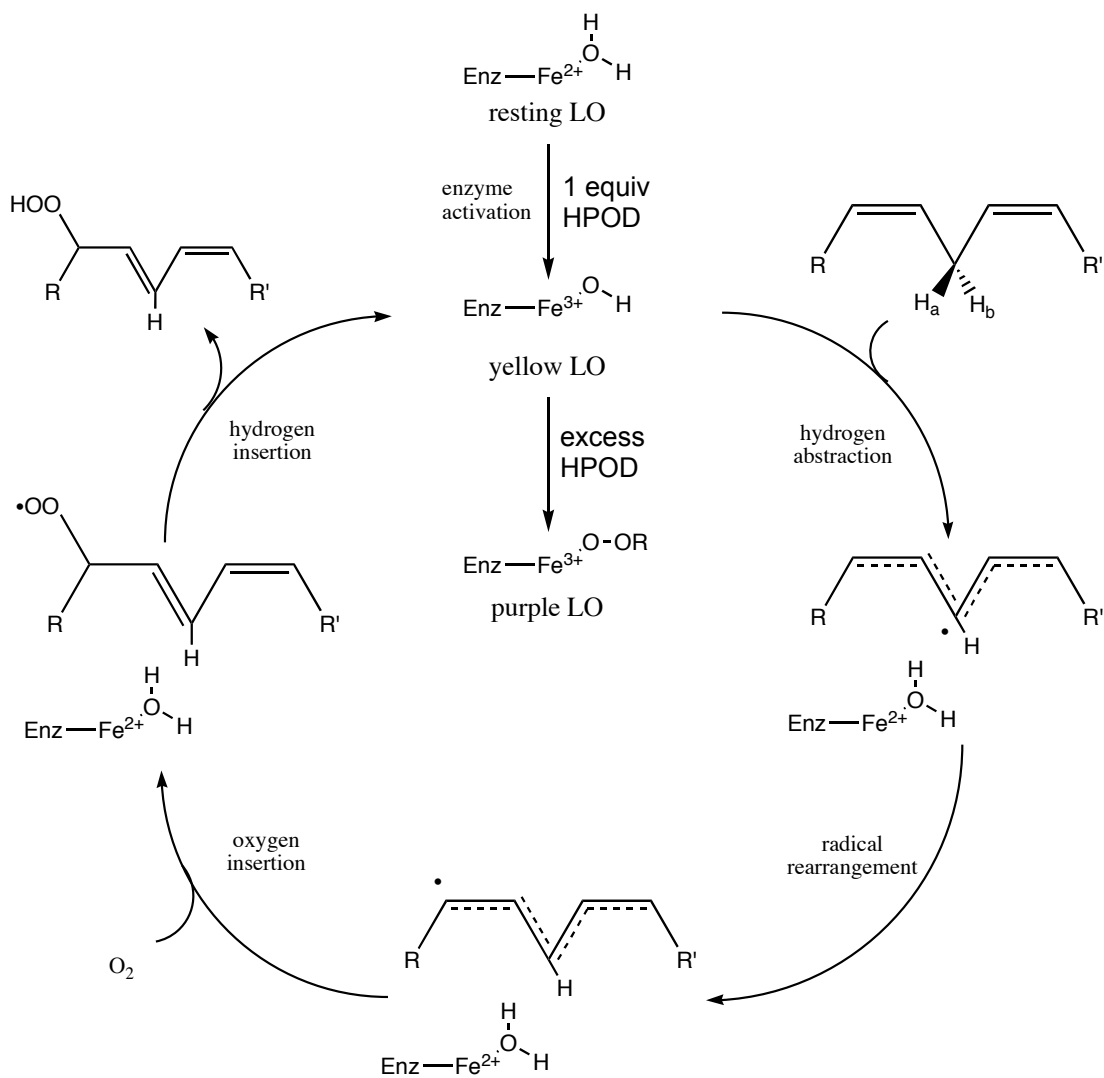


Table 1. *The data collection and refinement statistics for N694G. The data for the highest resolution shell (2.02-1.95 Å) are reported in parentheses.*

Data collection	
Wavelength (Å)	1.0311
Resolution range (Å)	50.00 – 1.95
Unique reflections	60260
R _{merge}	0.044 (0.333)
Completeness (%)	95.9 (91.0)
Average redundancy	2.8 (2.7)
Average I/σ(I)	21.3 (3.1)
Refinement	
Resolution (Å)	33.00 - 1.95
Unique reflections	56381
Completeness (%)	96.1 (91.0)
R (%)	18.0 (20.5)
R _{free} (%)	23.2 (28.3)
No. non-H atoms	7191
No. water molecules	676
R.m.s.d. bond lengths (Å)	0.019
R.m.s.d. bond angles (°)	1.68
R.m.s.d. torsion angles (°)	6.49
Average B factor (Å ²)	28.84
Ramachandran plot	
Residues in most favored region (%)	90.7
Residues in additional allowed regions (%)	9.2
Residues in not allowed regions (%)	0.1

Table 2. Comparison of Activation Parameters for WT and N694G sLO-1^a

	K_D^b (μM)	k_2^b (s^{-1})
WT	39(14)	349(31)
N694G	41(5)	606(18)

^aNumbers in parentheses represent errors as determined from replicate determinations.

^bData were collected at 25 °C.

Table 3. Comparison of Kinetic Parameters for WT, N694G, and N694H sLO-1^a

	$k_{\text{cat}}^{\text{b}}$ (s ⁻¹)	$k_{\text{cat}}/K_{\text{M}}^{\text{b}}$ ($\mu\text{M}^{-1} \text{s}^{-1}$)	$k_{\text{cat}}/K_{\text{M(O}_2\text{)}}^{\text{c}}$ ($\mu\text{M}^{-1} \text{s}^{-1}$)
WT	266(22)	18(5)	7.5(0.9) ^e
N694G	1.17(0.02)	0.4(0.2)	$\leq 0.7^{\text{d}}$
N694H(20)	10(2)	2.5(1.1)	nd

^aNumbers in parentheses represent errors as determined from replicate determinations.

^bData were collected at 25 °C.

^cData were collected at 20 °C.

^d $K_{\text{M(O}_2\text{)}}$ too low to be measured ($\leq 1 \mu\text{M}$).

^e Ref. (13)

Table 4. Product Distributions for WT and N694G sLO-1

	% HPOD ^a			
	<i>13(S)</i>	<i>13(R)</i>	<i>9(S)</i>	<i>9(R)</i>
WT	83	6	8	3
N694G	81	6	8	5

^aAverage of two separate experiments. The estimated error ((run 1 – run 2)/2) is < 0.1%.

Table 5. Theoretical EPR simulation parameters

	E/D	$g_x (\Delta g)^a$	$g_y (\Delta g)^a$	$g_z (\Delta g)^a$
Sim Axial	0.0	6.0 (19)	6.0 (155)	2.0 (170)
Sim Inter. Rhombic 1 (yellow, pH 9.2)	0.05	7.0 (77)	5.0 (211)	1.9 (170)
Sim Inter. Rhombic 2 (yellow, pH 7.2)	0.05	7.0 (35)	5.0 (270)	1.9 (170)
Sim Inter. Rhombic 3 (purple, pH 9.2 & 7.2)	0.05	7.0 (49)	5.0 (69)	1.9 (170)
Sim Rhombic 1	0.33	4.3 (30)	4.3 (30)	4.3 (30)
Sim Rhombic 2	0.33	4.5 (33)	4.3 (22)	4.0 (42)

^aLinewidths in gauss at half-height of the absorption curve are given in parentheses.

Table 6. Iron-ligand Bond Distances of WT sLO-1 and N694G, in angstroms.^a

	WT	N694G
	(1F8N)	(1Y4K)
Nε2 His499	2.21; 2.24	2.35
Nε2 His504	2.34	2.19
Nε2 His690	2.29	2.31
Oδ1 Asn694	2.87	2.14 (OH ₂ 1003)
O1 Ile839	2.28	2.20
OH ₂ 841	2.11	2.27 (OH ₂ 1002)

^aEquivalent residues noted in parentheses.

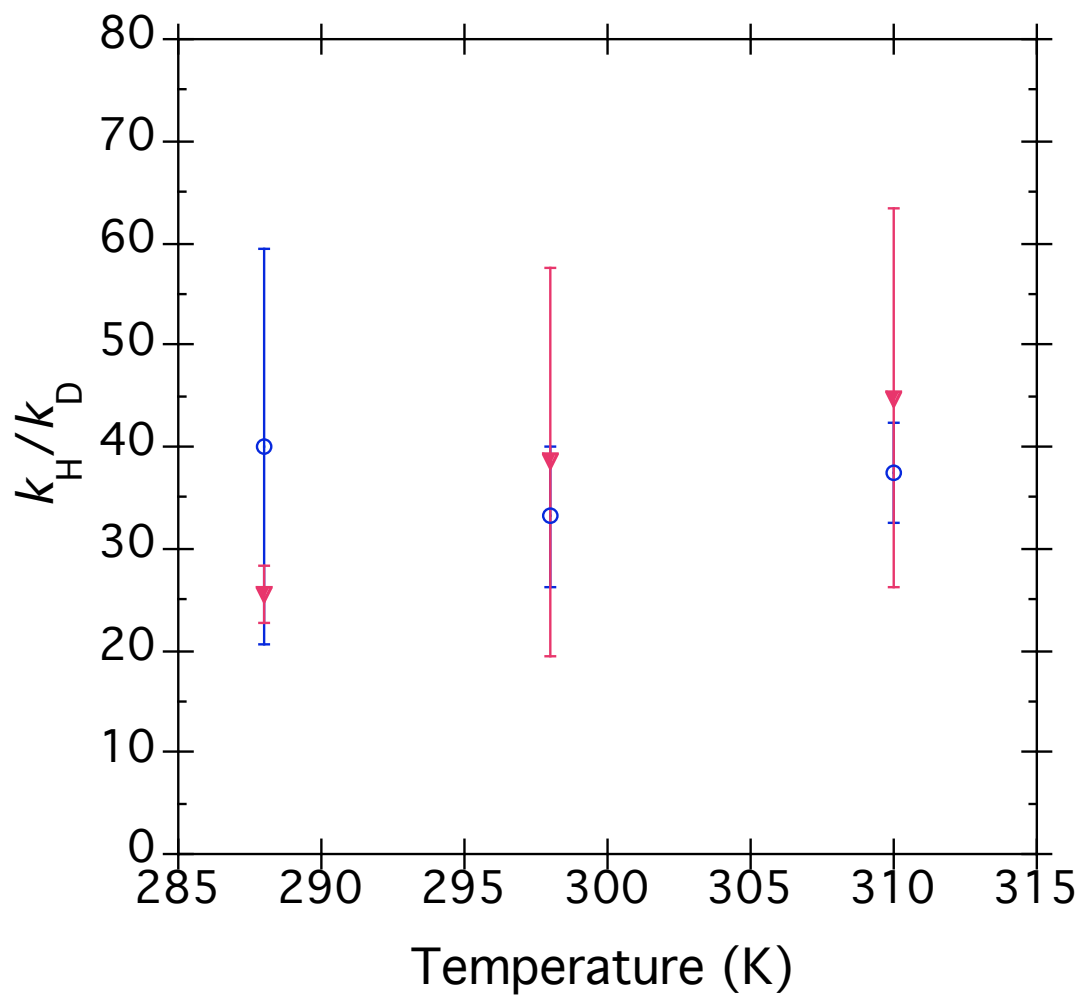


Figure 1. Apparent KIE of k_{cat} (open circles) and k_{cat}/K_M (inverted triangles) for N694G.

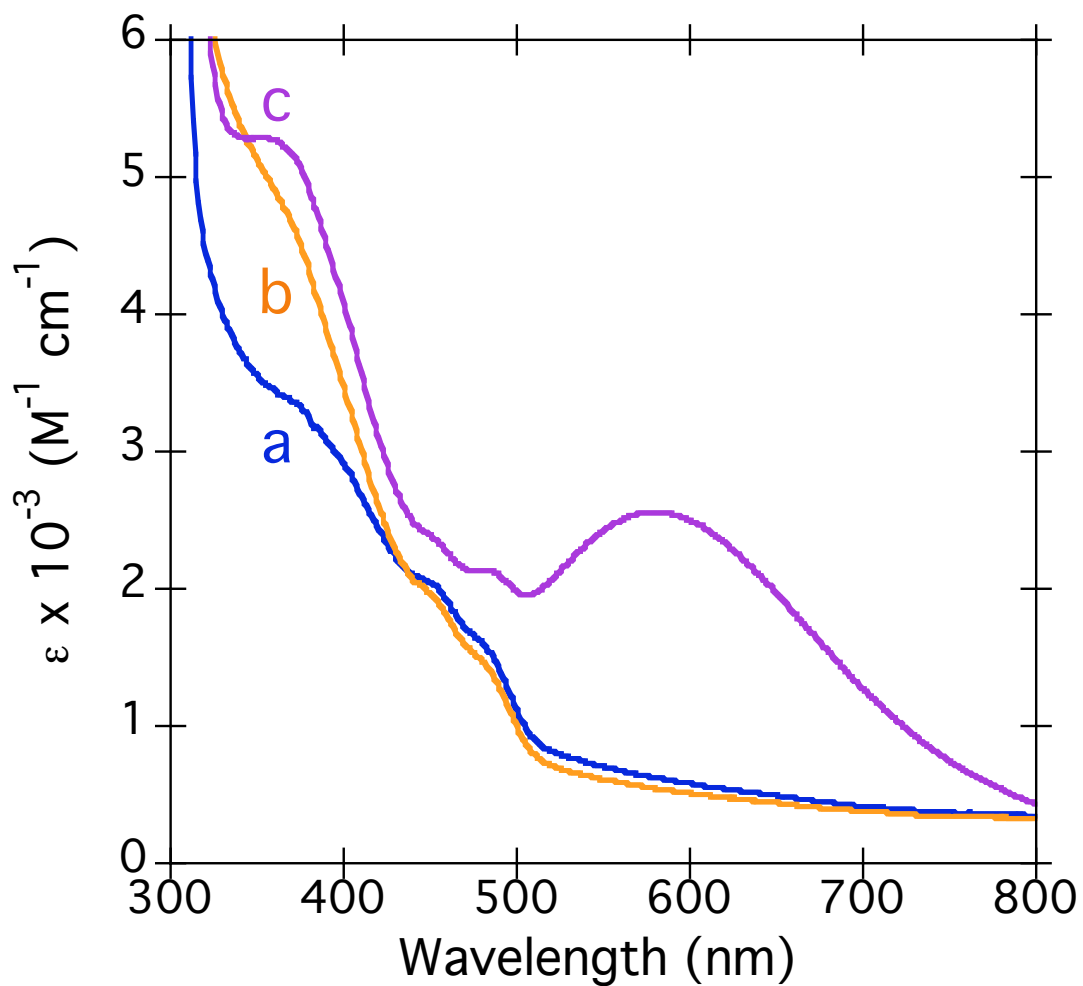


Figure 2. UV-vis absorbance spectra of a) ferrous N694G, b) yellow N694G, and c) purple N694G (180 μM) at 4 $^{\circ}\text{C}$

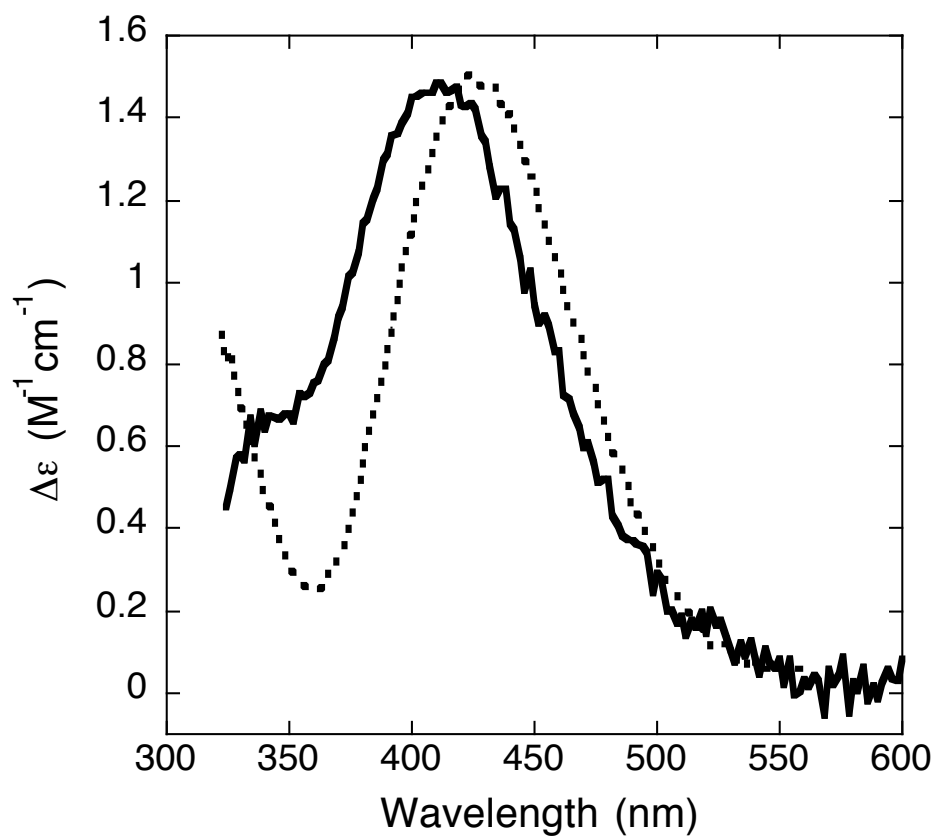


Figure 3. CD Spectra of yellow WT sLO-1 (200 μ M) (dashed line) and yellow N694G (150 μ M) (solid line) at 4 °C. Samples were corrected for iron content.

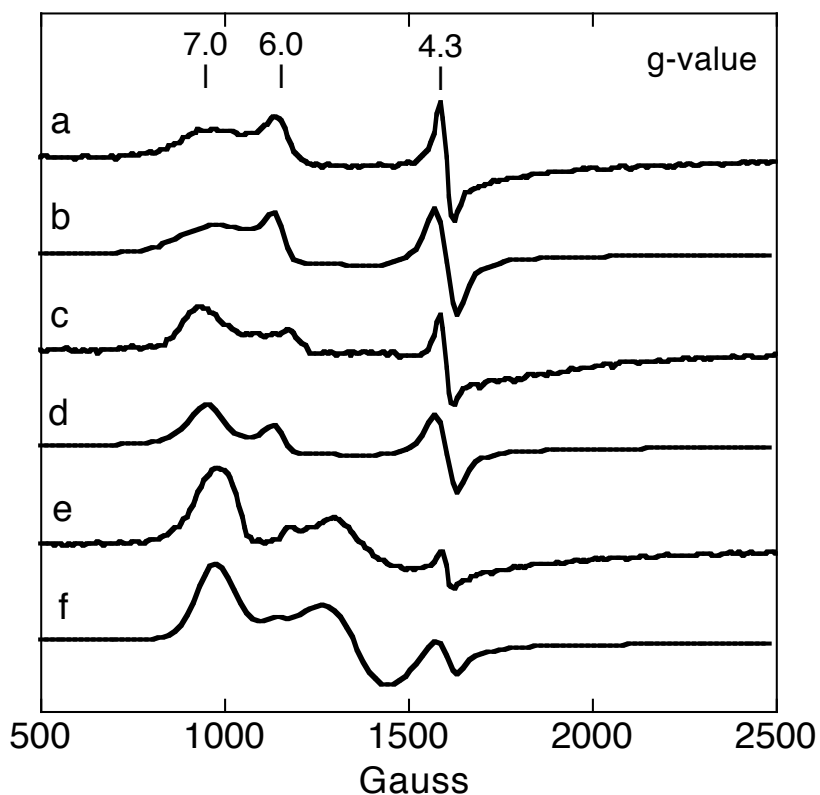


Figure 4. EPR spectra of the N694G: a) Yellow form, 0.1 M Borate, pH 9.2; b) Simulation with $64 \pm 6\%$ intermediate rhombicity, $28 \pm 3\%$ axial, and $8 \pm 2\%$ rhombic; c) Yellow form, 0.1 M Tris, pH 7.2; d) Simulation with $76 \pm 7\%$ intermediate rhombicity, $18 \pm 2\%$ axial, and $6 \pm 2\%$ rhombic; e) Purple form, 0.1 M Borate, pH 9.2; f) Simulation with $96 \pm 2\%$ intermediate rhombicity, $2 \pm 1\%$ axial, and $2 \pm 1\%$ rhombic. Experimental parameters for all spectra: Concentration = $150 \mu\text{M}$ Fe(III), Ionic strength = 0.1 M, Temperature = 4 K, field set = 4000 G, scan range = 8 kG, time const. = 82 ms, mod. Amplitude = 8 G, microwave power = $200 \mu\text{W}$, freq. = 9.63 GHz, gain = 60.



Figure 5. Stereo view of WT sLO-1 (1F8N, red) and N694G (1Y4K, blue), superimposed in the area of the active site. For WT sLO-1 (1F8N), double conformation of the His499 is shown. The residues are named according to the N694G structure.

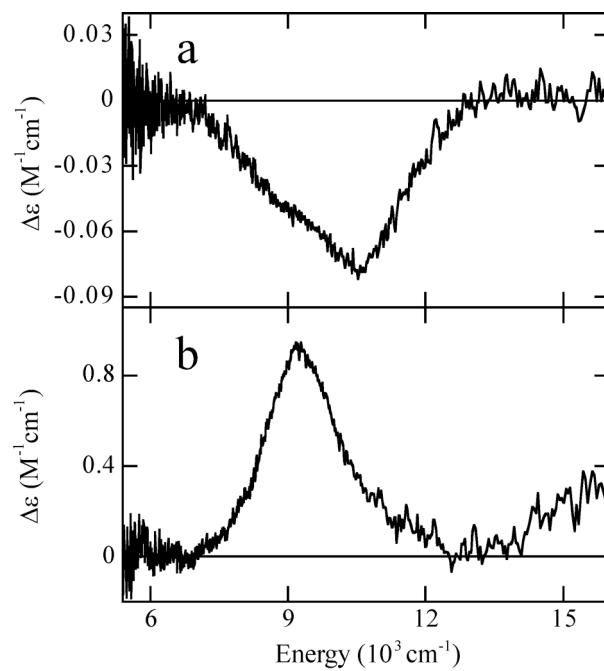


Figure 6. a) The 278 K near-infrared CD spectrum and b) the 5K, 7T near-infrared MCD spectrum of N694G.

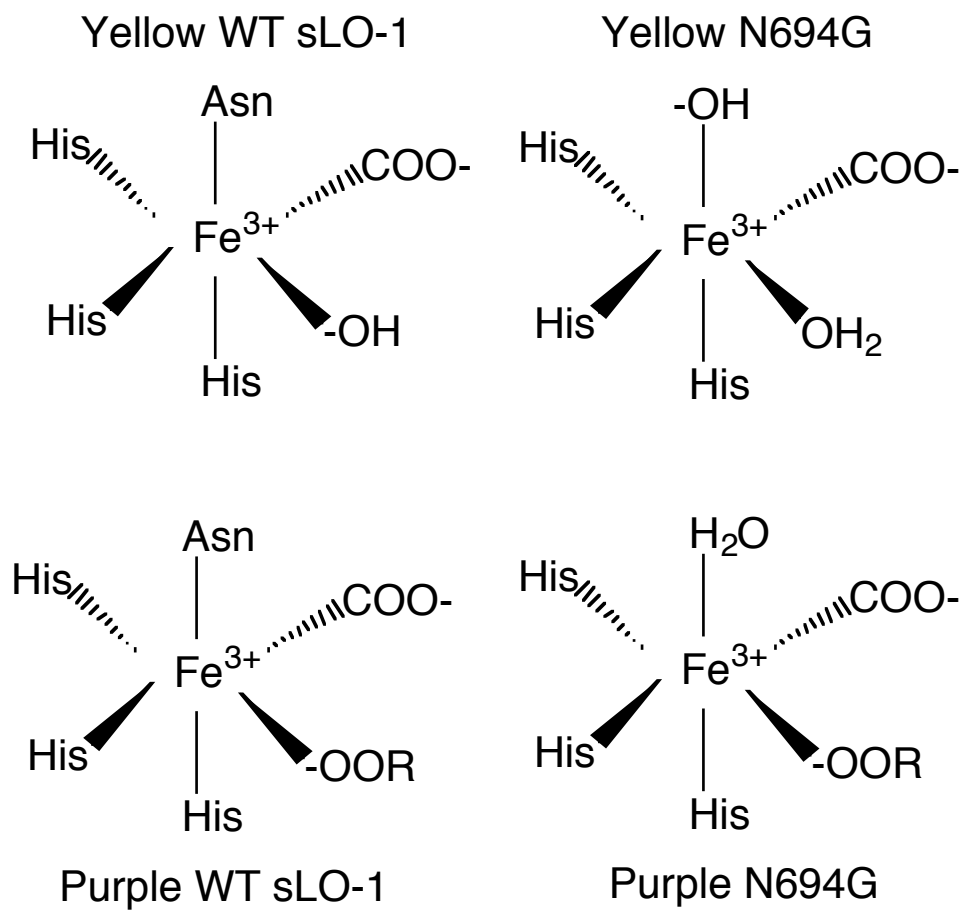


Figure 7. Proposed coordination geometry of the Fe(III) center for WT sLO-1 and N694G.

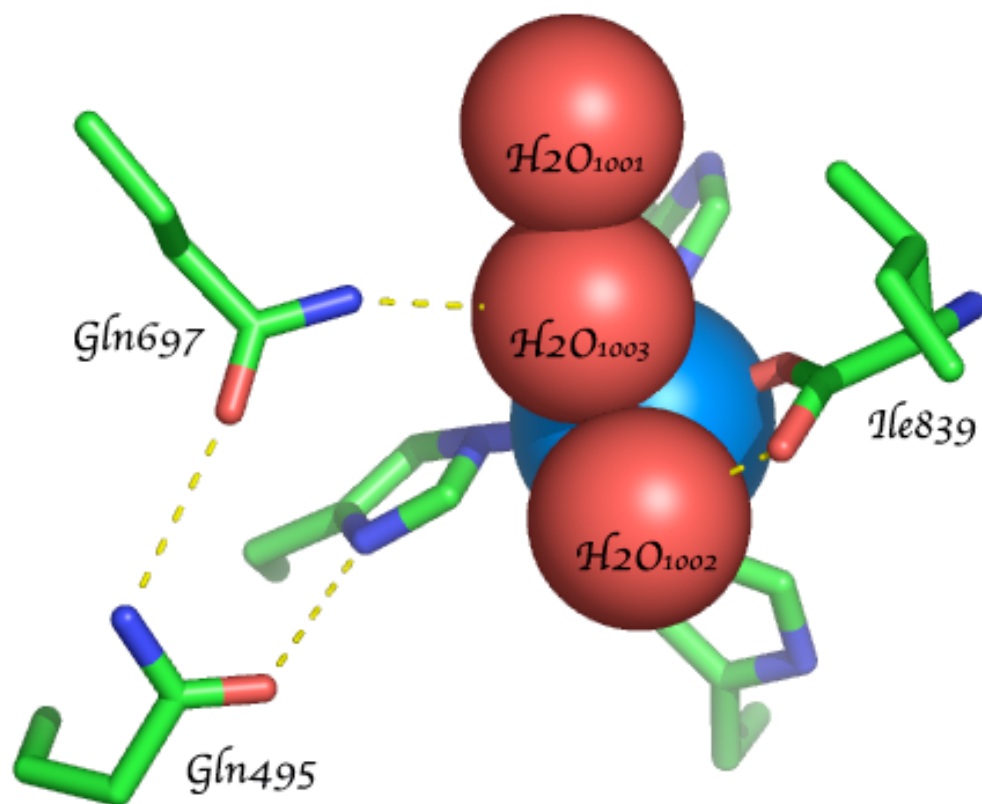


Figure 8. Coordination geometry of the Fe(II) center of N694G. Bonds between the iron and first sphere ligands are drawn as solid lines and hydrogen bonding interactions are drawn as dashed lines between Water1003, Gln697, Gln495 and His499. Oxygen atoms are drawn in red, nitrogens in blue, carbons in green, and the iron atom in cyan.