5-7-2016

Spectroscopic and Thermodynamic Characterization of Human 3-Hydroxyanthranilate-3,4-Dioxygenase

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SPECTROSCOPIC AND THERMODYNAMIC CHARACTERIZATION OF HUMAN 3-HYDROXYANTHRANILATE-3,4-DIOXYGENASE

by

CALMOUR HENRY

Under the direction of Aimin Liu, Ph.D.

ABSTRACT

3-Hydroxyanthranilate-3,4-dioxygenase (HAO) is a non-heme iron dependent enzyme that catalyzes the oxidative ring opening of 3-hydroxyanthranilate (HAA) which is an intermediate in the kynurenine pathway and its ring opening is the final enzymatic step from tryptophan to quinolinic acid (QUIN). QUIN functions as an *N*-methyl-D-aspartate (NMDA) receptor agonist and elevated brain levels of QUIN have been observed in neurodegenerative diseases. Reducing QUIN levels is of pharmacological importance. Kinetic and calorimetric studies were performed on human HAO using UV/Vis spectroscopy and isothermal titration calorimetry to understand its stability and behavior using its natural substrate (3-HAA) and neurological inhibitors such as acetylsalicylic acid (aspirin). This study reveals the first analysis of human HAO and facilitates understanding of its binding dynamics and enzymatic activity which will later support discovering suitable pharmacological compounds.

INDEX WORDS: Dissociation constant, Acetylsalicylic acid, 2-amino-3-carboxymuconic acid semialdehyde, Picolinic acid, Michaelis-Menten constant, Kynurenine pathway

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A Thesis Submitted in Partial Fulfillment of the Requirement for the Degree of

Master of Science

In the College of Arts and Sciences

Georgia State University

2016

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May 2016

DEDICATION

This paper is dedicated to my loving mother, Cecile De Silva and to my loving wife Dena Phillips Henry. They are both the inspiration that contributed to my resolve and perseverance I displayed through my graduate career.

ACKNOWLEDGMENTS

I would like to thank Dr. Aimin Liu for the opportunity to work with his group. Also, I would like to thank my senior lab mates, Kenderlin Dornevil and Ian Davis for their hard work and advisement they've giving me in the past couple of years. Lastly, a great thank you to those individuals who didn't believe in me.

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1 Non-Heme iron enzymes vs. Heme iron enzymes

There are a number of chemical reactions that certain enzymes perform with the assistance of an organic or inorganic molecule called a prosthetic group, a tightly bound coenzyme (1). Specifically, organic prosthetic groups may be a vitamin, sugar or lipid. Moreover, inorganic prosthetic groups can simply be a metal ion. A heme prosthetic group consists of a protoporphyrin ring and a central iron atom. The central iron (Fe) atom can either be in the ferrous (Fe^{2+}) or the ferric (Fe^{3+}) oxidation state. The protoporphyrin ring is made up of four pyrrole rings linked by methine bridges which comprises of four methyl, two vinyl, and two propionate side chains attached (1). Non-enzymatic and enzymatic proteins that utilize the heme prosthetic group are called hemoproteins. Hemoproteins are able to conduct various biochemical functions which includes oxygen transport and storage, gas sensing, electron transfer, and chemical catalysis. In nature, the usage of heme iron for dioxygen activation and oxygen insertion into organic substrates is common. For example, enzymatic protein such as tryptophan 2, 3-dioxygenase has the ability to bind to oxygen only when there is a bound prosthetic heme group present. The iron atom is positioned in the middle of the protoporphyrin, bonded to the four pyrrole nitrogen atoms. Furthermore, only the ferrous state Fe^{2+} has the ability to bind to molecular oxygen which renders ferric state Fe^{3+} inactive due to its inability to bind. The Fe^{2+} has the capability to form two additional bonds on each side of the heme plane and these binding sites are called the fifth and sixth coordination sites (1). Myoglobin, a non-enzymatic protein, its fifth coordination site is engaged by the imidazole ring of a histidine from within, it is also known as the proximal histidine. The sixth coordination site is where oxygen binding occurs within the myoglobin protein. Upon binding oxygen to the sixth coordination site, reassembly of the electrons within the iron atom allows it to be efficiently smaller, thus promoting its

movement within the plane of the porphyrin. The three dimensional structure of myoglobin has shown a significant change when it binds to the iron atom. The interaction between iron and oxygen in myoglobin is viewed as a composite of resonance structures, one with Fe^{2+} and dioxygen and the other with Fe^{3+} and superoxide ion (1). Electron transfer from the ferrous ion in heme to oxygen is a binding parameter which creates a ferric ion and superoxide anion complex. The distal histidine stabilizes the oxygen complex by donating a hydrogen bond to the oxygen which prevents the release of superoxide because this can be damaging to biological material. Hemoglobin is a non-enzymatic hemoprotein that is found in red blood cells and consists of four myoglobin-like subunits. Specifically, two identical alpha chains and two identical beta chains are the four different polypeptide chains that give the tetramer composition of hemoglobin. Each subunit holds a heme prosthetic group that allows for efficient transportation and release for oxygen molecule throughout the body. Cooperative binding and release of oxygen is the essential reason why hemoglobin is one of the most important heme-proteins in the body, it delivers 10 times as much oxygen as could be delivered by myoglobin and more than 1.7 times as much as could be delivered by any non-cooperative protein (1).

Cytochrome P450 and its large family are cysteinato-heme enzymes which contain a prosthetic group constituted of an (Fe^{3+}) protoporphyrin IX linked to the protein by the sulfur atom of a proximal cysteine ligand. Consistent with their dioxygen activation, cytochrome P450 natural function is to catalyze the hydroxylation of saturated hydrogen bonds, the epoxidation of double bonds and the oxidation of aromatics. Cytochrome P450 performs oxidation with use of molecular oxygen by inserting one of the oxygen atoms into a substrate and reduces the second oxygen to a water molecule by way of NAD(P)H and a reductase (2). Molecular oxygen by itself is unreactive toward organic molecules at low temperatures due to high energy barriers (3).

Nature has found another route for inserting oxygen atoms into compounds via enzymes which performs the desired oxidation reaction. Enzymes such as cytochrome P450 are metal-dependent oxygenases. There are three classes of cytochrome P450. Class I is designated to mammalian mitochondrial enzymes which are involved in steroid syntheses, furthermore, iron-sulfur cluster is utilized for the transfer of electrons. Class II refers to mammalian enzymes located in the endoplasmic reticulum of liver cells, which are involved in drug metabolism and utilizes FAD and FMN to transfer electrons from NADPH to the monooxygenase protein. Lastly, all cytochrome P450s are part of a multienzymatic assembly that performs numerous biological functions. Tryptophan 2,3-dioxygenase (TDO) is a heme-dependent dioxygenase in which it utilizes a histidine-coordinated iron to facilitate dioxygen activation and oxygen insertion reactions. TDO plays a significant role in tryptophan metabolism, by employing a b-type ferrous heme to catalyze the oxidative cleavage of the indole ring of L-tryptophan (L-Trp), converting it to *N*-formylkynurenine (NFK) (4-6). TDO is the first enzyme in the kynurenine pathway and designated as the initial and rate limiting step, furthermore, this is the primary route for L-Trp degradation in mammals, specifically within the liver organ. Addition to the kynurenine pathways role in L-Trp degradation, it constitutes the major part of the de novo biosynthesis of nicotinamide adenine dinucleotide (NAD), a redox cofactor that is essential for life sustainment, in eukaryotic organisms and in a few bacterial species (7, 8).

Heme systems can be studied spectroscopically due to their intense spectral feature characteristics of the porphyrin ligand. Hemeproteins electromagnetic properties allows for easier understanding of catalysis whereas non-heme enzymes become more difficult to study because they do not exhibit the intense spectral features characteristic of the porphyrin ligand. A widely used spectroscopic technique for delineating mechanistic properties of heme proteins is

EPR (Electron Paramagnetic Resonance) spectroscopy. EPR detects species that have unpaired electrons, this includes free radicals and many transition metals ions. Because of the EPR sample sensitivity to local environments, it provides information of the molecular structure near the unpaired electron. An important feature found in heme systems is the π-π^* transition characteristic of the porphyrin ligand when combined with valance state and coordination of the metal allows for efficient study of the hemeprotein in question (9). However, active sites that are within a ferrous state are often EPR silent and cannot be investigated through conventional EPR affiliated techniques. The most popular non-heme proteins Fe^{2+} active sites are composed of two His, one monodentate carboxylate, and two to three water ligands (10). In comparison to heme sites which have the protoporphyrin that has only one axial position for molecular oxygen to bind and proceed with activation, there are additional and also exchangeable positions that can facilitate the possibility of substrate and/ or cofactor binding to the ferrous iron and new coordination modes for oxygen activation observed in the non-heme Fe^{2+} active sites. Furthermore, the electronic structure of the non-heme Fe^{2+} active site can vary greatly due to the His/carboxylate/H_2O ligation as opposed to heme sites, this contributes to why it exhibits limited π-interactions with iron.

2 Dioxygen activation by Mononuclear Non-Heme enzymes

Mononuclear non-heme iron active sites that require dioxygen activation are prevalent in many metabolically important reactions, each shows some type of pharmaceutical significance. The dioxygen activation is quite unique. Dioxygen serves multiple roles that have a strong impact on aerobic life. Oxidative phosphorylation is facilitated by dioxygen functioning as the terminal electron acceptor which produces the energy rich molecule ATP that is needed through a number of metabolic processes. Insertion of the oxygen atom into a number of essential

biological molecules is another role of the dioxygen molecules serves; steroid hormones, aromatic amino acids, neurotransmitters, signaling molecules, and regulatory factors require dioxygen to be formed (16).

Dioxygen is a stable molecule with a high potential reactivity which makes it the desirable reagent that so many biological systems use. Its molecular structure, triplet ground state of dioxygen is the product two unpaired electrons in degenerate molecular orbitals makes the direct reaction with singlet molecules which is the spin-paired state of most potential reaction partners a unfavorable process (18). The understanding of how oxygen activation is conducted is relevant to number of areas in medical and industry field. Living in a sea of oxygen that is part of various biological reactions promotes inquiry to how the dioxygen is activated and incorporates the oxygen atom into these molecules. This information can be advantageous to developing new compounds that can combat variety of diseases and aid in cultivation of new biofuels that can be essential to the environment.

Oxidase and oxygenase enzymes activates dioxygen from its common triplet ground state to the reactive singlet or doublet species. These reactions are dependent on the specific arrangement or orientation of the insertion molecule reacting with oxygen. Generally, enzymes are aided by transition metals such iron, copper, or manganese and even organic cofactors such as heme molecules. The advance in biophysical instruments allows for further understanding of the activation of biological oxygen activation through the systematic use of computation, spectroscopic, and structural techniques (19). Oxygen-activating enzymes with mononuclear non-heme iron active sites are prevalent in the environment, pharmaceutical development, and medical field due to the fact they participate in metabolically important reactions. Every mononuclear non-heme iron enzyme is functionally and geometrically unique to each other due

to their different requirements for activity, various endogenous ligand set, and their coordination and geometry differ across the spectrum. For example, lipoxygenase requires Fe(III) to be active and holds 3 histidines, 1 isoleucine bound to carboxylate group, and asparagine for its endogenous ligand set (21). Lipoxygenases are responsible for oxidizing unsaturated fatty acids into precursors of ketotrienes and lipoxins, potential targets for anti-inflammatory drugs. Dioxygen activation is specific to the mononuclear non-heme iron being used and results in various but similar reaction mechanisms for this group.

3 Extradiol catecholic dioxygenases

Catechol dioxygenases are responsible for nature's ability to degrade aromatic molecules in the environment, moreover, the transformation from aromatics into aliphatic products is considered the last step in this degradation process. Extradiol catecholic dioxygenases are part of the catechol dioxygenase family that utilizes an Fe(II) ion for catechol cleavage of aromatic compounds, while intradiol catecholic dioxygenases utilize an Fe(III) ion for oxidative ring cleavage (22). Both extradiol and intradiol dioxygenases cleave the aromatic ring by way of inserting both atoms of dioxygen which produces muconic semialdehyde adducts (23). In recent years structural evidence shows that the active site is composed of Fe(II) with a 2-His+Asp/Glu facial triad coordination. During the catalytic process, aromatic compounds has been suggested to provide the necessary electrons to activate dioxygen due to the simple fact there is no reductant required for catalysis. The extradiol dioxygenase 2-His+Asp/Glu ligand coordination is prevalent throughout its family with similar mechanistic approach. Catecholic substrate forms a chelate complex with the extradiol dioxygenase Fe(II) active site, subsequently binds dioxygen to an adjacent metal ligand site (24). Substrate and dioxygen are activated in concerted mechanism by transfer of an electron through the metal cofactor. Alkylperoxo intermediate is

created due to the enforced geometry of the metal complex, subsequently, Criegee rearrangement is observed and the alkyperoxo intermediate is broken down to a lactone form. Before the final product is formed, heterolytic O-O bond cleavage during the catalytic process would be enabled with the protonation of the oxygen atom bound to the iron by an active site acid mechanism. Hydrolysis of the lactone is conducted by the remaining oxygen atom from dioxygen that bound to Fe(II) in the initial catalytic process, which allows for the release of open ring product. This mechanism was recently highlighted by a review article (25). Support has been derived from the combination of spectroscopic studies and crystal structures of specific enzymes within the extradiol dioxygenase family. For example, *Brevibacterium fuscum* homoprotocatechuate 2,3-dioxygenase (HPCD) has been trapped within a single crystal, following this was reacting the crystals to soak in a substrate analogue, 4-nitrocatechol, in the presence of low concentration of dioxygen before cryo-freezing (26). In this study, three intermediates were found, specifically in various subunits of the enzyme HPCD. HPCD is a homotetrameric enzyme which the above study described one subunit contained the chelated substrate-Fe(II)-O_2 complex, this type of side on configuration places the oxygen in correct alignment for reacting with the specific carbon of the 4-nitrocatechol. Furthermore, 4-nitrocatechol suggested to transfer electron to the oxygen to form superoxide which is supported by the long Fe-O bond lengths and its sustained Fe(II) state. Alkylperoxo intermediate were found in two other subunits of HPCD crystals and Fe(II)-product complex was found in the fourth subunit of HPCD. Further structural studies within the above study details the two important residues within the HPCD mechanism, Tyr257 and His200. Tyr257 primarily role is to draw substrate into the reaction intermediates and His200 performs as an acid catalyst, ensuring activated oxygen species is unstable which it allows it to utilized in the catalytic process of HPCD. There are a number of enzymes that have the similar 2-His+Asp/Glu

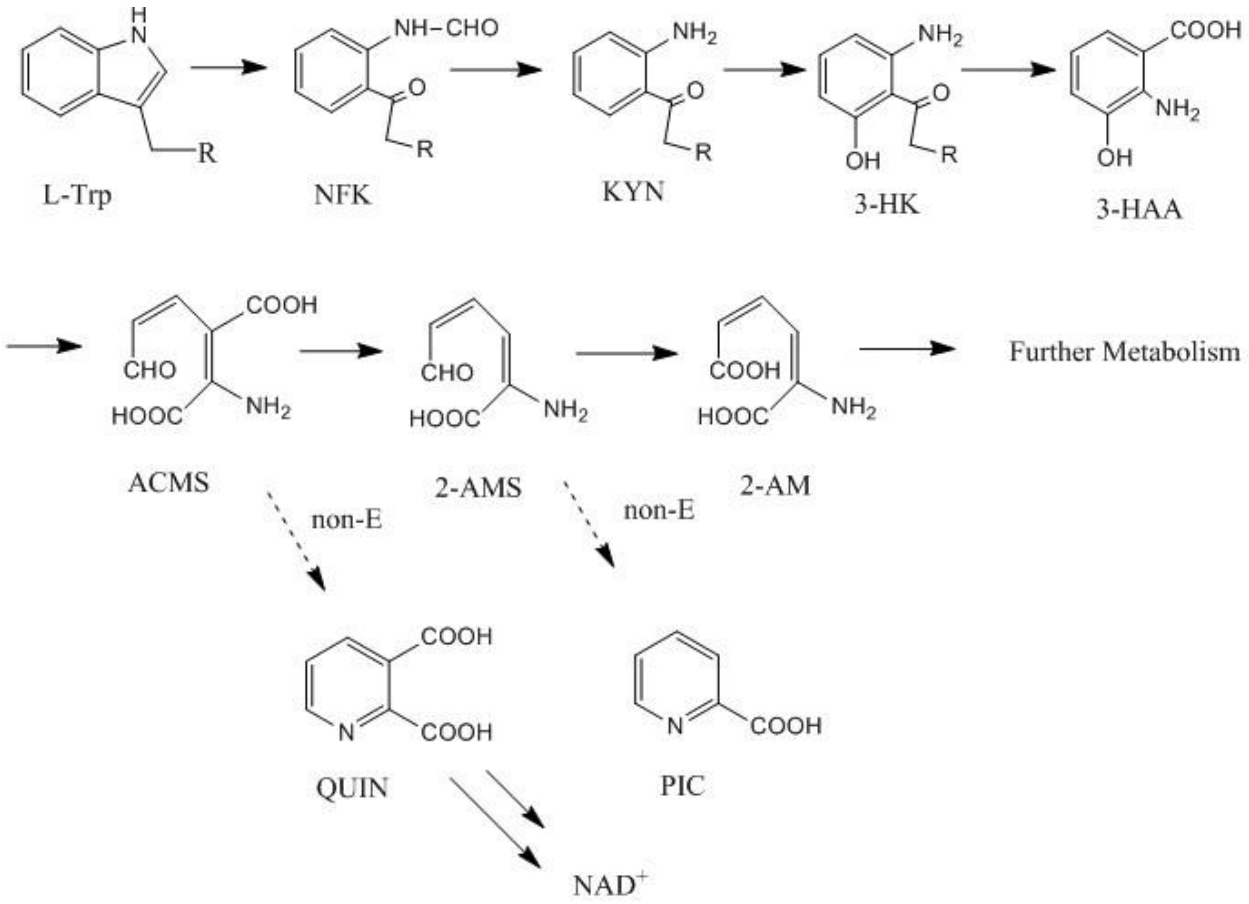
binding motif as extradiol catechol dioxygenase, which is essential to oxygen activation and allows for oxygen insertion. Further studies should focus more on the regulation and manipulation of the 2-His+Asp/Glu to harness the dioxygen activation power.

4 Kynurenine Pathway: Tryptophan Metabolism

There are 20 amino acids that proteins are composed of, 9 of these amino acids are essential to the human body due to the inability to synthesize which means external intake is required. Tryptophan is one of the 9 essential amino acids that is utilized in the biosynthesis of proteins and serves as a precursor to several neurological compounds. After the initial process of absorption through the body, tryptophan travels throughout the periphery circulation via bound to albumin or in its free form, however the two states are in equilibrium but the majority of tryptophan is bound to albumin (27). Moreover, passing through the blood-brain barrier requires facilitation with a large neutral amino acid transporter (28). Upon arriving in the central nervous system, tryptophan acts as a precursor to several metabolic processes such as the kynurenine pathway and the serotonin pathway. Within the peripheral nervous system and the central nervous system, 95% of tryptophan is metabolized through the kynurenine pathway (29).

Based on the schematic (Scheme 1) of the kynurenine pathway, tryptophan (L-Trp) is oxidized by cleavage of the indole ring which is initiated by tryptophan 2, 3-dioxygenase (TDO), indoleamine 2, 3-dioxygenase 1(IDO-1) or IDO-2 (30-32). All three are identified as rate limiting enzymes of tryptophan metabolism that produces the *N*-formyl-$_{L}$-kynurenine (NFK). Furthermore, each are induced differently throughout the body, TDO is found in the liver and is induced by tryptophan or corticosteroids, as opposed to IDO-1 which resides in numerous cells, including macrophages, microglia, neurons, and astrocytes. (33-35). Furthermore, IDO-1 is induced by immunological responses or in the presence of cytokines, inflammatory molecules

and more emphatically response is viewed by interferon gamma (IFN-γ) (36-37). With the production of *N*-formyl-L-kynurenine, it's then converted to L-kynurenine (KYN) by the second enzyme within the kynurenine pathway, formamidase. L-kynurenine is metabolized by three different routes within the kynurenine pathway to form kynurenic acid, 3-hydroxy-L-kynurenine, and anthranilic acid by the function of kynurenine aminotransferase, kynurenine 3-monooxygenase, and kynureninase enzymes respectively.



Scheme 1 The Kynurenine pathway is the catabolic processes of L-tryptophan.

Along the pathway, formation of 3-hydroxyanthranilic acid (3-HAA) is produced by kynureninase from 3-hydroxy-L-kynurenine (3-HK) or by nonspecific hydroxylation of

anthranilic acid. 3-hydroxy-3,4-dioxygnase converts 3-hydroxyanthranilc acid to 2-amino-3-carboxymuconate-semialdehyde (ACMS) which is an unstable compound that non-enzymatically converts to quinolinic acid (QUIN), a precursor to NAD^+ and $NADP^+$, known nicotinamide cofactors. Lastly, ACMS converts to 2-aminomuconic semialdehyde (2-AMS) by α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase, which 2-AMS is converted to picolinic acid (PIC) via a non-enzymatic process (38).

Quinolinic acid is a structural analogue of neurotransmitters such as glutamate and aspartate that functions as an agonist to N-methyl-D-aspartate (NMDA) receptors (39). Excessive activity of the NMDA receptors have been attributed to the activation of the kynurenine pathway which produces above basal level of quinolinic acid that has been confirmed to be associated with several neurological diseases such as Alzheimer's disease, anxiety, depression, epilepsy, human immunodeficiency virus-associated neurocognitive disorders and Huntington's disease (40-42). Quinolinic acid has been located within macrophages, microglia and dendritic cells when inflammatory conditions in the brain are induced (43). Through *in vitro* study, the synthesis of quinolinic acid by CD8 dendritic cells induced apoptosis in Th1 target cells and quinolinic acid can also selectively inhibited the proliferation of CD4 and CD8 T lymphocytes and natural killer cells undergoing activation (44, 45). Axon-sparing lesions in rat models have been induced by the injection of quinolinic acid into discrete areas of the brain (46). There has been an increasing amount of data in the past half century that continues to support that quinolinc acid plays a major role in neurological diseases and immunological disorders. Thus, therapeutic methods should concentrate its effects on inhibiting/ regulating it production. In the central and peripheral nervous system, quinolinic acid is formed by 3-hydroxyanthranilinc acid 3,4-dioxygenase (HAO), which catalyzes the oxidative ring opening of the kynurenine

pathway metabolite 3-hydroxyanthranilinic acid, leading to the unstable intermediate 2-amino-3-carboxymuconic semialdehyde (ACMS) (47). Due to ACMS instability, it spontaneously cyclizes to quinolinic acid, which is then further converted to NAD^+. Efforts to regulate the quinolinic acid levels can be established with detail study of HAO and its mechanistic process of ACMS production, therapeutically making it a primer pharmacological target.

5 3-Hydroxyanthranilate-3,4-dioxygenase

The final enzymatic step in tryptophan catabolism to quinolinic acid is conducted by 3-hydroxy-3,4-dioxygenase (HAO) by catalyzing the oxidative ring opening of 3-hydroxyanthranilic acid. The biosynthesis of quinolinic acid demands interest due to its role in neurological disorders. HAO is a non-heme iron (II)-dependent extradiol dioxygenase that requires the activation of a dioxygen molecule to perform its catalytic function (49). It has been well documented that HAO plays a significant role in the kynurenine pathway in tryptophan metabolism, but HAO has also been found in the prokaryotic 2-nitrobenzoic acid biodegradation pathway (50). The crystal structure HAO was first determined from *Cupriavidu metallidurans*, which elucidated that this enzyme is part of the cupin superfamily (51, 52). The Ealick group discovered each monomer of HAO contains two iron binding sites which one is responsible for catalytic function and another resembling a rubredoxin site (51). Furthermore, the active site was reported to have a complex functionality with residues that play key significant roles in the catalytic mechanism of HAO: iron binding residues consisted of 2-His-1-carboxylate facial triad that anchored the catalytic iron, substrate binding residues that consisted of Asn27, Arg99, Glu110, and oxygen binding residue Arg47, which stabilizes the position of oxygen for Fe-O bond (51). Rubredoxin site found within HAO crystal structure was 24 angstroms away from the catalytic iron atom and it is coordinated by sulfur atoms derived from four conserved cysteine

residues at the C terminus, which forms somewhat tetrahedral FeS_4 center (51). The role of the FeS_4 centers has been suggested to function as electron carriers in most catalytic mechanisms (53).

For some time it has been unclear what role FeS_4 plays in HAO activity or even does it have some significance structurally has been the two questions that many studies couldn't answer. Recently it has been discovered that the role of the FeS_4 is to function as a "spare tire," or an iron reservoir (54). The above study provided theoretical and experimental data to support once the catalytic iron has been removed by downstream metabolite, intermolecular iron shuttling from the FeS_4 to the active site restores catalytic function within an apoenzyme (54). The binding affinity of the catalytic and FeS_4 site has a binding energy difference of ~10 kcal/mol, based on metal-binding dynamics, redistribution of the iron atom in FeS_4 to catalytic site is confirmed (54).

Human HAO (hHAO) is a monomeric bicupin protein consisting of 280 amino acids and a molecular weight of 33 kDa. Like many of its members within the HAO family, the enzyme becomes inactive after purification but activity can be restored through reconstitution/incubation with Fe^{2+}. hHAO lacks the rubredoxin like site that is so famously known for in prokaryotic HAO structures (51) (PDB entry 2QNK). Furthermore, its catalytic function has been consistent with the rest of the HAO family, catalyzing the ring opening of 3-hydroxyanthranilate acid (3-HAA) within the kynurenine pathway to facilitate the non-enzymatic production of quinolinic acid (46). A recent computationally study of hHAO suggests the significance of the Arg43, Arg95 and Glu105, which are conserved active site residues also found in the bacterial HAO proteins (55). Another significant fact was the catalytic mechanism proposed by Zhang et al. coincided with the modeling study, which suggested the reaction to exergonic with the final

energy of the system to be 59.8 kcal mol^{-1} lower than the initial and highest energy barriers (55). The point of this current study is to understand hHAO binding dynamics, its potential inhibition with known neurological inhibitors, and whether it's a regulatory site within the kynurenine pathway.

6 Experimental Data

Chemicals- 3-hydroxyanthranilate acid, picolinic acid, acetylsalicylic acid, ammonium ferrous sulfate hexahydrate, ascorbate, EDTA, Tris base, and glycerol were purchased from Sigma at the highest grade available.

Protein Preparation- hHAO plasmid was purchased from DNASU plasmid repository. Overexpression and protein purification conducted according to a published method (46). All catalytic activity assays were performed in 50 mM Tris-HCl, pH 7.6, and 5% glycerol. The metal-free, apo-form of hHAO was prepared by overnight treatment with EDTA (10 mM) at 4°C, followed by dialysis and gel filtration chromatography for removal of EDTA. The fully iron-loaded hHAO (holo-hHAO) was obtained by adding 2 eq of Fe^{2+} (from a fresh O_2-free solution of ammonium ferrous sulfate) to apo-hHAO under anaerobic conditions. Excess iron ions were removed by gel-filtration chromatography using argon-saturated buffer.

Inhibition Assays - The inhibition assays were performed in 50 mM Tris-HCl buffer (pH 7.6, 5% glycerol) as previously described using an Agilent 8453 spectrophotometer. The rate of enzymatic reactions was monitored based on formation of deoxygenation product, 2-amino-3-carboxymuconic semialdehyde (ACMS), at 360 nm $\varepsilon_{360\ nm}$ = 47,500 M^{-1} cm^{-1} (46). A typical reaction mixture consisted of 5-100 μM 3-HAA, fixed concentration of acetylsalicylic acid (ASA) for each Michaelis-Menten (MM) plot ranging from 50-150 μM, and 500 ng of

reconstituted hHAO diluted to 500 μL with 50 mM Tris-HCl pH 7.6 buffer. The above procedure was conducted in the same manner for picolinic acid (PA) at fixed concentration for each MM plot. For the determination of kinetic parameters, initial rates of product formation were plotted versus the substrate concentration and fitted to the Michaelis-Menton equation using Origin 8.5. Competitive, Mixed-typed, and Non-competitive inhibition equations were tested using Origin 8.5 to identify PA and ASA mode of inhibition.

Oxygen Affinity Assay- The activity of hHAO was measured by monitoring the decay of dioxygen within a finite chamber using a Perspex oxygen electrode. The chamber was saturated with a specific concentration of O_2 ranging from 100-700 μM with a fixed concentration of 100 µM of 3-HAA. Once the chamber was stabilized with the specific O_2 concentration, reconstituted hHAO was injected into the chamber with a fixed concentration of 1 μg diluted to 1 mL with 50 mM Tris-HCl 7.6 pH buffer. For the determination of kinetic parameters, initial rates of O_2 decay were plotted versus the O_2 concentration and fitted with to a Hill/MM equation using Origin 8.5.

Isothermal Titration Calorimetry Assay- ITC experiments were conducted in an oxygen-free environment and performed at 10°C on a MicroCal VP-ITC. The sample cell contained 20 μM of hHAO with a volume of 1.5 mL using a 10 mM potassium phosphate pH 7.8 5% glycerol and .01% dimethyl sulfoxide (DMSO). 3-HAA was dissolved in DMSO and diluted to 100 μM in 280 μL syringe, ASA was also dissolved in DMSO and diluted to 200 μM for the second assay, and each ligand used the same buffer as the sample cell. The ligand solution was titrated into the protein solution; 28 injections (10 μL) of 4.8 seconds duration were made at 200 s intervals. Control experiments of ligand to buffer showed insignificant heats ensuring the DMSO percentage was matched in both sample cell and syringe. The data were processed and

thermodynamic parameters obtained by fitting the data to single-site-binding model using Origin 7.0 and fixing the stoichiometry as 1.0 for 1 binding site. All data from ITC measurements are shown in figures 2 and 3.

7 Results

Inhibition Assay using Acetylsalicylic acid. ASA inhibited the activity in hHAO production of 3-HAA to ACMS. Various inhibition equations were used for the fitting with Origin 8.5 (56). The experimental data fit well with both mixed-type and non-competitive inhibition with an adjusted chi square of .97505 and .97, respectively. The adjusted chi-square values represents how well the data fits with its respective enzyme inhibition equation. Ultimately, using ASA as an inhibitor decreases the activity of hHAO significantly with inhibition constant (K_i) of 168 μM, which is shown in figure 1 and 2.

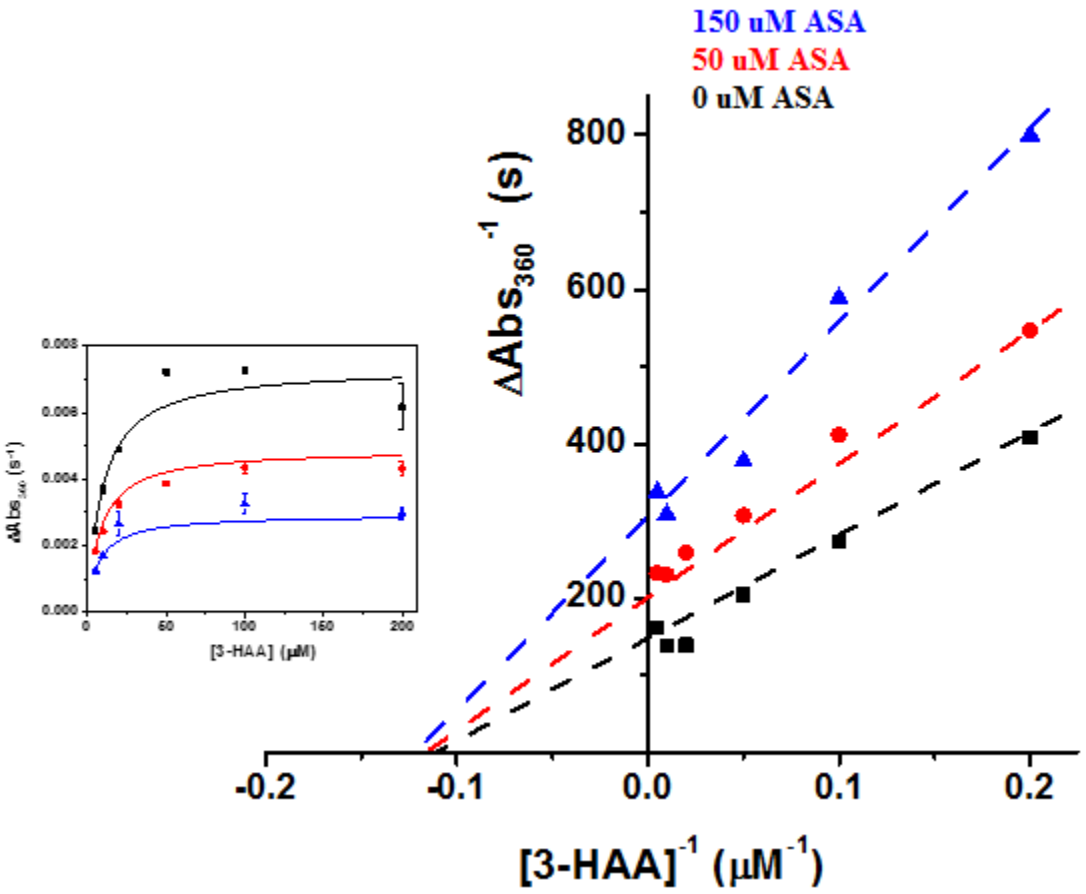


Figure 1 Michaelis-Menton plot to the left and Lineweaver-Burk plot in the center.

ASA was fitted to the Mix-type inhibition equation using Origin 8.5 and produced an adjusted chi-square of .97505. Each color trace represents the fixed concentration that ASA was used to conduct the assay.

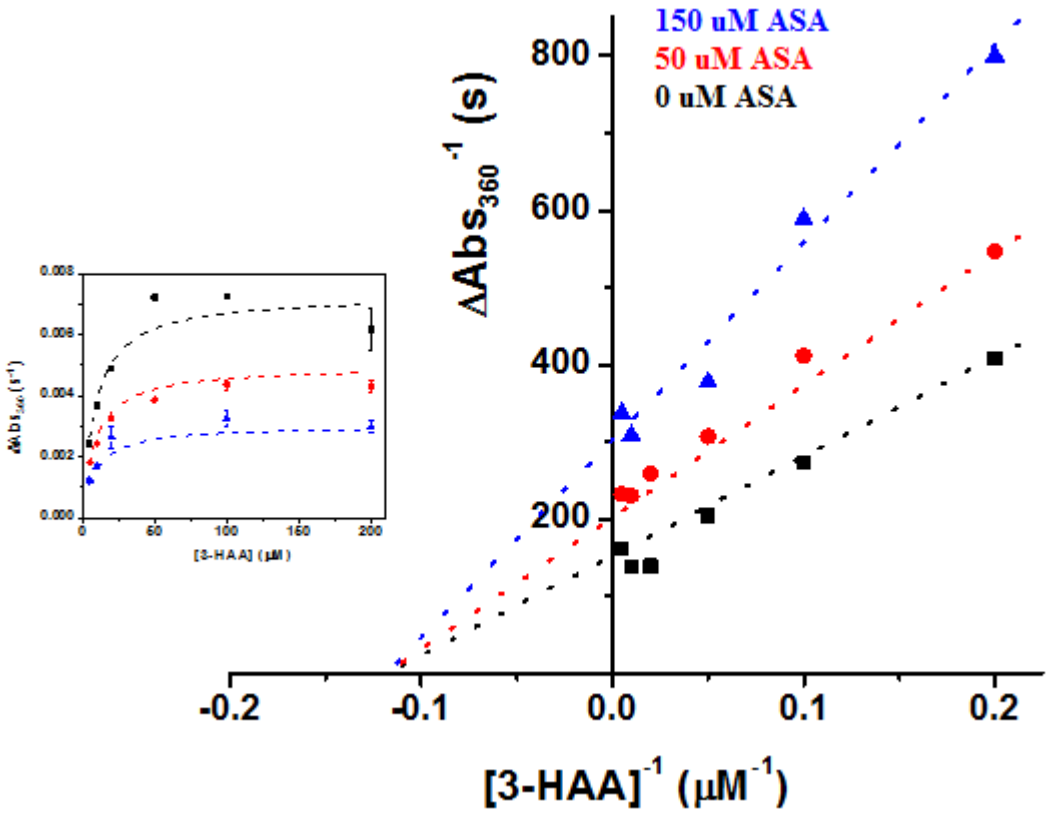


Figure 2 Michaelis-Menton plot to the left and Lineweaver-Burk plot in the center.

ASA was fitted to the non-competitive inhibition equation using Origin 8.5 and produced an adjusted chi-square of .97. Each color trace represents the fixed concentration that ASA was used to conduct the assay.

Inhibition Assay using Picolinic Acid. As evident in Fig. 3, picolinic acid performs as a competitive inhibitor, which decreases the activity of hHAO significantly. The inhibition constant is 131 μM bring activity down by 30% when compared to absence of picolinic acid in the assay. The experimental data was fit with the competitive inhibition equation with a reduced chi square of .9919.

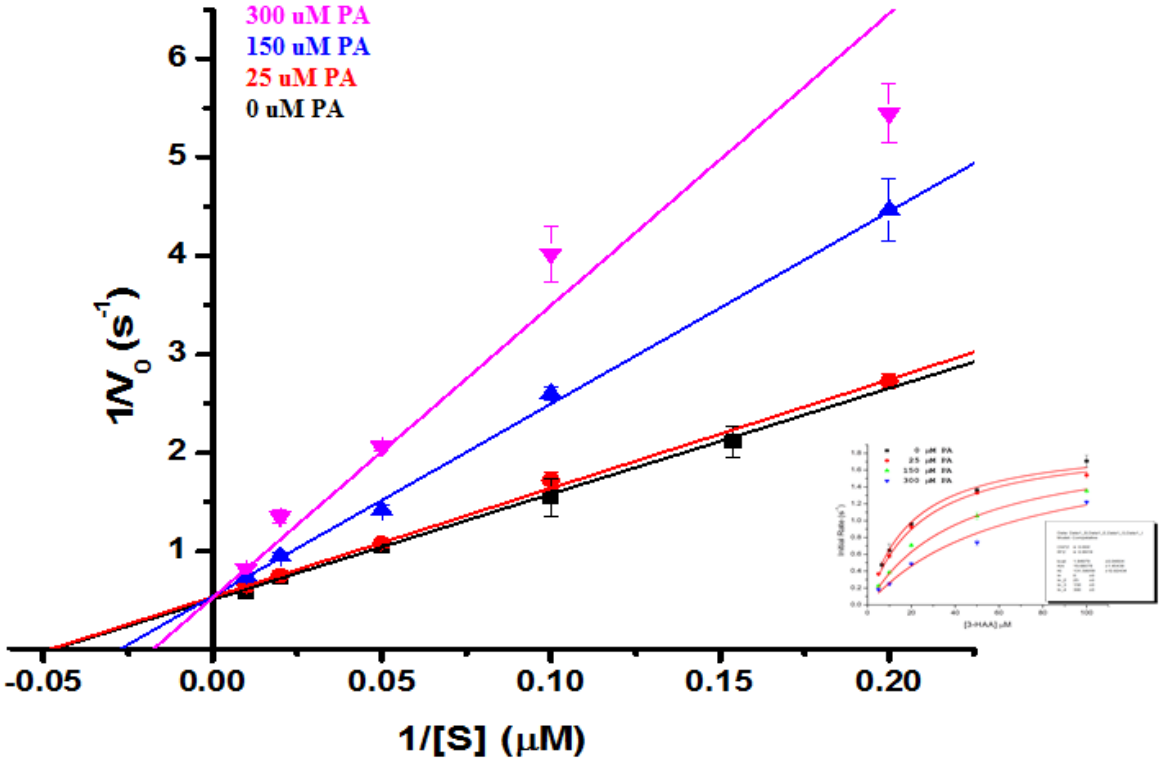


Figure 3 The inhibitory effect that Picolinc acid has on hHAO activity is shown in the Lineweaver-Burk plot and in the Michaelis-Menton plot in right corner. Competitive inhibition constant was shown to be 131 μM.

Oxygen Affinity Assay. The theoretical Michaelis-Menton constant (K_m) for oxygen is estimated to be 225 μM. hHAO becomes saturated between 400 to 500 μM of oxygen. Furthermore, the catalytic turnover (K_{cat}) is calculated to be ~2 per second within this experiment. Oxygen concentration exceeding 500 μM shows to decrease hHAO activity.

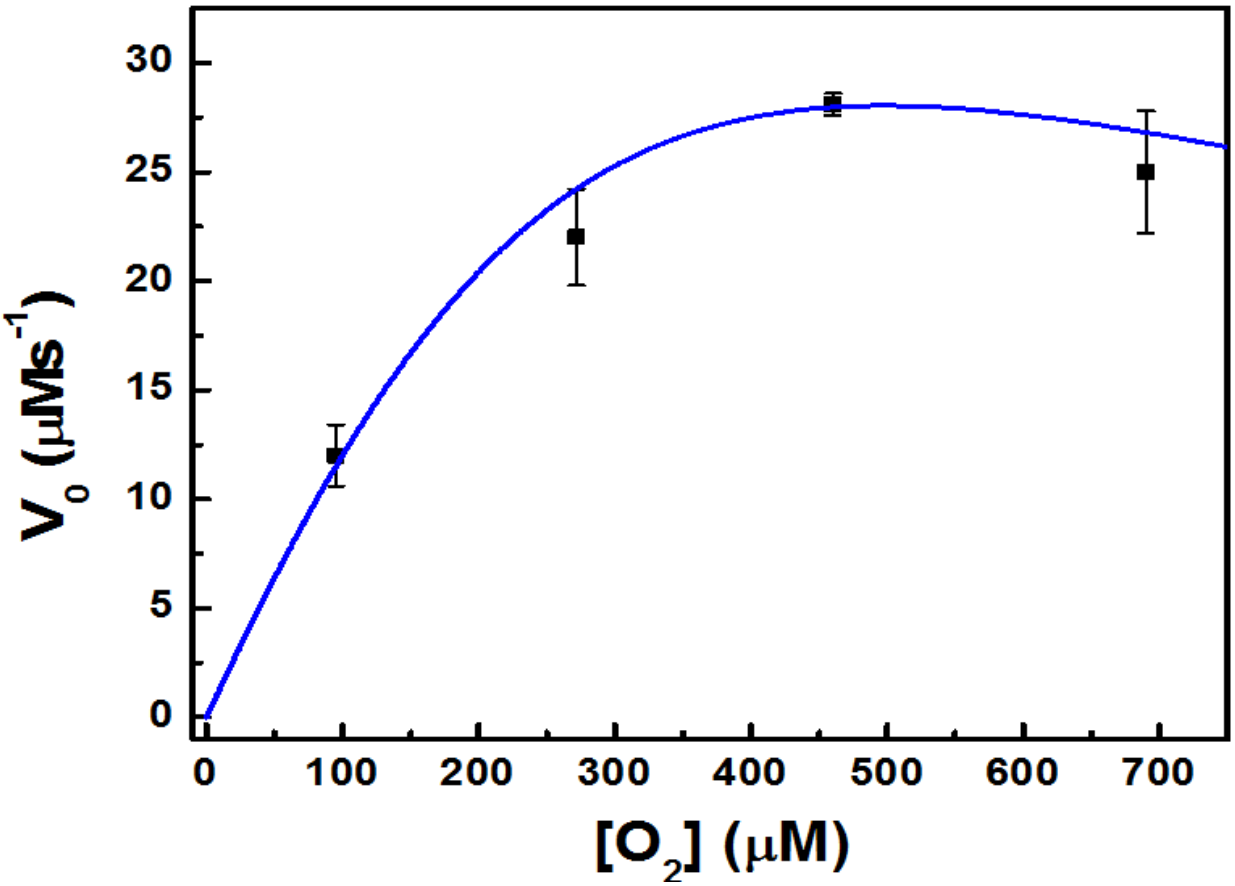


Figure 4 A spline curve is shown in the Michaelis-Menton plot for O_2 affinity at standard temperature and pressure.

Isothermal Titration Calorimetry Assay. ITC data shows (Fig. 5) that binding of 3-HAA to hHAO is exergonic process by which producing heat energy of -70 kcal per mol. The N-value is .966 and C value was calculated as 369 (57). The N value represents the average number of binding sites per mole of protein in the solution. The C value is the critical parameter that determines the shape of the binding isotherm and is the product of the binding constant, initial macromolecule concentration in the cell and the stoichiometry parameter according to MicroCal. C values between 10 and 500 are considered optimal for determining the accuracy of an isotherm. The entropy was calculated as -215 cal per mole degree and the equilibrium constant (K_A) was measured as 2.19×10^{-7} M^{-1}

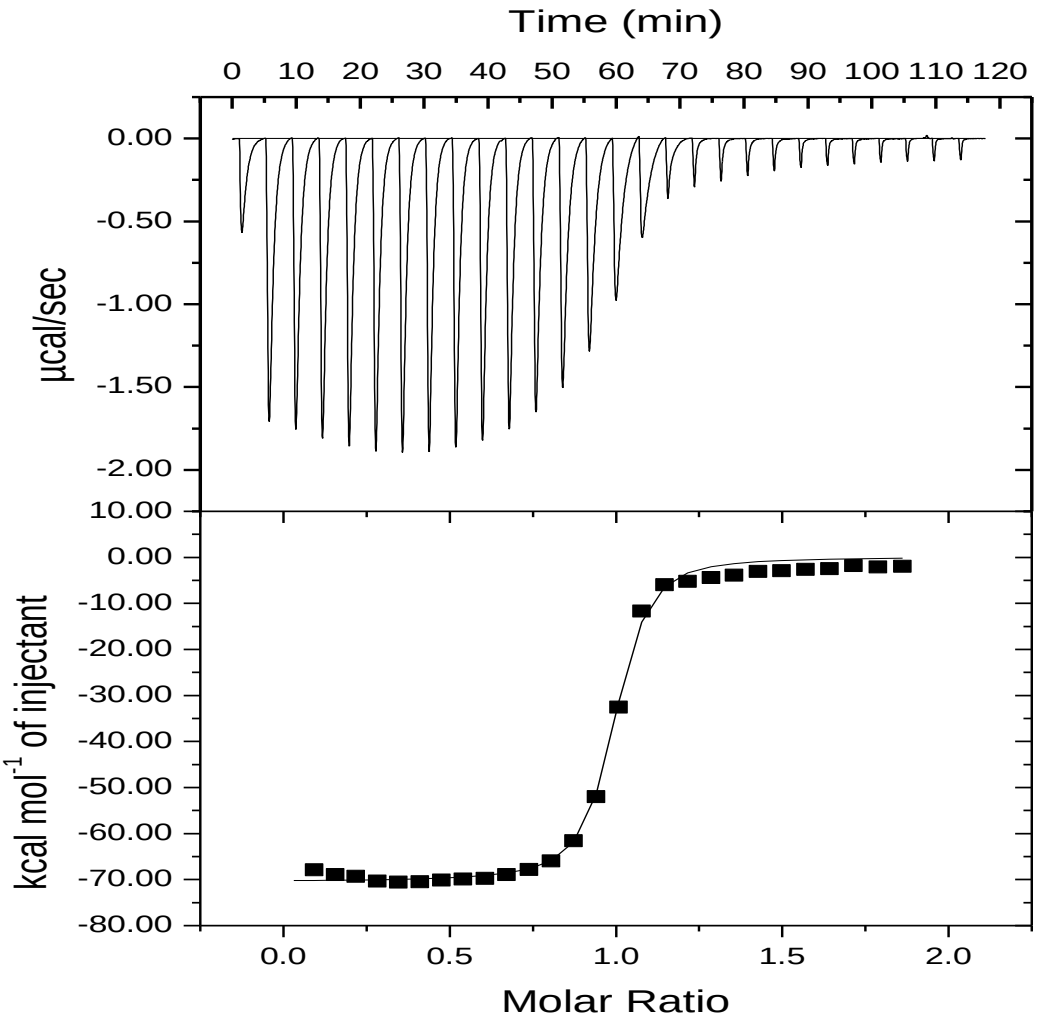


Figure 5 Shows the isotherm profile of hHAO being titrated with 3-HAA.

Upon the first injection heat is released in the amount of -70 kcal per mol. Saturation begins at 1 molar ratio with a decline in heat production.

Isothermal Titration Assay using Acetylsalicylic Acid hHAO was titrated with ASA producing heat in the amount of -2 kcal per mol. Saturation begins at .7 molar ratio and continues until reaching 2.0 molar ratio where it starts to plateau.

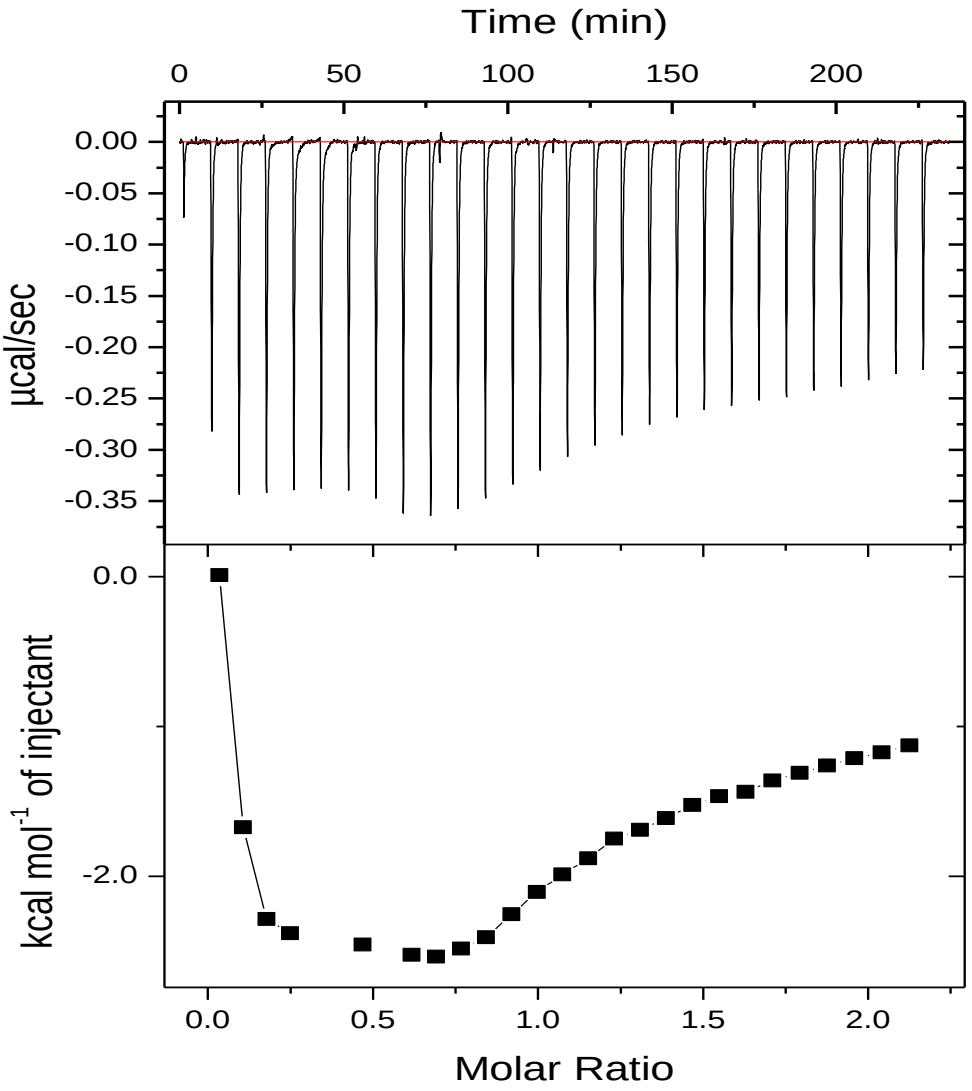


Figure 6 Isotherm profile of hHAO titrated with acetylsalicylic acid shows binding between the two macromolecules with a ΔH of -2.0 kcal per mol.

8 Discussion

Inhibition of hHAO using ASA. Acetylsalicylic acid also known as aspirin, the nonsteroidal anti-inflammatory drug, has found a new function within this study. Its ability to reduce hHAO activity has been confirmed. Inhibition constant (K_i) was calculated to be 169 μM. The non-competitive and mixed-typed inhibition models both fit well with the experimental data.

ASA has been shown in previous studies to possess antioxidant activity at inhibiting quinolinic acid induced superoxide anion generation, lipid peroxidation and maintained cell integrity in rat hippocampus (58). In addition to the oxidative protection, the above study has shown both acetylsalicylic acid and acetaminophen can inhibit the activity of rat liver HAO individually, moreover, enzyme activity is reduced by 77% when both compounds are combined the assay (58). ASA has the ability to bind to iron coordinated complexes such as Fe^{2+} and Fe^{3+} (59), suggesting that its mechanism of inhibition is ligation of the catalytic iron ion within the active site of HAO enzyme, contributing to the reduced activity in the presence of ASA. The theory of ASA binding to the active site in some facet is supported by the ITC data in Fig. 5. Once ASA is injected into the sample cell with hHAO, there is a production of heat in the amount -2 kcal per mol which indicates binding between the two macromolecules. These findings support further research into ASA as a scaffold in which new pharmacological compounds can be derived from to effectively inhibit hHAO activity.

Picolinic acid inhibitory effect. Based on the Fig. 3, picolinic acid has the ability to inhibit the activity of hHAO. The calculated inhibition constant (K_i) was calculated as 132 μM during this assay and according to the Lineweaver-Burk plot, picolinic acid performs as competitive type inhibitor. As a competitive inhibitor, picolinic acid competes for the active site of hHAO against 3-HAA. Picolinic acid and 3-HAA are very similar in structure and contribute to the competitive inhibition that is observed in this study. It has been recently discovered that picolinic acid has the ability to chelate and remove the catalytic iron from a bacterial HAO (54). The above study suggested that picolinic acid has taken advantage of the HAO active site which holds two weakly associated water ligands, thus allowing for binding followed by removal of the catalytic metal (54).

Picolinic acid is a downstream metabolite within the kynurenine pathway and its mechanism of inhibition, which effectively chelates and removes catalytic iron from the active site can be categorized as feedback inhibition. Within the kynurenine pathway, nonenzymatic cyclization of 2-aminomuconate-6-semialdehyde produces picolinic acid (Scheme 1). Picolinic acid is a dead end product within the kynurenine pathway and its accumulation will lead to inhibition of HAO enzyme, which suggests that HAO is a controlled regulatory site within the kynurenine pathway during high metabolic activity.

hHAO affinity for O_2. The Michaelis-Menton (MM) constant (K_m) for O_2 was measured at ~150 μM. This was a significant value since oxygen solubility in liquid is 258 μM at standard temperature and pressure. MM plot shows saturation of hHAO occurring at 470 μM, which is more than double the K_m for this reaction (Figure 4). Furthermore, there is possible substrate inhibition being seen after O_2 concentration exceeds 500 μM. Excess oxygen not being used in the incorporation can inhibit the activity of hHAO is supported by a previous EPR study where exposure HAO to oxygen results in metal ion oxidation and enzyme auto-inactivation (60). This suggests that excess oxygen which is not activated due to saturation of the active site can become toxic to the catalytic iron and reduce activity of the enzyme.

Binding affinity of 3-hydroxyanthranilic acid. hHAO binding with 3-HAA was observed in a 1 to 1 ratio and shown in the isotherm profile in (Fig. 5). N value of .966 and C value of 369 (57) indicates that the thermodynamic parameters calculated are in reasonable measure and can be used to elucidate additional information about the reaction between hHAO and its substrate 3-HAA which produces the enzyme-substrate (ES) complex. The equilibrium constant $[E][S]/[ES]= K_{eq}$ was calculated using Origin 8.5 as 2.19×10^{-7} M^{-1} With the inversion of K_{eq}, this produces $1/K_{eq}$, which equals the dissociation constant (K_d) of 46 nM. The dissociation

constant is the value of affinity between enzyme and its substrate without incorporating the complex rates of various intermediate formations and product release (61). This approach of viewing Michaelian kinetics allows for the isolation of [E] + [S] → [ES], which can help elucidate further information into the thermodynamics of hHAO and 3-HAA binding.

Upon hHAO binding to its substrate, there is a release of heat at -70 kcal per mol, indicating the binding of this reaction is exergonic process (see Fig 5). In addition to the enthalpy change (ΔH) measured at -70 kcal per mol, entropy (ΔS) was measured at -215 cal per mol degree, indicating the degree of freedom has been lost upon binding. Obtaining values of ΔH, ΔS, using the correct gas constant, and using the temperature the reaction was conducted in, Gibbs free energy (ΔG) was calculated at -9.2 kcal per mol.

9 Conclusion

To summarize, on the basis of spectroscopic analysis, this study has suggested the ability of aspirin to perform as a mixed type inhibitor by chelating the catalytic iron within the active site inducing inactivation of hHAO. In addition, a downstream metabolite has been found to inhibit the activity of hHAO by acting as a competitive type inhibitor that performs in similar action as aspirin, using chelation as a method of inactivation. Aspirin, 3-HAA, and picolinic acid have similar structures, combining this with the recent data, this warrants investigation into these inhibitory molecules to which can facilitate innovative neurological therapies that can regulate hHAO activity in various neurological disorders. Lastly, the binding parameters found within this study can contribute to the understanding of the reaction mechanism of hHAO. To the best of my knowledge, this is first time that the binding affinity of hHAO has been reported. Already this data has affirmed that the rate of catalysis in this reaction is faster than the rate of dissociation within this reaction due to K_m value 20 μM being significantly larger than the K_d 46

nM, this theory has established by understanding the meaning of these values (61). It is my hope that further investigation of hHAO is elicited from this study.

Work Cited

1. Berg, J.M., Tymoczko, J.L., Stryer, L. *Biochemistry sixth edition* 184-185 W.H. Freeman and Company., New York, 2007
2. Meunier, B., Visser, S.P., Shaik, S. *Chem. Rev* 2004, **104**, 3947-3980
3. Filatov, M., Reckien, W., Peyerimhoff, S.D., Shaik, S. *J. Phys. Chem. A* 2000. 104. 12014
4. Y. Kotake, I. Masayma, Z. *Physiol. Chem*. **243** (1936) 237-244.
5. Hayaishi, O., Rothberg, S., Mehler, A.H., Saito, Y. *J. Biol. Chem*. 229 (1957) 889-896.
6. Tanaka, T., Knox, W.E. *J. Biol. Chem*. **234** (1959) 1162-1170.
7. Kurnasov, O., Meininger, V., Brew, B.J. *Neurodegner. Dis.* 2 (2005) 166-176.
8. Magni, G., Amici, A., Emanuelli, M., Raffaelli, N., Ruggieri, S. *Adv. Enzymol. Relat. Areas Mol. Biol*. **73** (1999) 135-182.
9. Solomon, E., Decker, A., Lehnert, N. *PNAS* 10.1073 (2003)
10. Hegg, E.L., Que, L., Jr. (1997) *Eur. J. Biochem*. **250**, 650-629.
11. Pavel, E.G., Kitajima, N., Solomon, E.I. (1998) *J. Am. Chem. Soc.* **120**, 3949-3962
12. Solomon, E.I., Zhang, Y. *Acc. Chem. Res*., **25** (1992) 343.
13. Mabrouk, P.A., Orville, A.M., Lipscomb, J.D., Solomon, E.I. *J. Am. Chem. Soc*., **113** (1991) 4053
14. Schlafer, H.L., Gliemann, G. Basic Principles of Ligand Field Theory. Wiley Interscience: NY; 1969
15. Piepho, S.B., Schatz, P.N. Group Theory in Spectroscopy: With Application to Magnetic Circular Dichroism, John Wiley, New York, 1983.
16. Ozer, A. & Bruick, R.K. Non-heme dioxygenases: cellular sensors and regulators jelly rolled into one? *Nat. Chem. Biol.* **3** 144-153 (2007)

17. Kirk, T.K. In Microbial Degradation of Organic Compounds **13** 399-438 (Marcel Dekker, Inc., New York, 1984)

18. Pau, M.Y.M., Lipscomb, J.D. & Solomon, E.I. Substrate activation for O_2 reactions by oxidized metal centers in biology. *Proc. Natl. Acad Sci. USA* **104**, 18355-18362 (2007)

19. Nam, W. Dioxygen activation by metalloenzymes and models. *Acc. Chem. Res.* **40**, 465 (2007)

20. Gibison, D.T. Microbial Degradation of Organic Molecules; Ed.; Marcel Dekker: New York, 1984, pp 535.

21. Que, L. & Ho, R.Y.N., Dioxygen Activation by Enzymes with Mononuclear Non-Heme Iron Active Sites. *Chem. Rev*. 1996, **96**, 2607-2624

22. Lipscomb, J.D., Orville, A.M. Metal Ions Biological Systems. 1992, **28**, 243-298

23. Kovaleva, E.G. & Lipscomb, J.D. Versatility of Biological non-heme Fe(II) centers in oxygen activation reactions. Nat Chem Bio 2008 **4** 186-193

24. Shu, L. et al. X-ray absorption spectroscopic studies of the Fe(II) active site of catechol 2,3-dioxygenase. Impications for the extradiol cleavage mechanism. Biochemistry **34**, 6649-6659 (1995)

25. Kovaleva, E.G. & Lipscomb, J.D. Versatility of Biological non-heme Fe(II) centers in oxygen activation reactions. Nat Chem Bio **4** 186-193 2008

26. Kovaleva, E.G. & Lipscomb, J.D. Crystal structures of Fe^{2+} dioxygenase superoxo, alkylperoxo, and bound product intermediates. Science **316**, 453-457 2007

27. Mcmenamy, R.H. Binding of indole analogues to human serum albumin. Effects of fatty acids. *J Biol Chem*. **240** 4235-4300 1965

28. Pardridge, W.M. Blood-brain barrier carrier mediated transport and brain metabolism of amino acids. *Neurochem. Res*. **23**, 635-644 1998

29. Wolf, H. The effect of hormones and vitamin B_6 on urinary excretion of metabolites of the kynurenine pathway. *Scand. J. Clin Lab. Invest. Suppl*. **136**, 1-186 1974

30. Salter, M., Pogson, C.I. The role of tryptophan 2,3-dioxygenase in the homornal control of tryptophan metabolism in isolated rat liver cells. Effects of glucocorticoids and experimental diabetes. *Biochem J*. **229**, 499-504 1985

31. Takikawa, O. Biochemical and medical aspects of the indoleamine 2,3-dioxygenase intiated L-tryptohan metabolism. *Biochem Biophys Res. Commun*. **338** 12-90 2005

32. Metz, R., Duhadaway, J.B., Kamasani, *et al.* Novel tryptophan catabolic enzyme IDO2 is the preferred biochemical target for the antitumor indoleamine 2,3-dioxygenase inhibitory compound D-1-methly-tryptophan. *Cancer Res*. **67** 7082-7 2007

33. Guillemin, G.J., Smythe, G., Takikawa, O., Brew, B.J. Expression of indoleamine 2,3-dioxygenase and production of quinolinic acid by human microglia, astrocytes, and neurons. *Glia*. **49** 15-23 2005

34. Guillemin, G.J., Cullen, K.M., Lim, C.K., et al. Characterization of the Kynurenine Pathway in Human Neurons. *J Neuro sci* **27** 128884-92 2007

35. Guillemin, G.J., Kerr, S.J., Smythe, G.A., et al. Kynurenine pathway metabolism in human astrocytes: a paradox for neuronal protection. *J Neuro chem* **78** 1-13 2001

36. Takikawa, O. Biochemical and medical aspects of the indoleamine 2,3-dioxygenase initiated L-tryptophan metabolism. *Biochem Biophys Res Commun* **338** 12-9 2005

37. Fujigak, S., Saito, K., Sekikawa, K et al. Lipopolysaccharide induction of indoleamine 2,3-dioxygenase is mediated dominantly by an IFN-gmma-independent mechanism. *Eur J Immunol*. **31** 2313-8 2001

38. Davis, I., Liu, A., What is the tryptophan kynurenine pathway and why is it important to neurotherapeutics? *Expert Rev. Neurother*. **15** 2015

39. Guillemin, G.J. Quinolinic acid, the inescapable neurotoxin. *FEBS J*. **279**, 1356-1365 2012

40. Stone, T.W., and Perkins, M.N. Quinolinic acid: a potent endogenous excitant at amino acid receptors in CNS, *Eur. J. Pharmacol*. **72**, 411-2

41. Miller, A.H. Conceptual confluence: The Kynurenine pathway as a common target for ketamine and the convergence of the inflammation and glutamate hypothesis of depression *Neuropsychopharmacology* **38** 1607-8 2013

42. Beal, M.F., Matson, W.R., Swartz, K.J. et al. Kynurenine pathway measurements in Huntington's disease striatum: evidence for reduced formation of kynurenic acid. *J Neuochem* **55**(4) 1327-39 1990

43. Heyes, M.P., Achim, C.L., Wiley, C.A. et al. Human micorglia convert 1 tryptophan into the neurotoxin quinolinic acid *Biochem J*. **320**(pt2) 595-7 1996

44. Guillemin, G.J., Smith, D.G., Kerr, S.J. et al. Characterization of kynurenine pathway metabolism in human astrocytes and implications in neuropathogenesis. *Redox Report*. **5** 108-111 2000

45. Wolfensberger, M., Amsler, U., Cuenod, M. et al Identification of quinolinic acid in rat humanbrain tissue. *Neurosci Lett* **41** 247-52 1983

46. Belladonna, M.L., Grohmann, U., Guidetti, P. et al. Kynurenine pathway enzymes in dentric cells intiate tolerogenesis in the absence of functional IDO. *J Immunol* **111** 130-7

47. Zhang, Y., Colabroy, K.L. et al Structural Studies on 3-Hydroxyanthranilate-3,4-dioxygenase: The catalytic mechanism of a complex oxidation involved in NAD biosynthesis Biochemistry 44 7632-76

48. Foster, A.C., Zinkand, W.C., Schwarcz, R. Quinolinc acid phosphoribosyltransferase in rat brain. J. Neurochem. **44** 446-454 1985

49. Ichiyama, A., Nakamura, S., Kawat, H. et al Studies on the meatbolsim of the benzene ring of tryptophan in mammalian tissues: II. Enzymatic function of a-amino-muconic acid from 3-hydroxyanthranilinic acid. *J. Biol. Chem*. **240** 740-749 1965

50. Muraki, T., Taki, M., Hasegawa, Y., Iwaki, H., and Lau, P.C. Prokaryotic homologs of the eukaryptic 3-hydroxyanthanilate 3,4-dioxygenase and 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase in the 2-nitrobenzoate degradation pathway of *Pseudomonas fluorescens* strain KU-7. *Appl. Environ. Microbiol*. **69** 1564-1572 2003

51. Zhang, Y., Colabroy, K.L., Begley, T.P., Ealick, S.E. Structural Studies on 3-Hydroxyanthanilate-3,4-dioxygenase: The Catalytic Mechanism of a Complex Oxidation Involved in NAD Biosynthesis. *Biochemistry* **44** 7632-7643 2005

52. Dunwell, J.M., Purvis, A., and Khuri, S. Cupins: the most functionally diverse protein family?, *Phytochemistry* **65** 7-17 2004

53. Sieker, L.C., Stenkamp, R.E., and LeGall, J. Rubredoxin in crystalline state. *Methods Enzymol*. **243**, 203-16 1994

54. Liu, F., Geng, J., Gumpper, R.H., Barman, A., Davis, I., Ozarowski, A., Hamelberg, D., Liu, A. An Iron Reservoir to the Catalytic Metal: The rubredoxin iron in an extradiol dioxygenase. *J. Biol Chem* **290**(25) 15621-34 2015

55. Brkic, H., Kovacevic, B., and Tomic, S. Human 3-hydroxyanthranilate 3,4-dioxygenase (3HAO) dynamics and reaction, multilevel computational study. *Mol. BioSyt*. **11** 898-907 2015

56. Henderson, P.J.F. A linear equation that describes the steady-state kinetics of enzymes and subcellular particles interacting with tightly bound inhibitors. *J. Biochem* **127** 321-333 1972

57. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. Rapid measurement of binding constants and heats of binding using a new titration calorimeter. Anal. Biochem. 179, 131–137 1989

58. Maharaj, H., Maharaj, D.S., Daya, S. Acetylsalicylic acid and acetaminophen protect against oxidative neurotoxicity. *Metab Brain Dis* **21** 189-199 2006

59. Kotrly, S., Sucha, L. Handbook of Chemical Equilibria in Analytical Chemistry, Ellis Horwood Limited, Chichester, UK, p 163.

60. Colabroy, K.L., Zhai, H., Li, T., Ge, Y., Zhang, Y. Liu, A. Structural studies on 3-hydroxyanthranilate-3,4-dioxygenase: the catalytic mechanism of a complex oxidation involved in NAD biosynthesis. *J Biochem* 44 7623-7631 2005

61. Northrop, D.B. On the Meaning of K_m and V/K in Enzyme Kinetics. J Chem Edu Vol 75 **9** 1153-1157 1998