A Look at the Catalytic Efficiency of E Coli.Catalase HPII using Native Tyr-415 and Inductive ncAAs: 3-Nitrotyrosine and 4-Iodophenylalanine

Author: Sean Murphy
Co-Investigators: Heather Kahalehili, Quentin Hahn, Adrianna Parent
Oregon State University Department of Biochemistry

Abstract

Escherichia coli Catalase HPII is a monofunctional catalase that is responsible for the decomposition of peroxide into water and oxygen. Heme containing catalases have been heavily studied in order to understand the reactions that drive the decomposition of hydrogen peroxide.Therefore, Tyr - 415 has been identified to play a critical role in the modulation of heme iron reactivity by stabilizing higher oxidation states.To further explore the catalytic importance of Tyr - 415 we replaced native Tyr - 415 with non native nCAA: 3-NY - 415 (3-nitrotyrosine) and 4pIF - 415 (4-Iodophenylalanine).We conducted quantitative and qualitative assays on native Catalase HPII ,3-NY - 415 variant, and 4pIF - 415 variant in order to explore the catalytic activity of mutants and wild type Catalase HPII. By conducting this investigation we believe to have achieved successful incorporation of nCAA 3-NY into the critical Tyr-415 residue of Catalase HPII. It should be noted that to date no literature describing a successful incorporation of mutants at Tyr415 has been published[11]. Therefore, achieving successful incorporation of critical tyrosine residues could allow for a better understanding of how monofunctional catalases operate in regards to critical Tyr residues.

Introduction

Catalases are a special class of ubiquitous enzymes that can be found in both eukaryotic and prokaryotic cells[4].The primary function of these catalases is to breakdown hydrogen peroxide in the cell and protect the cell from the toxic effects of small peroxides[4]. As a result of this breakdown, catalase yields molecular water and oxygen through a two step mechanism (Reaction Mechanisms 1.). This reaction is integral in many species because it yields beneficial chemical species while disposing of potential chemical waste. One of the major types of catalases is *Escherichia coli* Catalase HPII, which this study will be centered around. Catalase HPII is a monofunctional catalase that is encoded and regulated through the katE[4] gene.

Escherichia coli Catalase HPII, is a relatively large catalase containing 753 residues per subunit[4] and has a molecular weight of roughly 84 kDa. Catalase HPII is large in comparison to most catalase species but is still heavily observed within the scientific community.This comes as no surprise because most catalase species tend to have structural similarities. One known feature that catalase species have in common with one another; is that they are responsible for the disposal of free radicals that form in organisms that rely on oxygen.These reactive oxygen species have been known to have cytotoxic effects.If these reactive species are allowed to linger

they can cause damage to lipids, proteins and nucleic acids.Catalase species might have similarities; but catalases have been studied for several years but are not completely understood.Several questions concerning the structure of different catalase species as well as their physiological role remain unanswered[10]. Therefore, obtaining the answers to how catalase species operate at the physiological level could open new therapeutic pathways for human diseases that generate oxygen radicals[5].

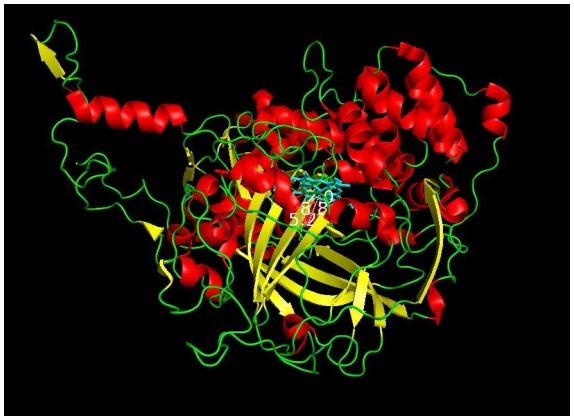
(1) Step 1: $H_2O_2 + \text{Por-Fe}^{III} \rightarrow \text{Por}^{+\bullet}\text{Fe}^{IV}\text{= O} + H_2O$
(2) Step 2: $H_2O_2 + \text{Por}^{+\bullet}\text{Fe}^{IV}\text{= O} \rightarrow H_2O + O_2$

Reaction Mechanisms 1.

Escherichia coli Catalase HPII functions as a two step mechanism. The initial oxidation reaction (1) generates a FeIV, and a Por group (porphyrin ring) that contains electrons that are delocalized on the group. This part of the reaction is considered to be an intermediate state of reaction (1). A water molecule is then yielded from the reaction (1). During step two a reduction reaction occurs that results in the leaving of a water and oxygen molecule. As a result of research that has been completed in the past, it is believed that step one is the rate-limiting step of the reaction[2]. Therefore, making the first step of this reaction a good target when trying to alter catalytic speed.

Catalase HPII is comprised of four monomeric subunits(tetramer)(Figure1) and contains one *cis*-heme d *prosthetic* group per subunit[4]. The active site of the heme is buried deep inside the homotetrameric structure of catalase requiring the use of different selective channels within the protein to mediate the flow of substrate[6]. The reactants enter HPIIs structure via the main channel and products are then pushed out through the lateral channel.The heme component of Catalase HPII is believed to be different in comparison to other catalase species; It is speculated that HPII itself carries out post-translational modifications that result in the oxidation of heme b to heme d[12]. In fact we have reason to believe that Catalase HPII is unique among catalases due to its nature of carrying out two posttranslational modifications in the vicinity of the active center[6]. Therefore, we can speculate that Tyr 415 which is covalently bonded to the heme active site of Catalase HPII plays a critical role in the regulation of iron reactivity by stabilizing higher oxidation states.

Another important thing to note is that the Catalase HPIIs heme is believed to be roughly 2Å from Tyr-415 and is roughly 8.8Å from His-392. Past research on Catalase HPII has revealed that there is a critical bond between the beta carbon of Tyr-415 and the imidazole ring of His-392[3]. Crystal structures of His 392-Tyr-415 bond have shown that the imidazole ring of His-392 rotates 80° in relation to the position of HPII[3]. This suggests that there is a relationship between the heme b to heme d conversion that catalase carries out and the formation of the His-Tyr formation[3].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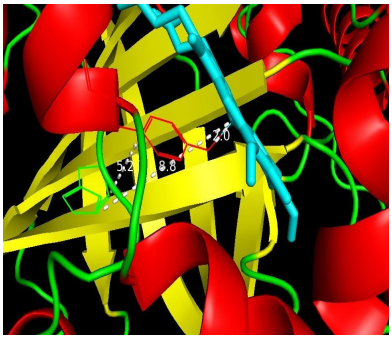

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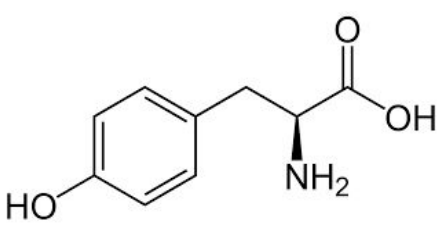
Figure 1: (a) 3D Structure of *Escherichia coli* Catalase HPII generated from x-ray diffraction experiment and studied via pymol tool Protein ID:1IPH[1]. (a) Only depicts a single monomer from catalases 4-homo-mer structure.(b) Displays a close up image of where Try - 415 is located, as well as what is believed to be catalases active site[3] region.Note:the blue is the heme group, red is the tyrosine and the green is histidine.



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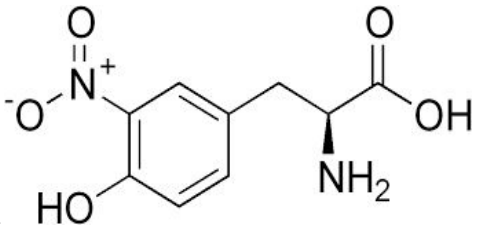

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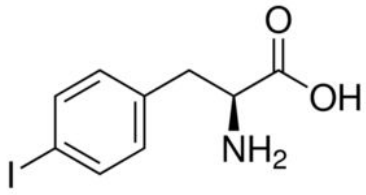

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Figure 2: (a) Native Tyrosine contains a pKa value of 10.46, Tyr - 415 plays a critical role in the regulation of heme iron reactivity by carrying out stabilizations at higher oxidation states.(b) 3- Nitrotyrosine contains a pKa value of 9.46, the nitrate group acts a strong electron withdrawing group.(c) 4-Iodophenylalanine contains a pKa value of 9.44, iodine acts as a strong electron withdrawing group.

The heme that is attached to Catalase HPII allows the enzyme to react with hydrogen peroxide and initiate the breakdown of H_2O_2 .Since we believe that the breakdown of hydrogen peroxide is the rate limiting step from the literature[2] we intend to utilize highly inductive nCAA species in our research. Highly inductive subunits are said to help with stabilization of the first compound that is generated in a reaction.The inductive effect is an electrical effect that is due to the polarization σ bonds within a molecule. We intend to attempt to utilize the inductive effect in order to obtain better stability during the rate limiting step in the hopes that this will increase the catalytic efficiency of Catalase HPII. We have chosen 3-NY and pIf (Figure 2) because we

believe both variants have a larger inductive effect in comparison to the wild type Tyr-415 due to the addition of halogenated groups.In order to test this we will incorporate these nCAA's into the Tyr 415 position and uncover catalytic data through experimental comparison between wild type and variants. However, a potential issue with this experiment is that incorporation of mutants at Tyr 415 has never been done successfully. If we are able to achieve positive incorporation of nCAA it would fill a potential gap in our knowledge about Catalase HPII.

Ultimately, the overarching goal of this study is to determine if catalase will obtain improved catalytic efficiency through the replacement of wild type Tyr-415 with non native nCAAs (3-NY and pIf) and to see if incorporation of nCAA is possible at Tyr-415. The results we hope, will create a better understanding of the importance of the covalently bound Try 415 to active site complex. In fact, we believe that the activity of HPII will result in a positive increase over a large temperature range when using the halogen and nitrate groups contained in the selected unnatural amino. This is primarily because if incorporated properly, these nCAA'S should increase the stability of the rate limiting step of Catalase HPII reaction.Hopefully, this research will expand our current understanding of how Catalase HPII operates.

Materials and Methods

Protein Expression:

For our expressions we used *Escherichia coli* DH10B cells which contained a pBAD plasmid. The pBAD plasmid contained a His-tagged catalase gene, β-lactamase gene, and ampicillin resistance[1](Figure 3). Our expressed cells contained a pDULE plasmid which provided the necessary translational machinery, as well as the protein that encoded for tetracycline resistance[1]. We utilized the expression plasmid: pBad HPII TAG-415 for both 3-NY and pIF mutants(Figure 3).A specific machinery plasmid was also used for our 3-NY and pIF mutants; 3-NY: utilized pDule-3-nitrotyrosine machinery plasmid and pIF utilized the: pDule-pCNF machinery plasmid.Our expressed cells where prepared for a dilution 1:100 in 75 mL of autoinduction media(AIM)[13](Table 1) or alternatively (1:100) in 5 mL autoinduction media as controls. Six cultures were prepared in order to produce: WT HPII, 3-Nitro-Tyr-415 catalase (3-NY), 4-Iodo-Tyr-415 catalase (pIF), 3-Nitro-Tyr-415 catalase with no UAA added, 4-Iodo-Tyr-415 with no UAA added. All cultures except our native catalase(WT) received the UAA translational machinery.We added UAAs to both the 3-NY-415 and pIF-415 cultures. Plasmid cultures without UAA's were grown as our controls in order to confirm the integrity of the translational machinery. Because our controls had an absence of UAA's we would expect to observe a truncated protein.Cultures where incubated at 37°C for 48 hours while placed on a slow shaker. After the incubation period 250μL of each of the cultures was collected and centrifuged at 4000 rpm (5min), and resuspended in 50 mL Milli Q H_20. The leftover cultures were then split up into sample tubes and centrifuged at 7000 rpm for 10 min. Cell pellets were then collected, labelled and stored at -80°C.

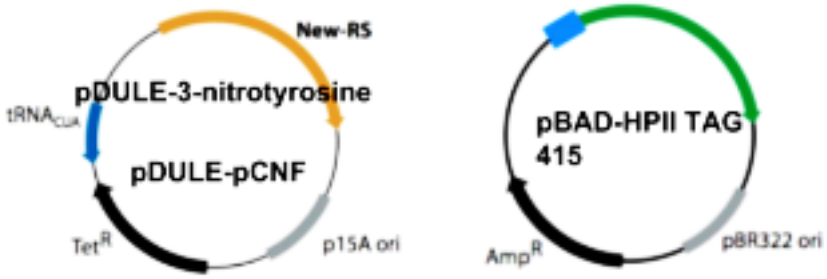


Figure 3: Plasmid Vectors used for the Catalase HPII gene displayed on the right hand side(pBAD-Tyr 415 TAG) and translational machinery is displayed to the left (pDULE-pCNF and pDULE-3-nitrotyrosine) used for the incorporation of nCAAs.

Protein Purification:

All cell lines were resuspended by individual cell pellets in the Talon equilibration buffer. The cell pellets were then processed by a microfluidizer which is a thermostable method to lyse cells. Cell lysates were recovered and centrifuged at 15,000 rpm at 4°C for 20 minutes; This step allowed for the removal of all cellular debris. 600µL of washed resin was placed in each of the samples in a 50mL tube.The samples were then incubated on ice and placed on a rocker for 20 minutes.Samples bound to resin were then centrifuged 700g for 5 minutes, the wash was repeated with 15 mL of wash buffer. We then re-suspended our samples in 1.5mL equilibration buffer and then transferred to a gravity flow column.Protein samples where then eluted from the resin using fractions of 500 µL of elution buffer and stored in the fridge at 4˚C; each of our protein fractions yielded 3-5 fractions which were then used during experimentation.We did not desalt our samples this step was unnecessary for the assays ran.

Sample Preparation:

In order to prepare crude extract samples for SDS-PAGE we obtained 250 µL of each cell pellet (pIF, 3-NY,WT,GFP +NCAA, GFP -NCAA) and stored it at -20°C. The cells were then resuspended in distilled H2O, following that 50 µL of 2X SDS dye was added to each sample. Each sample was then heated to 100 °C for roughly 10 min and vortexed as needed. To finalize our sample we centrifuged each sample at 10,000 rpm for 5 min. An SDS-PAGE (10%)gel was used to analyze protein concentration. Then, 20µL of Crude and purified protein samples where loaded onto a SDS-PAGE gel that ran at 110V for 45 min. 5 µL of Dual Color ladder was added to the first lane on the gel to be used as the measurement system for molecular weight of our protein. Blue Coomassie dye was used to see the resulting proteins.

Assay Preparations:

Qualitative Assay: A purely observational approach to validating the activity of Catalase HPII was performed at room temperature. It was carried out by taking 20 µL of purified Catalase (from each elution strain) and adding it to 30 µL of 10% H_2O_2 + .5M tris buffer in order to get a 1% concentration of H_2O_2 in, 1mL final volume. If the resulting mixture resulted in bubbles forming this indicated that there was activity between the enzyme and substrate.

Quantitative Pressure Probe Assay: We used a Vernier Pressure Probe to measure the rate of oxygen gas production. This was done by adding 100µL of purified Catalase (from each elution strain) to a 1 mL mix of 30% stock hydrogen peroxide + .5M tris buffer at a PH of 7.5.

This assay was performed for each of the elution strains and was measured over the course of 6 minutes. The goal of the Quantitative Assay was to obtain information that could be used to observe protein kinetics. Note: The amount of hydrogen peroxide to Tris varied depending on the desired final concentration of H_2O_2.

Protein Concentration Determination

In order to determine our protein concentration we used a Bradford Assay on each of our fractions with the absorbance set to 595 nm. Each of our samples was prepared by adding 20 μL of eluted sample to 1 mL of 1X Bradford dye. Bovine serum albumin was used as a standard and the standards in this assay ranged from 0-2000 μg/mL concentration.

Table 1: Components of autoinduction media[13].

Aspartate (5%, pH 7.5)	12.5 mL
Glycerol (10%)	12.5 mL
25x Mineral Salts	10.0 mL
Glucose (40%)	0.3125 mL
MgSO4 (1M)	0.5 mL
Arabinose (20%)	0.625 mL
Trace Metals (5000x)	0.05 mL
18 AA Mix (25x)	10.0 mL
Ampicillin (100ug/μL)	0.250 mL
Sterile Water	To final volume of 250 mL (203.2 mL)

Table 2: Compositions of buffers used:

Equilibration Buffer (5X)	250 mM sodium phosphate, 1.5 M NaCl pH 7.0
Elution Buffer	50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 7.0
Sodium Phosphate Buffer	0.1M, pH 7.0
SDS-PAGE Running Buffer (10X)	125 mM Tris base, 960 mM glycine, and 0.5% SDS in DI H2O, pH8.3

Results and Discussion

In order to obtain the purity and confirm the integrity of our samples an SDS-PAGE gel was run. We obtained a highly resolved image that we believe confirmed the production of purified (i.e His-Tagged catalase bound to resin) Catalase HPII, of our WT and 3-NY mutants. The resulting gel (Figure 4b) yielded surprising results; we obtained bands between the 100 kDa and 75 kDa makers for our purified WT2, 3NY2 and 3NY3 sample strains.According to the literature on HPII its monomeric molecular weight is roughly 84.2 kDa[4]. Therefore we would expect the bands for our catalase monomers to lie somewhere between the 100 kDa and 75 kDa makers as can be seen in (Figure 4b). Though it should be noted that we might have obtained a distinct band for WT1 if there was not an unexpected bleed over of ladder into WT1. No distinct bands can be seen on (Figure 4b) for the pIF mutants.This led us to believe that our attempts to incorporate the pIF mutants were unsuccessful.There are several reasons as to why the incorporation could have been unsuccessful. One such reason for the unsuccessful incorporation could be that pIF strains were truncated and never incorporated. Another reason could be due to the fact that the size of pIF was much too large to successfully incorporate at the Tyr-415 residue.The Iodo group that pIF brings is large in retrospect to the WT-415 and 3-NY; This led us to believe that incorporation of pIF either stopped very early on or pIF incorporated but protein functionality was lost.

When looking at our crude samples (Figure 4a) we were unable to obtain any clear images from these experiments. For each crude SDS-PAGE gel similar results as to what can be seen in (Figure 4a) was obtained. The reason for this could have been from an overloading of 2x dye when running these gels or just consistent lack of procedural caution in order to obtain a clear image of the crude.As a result, this could be a potential issue because we can't make any claims about our -nCCA control groups integrity in this study.

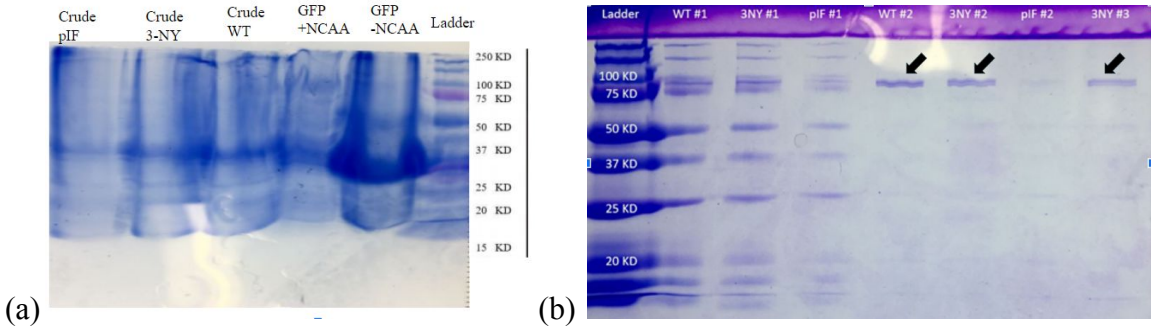


Figure 4: SDS-PAGE gel representing crude samples cell lysate(A) .From this image we can observe that the wild type catalase (monomer) has a thick dark blue band showing up at molecular weight of around 23 KDa.We would expect this band to be much higher according the literature at roughy 84.2 kDa. Though it should be noted that image resolution is extremely low.(B) SDS-PAGE gel representing purified protein samples at (90% purity). As can be seen WT2, 3-NY2,and 3-NY3 display clear bands at where we would expect to see Catalase HPII monomer (84.2 kDa). Based off this image we can confidently say we obtained WT2, 3-NY2,and 3-NY3 at a purity of about (90%).We can also see faint bands for pIF mutants but this is not enough information to make any inferences.

After obtaining the results from our crude SDS-PAGE gel (Figure 4.a) we decided to do an additional confirmation experiment.This allowed use to confirm activity between enzyme and substrate. We took 20µL of crude 3-NY, crude native and crude pIF samples that were left over from our gel experiment and placed them in a 1% hydrogen peroxide .5 M tris buffer mix. The results from this experiment were surprising because in all three cell lines, bubbles started to generate after the 1-2 min markers. Some samples were slower than others in terms of bubble production, which could be due to inconsistency in sample purity. However, this experiment did confirm that we did indeed have Catalase HPII for all three generated cell lines of crude. Though it should be noted for the pIF samples bubbling was not sufficient enough to justify any total claim of activity.

A second qualitative assay was then conducted using the purified samples. All samples after 5 minutes displayed noticeable levels of activity, except for the pIF mutants (Figure 5.) These result made sense from the data that was obtained in (Figure 4b) past experimentation.Our results indicated that we had successfully generated functional HPII for both our WT and 3-NY cell lines. One thing to note about the results displayed in (Figure 5) is that 100µL of each elution was added in this round of the qualitative assay. Though the added amount of each elutions was the same; the concentration of each elution was different.Therefore it made complete sense to observe large variations in each sample activity.

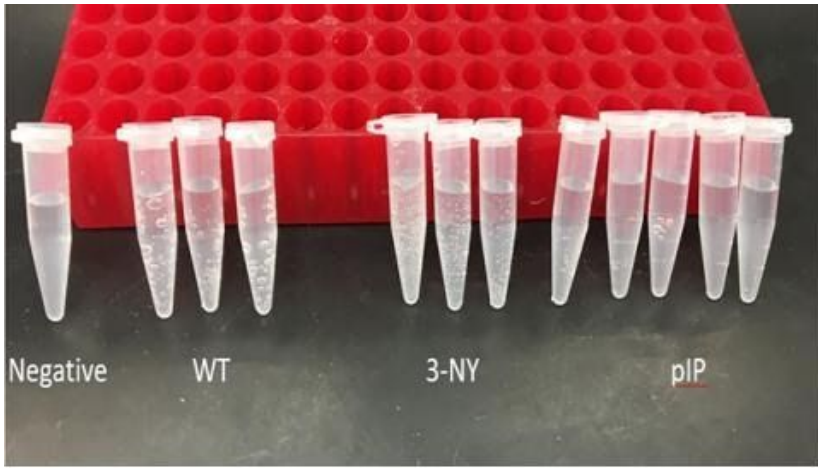


Figure 5: Image qualitative protein validation study using purified protein elutions. We can see from this image the wild type (WT) cell line has the most activity while pIF had little to no activity. This could be due to a large difference in the percent purity of our proteins.

In order to obtain the total concentrations of our purified samples we conducted a Bradford Protein Assay. Our Bradford Assay (Table 3) revealed results that were consistent with data we obtained in past assay's and experiments. We found that the WT purified strains yielded the largest total concentrations followed by 3-NY and pIF being the least concentrated.These results confirm a difference in concentration and help explain why both the Wild Type and 3-NY strains displayed better activity during the qualitative assay (Figure 5.). One interesting thing to note is that we still obtained a positive concentration amount for all pIF mutants. We would expect to see the pIF mutants concentrations to be closer to zero rather than what was observed.These positive pIF concentrations could be the result of potential contamination of the pIF mutants or due to successful incorporation of pIF into Tyr-415. Though if incorporation of pIF was successful the generated mutants were most likely non-functional/slightly functional mutant protein based off of prior assay results.

Table 3: Bradford Assay results at absorbance of 595 nm and concentrations of different elution fractions.Concentrations calculated through the use of a the standard Bradford Curve.

Sample type	Absorbance 595 nm	calculated concentration (mg/L)
WT1	0.225	125
WT2	0.235	130.5555556
WT3	0.233	129.4444444
3NY1	0.12	66.66666667
3NY2	0.124	68.88888889
3NY3	0.159	88.33333333
pIF1	0.024	13.33333333
pIF2	0.028	15.55555556
pIF3	0.045	25

Table 4. Final Concentrations of protein strains used in Qualitative Assay.

Protein	Concentration (mg/L)
WT	128.334
3-NY	74.630
pIF	17.963

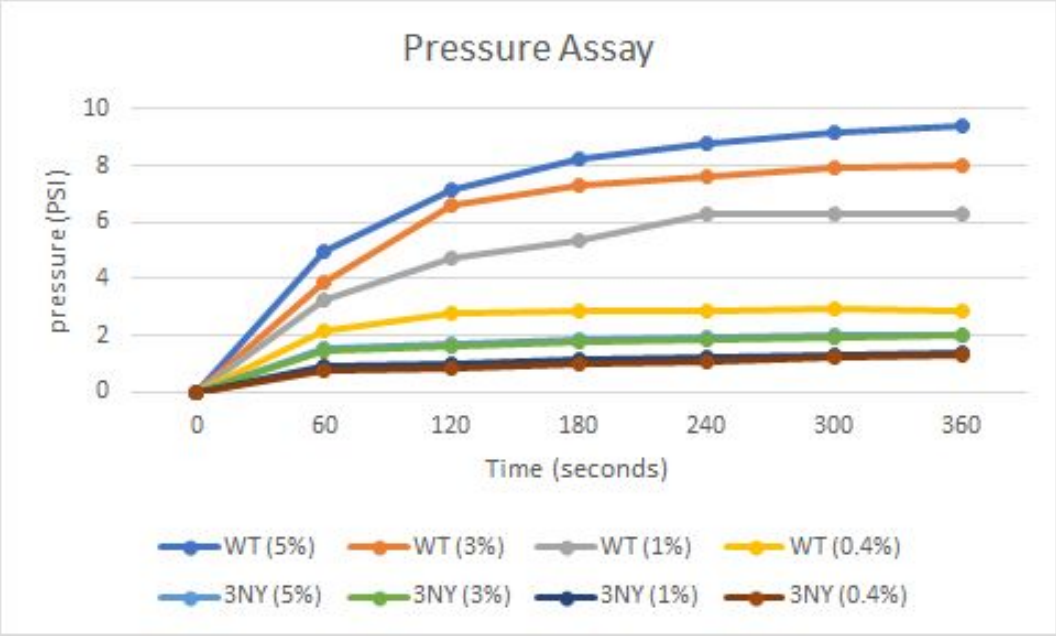
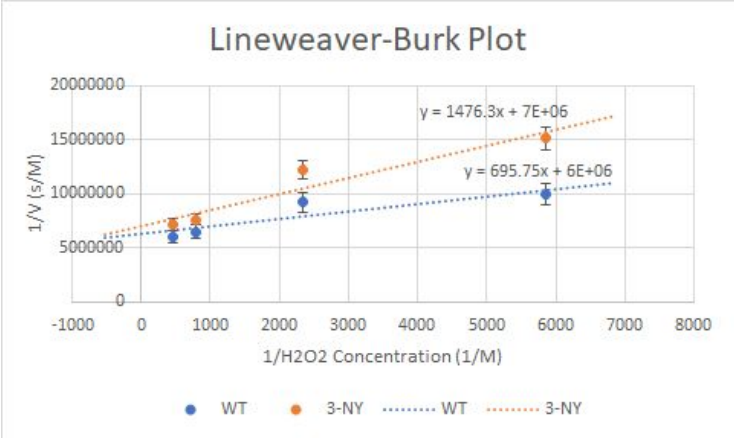


Figure 6: Results from quantitative Vernier pressure probe assay conducted with WT and 3NY samples(Table 4). Before the assay was ran the device was blanked to zero psi before obtaining data. This graph shows the rate of consumption of substrate at different concentration of H_2O_2 over a 6 min time period.

A final Quantitative Assay was conducted using a vernier pressure probe to measure the rate of enzymatic consumption of substrate over a 6 min. period of time (Figure 6). The assay time was chosen because it allowed for a good depiction of the steady state of the reaction.Our Quantitative Assay was only conducted with 3-NY and WT sample strains (Table 4).We decided against using pIF in this assay due to poor results that were yielded throughout the assay optimization processes.The results from our Quantitative Assay showed that, WT catalase displayed more activity at all levels of H_2O_2 concentration when compared to the 3-NY mutant.

These results came as no surprise since we only added 100μL of each strain to the peroxide, tris mixture (Table 4). This indicates that the concentrations of WT catalase and 3-NY that were added to this assay were different. The 3-NY sample that was used in this assay has roughly 59% the concentration of the WT sample.Which would potentially mean that the 3-NY mutant is actually much closer to the WT in consumption rate than is shown (Figure 6). Therefore, this could indicate that the WT catalase and 3-NY mutant display somewhat similar catalytic rate. However, further studies using proper protein concentrations should be conducted before making these claims.

(a)



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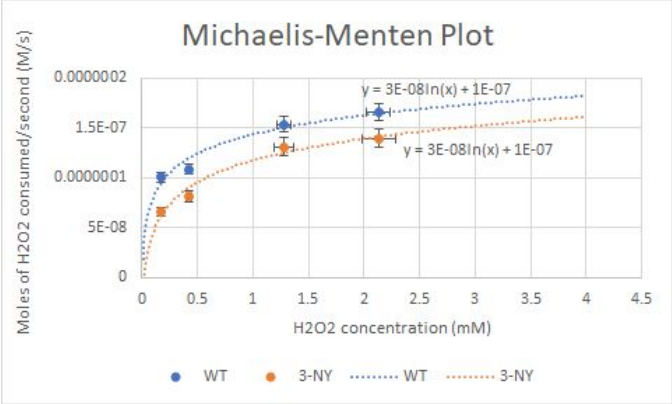

Figure 7: Theoretical Kinetics data generated after correcting protein concentrations. (a) displays the Lineweaver-Burk plot which is a graphical display of basic enzyme kinetics of the reactions shown in (figure 6). (b) displays the Michaelis menten plot which shows the predicted reaction velocity as a function of substrate concentration and is a graphical depiction of V_{max} and K_M.

Table 5: Table of theoretical kinetics values after factoring in protein concentration error for WT, 3-NY as well as literature referenced values.

	$K_{M\,(app)}$ (mM H_2O_2)	k_{cat} ($s^{-1} \times 10^{-7}$)	k_{cat}/K_M ($s^{-1}mM^{-1}$)
WT	0.116 ± 0.032	750 ± 15	6.46×10^{-4}
3-NY	0.211 ± 0.044	997 ± 29	4.73×10^{-4}
Literature[14] (pH = 7, T = 37 °C)	86.5 ± 1.5	5.0 ± 0.1	5.78×10^{-6}

Because we used different concentrations for 3-NY and WT Catalase HPII in the Quantitative Assay we generated theoretical kinetics data through an adjustment that can be seen on (Figure 7) and (Table 4). We adjusted the 3-NY sample concentration up by roughly 59% to be equal to the concentration of our WT catalase for this data.

From the kinetics data displayed (Table 5) we can see that both our WT catalase and 3-NY have a very low K_M value in comparison to the recorded literature[14] value for catalase. Therefore, this indicates that we generated protein that has a higher affinity with its substrate.It should be noted our WT and 3-NY where not studied under the same conditions as the literature values that we will be referring too.When comparing WT catalase to the 3-NY mutant it has a slightly lower K_M value; This means that the WT Catalase HPII can easily achieve V_{max} in comparison to the 3-NY mutant.Our 3-NY mutant will require more substrate in order to achieve V_{max} which makes sense based off past observations (Figure 6). 3-NY in every instance of this study displayed lower efficiency than the WT Catalase HPII (Figure 6) (Figure 7).

For both WT and 3-NY we obtained much larger values of K_{Cat} (Table 5)than the literature has recorded[14]. This could indicate that the WT and the 3-NY mutant have a much larger turnover rate than what we would expect to see in WT Catalase HPII.However, we suggest in order to further explore the significance of these kinetics values it is critical to run experimentation under similar reaction conditions that were conducted in this study. This will allow for more concrete evidence to make claims rather than using our theoretical model where any claims in nature are difficult to make.

Conclusion

In sum, then the pIF mutant displayed little to no activity throughout the course of experimentation. The results could have been due to the pIF being unsuccessfully incorporated at the critical Try-415 ligand.In most cases when incorporation has been attempted at this residue it resulted in an non functional protein[11]. Replacing native Tyr-415 has been recorded to affect the folding of Catalase HPII[11]. Therefore, it makes sense that incorporation wouldn't have worked with the addition of the iodo group to Tyr-415.It should be noted that we did see some activity from our pIF mutants and obtained non negative protein concentrations; However, we fell short in obtaining an SDS images that would have help further a claim of incorporation. As a result of this, we believe pIF failed to incorporate successfully. Despite this,we do think that further experimentation with pIF at Tyr-415 should be conducted in order to confirm faulty incorporation.

Furthermore, when looking at the 3-NY mutant we potentially saw successful incorporation. In fact, from the results we presented we generated a functional Catalase 3-NY-415 mutant.If incorporation was successful this would prove that incorporation at the critical Tyr-415 is possible.However, I believe further experimentation should be conducted before solidifying a claim of successful incorporation. One suggestion for future works is Analytical Ultracentrifugation (AUC) which could be used in order to confirm the molecular weight of 3-NY. Ultimately, this information would help support claims of incorporation of 3-NY at the critical Try-415.

When looking at our kinetics data we found that the WT catalase was actually more favorable in terms of catalytic efficacy in comparison to the 3-NY mutants.The 3-NY mutant in turn had a much faster turnover rate in comparison to the WT. It should be noted that the kinetics data obtained was done using an Applied Theoretical Kinetics Model we constructed. Also, in order to prove any of our kinetics claims it is critical that these experiments be run in similar conditions in order to obtain non theoretical kinetics values.

As a results of this investigation, we were potentially able to display that incorporation of 3-NY at the critical Tyr-415 residue of Catalase HPII is possible; This has never been reported in the literature[11].Along with this we were also able to confirm that pIF is unlikely to incorporate at HPII when replacing the critical Tyr ligand. Nevertheless, no claim can be made from this study about the rate limiting step and the effects of inductivity on it;the reason being, that all of our kinetics data is theoretical. So, in order to make any claims on these topics further experimentation should be conducted.

References

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Appendix (supporting materials)