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Hydrogen Peroxide Inhibition and Inactivation of Bicupin Oxalate Oxidase

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HYDROGEN PEROXIDE INHIBITION AND INACTIVATION OF BICUPIN
OXALATE OXIDASE

By

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ABSTRACT

Oxalate oxidase is a manganese containing enzyme that catalyzes the oxygen dependent oxidation of oxalate to carbon dioxide and hydrogen peroxide. Oxalate oxidase from Ceriporiopsis subvermispora (CsOxOx) is the first characterized bicupin oxalate oxidase. Potential applications of oxalate oxidase in cancer treatment, paper pulping, and biofuel cells highlight the need to study the effects of hydrogen peroxide on the CsOxOx catalyzed oxidation of oxalate. Membrane inlet mass spectrometry (MIMS) was used to directly measure initial rates of carbon dioxide formation and oxygen consumption in varying initial concentrations of hydrogen peroxide. This work demonstrates that hydrogen peroxide is both a reversible noncompetitive inhibitor of the CsOxOx catalyzed oxidation of oxalate and an irreversible inactivator. CsOxOx is irreversibly inactivated under turnover conditions and hydrogen peroxide plays a key role in this turnover dependent inactivation. The introduction of catalase to reaction mixtures protects the enzyme from inactivation allowing reactions to proceed to completion. Circular dichroism spectra suggest that hydrogen peroxide does not induce changes in CsOxOx global protein structure. Additionally, we show that the CsOxOx mediated mesoxalate reaction consumes oxygen and produces hydrogen peroxide, which is in contrast with previous proposals that the enzyme catalyzed the non-oxidative decarboxylation of this three carbon substrate. Finally, we observe a $^{13}$C kinetic isotope effect on the CsOxOx catalyzed reaction using MIMS which supports that the carbon-carbon bond cleavage of oxalate is at least partially rate limiting.
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LIST OF ABBREVIATIONS

OxOx Oxalate oxidase
CsOxOx Ceriporiopsis subvermispora oxalate oxidase
BsOxDC Bacillus subtilis oxalate decarboxylase
EPR Electron paramagnetic resonance
TEMPO 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl
PDA Pancreatic ductal adenocarcinoma
KRAS Kirsten rat sarcoma
MS Mass spectrometry
MIMS Membrane inlet mass spectrometry
MALDI Matrix assisted laser desorption ionization
ESI Electrospray ionization
EII Electron impact ionization
TOF Time of flight
HRP Horseradish peroxidase
KIE Kinetic isotope effect
CHAPTER 1: INTRODUCTION

Chapter 1.1: Oxalate Oxidase

Oxalate oxidases (EC 1.2.3.4) are a group of enzymes that catalyze the oxidation of oxalate to carbon dioxide and hydrogen peroxide\(^1\)\(^3\) (Figure 3). All oxalate oxidases (OxOx) characterized thus far belong to the cupin superfamily of proteins\(^4\)\(^6\). The cupin superfamily is a functionally diverse group of proteins that includes seed storage proteins, nuclear transcription factors, epimerases, dioxygenases, decarboxylases, oxidases, synthases, isomerases, and others\(^5\)\(^7\). Although functionally different, proteins belonging to the cupin superfamily share a common structural beta-barrel motif\(^5\)\(^8\). The beta-barrel fold in the cupin domain of all known oxalate oxidases contains a manganese ion coordinated to three histidine residues and one glutamate residue\(^9\)\(^10\) (Figures 1, 2 and 5). Most known oxalate oxidases are plant derived monocupins containing only a single cupin domain (Figure Error! Reference source not found.) with the exception of the bicupin enzyme derived from the white wood rot fungus Ceriporiopsis subvermispora.

![Ribbon structure of monocupin oxalate oxidase derived from barley (PDB code: 2et1) showing the beta-barrel fold typical of the cupin superfamily.](image)

Figure 1: Ribbon structure of monocupin oxalate oxidase derived from barley (PDB code: 2et1) showing the beta-barrel fold typical of the cupin superfamily.
Figure 2: Ribbon homology structure of bicupin oxalate oxidase derived from Ceriporiopsis subvermispora.

Figure 3: Reaction catalyzed by oxalate oxidase.
Figure 4: The proposed mechanisms of oxalate oxidase and oxalate decarboxylase. Modified from Moomaw et al\textsuperscript{6}.

The oxalate oxidase derived from \textit{Ceriporiopsis subvermispora} (CsOxOx) is of particular interest to this study as it produces a unique bicupin form of oxalate oxidase containing two beta-barrel folds\textsuperscript{11,12}. Interestingly, this enzyme has a high degree of amino acid sequence identity with another bicupin and manganese dependent oxalate degrading enzyme, oxalate decarboxylase from \textit{Bacillus subtilis}\textsuperscript{1,8} from whose crystal structure the homology model of CsOxOx is derived\textsuperscript{12} (Figures 2 and 5). The oxalate decarboxylase from \textit{Bacillus subtilis} (BsOxDC) catalyzes the non-oxidative, but oxygen dependent decarboxylation of oxalate to formate and carbon dioxide. Previous
work with both CsOxOx and BsOxDC have demonstrated that catalysis takes place in
the N-terminal manganese binding site\textsuperscript{6,13}, but as of yet there is no evidence for either
enzyme that precludes catalysis from also taking place in the C-terminal domain\textsuperscript{14}.
Furthermore, other studies on BsOxDC suggest that the C-terminal Mn(II) site does
catalyze decarboxylation\textsuperscript{15}. No such evidence yet exists for CsOxOx.

The current proposed catalytic mechanisms (Figure 4) for CsOxOx pools observations
from studies on barley oxalate oxidase, CsOxOx, and BsOxDC and, therefore, shares
similarities. EPR and metal analysis data and the observation that specific activity is
correlated with manganese metal incorporation suggest the enzyme bound metal
component to be Mn(II)\textsuperscript{10,11,16} in the resting state. Addition of oxalate was found to
perturb the x-band EPR generated Mn(II) spectrum strengthening the hypothesis
oxalate binds the enzyme bound Mn(II) ion directly during turnover\textsuperscript{16}. Crystal
structures confirm this as well as demonstrate that oxalate reversibly binds the
manganese ion in a monodentate fashion\textsuperscript{17}. It is assumed that the binding of oxalate
perturbs the metal redox potential enough to facilitate the binding of diatomic oxygen
and the oxidation of the Mn(II) center to Mn(III)\textsuperscript{18}. Chemical precedent from the
permanganate reaction with oxalate suggests that Mn(III) is the active species that
facilitates the decarboxylation of oxalate ligand leaving the formyl bound free radical
anion\textsuperscript{19}. The existence of this intermediate is supported by EPR studies on CsOxOx
and BsOxDC\textsuperscript{11,16}. It is believed that the fate of the intermediate species is where the
proposed mechanisms for the oxalate oxidases and oxalate decarboxylase diverge\textsuperscript{14,18}.
In the oxalate decarboxylase mechanism, the radical anion is protonated by a general
acid and released from the manganese complex as formate\textsuperscript{11,20}. In the oxalate oxidase
mechanism, the formation of the radical anion intermediate is succeeded by the
formation of a, as of yet unobserved, percarbonate intermediate species prior to decomposition of the intermediate into carbon dioxide and hydrogen peroxide.\(^{18}\)

**Figure 5:** Homology models of the N-terminal (left) and C-terminal (right) manganese coordination centers of *Ceriporiopsis subvermispora* derived bicupin oxalate oxidase. Manganese ions (purple) and active site residues (carbon, cyan; nitrogen, blue; oxygen, red) are labelled. Swiss-Model (the Swiss Institute of Bioinformatics) was used to generate the homology model from the amino acid sequence of CsOxOx and the available structural data of BsOxDc (PDB ID 1UW8). The Pymol software (The PyMOL Molecular Graphics System, Schrödinger, LLC)\(^6\).

Much of this mechanism is still disputed. A recent study by Whitaker *et al.* suggests an alternative mechanism in which only enzyme containing Mn(III) ions are active and react with oxalate directly without the need for oxygen binding\(^{21}\). Oxygen is then consumed by the carbon dioxide radical anion forming unbound superoxide which then regenerates the Mn(III) ion for further turnover producing hydrogen peroxide in the process\(^{21}\).

Chapter 1.2: Oxalate oxidase as a component in biofuel cells

Fuel cells convert the latent chemical energy from a fuel molecule, typically hydrogen or hydrocarbons, into electrical potential. The typical fuel cell has three main parts; a cathode, an anode, and an electrolyte connecting the two electrodes\(^{22}\). Fuel molecule
oxidation takes place at the anode of the fuel cell and is coupled with the reduction of an oxidant at the cathode. Redox chemistry taking place at both electrodes is made favorable by the addition of one or more electrocatalysts. Most of the fuel cells in use today utilize metal catalysts; however, the use of biocatalysts in a subcategory of fuel cells called biofuel cells shows great promise.

Enzymes have a number of considerable advantages over conventional electrocatalysts. For instance, enzyme active sites are specific for their substrate and are not easily inactivated by impurities found in fuels. Also, modern fuel cells typically utilize costly precious metals as the catalysts for redox chemistry while biocatalysts can be inexpensively produced in mass using bioreactors. Additionally, advances in biofuel cell design combined with biocatalyst specificity obviate the need for the insulating membranes present in most liquid fuel cells significantly simplifying design. However, one major barrier to practical application of biocatalysts in fuel cell design is the biocatalysts short life span; they are typically not as stable as their metal counterparts and tend to denature or degrade more rapidly. Therefore, techniques that extend the active lifespan of a biological redox catalyst are critical. Hickey et al. have published a study demonstrating the complete oxidation of glycerol to carbon dioxide and hydrogen peroxide using a combination of the organic catalyst 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) and the oxalate oxidase enzyme (from Hordeum vulgare) (Figure 6).
Chapter 1.3: Oxalate oxidase as a potential cancer treatment

Pancreatic ductal adenocarcinoma (PDA) is a deadly pancreatic cancer that undergoes metastasis early, presents late, and is resistant to chemo and radiotherapies\(^{25-28}\). These factors all contribute to the low 5-year survival rate after diagnosis of less than 6\(^{29}\). Interestingly, the Kirsten rat sarcoma viral oncogene homolog (KRAS) is present in 95\% of PDA cases\(^{29}\) and is an attractive drug target. However, the KRAS protein itself has proven to be a difficult drug target\(^{26,30-32}\).

A recent study found that KRAS positive pancreatic cancer cells have significantly higher concentrations of ascorbate derived oxalic acid compared to those concentrations in normal cells\(^{33}\). This prompted a study exploring the cytotoxic effects of CsOxOx on KRAS pancreatic cancer cells in the laboratory of Dr. Gaurab Chakraborti. Data (Figure 7) shows that cancer cells exposed to CsOxOx had significantly lower survivability compared to the buffer treated controls, and that the
CsOxOx treatment did not have a significant effect on the survivability of the normal cell line. These early results demonstrate the therapeutic potential for oxalate oxidase as a pancreatic cancer drug.

Figure 7: Thirty hour MiaPaCa2 and HPDE (Human Pancreatic Duct Epithelial Cell Line) cell survival plots with and without CsOxOx. Points represent means. Error bars denote standard deviations. Those points marked with *** represent plots that are significantly different from the control values (p value < 0.001).

Chapter 1.4: Membrane inlet mass spectrometry

Mass spectroscopy (MS) is a powerful analytical tool capable of both quantitative and qualitative analysis of a variety of compounds. However, the inherent aqueous nature of biochemistry presents a barrier to the utility of this technique, and therefore MS is not often used for enzymatic assay and the determination of enzyme kinetic parameters. Mass spectrometry requires the maintenance of a strong vacuum within the analytical assembly in order to function properly and therefore only small gaseous
samples are compatible for analysis\textsuperscript{35}. A number of sampling techniques have been developed that overcome this barrier\textsuperscript{34}. The MS employed in this study uses a membrane (hence the name membrane inlet mass spectrometry or MIMS) through which gaseous and/or volatile samples are introduced into the high vacuum of the instrument via “pervaporation” (Figure 8). A sample undergoing pervaporation is adsorbed from the liquid matrix onto the surface of the sampling membrane, diffuses through the membrane, and then desorbs from the opposite side and into the high vacuum environment of the mass spectrometer\textsuperscript{36}.

\textbf{Figure 8}: The Hiden Analytical HPR40 Membrane Inlet Mass Spectrometer at Kennesaw State University.

As the technique of mass spectrometry relies heavily on the principles of ion optics, sampled substances must be ionized prior to filtration and analysis. There are a number of techniques useful for sample ionization. Techniques include Matrix Assisted Laser Desorption Ionization (MALDI), Electrospray Ionization (ESI), and Electron Impact Ionization (EII). MALDI and ESI are modern techniques that provide
users with the ability to ionize and study large macromolecules (i.e. proteins). EII is an older and simpler technique suitable for gaseous or volatile small molecules. EII is the technique used in this study and will be the focus of further discussion. The EII technique ionizes the volatilized sample by direct bombardment with an electron beam perpendicular to sample flow. The electron beam is generated by a heated filament providing a source of electrons that travel across the sample inlet stream and into an electron cage. Some portion of sample particles struck by electrons is ionized and is accelerated further into the instrument where they are then filtered according to their respective mass and charge.

Figure 9: Diagram of the quadrupole mass filter. Modified from.
Ion filtering is accomplished by measuring the time the ions take to travel a known distance (Time of Flight, TOF), or by deflection in a magnetic and/or electric field. A particularly powerful filtering tool is the quadrupole mass filter (Figure 9). The quadrupole mass filter is composed of two sets of two similarly charged poles; a pair of positive poles directly across from each other and a pair of negative poles directly opposite each other. The charges are maintained via a direct current supplied to each pole. An alternating current is also supplied to each pair of poles. In this way, the positive poles act as a high mass filter while the two negative poles function as a low mass filter. The voltages of the currents as well as the frequency of the alternating currents can be altered to fine tune the filtering functions of the quadrupole attenuating the window of allowed masses to a width of 1 amu. Filtered ions are then detected using a faraday cup or single channel electron multiplier detector. In applications where ionized sample pressures are high, faraday cup detectors are more appropriate due to their durability and long life time. Single channel electron multiplier detectors are capable of detecting relatively small sample pressures, but are far more fragile and expensive. The Hiden Analytical HPR-40 membrane inlet mass spectrometer is equipped with both a single channel electron multiplier detector and a faraday cup.

Past studies of CsOxOx enzyme kinetics utilize a UV/Vis spectrophotometer assay coupling the production of hydrogen peroxide to the catalytic oxidation of 2, 2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) dye by horseradish peroxidase (HRP). The chemistry of the HRP presents an obvious barrier to inhibition studies using initial concentrations of hydrogen peroxide as the initial hydrogen peroxide concentration will also be used as substrate and therefore prevents an accurate rate determination by this assay. Therefore, MIMS was utilized to assay CsOxOx activity
by continuously measuring in real time the production of carbon dioxide and the consumption of oxygen by CsOxOx catalyzed reaction\textsuperscript{38}.

Chapter 1.5: Circular dichroism

Circular dichroism (CD) is the difference between left-handed and right-handed polarized light absorbed from a chiral molecule. Absorbance of the two types of rotating radiation is also anisotropic; its degree is dependent on the orientation of the molecule to the incident radiation. Large biological molecules such as proteins contain many chiral molecules (amino acid residues) chained together and folded into some definite structure. Due to anisotropy, the CD of a large biological macromolecule is dependent on its three-dimensional structure and therefore CD measurements can be used to observe global structural changes.

Circular dichroism spectroscopy involves scanning the CD values of a sample within a range of radiation wavelengths specific to the sample. Typically, protein sample CD spectra are obtained between the wavelengths of 170 nm to 250 nm or 250 nm to 320 nm. The first group of wavelengths which includes the far UV are those at which the peptide backbone absorbs and can observe protein secondary structure such as beta-sheet and alpha helices\textsuperscript{39-42}. The later range corresponds to aromatic amino acids and CD measurements within it are able to discern tertiary protein structure changes\textsuperscript{39}. There are a number of different ways to present the data from a CD experiment. The most basic CD value is simply the difference in absorbance between the left and right-handed light (Equation Error! Reference source not found.).\textsuperscript{40,43}

\[ \text{CD} = A_L - A_r \]

\text{(Eq. 1)}
Here, CD is the CD value, A_l is the absorbance of left-handed polarized light, and A_r is the absorbance of right-handed polarized light. However, this value can be rather vague. A more specific way to present CD data is molar CD which is derived directly from Beer-Lambert. Molar CD is calculated according to Equation Error! Reference source not found.40-42,

\[
[CD] = \frac{CD}{C \times l} \quad \text{(Eq. 2)}
\]

where CD is defined as in Equation Error! Reference source not found., C is the molar concentration of species of interest, and l is the pathlength in centimeters. For protein measurements, the species of interest are the individual peptide residues, and is the product of the molar concentration of a specific protein and the number of residues present per protein molecule.

Classically, CD data can be presented as degree of ellipticity, which is the tangent of the smallest over the largest possible combinations of the magnitudes of the right-handed and left-handed electrical field vectors (Equation 3)\textsuperscript{39,43}. In equation 3,

\[
\theta = \tan \frac{E_R - E_L}{E_R + E_L} \quad \text{(Eq. 3)}
\]

\(\theta\) is the ellipticity in radians, \(E_R\) is the magnitude of the electric field vector for the right-handed polarized light, and \(E_L\) is the magnitude of the electric field vector for the left-handed polarized light. Assuming that the difference in absorption of left-handed versus right-handed light is small and converting from radians to degrees it is possible to derive a constant of proportionality between molar ellipticity circular dichroism (Equation 4)\textsuperscript{39,43}.

\[
\theta = 32.982 \times CD \quad \text{(Eq. 4)}
\]
Here, $\theta$ is the ellipticity in degrees and CD is the circular dichroism as defined in Equation 1. It is also typical to report CD data in molar ellipticity which is the degrees of ellipticity divided by the product of the molar concentration of sample species and pathlength in meters.

Chapter 1.6: Enzyme kinetics

Enzyme kinetics aims to model mathematically an enzyme catalyzed reaction in order to gain mechanistic insight by comparing the behavior of the enzyme under study to the theoretical behavior of the model. The overall catalytic velocity of an enzyme under constant conditions is a function of the concentrations of all relevant enzymes, substrates, products, activators, and inhibitors. Equation 5, 

$$v_i = F([E], [S], [P], [A], [I])$$  \hspace{1cm} (Eq. 5)

represents a simplified mathematical description of the overall catalytic velocity where $v_i$ is equal to the change in the concentration of the species of interest (typically product) per unit time, $[E]$ is the total concentration of enzyme, $[S]$ is the total concentration of substrate, $[P]$ is the total concentration of product, $[A]$ is the concentration of activator, and $[I]$ is the concentration of inhibitor.

Typically, kinetic models are distinguished by first studying the relationship between substrate concentration and reaction velocity. Leonor Michaelis and Maud Menten were the first to mathematically describe a system in which a single substrate reversibly binds the enzyme and then is turned into product$^{44-46}$. This simplest kinetic mechanism is shown in Figure 10.
In this model, the assumption is made that the binding and dissociation constants for the initial step are far faster than the chemical step in which the substrate is transformed to product. The conversion of [ES] to [P] is shown as irreversible and initial rate data implies that there is no significant concentration of product formed or reactant consumed during the period of assay. From these assumptions, they were the first to describe this type of system mathematically (Equation 6).

\[ v_0 = \frac{V_{max} \times [S]}{K_M + [S]} \]  
(Eq. 6)

Here, \( v_0 \) is equal to the initial reaction rate, \( V_{max} \) is equal to the maximal rate at which the enzyme is completely saturated with substrate, \( K_M \) is equal to the substrate concentration which produces one-half the \( V_{max} \) rate, and \([S]\) is equal to the concentration of substrate. Later Briggs and Haldane suggested that there was no \textit{a priori} reason that the substrate dissociation step should be very much faster than the enzymatic chemical processing step and therefore derived a more inclusive model assuming that there exists a steady state at which the concentrations of all intermediate species remains largely unchanged and that this steady state is achieved before any significant product formation has occurred\(^{45}\). Interestingly, both approaches yield the same characteristic rectangular hyperbola (Equation 6). The only difference between them is the meaning of the \( K_M \) constant. In the Michaelis-Menten equation the \( K_M \) is equal to the rate constant of substrate dissociation over the rate constant of substrate binding. While in the Briggs-Haldane model, the \( K_M \) is equal to
the sum of the substrate dissociation constant and the rate of substrate conversion to product divided by the rate constant of substrate binding. The equation takes the same form but the definition of the constants change. In this way, most kinetic models describing enzyme catalysis are degenerate when looking at the effects of substrate concentration. Therefore, it is often necessary to perturb the systems using initial concentrations of inhibitors or activators to narrow down an appropriate kinetic model.

Enzyme kinetic studies are typically illustrated graphically by plotting initial enzymatic reaction rate versus substrate concentration. If the resulting plot is a rectangular hyperbola, the data is consistent with Michaelis-Menten kinetics. The asymptote of the rectangular hyperbola is equal to the maximal velocity ($V_{max}$) obtained under saturating concentrations of substrate. The substrate concentration that gives an initial reaction rate equal to one-half the asymptote represents the $K_M$ constant of the Michaelis-Menten equation. Plotting the reciprocal of the initial reaction rate versus the reciprocal of the substrate concentration linearizes the classic Michaelis-Menten equation. This reciprocal plot is called the Lineweaver-Burk plot and provides visual insight into the kinetic mechanism. The y-intercept of the line is equal to the reciprocal of the maximum velocity, the x-intercept is equal to the negative reciprocal of the $K_M$ constant, and the slope of the line is equal to the ratio of the $K_M$ to $V_{max}$ values.

Chapter 1.7: Enzyme Inhibition Kinetics

Enzyme inhibitors affect the kinetic parameters in ways dependent on the inhibition type. In a simple unireactant enzyme kinetic model, an inhibitor can alter the observed
kinetic parameters illustrating three broad types of inhibition. In the first type of inhibition, the inhibitor reversibly binds to the same enzyme species that binds the substrate, and therefore blocks the binding of substrate\textsuperscript{45}. This increases the apparent $K_M$ value as additional substrate is needed to achieve the respective uninhibited velocity. The maximum reaction velocity ($V_{\text{max}}$) is not affected at high substrate to inhibitor concentrations. The effect on the concentration of enzyme-substrate complex is insignificant. Uncompetitive inhibitors bind the enzyme-substrate complex only and prevent product formation. As less enzyme-substrate complex is able to form product, the maximal velocity is lowered in uncompetitive inhibition. Also, the removal of enzyme-substrate complex pushes the total pre-product equilibrium to the right reducing the apparent $K_M$ parameter. Noncompetitive inhibitors may reversibly bind a form of the enzyme that the experimentally varied substrate ($S_2$ in Figure 11) does not, but there must be a reversible connection between the substrate binding species and the inhibitor binding species. ($S_1$ in Figure 11):

\[
[E] + [S_1] \rightleftharpoons [ES_1] + [S_2] \rightleftharpoons [ES_1S_2] \rightarrow [E] + [P] + [I] \]

\[
[E]I
\]

**Figure 11**: General kinetic scheme for a simple bireactant enzyme illustrating one possible mechanism of noncompetitive inhibition.
Alternatively, a noncompetitive inhibitor may bind to the enzyme form that the substrate binds and a different enzyme species (E and ES in Figure 12). In this “classic noncompetitive” case, the equilibrium constants for the binding of inhibitor with enzyme alone and inhibitor with another enzyme species are equal. When the two equilibrium constants are different, traditional inhibition nomenclature becomes ambiguous\textsuperscript{45,48} with this case being defined as a “mixed-type” inhibitor and not a noncompetitive inhibitor. However, there is no a priori reason that the equilibrium constants should be equal. For these reasons, it has been proposed that mixed inhibition be reclassified as noncompetitive inhibition, and in this study we refer to mixed inhibition simply as noncompetitive inhibition. Under this definition, a noncompetitive inhibitor will reduce the apparent maximal velocity, but can reduce, increase or have no effect on the $K_M$ parameter.

*Product inhibition* describes a product molecule that is also an inhibitor of the enzyme that catalyzes its formation. The effects of product inhibition are observable from the overall velocity equation that accounts for the reverse reaction and the related product-enzyme complex species (Equation 7)\textsuperscript{45,46}. In Equation 7, $v_i$ is the initial increase in concentration of product over time, [P]. [S] is the substrate concentration,
[P] is the product concentration, $V_1$ is the maximal rate of product formation, $V_2$ is the maximal rate of substrate formation, $K_a$ is the equilibrium constant relating the rate constants of substrate binding and dissociation to and from the enzyme complex, and $K_p$ is the equilibrium constant relating the rate constants of product dissociation and association to the enzyme complex. Each denominator term represents an intermediate enzyme species. The term representing the enzyme product complex(s) changes depending on the kinetic mechanism and allows one to differentiate between kinetic mechanisms that appear degenerate varying substrate concentration(s) alone.

\[
\nu_i = \frac{V_1[S] - V_2 \left( \frac{K_a}{K_p} \right) [P]}{K_a + [A] + \left( \frac{K_a}{K_p} \right) [P]}
\]  
(Eq. 7)

Inhibitors can be further subdivided into complete and partial inhibitors. Complete inhibitors bind their target species and prevent progression towards product formation until the inhibitor has dissociated (rate constant $k_p$ in Figure 13). Partial inhibitors do not completely prevent progression through the main forward kinetic mechanism. Partial inhibitors only slow forward progress. Noncompetitive partial inhibitors bind to the free enzyme species and an enzyme complex species on the main scheme towards product release. The catalytically active inhibitor bound enzyme complex still produces product but at a slower rate. The reduction in the catalytic kinetic constant is captured by a factor, $\beta$ (Figure 13).
All of the classical inhibitor types (noncompetitive, uncompetitive and competitive) reversibly bind their targets. Inhibitors exist that irreversibly bind their targets and therefore completely remove enzyme from the equilibrium. Removal of enzyme causes a decrease in $V_{\text{max}}$ but no change in $K_M$, and is therefore indistinguishable from a noncompetitive inhibitor if one studies only the effects of inhibitor and substrate concentration on reaction velocity. To distinguish reversible noncompetitive inhibition from irreversible inhibition one needs to observe how maximal velocity changes in relation to assumed total enzyme concentration. A $V_{\text{max}}$ versus total enzyme concentration plot of an irreversibly inhibited enzyme will have a slope that is identical to that of the plot of the uninhibited enzyme. The x-intercept of the irreversibly inhibited enzyme plot will be shifted to the right of the uninhibited enzyme plot. The difference between the two x-intercepts is equal to the amount of enzyme that has been removed from the equilibrium. An enzyme concentration versus $V_{\text{max}}$ plot for a reversibly inhibited enzyme will have a lower slope than that of the uninhibited plot, and the two lines will intercept the x-intercept at the exact same value. This plot is a powerful tool for visualizing both irreversible and reversible inhibition and allows one to comment on the degree of irreversible inactivation.
Chapter 1.8: The motivation and novelty of this work

This study seeks to better understand the role of CsOxOx as a biocatalyst in biofuel cell, cancer, and other applications while leveraging observations to gain mechanistic insights. Data suggesting a potential role for CsOxOx as a cancer therapeutic beget questions regarding the effects of hydrogen peroxide build up during enzymatic turnover. There is very little literature characterizing the effects of hydrogen peroxide on oxalate degrading enzymes. Initial rate inhibition studies on oxalate decarboxylase suggest that hydrogen peroxide is not an inhibitor of the enzyme\(^\text{13}\). Observations by EPR found that the addition of hydrogen peroxide to barley oxalate oxidase did not perturb the manganese center\(^\text{16}\). The only study looking at the inhibitory effects of hydrogen peroxide on oxalate oxidase examined a broad spectrum of compounds found in paper bleaching filtrates\(^\text{49}\). This study reported that barley oxalate oxidase activity is not significantly reduced up to concentration of 1 mM hydrogen peroxide, but only 30% of the activity remains at 20 mM hydrogen peroxide. As hydrogen peroxide is a product of the CsOxOx catalyzed reaction any application utilizing CsOxOx as a biocatalyst will benefit from a more complete understanding of the effects of hydrogen peroxide on the enzyme. Additionally, the mechanism employed by CsOxOx in the oxidation of oxalate has been well studied, but questions still remain regarding the existence and fate of the percarbonate radical and how the resulting products influence enzyme activity. Enzyme product inhibition studies are commonly used to gain insight into enzyme mechanisms and, among other things, to identify abortive enzyme product complexes. Therefore, efforts to characterize hydrogen peroxide product inhibition of CsOxOx will lead to further mechanistic insight.
Efforts were also made to further characterize CsOxOx enzyme promiscuity. Enzyme promiscuity (for the purposes of this study is defined as substrate ambiguity) is a common phenomenon and useful in a large number of applications. The application of isothermal titration calorimetry (ITC) has demonstrated the ability of CsOxOx to utilize a number of small di-carboxylic acid molecules as substrates\textsuperscript{50}. Additionally, the three-carbon molecule, mesoxalate (oxopropanedioic acid), has been shown to be a substrate for the CsOxOx-catalyzed reaction, with a kinetic parameters comparable to that of oxalate. The usefulness of OxOx as a biocatalyst in biofuel cells\textsuperscript{23} may inform efforts to modify the properties of CsOxOx through rational design/directed evolution. Characterizing the extent of promiscuity of CsOxOx is important as it may provide a basis for these modifications. Furthermore, elucidation of the reaction products of the CsOxOx catalyzed reaction with mesoxalate is important to inform the type of reaction that is catalyzed (oxidative or nonoxidative) and could lead to further mechanistic insight.
Chapter 2: Experimental

Chapter 2.1: Materials

$^{13}$C$_3$-glycerol and $^{13}$C$_2$-oxalate were obtained from Cambridge Isotope Laboratories. Lyophilized catalase powder from bovine liver was a purchased from Sigma Aldrich. All other reagents described were purchased from Sigma Aldrich or Fisher Scientific and were of the highest purity available unless otherwise stated.

Chapter 2.2: Expression/purification of CsOxOx

CsOxOx was recombinantly expressed in *Pichia pastoris* as previously described$^{11}$. The CsOxOx sequence (GenBank accession number: AJ746412) was amplified using engineered primers incorporating an XbaI restriction site and Kex2 protease cleavage site at the 5’ end. At the 3’ end an XhoI site was added. After digestion with XhoI and XbaI, the resulting fragments were ligated into pPICZαA vector (Invitrogen) also previously digested with XhoI and XbaI. The Invitrogen EasyComp transformation kit was then used to introduce the recombinant plasmid into *Pichia pastoris* X-33. The resulting recombinant cells, capable of growth on plates containing methanol as the sole carbon source and capable of growth in the presence of Zeocin, were inoculated into 5 baffled flasks containing 500 mL of MGY media (1.34% yeast nitrogen base, 1% glycerol, $4 \times 10^{-5}$ % biotin, 5 mM MnCl$_2$) and incubated overnight at ~29°C under constant agitation. The yeast cells were then harvested by centrifugation at 8000 RPM for 8 minutes. The supernatant was poured off and the pellets were each resuspended in 500 mL of MM media (1.34% yeast nitrogen base, $4 \times 10^{-5}$ % biotin, 0.5% methanol, 5 mM MnCl$_2$). The suspensions were then incubated at ~29°C under
constant agitation for 5 days. At the beginning of each day of incubation an additional aliquot of methanol (0.5%) was added. After the fifth day, the cells were removed by centrifugation at 10,000 RPM and the supernatant was concentrated to a volume of roughly 50 mL using both the Pellicon XL and Amicon ultra filtration devices. The concentrate was then dialyzed exhaustively against 25 mM imidizole chloride buffer (pH 7.0). Dialyzed concentrate was then further purified using a DEAE-Sepharose Fast Flow column. The concentration of sodium chloride in the mobile phase buffer A (50 mM imidazole-Cl, pH 7.0) was slowly increased using a gradient pump, and eluent was collected in 10 mL fractions. Fractions containing oxalate oxidase activity were then pooled and ammonium sulphate was then added to the pooled fractions to concentration of 1.7 M. This solution was then applied to a Butyl-Sepharose column and protein was eluted using a linear gradient from 100% buffer B (50 mM Imidazole-Cl, 1.7M ammonium sulfate) to 100% buffer A. Fractions were again tested and those containing oxalate oxidase activity were pooled and concentrated to a target specific activity. This method has been previously described and multiple times\textsuperscript{6,11,38,51}.

Chapter 2.3: Chromatographic separation of small carboxylic acid molecules by HPLC

HPLC separation of small carboxylic acid molecules was achieved on a 300 mm x 7.8 mm Aminex HPX-87H column, using sulfuric acid solution as the mobile phase (4 mM, 0.6 mL/min)\textsuperscript{23,52}. Samples were injected (one 25 µL sample injection every thirty minutes) into the HPLC system. The absorbance of the eluent at a wavelength of 230 nm was recorded over time and the data was analyzed using the Dionex PeakNet software.
Chapter 2.4: Observation of global structural protein changes by circular dichroism

Structural changes of the wild type CsOxOx enzyme brought on by increasing hydrogen peroxide concentrations were measured using circular dichroism spectroscopy. The JASCO J-1500 Spectropolarimeter (JASCO Inc.) was used to measure the molar ellipticity of the samples. A sample of purified CsOxOx enzyme was exchanged into 25 mM potassium phosphate (pH 7.0), and diluted with 25 mM potassium phosphate buffer until its CD spectrum reached a minimum at 207 nm. Aliquots of concentrated hydrogen peroxide were then added to the protein sample resulting in final concentrations of 0, 4, 8, 12, 16, and 20 mM hydrogen peroxide. A Teflon stopper was used to retard evaporation. The scan rate, time constant and numbers of scans were 10 nm/min, 2 s, and 3, respectively. Wavelengths were recorded over the range of 195-250 nm. The scan rate and bandwidth were 10 nm/min and 1 nm, respectively, and each spectrum was an accumulation of five scans. A blank spectrum was performed with buffer and subtracted from the spectra.

Chapter 2.5: UV-Vis horseradish peroxidase coupled CsOxOx kinetic assay

Samples were assayed on a UV-Vis assay that utilizes horseradish peroxidase to couple the formation of oxalate derived hydrogen peroxide to the oxidation of 2,2’-azinobis- (3-ethylbenzthiazoline- 6-sulfonic acid) (ABTS) dye\(^1\). The spectrometer is first blanked with reaction solution (50 mM potassium oxalate, 5 mM ABTS, 25 U horseradish peroxidase and 50 mM sodium succinate, pH 4.0) and then the sample is
added prior to measuring the increase in absorption at 650 nm over 20 seconds. A molar extinction coefficient of 10,000 M⁻¹cm⁻¹ for the ABTS dye was assumed.

Chapter 2.6: MIMS setup

The inlet probe comprised a length of tubular silastic membrane (1.96 mm OD and 1.47 mm ID) sealed at one end by a ruby/sapphire ball (2 mm) and attached at the other end to a piece of glass tubing (120 mm length, 2.3 mm ID, and 6.35 mm OD). A stainless steel membrane support spring (316SS) was used to support the tubular silastic membrane at running vacuum pressures. The length of silastic membrane from the ruby/sapphire ball to the beginning of the glass tubing was 3.3 mm. The glass tubing is connected quartz tubing was attached to an elbow joint that exited directly into the main vacuum chamber of a Hiden Analytical HPR-40 DSA Membrane Inlet Mass Spectrometer.

Figure 14: Close-up of the probe mass spectrometer fittings.
Chapter 2.7: MIMS oxalate oxidase assay

To determine the rate of catalysis by CsOxOx, a solution of sodium succinate buffer (50 mM, pH 4.0) and potassium oxalate (50 mM, pH 4.0) was incubated at room temperature for 5 minutes with the MIMS probe submerged and data collected at 32 m/z and m/z 45 or 44. After a stable baseline reading was achieved, a sample of CsOxOx of known concentration was added to the buffered substrate solution. The ion currents were then recorded for another 20 minutes.

The rate of MIMS signal change after enzyme addition was determined by plotting the first minute of linear increase in the m/z 45 signal versus time in minutes and the slope of the linear best fit line as determined using Microsoft Office Excel 2010. The baseline slope was subtracted from the sample rate and the difference was then divided by the conversion factor from the standard curve and the mass of CsOxOx enzyme (in mg) utilized in the reaction to obtain a specific activity in millimoles CO₂ per minute per milligram of CsOxOx.

Chapter 2.8: Determination of $K_M$ and $V_{max}$

The $K_M$ values for the CsOxOx catalyzed oxidation of oxalate was determined by plotting the initial rates at various oxalate substrate concentrations (0.1 to 10 $K_M$) using the MIMS assay previously described in Section 2.6. Specific activities were plotted versus millimolar oxalate substrate concentration and the plot was fit to the typical Michaelis-Menten rectangular parabola using the KaliedaGraph 4.1 software, and the $K_M$ was determined from the fit parameters. For product inhibition studies, the $K_M$ and $V_{max}$ was determined by measuring the specific activities of MIMS assay solutions containing oxalate concentrations of 20, 10, 5, 2.5, 1, 0.5, 0.25, 0.1, and 0.05
mM. Again, the specific activities were plotted versus millimolar oxalate concentrations and the plot was fit to the typical Michaelis-Menten rectangular parabola using the KaliedaGraph 4.1 software, and the kinetic constants were calculated directly from the fit parameters. Additional $K_M$ and $V_{max}$ determinations were obtained for the oxalate reaction at varying initial hydrogen peroxide concentrations (2 mM, 4 mM, 6 mM, 10 mM, 14 mM, and 20 mM). All Lineweaver-Burk plots were obtained by plotting the reciprocal specific activities versus the respective reciprocal substrate concentration using the KaliedaGraph 4.1 software.

Chapter 2.9: Determining reversibility of inhibition
The $V_{max}$ in µmol/minute was determined as previously described for solution containing 0 and 10 mM hydrogen peroxide, and total enzyme concentrations of 104.5, 69.7, 34.8, and 17.4 nM. $V_{max}$ determinations at both initial concentrations of hydrogen peroxide were plotted against total enzyme concentrations, and the two plots were fit to linear functions using the KaliedaGraph 4.1 software.

Chapter 2.10: Characterizing turnover dependent inactivation
In order to explore further the inactivation of CsOxOx during turnover, 772 nM enzyme was incubated at room temperature with 10 mM potassium oxalate in 50 mM sodium succinate, pH 4.0 in the presence and absence of 1.0 mg/mL bovine catalase. To test the stability of the enzyme, control incubations without oxalate present and with and without 10 mM hydrogen peroxide were also maintained at room temperature. Aliquots were removed at time 0, 2 hours, and 24 hours and assayed to determine initial rates under saturating substrate conditions ($>10 K_M$) as described in
Chapter 2.7: MIMS oxalate oxidase assay, above. Total enzyme concentrations of 128.8, 103.0, 77.2, and 51.5 nM were measured in duplicate. Rates under these conditions were plotted against total enzyme concentration for the 0 and 2-hour time points, as previously described\textsuperscript{53}.

Chapter 2.11: Determining the $K_i$ and $\alpha$ kinetic constants for the characterization of complete inhibition

Our data is suggestive of an ordered catalytic mechanism and the use of the terms $K_i$ and $\alpha K_i$ is most appropriate. Alternative terms ($K_{ii}$ and $K_{is}$) are sometimes seen in the literature when less information is known about the order of binding\textsuperscript{45,48}. In an effort to identify better define the kinetic constants, $K_i$ and $\alpha$ (Figure 13), as well as to discern complete versus partial noncompetitive inhibition, CsOxOx was assayed in 10, 2.5, 1.43, and 1 mM concentrations of oxalate in 40, 20, 10, and 0 mM initial concentrations of hydrogen peroxide. $K_i$ and $\alpha$ were estimated from the slope and intercept secondary plots of the reciprocal plots versus hydrogen peroxide concentration\textsuperscript{46}. A separate calculation of the $\alpha$ value was also determined by finding the reciprocal velocity value at which the reciprocal plots intersect.

\[
\frac{1}{v} = \frac{1}{V_{max}} \left( \frac{\alpha - 1}{\alpha} \right)
\]  
(Eq. 8)

Here, $\nu$ is the initial velocity at the intersection of the plots, $V_{max}$ is the velocity at saturating oxalate concentrations, and $\alpha$ is the factor that captures the relationship between the equilibrium constants of the two inhibitor binding steps. In order to find a value and uncertainty for the intersection point the average of unique intersection points calculated as the intersection of each line with every other line was determined.
Chapter 2.12: Effects of anaerobic incubation of CsOxOx with oxalate

Previous literature from Whittaker et al. demonstrates complete inactivation of barley oxalate oxidase by incubation of the enzyme with substrate in the absence of oxygen supporting Mn(III) containing enzyme as the active form that directly acts on oxalate. A similar experiment was performed to determine if the same is true in CsOxOx. A solution containing sodium oxalate (10 mM, pH 4.0) and sodium succinate (50 mM, pH 4.0) was purged with nitrogen for one hour. After one hour, CsOxOx was added to the, still purging, solution (77 nM). The solution was then allowed to incubate under nitrogen for another 40 minutes before being opened to the air. The whole process was observed in real time using membrane inlet mass spectrometry measuring particles with mass-to-charge ratios of 45 and 32.
Chapter 3: RESULTS AND DISCUSSION

Chapter 3.1: Kinetic plots for hydrogen peroxide are consistent with noncompetitive inhibition

The Michaelis-Menten and Lineweaver-Burk plots for varying concentrations of hydrogen peroxide are shown in Figure 15 and 16, respectively. The observed $V_{max}$ is represented as a percentage of the uninhibited $V_{max}$ and the $K_M$ values determined from the nonlinear regression of the Michaelis-Menten plots for the CsOxOx catalyzed oxidation of oxalate at varying initial concentrations of hydrogen peroxide obtained using the MIMS assay is presented in Table 1. With an initial hydrogen peroxide concentration of zero, the $V_{max}$ was estimated to be $2.03 \pm 0.03$ U/mg and the $K_M$ was estimated at 0.47 mM. Similarly, 2 mM initial hydrogen peroxide concentration the $V_{max}$ is $1.65 \pm 0.03$ U/mg and the $K_M$ is 0.47 mM, at 4 mM initial hydrogen peroxide concentration $V_{max}$ is $1.35 \pm 0.02$ U/mg and the $K_M$ is 0.56 mM, and at 10 mM initial hydrogen peroxide concentration the $V_{max}$ is $0.87 \pm 0.02$ U/mg and the $K_M$ is 0.76 mM. Initial concentrations of hydrogen peroxide reduce the apparent $V_{max}$ and appear to increase the observed $K_M$ value. Further investigation into the effects of hydrogen peroxide on the apparent $K_M$ is described in Chapter 3.4. These data suggest that hydrogen peroxide behaves as a mixed-type or noncompetitive inhibitor of CsOxOx. All models consistent with this observation have hydrogen peroxide reversibly binding both CsOxOx alone and another CsOxOx complex.
Figure 15: Michaelis-Menten plots of CsOxOx activity on oxalate at 0 mM (black, closed diamonds), 2 mM (red, closed triangles), 4 mM (blue, closed circles), and 10 mM (green, closed squares) initial concentrations of hydrogen peroxide. Coefficients of determination for the non-linear fits are as follows: 0.994 for the 0 mM hydrogen peroxide plot, 0.990 for the 2 mM hydrogen peroxide plot, 0.994 for the 4 mM hydrogen peroxide, and 0.990 for the 10 mM hydrogen peroxide plot.
Figure 16: Lineweaver-Burk plots of CsOxOx activity on oxalate at 0 mM (black), 2 mM (red), 4 mM (blue), and 10 mM (green) initial concentrations of hydrogen peroxide. Coefficients of determination for the linear fits are as follows: 0.997 for the 0 mM hydrogen peroxide plot, 0.995 for the 2 mM hydrogen peroxide plot, 0.993 for the 4 mM hydrogen peroxide, and 0.996 for the 10 mM hydrogen peroxide plot.
Table 1: Percent of the uninhibited specific activity and $K_M$ values for the CsOxOx catalyzed oxidation of oxalate in the presence and absence of hydrogen peroxide measured by MIMS.

<table>
<thead>
<tr>
<th>Substrate ± H$_2$O$_2$</th>
<th>% of Uninhibited Spe. Act., U/mg</th>
<th>$K_M$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalate</td>
<td>100</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>+ 2 mM H$_2$O$_2$</td>
<td>81.3</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>+ 4 mM H$_2$O$_2$</td>
<td>66.5</td>
<td>0.56 ± 0.04</td>
</tr>
<tr>
<td>+ 10 mM H$_2$O$_2$</td>
<td>42.9</td>
<td>0.76 ± 0.08</td>
</tr>
</tbody>
</table>

Chapter 3.2: [E] vs $V_{max}$ plots are consistent with hydrogen peroxide as a reversible inhibitor

Figure 17 shows the plots of total enzyme concentration, [E], versus oxalate saturated enzyme reaction velocity ($V_{max}$) for CsOxOx incubated with a 10 mM initial concentration of hydrogen peroxide and with no initial concentration of hydrogen peroxide. The best fit linear equation to the plot of $V_{max}$ versus total enzyme concentration for those experiments with no initial hydrogen peroxide concentration had a slope of 0.00050 U/nM ± 0.00004 U/nM, a y-intercept of -0.002 U ± 0.003, and the coefficient of determination was 0.95. The best fit linear equation to the plot of $V_{max}$ versus total enzyme concentration for those experiments with an initial hydrogen peroxide concentration of 10 mM had a slope of 0.00023 U/nM ± 0.00002 U/nM, a y-intercept of -0.001 U ± 0.001 U, and the coefficient of determination was 0.94. The slope of the line fit to the data without hydrogen peroxide was significantly greater that the slope of the line fit to the data obtained with an initial concentration of hydrogen peroxide suggesting that hydrogen peroxide is affecting the number and
distribution of intermediate substrate species at steady state. The x-intercept for the uninhibited plot was 7 nM ± 8 nM and the x-intercept for the inhibited plot was 8 nM ± 9 nM. The x-intercepts from both linear fits are not significantly different and appear to intersect the x-axis at approximately the same point suggesting that enzyme is not inactivated upon addition of hydrogen peroxide within the time and chemical restraints of the kinetic assay. From this plot, it seems all or most of the inhibition of CsOxOx by hydrogen peroxide under the constraints of the kinetic assay is reversible, and this is consistent with hydrogen peroxide as a reversible noncompetitive inhibitor. The lines do intersect at a non-zero point to the right of the principal axis. An explanation of this is likely that the assumed protein concentration determined using the Lowry protein determination slightly overestimated the protein concentration. The shift in the x-intercept from 0 only represents an overestimation of 4 nM ± 4 nM, or 0.6% of the total concentration of protein. Notably, one standard deviation from the x-intercept for both best fit lines includes zero.
Figure 17: The $V_{\text{max}}$ versus total CsOxOx enzyme concentrations at initial hydrogen peroxide concentrations of 0 mM (black) and 10 mM (blue). Each point represents an entire $V_{\text{max}}$ determination using oxalate concentrations of 0.2, 0.5, 1.0, 5.0, and 10.0 mM. Coefficients of determination for the linear fits are as follows: 0.97 for the 0 mM hydrogen peroxide plot, and 0.97 for the 10 mM hydrogen peroxide plot.
Chapter 3.3: CD spectra of CsOxOx show no change in global structure in hydrogen peroxide

Circular dichroism spectroscopy was implemented to assess the effects of hydrogen peroxide on the global protein structure of CsOxOx. Image A in Figure 18 shows overlaid CD spectra of CsOxOx at varied hydrogen peroxide concentrations (no H$_2$O$_2$, light blue; 4 mM H$_2$O$_2$, orange; 8 mM H$_2$O$_2$, grey; 12 mM H$_2$O$_2$, yellow; 16 mM H$_2$O$_2$, dark blue; 20 mM H$_2$O$_2$, green). In all hydrogen peroxide concentrations, the CsOxOx spectrum maintains most of its characteristic shape. The average global minimum was 208.6 nm ± 0.4 nm. A Pearson coefficient of 0.714 was found between hydrogen peroxide concentrations and the associated wavelength at the global spectrum minima suggesting that as hydrogen peroxide concentration increases the wavelength at the minima shifts to the right. The average value of the minima was -7592.6 deg·cm$^{-2}$·dmol$^{-1}$ ± 182.7 deg·cm$^{-2}$·dmol$^{-1}$. A Pearson coefficient of 0.995 was found between the hydrogen peroxide concentration and the magnitude of the global minima suggesting that as hydrogen peroxide concentration increases the minima increases in value (decreases in magnitude). Images B and C in Figure 18 demonstrate the effects of thermal denaturation and protein concentration on the observed CD spectrum, respectively. These data, reported previously by our lab have been included for comparison with those data obtained using hydrogen peroxide$^{51}$. All spectra display a similar shape with a single minimum. As temperature increases the value of the minima decreases significantly (Pearson coefficient -0.967) and the wavelength at the minima shifts to the right (Pearson coefficient 0.873). The minima value increases significantly with increasing enzyme concentration (Pearson coefficient 0.892) as does the wavelength at the minima (Pearson coefficient 0.967). Both temperature and CsOxOx concentration appear to have far greater effects on the minima value than
hydrogen peroxide concentration. Hydrogen peroxide by contrast does not appear to significantly shift the minimum wavelength value. Therefore, we conclude that hydrogen peroxide concentrations up to 20 mM have little effect on the overall secondary structure of the CsOxOx protein.

Figure 18: CD spectra of CsOxOx with (A) increasing concentration of hydrogen peroxide (light blue, no H$_2$O$_2$; orange, 4 mM H$_2$O$_2$; grey, 8 mM H$_2$O$_2$; yellow, 12 mM H$_2$O$_2$; dark blue, 16 mM H$_2$O$_2$; green, 20 mM H$_2$O$_2$), (B) at varying temperatures (0.68 mg/ml CsOxOx in 25 mM potassium phosphate (pH 7.0) at different temperatures: grey, 90 °C; burgundy, 80 °C; dark blue, 70 °C; green, 60 °C; blue, 50 °C; yellow, 40 °C; light grey, 30 °C; orange, 20 °C; light blue, 10 °C), and (C) at varying protein concentrations: light blue, 0.33 mg/ml; orange, 0.5 mg/ml; grey, 0.7 mg/ml; yellow 1.0 mg/ml; dark blue, 1.5 mg/ml; green, 3.0 mg/ml).

Chapter 3.4: Characterization of complete noncompetitive inhibition

In an effort to better characterize the noncompetitive inhibition observed in Figures 19 and 20, experiments at substrate concentrations resulting in Lineweaver-Burk plots of equidistance and equal weight were carried in a larger range of hydrogen peroxide concentrations. Plots of reciprocal initial oxalate oxidase activity versus reciprocal
oxalate concentration for CsOxOx are shown in Figure 19. Secondary plots of the slopes and intercepts of the reciprocal plot linear best fit lines versus hydrogen peroxide concentration are shown Figure 20. The $\alpha K_I$ and $K_I$ values are estimated to be $7.91 \pm 1.12$ and $2.84 \pm 1.06$ from the x-intercepts of the reciprocal plot intercept and slope versus hydrogen peroxide concentration secondary plots. The slope and intercept secondary plots are linear ($R^2$ values greater than 0.99) suggesting that the hydrogen peroxide inhibition of CsOxOx is complete and not partial (up to 40 mM hydrogen peroxide). From these data, $\alpha$ was estimated to be $2.79 \pm 1.12$. A way to mathematically check this value is to use the regression lines from the reciprocal plots. These constellate around the point $(-0.207\pm0.011, 0.195\pm0.042)$. This corresponds to an $\alpha$ value of $2.25 \pm 0.59$. The values for the $\alpha$ constant determined using two different methods agree well with one another and demonstrate that hydrogen peroxide is a noncompetitive inhibitor. It is assumed here that hydrogen peroxide inhibition of oxalate oxidase takes a form similar to the scheme shown in Figure 12.
Figure 19: Plots of reciprocal initial oxalate oxidase activity versus reciprocal oxalate concentration for CsOxOx in 0 (circle), 10 (square), 20 (diamond), and 40 (triangle) mM hydrogen peroxide. Coefficients of determination for the linear fits are as follows: 0.969 for the 0 mM hydrogen peroxide plot, 0.992 for the 10 mM hydrogen peroxide plot, 0.984 for the 20 mM hydrogen peroxide plot, and 0.864 for the 40 mM hydrogen peroxide plot.

Figure 20: Secondary plots of the slopes and intercepts from the reciprocal plot linear best fit lines (Figure 19) versus hydrogen peroxide concentration. Coefficients of determination for the linear fits are as follows: 0.998 for the slope secondary plot, and 0.998 for the intercept secondary plot.
Oxygen is assumed to be at saturating concentrations and therefore the binding of oxygen and subsequent decarboxylation can be simplified into one single irreversible step\textsuperscript{38}. Under these conditions the CsOxOx enzyme is effectively a unireactant system and can be modelled as such. Literature suggests that oxygen binds CsOxOx after the binding of oxalate. If this is true, the simplest model for hydrogen peroxide inhibition is that of a noncompetitive inhibitor that binds both the enzyme alone and the enzyme substrate complex. Linear secondary plots suggest that inhibition is complete and inhibitor bound enzyme species are not directly capable of catalysis. With this model, one can then assign meaning to the determined $\alpha K_I$ and $K_I$ values. Under these assumptions, the $K_I$ is the equilibrium constant describing the dissociation of the enzyme-inhibitor complex, and the $\alpha K_I$ is the equilibrium constant describing the dissociation of inhibitor from the enzyme-substrate-inhibitor complex. The $K_I$ value is significantly smaller than the $\alpha K_I$ value suggesting that hydrogen peroxide binds the CsOxOx enzyme alone stronger than it associates with the CsOxOx-oxalate complex, and is consistent with hydrogen peroxide as a product inhibitor that is formed from catalysis, remains bound to the CsOxOx enzyme, and then dissociates into bulk solution. The $\alpha K_I$ value suggests that while oxalate binding weakens the association of the hydrogen peroxide inhibitor it does not prevent its binding suggesting that hydrogen peroxide binds CsOxOx at a site other than the oxalate binding site. That oxygen has a binding site other than the active site manganese ion has been suggested for OxD\textsuperscript{54}. Experiments describing the effects of varying concentrations of oxygen on hydrogen peroxide product inhibition would allow further comment regarding the binding of oxygen with respect to hydrogen peroxide.
Chapter 3.5: HPLC and MIMS analysis show that CsOxOx is inactivated during turnover

Figure 21 shows the degree of oxalate oxidation by CsOxOx over time with (Figure 21B) and without (Figure 21A) catalase. Succinate, oxalate, and hydrogen peroxide standards eluted at 12.4, 6.9, and 6.3 minutes and informed the assignment of peaks in Figure 21. In Figure 21A, the peak at 6.7 minutes decreases in area over time starting at 60.85 mAU/minutes then slowly decreasing to 50.76 mAU/minutes after 24 hours of reaction. Roughly 83% of the oxalate (initially 10 mM) remained after 24 hours. The succinate peak eluting at 12.2 minutes remains largely the same for all time points consistent with its role as a buffer (22.85 mAU/minutes at 0 minutes, 24.33 mAU/minutes at 20 minutes, 23.51 mAU/minutes at 1 hour, 21.88 mAU/minutes at 2 hours, and 22.61 mAU/minutes at 24 hours). The peak representing hydrogen peroxide, eluting at 6.4 minutes, is apparent at time points 20 minutes, 1 hour, 2 hours, and 24 hours. The area of this peak increases over time starting at 3.37 mAU/minutes at 20 minutes and increases to 13.71 mAU/minutes after 24 hours consistent with the progression of the oxidative decarboxylation of oxalate. The hydrogen peroxide and oxalate peaks had some degree of overlap and confounded precise estimation of peak area and, by extension, chemical concentrations.
Figure 21: HPLC stacked chromatograms of the products of the CsOxOx catalyzed oxalate reaction. An aliquot of the incubation reaction without (A) or with (B) recombinant bovine catalase was analyzed at 0 minutes, 20 minutes, 1 hour, 2 hours, and 24 hours.

Stacked HPLC chromatograms in Figure 21B demonstrate oxalate oxidation by CsOxOx over 24 hours in the presence of catalase. As in the data shown in Figure 21A, the succinate peak eluting at 12.2 minutes remained roughly the same over the time tested (22.53 mAU/minutes at 0 minutes, 24.20 mAU/minutes at 20 minutes, 24.01 mAU/minutes at 1 hour, 24.31 mAU/minutes at 2 hours, and 24.31 mAU/minutes at 24 hours). The oxalate peak area began at 57.08 mAU/minutes at 0 minutes then decreased to 1.63 mAU/minutes after 24 hours. Roughly 3% of the oxalate substrate remains after 24 hours suggesting that the oxidation of the oxalate substrate (initially 10 mM) is nearly complete. Additionally, hydrogen peroxide generated during turnover does not build up in concentrations observed in the reaction without catalase. There is no apparent build-up of hydrogen peroxide over the 24 hours consistent with the presence of active catalase.
In analogous experiments employing the MIMS, samples of CsOxOx after 24 hours of turnover without catalase were assayed according to the method described in Chapter 2.7. CsOxOx was observed to be relatively stable under the conditions of the assay in the absence of substrate, retaining 73% of the original enzyme activity after 24 hours at room temperature diluted in buffer. CsOxOx incubated for 2 hours in 10 mM oxalate had only 20 percent of its original activity (not shown), and no activity was detected in enzyme incubated for 24 hours in 10 mM oxalate (Table 2). CsOxOx incubated for 24 hours in the presence of 10 mM oxalate and bovine catalase retained approximately 80% of its original activity; preventing the build-up of hydrogen peroxide prevented the previously observed inactivation. The inactivation of CsOxOx is only observed after pre-incubation with oxalate. Pre-incubation in buffer or buffer and hydrogen peroxide show no inactivation. Furthermore, addition of catalase to the turnover inactivated enzyme does not recover any significant amount of activity.

Table 2: MIMS measurements of CsOxOx with no prior turnover with and without hydrogen peroxide, and turnover in the absence and in the presence of 1.0 mg/mL bovine catalase. Uncertainties are presented as standard deviations from the mean value. Samples that had undetectable activity are signified by “nd”.

<table>
<thead>
<tr>
<th>Time, hours</th>
<th>No prior turnover, U</th>
<th>No prior turnover in 10 mM hydrogen peroxide, U</th>
<th>Turnover treated, U</th>
<th>Turnover treated plus catalase, U</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.026 ± 0.001</td>
<td>0.023 ± 0.001</td>
<td>0.026 ± 0.001</td>
<td>0.030 ± 0.001</td>
</tr>
<tr>
<td>24</td>
<td>0.019 ± 0.001</td>
<td>0.01 ± 0.001</td>
<td>nd</td>
<td>0.024 ± 0.003</td>
</tr>
</tbody>
</table>

In order to further investigate the CsOxOx turnover dependent inactivation, a plot of reaction velocities versus total enzyme concentration was prepared for enzyme treated with two hours of turnover prior to assay and enzyme incubated under similar conditions for 2 hours but without turnover (Figure 22). The slope of the line generated from turnover treated enzyme data is shallower than the line generated from
untreated enzyme suggesting some effect on the distribution or number of enzyme intermediate complexes at steady state. This is likely due in part to the build-up of hydrogen peroxide and its previously described reversible inhibitory effect. The x-intercept of the line generated from the turnover treated data is shifted significantly to the right of the intercept of the line generated from the untreated enzyme. This suggests that enzyme has been irreversibly inactivated during the two hours of turnover prior to assay.

![Graph](image)

**Figure 22**: Plot of total enzyme concentration versus saturated initial enzyme velocity. Data points in blue represent those points treated with two hours in oxalate prior to assay. Points in black represent samples treated with two hours of incubation without oxalate prior to assay. Coefficients of determination for the linear fits are as follows: 0.989 for the 0 mM hydrogen peroxide plot, and 0.949 for the 10 mM hydrogen peroxide plot.

Taken together, these data suggest that hydrogen peroxide is having two separate effects on the CsOxOx enzyme. That hydrogen peroxide behaves as a reversible
noncompetitive inhibitor as described in Chapter 3.1, but also the generation of hydrogen peroxide is linked to the turnover dependent irreversible inactivation of CsOxOx.

The mechanism of hydrogen peroxide, turnover dependent inactivation of CsOxOx is not entirely clear. An alternative mechanism proposed in the literature suggests that the fraction of the enzyme containing Mn(III) represents the catalytically active enzyme form contrary to other proposed mechanisms\textsuperscript{6,14,20,55}. In this alternative proposal, oxalate binds to the metal center followed by the subsequent decarboxylation of oxalate without the binding of diatomic oxygen to the metal center. The generated carbon dioxide anion radical then reacts with diatomic oxygen (possibly in bulk solution) to form carbon dioxide and a hydroperoxyl radical, a reaction for which there is chemical precedence\textsuperscript{56}. EPR studies observing the persistence of free hydroperoxyl species are consistent with this proposal\textsuperscript{57}. This proposal also suggests that the hydroperoxyl free radical is responsible for the reoxidation of Mn(II) to Mn(III), restarting the catalytic cycle. Loss of hydroperoxyl radical could thus prevent reoxidation of the metal center rendering it inactive. A similar mechanism of inactivation was proposed in an attempt to understand the observation of a spin trapped free-radical by EPR\textsuperscript{57}. This mechanism proposes a “leaky” active site from which the intermediate carbon species can enter “bulk” solution. It was postulated in the literature that loss of the electron sink at the metal center could also result in the inactivation of the enzyme\textsuperscript{54}. 

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Chapter 3.6: CsOxOx is not inactivated after anaerobic incubation in oxalate

Discrepancies exist in our observations on CsOxOx and observations on barley oxalate oxidase from literature\textsuperscript{21}. One example of this is the observation that barley oxalate oxidase is inactivated by catalase, while it has been shown here that catalase protects CsOxOx from inactivation. Additionally, Whitaker \textit{et al.} observed irreversible inactivation of barley oxidase after anaerobic incubation in oxalate.

Figure 23 shows the MIMS trace of the anaerobic incubation of CsOxOx in oxalate and the subsequent reintroduction of oxygen to the sample.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure23.png}
\caption{Reaction trace highlighting the subsequent production of labelled carbon dioxide following oxygenation of buffered, nitrogen purged solution containing oxalate and CsOxOx. Signals for O\textsubscript{2} and CO\textsubscript{2} are shown here in orange and blue respectively.}
\end{figure}

After reintroduction of oxygen, an increase is observed in the m/z 32 measurement shown in Figure 23. The generation of \textsuperscript{13}C labelled carbon dioxide immediately followed upon oxygen reintroduction suggesting that, while inactive under anaerobic conditions, CsOxOx retains its activity. The mechanism of deactivation of barley
oxalate oxidase described previously follows directly from Whitaker’s proposed catalytic mechanism\textsuperscript{21}. In it oxalate directly reacts with the active Mn(III) center resulting in the carbon dioxide anion species. Without oxygen the intermediate species is unable to generate the hydroperoxyl radical proposed to regenerate the active enzyme form and the enzyme is stuck in the inactive reduced form. However, the observations of CsOxOx are not consistent with this mechanism; CsOxOx remains active after treatment with oxalate under anaerobic conditions. This supports the role of Mn(II) as the resting enzyme center species whose job is the reductive activation of diatomic oxygen. Furthermore, our results suggest a completely different mechanism of inactivation. One that requires enzymatic turnover in the presence of hydrogen peroxide reminiscent of the inactivation of catalase by hydrogen peroxide\textsuperscript{58,59}.

Chapter 3.7: Observed kinetic isotope effect on the CsOxOx mediated reaction with oxalate

The change in reaction rate due to an isotopic substitution is called a kinetic isotope effect (KIE). There can be a number of factors that cause this observed effect including changes in symmetry of the reactant or its transition states due to isotope substitution, changes in moments of inertia, and/or changes in quantum tunneling. However, typically the most common explanation of the KIE is due to the change in the zero point energy of the vibrational energy bond under observation. This effect derives from the fact that the vibrational energy of two bonded species is dependent on the square root of the inverse of the reduced mass of the two bound species\textsuperscript{60}. KIEs from hydrogen to deuterium or tritium exchange tend to be large due to the dramatic percent change in the reduced mass of the system. Quantum tunneling effects are more commonly observed in KIEs from hydrogen to deuterium or tritium\textsuperscript{21,45}. Larger
isotopically labelled molecules have a much smaller KIE and quantum tunneling becomes far less likely and the relative change in reduced mass after isotopic substitution is small. For this reason, only highly sensitive techniques can precisely measure heavier molecule KIEs. To demonstrate the sensitivity of the MIMS as well as gain further mechanistic insight, a KIE was observed for the CsOxOx mediated oxidative decarboxylation of oxalate using an intermolecular competition experiment. Figure 24 shows an example of a continuous reaction trace of the intermolecular competition experiment measured using MIMS. The black trace follows the oxidation of $^{12}\text{C}$ oxalate over time via the measurement of $^{12}\text{C}$ carbon dioxide change at m/z 44. The blue trace follows the oxidation of $^{13}\text{C}$ oxalate over time by the measurement of $^{13}\text{C}$ carbon dioxide change at m/z 45. The V/V kinetic isotope effect of 1.05 was observed between the $^{13}\text{C}$ labelled and unlabeled oxalate substrates, and is a normal estimate for the doubly labelled $^{13}\text{C}$ kinetic isotope effect. This suggests that the carbon-carbon bond breaking step during catalysis is at least partially rate limiting.
Figure 24: MIMS reaction trace of an example intermolecular competition experiment in the estimation of the V/V kinetic isotope effect. The trace in black represents the increase in carbon dioxide from the $^{12}$C oxalate and the trace in blue represents the increase in carbon dioxide from the doubly labelled $^{13}$C oxalate.

Chapter 3.8: Non-stoichiometric consumption of oxygen and production of carbon dioxide

Studies reporting measurements of the stoichiometry of the oxalate oxidase catalyzed oxidative decarboxylation of oxalate are non-existent to the best of our knowledge. Figure 25 represents the first effort to study the observed stoichiometry of the oxalate oxidase mediated reaction as it relates to substrate concentration. Throughout the range of oxalate concentrations tested, the ratio of the production of carbon dioxide to the consumption of diatomic oxygen remains consistently at approximately 1.3 carbon dioxide molecules produced per diatomic oxygen consumed. This value is less than the theoretical value of 2 carbon dioxide molecules per oxygen molecule consumed. The consumption of additional oxygen per carbon dioxide molecule consumed suggests an alternative fate of an oxygen derived species. The generation of a hydroperoxyl radical is postulated in both proposed oxalate oxidase mechanisms and
has been observed in spin-trapping experiments on OxDC suggesting hydroperoxyl radicals are available in bulk solution\textsuperscript{57}. Hydroperoxyl radicals are highly reactive and can cause oxidative damage to proteins and other biological macromolecules. In one proposed mechanism (Figure 3) the hydroperoxyl radical couples to the carbon dioxide radical anion forming hydrogen peroxide and carbon dioxide. In the event that the hydroperoxyl radical species reacts with a species other than the carbon dioxide anion, the carbon dioxide anion would be free to react with another molecule of diatomic oxygen resulting in the formation of an additional hydroperoxyl radical and, possibly, further oxidative damage. In an alternatively proposed mechanism\textsuperscript{21}, the hydroperoxyl radical is generated from the reaction of carbon dioxide radical with diatomic oxygen. A pathway that accounts for the additional consumption of oxygen is not immediately apparent from this second proposal.
Chapter 3.9: CsOxOx catalyzes the oxidative decarboxylation of mesoxalate

A proposal exists in the literature that OxOx non-oxidatively decarboxylates mesoxalate to yield glyoxylate and CO$_2$\textsuperscript{23}. Figure 26 shows the reaction trace of the CsOxOx mediated mesoxalate reaction. The blue line follows the progression of the reaction by MIMS measuring the production of carbon dioxide at m/z 44. The orange line follows the consumption of diatomic oxygen throughout the OxOx mediated reaction. The black arrow indicates the time point at which CsOxOx was added to the reaction mixture. Subsequent to enzyme addition, carbon dioxide concentration increased and oxygen concentration decreased over time which is consistent with the production of carbon dioxide and the consumption of oxygen.
Figure 26: Reaction trace of the CsOxOx catalyzed mesoxalate reaction. The orange trace follows the consumption of diatomic oxygen at m/z 32. The blue trace follows the production of carbon dioxide at m/z 44. The black arrow signifies the addition of CsOxOx enzyme.

Figure 27 shows the time course of the CsOxOx mediated mesoxalate reaction as a series of stacked HPLC chromatograms. Chromatograms at time points 0 minutes, 20 minutes, 1 hour, 2 hours, and 24 hours contain peaks eluting at 6.8 minutes and 12.3 minutes representing mesoxalate and succinate chemical species, respectively. Glyoxylate standards informed an expected retention time of 9.4 minutes. No peak eluting at 9.4 minutes was observed in any of the chromatograms.
Reaction rates were observable using the horseradish peroxidase coupled assay. Average rates are consistent with the rates observed using the MIMS assay. The observation of a rate using the coupled assay is dependent on the production of hydrogen peroxide by the reaction.

The production of carbon dioxide, and hydrogen peroxide, and the consumption of oxygen by the CsOxOx mediated mesoxalate reaction are consistent with oxidative decarboxylation contrary to suggestions from literature suggesting that CsOxOx catalyzes the nonoxidative decarboxylation of mesoxalate to glyoxylate and carbon dioxide$^{23}$. 

**Figure 27:** HPLC stacked chromatograms following the CsOxOx mediated mesoxalate reaction over a period of 24 hours.
CHAPTER 4: CONCLUSIONS

Observations from MIMS kinetic experiments found that hydrogen peroxide is a reversible noncompetitive inhibitor of the CsOxOx mediated oxidative decarboxylation of oxalate. Additionally, HPLC and further MIMS experimentation found that CsOxOx undergoes a turnover dependent irreversible inactivation, and that catalase protects the enzyme from the described inactivation. CD spectra suggest hydrogen peroxide does not cause global structural change to CsOxOx in concentrations up to 20 mM. Additionally, anaerobic incubation of CsOxOx in oxalate does not inactivate the enzyme as was observed in barley oxalate oxidase. Studies observing the stoichiometry of the CsOxOx catalyzed oxidative decarboxylation of oxalate found that only 1.3 moles of carbon dioxide are produced per mole of oxygen consumed, and this observation differs from the theoretical stoichiometry in literature. An observed V/V kinetic isotope effect greater than 1 was observed for the CsOxOx mediated oxalate reaction suggesting that the carbon-carbon bond breaking step during catalysis is rate limiting. Finally, the CsOxOx mediated mesoxalate reaction consumes oxygen and produces hydrogen peroxide and carbon dioxide consistent with CsOxOx catalyzing the oxidative decarboxylation of mesoxalate.
APPENDIX A: CALIBRATION OF MIMS

Calibration curves for each measured gas were generated. For carbon dioxide calibration, a stock solution of sodium carbonate/potassium bicarbonate (1 mM, pH 10.2) is added into an acetic acid solution (50 mM) to produce a proportional concentration of CO$_2$ \textit{in situ}, and this entire process is monitored on the MIMS at m/z 44. This experiment is repeated through a range of final CO$_2$ concentrations, and the instrument response at m/z 44 at the height of the sample response peak is recorded at each concentration of CO$_2$. The instrument response is then subtracted from the baseline, and the difference is then plotted against the associated concentration of CO$_2$.

Figure A1 shows an example calibration curve relating ion current response at m/z 44 to carbonate generated carbon dioxide. In order to test the reproducibility, the slopes of the lines of fit of seven standard curves generated from carbonate (Method 2 above), calibration plots were 2.49e-6, 2.31e-6, 2.11e-6, 1.7e-6, 2.23e-6, 2.22e-6, and 2.42e-6 torr/mM. Respective intercepts were 1.20e-9, 4.68e-9, 3.27e-9, 7.18e-9, 2.07e-9, 1.86e-9, and 2.32e-9 torr. Intercepts are all very much smaller than the slope of the calibration curve and their effects on the predicted number were assumed to be insignificant. Therefore, the slope of the calibration curve only was used to relate ion current to carbon dioxide concentration. Standard curves depend on a number of experimental variables including barometric pressure, temperature, stir rate and must be measured daily.

Figure A2 shows an example calibration curve relating ion current response at m/z 32 to dissolved oxygen made from mixtures of saturated oxygen and nitrogen solutions.
Figure A1: Example calibration curve relating carbonate generated carbon dioxide to ion current.
A nitric oxide standard curve was generated via two reactions. The first reaction used was the decomposition of MAHMA nonoate at pH 4.0. Aliquots of a known concentration of MAHMA nonoate were added to a solution of succinate buffer at pH 4.0 to achieve a range of nanomolar concentration of nitric oxide. The second method utilized the reaction in which nitrite is oxidized to nitric oxide by potassium iodide under acidic conditions. Aliquots of a known concentration of sodium nitrite solution were added to a solution of 0.1M sulfuric acid to achieve a range of nanomolar concentration of nitric oxide detectable on the MIMS at m/z 30.

Figure A3 shows an example calibration curve relating nonoate derived nitric oxide concentration and ion current at 30 m/z. The average slope of the best fit line to the calibration curve was $1.33 \times 10^{-12} \text{ nM}^{-1} \pm 0.32 \times 10^{-12} \text{ nM}^{-1}$. The intercepts were small.
compared to the ion current reading at 0 nM nitric oxide. The nonoate standard curves varied in shape from linear to hyperbolic. The diminishing returns in signal for higher nitric oxide concentrations is likely due to the combination of nitric oxide reacting with oxygen and water to form nitrite as well as the escape of nitric oxide from the solution.

![Graph showing MIMS calibration curve](image)

**Figure A3:** Example MIMS calibration curve relating MAHMA nonoate derived nitric oxide to ion current.

Figure A4 shows an example calibration curve relating nitrite derived nitric oxide concentration and ion current at 30 m/z. The average slope of the best fit line to the calibration curve was $1.68 \times 10^{-12} \text{nM}^{-1} \pm 0.06 \times 10^{-12} \text{nM}^{-1}$. Intercepts were small compared to the 0 nM nitric oxide blank. The nitrite derived nitric oxide calibration curves are far more reproducible than those generated from nonoate. Under the
conditions of the measurement the nitrite produced by nitric oxides reaction with oxygen and water is converted back to nitric oxide by potassium iodide.

Figure A4: Example MIMS calibration curve relating nitrite derived nitric oxide to ion current.
APPENDIX B: DETECTION OF NITRIC OXIDE BY MIMS PROVIDES A BASIS FOR A NITRIC OXIDE SYNTHASE ASSAY

Nitric oxide synthases (NOS, EC 1.14.13.39) catalyze the oxygen and NADPH-dependent oxidation of L-arginine to citrulline and NO\(^6\). These enzymes possess different biochemical and regulatory attributes depending on the tissue from which they are derived. Understanding these enzymes is essential to understanding cellular signaling, vasodilation, and oxidative stress. Despite much effort and numerous available methods, detection of NO in biological samples remains a difficult task with each method possessing limitations and complex considerations. Methods available to assess NOS synthase activity include the Griess reaction\(^6\) and the measurement of the conversion of oxyhemoglobin to methemoglobin\(^3\). Membrane inlet mass spectrometry (MIMS) has been applied to the detection and measurement of NO\(^4\). Here we further the application of MIMS to the detection of NO by using a commercially available instrument and probe assembly and by applying this method to the assay of NOS.

Nitric oxide synthase (inducible from mouse, iNOS) and MAHMA nonoate were purchased from Sigma Aldrich. Other reagents were of the highest purity available and were purchased from either Sigma Aldrich or Fisher Scientific. Figures A2 and A3 in Appendix A show the calibration of the MIMS ion current to the concentration of NO generated from MAHMA nonoate and sodium nitrite, respectively. As may be seen from the calibration curves, the detection of NO by MIMS is linear over a range from 60 to 2000 nM. The lower level of detection of the spectrophotometric Griess reagent system is 1.0 \(\mu\)M.
We demonstrate the utility of MIMS in the assay of NOS by an end point assay (Figure B1) and a direct, continuous assay (Figure B2). Reaction mixtures contained 1 mM arginine, 1 mM magnesium acetate, 0.15 mM NADPH, 180 µM DTT, 18 µM THB, and 50 mM HEPES, pH 7.4. For the end point assay, samples were quenched at 20, 30, 40, and 50 minutes by addition of an aliquot of the reaction mixture into the 0.1 M H$_2$SO$_4$/0.1 M KI solution and the m/z 30 was measured. For the direct, continuous method, the MIMS probe was placed directly into a cuvette containing the stirred reaction mixture (maintained at 37°C) and the reaction was initiated by the addition of enzyme. Results from these two assays methods yield comparable results (0.038 U/mg for the end point assay and 0.024 U/mg for the continuous assay). These experiments provide a basis for the application of MIMS for the assay of NOS.
Figure B2: Reaction trace measuring the production of nitric oxide at m/z 30 of the nitric oxide synthase mediated reaction. The black arrow indicates the addition of nitric oxide synthase enzyme.
APPENDIX C: TARTRONIC ACID AND DIETHYL KETOMALONATE AS ALTERNATIVE SUBSTRATES

As introduced in Chapter 1.8, understanding the degree of promiscuity (substrate ambiguity) of CsOxOx is important to provide insights into the molecular features that allow some enzymes to utilize a broad range of substrates, the role substrate ambiguity may play in enzyme evolution, and to guide efforts to tailor enzymes as biocatalysts. In the HRP coupled assay, tartronic acid and diethyl ketomalonate appear as weak inhibitors, each with $K_I$ values of approximately 10 mM (data not shown). Assessing whether or not these three-carbon molecules that share structural similarities with mesoxalate, serve as substrates was not possible using the HRP coupled assay. We, therefore, employed the MIMS assay (as described in Chapter 2.7) to determine whether or not they serve as alternative substrates and to acquire initial rate data under saturating conditions.

Representative traces are shown in Figure C1 (tartronic acid) and Figure C2 (diethyl ketomalonate). The results are summarized as activities relative to oxalate in Table B1. That tartronic acid only possesses 4% of the activity relative to oxalate and approximately 10% relative to mesoxalate emphasizes the importance of the alpha carbonyl oxygen in mesoxalate in contrast to the hydroxyl group in that position in tartronic acid. That diethyl ketomalonate (diethyl mesoxalate) serves as a substrate with a significant rate (12% relative to oxalate and 28% relative to mesoxalate) raises interesting questions about how this molecule fits into the active site and binds to the metal center. Proton NMR studies were carried out (data not shown) that demonstrate that the aqueous samples tested for activity had not been hydrolyzed to the carboxylic acid. These data showing that tartronic acid and diethyl ketomalonate serve as
alternative substrates expand our understanding of the degree of substrate promiscuity of the CsOxOx catalyzed reaction\textsuperscript{51} and may motivate future work to identify the amino acid residues involved in this promiscuity that may, in turn, provide insight into the evolutionary path that CsOxOx has taken and delineate a path toward its modification for its use as a biocatalyst.

Figure C1: Reaction trace measuring the production of carbon dioxide at m/z 44 of the CsOxOx mediated reaction using tartronic acid as a substrate. The black arrow indicates the addition of CsOxOx enzyme.
Figure C2: Reaction trace measuring the production of carbon dioxide at m/z 44 of the CsOxOx mediated reaction using diethyl ketomalonate as a substrate. The black arrow indicates the addition of CsOxOx enzyme.

Table C1: Relative activities of the CsOxOx catalyzed reaction utilizing mesoxalate, tartronic acid, and diethyl ketomalonate. Activities are relative to that with oxalate in 50 mM sodium succinate, pH 4.0.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative Activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalate</td>
<td>100</td>
</tr>
<tr>
<td>Mesoxalate</td>
<td>43</td>
</tr>
<tr>
<td>Tartronic acid</td>
<td>4</td>
</tr>
<tr>
<td>Diethyl ketomalonate</td>
<td>12</td>
</tr>
</tbody>
</table>
APPENDIX D: OXIDATION OF $^{13}$C GLYOXYLATE TO $^{13}$C MESOXALATE

In order to improve the sensitivity of the MIMS assay of the CsOxOx catalyzed mesoxalate reaction, an effort was made to synthesize $^{13}$C$_3$-mesoxalate. Triply labelled $^{13}$C mesoxalate is unavailable commercially and, therefore, we attempted to synthesize it from $^{13}$C$_3$-glycerol prior to kinetic studies. A literature method demonstrated a one-pot, high yield, mesoxalate synthesis procedure starting from glycerol, utilizing TEMPO as a catalyst, and bleach as the distal oxidant$^{52,65}$. Mesoxalate synthesis was carried out on triply label $^{13}$C glycerol according to the literature procedure. The identity and purity of $^{13}$C mesoxalate was determined using HPLC and $^{13}$C NMR. Two sample solutions of synthesis product were dissolved in mobile phase (2.2 g/L). One sample was spiked with a concentrated solution of sodium mesoxalate monohydrate (Sigma-Aldrich) (0.1 mM) prior to both samples being filtered through 0.45 micron syringe filter and extensively degassed under vacuum. The HPLC chromatogram of synthesized product showed a strong peak at 6.7 minutes that agrees with the spiked sample chromatogram peak at 6.9 minutes and is consistent with a mesoxalate product (Figure D1). However, it should be noted that mesoxalate co-elutes with oxalate.
13C NMR spectra were used to confirm the identity of the reaction products. Figure D2 shows the NMR spectrum of the synthesis product(s). The doublet at 174.8 ppm is consistent with a highly deshielded carboxylate 13C nucleus adjacent to another carbon nucleus, and is also consistent with a mesoxalate product. The triplet at 73.4 ppm is consistent with a moderately shielded 13C nucleus adjacent to two different 13C nuclei; peak of the correct multiplicity but shifted further up field than what would be expected for a mesoxalate product. The strong singlet at 170.7 ppm is consistent with a one unique 13C nucleus or a group of identical 13C nuclei and is not consistent with a mesoxalate product. Figure D3 shows the 13C NMR spectrum of a store-bought 13C saturated sodium oxalate sample at pH 4.0. This spectrum shows a strong singlet at 169 ppm. Furthermore, the singlet peaks overlap in a 13C NMR of the synthesis product spiked with store-bought 13C oxalate (Figure D4), consistent with an oxalate product.
synthesis product. Thus, TEMPO and sodium hypochlorite oxidize glycerol to oxalate as the major product.

**Figure D2:** $^{13}\text{C}$ NMR of the TEMPO mediated glycerol reaction product at pH 4.0.
Figure D3: $^{13}$C NMR of saturated C$^{13}$ sodium oxalate at pH 4.0.
Figure D4: $^{13}$C NMR of the TEMPO mediated glycerol reaction product spiked with $^{13}$C sodium oxalate at pH 4.0.
References


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