THE PENNSYLVANIA STATE UNIVERSITY

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Structural and Functional Analysis of Two Novel Iron- and Oxygen-Dependent Halogenases

ADAM WERTZ
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Reviewed and approved* by the following:

Amie Boal
Professor of Biochemistry
Thesis Supervisor

David Gilmour
Professor of Biochemistry
Honors Advisor

* Electronic approvals are on file.

ABSTRACT

In this thesis, we seek to gain a better understanding of structure and mechanism for halogenases that target sp3-hybridized carbons. Halogenation is important in synthesis and in the properties of pharmaceuticals and agrochemicals. Enzymatic halogenation provides a cheap and safe way to perform this highly desired chemistry. Here we explore the properties of two recently reported enzymatic halogenases. We performed an analysis of substrate binding in a newly discovered WelO5 homolog, reported to be active against a synthetically tractable substrate analog. I cloned and purified the enzyme to enable electron paramagnetic resonance analysis of its interaction with the synthetic substrate analog. While we did not initially detect a binding event, future work will explore improvements to sample preparation to enhance any interaction that might exist. We also performed a bioinformatic analysis of another newly reported halogenase family and generated homology models to make predictiosn about substrate specificity. The goal of this work is to identify new halogenases for structural and biochemical study.

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ACKNOWLEDGEMENTS

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Also, I would like to thank Dr. Jeffrey Slater a post-doc in the Bollinger-Krebs lab who acted as a mentor to me. He helped me through my experiments regarding Wel_05 and taught me many valuable lessons within the lab.

Introduction

Halogen substituents are important functional groups in synthetic and naturally-occurring compounds, both for their inherent properties and as good leaving groups in synthetic intermediates. Many pharmaceuticals and industrially-relevant agricultural chemicals contain at least one halogen and, often, this substituent is critical for the activity of the molecule a hydrogen in that same position.

Halogens offer a wide range of benefits for drug compounds. These larger size of halide groups compared to hydrogen can allow for improved fit and affinity for binding sites for macromolecular drug targets. Halogenation can also inhibit oxidative detoxification of xenobiotic compounds by P450 liver enzymes in humans. This phenomenon leads to a drug that can be used a much lower dosage in patients.

While halogens are broadly useful, they are challenging to install, particularly at late stages of chemical or biological synthesis. chemical halogenation, poor atom economy is observed, with toxic byproducts and left-over halogen reactants that must be handled. However, biological systems have evolved highly efficient selective enzymatic halogen installation systems, termed halogenases, that use environmentally friendly halide salts as reactants. In principle, biologically derived halogenases can give the same end product as chemical halogenation methods but do so in a way that is biologically safe and relatively efficient.

Recently, a family of enzymes that catalyze halogenation of inactivated sp3-hybridized C-H bonds was discovered.

To perform the complex chemistry required to make these strong halogen-to-carbon bonds, these enzymes utilize iron(II), oxygen, and a 2-oxo-glutarate (2OG) as cofactors or cosubstrates. These halogenases are part of a larger enzyme superfamily, termed Fe(II)- and 2OG-dependent (Fe/2OG) oxygenases, that can functionalize inactivated C-H bonds in a variety of ways. Known reaction outcomes include hydroxylation, 1,2-dehydrogenation, 1,3/5-dehydrogenation, and stereo inversion. Hydroxylation

is by far the most common chemistry enabled in this superfamily, and it is often observed as a side reaction in halogenases, particularly when the substrate is structurally altered (Bollinger et al.).

All Fe/2OG enzymes use a conserved mechanism of reaction with substrate. Reaction of a 2OG-coordinated Fe(II) cofactor with O2 yields a highly reactive Fe(IV)-oxo (ferryl) intermediate that is capable of abstracting an H-atom from aliphatic C-H bonds on substrates. This step yields a substrate C• intermediate, which can then undergo further functionalization. In hydroxylases, the substrate radical couples to the O2-derived -OH group coordinated to the iron cofactor. In the halogenases, a conserved substitution in a common metal binding motif (D/E-X-H…H in the hydroxylases becomes A/G-X-H…H in the halogenases) opens a coordination position for a halide ligand. This halide instead couples to the substrate radical. A key question regarding Fe/2OG halogenase reactivity is which features of the enzymes structure promote halide transfer to substrate and suppress -OH transfer after ferryl-mediated substrate activation.

To address this question, we hypothesize that the structure of the ferryl intermediate differs in halogenases and hydroxylases. To test this idea, we have developed a mimic of the normally fleeting and reactive ferryl intermediate.

Fe(IV) used in Fe/2OG enzymes for reaction with substrate. A mimic of the ferryl intermediate has been developed in which vanadium (IV) is substituted for iron(IV), yielding a complex that is structurally very similar to the reactive form of Fe/2OG enzymes but with indefinite stability and favorable properties for spectroscopic analysis.

In this thesis, I will investigate these questions in two newly identified Fe/2OG halogenases. My systems of interest include Wel05_2, which targets a complex natural product but was recently shown to be active against a synthetically accessible substrate analog, and homologs of BesD, a newly discovered halogenase that operates on a simple amino acid substrate, L-lysine.

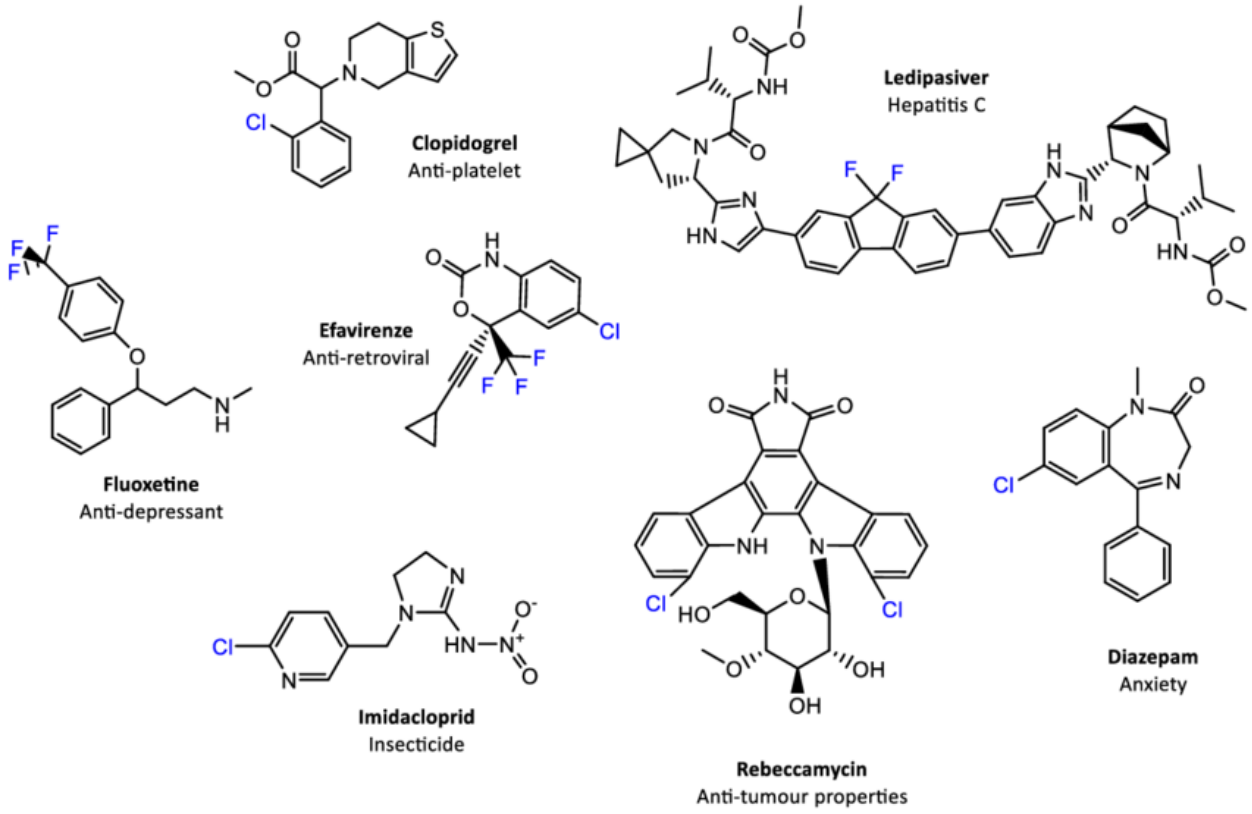

Figure 1. Examples of different halogen substituents in drugs, bioactive natural products, and agriculturally relevant compounds (public domain).

Electron paramagnetic resonance spectroscopy

The aforementioned vanadyl EPR-active ferryl mimic was used to design a simple assay for substrate binding that we could use in our initial assessments of novel halogenases and their utility in studying the mechanism of Fe/2OG-mediated halogenation. In the well-characterized Fe/2OG halogenase, SyrB2, we have shown that vanadyl substitution gives rise to a characteristic spectrum. And addition of substrate, SyrB1, changes certain features, indicating close interaction between substrate and the intermediate mimic.

Electron paramagnetic resonance spectroscopy (EPR) probes interactions between unpaired electrons and an externally-applied magnetic field.

At certain field strengths, the electrons are excited, and their spin state changes, which gives rise to an absorption feature. This approach can be used to obtain detailed information about the environment of unpaired spins in organic radicals or transition metal complexes. Here, we will use EPR to test whether substrate can perturb the spectrum of vanadyl-substituted halogenases as a first step to understanding structure-function relationships in key intermediate states of catalysis in these enzymes.

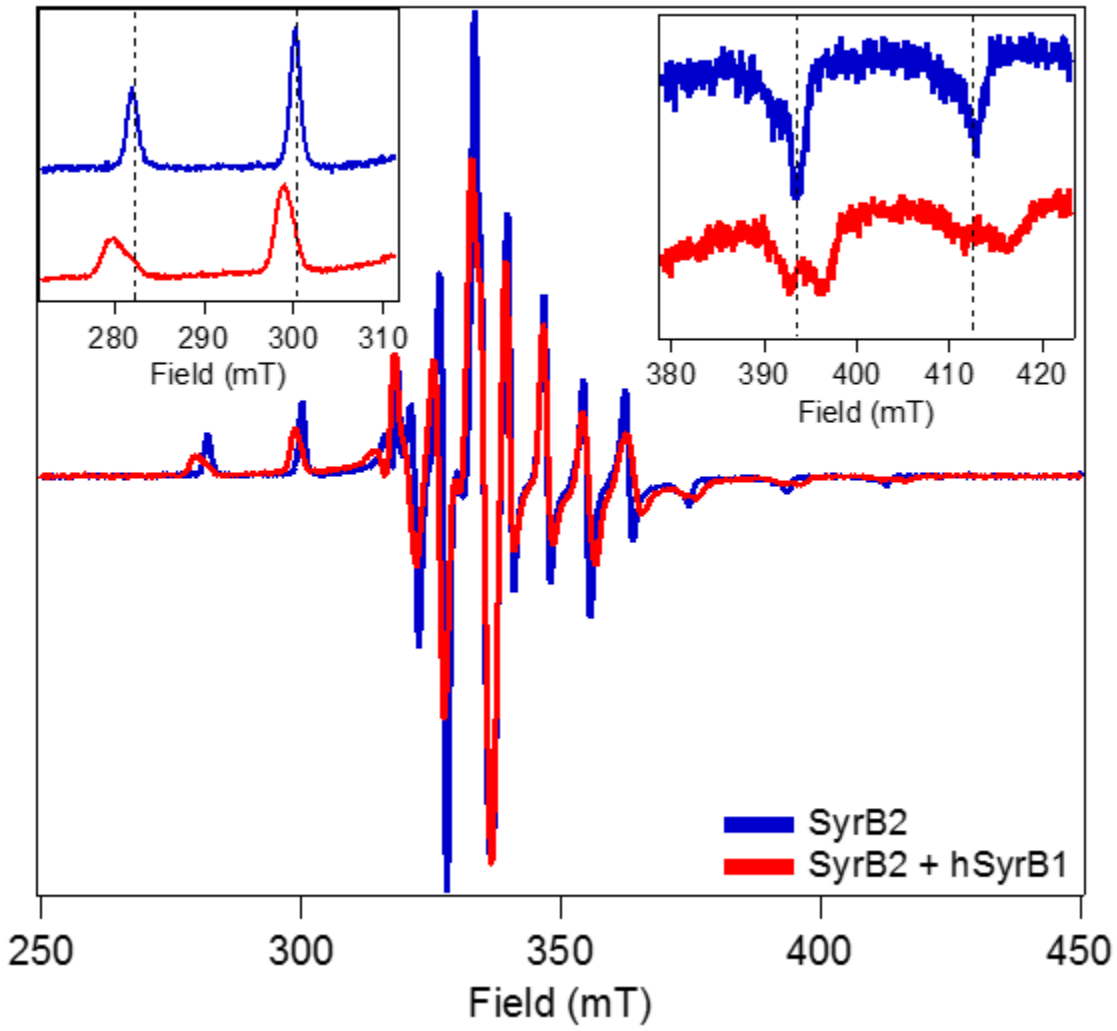

Figure 2. Example EPR spectrum for vanadyl-substituted Fe/2OG halogenase SyrB2 in the absence (blue trace) and presence (red trace) of its substrate, hSyrB1. Insets show zoomed-in views of regions of the spectrum that change the most when substrate is bound.

Bioinformatic analysis to identify new halogenases

All Fe/2OG halogenases identified to date have certain limitations that prevent us from thoroughly investigating our hypothesis about an alternative structure for the ferryl intermediate. However, recent developments in bioinformatic tools used to analyze unannotated protein sequences in publicly available databases have provided new opportunities to identify novel enzymes. Here we apply these approaches to identification of new Fe/2OG halogenases that might be similar to recently reported amino acyl halogenase, BesD. This work builds on a similar effort published last year by Neugebauer et. al. but updates the analysis to include recently deposited protein sequences. Our approach involves use of the Enzyme Function Initiative (EFI) enzyme similarity and genome neighbor tools. These resources can be used to calculate the sequence similarity between any two protein sequences in a given dataset. Using these relationships, one can infer different functional properties for a group of uncharacterized enzymes.

We generated sequence similarity networks (SSNs) of BesD homologs and used these as pictoral representations of the similarity between different clusters of enzymes to identify new enzymes for future study. A representative generic SSN is shown in Figure 3. In this example, each box represents a sequence of a protein in the network (or a set of very similar sequences). The lines connecting nodes represent the level of sequence similarity to all other nodes in the network. SSNs are filtered a certain threshold (alignment score) to yield isofunctional clusters of sequences.

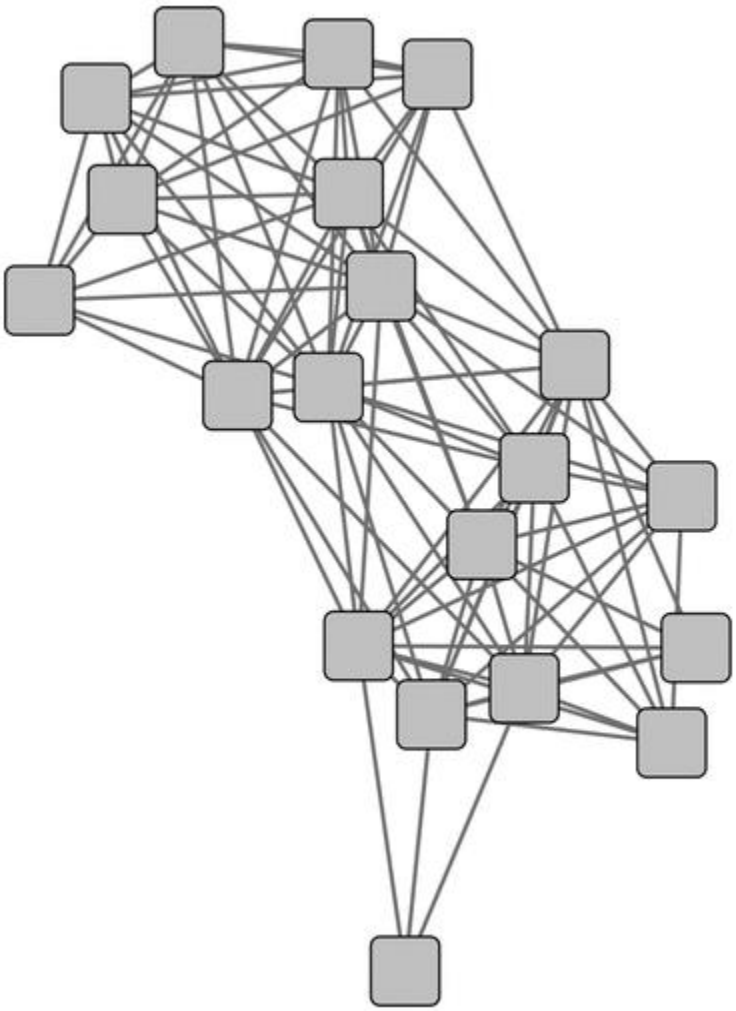

Figure 3. Example of SSN cluster for with different nodes. (Gerlt 2017).

Experimental Methods

WelO5_2 overexpression and protein purification

The DNA sequence encoding halogenase Wel_O5_2 was synthesized (Hayashi et a.) and cloned into a pET-28 DNA vector for overexpression by using restriction digest and ligation of the resulting DNA fragments. Two separate restriction digests were prepared to generate linear fragments of the

WelO5_2 gene and the pET-28a vector. A solution of 1 µg pET-28 vector was prepared at a concentration of 90.1 µg/µL in 1X CutSmart buffer (New England Biolabs) The restriction enzymes XhoI and NdeI were added to the mixture and The restriction digest was allowed to proceed for 2 hours at 37 °C.

The Wel_05* insert was resuspended at a concentration of 10 ng/µL in water at 50 °C for 20 min.l A total of100 ng insert DNA was prepared in a solution of 1X CutSmart buffer. Restriction enzymes XhoI and NdeI were added to the and incubated for 2 hours at 37 °C. For each reaction, the restriction enzymes were heat inactivated after the 2-hour incubation period.

The digested products of each reaction were ligated together using the following procedure. T4 ligase (1 µL) was added to a solution containing 37.5 ng of digested WelO5_2 insert and 50 ng of the digested pET-28 vector in 1X T4 DNA ligase bufferhe mixture was allowed to 16 °C.

The ligation products were used to transform DH5a E. coli via at 42°C.Successful transformants were selected on plates containing Luria-Bertani (LB) medium supplemented with kanamycin (50 g/mL).The plates were incubated overnight at 37 °C. The sequence of the WelO5_2/pET28a construct was verified by Sanger sequencing.

The WelO5_2 protein was overexpressed in BL21(DE3) E. coli cells, transformed as described above. Bacterial cell paste containing the WelO5_2 protein was resuspended in 20 mM HEPES at pH 7.5. The cells were disrupted by sonication and centrifuged to remove insoluble debris. The resulting supernatant was applied to a Ni-NTA affinity column. The WelO5_2 protein contains an N-terminal hexahistidine tag, which allows it to bind to the Ni(II) column. Bound proteins were eluted with a buffer containing imidazole, a competitive ligand for the Ni(II) column. The eluted protein fraction was dialyzed against 20 mM HEPES, pH 7.5, 10 mM EDTA to remove adventitiously bound metal ions. Wel_05_2 was further purified using anion exchange chromatography. The resulting purified protein fractions were collected and concentrated using a 10K MWCO centrifugal filter (Pall Corporation). The concentration of purified

Wel_05_2 was obtained by UV-visible absorbance at 280 nm. The purity of the sample was assessed by SDS-PAGE.

Analysis of substrate binding by WelO5_2

WelO5_2 substrate compound 1 (C1) was synthesized by our collaborator, Dr. Bo Wang. The structure of the final compound can be seen in (Figure 5.). A sample of C1 was dissolved in a solution of DMSO prior to addition to WelO5_2.

To assess whether Wel_05_2 can bind to C1, we substituted the metal binding site in WelO5_2 with an EPR-active mimic of the reactive ferryl intermediate, vanadyl. In other Fe/2OG enzymes, the EPR spectrum of vanadyl changes upon binding the substrate. The reaction mixture for EPR analysis contained .750 □M Wel_05, 10 mM C1, DMSO, .700 □M Vanadyl, 10 mM succinate, 50 mM NaCl in 20 mM HEPES, pH 7.5, .4M sucrose. Continuous wave-EPR was performed at 35 K with a modulation amplitude of 0.5mT, a microwave power of 20 dB, and frequency of 9.43529 GHz. We compared the WelO5 results to a positive control for another halogenase, SyrB2, bound to its substrate, SyrB1.

Bioinformatics analysis of amino acid targeting halogenases

To identify and analyze homologs of the L-Lys halogenase, BesD, we used the BLAST function of the Enzyme Function Initiative (EFI) Enzyme Similarity Tool to retrieve 232 sequences. We generated a sequence similarity network (SSN) for this dataset using an alignment score of 95. The SSN was visualized and analyzed in Cytoscape. For the top 10 sequence clusters in the network, we generated sequence alignments in Clustal OMEGA to verify that all contain the predicted substitution in the metal binding motif characteristic of halogenases. We also generated representative homology models for each cluster. using SWISS-MODEL. The BesD structure (PDB accession code 6NIE) was used as the template. Structure figures were generated in PyMOL (Schrödinger, LLC). All sequence clusters were

further analyzed using the EFI Genome Neighbor Tool (Carl R. Woese Institute for Genomic Biology) to identify patterns in neighboring genes that could help infer substrate specificity and function.

Results and Discussion

Purification of WelO5_2 and analysis of substrate binding

WelO5 is an Fe/2OG halogenase that chlorinates a complex natural product shown below in Figure 4. WelO5 was the first halogenase in this family reported to act on a free-standing small molecule substrate, allowing for structural characterization of the enzyme bound to its substrate in the reactant (Fe(II)•2OG•WelO5) form. However, the complexity of the substrate and its availability in low quantities (< 1 mg) in this system has hindered efforts to further characterize the enzyme. Additionally, it has been difficult to substitute the enzyme with vanadyl to probe the structure of intermediate states. Recently, a homolog of WelO5, called WelO5_2, was identified to have the ability to modify a simpler substrate analog, compound 1 (Figure 5), that can be produced synthetically in larger quantities. Here we describe production and purification of WelO5_2 and study of its interaction with compound 1 by EPR methods. This work will help establish whether WelO5_2 can serve as an appropriate platform for further study.

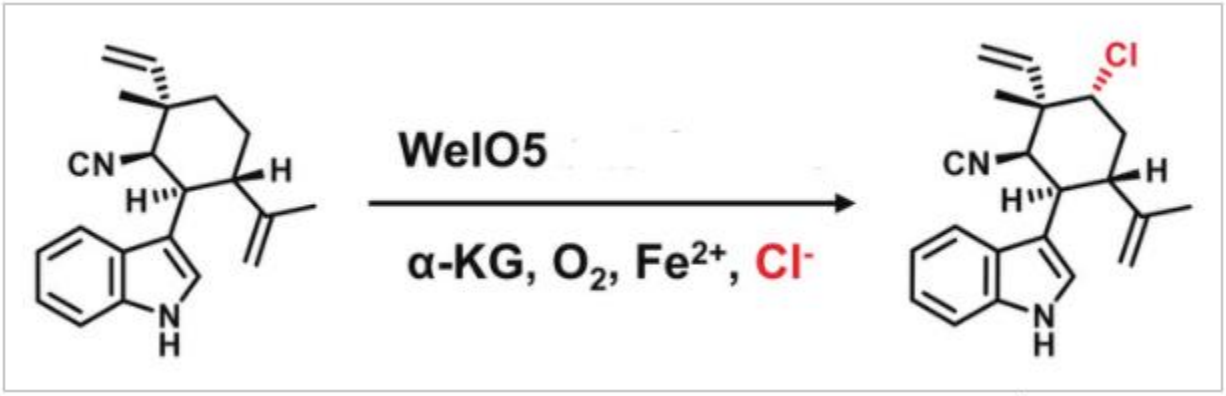

Figure 4. halogenation of an Sp3 hybridized carbon by Wel05 in the presence of chloride ion alpha-KG, oxygen and ferryl ion. Please note this is not the substrate used within the context of this experiment and is meant to give an overall idea of Wel05 halogenation (Hayashi et al.)

The gene encoding WelO5_2 was synthesized and incorporated into an overexpression vector. E. coli cells overproducing WelO5_2 were harvested and lysed. WelO5_2 was purified via affinity and anion-exchange chromatography. The purity of the resulting Wel_05 solution was assessed by SDS-PAGE gel electrophoresis (Figure 6).

Compound 1, an active substrate analog for WelO5_2 chlorination, was synthesized by our collaborator, Dr. Bo Wang using the protocol detailed in Hayashi et al.

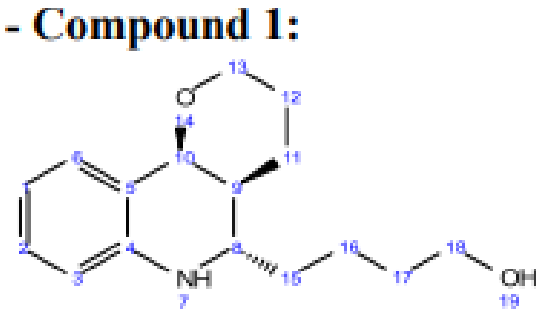

Figure 5. Compound 1 (C1) substrate for Wel_05. (Hayashi et al.)

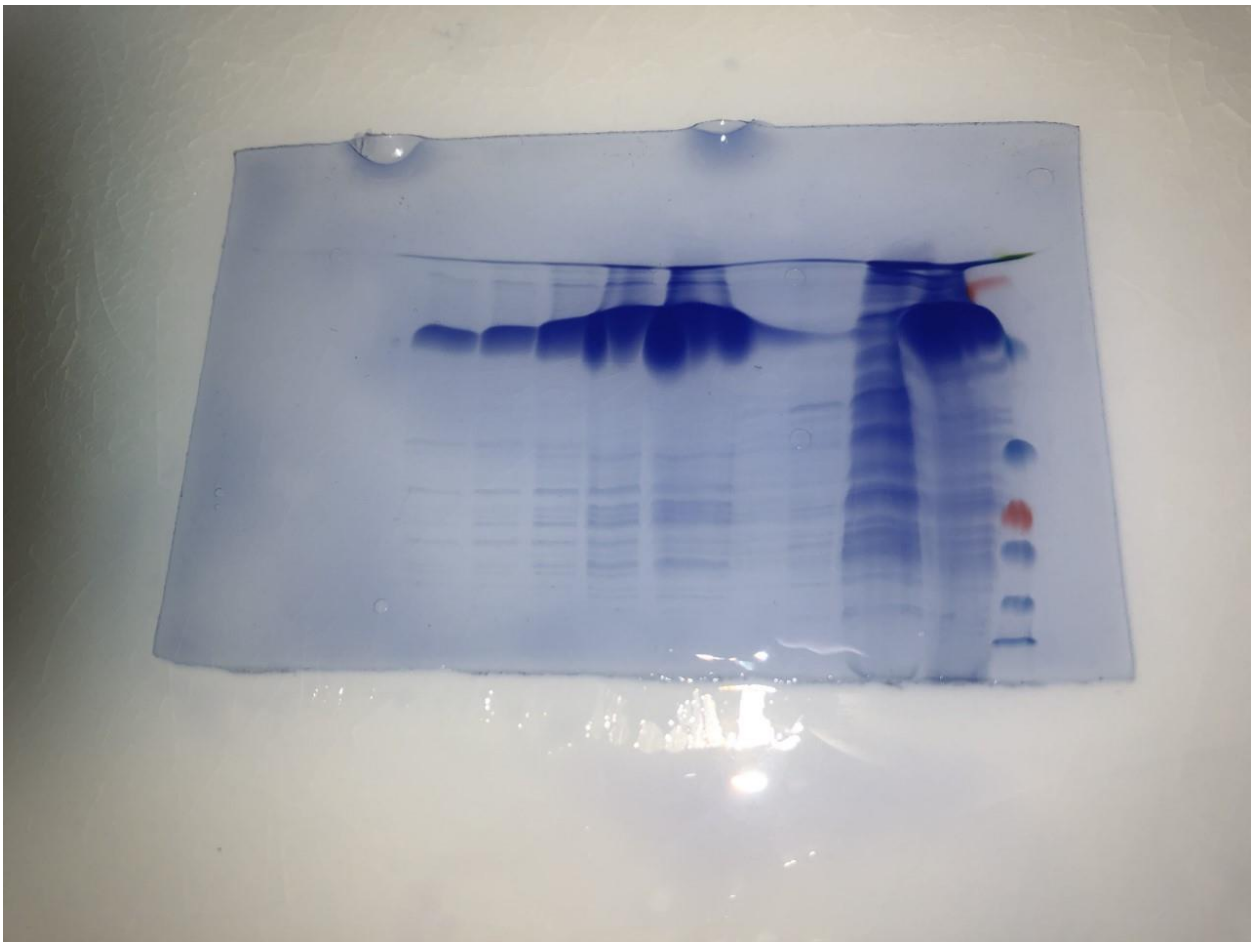

Figure 6. SDS-PAGE gel of Wel_05 enzyme Each lane contains newly made Wel_05 enzyme and final lane contains protein MW marker.

To assess the interaction between WelO5_2 and compound 1, we performed an EPR analysis of vanadyl-loaded protein with and without the substrate analog. Experimental details for sample preparation and instrument setup are described in the experimental methods section. The sample also contained the cryosolvent sucrose (4 M concentration) which we initially presumed will not affect substrate binding or enzyme function. In Figure 7, we show the results of this experiment in comparison to a control with a different halogenase, SyrB2. Unlike the results of the experiment with SyrB2, the vanadyl EPR spectrum for WelO5_2 does not change when compound 1 is added in 1:1 ratio, suggesting that the substrate analog either does not bind appreciably to the active site or it does not perturb the vanadyl cofactor sufficiently to observe spectral changes.

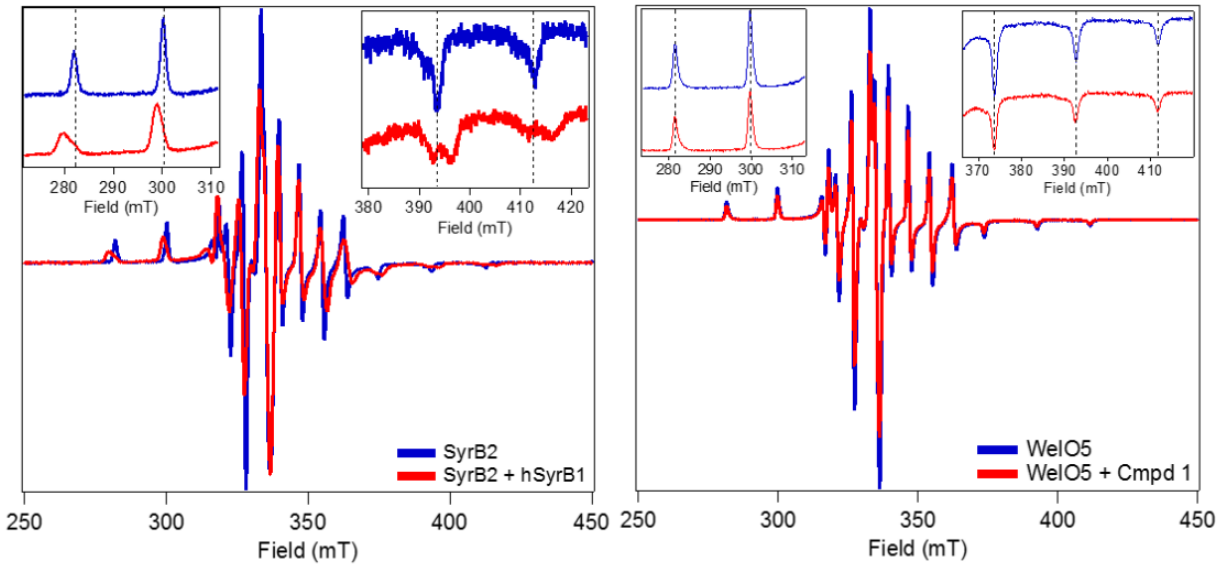

Figure 7. Positive data (left) compared to experimental Wel_05 data (right). Red lines show presence of substrate and enzyme while blue lines show presence of just enzyme without substrate present in the reaction mixture.

The next steps for this research direction involve more detailed investigation of the failure of compound 1 to perturb the WelO5_2 vanadyl EPR spectrum.

Future directions include additional purification of WelO5_2 to minimize off-target interactions between compound 1 and other impurities in the sample. We also plan to more comprehensively validate the

structure of compound 1 synthesized by our collaborator by, for example, NMR analysis. These results could be compared to results of previous NMR validation of structure in Hayashi et al.

Another potential source of error could be from the cyrosolvent, sucrose, that was used during the binding assay. While this solvent was required to allow for EPR analysis at low temperatures, it could have interfered with the binding of C1 to Wel_05. In the future we will decrease the concentration of sucrose to understand the role that this component may have played in the binding of C1 to Wel_05.

Finally, we will also mutate WelO5_2 as described in Hayashi et al. to increase the activity of the enzyme towards compound 1. Two mutations were found to afford large increases in turnover number and kcat for this reaction. These mutations were V81S and I161V. We plan to make the corresponding

mutations in our overexpression construct and repeat the experiments described here for the wild-type protein to see whether these substitutions enhance affinity for compound 1. We could also attempt to solve x-ray crystal structures of substrate complexes for all proteins to achieve our goal of determining whether this system would be appropriate for detailed study of vanadyl complexes.

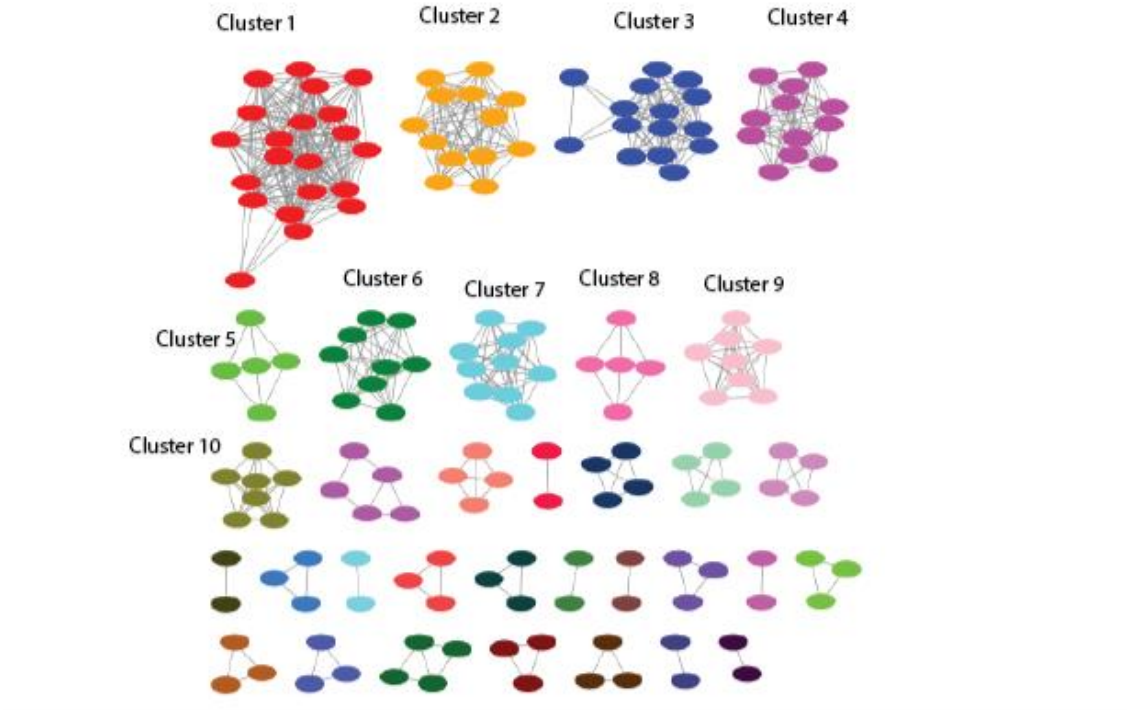

Figure 8. Cluster data for sequences related to BesD halogenase.

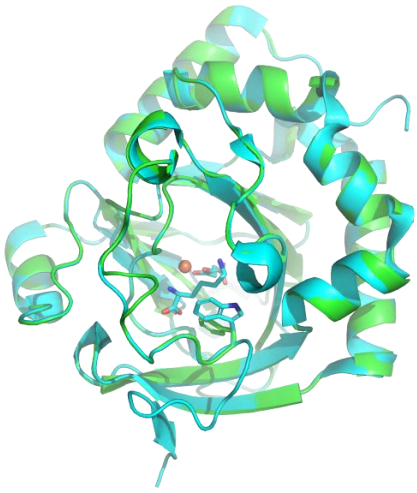

Figure 9a. BesD (blue) superimposed to cluster one representative (green). Amino acid Trp239 clearly in display with a comparison at the same position of cluster one representative.

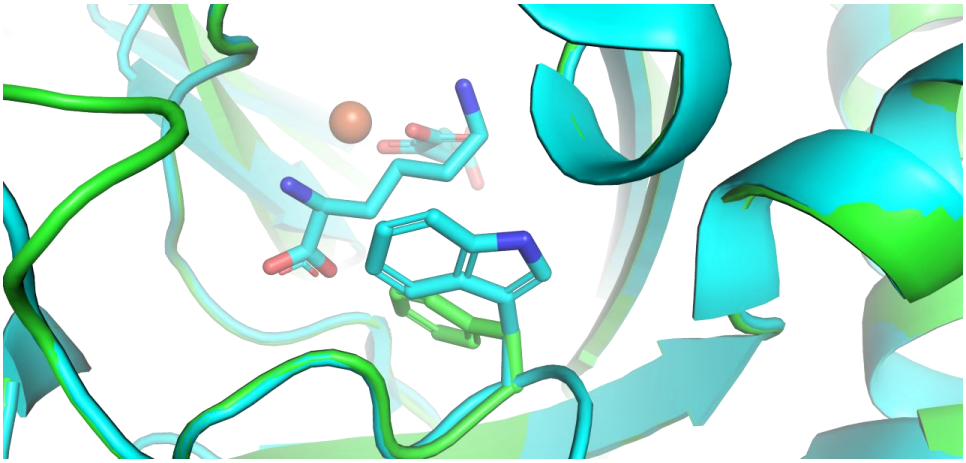

Figure 9b. Zoomed in BesD (blue) superimposed to cluster one representative (green). Amino acid Trp239 clearly in display with a comparison at the same position of cluster one representative.

Bioinformatic analysis of BesD homologs

BesD is the second example of Fe/2OG halogenease with a standalone substrate. Unlike WelO5, the substrate for this halogenase is a simple amino acid, L-lysine. BesD is also distinct from WelO5 in terms of sequence (11%sequence identity) and structure. Identification of BesD and the other halogenases that might be similar in substrate specificity offers a new and exciting range of tools for selective halogenation and biochemical study. In this section, we use the BesD sequence to identify other sequences of potential halogenases with distinct substrate specificities that might be good candidates for such studies.

BesD has been structurally characterized in complex with its substrate and analyzed initially via bioinformatics methods. The structure reveals several important substrate binding determinants, including a C-terminal Trp239 residue that is used to cap L-Lys in its binding pocket near the active site. The structure also shows that His134 and Asn219 are major players within the enzyme's second sphere interactions. His134 hydrogen-bonds to the carboxylate of L-Lys to properly orient it within the binding pocket to allow for halogenation to take place. Asn219 interacts with 2OG to help maintain its orientation

in the active site. We propose that this interaction could be important for preventing hydroxylation and favoring the halogenation product. Bioinformatic detection of BesD homologs revealed five sequence clusters containing representatives shown to modify other amino acid side chains (Figure 11). All of these homologs contain expected sequence characterisitics for Fe/2OG halogneases, including the conserved HWG motif, which differs from the pattern for hydroxylases, an HXD motif in the same position. In this section, we will update this bioinformatics analysis to include newly deposited BesD homologs and generate homology models of representative sequences to assess whether the interactions described above are conserved, allowing us to make predictions about substrate specificity for future biochemical study.

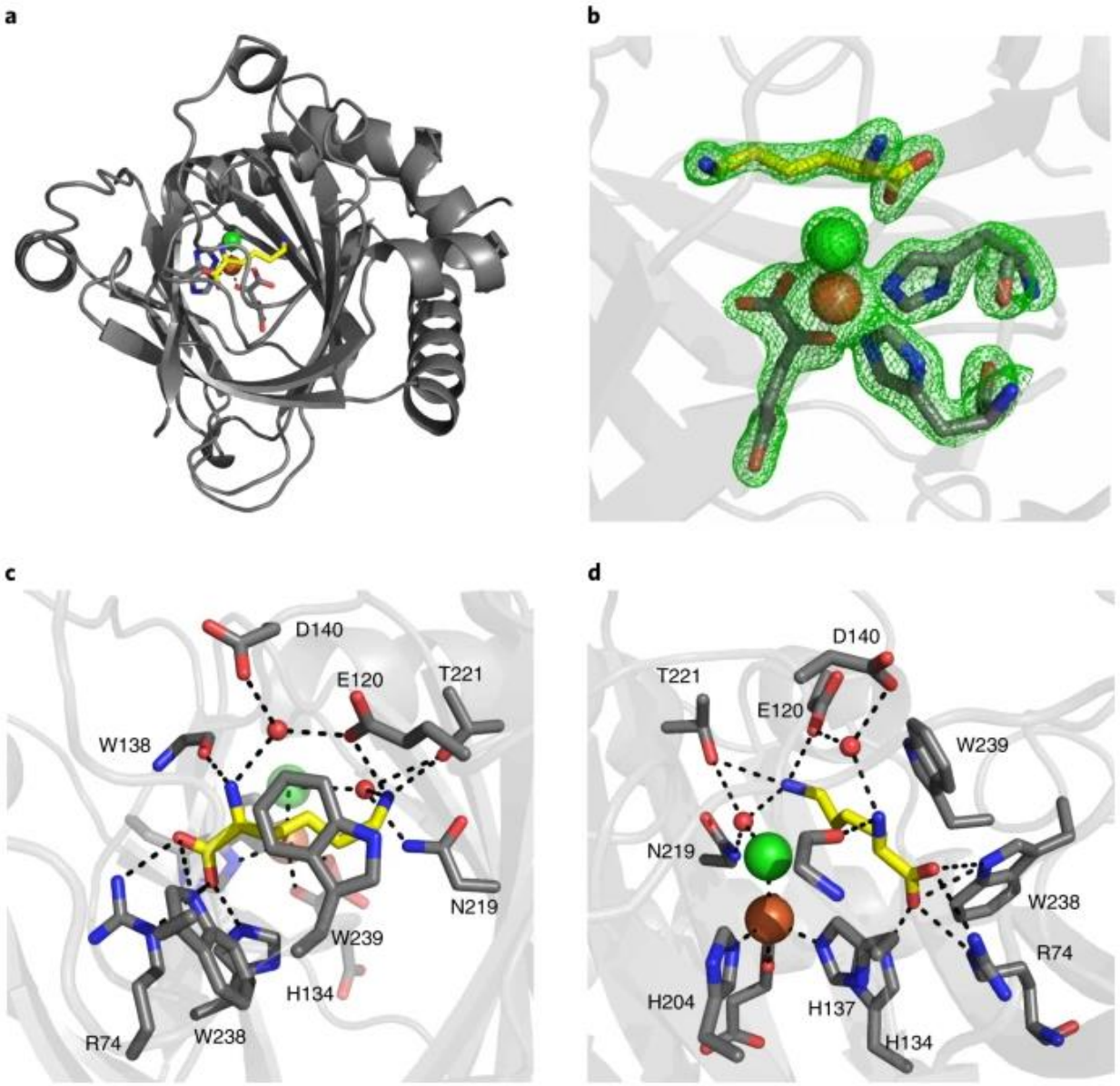

Figure 10. BesD binding lysine with residues important for substrate binding shown in stick format. (Neugebauer et al.). H-bonding interactions are shown as dashed lines. A. Folding of substrate-bound BesD B. Omit map for L-Lys (yellow) and iron-bound in complex with two histidine residues, Cl (green), iron (brown), alpha-KG. omit map was calculated by removing ligands before refinement and is contoured at ±3σ. C and D. two different images of lysine binding, Front (C) and back (D).

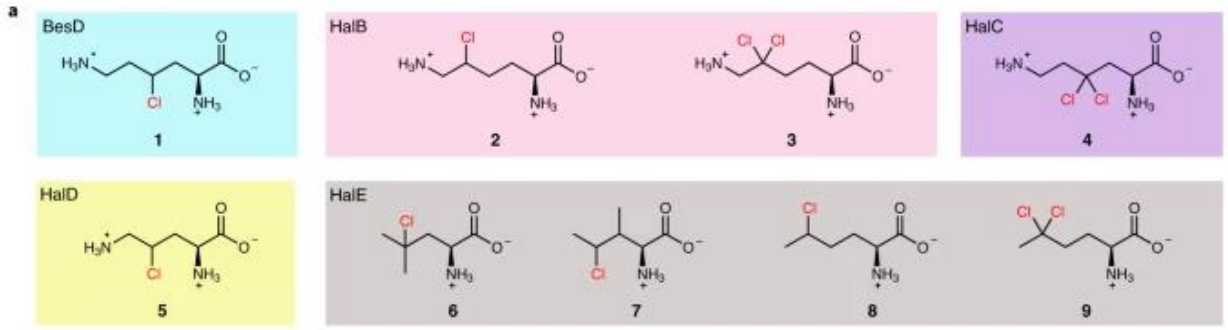

Figure 11. Different substrate preferences of BesD homologs identified bioinformatically (Neugebauer et al.).

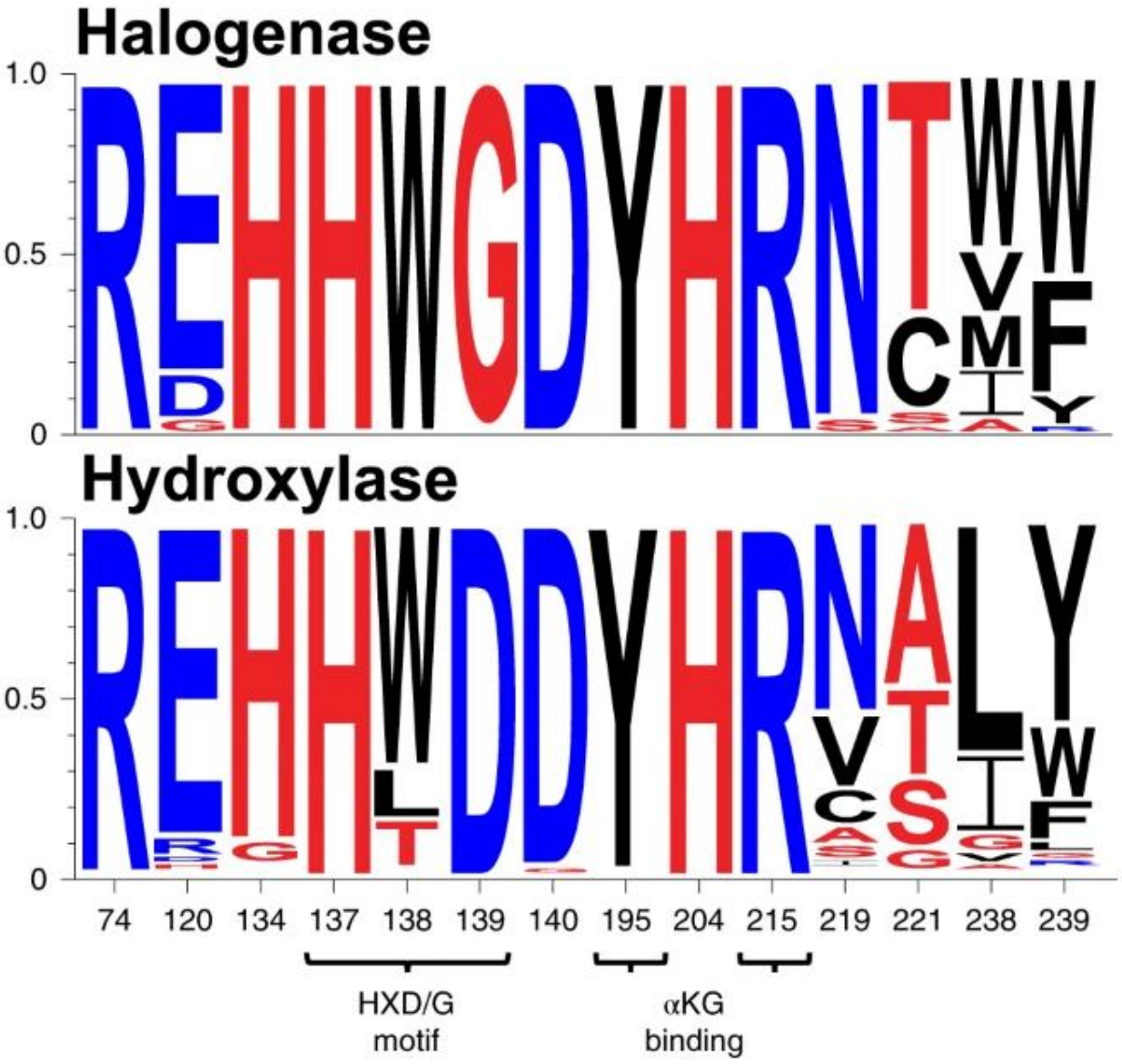

Figure 12. Sequence logo diagram for selected positions in BesD halogenase and hydroxylase homologs. (Neugebauer et al.).

To update our pool of BesD homologs, we queried the NCBI BLAST database using the sequence of BesD, retrieving XX sequences. We generated an SSN of this sequence pool using an alignment score of XX, yielding a network containing ~30 different sequence clusters. We subjected this SSN to genome neighbor analysis, which showed that each cluster had a distinctive set of neighboring genes, suggesting different biological roles for each set of proteins within a given cluster. Our next objectives would be to generate homology models for the top 10 sequence clusters and align them with the BesD•L-Lys complex structure to assess whether any differences might exist in amino acids near the active site. We completed this analysis for a sequence in cluster 1. Excitingly, we detected a key difference near the C-terminus of the cluster 1 representative. The cluster 1 halogenase is shorter and it lacks the capping Trp side chain found in BesD. We predict that this change could indicate preference for a different amino acyl substrate or a different substrate entirely. In future work, we plan to extend this analysis to the other BesD homologs shown in our SSN. Any representatives, like the sequence in cluster 1, with predicted differences in substrate specificity will be biochemically characterized and assessed as platforms for structure-function analysis as described for WelO5_2.

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ACADEMIC VITA

ADAM G. WERTZ

EDUCATION

The Pennsylvania State University (Schreyer Honors College) - University Park — **University Park, PA**
College of Science | B.S. in Biochemistry and Molecular Biology (Biochemical Option)
College of Engineering | Minor in Biomedical Engineering

Penn State Dean's List: *Fall 2017, 2018, 2019. Spring 2018,2019*

Pennsylvania State University - *Schreyer Honors College*

ADDITIONAL ACADEMIC EXPERIENCE

Learning Assistant for General Chemistry — **University Park, PA** *August 2018- present*

- Helped students in Penn State's general chemistry course understand difficult concepts.
- Facilitated discussions between students using techniques such as active learning.
- Worked under Dr. Philip Bevilaqua of the Penn State Chemistry Department.

Undergraduate Researcher at Penn State University department of Chemistry - Boal Lab — **University Park, PA** *fall 2019- present*

- Work on novel halogenases and their activity.
- Personal project under the guidance of Dr. Jeffrey Slater on WEL05 halogenase and its significance to medicine and engineering.

Undergraduate Teaching Assistant - Sociology — **University Park, PA** *fall 2019- present*

- Worked under Dr. Jonathan Fulton of the Penn State Sociology Department.
- Manged students and assignments done by students in a orderly manor.
- Led discussions on complex sociological topics to enhance student learning.

CLINICAL EXPERIENCE/CLINCAL VOLUNTEERING

Latrobe Hospital Patient Care Team — **Latrobe, PA** *June 2019 – present*

- Helped Doctors and Nurses do daily tasks around the hospital while learning key skills.
- Worked with patients onw-on-one to meet their needs in a hospital setting.
- Developed relationships with doctors, nurses, and patients within the hospital.

Forbes Regional Hospital - Hospice Care — **Pittsburg, PA** *June 2016 – present*

- Was able to volunteer alongside chaplains and palliative care specialists to give terminal patients care and comfort.
- Helped set up fundraisers to be able to give back to the hospice care center.
- Experience firsthand the trials of dealing with end of life care.

SKILLS, HONORS & INTERESTS

- **Skills:** Familiarity with Microsoft Excel, Word, PowerPoint, and Google Equivalents, Java and MATLAB Programming Knowledge
- **Honors:** National Honor Society, Spanish National Honor Society, Penn StateDean's List
- **Interests:** Research, Medicine, Tissue Engineering.