2022

Investigation of Catalysis of Nitration by Cytochrome P450s

Lannika Johnson
University of Central Florida

INVESTIGATION OF CATALYSIS OF NITRATION BY CYTOCHROME P450s

by

LANNIKA JOHNSON

A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Chemistry, Biochemistry Track
in the College of Sciences
and in the Burnett Honors College
at the University of Central Florida
Orlando, Florida

Spring Term
2022

Thesis Committee Chair: Jonathan Caranto, Ph.D.

ABSTRACT

TxtE is a protein related to cytochrome P450 enzymes, which catalyze a number of reactions that typically involve oxygen and not nitrogen. It has been discovered that TxtE can nitrate tryptophan through an unusual reaction in which it uses nitric oxide (NO) as a nitrogen donor to install the nitro group despite NO typically being considered toxic to bacteria. This project will determine if all cytochromes P450 can catalyze nitration as long as they are given NO. This will have an impact on understanding drug delivery and metabolism for which nitration is important.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Caranto and Chris Martin for supporting me through this project. Thank you to my fellow NOx Nation members for your kindness and support these past few years. I would like to give another special thanks to Dr. Caranto for allowing me to join his research group, granting me an experience I will remember for lifetimes to come.

Thank you to my parents for their love, support, and understanding as I worked on this project. I am eternally grateful.

DEDICATION

To my parents – Veranika and Landry Johnson, and my grandmother, Momma

Thank you for supporting me in all of my crazy endeavors and allowing me to be me.

Love you,

Laney

TABLE OF CONTENTS

TABLE OF FIGURES

INTRODUCTION

Cytochromes P450 (CYPs) catalyze the biodegradation of drugs and foreign substances called xenobiotics. Many drugs function as CYP inhibitors (to increase the potency of a drug) or, CYP inducers (to increase metabolism of a drug by influencing a metabolic pathway), or CYP substrates (to eliminate drugs and other metabolites). Fifty-seven CYPs have been characterized for their drug metabolism activities in humans. Much of this metabolism occurs in the liver where CYP3A4 is the predominant enzyme.[1] There is evidence that during the liver disease cirrhosis, drug metabolism is inhibited due to changes in the liver decreasing the number of hepatocytes and drug metabolizing enzymes like CYPs. Interestingly, there is also a strong correlation between reduction in CYP activity and increased severity of cirrhosis, specifically in the content and activity of CYP1A, 2C19 and 3A, implicating the importance of CYP mediation in preventing the onset and progression of diseases.[2,3]

Some well-known CYP inhibitors include antibiotics (clarithromycin, erythromycin), calcium channel blockers (diltiazem, verapamil), antidepressants (fluvoxamine, fluoxetine, and sertraline, also known as Zoloft), and even anti-HIV agents (delavirdine, ritonavir).[4] CYP inducers include rifampin (used to treat tuberculosis), carbamazepine, and phenytoin (anticonvulsants used to treat seizures and epilepsy). Similarly, CYP substrates include caffeine and ibuprofen, in which CYPs eliminate the inactive metabolites via oxidative metabolism.[5,6]

Significance

The potential of CYPs being able to perform nitration chemistry when put under the same conditions as TxtE (the addition of nitric oxide and removal of an electron) has applications in the creation of disease-fighting pharmaceutics. Many drugs contain nitro groups, which assist in the synthesis of complex drug compounds. Typical nitration methods used industrially involve nitric acid (Scheme 6) to add the nitro group but it is severely limited as it is non-selective and creates large amounts of dangerous acidic waste. The potential of CYPs to function as a nitration biocatalyst presents a highly selective and non-toxic alternative.[7]

Cytochrome P450 Typical Behavior: Hydroxylation

CYPs are heme-dependent enzymes that usually function as monooxygenases. When given a substrate and dioxygen (O_2), one of the oxygen atoms is incorporated into the substrate while the other is reduced to water.[8] The most common example of mono-oxygenase activity is hydroxylation in which an R-OH group is added to substrates (Figure 1). The reaction requires the input of two electrons.

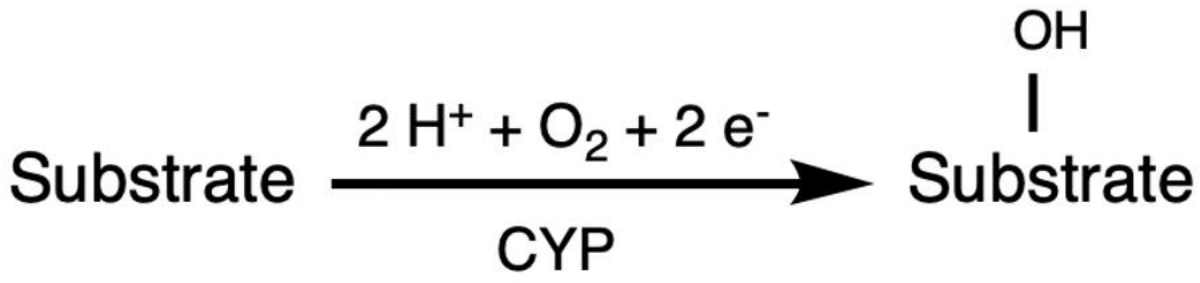

Figure 1. *Balanced equation for the hydroxylation of substrate by CYPs.*

Decades of mechanistic research have provided detailed insight into the reaction pathway of substrate hydroxylation by CYP.[9] The accumulated findings are summarized in Figure 2. A substrate, depicted as RH in Figure 2, binds to the CYP, followed by the donation of a single electron to Fe^{3+} from an electron source, reducing it to Fe^{2+}. A molecule of O_2 then quickly binds Fe^{2+} and forms the ferric-superoxo (Fe^{3+}-O_2^-) complex. A second electron then reduces the heme bound O_2 to form a ferric-peroxo complex (Fe^{3+}-O_2^{2-}). Two protonation events result in cleavage of the O–O bond, resulting in the formation of water and a highly oxidizing intermediate called Compound I. The reactivity of Compound I enables transfer of a hydrogen atom from the substrate to form Compound II and a substrate radical. Radical rebound to the substrate radical results in formation of the hydroxylated product.

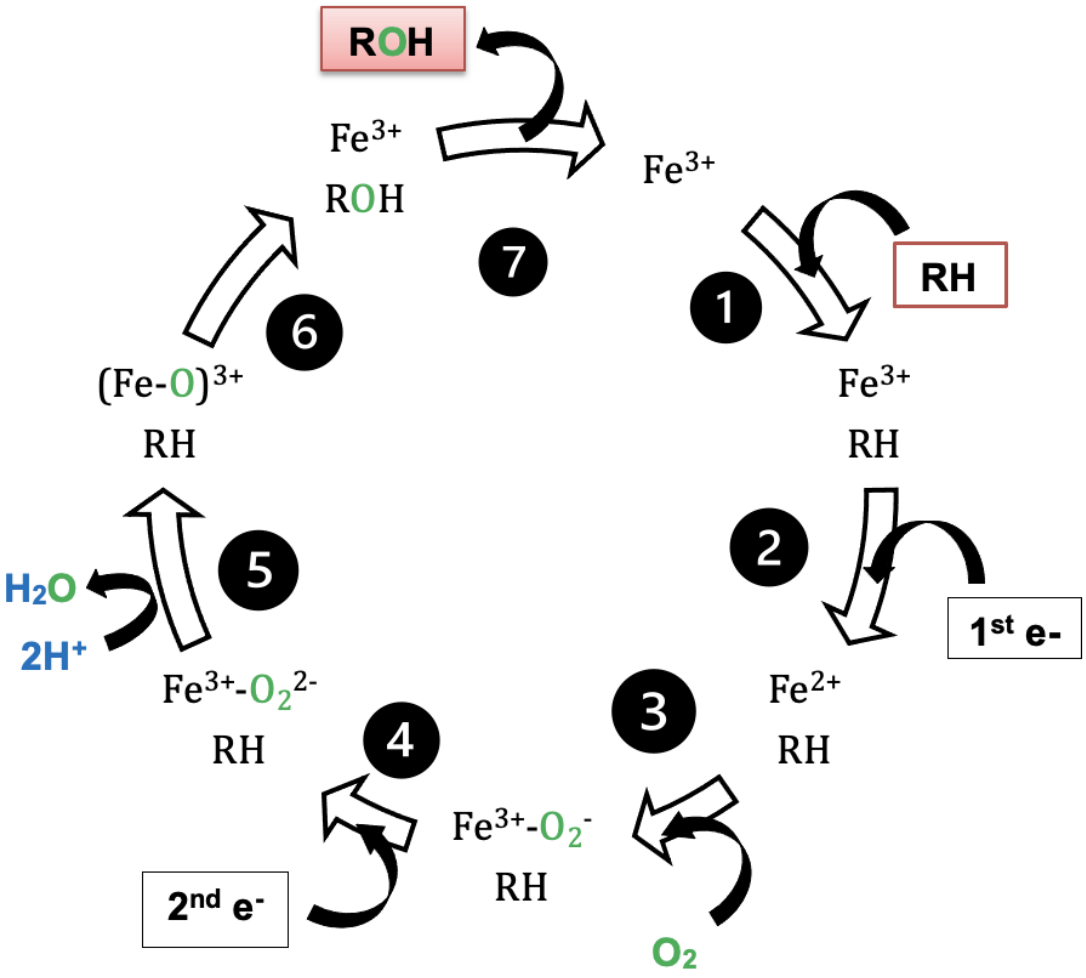

Figure 2. *A simplified general catalytic cycle of Cytochrome P450 depicting the hydroxylation of a substrate* RH.[10] *Step **1** shows substrate-binding while steps **2** and **4** demonstrate the reduction steps of the cycle. Step **3** is the binding of O_2, which is then cleaved in step **5** resulting in the release of a water molecule. The remaining oxygen atom is then transferred to the substrate in step **6** resulting in the release of the hydroxylated substrate in step **7**.*

TxtE Can Nitrate L-Tryptophan

It has been discovered that a cytochrome P450 homolog, TxtE, can catalyze nitration in the first step of the biosynthesis of thaxtomin A, a phytotoxin produced by Streptomyces species.[11] This biosynthesis was elucidated via examination of potatoes infected with scab disease, which causes lesions, or scabs, on the potato tubers. Pathogens reside and reproduce in these lesions and are caused by Streptomyces species. Evidence shows that thaxtomin A biosynthesis allows these lesions to form and be plant pathogenic. [12]More specifically, this molecule inhibits cellulose biosynthesis in plants It involves TxtA and TxtB, which encode non-ribosomal peptide synthases that produce its N-methylated cyclic dipeptide backbone, and TxtC, a P450 monooxygenase that hydroxylates the backbone.[11] The nitro ($R\text{-}NO_2$) group present on thaxtomin is required for its phytotoxicity and originates from the precursor L-4-nitrotryptophan, which is incorporated into the N,N'-dimethyldiketopiperazine core of thaxtomin A by TxtA and TxtB. Typically, oxidation of an amino group ($\text{-}NH_2$) is the source of nitro groups in natural products, but in thaxtomin A, the 4-nitrotryptophan precursor is generated by the enzyme TxtE, which catalyzes the regioselective nitration, or addition of a nitro group, onto L-tryptophan. This enzyme obtains electrons from NADPH via electron transfer proteins and both O_2 and NO are used as co-substrates (Figure 3). TxtD, a bacterial nitric oxide synthase, oxidizes arginine present in the thaxtomin, resulting in the formation of citrulline and nitric oxide.[13] This is unusual, as NO is typically toxic to bacteria.

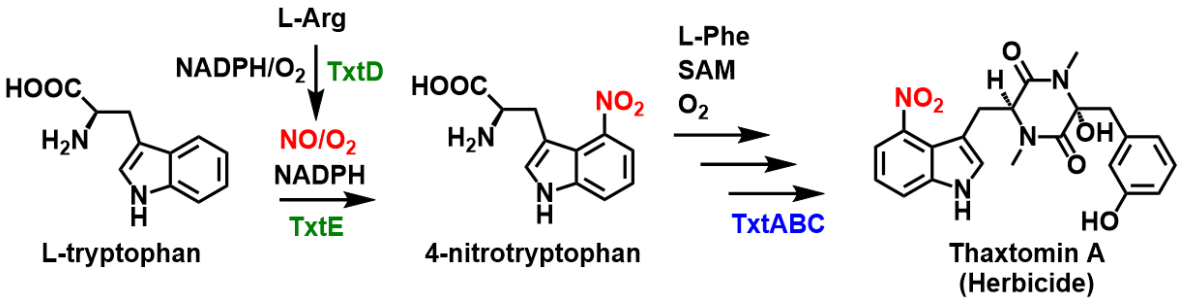

Figure 3. *Canonical nitration of L-tryptophan by TxtE in the biosynthesis of thaxtomin A.*

HYPOTHESIS

We predict that if we introduce a canonical CYP to the reaction conditions encountered by TxtE during nitration of L-tryptophan—i.e., addition of NO limiting the reducing equivalents to a single electron--that the any canonical CYP enzyme will catalyze nitration of its substrate. In the case of CYP101A1, the native substrate is camphor. Therefore, we propose that under nitration conditions, the proposed reaction will form nitrated camphor (Figure 4).

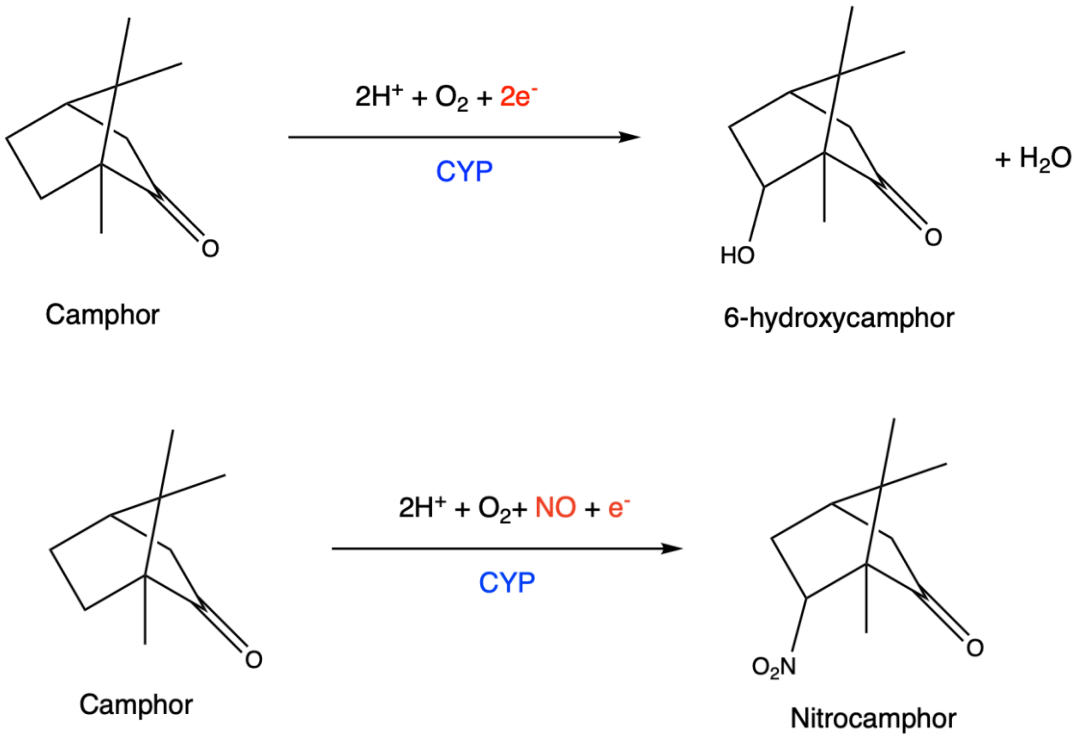

Figure 4. *Typical hydroxylation of camphor performed by CYPs (top). The bottom Figure depicts the predicted nitration of camphor by CYPs, whose regiospecificity is unknown (bottom).*

EXPERIMENTAL

The cytochrome P450 that was used to test for substrate nitration is CYP101A1. It originates from *Pseudomonas putida* and is one of the most well characterized CYPs. To express and purify CYP101A1, a previously reported protocol was followed.[14] E. *coli* cells containing pP450cam, a plasmid with a pET-28b(+) vector backbone with a 6x His-tagged CYP101A1 gene inserted into it,were grown in a starter culture of TB media containing ampicillin and then lysed by lysozyme. Unwanted protein was separated from the protein of interest, CYP101A1, via heat denaturation (sitting in a water bath of 75℃ for about 15 minutes) then removed via centrifugation. CYP101A1 was then be salted out of the remaining supernatant via ammonium sulfate precipitation. The remaining ammonium sulfate pellet was resuspended in 20 mM potassium phosphate buffer and isolated on a cobalt IMAC column. Gel filtration was performed to isolate the protein by size followed by purity analysis using SDS-PAGE. Pure fractions were pooled and concentrated to ensure proper expression and purification of the protein. Samples of CYP101A1, (substrate), 50 mm Tris buffer, and PROLI-NONOate (source of NO) were prepared under anaerobic conditions in a glove box and then exposed to O_2 via removal from the glove box to perform nitration. Liquid chromatography–mass spectrometry (LC-MS) were used to identify nitrated camphor via its molar mass after the removal of an H and the addition of a NO_2 group (~198 g/mol).

Results

The CYP101A1 protein was purified using literature protocols. SDS-PAGE analysis (Figure 5) shows that protein samples exhibit one major band indicative of the obtainment of pure protein. In addition, the band migrates at the expected size of CYP101A1 (~47 kDa). Fractions containing the protein were pooled and determined to have a concentration of 1.02 mM as determined by BCA assay.

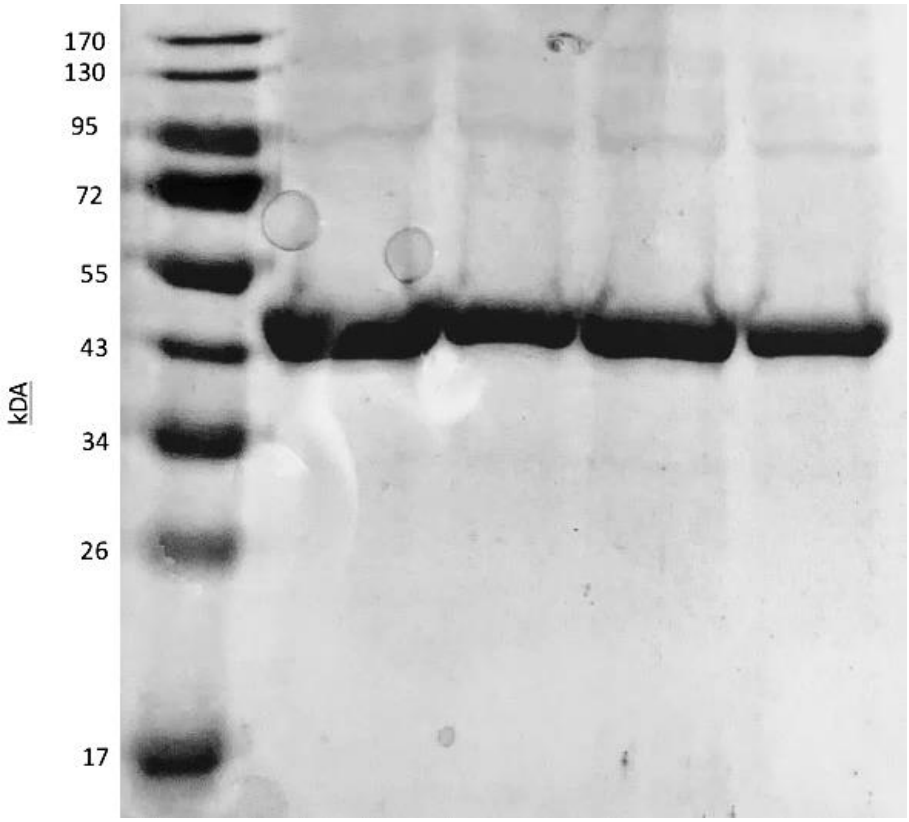

Figure 5. *SDS-PAGE of CYP101A1. Fractions of CYP101A1 collected from the cobalt IMAC column were loaded into lanes of 8% stacking and 12% resolving polyacrylamide gel.*

Next, the protein was used to test the hypothesis that CYP101A1 could perform nitration of camphor. Samples mixtures were prepared containing 50 mM Tris buffer, 400 mM camphor, 6 µM sodium dithionite, 100 mM CYP101A1, and 3.5 mM PROLI NONOate. Oxygen was added via removal of the sample mixture from the anaerobic glovebox. LC-MS analysis showed no peaks of *m/z* 198 in the extracted ion chromatogram (Figure 6), whose presence would indicate the formation of nitrocamphor. This result indicates that CYP101A1 is not capable of nitrating camphor to nitrocamphor.

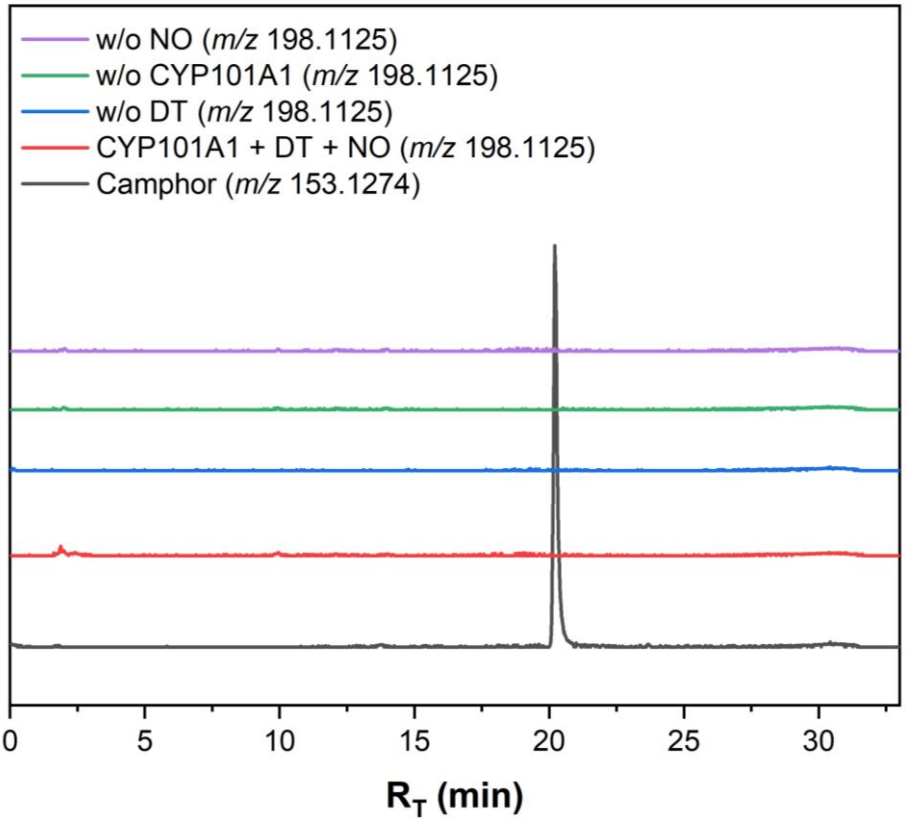

Figure 6. *Liquid chromatography coupled to mass spectrometry extracted ion chromatogram of samples with camphor, CYP101A1, NO, and O_2. Controls without each of the following components were measured: 3.5 mM PROLI NONOate (purple), 100 mM CYP101A1 (green), and 50 µM sodium dithionite (blue). Reaction mixture of PROLI NONOate, CYP101A1, sodium dithionite, 50 mm Tris buffer, and oxygen (added via removal of samples from anaerobic glove box).*

Discussion

Despite being structural homologs, TxtE and CYP101A1 are not both capable of nitration. CYP101A1, which can hydroxylate camphor, was unable to nitrate camphor when put under the same experimental conditions as TxtE (Figure 4). The collected data disproves the hypothesis introducing a canonical CYP to the same nitration conditions as TxtE (adding NO and limiting the reducing equivalents to a single electron) would result in nitration of camphor.

The results imply that there are structural differences between TxtE and CYP101A1 that commits P450s to hydroxylation and TxtE to nitration. Further investigation of the active site (Figure 7) shows that the inner spheres of the enzymes' active sites are similar, both containing a cysteine and heme group. By contrast, there are clear differences in the outer coordination sphere of the active site. TxtE's outer sphere contains proline residues in place of a critical acid-alcohol pair (D251 and T252 in CYP101A1) that are essential for camphor hydroxylation. Future experiments should focus on if the H-bond network mediated by the acid-alcohol pair prevents nitration or how the homologous proline residues in TxtE allow for nitration. Alternatively, there is an inherent difference in the structure of the substrates. The TxtE substrate, tryptophan is aromatic while camphor, the CYP101A1 substrate, is not. The inability of CYP101A1 could result from these different substrates. Nitration in CYP101A1 or similar monooxygenase enzymes could be tested with aromatic substrates to test if the reaction is substrate dependent.

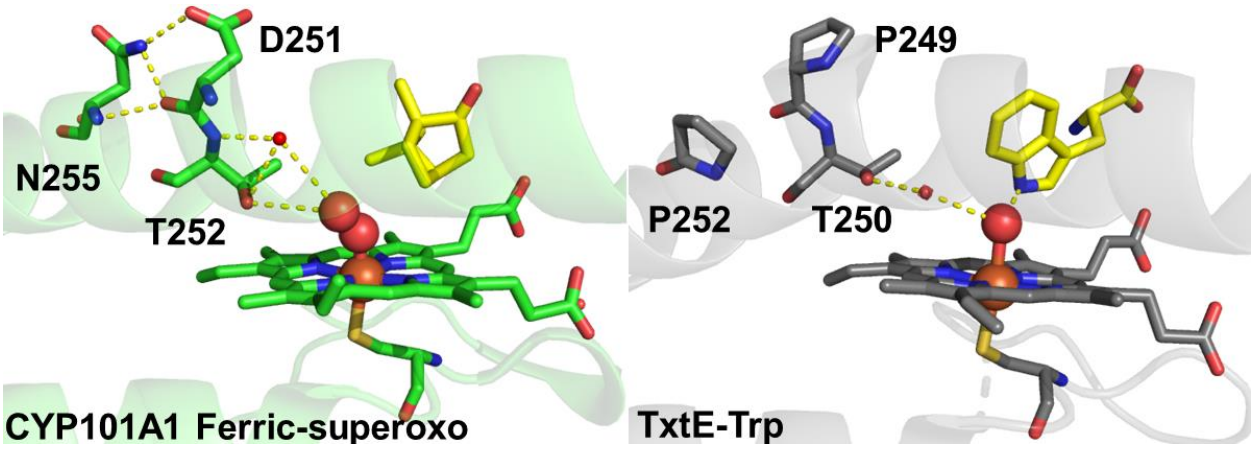

Figure 7.[15] *Comparison of the the active site of CYP101A1 and camphor and TxtE bound to Tryptophan. D251 is analogous to P249, N255 is analogous to P252, and T252 is analogous to T250.*

Conclusion

The canonical CYP, CYP101A1, was unable to nitrate camphor when put under the same experimental conditions as TxtE. These findings could lead to further experiments to gain insight into how the structures of TxtE and CYP101A1 influence their function. Examination of the active sites of TxtE and CYP101A1 (Figure 7) shows similarity in the inner sphere of the active sites, but differences in the outer spheres. The outer sphere of the active site of TxtE, for instance, contains proline residues in place of a critical acid-alcohol pair (D251 and T252 in CYP101A1) that are essential for camphor hydroxylation. Future work could use mutagenesis to change the branched amino acid residues of CYP101A1 to cyclic amino acid residues similar to the proline residues in TxtE. Similarly, catalysis of nitration could be tested with other substrates to determine if the reaction is substrate dependent. TxtE nitrates tryptophan, a cyclic compound, but CYP101A1 favors camphor, which has a bridged structure, as a substrate. A cytochrome P450 that binds a cyclic compound like tryptophan could be used to test for catalysis of nitration.

REFERENCES

(1) Zanger, U.; Schwab, M. Cytochrome P450 Enzymes In Drug Metabolism: Regulation Of Gene Expression, Enzyme Activities, And Impact Of Genetic Variation. *Pharmacology & Therapeutics* 2013, *138* (1), 103-141.

(2) Villeneuve, J.; Pichette, V. Cytochrome P450 And Liver Diseases. *Current Drug Metabolism* 2004, *5* (3), 273-282.

(3) Frye, R. F.; Zgheib, N. K.; Matzke, G. R.; Chaves-Gnecco, D.; Rabinovitz, M.; Shaikh, O. S.; Branch, R. A. Liver Disease Selectively Modulates Cytochrome P450--Mediated Metabolism. *Clin. Pharmacol. Ther.* **2006**, *80* (3), 235–245.

(4) Zhou, S.-F.; Xue, C. C.; Yu, X.-Q.; Li, C.; Wang, G. Clinically Important Drug Interactions Potentially Involving Mechanism-Based Inhibition of Cytochrome P450 3A4 and the Role of Therapeutic Drug Monitoring. *Ther. Drug Monit.***2007**, *29* (6), 687–710.

(5) Lynch, T.; Neff, A. P. The Effect of Cytochrome P450 Metabolism on Drug Response, Interactions, and Adverse Effects https://www.aafp.org/afp/2007/0801/p391.html (accessed Dec 30, 2021).

(6) Mazaleuskaya, L. L.; Theken, K. N.; Gong, L.; Thorn, C. F.; FitzGerald, G. A.; Altman, R. B.; Klein, T. E. PharmGKB Summary: Ibuprofen Pathways. *Pharmacogenet. Genomics* **2015**, *25* (2), 96–106.

(7) Zuo, R.; Zhang, Y.; Jiang, C.; Hackett, J.; Loria, R.; Bruner, S.; Ding, Y. Engineered P450 Biocatalysts Show Improved Activity And Regio-Promiscuity In Aromatic Nitration. Scientific Reports 2017, 7 (1).

(8) Louka, S.; Barry, S.; Heyes, D.; Mubarak, M.; Ali, H.; Alkhalaf, L.; Munro, A.; Scrutton, N.; Challis, G.; de Visser, S. Catalytic Mechanism Of Aromatic Nitration By Cytochrome P450 Txte: Involvement Of A Ferric-Peroxynitrite Intermediate. *Journal of the American Chemical Society* 2020, *142* (37), 15764-15779.

(9) Ortiz de Montellano, P. R. Hydrocarbon Hydroxylation by Cytochrome P450 Enzymes. Chemical Reviews 2009, 110 (2), 932–948.

(10) The P450 Catalytic Cycle. http://www.tcm.phy.cam.ac.uk/~mds21/thesis/node49.html (accessed Dec 3, 2021).

(11) Barry, S.; Kers, J.; Johnson, E.; Song, L.; Aston, P.; Patel, B.; Krasnoff, S.; Crane, B.; Gibson, D.; Loria, R.; Challis, G. Cytochrome P450–Catalyzed L-Tryptophan Nitration In Thaxtomin Phytotoxin Biosynthesis. *Nature Chemical Biology* 2012, *8* (10), 814-816.

(12) Loria, R.; Bignell, D. R.; Moll, S.; Huguet-Tapia, J. C.; Joshi, M. V.; Johnson, E. G.; Seipke, R. F.; Gibson, D. M. Thaxtomin Biosynthesis: The Path to Plant Pathogenicity in the Genus Streptomyces. Antonie van Leeuwenhoek 2008, 94 (1), 3–10.

(13) Caranto, J. D. The Emergence of Nitric Oxide in the Biosynthesis of Bacterial Natural Products. *Curr. Opin. Chem. Biol.***2019**, *49*, 130–138

(14) Tan, C. Y.; Hirakawa, H.; Suzuki, R.; Haga, T.; Iwata, F.; Nagamune, T. Immobilization of a Bacterial Cytochrome P450 Monooxygenase System on a Solid Support. Angewandte Chemie 2016, 128 (48), 15226–15230.

(15) Martin, C.; Chen, M.; Martinez, M.; Ding, Y.; Caranto, J. The Ferric-Superoxo Intermediate Of The Txte Nitration Pathway Resists Reduction, Facilitating Its Reaction With Nitric Oxide. *Biochemistry* 2021, *60* (31), 2436-2446.