SUPPORTING INFORMATION

Structural and biochemical characterization of the early and late enzymes in the lignin β-aryl ether cleavage pathway from *Sphingobium* sp SYK-6

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Running title: Structural studies of β-aryl ether bond cleaving enzymes

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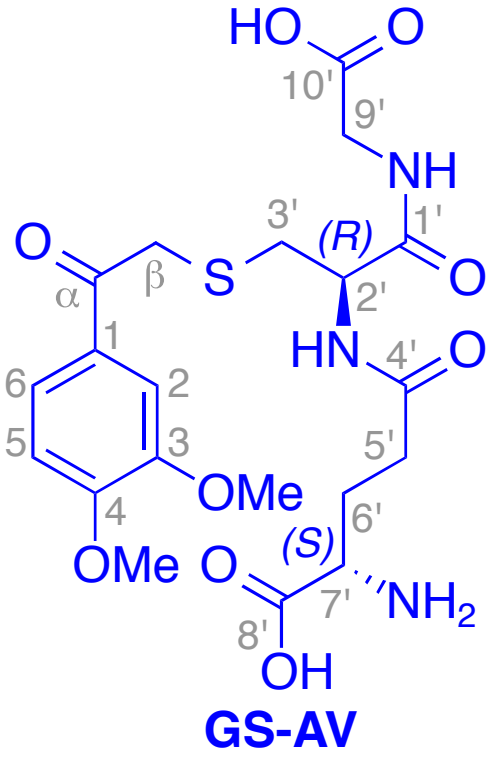
Supporting Figure 1



Supporting Figure 1 - a) Structure of the GS-HPV analog substrate, GS-AV, which was used in the crystallization of LigG.

Supporting Figure 2

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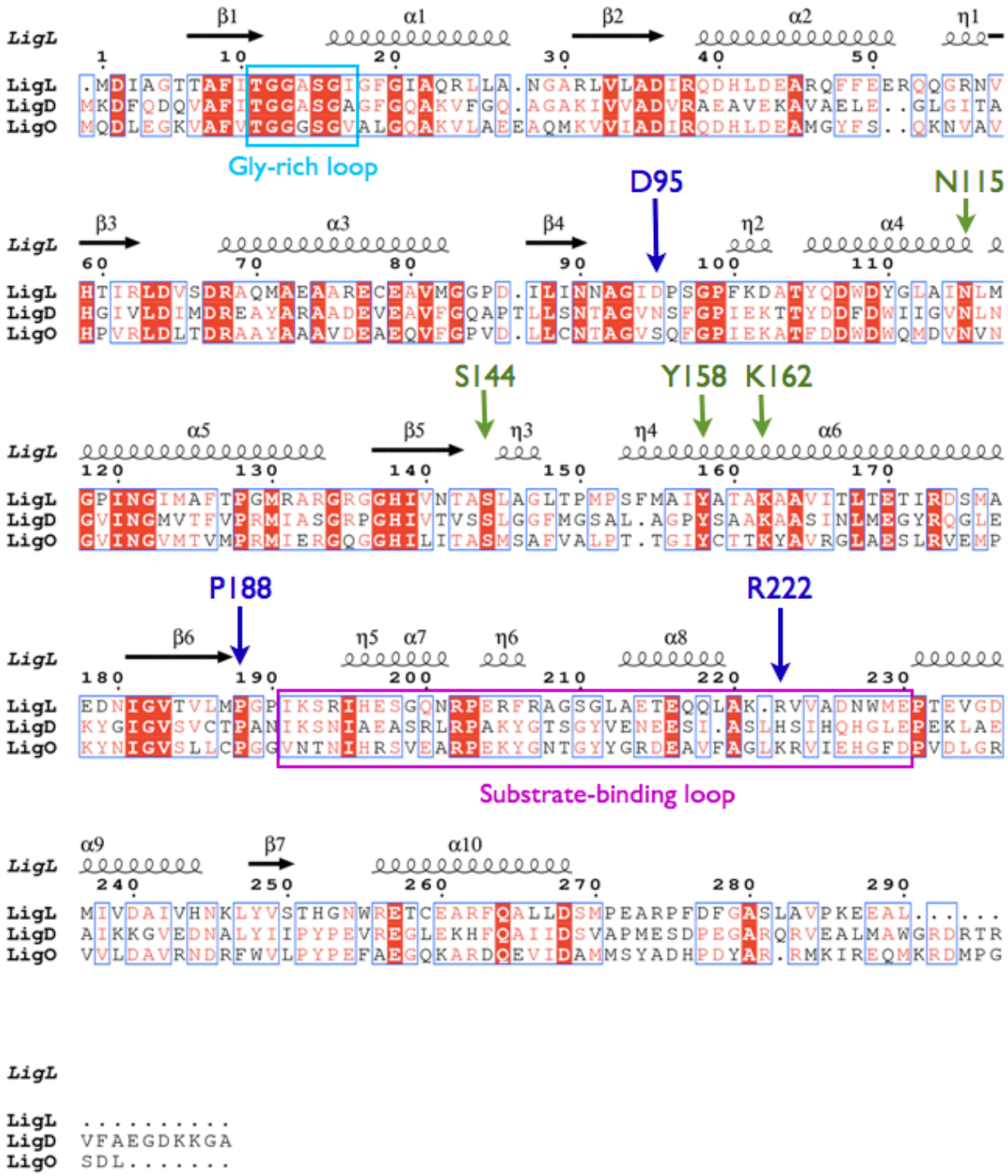
Supporting Figure 2 - Gene construct sequence for pCPD-LigL that makes use of the *Vibro cholera* MARTX toxin cysteine protease domain (CPD). LigD, LigO and LigG were cloned into this same vector in place of the LigL gene. The original plasmid was constructed such that a leucine was placed in between the protein of interest and CPD; upon activation by InsP6, CPD cleaves the peptide bond after this leucine. CPD is known to be promiscuous and, during protein purification, it was found that the protease cleaved at two additional leucine residues at the C-terminal end of the protein; L203 (numbering refers to the truncated CPD gene) and a leucine inside of a TEV cleavage site in the flexible spacer. Both leucines were mutated; L203 to isoleucine and the leucine in the TEV site to an alanine. These mutations eliminated the observed self-cleavage.

Color legend:

- Green: LigL gene
- Yellow: Leucine cleavage site introduced in between LigL gene and CPD module
- Cyan: CPD (L203I is indicated in bold, underlined, capitalized text)

- Magenta: Flexible linker comprised of a mutated TEV cleavage site and a *StreptII* tag (the leucine to alanine mutation is indicated with capitalized bold text)
- 10X his tag indicated by underlined, capitalized text
- Red: Stop codon

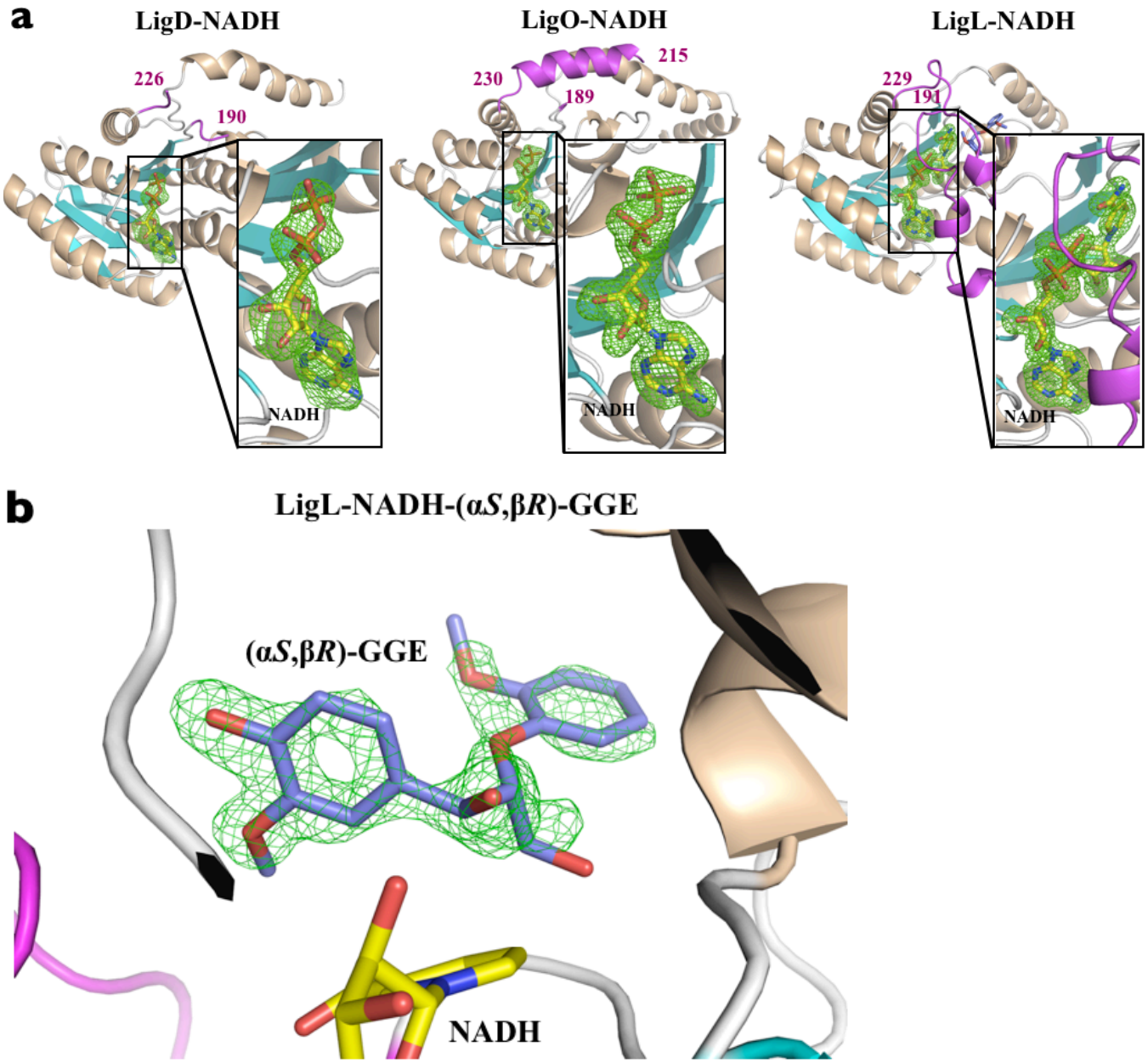
Supporting Figure 3



Supporting Figure 3 - The sequence alignment between the Cα dehydrogenases LigD, LigO and LigL shows an identity between LigD-LigO, LigD-LigL and LigO-LigL of 40%, 38% and 37% respectively. The Glycine-rich loop consensus sequences (cyan box) located at N-terminal region $_{11}$TGXXXGXG/A$_{18}$ observed in all SDR family members are in contact with pyro-

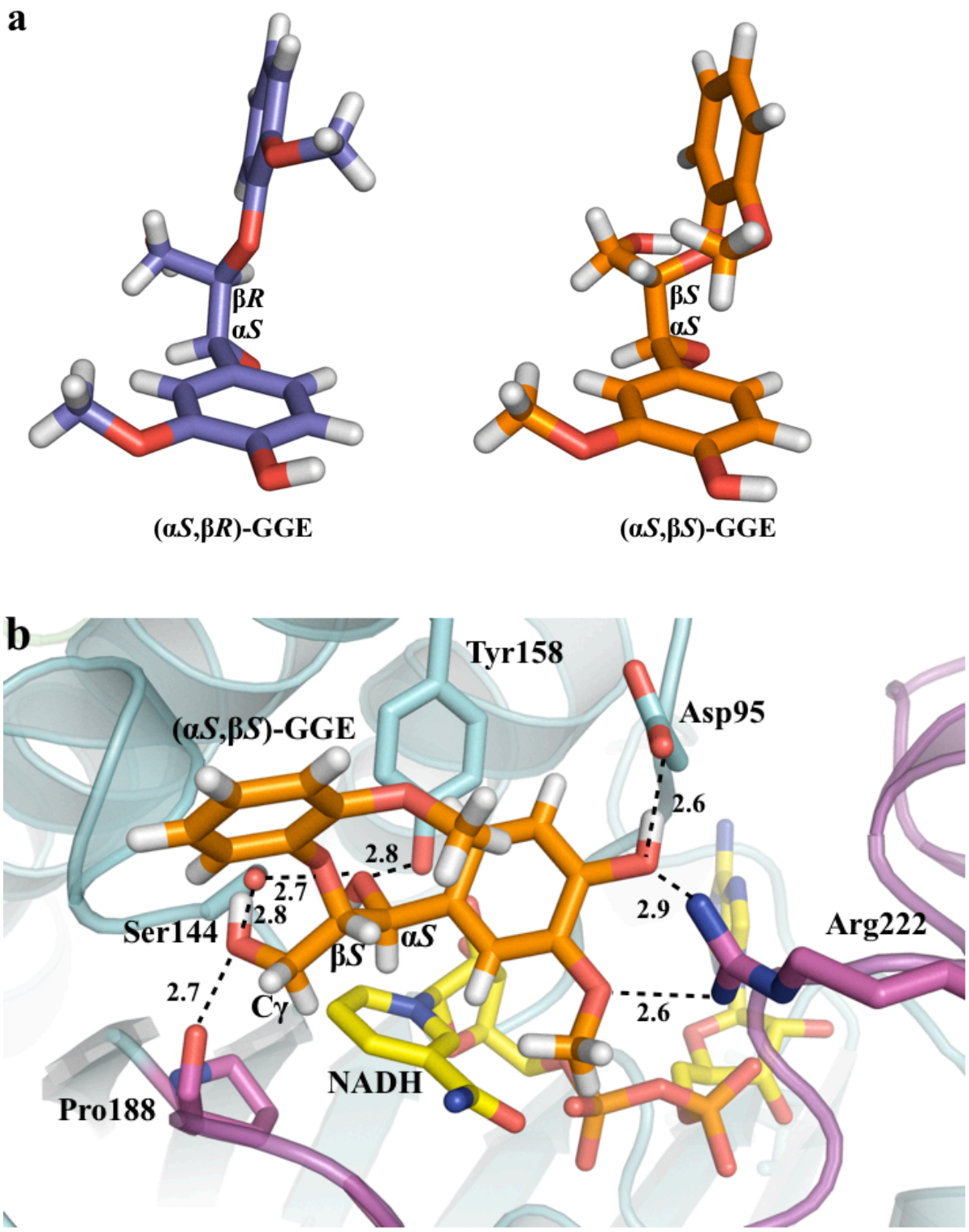
phosphate group of NADH cosubstrate. Green arrows indicate the catalytic tetrad of SDR members N115-S144-Y158-K162. Blue arrows indicate the residues D-95, P-188 and R-222 of LigL that make direct contacts with GGE substrate. The most sequence-divergent region is the substrate-binding loop shown inside the magenta box.

Supporting Figure 4



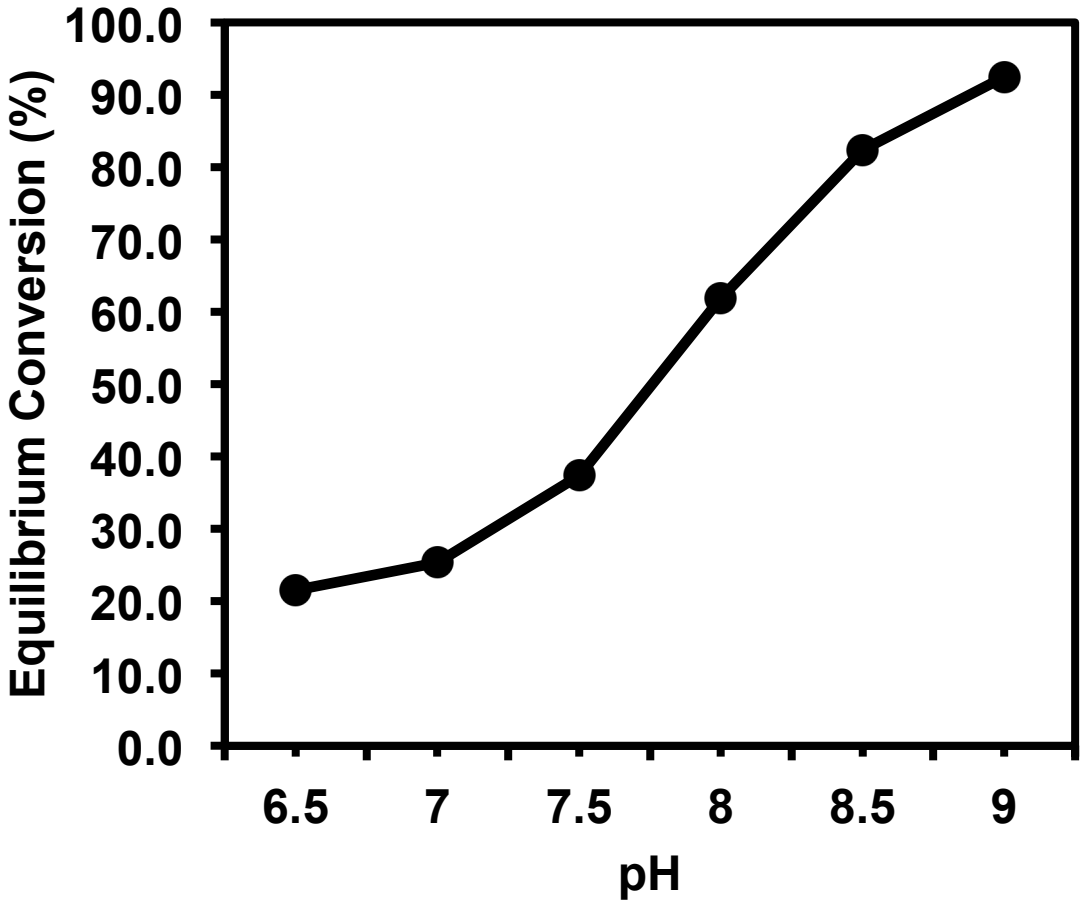
Supporting Figure 4 - a) A mF_o-DF_c electron density map omitting the NADH nucleotide molecule contoured at 3.0 σ is shown in green. The adenine part of cosubstrate was defined on the electron density maps whereas the nicotinamide portion is missing from LigD-NADH and LigO-NADH structures. **b)** The ternary complex structure of LigL-NADH-(α*S*,β*R*)-GGE show a clear mF_o-DF_c electron density map contoured at 2.8 σ in green omitting the (α*S*,β*R*)-GGE substrate.

Supporting Figure 5



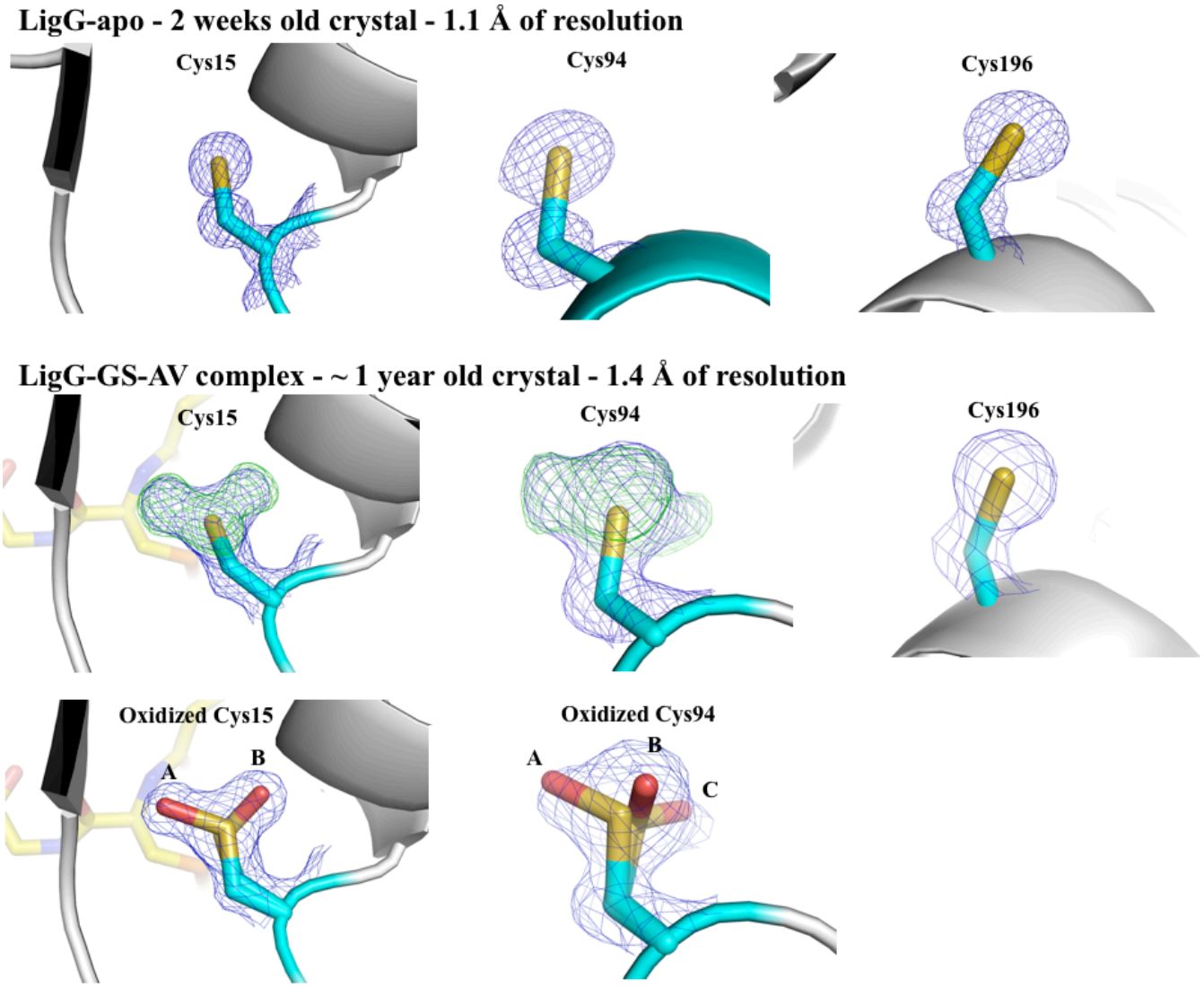
Supporting Figure 5 - a) Schematic representation of α(*S*)-configured substrates (α*S*,β*R*)-GGE and (α*S*,β*S*)-GGE showing the two alternative chiral centers, *R* and *S,* present at the Cβ of the GGE molecule. **b)** Substrate binding site for the molecular model of the LigL-NADH-(α*S*,β*S*)-GGE complex showing residues Asp95, Ser144, Tyr158, Pro188 and Arg222 that interact directly with the GGE substrate. An extra hydrogen bond between Ser144 and a hydroxyl group attached to C**γ** of (α*S*,β*S*)-GGE is observed in this model compared to the crystal structure of LigL-NADH-(α*S*,β*R*)-GGE. Broken lines represent hydrogen bonds and distances are shown in Angstroms.

Supporting Figure 6



Supporting Figure 6 - The dehydrogenation of GGE to MPHPV by LigL is strongly governed by solution pH, with equilibrium conversion reaching 90% at pH 9 but only 25% at pH 7. A phosphate buffer was used for pH 6.5 through 7.5, and a Tris buffer was used for pH 8 through 9.

Supporting Figure 7



Supporting Figure 7 - A LigG-apo $2mF_o$-DF_c electron density map contoured at 1.0 σ (blue) from a 2-weeks old crystal shows no additional density around the sulfur atoms of Cys15 (catalytic residue), Cys94 and Cys196. Approximately 1-year old crystals of apo-LigG were used for soaking experiments using GS-AV ligand. These older crystals showed clear additional mF_o-DF_c electron density in maps contoured at 3.0 σ (green) around the sulfur atoms of Cys15 and Cys94 indicating the oxidation of these residues, but not for Cys196. Two alternative conformation (A and B) for oxidized Cys15 and three

alternative conformations (A, B and C) for oxidized Cys94 were modeled in the final LigG-GS-AV complex, and are shown with the final $2mF_o$-DF_c electron density map contoured at 1.0 σ (blue).