Biochemical Characterization of 2-Nitropropane Dioxygenase from Hansenula MRAKII

Slavica Mijatovic

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BIOCHEMICAL CHARACTERIZATION OF 2-NITROPROPANE DIOXYGENASE

FROM HANSENULA MRAKII

by

SLAVICA MIJATOVIC

Under the Direction of Dr. Giovanni Gadda

ABSTRACT

2-Nitropropane dioxygenase from Hansenula mrakii is a flavin-dependent enzyme that catalyzes the oxidation of anionic nitroalkanes into the corresponding carbonyl compounds and nitrite, with oxygen as the electron acceptor. Although nitroalkanes are anticipated to be toxic and carcinogenic, they are used widely in chemical industry for a quick and effective way of synthesizing common reagents. Consequently, the biochemical and biophysical analysis of 2-nitropropane dioxygenase has a potential for bioremediation purposes. In this study, recombinant enzyme is purified to high levels, allowing for detailed characterization. The biochemical analysis of 2-nitropropane dioxygenase presented in this study has established that enzyme utilizes alkyl nitronates as substrates by forming an anionic flavosemiquinone in catalysis. The enzyme is inhibited by halide ions, does not contain iron and has a positive charge located close to the N(1)-C(2)═O locus of the isoalloxazine moiety of the FMN cofactor.

INDEX WORDS: 2-Nitropropane Dioxygenase, Nitronate, Sulfite, Flavoprotein, FMN, Enzyme kinetics, Flavin semiquinone, Hansenula mrakii.
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SLAVICA MIJATOVIC

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Georgia State University

2008
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FROM HANSENULA MRAKII

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Office of Graduate Studies
College of Arts and Sciences
Georgia State University
February 2008
DEDICATION

To my late grandfather Tomislav Vidakovic
ACKNOWLEDGEMENTS

To stand where I am today would not be possible without my parents Slavko and Edis Mijatovic. There are never enough adequate words to express my gratitude for your support and encouragement in pursuit of my education. My “rock”, Muamer Rustempasic, thank you for the support, listening and understanding. Without you where would I find the courage to go through life and a safe place at the end of the day. I am thankful to my brother Tomislav, niece Adriana, nephew Dario and grandmother Kata for providing the emotional support and laughter.

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# TABLE OF CONTENTS

DEDICATION..............................................................................................................iv

ACKNOWLEDGEMENTS..............................................................................................v

LIST OF TABLES..........................................................................................................xi

LIST OF FIGURES........................................................................................................xii

LIST OF SCHEMES....................................................................................................xiii

Chapter I INTRODUCTION............................................................................................1

1. Nitroalkanes..............................................................................................................1
   1.1. Physical properties..............................................................................................1
   1.2. Toxicity and carcinogenicity..............................................................................4

2. Previous studies of 2-nitropropane dioxygenase from *Hansenula mrakii*..............6
   2.1. Purification from the original source.................................................................6
   2.2. Biochemical characterization............................................................................6
   2.3. Kinetic studies...................................................................................................8
   2.4. Inhibition of 2-nitropropane dioxygenase by superoxide scavengers.............9
   2.5. Stoichiometry of the reaction............................................................................10

3. Flavin dependent nitroalkane-oxidizing enzyme.....................................................10
   3.1. Nitroalkane oxidase............................................................................................12
   3.2. 2-Nitropropane dioxygenase from *Neurospora crassa*....................................15
   3.3. Glucose oxidase................................................................................................18
   3.4. D-Amino acid oxidase.....................................................................................20
   3.5. Propionate-3-nitronate oxidase........................................................................22

4. Goals......................................................................................................................23
Chapter II  OXIDATION OF ALKYL NITRONATES CATALYZED BY 2-NITROPROPANE DIOXYGENASE

Abstract .......................................................... 39
Introduction ....................................................... 41
Materials and methods ........................................ 43
  Materials ......................................................... 43
  Instruments .................................................... 43
  Cloning of 2-Npd into pET20b(+) .......................... 44
  Expression and purification of 2-nitropropane dioxygenase .......................... 44
  Biochemical methods ........................................ 45
  Enzyme kinetic assays ...................................... 46
  Data analysis ................................................. 47
Results .......................................................... 48
  Cloning, expression and purification of 2-nitropropane dioxygenase .............. 48
  Cofactor content ............................................. 48
  Alkyl nitronates as substrates for 2-nitropropane dioxygenase ...................... 50
  Effect of superoxide dismutase and catalase on the enzymatic activity .......... 53
  Substrate specificity ........................................ 54
  Formation of an N(5)-flavin adduct with sulfite .................................... 55
Discussion ..................................................... 58
References ..................................................... 63

Chapter III  STOICHIOMETRY OF THE REACTION CATALYZED BY 2-NITROPROPANE
Chapter IV  INHIBITION OF 2-NITROPROPANE DIOXYGENASE WITH CHLORIDE IONS

Abstract..............................................................................................................83
Introduction.........................................................................................................84
Experimental Procedure.....................................................................................87
Materials...........................................................................................................87
Methods...........................................................................................................87
Data analysis.....................................................................................................87
Results and Discussion......................................................................................89
Chapter V  pH STUDIES ON 2-NITROPROPANE DIOXYGENASE

Abstract

Introduction

Experimental procedure

Materials

Methods

Preparation of substrates

Data analysis

Results and discussion

References

Chapter VI  GROWING THE CRYSTALS OF RECOMBINANT 2-NITROPROPANE DIOXYGENASE

Abstract

Introduction

Experimental Procedures

Materials and Methods

Crystallization

Results and discussion

References

Chapter VII  GENERAL DISCUSSION

References

Appendix 1
LIST OF TABLES

Table 1.1. pK\textsubscript{a} values of nitroalkanes.................................................................3

Table 2.1. Inductively coupled plasma mass spectroscopic analysis of \textit{H. mrakii} 2-nitropropane dioxygenase.................................................................50

Table 2.2. Enzymatic activity of \textit{H. mrakii} 2-nitropropane dioxygenase with 1 mM ethynitronate as a substrate in the absence and presence of nitroethane in 50 mM potassium phosphate pH 7.4 and 30 °C......................................................53

Table 2.3. Effect of superoxide dismutase and catalase on the enzymatic activity of \textit{H. mrakii} 2-nitropropane dioxygenase.................................................................54

Table 2.4. Apparent steady state second order rate constants, \(k_{cat}/K_M\), for alkyl nitronates as substrates for \textit{H. mrakii} 2-nitropropane dioxygenase in 50 mM potassium phosphate pH 6 and 30 °C.................................................................55

Table 3.1. Oxygen consumption and nitrite production during 2-nitropropane dioxygenase turnover with butyl-1-nitronate as substrates........................................74

Table 3.2. Oxygen consumption and nitrite production during 2-nitropropane dioxygenase turnover with 100 µM pentyl-1-nitronate as substrates..............................75

Table 3.3. Effect of superoxide dismutase and catalase on oxygen consumption and nitrite production during 2-nitropropane dioxygenase turnover with 10 mM alkyl nitronates as substrates.................................................................76

Table 4.1. Apparent steady state second order rate constants, \(k_{cat}/K_M\), in absence and presence of potassium chloride in 50 mM potassium phosphate pH 6 and 30 °C..........................90

Table 6.1. Crystal screen HR2-110 reagent formulation.........................................................117

Table 6.2. Crystal screen lite HR2-128 reagent formulation.....................................................118
LIST OF FIGURES

Figure 2.1. Purification of recombinant 2-nitropropane dioxygenase from *Hansenula mrakii*….48
Figure 2.2. UV-visible absorbance spectrum of 2-nitropropane dioxygenase as purified……….49
Figure 2.3. Anaerobic substrate reduction of 2-nitropropane dioxygenase with alkyl nitronates.52
Figure 2.4. Apparent steady state kinetics of 2-nitropropane dioxygenase with ethynitronate or propyl-1-nitronate as determined in 50 mM potassium phosphate, pH 6 and 30 °C……..55
Figure 2.5. Reaction of 2-nitropropane dioxygenase with sodium sulfite……………………56
Figure 3.1. Betaine-aldehyde and hexanal standard curve …………………………………..77
Figure 4.1. Oxygen consumption of 2-nitropropane dioxygenase with 20 mM ethynitronate during turnover………………………………………………………………………………..88
Figure 4.2. Effect of KCl on $k_{cat}/K_M$ of 2-nitropropane dioxygenase……………………..90
Figure 4.3. Apparent steady state kinetics of 2-nitropropane dioxygenase with ethynitronate in presence of 1 mM potassium iodide, potassium bromide or potassium fluoride as determined in 50 mM potassium phosphate, pH 6 and 30 °C…………………………..92
Figure 5.1. pH dependence of the $k_{cat}/K_m$ value of 2-nitropropane dioxygenase from *Neurospora crassa* with nitroalkanes………………………………………………………………………98
Figure 5.2. pH dependence of the $k_{cat}$ value of 2-nitropropane dioxygenase from *Neurospora crassa* with alkyl nitronates………………………………………………………………………98
Figure 5.3. Steady state kinetic with ethynitronate and oxygen as substrates in 50 mM potassium phosphate pH 6………………………………………………………………………………102
Figure 5.4. Steady state kinetic with ethynitronate and oxygen as substrates in 50 mM potassium phosphate pH 8………………………………………………………………………………103
Figure 5.5. Steady state kinetic with ethylnitronate and oxygen as substrates in 50 mM potassium pyrophosphate pH 10.5……………………………………………………………103

Figure 5.6. pH dependence on $k_{cat}/K_m$ with ethylnitronate and mixture of ethylnitronate and nitroethane………………………………………………………………………………104

Figure 5.7. pH dependence on $k_{cat}/K_m$ with butyl-1-nitronate and mixture of butyl-1-nitronate and nitrobutane………………………………………………………………………………105

Figure 6.1. Crystal structure of nitroalkane oxidase from *Fusarium oxysporum*………………110

Figure 6.2. Active site of nitroalkane oxidase illustrating an alternative orientation of Asp402 and Ser276………………………………………………………………………………111

Figure 6.3. Overall structure of 2-nitropropane dioxygenase from *Pseudomonas aeruginosa*...112

Figure 6.4. Active site of 2-nitropropane dioxygenase from *Pseudomonas aeruginosa*………..113
LIST OF SCHEMES

Scheme 1.1 Nitroalkanes in aqueous solution...............................................................5
Scheme 1.2. The ionization of nitroethane (left) to yield ethyl nitronate (right) in solution.....7
Scheme 1.3. Denitrification reaction catalyzed by oxidase and dioxygenase......................12
Scheme 1.4. Kinetic mechanism of nitroalkane oxidase...............................................13
Scheme 1.5. Proposed mechanism for nitroalkane oxidase..........................................14
Scheme 1.6. Proposed mechanism for 2-nitropropane dioxygenase from Neurospora crassa....17
Scheme 1.7. The enzymatic reaction catalyzed by glucose oxidase..............................18
Scheme 1.8. Reaction catalyzed by flavin dependent D-amino acid oxidase....................20
Scheme 2.1. The ionization of nitroethane (left) to yield ethyl nitronate (right) in solution....41
Scheme 3.1. Reaction catalyzed by 2-nitropropane dioxygenase.................................69
Scheme 4.1. Reaction catalyzed by 2-nitropropane dioxygenase.................................83
Chapter I

INTRODUCTION

1. Nitroalkanes

1.1. Physical properties. One of the fundamental classes of substances in organic chemistry, nitroalkanes are polar and acidic compounds [1]. They exist as the neutral form in organic solvents, whereas in aqueous solutions they are in equilibrium between protonated, non-protonated nitronic acid and the anion of the nitronate (Scheme 1.1)[2].

\[
\begin{align*}
\text{H} & \quad \text{R-CH} & \quad \text{R-CH} \\
\text{N}^+ & \quad \text{O} & \quad \text{N}^+ \\
\text{O} & \quad \text{O} & \quad \text{OH}
\end{align*}
\]

Scheme 1.1 Nitroalkanes in aqueous solution.

Nitroalkanes can be generated from a wide variety of organic compounds. Direct nitration of aliphatic hydrocarbons via anionic intermediates, alkenes or ketones (\(\text{\(\mathfrak{N}\)nitration}) yields nitroalkanes [3; 4]. In addition, nitroalkanes can be generated by conversion of other functionalities, such as: carbonyls, oximes and azides [5; 6; 7] or by nitration of the alkyl halides with metal nitrites, such as silver nitrite in diethyl ether (Victor-Meyer reaction), potassium nitrite, or sodium nitrite in \(N,N\)-dimethylformamide or in dimethyl sulfoxide (Kornblum reaction) [8; 9]. The most common method used in preparation of nitroalkanes is the conversion of alkyl halides to nitro compounds.

Alkyl nitronates or anionic nitroalkanes can be generated from nitroalkanes by adding any of the bases that act as carbon nucleophiles including haloalkanes [10], aldehydes [11; 12] and Michael acceptors [13].
The nitro group can be converted into a carbonyl group by treating conjugate bases with sulfuric acid via a reaction known as the Nef reaction, which involves hydrolysis of the C=N double bond [14; 15]. Also, there are several other methods for converting nitroalkanes to carbonyl compounds, such as reaction of aliphatic nitro compounds with aqueous TiCl₃, cetyltrimethylammonium permanganate, tin complexes and NaHSO₃, activated dry silica gel or 30 % H₂O₂-K₂CO₃ [16]. In addition, conversion of nitroalkanes to carbonyl compounds can be achieved by treatment of the nitro compound with KMnO₄, t-BuOOH and a catalyst, ceric ammonium nitrate, MoO₅-pyridine-HMPA, ozone, or singlet oxygen [16].

Scheme 1.2. The ionization of nitroethane (left) to yield ethynitronate (right) in solution.

Since nitroalkanes undergo a variety of carbon-carbon bond-forming processes and the nitro group can be converted into several other functional groups, nitroalkanes have been important for production of explosives and as precursors for azo dyes [14] [4]. Due to the high electron-withdrawing power of the nitro group that provides an enhancement of the hydrogen acidity at the α-position, nitroalkanes are source of stabilized carbanions [5; 17; 18]. Today, because of the low pKₐ values for deprotonation of the α-carbon (Scheme 1.2) [19], nitroalkanes play an important role as synthetic intermediates in the preparation of perfumes, pharmaceuticals, dyes, plastics and many natural products [5; 20; 21; 22; 23]. In addition to being synthesized in the chemical industry, leguminous plants produce nitro toxins such as 3-nitro-1-propionic acid and 3-nitro-1-propanol [24; 25; 26].
Nitroalkanes are unusual acids because the protonation of the $\alpha$-carbon is much slower than expected for the majority of the acids. Consequently, proton transfer reactions involving nitroalkanes have generated interest for many years [27; 28; 29; 30; 31; 32; 33; 34; 35; 36]. Usually, increased carbanion basicity is generally observed with each addition of the substituent that increases nucleophilicity [28; 37]. In contrast (Table 1.1), the $pK_a$ value of $\text{RCH}_2\text{NO}_2$ decreases in the order $\text{CH}_3\text{NO}_2 > \text{CH}_3\text{CH}_2\text{NO}_2 > (\text{CH}_3)_2\text{CHNO}_2$ in water, but the rate of proton abstraction by hydroxide ion decreases in the same order [38]. Therefore, the reaction is slower for the more acidic substrate.

<table>
<thead>
<tr>
<th>Nitroalkane</th>
<th>$pK_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitromethane</td>
<td>10.21</td>
</tr>
<tr>
<td>Nitroethane</td>
<td>8.5</td>
</tr>
<tr>
<td>1-Nitropropane</td>
<td>8.98</td>
</tr>
<tr>
<td>2-Nitropropane</td>
<td>7.7</td>
</tr>
</tbody>
</table>

Table 1.1. $pK_a$ values of nitroalkanes in water at 25 °C[19].

Bernasconi attributes the slow deprotonation of the nitroalkanes to the imbalance of the transition state, where the degree of charge delocalization into the $\pi$-acceptor group lags behind proton transfer [39]. Therefore, the rate constant is decreased when product-stabilizing factors lag behind the transition state. Because of that imbalance, as Bernasconi further rationalizes, the transition state cannot take advantage of the resonance stabilization of the carbanion because stabilization is barely developed during the transition state. This unusual relationship between rates and equilibria for deprotonation of nitroalkanes is known as the nitroalkane anomaly [38]. The anomaly has been analyzed in terms of four interactions: electrostatic and conjugative effects of the substituent which operate both at the product and transition state, and interactions between the substituent and the base, and between the substituent and a partial negative charge localized at $C_\alpha$ which are effective only at the transition state [38].
1.2. Toxicity and carcinogenicity. Nitroalkanes have been anticipated to be toxic [40] and some to be carcinogenic [41; 42; 43; 44; 45; 46; 47]. Nitromethane was shown to have carcinogenic activity in mice based on a 2-year inhalation study that showed increased incidences of mammary gland fibroadenomas and carcinomas [48]. Additionally, 1-nitropropane has been shown to be a mutagen in cells, since it induces unscheduled DNA synthesis in rats but does not induce tumors in rats following chronic exposure [49]. The secondary nitroalkanes, 2-nitrobutane and 3-nitropentane, produce a highly significant incidence of hepatocarcinoma with metastases to the lungs, whereas the primary nitroalkane 1-nitrobutane was not found to be carcinogenic [50]. The nitronates of the secondary nitroalkanes 2-nitropropane, 2-nitrobutane, 3-nitropentane, 2-nitroheptane, nitrocyclopentane and nitrocyclohexane were found to be substrates for the aryl sulfotransferase-catalyzed production of 8-aminoguanosine and 8-oxoguanosine from guanosine in vitro [50]. None of the primary nitronates of 1-nitropropane, 1-nitrobutane, 1-nitropentane and 1-nitroheptane were found to be substrates for the aryl sulfotransferase in vitro [50].

A number of compounds have been used to induce oxidative DNA damage in experimental animals [51]. As a potent hepatocarcinogen, 2-nitropropane and its anionic form propyl-2-nitronate have been used in many studies for induction of carcinogenic cells in rats [52; 53; 54; 55; 56; 57; 58; 59; 60; 61; 62]. In addition, 2-nitropropane has been found to be mutagenic in a number of short-term mutagenicity assays both in vitro and in vivo [63] and to cause point mutations in a microbial test system [64].

Although many studies have been focused on the metabolic pathway of 2-nitropropane and propyl-2-nitronate in the cell, no mechanism for DNA and RNA modification by 2-nitropropane has been generally accepted. It has been hypothesized that the genotoxicity of
propyl-2-nitronate may be due to the generation of DNA-damaging reactive forms of oxygen or free radicals [65]. The nitrite radicals, formed after the nonenzymatic degradation of 2-nitropropane or propyl-2-nitronate through peroxidative chain reaction, react appreciably fast with ribonucleosides, deoxyribonucleosides, and guanosine nucleotides, inducing DNA damage [66]. On the contrary, it was also proposed that metabolic sequence from 2-nitropropane to the reactive species causing DNA and RNA modifications does not involve the removal of the nitro group [67]. Additionally, it was found that the DNA damage was also caused by radicals generated via hydrogen peroxide species in metabolism of 2-nitropropane [68]. Secondly, the stimulatory effect of 2-nitropropane on the cellular proliferation and the rate of DNA synthesis in the liver has been hypothesized as the mechanism by which the carcinogenic action is induced [63].

The genotoxicity of 2-nitropropane in rats has been attributed to sulfotransferase-mediated formation of DNA-reactive nitrenium ions from the anionic form of 2-nitropropane [69]. The sulfotransferase-mediated pathway, in the case of the formation of 8-aminoguanine in both DNA and RNA, included converting the carcinogen into species capable of aminating nucleic acids and proteins involving oxime- and hydroxylamine-O-sulfonates as intermediates [70]. Neutral form of 2-nitropropane is metabolized to hydroxylamine-O-sulfonate or acetate, which yields the reactive nitrenium ion as well [71]. 2-Nitropropane has been found to induce aryl sulfotransferase-mediated liver DNA and RNA base modifications identified as 8-aminoguanine, 8-oxoguanine, 8-hydroxyguanine, 8-oxo-2'-deoxyguanosine [72]. Two rat sulfotransferases were identified of being capable of catalyzing the metabolic activation step of propyl-2-nitronate [73]. In addition, the human phenol sulfotransferases were capable of metabolically activating propyl-2-nitronate and are apparently identical to sulfotransferases in rat
liver, where 2-nitropropane causes carcinomas [74]. In another study authors concluded that species other than the rats and organs other than the liver can be targets for the genotoxicity and carcinogenicity of secondary nitroalkanes [75]. In conclusion, 2-nitropropane in neutral and anionic form should be regarded as a potential human carcinogen, either because of radical formation or the formation of intermediates in sulfotransferase-mediated pathways.

2. Previous studies of 2-nitropropane dioxygenase from *Hansenula mrakii*

2.1. Purification from the original source. In 1975, Kido and Soda found that some intercellular enzymes of the yeast *Hansenula mrakii* can oxidatively denitrify 2-nitropropane, 1-nitropropane and nitroethane [76]. A year later, 2-nitropropane dioxygenase was isolated for the first time from *Hansenula mrakii* by the same scientific team [77]. The yeast extract was grown in a medium containing nitroethane as the sole nitrogen source. The purification procedure involved fractionation with ammonium sulfate, followed by column chromatography using the ionic exchanger diethylamminoethylcellulose (DEAE) and hydroxyapatite (HA), as well as gel filtration through a Bio-Gel P-150 column. This procedure allowed for a 1300-fold purification of the enzyme, with an overall yield of 22%. The resulting enzyme of two non-identical subunits showed to be homogeneous by disc-gel electrophoresis and ultracentrifugation techniques [77; 78; 79]. Subsequently, the same group purified the enzyme consisting of a single polypeptide by utilizing the same procedure in addition to another DEAE-cellulose chromatography step [80]. Overall, 41 mg of purified enzyme could be obtained from 18 kg wet cell paste of the original host *Hansenula mrakii*.

2.2. Biochemical characterization. The purified *H. mrakii* enzyme was characterized as a non-heme iron flavoprotein based on the observation that the UV-visible absorbance
spectrum maxima at 370, 415 and 440 nm resembled the spectra of dihydroorotic acid dehydrogenase and xanthine oxidase [77; 79]. The molecular mass of the isolated enzyme was 62,000 Da, comprised of two non-identical subunits of 39,000 and 25,000 Da, as determined by gel filtration, sedimentation equilibrium methods and disc gel electrophoresis. The flavin species in 2-nitropropane dioxygenase was identified as FAD rather than FMN due to the observation that apo-D-amino acid oxidase can be activated with the flavin isolated from 2-nitropropane dioxygenase by acid denaturation and centrifugation of the protein [77]. In addition, a 1:1 ratio of flavin to protein was determined by comparing the fluorescence intensity of the flavin isolated from 2-nitropropane dioxygenase to the fluorescence of free FAD [79]. A 1:1 ratio of iron to protein was determined by atomic absorption method [77]. Based on this data, the authors initially proposed that 2-nitropropane dioxygenase contains two non-identical subunits with FAD and iron for cofactors. However, subsequent purification methods, which included additional DEAE-cellulose chromatography, yielded an enzyme form that consisted of a single polypeptide with a molecular weight of 42,000 [80]. In addition to performing the disc-gel electrophoresis and sedimentation equilibrium analysis, the atomic absorption analysis and UV-visible absorbance spectra confirmed the absence of the previously reported smaller subunit and iron. With this study, the authors concluded that an iron-containing protein, identified as a smaller 25,000 Da unit, rapidly bound to the 2-nitropropane dioxygenase. Therefore the two-protein complex, exhibited characteristics of iron-containing protein in all of the previous studies. By comparing the amino acid sequence of old and new purifications, the authors identified additional 200 amino acids in the initial two purifications [80]. In addition, amino acid analysis of 2-nitropropane dioxygenase revealed that the enzyme has flavin and NADH binding sites [81].
2.3. **Kinetic studies.** The oxidation of nitroalkanes by 2-nitropropane dioxygenase was observed upon initial purification of the enzyme [77]. The enzymatic assays were performed by incubating the nitroalkane substrates in 100 µmol potassium phosphate pH 7 or 8 with the enzyme aerobically at 30 °C and measuring the oxygen consumption or nitrite production after 60 minutes of incubation [77]. The enzyme was found to be catalytically active with 2-nitropropane, 1-nitropropane and nitroethane but not with nitromethane [77]. Additionally, the enzyme was found to utilize alcohols as substrates, such as 3-nitro-2-pentanol, 3-nitro-2-butanol and 2-nitro-1-butanol, by converting them into nitrite and the corresponding carbonyl compounds [77]. Spectrophotometric studies on the enzyme incubated with 2-nitropropane confirmed that the enzyme is capable of oxidizing nitroalkanes [77; 79]. In later studies by the same group, the enzyme was found to be more efficient with the anionic form of nitroalkanes [80]. The kinetic parameters of the enzyme with 1-nitropropane, 2-nitropropane, nitroethane, 3-nitro-2-butanol, 3-nitro-2-pentanol and their anionic counterparts showed that the second-order rate constant $k_{cat}/K_M$ is significantly higher and $k_{cat}$ values were 20 to 70 fold larger for anionic substrates compared to their neutral counterparts. The enzyme was most efficient at pH 8 with neutral and pH 6.5 with anionic species, as supported by nitrite formation or oxygen consumption studies. The authors did not list all of the conditions needed to examine enzymatic activity at various pH with anionic and neutral nitroalkanes as substrates, but just stated the optimum conditions for denitrification of these substrates [80]. Based on these data, the authors proposed that the neutral nitroalkanes are enzymatically converted into the anionic form prior to the oxidation and that the deprotonation is the rate-limiting step in denitrification of neutral nitroalkanes [80]. A bi bi ordered kinetic mechanism was determined for 2-nitropropane dioxygenase, where 2-nitropropane binds to the enzyme first, followed by the oxygen to form an
oxygenated ternary complex. The subsequent release of the nitrite and acetone occurs with the concomitant reoxidation of the reduced flavin. In addition, the conclusion of the kinetic mechanism was further supported by product inhibition studies. Acetone and nitrite were found to be competitive and noncompetitive inhibitors of the enzyme with respect to 2-nitropropane, whereas both products inhibited the enzyme non-competitively with respect to molecular oxygen when enzyme was not saturated by 2-nitropropane, results consistent with sequential kinetic mechanism [78].

2.4. Inhibition of 2-nitropropane dioxygenase by superoxide scavengers. Since the initial purifications contained the protein with iron, the inhibition studies were performed with chelating agents as well as several other compound [77]. Tiron, cysteine and glutathione were found to inhibit 2-nitropropane dioxygenase completely while HgCl₂ and oxine were found to be inhibitors. In contrast, chelating agents for divalent and trivalent metals such as α,α'-dipyridyl and o-phenanthroline acted as weak activators. Based on these studies authors concluded that inhibition of enzymatic activity is not due to chelating of the iron. Since the compounds such as tiron, oxine and various thiols were known to trap superoxide anions effectively, formation of the superoxide intermediate was hypothesized. Further studies on enzymatic activity in the presence of superoxide dismutase and various superoxide scavengers such as cytochrome c, epinephrine, NADH, thiols and polyhydric phenols confirmed the formation of superoxide during the denitrification of 2-nitropropane [78]. The observation that addition of superoxide to the enzymatic reaction induced the oxidation of nitroalkanes further, confirmed the formation of superoxide during catalysis. These studies led to the hypothesis of superoxide function as an essential intermediate in the oxygenation, which is formed from organic substrate and oxygen [78; 82].
2.5. Stoichiometry of the reaction. To determine whether 2-nitropropane dioxygenase catalyzes the denitrification of nitroalkanes via oxidase or oxygenase mechanism, formation of hydrogen peroxide was monitored due to ability of oxidases and not oxygenases to produce hydrogen peroxide [77]. The lack of hydrogen peroxide formation during catalysis indicated that the nitroalkane-oxidizing enzyme acts as an oxygenase rather than an oxidase. Additionally, incorporation of \(^{18}\text{O}_2\) into acetone during the oxidation of 2-nitropropane confirmed that both atoms of molecular oxygen are incorporated into acetone [79]. In contrast, replacing the 2-nitropropane with 1-nitropropane and nitroethane as substrates, no incorporation of \(^{18}\text{O}_2\) was observed in final products propionaldehyde and acetaldehyde, respectively. Based on these studies, the authors hypothesized that exchange of oxygen between aldehydes and water is far more rapid compared to the exchange between acetone and water. Therefore, 2-nitropropane dioxygenase was classified as oxygenase. Furthermore, oxygen consumption as well as nitrite and acetone formation was determined in order to elucidate the stoichiometry of the enzymatic reaction. Reaction mixture was constructed by incubating 2-nitropropane in 13 mM potassium phosphate pH 8 with the enzyme at 30 °C for 60 minutes. This study suggests that 2-nitropropane dioxygenase is an intermolecular dioxygenase capable of denitrifying 2 molecules of 2-nitropropane by forming two molecules of nitrite while incorporating two atoms of oxygen into two molecules of the acetone.

3. Flavin dependent nitroalkane-oxidizing enzymes.

Since Little’s discovery of oxidative degradation of nitroethane and 2-nitropropane by extracts of *Neurospora crassa* and pea seedling, respectively, many enzymes have been characterized to have the ability to utilize nitrocompounds as substrates [83; 84]. Several
Streptomyces strains have been identified to catalyze the oxidation of 2-nitropropane to acetone and nitrite [85]. An enzyme found in Aspergillus flavus is capable of nitrite formation from β-nitropropionic acid [86]. Degradation of 3-nitropropanol by unidentified bacteria from the ruminal fluid of cattle have been reported [87]. In addition, the flavoenzyme propionate-3-nitronate oxidase can oxidatively denitrify branched anionic nitroalkanes [88].

Other flavin-dependent enzymes, such as glucose oxidase [89] and D-amino acid oxidase [90], have been found to oxidatively denitrify nitroalkane anions in addition to their physiological substrates. 2-Nitropropane dioxygenase from H. mrakii compared to D-amino acid oxidase and glucose oxidase was found to be 1200 and 1800 times more effective with anionic nitroalkanes, respectively [91]. In the attempt to elucidate the mechanism of flavoprotein oxidases with the physiological substrates, the analogs were used in mechanistic studies. Successfully, studies on D-amino acid oxidase with anionic nitroalkanes as substrates have provided evidence for N-5 flavin-carbanion adduct to be obligatory intermediate in oxidation reactions of D-amino acid oxidase with nitroalkanes [92]. In contrast, the reaction of DAAO with the physiological substrates proceeds via hydride transfer mechanism [120].

To date, two flavin-dependent enzymes, nitroalkane oxidase from Fusarium oxysporum and 2-nitropropane dioxygenase from Neurospora crassa, have been identified to utilize nitroalkanes as their substrates [93; 94]. The two enzymes differ greatly in their proposed mechanism, one is an oxidase and second is dioxygenase (Scheme 3.1.).
3.1. **Nitroalkane oxidase.** Among all the nitroalkane oxidizing enzymes, nitroalkane oxidase (NAO, EC 1.7.3.1.) is the most extensively characterized enzyme in its biochemical, structural, kinetic and mechanistic properties. Nitroalkane oxidase is a flavin dependent enzyme \[95\] that catalyzes the oxidation of nitroalkanes to aldehydes with the production of hydrogen peroxide and nitrite, with oxygen as an electron acceptor \[93\]. In 1978, an inactive form of nitroalkane oxidase was isolated from the fungus *Fusarium oxysporum* by Kido, Hashizume and Soda \[96\]. Upon addition of exogenous flavin adenine dinucleotide (FAD), the active form of the enzyme was capable of oxidizing primary and secondary nitroalkanes. Subsequent work by Fitzpatrick’s group provided a detailed biochemical characterization of the enzyme as well as a detailed mechanism. Nitroalkane oxidase consists of four subunits of identical molecular weight (47,000) \[95\]. Even though the enzyme requires FAD for activity, upon denaturation of the protein at the neutral pH a stoichiometric amount of oxidized flavin was observed spectroscopically \[97\]. The spectroscopic and chromatographic studies on the flavin cofactor revealed that the inactive form of the enzyme contains a 5-nitrobutyl-FAD adduct as a cofactor \[97\]. The authors proposed that formation of the adduct occurs in the fungal cell during the incubation with nitroethane, where formation of the adduct requires reaction of the flavin with a neutral nitroethane followed by the nucleophilic attack by the anionic nitroethane \[97\].

**Scheme 1.3.** Denitrification reactions catalyzed by oxidases and dioxygenases.
Moreover, the carbanion intermediate is involved in both catalysis and inactivation of the enzyme [97].

Nitroalkane oxidase utilizes a broad range of non-aromatic nitroalkanes as substrates, but it has a preference for primary unsubstituted nitroalkanes [98]. Monotonic increase of the second-order rate constant $k_{\text{cat}}/K_M$ with increasing length of the alkyl chain up to four carbon was observed. The two orders of magnitude smaller specificity constant for substrates containing hydroxyl group suggested that the enzyme has a hydrophobic active site [98]. In this context, nitroalkane oxidase utilizes hydrophobic interactions to stabilize both the binding of the substrate and the transition state developed during catalysis [99].

![Scheme 1.4. Kinetic mechanism of nitroalkane oxidase. Taken without permission [93].](image)

A ping-pong kinetic mechanism was determined for nitroalkane oxidase [93], in which following substrate oxidation and flavin reduction, the aldehyde product is released and the reduced flavin reacts with oxygen (Scheme 1.4.). The pH dependence studies on the kinetic parameters ($k_{\text{cat}}$ and $k_{\text{cat}}/K_M$) of nitroalkane oxidase with nitroethane showed the requirement for two ionizable groups: one must be unprotonated and the second protonated for catalysis [100]. The tyrosine 398, which participates in binding of the substrate by forming the hydrogen bond to
the nitro group, is an amino acid that needs to be protonated for catalysis, based on the inactivation studies with tyrosine directed reagent, tetranitromethane [101]. The oxidation of nitroalkanes by nitroalkane oxidase is initiated by a base-catalyzed proton abstraction from the α-carbon of the substrate by Asp402, as indicated by the crystallographic and mutagenesis studies, as seen in Scheme 1.5 [102; 103; 104].

![Scheme 1.5. Proposed mechanism for nitroalkane oxidase. Taken without permission [104].](image)

Proton abstraction is irreversible and fully rate-limiting for reduction as indicated by the kinetic isotope studies on $k_{cat}/K_M$ with nitroethane and reductive-half reaction studies [104]. Upon generation of the anionic substrate, the resulting carbonanion can attack the N(5)-position of the FAD and form a covalent adduct, as suggested by trapping experiments with cyanide [105]. The carbon-hydrogen bond cleavage on the substrate is the rate limiting step in catalysis,
as indicated by kinetic isotope effects studies [100]. The cleavage of carbon-nitrogen bond results in elimination of nitrite and formation of a cationic electrophilic imine that can be attacked by hydroxide [100]. The active site base is required for deprotonation of the water molecule to form the hydroxide, which reacts with the cationic imine. The imine can be trapped by anionic nitroethane to form the stable and inactive form of the flavin, 5-nitrobutyl-flavin adduct [97], or by elimination of aldehyde can yield reduced FAD [100]. The inhibition studies revealed that both oxidized and reduced flavin can form the dead-end complexes by either binding to the aldehyde as a product or the nitroalkane as the substrate, respectively [106].

3.2. 2-Nitropropane dioxygenase from *Neurospora crassa*. 2-nitropropane dioxygenase (EC 1.13.11.32) from *Neurospora crassa* is a flavin-dependent enzyme that catalyzes the oxidation of both anionic and neutral nitroalkanes to the corresponding carbonyl compounds and nitrite, with oxygen as an electron acceptor [94]. In 1951, 2-nitropropane dioxygenase was isolated from *Neurospora crassa* by Little and characterized as an oxidase [83]. At the later date the enzyme was reclassified as a dioxygenase based on $^{18}$O$_2$ isotope study observation that the oxygen atom of organic product formed during the oxidation of propyl-2-nitronate was derived from molecular oxygen and not from water [107]. Biochemical and mechanistic studies were possible upon obtaining large quantities of pure and stable enzyme using recombinant technology [94]. The purification procedure involved fractionation with ammonium sulfate followed by column chromatography using the anion exchanger DEAE-sepharose and octyl-sepharose columns. The recombinant enzyme is a homodimer of 80 kDa, with each monomer containing one tightly but not covalently bound FMN. The enzyme has broad substrate specificity, in that 2-nitropropane dioxygenase can oxidatively denitrify 2-nitropropane in addition to a number of primary nitroalkanes in anionic or neutral forms.
Furthermore, the size of the alkyl chain does not affect the overall enzymatic rate of turnover, with the enzyme being more specific for nitronates as compared to nitroalkanes [94].

**Scheme 1.6.** Proposed mechanism for 2-nitropropane dioxygenase from *Neurospora crassa*. Taken without permission [108].
A sequential steady state kinetic mechanism was determined for the recombinant enzyme, as indicated by steady state kinetic studies with either neutral or anionic form of 2-nitropropane, nitroethane, nitrobutane, and nitrohexane [94]. Oxidation of both neutral and anionic nitroalkanes proceeds in the same manner, in addition to the initial abstraction of the proton from the \( \alpha \)-carbon of the neutral nitroalkanes (Scheme 1.6) [94]. Mechanistically, the oxidation catalyzed by 2-nitropropane dioxygenase can be summarized as follows. First, during the reductive half-reaction, after the organic substrate binds to the free enzyme, the enzyme-bound flavin is reduced through a single electron transfer from the organic substrate, forming an anionic semiquinone [94]. Second, during the oxidative half-reaction, the anionic semiquinone reacts with molecular oxygen to form a superoxide anion, which in turn reacts with the nitro radical to yield a nitroperoxide anion species that is released from the active site. It was proposed that the nitroperoxide anion species undergoes non-enzymatic nucleophilic attack to yield nitrite and carbonyl product. A non-oxidative deprotonation/protonation pathway, in which the enzyme catalyzes the interconversion of nitroalkanes between their anionic and neutral forms was proposed based on the inverse \( \alpha \)-secondary kinetic isotope effects on \( k_{\text{cat}}/K_M \) with ethylnitronate and 1-[\(^2\text{H}\)]-ethylnitronate as substrates [108]. pH dependence studies on the kinetic parameters \( (k_{\text{cat}} \text{ and } k_{\text{cat}}/K_M) \) of 2-nitropropane dioxygenase showed the requirement of an acid and both acid and a base for catalysis, for anionic and neutral nitroalkanes, respectively [94]. A likely role for the catalytic base, proposed to be His 196, is to abstract the proton from the \( \alpha \)-carbon of the neutral nitroalkane substrate. In contrast, no solvent viscosity effect has been detected on the kinetic parameters \( (k_{\text{cat}} \text{ and } k_{\text{cat}}/K_M) \) with nitroethane or ethylnitronate as substrates, suggesting that the substrate and product bind to the enzyme in rapid equilibrium [108]. Overall, mechanistic studies suggest that enzymatic turnover with neutral substrates is limited by proton
abstraction at low pH and formation of the flavosemiquinone at the high pH. In contrast, the turnover with anionic substrates is limited by the non-oxidative tautomerization of ethylnitronate to nitroethane at high pH [108].

3.3. Glucose oxidase. An FAD-dependent glucose oxidase (E.C. 1.1.3.4) catalyzes the oxidation of β-D-glucose to δ-glucono-lactone (Scheme 1.7) [109].

Glucose oxidase has been used as a biosensor for the quantitative determination of glucose in bodily fluids, beverages, food and fermentation liquor [110; 111], as well as for the production of gluconic acid that serves as a food preservative [112]. The active form of glucose oxidase has been isolated from several molds as well as fungi, such as *Penicillium amagasakienase*, *Aspergillus niger*, *Phanerochaete chrosporium* and *Talaromyces flavus* [109; 113; 114; 115]. The majority of biochemical and mechanistic studies have been performed on glucose oxidase from *Aspergillus niger*; therefore biochemical characteristics of the enzyme in this chapter focus on this enzyme. The dimer has the molecular mass between 150 to 180 kDa depending on the degree of glycosylation; with each monomer containing tightly attached FAD near the interface [109; 114]. A presence of the negative charge at the N(1)-C(2)=O locus of the isoalloxazine moiety has been identified based on NMR studies on the anaerobically reduced species of glucose oxidase at pH 5.6 [116]. The crystallographic data showed the
presence of His516\textsuperscript{Ne2} located ~ 3.8 Å from N(1) locus of the flavin, therefore stabilizing in its protonated state the negative charge at the N(1)-C(2)=O locus of the flavin [110].

The oxidation of glucose by glucose oxidase proceeds via a ping pong kinetic mechanism [117]; where upon formation of enzyme substrate complex, glucose is oxidized to gluconolactone and subsequent reduction of flavin occurs. The oxidative half-reaction proceeds with the release of the product with the subsequent oxidation of the reduced FAD by molecular oxygen and production of the hydrogen peroxide [117]. Product dissociation is a first order rate-limiting step, with glucose as substrate. However, substrate oxidation becomes partially rate limiting in steady state, whereas the product release does not limit the enzyme turnover with 2-deoxyglucose as substrate [117].

While the oxidation of glucose by the enzyme has been extensively studied, denitrification of nitroalkanes by glucose oxidase has been only reported for nitroethane as a substrate by Porter and Bright in 1976 [89]. Prior to any kinetic studies, substrate specificity study revealed that glucose oxidase is most efficient with nitroethane, followed by nitromethane, 1-nitropropane and 2-nitropropane. In addition, anionic forms were 6 x 10\textsuperscript{3} times more reactive than their neutral counterparts. A ping-pong kinetic mechanism was determined for glucose oxidase with nitroalkanes as substrates [89]. The oxidation of the nitroalkanes by glucose oxidase involves formation of the semiquinone form of the flavin. For each equivalent ethynitronate and oxygen consumed, formation of an equivalent of acetaldehyde, nitrate and hydrogen peroxide were not observed. In addition, formation of dinitroethane and nitrite were observed as products of the enzymatic reaction with nitroethane as substrate. Formation of the dinitroethane was found to be inversely proportional to the oxygen consumed when concentration of oxygen was below 240 µM. The authors proposed that the ratio of 1:1 of
products to reactants was not measured due to the involvement of the semiquinone species of the flavin in the oxidation of the nitroalkanes, therefore glucose oxidase is incapable of stabilizing the radical pairs sufficiently in order to transfer the second electron to the flavin and thereby complete overall oxidation of the nitroalkanes [89].

3.4. D-Amino acid oxidase. An FAD-dependent D-amino acid oxidase (E.C. 1.4.3.3, DAAO) catalyzes the dehydrogenation of D-amino acids to the corresponding $\alpha$-imino acids that are subsequently hydrolyzed to $\alpha$-keto acids and ammonia [118; 119] (Scheme 1.8).

The oxidation of D-amino acids by DAAO proceeds via a sequential kinetic mechanism [120]; during the reductive half-reaction, the amino acid is oxidized with concomitant reduction of the bound flavin, followed by the oxidative half-reaction in which reduced flavin is oxidized by molecular oxygen with subsequent release of the products [118; 121]. In an attempt to better understand the reactivity of enzyme-bound FAD, studies on the oxidation of alternative substrate were performed [90; 92].

DAAO was found to be irreversibly inactivated by nitromethane [90]. Due to the inactivation of the enzyme being dependent on the concentration of nitromethane, the authors
concluded that nitromethane is a substrate for DAAO only at low concentrations whereas at concentration higher than 5 mM nitroethane inactivates the enzyme [90; 92]. Consequently, the second study involving oxidation of nitroalkanes by DAAO was performed with anionic nitroethane as a substrate [92]. The oxidation of nitroethane by DAAO involves the formation of a covalent adduct between the substrate carbanion and the N-5 position of the flavin, which rapidly eliminates nitrite, becomes hydrated, and finally rearranges to expel acetylaldehyde and fully reduced flavin. For each equivalent ethynitronate and oxygen consumed, one equivalent of acetaldehyde, nitrite and hydrogen peroxide is formed [92]. The oxidation of nitroethane anion by D-amino acid oxidase proceeds via ping-pong kinetic mechanism as supported by the steady state kinetic data.

It was also noted that enzyme is inactivated by the substrate when the concentration of the substrate exceeded 5 mM. The inhibition, as authors proposed, is due to the interactions of a second anion of nitroethane binding to the enzyme [92]. The formation of the modified coenzyme of the inactivated enzyme, N5-acetyl-1,5-dihydro FAD, did not require oxygen as supported by similar results obtained in anaerobic and aerobic conditions [123]. The inhibition of the DAAO by the anion species was further examined in the presence of 1-chloro-1-nitroethane, where the chloro and nitro groups were recovered as free Cl⁻ and NO₂⁻ upon complete inactivation of the enzyme by 1.5 flavin equivalents of 1-chloro-1-nitroethane [123]. Furthermore, the inhibition of the DAAO by N-chloro-D-leucine was studied with respect to the site and mechanism of chlorination [124]. In that study it was shown that flavin reduction slows down by the factor of 2x10³ due to chlorination of the tyrosine that is converted to 3,5-dichlorotyrosine, as supported by amino acid analysis and spectral titrations. The chlorination reaction is highly specific, as only chloro derivatives of D-leucine, D-isoleucine, and D-
norvaline are able to inactivate DAAO, while chloro derivatives of L-amino acids can tightly bind and perturb the spectrum of enzyme-bound FAD [124]. Further studies with N-chloro-D-leucine confirmed that the chlorination of the DAAO in the active site region is consistent with consecutive chlorination of an amino acid residue by the first 2 molecules of N-chloro-D-leucine [125]. Anaerobic and aerobic spectral studies of DAAO with D-alanine and β-chloro-D-alanine as substrates further supported that the chlorinated derivatives inhibit the enzyme reaction with D-alanine competitively. Furthermore the kinetic studies revealed that the enzyme reacts with β-chloro-D-alanine four times less efficiently than with D-alanine [126].

3.5. Propionate-3-nitronate oxidase. An FMN-dependent propionate-3-nitronate oxidase (EC 1.7.3.5) from *Penicillium atrovenetum* catalyzes oxidation of 3-nitropropionate to malonate semialdehyde, nitrite and hydrogen peroxide [88]. To date, no in-depth biochemical, structural or kinetic characterization has been performed on propionate-3-nitronate oxidase. Only one study on the purification, cofactor and substrate specificity of the enzyme has been published in 1987 by Porter and Bright [88]. In that study, propionate-3-nitronate oxidase has been purified from the original source, i.e. fungus *Penicillium atrovenetum*, in active and stable form using three chromatographic steps onto anion exchange, hydroxyapatite and blue-agarose columns. The homogeneous enzyme is a dimer with molecular mass of 73,000 Da as indicated by sodium dodecyl sulfate-gel electrophoresis and gel filtration methods. Oxidized flavin spectra of propionate-3-nitronate oxidase rapidly converts into the anionic semiquinone flavin spectra upon anaerobic addition of propionate-3-nitronate, followed by the slow decomposition of semiquinone to the fully reduced flavin, as indicated by the anaerobic spectrophotometric study. Substrate specificity study on the enzyme revealed that the enzyme is capable of catalyzing numerous branched nitronates such as butyrate-4-nitronate, 2-hydroxypropionate-3-nitronate,
propyl-amine-3-nitronate and 2-aminopropionate-3-nitronate. In the same study, a ping-pong mechanism has been assigned to the enzyme based on the steady state kinetics of the enzyme with propionate-3-nitronate.

4. Goals

2-Nitropropane dioxygenase from *Hansenula mrakii* is a flavin dependent enzyme that catalyzes the denitrification of anionic nitroalkanes into corresponding carbonyl compounds and nitrite, with oxygen as an electron acceptor. The study of this enzyme is of importance for applied reasons, since nitroalkanes have been used widely in chemical industry but have been found to be toxic and carcinogenic. The study of 2-nitropropane dioxygenase has the potential for the development of bioremediation agents that target the denitrification of toxic and carcinogenic nitrocompounds. From a fundamental standpoint, 2-nitropropane dioxygenase is an addition to two well-characterized flavin enzymes that catalyze neutral and both neutral and anionic nitroalkanes as intrinsic substrates, nitroalkane oxidase from *Fusarium oxysporum* and *Neurospora crassa* enzyme, respectively. For this reason, the goal of the research presented herein is to investigate the biophysical, biochemical, spectrophotometrical, kinetic, and mechanistic properties of the 2-nitropropane dioxygenase purified from recombinant source.

The biophysical, biochemical, structural, and mechanistic characterization of an enzyme usually requires large quantities of the pure, active and stable form of such enzyme. Consequently, the first step in this project will be aimed at the purification of the recombinant enzyme. Efficient approaches for protein purification, such as fractionation with ammonium sulfate and the ionic exchanger diethylamminoethylcellulose column, will be required in order to obtain large quantities of the pure enzyme.
For a newly purified enzyme, understanding the biochemical and kinetic properties is of the importance for possible use of the enzyme for applied reasons. This requires the establishment of the cofactor, steady state kinetics, pH-dependence, substrate specificity as well as identifying the compounds that can inhibit the enzyme. In addition, examining the stoichiometry of the reaction catalyzed by 2-nitropropane dioxygenase can shed further light into the mechanism of this enzyme.

An in-depth characterization of the function and mechanism of 2-nitropropane dioxygenase also calls for the determination of the three-dimensional structure through x-ray crystallography. Structural data can give insight into why the enzyme efficiently utilizes anionic but not neutral nitroalkanes. Therefore, obtaining the crystals of the 2-nitropropane dioxygenase as part of the structure determination will be performed.
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Chapter II

OXIDATION OF ALKYL NITRONATES CATALYZED BY 2-NITROPROPANE DIOXYGENASE

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Abstract

2-Nitropropane dioxygenase from Hansenula mrakii was expressed in Escherichia coli cells and purified in active and stable form using 60% saturation of ammonium sulfate and a single chromatographic step onto a DEAE column. MALDI-TOF mass spectrometric and spectrophotometric analyses of the flavin extracted by heat and acid denaturation of the enzyme indicated that FMN, and not FAD as erroneously reported previously, is present in a 1:1 stoichiometry with the protein. Inductively coupled plasma mass spectrometric analysis of the enzyme established that H. mrakii 2-nitropropane dioxygenase contains negligible amounts of iron, manganese, zinc, and copper ions, which are not catalytically relevant. Anaerobic substrate reduction and kinetic data using a Clark oxygen electrode to measure rates of oxygen consumption indicated that the enzyme is active on a broad range of alkyl nitronates, with a marked preference for unbranched substrates over propyl-2-nitronate. Interestingly, the enzyme reacts poorly, if at all, with nitroalkanes, as suggested by lack of both anaerobic reduction of the enzyme-bound flavin and consumption of oxygen with nitroethane, nitrobutane, and 2-nitropropane. Finally, both the tight binding of sulfite ($K_d = 90 \mu M$, at pH 8 and 15 °C) to the enzyme and the formation of the anionic flavosemiquinone upon anaerobic incubation with alkyl...
nitronates are consistent with the presence of a positively charged group in proximity of the N(1)-C(2)=O atoms of the FMN cofactor.
Introduction

Nitroalkanes are widely used in chemical industry because they provide a quick and effective method of synthesizing common reagents [1; 2]. This stems primarily from the low pK\textsubscript{a} values in the range from 7 to 10 and the slow rates for protonation and deprotonation of the \( \alpha \)-carbon [3; 4], which allow for the presence of either (anionic) alkyl nitronates or (neutral) nitroalkanes under mild conditions (Figure 1). However, many nitroalkanes, and their correspondent alkyl nitronates, are anticipated to be toxic [5; 6; 7; 8; 9; 10], mutagens in bacteria [11; 12], and hepatocarcinogens in rats [5; 6]. Consequently, the study of the biochemical and kinetic properties of enzymes with the ability of oxidizing nitroalkanes and alkyl nitronates has potential for bioremediation applications.

\[
\begin{align*}
\text{CH}_3\text{C} & - \text{C} - \text{H} \\
\text{O} & \text{N}^+ \\
\text{O} & \text{O} \\
\end{align*}
\]

\[ pK_a = 8.5 \]

\[
\begin{align*}
\text{H}_2\text{C} & - \text{C} - \text{H} \\
\text{O} & \text{N}^+ \\
\text{O} & \text{O} \\
\end{align*}
\]

\[
\begin{align*}
\text{H}_3\text{C} & - \text{C} - \text{H} \\
\text{O} & \text{N}^+ \\
\text{O} & \text{O} \\
\end{align*}
\]

\[ \text{Scheme 2.1. The ionization of nitroethane (left) to yield ethynitronate (right) in solution.} \]

Following the initial reports of Little in 1951 on the oxidative denitrification of nitro compounds carried out by using extracts of Neurospora crassa [13] and pea seedlings [14], two nitroalkane-oxidizing enzymes have been extensively characterized in the past decade in their biochemical and mechanistic properties, i.e., nitroalkane oxidase from Fusarium oxysporum [15], and 2-nitropropane dioxygenase from Neurospora crassa [16; 17; 18]. Nitroalkane oxidase has been shown to oxidize nitroalkanes, but not alkyl nitronates [19], via a carbanion mechanism of catalysis involving the formation of a transient, flavin N(5)-substrate adduct [20; 21]. N. crassa 2-nitropropane dioxygenase has been shown to effectively utilize both the neutral and anionic nitronate forms of the substrate [17], with catalysis occurring through a single-electron
transfer reaction involving the transient formation of an anionic flavosemiquinone [17]. Other enzymes with the ability to oxidize alkyl nitronates, such as horseradish peroxidase [22; 23], and the flavoenzymes glucose oxidase [24] and D-amino acid oxidase [25; 26], have been shown to be significantly more efficient with their inherent substrates, resulting in their nitronate-oxidizing activities being regarded as non-physiological reactions.

The flavin-dependent enzyme 2-nitropropane dioxygenase\(^1\) from *Hansenula mrakii* has been reported to catalyze the oxidative denitrification of both alkyl nitronates and nitroalkanes into their corresponding carbonyl compounds and nitrite, with oxygen as electron acceptor [27; 28]. Initial reports showing the presence of iron atoms in enzymatic preparations were followed by studies from the same authors indicating that *H. mrakii* 2-nitropropane dioxygenase is a flavin-dependent enzyme with no requirement for iron [29]. In the present study, we have expressed and purified to homogeneity recombinant 2-nitropropane dioxygenase from *H. mrakii*, and characterized the purified enzyme for its substrate specificity and coenzyme content. Our data indicate that the enzyme contains tightly, but non-covalently, bound FMN as cofactor, does not require metal atoms for activity, and utilizes alkyl nitronates for catalysis, but not nitroalkanes.

\(^1\) 2-Nitropropane dioxygenase may be a misnomer for the alkyl nitronate-oxidizing enzyme from *H. mrakii*, as suggested by the broad substrate specificity of the enzyme with alkyl nitronates and the poor reactivity with nitroalkanes reported in this study. However, since mechanistic studies aimed at the elucidation of the mechanism of catalysis and the stoichiometry of the enzymatic reaction have not yet been carried out, we prefer to use the official IUBMB name, 2-nitropropane dioxygenase, for the enzyme investigated here to ensure continuity with previous study reported by other authors.
Materials and methods

Materials. The plasmid pUC25-I3 containing the gene encoding for 2-nitropropane dioxygenase from *H. mrakii* was a kind gift from Dr. Nobuyoshi Esaki and Dr. Tatsuo Kurihara, Kyoto University in Japan. Isopropyl-1-thio-β-D-galactopyranoside (IPTG), calf intestinal alkaline phosphatase, T4 DNA ligase and restriction endonucleases *Nde* I and *BamH* I were obtained from Promega (Madison, WI). The plasmid vector pET20b(+) was from Novagen (La Jolla, CA). Primers and primer extension reaction products were purified using mini kits from Qiagen (Valencia, CA). *Escherichia coli* strains BL21(DE3) from Novagen and XL1-Blue from Stratagene (La Jolla, CA) were used for protein expression and cloning procedures, respectively. Both strains were stored at -80 °C as 7% dimethyl sulfoxide suspensions. Luria-Bertani agar and broth, phenylmethylsulfonylfluoride (PMSF), lysozyme, nitroalkanes, superoxide dismutase, catalase and chloromphenicol were from Sigma-Aldrich. DNase, RNAse and the Rapid DNA ligation kit were from Roche Applied Science (Indianapolis, IN). The DEAE-Sepharose resin used for packaging the DEAE column was obtained from GE Healthcare (Piscataway, NJ). All reagents were of the highest purity commercially available.

Instruments. An Applied Biosystem model ABI 377 DNA sequencer at the DNA Core Facility of the Biology Department of Georgia State University was used for DNA sequencing utilizing an Applied Biosystems Big Dye kit. An ABI Voyager DE-pro mass spectrophotometer was used for recording the matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) spectra. An Agilent Technologies diode-array spectrophotometer Model HP 8453 PC, with thermostated water bath was used for acquiring UV-visible absorbance spectra. A Hansatech Instruments computer-interfaced Oxy-32 oxygen-monitoring system was used for measuring enzyme activity.
Cloning of 2-Npd into pET20b(+). *E. coli* strain XL1-Blue competent cells were transformed directly using plasmid pUC25-I3 carrying the gene encoding for 2-nitropropane dioxygenase (GenBank accession code U13900), utilizing the heat shock method of Inoue *et al.* [30]. After isolation of the amplified pUC25-I3 plasmid using a QIAquick Spin miniprep kit, plasmid DNA was used as template for primer extension amplification of the gene encoding for 2-nitropropane dioxygenase by using oligonucleotide sense and antisense primers containing *Nde* I and *BamHI* restriction endonuclease sites designed to anneal to the 5’- and 3’-ends of the gene, respectively. The engineered restriction sites allowed directional cloning of gene encoding for 2-nitropropane dioxygenase into corresponding sites of pET20b(+) using a Rapid DNA Ligation kit (Roche Applied Science). *E. coli* strain XL-1Blue competent cells were directly transformed using the ligation mixture. The resulting plasmid pET/2NPdhm was sequenced in both directions by using oligonucleotide primers designed to bind to DNA regions of pET flanking the inserted gene. Competent *E. coli* strain BL21(DE3) cells were transformed for protein expression.

Expression and purification of 2-nitropropane dioxygenase. Permanent frozen stocks of *E. coli* strain BL21(DE3) cells harboring the pET/2NPdhm plasmid were used to inoculate 4 x 1 L of Luria-Bertani broth medium containing 50 µg/mL ampicillin and 34 µg/mL chloramphenicol at 37 °C under shaking conditions. Once the O.D.₆₀₀ nm reached 0.8, the bacterial cultures were induced with 0.2 mM IPTG for 22 hours at 20 °C. After harvesting the cells by centrifugation, the cell wet paste was suspended in 4 volumes of 1 mM EDTA, 0.2 mg/mL lysozyme, 0.1 mM PMSF, 10 mM of MgCl₂, 5 µg/mL of DNase, 20 µg/mL of RNase and 50 mM potassium phosphate, pH 7.4, before sonication. The resulting cell free extract was collected by centrifugation at 12,500 x g for 30 min, and was treated with 60% saturation with
ammonium sulfate by stirring on ice for 30 minutes. After centrifugation at 12,500 x g for 30 min the resulting supernatant (~ 150 mL) was dialyzed against 5 x 2 L changes of 5 mM potassium phosphate, pH 7.4, over 16 hours, and applied to a DEAE Fast Flow column (2.5 x 30 cm) previously equilibrated with 5 mM potassium phosphate, pH 7.4. The enzyme was eluted from the column using a linear gradient from 0 to 0.5 M sodium chloride in 5 mM potassium phosphate, pH 7.4, at a flow rate of 5 mL/min. Fractions of the highest purity were pooled, dialyzed against 50 mM potassium phosphate, pH 7.4, and stored at -20 °C.

**Biochemical methods.** The concentration of purified 2-nitropropane dioxygenase was determined with the Bradford assay [31], using the Bio-Rad protein assay kit with bovine serum albumin as a standard. SDS-PAGE was performed for all the purification steps with molecular weight markers (Sigma) containing proteins ranging from 6,500 to 200,000 Da. The molar ratio of flavin to protein was determined in duplicate from the concentration of flavin removed from the enzyme upon heat denaturation, using an $\varepsilon_{450\text{ nm}}$ value of 12,200 M$^{-1}$s$^{-1}$ for FMN free in solution [32], and the concentration of protein determined by Bradford assay. Identification of the flavin cofactor of 2-nitropropane dioxygenase was carried out using matrix-assisted laser absorption/ionization-time of flight (MALDI-TOF) mass spectroscopic analysis in the negative ion mode using a 50:50 methanol/acetonitrile matrix. The sample was prepared by denaturing the enzyme with heat treatment at 100 °C for either 10 or 30 min, followed by centrifugation and collection of the supernatant. Alternatively, denaturation of the enzyme was achieved by incubation with 10% cold TCA (v/v) for 20 min on ice. Determination of the metal content of 2-nitropropane dioxygenase was carried out by inductively coupled plasma (ICP) mass spectroscopic analysis at the Chemical Analysis Facility of the University of Georgia on a sample dialyzed against 5 x 2 L changes of MilliQ® water over a period of 24 h at 4 °C. For
denaturing experiments aimed at the determination of the molar extinction coefficient, 2-nitropropane dioxygenase was incubated at 100 °C for 15 or 30 min, followed by the removal of the denatured protein by centrifugation. The molar extinction coefficient of the flavin cofactor bound to the enzyme was determined by following the change in absorbance at 446 nm in 50 mM potassium phosphate, pH 7.4, before and after heat treatment to extract the flavin from the enzyme. Anaerobic substrate reduction studies were performed using an anaerobic cuvette with two side arms. One side arm was loaded with the nitroalkane buffered solution, whereas the other side arm was loaded with the corresponding alkyl nitronate. The cuvette was made anaerobic by repeated cycles of vacuuming and flushing with ultra-pure argon for at least 15 times, before mixing the enzyme with the nitroalkane solutions. After approximately 10 to 15 min of incubation, where no spectral changes were observed, the enzyme solution was further mixed with the alkyl nitronate to ensure that the enzyme was functional. Formation of an N(5)flavin-sulfite adduct was determined by acquiring the UV-visible spectra every 5 seconds over a 15-minute period after the addition of 55 µM to 130 µM of sodium sulfite to the enzyme solution in 50 mM Tris-Cl, pH 8, at 15 °C. Reversibility of the flavin-sulfite complex was determined by following the increase in absorbance at 455 nm upon removal of the unbound sulfite using gel filtration of Sephadex G-25 column (PD-10 column, GE Healthcare) equilibrated with 50 mM Tris-Cl, pH 8.

**Enzyme kinetic assays.** The enzymatic activity of the purified enzyme was measured in air-saturated 50 mM potassium phosphate, pH 8, using the method of the initial rates [33] by monitoring the rate of oxygen consumption at 30 °C. Enzyme concentration was expressed per enzyme bound FMN content, using the experimentally determined 446 nm value of 13,100 M⁻¹ cm⁻¹ (this study). Stock solutions of nitroalkanes were prepared in 100% ethanol; those of alkyl nitronate were prepared by allowing the nitroalkanes to react with a 1.2 molar excess of KOH for
24 h at room temperature in 100% ethanol. The final concentration of ethanol in each assay mixture was kept constant at 1% to minimize possible effects on enzymatic activity. The reactions were started by the addition of the organic substrate to pre-equilibrated reaction mixtures, in order to minimize changes in the ionization state of the nitroalkanes or alkyl nitronate substrates. The second-order rate constants for deprotonation of nitroalkanes (5 to 6 M\(^{-1}\)s\(^{-1}\) [4]) and protonation of alkyl nitronates (15 to 75 M\(^{-1}\)s\(^{-1}\) [3]) were taken into account, ensuring that the determination of initial rates (typically ~ 30s) was performed with fully protonated or unprotonated substrates, respectively. Formation of superoxide during catalysis was determined by measuring the rate of oxygen consumption with 10 mM organic substrate in either absence or presence of 150 units of superoxide dismutase. Production of hydrogen peroxide was monitored using a similar approach as for the superoxide dismutase experiments, but by using 170 units of catalase instead of superoxide dismutase.

**Data analysis.** Kinetic data were fit with KaleidaGraph (Synergy Software, Reading, PA) and Enzfitter (Biosoft, Cambridge, UK) software. Since the enzyme could not be saturated with the organic substrate, the apparent second order rate constants \((k_{cat}/K_m)^{app}\) in atmospheric oxygen were determined by fitting the initial reaction rates determined at varying concentration of organic substrate to equation 1.

\[
\frac{v}{e} = \left(\frac{k_{cat}}{K_m}\right)^{app} A
\]  

(1)
Results

Cloning, expression and purification of 2-nitropropane dioxygenase. As a first step towards characterizing the properties of 2-nitropropane dioxygenase from *H. mrakii*, the gene encoding for the enzyme was subcloned in a pET20b(+) plasmid expression vector, and the resulting enzyme was expressed to high levels. A fractionation step with 60% saturation of ammonium sulfate and a single chromatographic step using a DEAE column were required to obtain stable, active, and homogeneous preparations of the enzyme, as determined by SDS-PAGE (Figure 2.1). Typically, 33 g of cell wet paste yielded 85 mg of enzyme, with a specific activity of 380 μmol O₂ min⁻¹ mg⁻¹ using 20 mM ethylnitronate as substrate at 30 °C and pH 8.

![Figure 2.1.](image)

**Figure 2.1.** Purification of recombinant 2-nitropropane dioxygenase from *Hansenula mrakii*. Lane 1, marker proteins; lane 2, purified enzyme.

Cofactor content. The UV-visible absorbance spectrum of purified 2-nitropropane dioxygenase at pH 7.4 showed the typical features of a flavin-containing enzyme in the oxidized state [34], with two maxima centered at 372 nm and 446 nm (Figure 2.2). A MALDI-TOF spectrometric analysis of the flavin extracted from the enzyme upon denaturation with heat, acid,
or anionic detergent, yielded a peak with an m/z ratio of 455.1 (Figure 2.2), consistent with the flavin cofactor being FMN. The extinction coefficient for non-covalently bound flavin at 446 nm was determined to be 13,100 M⁻¹ cm⁻¹ at pH 7.4, based on the absorbance ratio of bound to free FMN [32]. Furthermore, a stoichiometry of 0.80 ± 0.01 mol of flavin per mol of protein was established from the ratio of the concentration of extracted flavin to the protein concentration obtained by the Bradford assay.

![UV-visible absorbance spectrum of 2-nitropropane dioxygenase as purified. The absorbance spectrum was recorded in 50 mM potassium phosphate, pH 7.4. Inset, MALDI-TOF mass spectrometric analysis of the flavin cofactor extracted from 2-nitropropane dioxygenase. The mass spectrometric spectrum was recorded in negative ion mode with a 50:50 methanol/acetonitrile matrix using a sample prepared by treating the enzyme with heat, centrifugation and collection of the supernatant.](image)

**Figure 2.2.** UV-visible absorbance spectrum of 2-nitropropane dioxygenase as purified. The absorbance spectrum was recorded in 50 mM potassium phosphate, pH 7.4. *Inset,* MALDI-TOF mass spectrometric analysis of the flavin cofactor extracted from 2-nitropropane dioxygenase. The mass spectrometric spectrum was recorded in negative ion mode with a 50:50 methanol/acetonitrile matrix using a sample prepared by treating the enzyme with heat, centrifugation and collection of the supernatant.

In addition to examining the properties of the flavin, an inductively coupled plasma (ICP) mass spectrometric analysis of the enzyme treated with extensive dialysis against MilliQ® water was performed to determine whether metal cofactors were present in the enzyme. As shown in
Table 2.1, negligible amounts of several metals were identified (e.g., ≤ 8%), with the iron content being only 6% with respect to the protein. All taken together, the data indicate that 2-nitropropane dioxygenase expressed in the heterologous bacterial system contains tightly, but non-covalently, bound FMN and is devoid of metal cofactors.

<table>
<thead>
<tr>
<th>Metal</th>
<th>concentration (µM)</th>
<th>% in protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe^{2+}</td>
<td>5.8</td>
<td>6</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>6.2</td>
<td>7</td>
</tr>
<tr>
<td>Mn^{2+}</td>
<td>2.4</td>
<td>3</td>
</tr>
<tr>
<td>Co^{2+}</td>
<td>0.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Ni^{2+}</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Zn^{2+}</td>
<td>7.6</td>
<td>8</td>
</tr>
<tr>
<td>Cu^{2+}</td>
<td>2.3</td>
<td>3</td>
</tr>
</tbody>
</table>

* The analysis was performed on a 94 µM 2-nitropropane dioxygenase treated with 5 x 2L dialysis against milliQ water. The last change of milliQ® water was used as a blank for analysis.

Table 2.1. Inductively coupled plasma mass spectroscopic analysis of *H. mrakii* 2-nitropropane dioxygenase.

**Alkyl nitronates as substrates for 2-nitropropane dioxygenase.** 2-Nitropropane dioxygenase was mixed anaerobically with either nitroalkanes or alkyl nitronates to determine whether in the presence of the organic substrate the enzyme-bound flavin is reduced to the semiquinone or hydroquinone state. As shown in Figure 4, upon anaerobic mixing of the enzyme with 1 mM ethyl nitronate at pH 7.4 two peaks at 372 nm and 481 nm immediately appeared in the UV-visible absorbance spectrum, consistent with the stabilization of an anionic semiquinone form of the flavin. Similar results were obtained at pH 8 with ethyl nitronate or butyl-1-nitronate (Figure 2.3B), or at pH 6 with ethyl nitronate, propyl-1-nitronate, propyl-2-nitronate (Figure 2.3C), butyl-1-nitronate, penty1-1-nitronate, or hexyl-1-nitronate, suggesting that the anionic
semiquinone was stabilized in the enzyme-substrate complex irrespective of the identity of the alkyl nitronate used and the pH. Surprisingly, anaerobic mixing of the enzyme with 1 mM nitroethane did not result in any significant spectral changes within the first 10 min of incubation at pH 7.4 (Figure 2.3A)

2. In a similar fashion, no reduction of the enzyme-bound flavin was observed when nitroethane was used at pH 6 or 8, or when the nitroalkane was replaced with nitrobutane or 2-nitropropane (Figures 2.3B and 2.3C), suggesting that the enzyme reacts poorly, if at all, with neutral nitroalkanes.

---

2 The slow formation of the semiquinone species of the enzyme-bound flavin was observed upon anaerobic incubation of the enzyme with nitroethane over times significantly longer than 10 min. This is due to the fact that in aqueous solution at pH 7.4, nitroethane is deprotonated slowly yielding over time increasing amounts of ethynitronate, which is a substrate for the enzyme.
Figure 2.3. Anaerobic substrate reduction of 2-nitropropane dioxygenase with alkyl nitronates. Panel A, enzyme upon anaerobic incubation with 1 mM ethyl nitronate (line 1) or 1 mM nitroethane (line 2) as substrate for the enzyme at a final concentration of 56 nM, in 50 mM potassium phosphate pH 7.4 and 15 °C. Inset, oxygen consumption during the enzymatic turnover with 1 mM ethyl nitronate (line 1) or 1 mM nitroethane (line 2) in 50 mM potassium phosphate pH 7.4 and 30 °C. Panel B, 2-nitropropane dioxygenase upon anaerobic incubation with 1 mM butyl-1-nitronate (line 1) or 1 mM nitrobutane (line 2) in 50 mM potassium phosphate pH 8 and 15 °C. Panel C, 2-nitropropane dioxygenase upon anaerobic incubation with 1 mM propyl-2-nitronate (line 1) or 1 mM 2-nitropropane (line 2) in 50 mM potassium phosphate pH 6 and 15 °C. All UV-visible absorbance spectra with nitroalkanes were recorded after 10 min of anaerobic incubation; those with alkyl nitronates were acquired immediately after anaerobic mixing of the enzyme with the substrate, i.e., ca. 15 s.

As an independent approach to examining whether 2-nitropropane dioxygenase from H. mrakii can use alkyl nitronates as substrates, but not nitroalkanes, the rates of oxygen consumption were measured with 1 mM ethyl nitronate or 1 mM nitroethane as substrate for the enzyme in air-saturated buffer at pH 7.4 and 30 °C. As illustrated in the inset of Figure 2.3A, the oxygen electrode traces showed that under these conditions oxygen was completely depleted
from the assay reaction mixture containing ethylnitronate within 4 min of incubation, consistent with ethylnitronate being oxidized by the enzyme. In contrast, no oxygen consumption was observed when nitroethane was used as substrate for the enzyme, suggesting that nitroethane is not a substrate for the enzyme. In this regard, the apparent rate of oxygen consumption with 1 mM ethylnitronate as substrate for the enzyme in air-saturated buffer at pH 7.4 and 30 °C was not affected when nitroethane was present in the assay reaction mixture at a concentration as high as 20 mM (Table 2.2), consistent with the enzyme not being able to bind nitroethane as either inhibitor or poor substrate.

Table 2.2. Enzymatic activity of \textit{H. mrakii} 2-nitropropane dioxygenase$^a$ with 1 mM ethylnitronate as a substrate in the absence and presence of nitroethane in 50 mM potassium phosphate pH 7.4 and 30 °C

<table>
<thead>
<tr>
<th>nitroethane, mM</th>
<th>rate, $^b$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50.4 ± 0.7</td>
</tr>
<tr>
<td>1</td>
<td>50.5 ± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>50.3 ± 0.1</td>
</tr>
<tr>
<td>20</td>
<td>50.7 ± 1.3</td>
</tr>
</tbody>
</table>

$^a$ Final concentration of the enzyme in the assay was 56 nM.

$^b$ Kinetic data are the average of two independent measurements.

**Effect of superoxide dismutase and catalase on the enzymatic activity.** The effect of superoxide dismutase or catalase on the enzymatic activity of 2-nitropropane dioxygenase with unbranched alkyl nitronates or propyl-2-nitronate was determined at pH 8 and 30 °C to establish whether superoxide or hydrogen peroxide were produced and released from the active site of the enzyme in turnover. As summarized in Table 2.3, the apparent rates of oxygen consumption with unbranched alkyl nitronates of various chain lengths were similar irrespective of the presence or absence of superoxide dismutase or catalase. With propyl-2-nitronate, a significantly lower
apparent rate of oxygen consumption was observed in the presence of superoxide dismutase, but not in the presence of catalase. The kinetic data indicate that with the exception of propyl-2-nitronate, for which a significant amount of superoxide is released, there is no release of superoxide or hydrogen peroxide from the active site of the enzyme during turnover with unbranched alkyl nitronates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>control ( \text{s}^{-1} )</th>
<th>+ superoxide dismutase ( b ) ( \text{s}^{-1} )</th>
<th>+ catalase ( b ) ( \text{s}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylnitronate</td>
<td>55 ± 1</td>
<td>56 ± 1</td>
<td>55 ± 2</td>
</tr>
<tr>
<td>propyl-1-nitronate</td>
<td>127 ± 1</td>
<td>126 ± 1</td>
<td>125 ± 2</td>
</tr>
<tr>
<td>propyl-2-nitronate</td>
<td>55 ± 1</td>
<td>19 ± 1</td>
<td>56 ± 1</td>
</tr>
<tr>
<td>butyl-1-nitronate</td>
<td>96 ± 1</td>
<td>95 ± 1</td>
<td>96 ± 1</td>
</tr>
<tr>
<td>pentyl-1-nitronate</td>
<td>95 ± 1</td>
<td>95 ± 2</td>
<td>95 ± 1</td>
</tr>
<tr>
<td>hexyl-1-nitronate</td>
<td>157 ± 2</td>
<td>158 ± 2</td>
<td>155 ± 1</td>
</tr>
</tbody>
</table>

\( a \) Enzyme activity was measured with 10 mM organic substrate in the absence or presence of 151 units of superoxide dismutase or 168 units of catalase in air saturated 50 mM Tris-Cl, at pH 8.0 and 30 °C.

\( b \) Kinetic data are the average of two independent measurements.

**Table 2.3.** Effect of superoxide dismutase and catalase on the enzymatic activity of *H. mrakii* 2-nitropropane dioxygenase

**Substrate specificity.** Apparent rates of oxygen consumption were measured as a function of the concentration of a number of alkyl nitronates in atmospheric oxygen to determine the substrate specificity of the enzyme. As illustrated in the examples of Figure 2.4 for ethylnitronate and propyl-1-nitronate, the initial rates of reaction were linearly dependent on the concentration of substrate up to 20 mM at pH 6 and 30 °C. Therefore the apparent second order rate constants \( k_{cat}/K_m \) could be estimated for the different substrates, but not the turnover numbers \( k_{cat} \) and the Michaelis constants \( K_m \). As summarized in Table 4, the enzyme showed similar \( (k_{cat}/K_m)^{app} \) values in the lower \( 10^5 \) M\(^{-1}\)s\(^{-1}\) range with unbranched alkyl nitronates of
various chain length from 2 to 6 carbon. In contrast, the \( \frac{k_{\text{cat}}}{K_m} \)\textsuperscript{app} value with propyl-2-nitronate was two orders of magnitude lower, suggesting that the enzyme has a marked preference for unbranched alkyl nitronates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( \frac{k_{\text{cat}}}{K_m} )\textsuperscript{app}, M\textsuperscript{-1}s\textsuperscript{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethynitronate</td>
<td>129,000 ± 1,600</td>
</tr>
<tr>
<td>propyl-1-nitronate</td>
<td>170,000 ± 2,300</td>
</tr>
<tr>
<td>propyl-2-nitronate\textsuperscript{a}</td>
<td>1,600 ± 5</td>
</tr>
<tr>
<td>butyl-1-nitronate</td>
<td>112,000 ± 1,600</td>
</tr>
<tr>
<td>Pentyl-1-nitronate</td>
<td>121,000 ± 2,000</td>
</tr>
<tr>
<td>Hexyl-1-nitronate</td>
<td>130,000 ± 2,000</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Measured in presence of 170 U of superoxide dismutase.

**Table 2.4.** Apparent steady state second order rate constants, \( \frac{k_{\text{cat}}}{K_m} \), for alkyl nitronates as substrates for *H. mrakii* 2-nitropropane dioxygenase in 50 mM potassium phosphate pH 6 and 30 °C.

**Figure 2.4.** Apparent steady state kinetics of 2-nitropropane dioxygenase with ethynitronate (●) or propyl-1-nitronate (○), as determined in 50 mM potassium phosphate, pH 6 and 30 °C. Data were fit with equation 1.

**Formation of an N(5)-flavin adduct with sulfite.** As shown in Figure 2.5, incubation of 2-nitropropane dioxygenase with sodium sulfite resulted in the bleaching of absorbance peak at
446 nm with concomitant formation of a novel peak at 320 nm. Such changes in the UV-visible absorbance spectrum of the enzyme-bound flavin are consistent with the formation of an N(5)-flavin adduct with sulfite [35]. The process was sufficiently slow at pH 8 and 15 °C to allow for the determination of the observed rate of bleaching of the flavin at 446 nm as a function of the concentration of sulfite using a spectrophotometer. The values for the rate of formation ($k_{on}$) and dissociation ($k_{off}$) of the flavin-sulfite adduct could then be estimated to be $64 \pm 6 \text{ M}^{-1}\text{s}^{-1}$ and $0.0056 \pm 0.0006 \text{ s}^{-1}$ from the plot of $k_{obs}$ versus [sulfite]. These values, in turn, were used to calculate a $K_d$ value for sulfite binding of $88 \pm 10 \text{ µM}$, consistent with tight binding of sulfite to the flavin. Formation of the flavin-sulfite adduct was fully reversible as established by the increase in absorbance at 446 nm slowly ensuing after the removal of excess sulfite by gel filtration using a PD-10 column.

Figure 2.5. Reaction of 2-nitropropane dioxygenase with sodium sulfite. Enzyme was incubated with varying concentrations of sodium sulfite in the range from 55 µM to 130 µM in air-saturated 50 mM Tris-Cl, pH 8, at 15 °C. UV-visible absorbance spectra were recorded every 15 seconds for 15 min after each
addition of sodium sulfite. For clarity only selected spectra are shown: curve 1, absorbance spectrum of 2-nitropropane dioxygenase recorded 10 s after the addition of 130 µM sulfite; curve 20, same sample after 15 min of incubation. The *inset* shows the observed rates of bleaching of the absorbance at 452 nm as a function of sulfite concentration.
Discussion

In this study, recombinant 2-nitropropane dioxygenase from the yeast *H. mrakii* was expressed and purified to high levels in *E. coli* cells, and characterized in regard to its substrate specificity, coenzyme content, and reactivity with sodium sulfite.

*H. mrakii* 2-nitropropane dioxygenase oxidatively denitrifies alkyl nitronates of various chain lengths, as suggested by results of both anaerobic substrate reduction and kinetic data with a number of different substrates. Under atmospheric oxygen conditions the enzyme has a marked preference for unbranched primary alkyl nitronates as compared to propyl-2-nitronate, as indicated by the apparent $k_{cat}/K_m$ values determined at pH 6 in the $10^5$ M$^{-1}$s$^{-1}$ range as compared to $10^3$ M$^{-1}$s$^{-1}$. Interestingly, the enzyme shows minimal discrimination among unbranched substrates with chain lengths spanning from 2 to 6 carbon atoms, for which the apparent $k_{cat}/K_m$ values were comprised between $1.1 \times 10^5$ M$^{-1}$s$^{-1}$ and $1.7 \times 10^5$ M$^{-1}$s$^{-1}$. In this regard, *H. mrakii* 2-nitropropane dioxygenase is similar to the well-characterized 2-nitropropane dioxygenase from *Neurospora crassa*, which showed similar substrate specificity [17]. With all the substrates tested, *H. mrakii* 2-nitropropane dioxygenase is immediately reduced to the one electron anionic flavosemiquinone species upon anaerobic mixing of the enzyme with the substrate. Moreover, no production and release of hydrogen peroxide or superoxide was observed during turnover of the enzyme with unbranched primary alkyl nitronates. The latter two features were also recently reported for the *N. crassa* 2-nitropropane dioxygenase [17], suggesting that the two enzymes may share similar catalytic mechanisms for the oxidation of nitronate substrates in which a transient flavosemiquinone is observed in turnover.

*H. mrakii* 2-nitropropane dioxygenase reacts poorly, if at all, with unbranched and secondary nitroalkanes, as suggested by several independent observations. First, upon incubating
anaerobically the enzyme with nitroethane, nitrobutane, or 2-nitropropane, there were no significant changes in the UV-visible absorbance spectrum of the oxidized enzyme-bound flavin, irrespective of the pH used. In contrast, the same enzyme was immediately reduced to the flavosemiquinone state upon addition of the corresponding alkyl nitronates in control experiments, consistent with the enzyme being functional in the presence of proper substrates.

Second, no oxygen was consumed over at least 180 seconds when the enzyme was incubated with 1 mM nitroethane in a reaction chamber of a Clark oxygen electrode system. In contrast, oxygen was completely depleted from a reaction mixture containing 1 mM ethyl nitronate instead of nitroethane under the same conditions, suggesting that the lack of reactivity with nitroethane is not due to the enzyme being inactive. Finally, the apparent rates of oxygen consumption with 1 mM ethyl nitronate as substrate for the enzyme were not changed when nitroethane up to a concentration of 20 mM was present in the enzyme reaction mixture, consistent with nitroethane not binding in the active site of the enzyme, either as a slow substrate or an inhibitor. The apparent incongruence of the results reported in this study with results showing that the enzyme can utilize nitroalkanes as substrate previously reported by other authors is readily explained upon considering that an end-point assay for the measurement of the nitrite produced upon turnover of the enzyme with 2-nitropropane for 20 minutes at pH 8.0 was used [27; 28]. Indeed, over such an extended time at pH 8.0 significant amounts of nitroalkane are being converted in a non-enzymatic reaction catalyzed by the buffer into the corresponding alkyl nitronate [4], which is then oxidized by the enzyme as it becomes available. The lack of reactivity of H. mrakii 2-nitropropane dioxygenase with nitroalkanes differentiates this enzyme from the other two well-characterized flavin-dependent enzymes with the ability to oxidize nitro compounds: 2-nitropropane dioxygenase from N. crassa, which oxidizes both alkyl nitronates and nitroalkanes
[17], and nitroalkane oxidase from *Fusarium oxysporum*, which oxidizes nitroalkanes, but not alkyl nitronates.

*H. mrakii* 2-nitropropane dioxygenase contains tightly, but non-covalently, bound FMN in a 1:1 stoichiometry with the protein, as indicated by the results of the MALDI-TOF mass spectrometric and the spectrophotometric analyses of the flavin extracted by denaturation of the enzyme with heat or acid. Consistent with FMN being the cofactor for *H. mrakii* 2-nitropropane dioxygenase the amino acid sequence of the enzyme (GenBank accession no. AAA64484) does not contain any consensus sequence of the type GXGXXG/A, which is typically found in FAD-binding proteins [36]. Moreover, no metal ions are present in amounts that are sufficiently large to be considered catalytically relevant, as suggested by the ICP mass spectrometric analysis of the enzyme. The determination of the flavin as FMN reported here apparently contrasts with data that are available in the literature, showing that FAD may be the cofactor of the enzyme [27; 28; 29]. In those studies, however, the identity of the flavin was proposed based on the alleged observation that the flavin extracted from the *H. mrakii* enzyme was able to confer enzymatic activity to an apoprotein form of D-amino acid oxidase [28]. Indeed, the experimental data were not presented and the proper control experiments showing either the presence or lack of enzymatic activity in D-amino acid oxidase reconstituted with FMN were not carried out [29]. Lack of iron in the recombinant enzyme from *H. mrakii* expressed in *E. coli* confirms results previously reported by other authors, showing that the initial reports describing *H. mrakii* 2-nitropropane dioxygenase as an iron-dependent flavoprotein were flawed by the presence of contaminant iron-containing proteins [27; 28]. Thus, to this date the only three 2-nitropropane dioxygenases for which biochemical or crystallographic data are available, i.e., the enzymes
from *H. mrakii*, *N. crassa* and *P. aeruginosa*, all require FMN for catalysis and do not contain iron atoms in their active sites [17; 37].

The oxidation of propyl-2-nitronate catalyzed by *H. mrakii* 2-nitropropane dioxygenase has a significant non-enzymatic component that occurs outside the active site of the enzyme, as suggested by the effect of superoxide dismutase on the rates of oxygen consumption with this substrate. Such an effect of the dismutase can be readily rationalized with superoxide being normally produced during turnover of the enzyme with organic substrates, but being released from the enzyme surface only when the active site is occupied with propyl-2-nitronate. A significant amplification of the rate of oxygen consumption is consequently observed in the absence of the scavenging effect of superoxide dismutase because the superoxide released in solution would initiate and propagate a well-characterized radical reaction of oxidation of alkyl nitronates [38]. In contrast, the lack of effect of superoxide dismutase with unbranched alkyl nitronates is consistent with the oxidation reaction occurring exclusively at the active site of the enzyme with no adventitious release of the superoxide intermediate. A similar mechanistic behavior was recently reported for the oxidation of propyl-2-nitronate catalyzed by another 2-nitropropane dioxygenase, i.e., the enzyme from *N. crassa*, for which a significant non-enzymatic radical component was established [17].

A positive charge is located close to the N(1)-C(2)=O locus of the isoalloxazine moiety of the FMN cofactor, as suggested by the stabilization of the anionic form of the flavin semiquinone and the reactivity of the enzyme with sulfite [35]. In the absence of structural data on the enzyme from *H. mrakii*, one can speculate that such a positive charge may be provided by either a positively charged amino acid residue or the electrical dipole of an α-helix. In this regard, the crystallographic structure of 2-nitropropane dioxygenase from *P. aeruginosa* at a
resolution of 2 Å shows the presence of a lysine residue in proximity of the N(1)-C(2)=O locus of the FMN cofactor [37].

In conclusion, the biochemical characterization of 2-nitropropane dioxygenase from \textit{H. mrakii} presented in this study has established that the enzyme requires FMN as cofactor for catalysis, does not contain iron atoms in its active site, utilizes alkyl nitronates as substrates, and reacts poorly, if at all, with nitroalkanes. Moreover, an anionic flavosemiquinone is formed upon anaerobic reduction of the enzyme with nitronate substrates. These results allow to compare, and to contrast, this enzyme with two other well-characterized flavin dependent enzymes with the ability to oxidize nitroalkanes/alkyl nitronates, i.e., 2-nitropropane dioxygenase from \textit{N. crassa} and nitroalkane oxidase from \textit{F. oxysporum}. The former is known to oxidatively denitrify both nitroalkanes and alkyl nitronates and to utilize mechanism for the oxidation of the organic substrate involving a one-electron transfer to the flavin [17; 18]. The latter is active only on nitroalkanes and has been shown to utilize a carbanion mechanism of catalysis proceeding through the formation of a flavin N(5)-substrate adduct [15]. The availability of large amounts of \textit{H. mrakii} 2-nitropropane dioxygenase will be instrumental for future biochemical and mechanistic studies aimed at the elucidation of the chemical mechanism of catalysis and the reasons for the poor reactivity of the enzyme with nitroalkanes.

\textbf{Acknowledgment:} The authors thank Dr. Nobuyoshi Esaki and Dr. Tatsuo Kurihara, Kyoto University, Japan, for the kind gift of plasmid pUC25-I3; Dr. Siming Wang for mass spectroscopic analysis of flavin cofactor; Mr. Dawit Seyfe for the initial characterization of the enzyme; and Ms. Baotran ‘Nguyen for carrying out the sodium sulfite experiment.
References


Abstract

In previous studies, nitroalkane-oxidizing enzyme from *Hansenula mrakii* was named 2-nitropropane dioxygenase based on the observation that the enzyme utilizes 2-nitropropane as a substrate most efficiently. Dioxygenase part of the name stems from the observation that hydrogen peroxide is not formed during catalysis and conclusion that one molecule of oxygen is incorporated into two molecules of carbonyl product when 2-nitropropane is used as a substrate. Same results were not obtained when 2-nitropropane was replaced by 1-nitropropane and nitroethane. Therefore, the focus of this study is to determine stoichiometry of the enzymatic reaction of the recombinant enzyme using several alkyl nitronates. The ratio of organic substrate to oxygen and nitrite of 2 to 1 and 3 to 1 was determined when hexyl-1-nitronate, pentyl-1-nitronate and butyl-1-nitronate were used as substrates. These data suggest that *Hansenula mrakii* is a dioxygenase as previously reported.
Introduction

2-Nitropropane dioxygenase from *Hansenula mrakii* catalyzes the conversion of anionic nitroalkanes into corresponding carbonyl compounds by removing the nitrite and utilizing oxygen as the electron acceptor (Scheme 3.1).

\[ \text{H}_2\text{C}_2\text{R}_2\text{NO}_2^- + \text{O}_2 \rightarrow \text{H}_2\text{C}_2\text{R}_2\text{CO} + 2\text{NO}_2^- \]

*Scheme 3.1. Reaction catalyzed by 2-nitropropanedioxygenase.*

The stoichiometry of the reaction was previously studied on the enzyme obtained from the yeast *H. mrakii*. Formation of hydrogen peroxide was monitored to determine whether 2-nitropropane dioxygenase catalyzes the denitrification of substrates via oxidase or oxygenase mechanism [1]. The lack of hydrogen peroxide formation during catalysis indicated that the enzyme acts as an oxygenase rather than an oxidase. Based on \(^{18}\text{O}_2\) mass fragmentation study both atoms of molecular oxygen were found to be incorporated into the organic product during the oxidation of 2-nitropropane by 2-nitropropane dioxygenase [2]. Consequently, the *H. mrakii* enzyme was classified as a dioxygenase. In contrast, replacing the 2-nitropropane with 1-nitropropane and nitroethane as substrates, no incorporation of \(^{18}\text{O}_2\) was observed in final products propionaldehyde and acetaldehyde, respectively. Furthermore, oxygen consumption, as well as nitrite and acetone formation studies, suggest that 2-nitropropane dioxygenase is an intermolecular dioxygenase capable of denitrifying 2 molecules of 2-nitropropane by forming
two molecules of nitrite while incorporating two atoms of oxygen into two molecules of the acetone [2].

The enzyme was classified dioxygenase based on observations made with 2-nitropropane as a substrate while the observations that no incorporation of $^{18}$O$_2$ was observed in final products when 1-nitropropane and nitroethane are used as substrates were ignored. Also, no data was shown in $^{18}$O$_2$ mass fragmentation studies to support these conclusions.

Therefore, in this study stoichiometry of the enzymatic reaction was the focus. The reasons for this study are to examine the stoichiometry of the enzymatic reaction by the recombinant enzyme and to gain a better understanding of whether the stoichiometry of the enzymatic reaction is consistent for all of the substrates used. The study was carried out in three steps by identifying the ratio of organic substrate to oxygen, carbonyl compound and nitrite. Furthermore, future studies will have to involve MALDI-TOF analysis on the enzymatic assay obtained in milliQ water in order to identify all the products of the 2-nitropropane dioxygenase reaction. Using a buffered assay for MALDI-TOF analysis yields useless results due to the interactions of the buffer with the matrix used for the analysis. Also, using HPLC protocol described in this chapter for determination of carbonyl compounds would allow for determination of nano molar concentration of the carbonyl product.
Experimental Procedures

Materials, enzyme and organic substrate preparations. Nitroalkanes were from Sigma-Aldrich (St. Louis, MO). Greiss reagent system was from Promega. All other reagents were of the highest purity commercially available. 2-Nitropropane dioxygenase was prepared as described in Chapter 2 and stored in 50 mM potassium phosphate pH 7.4 at -20 °C. Enzyme concentration was expressed per enzyme bound FMN content, using the experimentally determined ε<sub>445</sub> value of 13,130 M<sup>-1</sup> cm<sup>-1</sup>. Stock solutions of alkyl nitronates were prepared by allowing the nitroalkanes to react with a 1.2 molar excess of KOH for 24 h at room temperature in 100% ethanol. The final concentration of ethanol in each assay mixture was kept constant at 1% to minimize possible effects on enzymatic activity. The reactions were started by the addition of the organic substrate to pre-equilibrated reaction mixtures in order to minimize changes in the ionization state of the nitroalkanes or alkyl nitronate substrates. The second-order rate constants for deprotonation of nitroalkanes (5 to 6 M<sup>-1</sup>s<sup>-1</sup> [3]) and protonation of alkyl nitronates (15 to 75 M<sup>-1</sup>s<sup>-1</sup> [4]) were taken into account, ensuring that the determination of initial rates (typically ~30s) was performed with fully protonated or unprotonated substrates, respectively. The mass spectroscopy analysis was performed on the sample, containing the products and the enzyme, prepared by heating at 100 oC for 30 min and centrifuged to remove the protein.

Instruments. Oxygen consumption during turnover was measured polarographically with a Hansatech oxygen electrode (HansaTech Oxy-32) thermostated at 30 °C. UV-visible absorbance spectra were recorded on a Hewlett-Packard 8453A diode array spectrophotometer.

Determination of organic substrate to oxygen ratio. Consumption of oxygen during the turnover of the enzyme was measured by comparing the oxygen concentration in solution before and after the enzymatic reaction. The buffer solution of 50 mM potassium phosphate pH 7.4 or 6 and 50 mM sodium pyrophosphate pH 9 were equilibrated at 30 °C for a period of 15
minutes, in order to achieve a stable reading of the oxygen concentration. Enzymatic reaction went to completion due to the limited concentration of the organic substrate (100 µM, 80 µM or 60 µM), which was smaller than the concentration of the oxygen (~250 µM at 30 °C). The ratio of organic substrate to oxygen was calculated by comparing the already known concentration of the organic substrate to the concentration of the oxygen consumed.

**Determination of the organic substrate to nitrite ratio.** The amount of nitrite produced during the catalytic turnover of the enzyme with alkyl nitronates was determined spectrophotometrically using a Greiss method modifications [5]. Briefly, calibration curve was constructed for 0-20 µM nitrite in 2 µM increments with the nitrite solution provided in the Greiss reagent system. The incubation with both 1% sulfilamide and 1% of N-1-naphthylethyleneamine (NED) were performed on the 23 °C by protecting the samples from light with aluminum foil for a period of 5-10 minutes. The 900 µL of standard nitrite solutions as well as to the unknown were incubated first with a 50 µL of sulfilamide solution, provided in Greiss reagent system. Upon incubation of the samples with a 50 µL of NED solution, also provided in Greiss reagent system, UV-visible absorbance was measured. The standard nitrite curve was constructed comparing the concentration of nitrite to the absorbance at 450 nm.

**Determination of organic substrate to carbonyl compound ratio.** The amount of carbonyl compounds produced during the catalytic turnover of the enzyme with alkyl nitronates as substrates was determined spectrophotometrically using 2,4-dinitrophenylhydrazine by Jellineck et.al. [6] procedure with modifications. Briefly, 0.5 µM 2-nitropropane dioxygenase was incubated with 60, 80 or 100 µM hexyl-1-nitronate in 1 ml of 50 mM potassium phosphate (pH 7.4 or 6) or 50 mM sodium pyrophosphate (pH 9) and 30 °C. Ice-cold trichloroacetic acid at a final concentration of 12% (v/v) was added to the mixture and incubated on ice for 20 min before centrifugation at 14,000 × g for 15 min to remove the protein. After addition to the
resulting supernatants of 250 µL of 0.4% 2,4-dinitrophenylhydrazine prepared in 2 M HCl, the samples were heated at 100 °C for 5 min. After cooling the samples with tap water, 0.25 ml of supernatant was mixed with 1 ml of 1.2 M NaOH, and allowed to stand for 10 min at 25 °C before measuring the absorbance at 440 nm.

**Quantification of the products by MALDI-TOF.** For the identification of products of the catalytic turnover of the enzyme with hexyl-1-nitronate, MALDI-TOF mass spectrometry was used in both the positive and negative ion mode with 50:50 methanol/acetonitrile matrix. The sample was prepared by running the enzymatic reaction and boiling it at 100 °C for 10 min followed by centrifugation to remove the protein.

**Data Analysis.** Data were fit with KaleidaGraph software (Synergy Software, Reading, PA). The nitrite standard curve was fitted with line equation, where y is the absorbance at 450 nm corresponding to concentration of nitrite and m is the slope of the line.
Results

The ratio of butyl-1-nitronate to oxygen consumed during the enzymatic turnover with substrates is 2 to 1 (Table 3.1). The determined ratio is not dependent on the pH of the experiment, as supported by the independent studies performed at pH 6, 7.4 and 9. Similar results were obtained when the concentration of the butyl-1-nitronate was varied (60 µM, 80 µM to 100 µM). The ratio of organic substrate consumed to nitrite formed was determined to be 3 to 1 in 50 mM potassium phosphate pH 6. The expectation was that for every molecule of alkyl nitronate denitrified during the enzymatic reaction, one molecule of nitrite would be formed. The pH of the reaction or the concentration of the butyl-1-nitronate did not play a role, as similar results were obtained at varying concentrations of butyl-1-nitronate in three different buffers.

Table 3.1. Oxygen consumption and nitrite production during 2-nitropropane dioxygenase turnover with butyl-1-nitronate as substrates.  

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Organic substrate : O₂</th>
<th>Organic substrate : Nitrite</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM KPi pH 6</td>
<td>0.52 ± 0.01</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>50 mM KPi pH 7.4</td>
<td>0.51 ± 0.02</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>50 mM NaPPi pH 9</td>
<td>0.66 ± 0.1</td>
<td>0.24 ± 0.01</td>
</tr>
</tbody>
</table>

Data is the average of three independent measurements performed for 60, 80 and 100µM of butyl-1-nitronate.

The ratio of 2:1 for organic substrate to oxygen was also determined when pentyl-1-nitronate was used as a substrate, as seen in the table 3.2. The pH of the reaction did not play a role, as similar results were determined in 50 mM potassium phosphate pH 6 and 7.4 and 50 mM sodium pyrophosphate pH 9. For every three pentyl-1-nitronate molecules consumed one molecule of nitrite was determined, as opposed to determining the one molecule of nitrite for every molecule of the alkyl nitronate denitrified. The determined ratio of pentyl-1nitronate to
nitrite is not dependent on the pH of the experiment. Similar results for organic substrate to oxygen or nitrite ratio were obtained when hexyl-1-nitronate was used as a substrate (data not shown). Varying the concentration of the hexyl-1-nitronate and pH of the reaction yielded similar results.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Organic substrate : O₂</th>
<th>Organic substrate : Nitrite</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM KPi pH 6</td>
<td>0.50 ± 0.01</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>50 mM KPi pH 7.4</td>
<td>0.51 ± 0.02</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>50 mM NaPPi pH 9</td>
<td>0.51 ± 0.03</td>
<td>0.27 ± 0.01</td>
</tr>
</tbody>
</table>

aData is the average of three independent measurements.

**Table 3.2.** Oxygen consumption and nitrite production during 2-nitropropane dioxygenase turnover with 100 µM pentyl-1-nitronate as substrates.

Due to the published results on reaction of nitrite with anionic species, such as superoxide and peroxide [7; 8], and reports of the flavin dependent enzymes producing superoxide transiently [9; 10], determination of organic substrate to nitrite ratio was performed in absence and presence of superoxide dismutase. If superoxide, or hydrogen peroxide, is formed during catalysis and is released in solution, the nitrite could readily react with superoxide anions and the concentration of the nitrite would decrease. In the presence or the absence of superoxide dismutase, the ratio of hexyl-1-nitronate to oxygen and nitrite was determined to be 2 to 1 and 3 to 1, respectively (Table 3.3). Upon substitution of hexyl-1-nitronate with pentyl-1-nitronate and butyl-1-nitronate, similar results were obtained, indicating that the production and the release of the superoxide and the subsequent reaction with the nitrite do not occur. In addition to performing the experiment with superoxide dismutase, the effect of catalase was examined due to production of the hydrogen peroxide by many known oxidases [9]. No effect was observed when catalase was added into the assay with hexyl-1-nitronate as a substrate in 50 mM
potassium phosphate pH 7.4. Similar results were obtained upon substituting hexyl-1-nitronate with pentyl-1-nitronate or butyl-1-nitronate, as seen in the Table 3.3.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>+/- SOD (µL)</th>
<th>Organic substrate : O₂</th>
<th>Organic substrate : Nitrite</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexyl-1-nitronate</td>
<td>0</td>
<td>0.52</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.50</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.51</td>
<td>0.32</td>
</tr>
<tr>
<td>pentyl-1-nitronate</td>
<td>0</td>
<td>0.66</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.64</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.65</td>
<td>0.35</td>
</tr>
<tr>
<td>butyl-1-nitronate</td>
<td>0</td>
<td>0.59</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.63</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.63</td>
<td>0.33</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>+/- Catalase (µL)</th>
<th>Organic substrate : O₂</th>
<th>Organic substrate : Nitrite</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexyl-1-nitronate</td>
<td>0</td>
<td>0.49</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.48</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.49</td>
<td>0.33</td>
</tr>
<tr>
<td>pentyl-1-nitronate</td>
<td>0</td>
<td>0.68</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.72</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.71</td>
<td>0.39</td>
</tr>
<tr>
<td>butyl-1-nitronate</td>
<td>0</td>
<td>0.61</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.58</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.60</td>
<td>0.37</td>
</tr>
</tbody>
</table>

*Measured in 50 mM potassium phosphate, pH 7.4 and 30 °C.*

**Table 3.3.** Effect of superoxide dismutase and catalase on oxygen consumption and nitrite production during 2-nitropropane dioxygenase turnover with 10 mM alkyl nitronates as substrates.*

Determining the hexanal as a product of the enzymatic turnover with hexyl-1-nitronate by Jellineck *et al* method [6] was unsuccessful due to the insolubility of the hexanal in the aqueous solution (data not shown). Several independent attempts made with commercially available hexanal to construct the standard curve were unsuccessful. In order to confirm if the method is usable, two standard curves, one for betaine aldehyde and one for hexanal, were constructed by following the same protocol. The standard curve for betaine aldehyde yielded a high $R^2$ of 0.99953 while the hexanal standard curve did not even produce the line with the positive slope.
Figure 3.1. Standard curve constructed by comparing the concentration of the compound with the absorbance of 440 nm. (●) Standard curve for betaine-aldehyde and (○) hexanal.
Discussion

Measuring the oxygen concentration before and after catalysis and comparing it to the concentration of the organic substrate have yielded the ratio of organic substrate to oxygen of 2 to 1. This ratio is independent of the concentration of organic substrate, as well as the length of the carbon chain on the substrate, as suggested by studies performed on the hexyl-1-nitronate, pentyl-1-nitronate and butyl-1-nitronate at different concentrations. The pH did not play a role on the ratio of organic substrate to oxygen, as seen from the ratio determined at pH 6, 7.4 and 9.

The determined ratio of organic substrate to nitrite of 3 to 1 does not match the theoretically expected ratio of 1 to 1. Third of the nitrite can be determined regardless of length or concentration of the organic substrate or the pH used during catalysis. It is expected that denitrification of one molecule of alkyl nitronate would yield one molecule of nitrite. Since Greiss reagent is specific for nitrite, quantification of nitrate, nitrous or nitric acid molecules as a result of nitrite conversion would have to be determined with other methods. Therefore, at this stage, we are unable to account for 2/3 of the nitrite.

Anionic oxygen species such as superoxide O$_2^-$ can be produced by flavin-dependent enzymes in reduced state upon reaction with molecular oxygen [9; 11; 12]. In addition, hydrogen peroxide has been found to be a product of flavin-dependent enzymes [13; 14]. Both anionic species, superoxide O$_2^-$ and peroxide O$_2^{2-}$, have been identified to be stable in many ionic solvents [7]. Nitrite is capable of reacting with superoxide to produce nitrate and peroxide, in addition of being capable of reacting with peroxide [8]. The formation and release of superoxide or hydrogen peroxide was not observed during enzymatic reaction. The conversion of nitrite to nitrate or to other species will have to be tested with the methods specific for the mentioned compounds.
Inability to construct the standard curve with commercially available hexanal is due to precipitation of the compound in aqueous solution upon reaction with 2,4-dinitrophenylhydrazine. Future studies focusing on the determination of carbonyl products will have to be carried out by a method that allows determination of the aldehyde and ketone compounds in nanomolar amounts. Study on determination of propionaldehyde, formaldehyde and acetaldehyde in nanomolar amounts was carried out on the samples obtained by mixing the compounds with 2,4-dinitrophenylhydrazine and using the HPLC [15]. In that study, C18 (5 µm 4.6 mm i.D. x 150 mm length) reverse-phase column was used at a flow rate of 1 mL min\(^{-1}\) by eluting the samples with two mobile phases, phase A (70 % milliQ water, 20 % tetrahydrofuran (THF) and 10 % methanol) and phase B consisting of 90 % acetonitrile in milliQ water. The chromatography was carried out with an isocratic elution at 90 % B over 6 minutes, followed by a 90 % A to 60 % A gradient over 24 minutes, followed by 60 % A to 0 % A gradient over 10 minutes. The process was finished with an isocratic gradient of 90 % B for 10 minutes. The UV-visible absorption detector was operated at 365 nm.

In conclusion, MALDI-TOF analysis of the reaction mixture in milliQ water is required in order to identify the products of the 2-NPD enzymatic reaction. Furthermore, utilizing the HPLC method described in previous paragraph will allow for determination of the concentration of the carbonyl product.
References


Chapter IV

INHIBITION OF 2-NITROPROPANE DIOXYGENASE WITH CHLORIDE IONS

Abstract

The flavin-dependent enzyme 2-nitropropane dioxygenase from Hansenula mrakii catalyzes the oxidative denitrification of alkyl nitronates into their corresponding carbonyl compounds and nitrite, with oxygen as electron acceptor. Flavin-dependent enzymes, which denitrify nitroalkanes, such as glucose oxidase and D-amino acid oxidase, are known to be inhibited by the chloride ions or compounds containing chloride. In this chapter, the effect of chloride ions on the enzymatic activity of 2-nitropropane dioxygenase is examined. The inactivation of the 2-nitropropane dioxygenase by chloride ions was dependent on the concentration of the halide. The apparent second order rate constants \((k_{\text{cat}}/K_m)^{\text{app}}\) followed saturation curve, where saturation region was observed once the ratio of chloride ions to flavin was 2 to 1, suggesting that chloride ions do not act as competitive inhibitors when ethynitronate is used as a substrate. Inability to saturate enzyme with organic substrate prevented determination of the chloride ions inhibition pattern.
Introduction

2-Nitropropane dioxygenase (2NPD) from Hansenula mrakii catalyzes the conversion of anionic nitroalkanes into corresponding carbonyl compounds by removing nitrite and utilizing oxygen as the electron acceptor (Scheme 4.1).

\[
\begin{align*}
2 \text{R}-\text{C}^- + \text{O}_2 & \rightarrow 2 \text{R}-\text{C}=\text{O} + 2 \text{NO}_2^- \\
\end{align*}
\]

Scheme 4.1. Reaction catalyzed by 2-nitropropane dioxygenase.

With the initial characterization of 2-nitropropane dioxygenase, inhibitory effects of several compounds as well as few chelating agents have been tested, due to the presence of iron-dependent protein bounded to 2-nitropropane dioxygenase [1; 2; 3]. Chelating agents for divalent and trivalent metals such \(\alpha,\alpha'\)-dipyridyl and \(o\)-phenanthroline acted as activators for the enzyme. However, EDTA, \(p\)-chloromercuribenzoate, \(N\)-ethylmaleimide and iodoacetate have been found to inhibit enzymatic activity. Tiron, cysteine and glutathione were found to inhibit 2-nitropropane dioxygenase completely; while \(\text{HgCl}_2\) and oxine were found to be powerful inhibitors.

A similar study was also performed on nitroalkane oxidase from Fusarium oxysporum, where \(\text{HgCl}_2\) as well as \(p\)-chloromercuribenzoate were found to completely inhibit the enzyme [4]. Even with the initial characterization of the Neurospora crassa 2-nitropropane dioxygenase, Little identified \(\text{NaCl}, \text{KCl},\) and \(\text{NH}_4\text{Cl}\) as inhibitors of the enzyme [5]. Halides have been found
to be competitive inhibitors of glucose oxidase with the respect of D-glucose or 2-deoxy-D-glucose as substrate, so the purification steps as well as the solutions used for storing or studying the enzyme have to be devoid of chloride [6; 7]. The binding of the chloride ions to the oxidized form of glucose oxidase results in a perturbation of the UV-visible absorbption spectrum of the enzyme-bound flavin [6]. Chloride ions also perturb the pKₐ of the ionizable group on the protein that is responsible for the binding of the substrate, as indicated by steady-state kinetic studies [6].

The flavin dependent D-amino acid oxidase (DAAO) is completely inactivated by 1-chloro-1-nitroethane, by ratio of 1:1.5 of 1-chloro-1-nitroethane to flavin [8]. Furthermore, the inhibition of the DAAO by N-chloro-D-leucine was studied with respect to the site and mechanism of chlorination [9]. In that study it was shown that flavin reduction slows down by a factor of 2 x 10³ due to chlorination of the tyrosine that is converted to 3,5-dichlorotyrosine, as supported by the amino acid analysis and spectral titration study. Further studies with N-chloro-D-leucine confirmed that the chlorination of DAAO in the active site region is consistent with consecutive chlorination of an amino acid residue by the two molecules of N-chloro-D-leucine [10].

Anaerobic and aerobic spectral studies of DAAO with D-alanine or β-chloro-D-alanine as substrate further supported the notion that chlorinated derivatives inhibit the enzyme competitively. Furthermore, kinetic studies revealed that the enzyme reacts with β-chloro-D-alanine four times less efficiently that with D-alanine [11; 12].

Therefore, the objective of this study was to identify the pattern of inhibition by the chloride ions on the enzymatic activity of 2-nitropropane dioxygenase. Chloride ions inhibit 60 % of the enzymatic activity upon addition of 2:1 ratio of chloride to flavin. Chloride ions are not competitive inhibitors of 2-nitropropane dioxygenase with the respect to ethynitronate, since the inhibition effect of chloride ions on the apparent second order rate constants \((k_{cat}/K_m)_{app}\) followed
saturation curve. However, due to inability to saturate the enzyme with the organic substrate, distinguishing between the noncompetitive and uncompetitive pattern of the inhibition could not be determined.
Experimental Procedure

**Materials.** Nitroalkanes were from Sigma-Aldrich. Potassium phosphate and KCl were from Fisher. Tris base was from J.T Barker. All reagents were of the highest purity commercially available.

**Methods.** Enzyme, 2-nitropropane dioxygenase was prepared as described in Chapter 2 and stored in 50 mM potassium phosphate pH 7.4 at -20 °C. Enzyme concentration was expressed per enzyme bound FMN content, using the experimentally determined $\varepsilon_{445\text{nm}}$ value of 13,130 M$^{-1}$ cm$^{-1}$. Enzyme activity was measured with the method of the initial rates [13] in air saturated 50 mM potassium phosphate or Tris-Cl at pH 6 by monitoring the rate of oxygen consumption with a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument Ltd.) thermostated at 30 °C. Stock solutions of alkyl nitronates were prepared by allowing the nitroalkanes to react with a 1.2 molar excess of KOH for 24 h at room temperature in 100% ethanol. The final concentration of ethanol in each assay mixture was kept constant at 1% to minimize possible effects on enzymatic activity. The reactions were started by the addition of the organic substrate to pre-equilibrated reaction mixtures, in order to minimize changes in the ionization state of the nitroalkanes or alkyl nitronate substrates. The second-order rate constants for deprotonation of nitroalkanes (5 to 6 M$^{-1}$s$^{-1}$ [14]) and protonation of alkyl nitronates (15 to 75 M$^{-1}$s$^{-1}$ [15]) were taken into account, ensuring that the determination of initial rates (typically ~30 s) was performed with fully protonated or unprotonated substrates, respectively.

**Data analysis.** Kinetic data were fit with KaleidaGraph (Synergy Software, Reading, PA) and Enzfitter (Biosoft, Cambridge, UK) software. Since the enzyme could not be saturated with the organic substrate, the apparent second order rate constants ($k_{cat}/K_m$) in atmospheric oxygen were determined by fitting the initial reaction rates determined at varying concentration of
organic substrate to equation 1. Here, $K_a$ represents the Michaelis constants for organic substrate (A) and $k_{cat}$ is the turnover number of the enzyme (e).

$$\frac{\nu}{e} = \left( \frac{k_{cat}}{K_a} \right)^{app} A \quad (1)$$
Results and Discussion

The enzymatic activity of 2-nitropropane dioxygenase with 20 mM ethylnitronate in 50 mM potassium phosphate pH 6 and 50 mM Tris-Cl pH 6 (Fig 4.1) was measured to be 770 s⁻¹ and 200 s⁻¹, respectively. The experiment was performed with the same sample of the enzyme and the substrate.

Figure 4.1. Oxygen consumption of 2-nitropropane dioxygenase with 20 mM ethylnitronate during turnover. Line 1- in 50 mM potassium phosphate pH 6 and 30 °C; line 2- in 50 mM Tris-Cl pH 6 and 30 °C.

Furthermore, the apparent second order rate constants \( \frac{k_{cat}}{K_m}^{app} \) in absence and presence of different concentrations of potassium chloride were obtained. As seen in Table 4.1, the enzymatic activity of 2-nitropropane dioxygenase with ethylnitronate is inhibited in the presence of different concentrations of potassium chloride. In the range of 0.11- 1 nM of potassium chloride inhibition of the enzymatic activity increases linearly as the concentration of potassium chloride increases. The further increase of the chloride concentration up to 11 nM did not lead to
further inhibition of the enzyme. Furthermore, even when 0.9 mM potassium chloride was used in the assay similar results were obtained (data not shown). Therefore, chloride ions cannot be competitive inhibitors of 2-nitropropane dioxygenase since the degree of inhibition does not increase with the increase of the inhibitor concentration.

Table 4.1. Apparent steady state second order rate constants, $k_{cat}/K_M$, in absence and presence of potassium chloride in 50 mM potassium phosphate pH 6 and 30 °C. The final concentration of 2-nitropropane dioxygenase in the enzymatic assay was 1.1 nM.

<table>
<thead>
<tr>
<th>[KCl] nM</th>
<th>$(k_{cat}/K_M)_0$ a</th>
<th>$(k_{cat}/K_M)_{KCl}$ b</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.11</td>
<td>129 ± 2</td>
<td>97 ± 3.3</td>
</tr>
<tr>
<td>0.33</td>
<td>152 ± 3</td>
<td>109 ± 4.2</td>
</tr>
<tr>
<td>1</td>
<td>133 ± 4</td>
<td>88 ± 3.1</td>
</tr>
<tr>
<td>1.9</td>
<td>131 ± 5</td>
<td>77 ± 3.8</td>
</tr>
<tr>
<td>3.3</td>
<td>152 ± 3</td>
<td>87 ± 2.8</td>
</tr>
<tr>
<td>9</td>
<td>133 ± 4</td>
<td>77 ± 1.9</td>
</tr>
<tr>
<td>11</td>
<td>127 ± 3</td>
<td>75 ± 1.1</td>
</tr>
</tbody>
</table>

a $(k_{cat}/K_M)_0$ represents apparent steady state second order rate constant obtained in the absence of the potassium chloride.

b $(k_{cat}/K_M)_{KCl}$ represents apparent steady state second order rate constant obtained in the presence of the potassium chloride.

As it is easily seen in the Figure 4.2, maximum inhibition of 40 % by chloride ions on 2-nitropropane dioxygenase activity is observed even when only two chloride ions per every flavin molecule are present in the solution. It could be hypothesized, that there are two sites on the protein where chloride ions could bind and induce conformational changes that inhibit catalytic power of the enzyme. Being that enzymatic assays were performed in 50 mM potassium phosphate, the addition of only 0.11 to 11 nM of potassium chloride does not affect the ionic
strength. Therefore, the observed reduced rate of the 2-nitropropane dioxygenase cannot be assigned to the ionic strength of the solution.

**Figure 4.2.** Effect of KCl on $\frac{k_{cat}}{K_M}$ of 2-nitropropane dioxygenase in 50 mM potassium phosphate pH 7.4 and 30 °C. The [KCl]/[2NPD] represents the ratio of potassium chloride to enzyme concentration used in the enzymatic assays. $(\frac{k_{cat}}{K_M})_0$ represents apparent steady state second order rate constant obtained in the absence of the potassium chloride. $(\frac{k_{cat}}{K_M})_0$ represents apparent steady state second order rate constant obtained in the presence of the potassium chloride. $(\frac{k_{cat}}{K_M})_0 / (\frac{k_{cat}}{K_M})_{KCl}$ represents the ratio of second order rate constant in absence and presence of potassium chloride. The concentration of 1.1 nM of 2-nitropropane dioxygenase was used throughout all experiments.

If the chloride ions were competitive inhibitors of the enzyme with the respect to the ethynitronate as a substrate, the inhibition of the enzyme would be further increased with the increase of the chloride ion concentration larger than 1.9 nM. Therefore, chloride ions are not competitive inhibitors for 2-nitropropane dioxygenase with the respect to ethynitronate. In order to further distinguish between noncompetitive and uncompetitive inhibition pattern of chloride ions, saturation of the enzyme with the organic substrate is needed. Because organic substrate is
made in ethanol and the percentage of ethanol in the enzymatic assay is kept under 1%, the concentration of the organic substrate higher than 20 mM in enzymatic assay cannot be achieved. Steady-state kinetics measured with ethylnitronate as a substrate in 50 mM potassium phosphate pH 6 and 8 and 50 mM sodium pyrophosphate pH 10.5 at 30 °C have shown that the highest saturation of the enzyme with the organic substrate is achieved at pH 8. In order to determine inhibition pattern of chloride ions in future experiments 50 mM potassium phosphate pH 8 should be used. Additionally, alcohols containing the nitro group are more soluble in water than nitroalkanes, e.g. 2-nitro-1-propanol is more soluble in water than 2-nitropropane [16]. If 2-nitropropane dioxygenase can effectively denitrify these alcohols, using them as substrates might provide a way to saturate the enzyme with organic substrate and determine the inhibition pattern of the chloride ions.

![Figure 4.3. Apparent steady state kinetics of 2-nitropropane dioxygenase with ethylnitronate (●), in presence of (○) 1 mM potassium iodide, (■) potassium bromide or potassium fluoride (□) as determined in 50 mM potassium phosphate, pH 6 and 30 °C. Data were fit with equation 1.](image-url)
In addition, the apparent second order rate constants \((k_{\text{cat}}/K_m)^{\text{app}}\) in absence and presence of 1 mM potassium iodide, potassium bromide or potassium fluoride were determined (Figure 4.1). The apparent second order rate constants \((k_{\text{cat}}/K_m)^{\text{app}}\) of 2-nitropropane dioxygenase with ethylnitronate in 50 mM potassium phosphate pH 6 was determined to be 120,000 M\(^{-1}\) s\(^{-1}\), while in presence of 1 mM potassium iodide, potassium bromide or potassium fluoride was determined to be 99,000 M\(^{-1}\) s\(^{-1}\), 51,000 M\(^{-1}\) s\(^{-1}\) or 97,500 M\(^{-1}\) s\(^{-1}\), respectively. The enzymatic activity of 2-nitropropane dioxygenase with ethylnitronate is inhibited 18 %, 57.5 % or 19 % in presence of potassium iodide, potassium bromide or potassium fluoride.

Halide ions can possibly induce conformational changes in 2-nitropropane dioxygenase and therefore influence the activity of the enzyme. In the case of cytochrome P450 it was established that conformation of the protein is highly dependent on the concentration of NaCl, as \(\alpha\)-helix content increased and \(\beta\)-sheet decreased with the increase in the salt concentration, coinciding with higher activity of the enzyme [17]. In addition, conformational changes by chloride ions are responsible for inhibition of shikimate kinase from \textit{Mycobacterium tuberculosis} [18]. In this context, future studies focusing on the effect of halide ions on the enzymatic activity of 2-nitropropane dioxygenase will have to include examining possible conformational changes of the enzyme in the presence of halide ions.

In conclusion, using nitro compounds, which are readily soluble in water, as substrates could lead to saturation of the enzyme with the organic substrate and therefore allow for the determination of the inhibition pattern of halide ions. In addition, future studies focused on reasons why 2NPD is inhibited by halide ions will have to include studies on conformation changes.
References


Chapter V

PH STUDIES ON 2-NITROPROPANE DIOXYGENASE

Abstract

The studies on pH dependence of the kinetic parameters could provide the valuable insight into the enzymatic reaction. These studies have provided the evidence supporting the need for protonated and unprotonated group for catalysis for both nitroalkane oxidase and 2-nitropropane dioxygenase from *Neurospora crassa*. Even though crystallographic and mutagenesis studies helped with identifying the ionizable groups, the pH profiles of these two nitroalkane-oxidizing enzymes were first evidence in establishing the need for acid or a base in catalysis. In this study, the pH dependence of apparent second order rate constants \(k_{cat}/K_m\)\text{app} with ethynitronate as substrate showed two plateau regions, consistent with the requirement of two ionizable groups that have to be protonated for catalysis. With mixture of nitroethane / ethynitronate as substrates the \(k_{cat}/K_m\)\text{app} values yielded bell-shaped pH profile. Similar results were obtained for pH dependence of apparent second order rate constants \(k_{cat}/K_m\)\text{app} for butyl-1-nitronate and the mixture of butyl-1-nitronate / nitrobutane.
Introduction

The studies on pH dependence of the kinetic parameters can reveal how many ionizable groups are needed in catalysis and if those groups need to be protonated or deprotonated for catalysis to occur. The pH dependence studies have been carried out for both well-characterized nitroalkane oxidizing enzymes, nitroalkane oxidase [1] and 2-nitropropane dioxygenase from Neurospora crassa [2].

The pH dependence studies on the kinetic parameters of nitroalkane oxidase with nitroethane as substrate showed the requirement for two ionizable groups, one that must be unprotonated and the second protonated for catalysis [1]. The amino acid that needs to be protonated for catalysis was later identified as tyrosine residue that participates in substrate binding based on the inactivation studies with tyrosine directed reagent, tetranitromethane [3]. Based on crystallographic and mutagenesis studies, the group that needs to be unprotonated for catalysis was identified to be Asp402, which abstracts the α-carbon of nitroalkane substrates [4-6].

In the case of 2-nitropropane dioxygenase from Neurospora crassa, oxidation of the neutral nitroalkane substrates requires a catalytic base, as supported by the pH profiles of the kinetic parameters obtained with nitroethane and nitrobutane as substrates (figure 5.1). In addition, the pH profiles of the kinetic parameters with both alkyl nitronates and nitroalkanes showed the requirement of ionizable group that needs to be protonated for catalysis, as shown in Figure 5.2 for alkyl nitronates [2].
Figure 5.1. pH dependence of the $k_{cat}/K_m$ value of 2-nitropropane dioxygenase from *Neurospora crassa* with nitroalkanes. Taken without permission [2].

Figure 5.2. pH dependence of the $k_{cat}$ value of 2-nitropropane dioxygenase from *Neurospora crassa* with alkyl nitronates. Taken without permission [2].

The focus of this study is on obtaining the pH dependence of second order rate constants $(k_{cat}/K_m)_\text{app}$ with two alkyl nitronates and the mixture of nitroalkane / alkynitronate.
Experimental procedure

**Materials.** Nitroalkanes were from Sigma-Aldrich. Potassium phosphate and potassium pyrophosphate were from Fisher. All reagents were of the highest purity commercially available.

**Methods.** Enzyme, 2-nitropropane dioxygenase was prepared as described in chapter 2 and stored in 50 mM potassium phosphate pH 7.4 at -20 °C. Enzyme concentration was expressed per enzyme bound FMN content, using the experimentally determined $\varepsilon_{445\ nm}$ value of $13,130\ \text{M}^{-1}\ \text{cm}^{-1}$. Enzyme activity was measured with the method of the initial rates [7] in 100 mM sodium pyrophosphate (in the range 5.5-6 and 8.5-10.5) and potassium phosphate (in the range of 6.5-8 and 11) by monitoring the rate of oxygen consumption with a computer-interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument Ltd.) thermostated at 30 °C.

**Preparation of substrates.** Stock solutions of alkyl nitronates were prepared by allowing the nitroalkanes to react with a 1.2 molar excess of KOH for 24 h at room temperature in 100% ethanol. The final concentration of ethanol in each assay mixture was kept constant at 1% to minimize possible effects on enzymatic activity. The reactions were started by the addition of the organic substrate to pre-equilibrated reaction mixtures, in order to minimize changes in the ionization state of the nitroalkanes or alkyl nitronate substrates. The second-order rate constants for deprotonation of nitroalkanes (5 to 6 M$^{-1}$s$^{-1}$ [8]) and protonation of alkyl nitronates (15 to 75 M$^{-1}$s$^{-1}$ [9]) were taken into account, ensuring that the determination of initial rates (typically ~ 30 s) was performed with fully protonated or unprotonated substrates, respectively. Mixtures of nitroalkanes /alkyl nitronates were prepared by diluting the nitroalkanes in buffer and adjusting the pH prior to performing the assay.
**Data analysis.** KaleidaGraph (Synergy Software, Reading, PA) or Enzfitter (Biosoft, Cambridge, UK) software was used to analyze the data. Since the enzyme could not be saturated with the organic substrate, the apparent second order rate constants \((k_{cat}/K_m)^{app}\) in atmospheric oxygen were determined by fitting the initial reaction rates to Michaelis-Menten equation for one substrate. Here, \(K_a\) represents the Michaelis constants for organic substrate (A) and \(k_{cat}\) is the turnover number of the enzyme (e).

\[
\frac{v}{e} = \left(\frac{k_{cat}}{K_a}\right)^{app} A
\]  

When both oxygen and the organic substrate concentration were varied kinetic parameters were determined by fitting the data with equations 2 and 3, describing a sequential and a ping-pong steady state kinetic mechanism. Here, \(K_a\) and \(K_b\) represent the Michaelis constants for organic substrate (A) and oxygen (B), respectively and \(k_{cat}\) is the turnover number of the enzyme (e). Apparent kinetic parameters in atmospheric oxygen were determined by fitting the data with equation 3, describing Michaelis-Menten equation, where \(K_a\) represents the Michaelis constant for organic substrate (A) and \(k_{cat}\) is the turnover number of the enzyme (e).

\[
\frac{v}{e} = \frac{k_{cat}AB}{K_aB + K_bA + AB + K_{ia}K_b}
\]  

\[
\frac{v}{e} = \frac{k_{cat}AB}{K_aB + K_bA + AB}
\]  

When ethylnitronate was used as substrate, the pH dependence of the apparent steady state kinetic parameters was determined by fitting initial rate data obtained at varying concentrations of organic substrate to equations 2, which describe a curve with a slope of –1 and a plateau region at low pH.
When mixture ethynitronate / nitroethane was used, the pH dependence of the apparent steady state kinetic parameters was determined by fitting initial rate data obtained at varying concentrations of organic substrate to equations 3 which describe a bell-shaped curve with a slope of +1 at low pH and a slope of −1 at high pH. C is the pH independent value of the kinetic parameter of interest.

\[
\log Y = \log \left( \frac{C}{1 + \frac{10^{-pK_{a2}}}{10^{-pH}}} \right)
\]

(4)

\[
\log Y = \log \left( \frac{C}{1 + \frac{10^{-pK_{a2}}}{10^{-pH}} + \frac{10^{-pK_{a1}}}{10^{-pH}}} \right)
\]

(5)
Results and discussion

The steady state kinetic parameters for 2-nitropropane dioxygenase were determined with ethylnitronate by varying both concentration of oxygen and organic substrate at pH 6, 8 and 10.5 and 30 °C. As shown in Figure 5.3, 5.4 and 5.5, the initial rates of oxygen consumption as a function of substrate concentration overlap with varying oxygen. Plots for varying organic substrate and oxygen were fitted with both equation 2 and 3 describing a sequential and ping pong mechanism. Using this approach steady state mechanism was not distinguishable between sequential and ping pong. The $K_{O_2}$ was determined to be 5 µM or below for experiments determined at pH 6, 8 and 10.5, therefore obtaining the kinetic parameters with ethylnitronate as a substrate at atmospheric oxygen would be a good estimate of true kinetic parameters measured at saturating oxygen. Subsequently, determining the substrate specificity for 2-nitropropane dioxygenase with anionic substrates of different alkyl chain lengths was performed by measuring the initial rates of enzymatic activity in air-saturated oxygen.

![Figure 5.3. Steady state kinetic of 2-nitropropane dioxygenase catalyzed reaction with ethylnitronate and oxygen as substrates. Enzymatic activity was measured by method of initial rates by varying](image-url)
concentrations of both ethylnitronate and oxygen in 50 mM potassium phosphate pH 6 and 30°C. Concentration of oxygen was: (●) 82 µM, (○) 102 µM and (■) 130 µM. Data fit with equation 1.

Figure 5.4. Steady state kinetic of 2-nitropropane dioxygenase catalysed reaction with ethylnitronate and oxygen as substrates. Enzymatic activity was measured by method of initial rates by varying concentrations of both ethylnitronate and oxygen in 50 mM potassium phosphate pH 8 and 30°C. Concentration of oxygen was: (●) 58 µM, (○) 41 µM and (■) 20 µM. Data fit with equation 1.

Figure 5.5. Steady state kinetic of 2-nitropropane dioxygenase catalysed reaction with ethylnitronate and oxygen as substrates. Enzymatic activity was measured by method of initial rates by varying
concentrations of both ethyl nitronate and oxygen in 50 mM sodium pyrophosphate pH 10.5 and 30°C. Concentration of oxygen was: (●) 69µM, (○) 82 µM and (■) 98µM. Data fit with equation 1.

The pH dependence of the apparent second order rate constants \((k_{cat}/K_m)^{app}\) of 2-nitropropane dioxygenase with ethyl nitronate and butyl-1-nitronate as substrates were measured in air-saturated buffer in the accessible pH range. With ethyl nitronate as a substrate \((k_{cat}/K_m)^{app}\) values increase with decreasing pH (Figure 5.6). Two plateau regions were observed, consistent with the requirement of a two ionizable groups that have to be protonated for catalysis. The participation of an acid with \(pK_a\) of 6.5 and 9.9 is suggested by the pH-dependence of the \((k_{cat}/K_m)^{app}\) values with ethyl nitronate, showing the involvement of two protonated groups in catalysis.

**Figure 5.6.** pH dependence on \(k_{cat}/K_m\) with ethyl nitronate and mixture of ethyl nitronate and nitroethane. Initial rates were measured in air saturated 100 mM sodium pyrophosphate in the range 5.5-6 and 8.5-10.5 and potassium phosphate in the range of 6.5-8 and 11 at 30 °C. (○) pH dependence when ethyl nitronate was used as a substrate, (●) pH dependence when mixture of ethyl nitronate and nitroethane was used for a substrate. Data were fit with equation 4.
With mixture of nitroethane / ethylnitronate as substrate the \((k_{\text{cat}}/K_m)^{\text{app}}\) values yielded bell-shaped pH profile (figure 5.6). This result can be interpreted as being consistent with involvement of two ionizable groups that must be protonated and unprotonated for the catalysis. This pH profile is consistent with the requirement of a single ionizable group on the enzyme that must be protonated for catalysis. As pointed out in chapter 2, 2-nitropropane dioxygenase utilizes alkynitronates as substrates but is incapable of oxidizing the neutral nitroalkanes. Therefore, the \(pK_a\) of 8.4 determined for the pH profile with mixture of nitroethane / ethylnitronate corresponds to the \(pK_a\) of the substrate, as supported by the \(pK_a\) of 8.5 for nitroethane determined in solution [9]. The second \(pK_a\) of 9.6 is similar to the \(pK_a\) of 9.9 determined from the pH profile with ethylnitronate. The group on the enzyme that can correspond to the \(pK_a\) with value of 9.9 could be the N3 on the isoaloxazine ring of the flavin. Similar results were obtained when ethylnitronate was substituted with butyl-1-nitronate or when the mixture of ethylnitronate / nitroethane was replaced with butyl-1-nitronate / nitrobutane, as seen in figure 5.4. Two \(pK_a\) values for the pH profile of butyl-1-nitronate are not clearly defined as for ethylnitronate pH profile due to the quality of data.

**Figure 5.7.** pH dependence on \(k_{\text{cat}}/K_m\) with butyl-1-nitronate and mixture of butyl-1-nitronate and nitrobutane. Initial rates were measured in air saturated 100 mM sodium pyrophosphate in the range 5.5-6
and 8.5-10.5 and potassium phosphate in the range of 6.5-8 and 11 at 30 °C. (○) pH dependence when butyl-1-nitronate was used as a substrate, (●) pH dependence when mixture of butyl-1-nitronate and nitrobutane was used for a substrate. Data were fit with equation 4.

Two pKa values for the pH profile of the mixture of butyl-1-nitronate / nitrobutane are 8.7 and 9.9. In this case the pKa of 8.7 does not correspond to the pKa of the substrate due to the masking of the substrate’s intrinsic pKa by the group on the enzyme that needs to be protonated for catalysis.

In order to identify the groups involved in catalysis future mutagenesis studies will have to be performed. In addition, group with the pKa ~ 9.9 in pH present in all pH profiles could possibly be assigned to the N3 on the isoaloxazine ring of the flavin. To further support this claim pH profile of the enzyme has to be performed spectrophotometrically.
References


Chapter VI

GROWING THE CRYSTALS OF RECOMBINANT 2-NITROPROPANE DIOXYGENASE

Abstract

Crystallographic analysis can be a powerful tool used to gain the mechanistic insights into the enzymes. The focus of this chapter is on obtaining the crystals for recombinant 2-nitropropane dioxygenase from *Hansenula mrakii*. Needle-shaped yellow crystals grew in 15% (v/v) (+/-)-2-methyl-2,4-pentanediol (MPD), 0.02 M calcium chloride dehydrate and 0.1 M sodium acetate trihydrate pH 4.6. By repeating the same conditions and re-seeding the crystals, bigger single crystals were obtained. Heavy precipitation and subsequent formation of the skin in the vapor drop were addressed by adding the glycerol to crystallization conditions.
Introduction

The crystal structure of nitroalkane oxidase (NAO) from *Fusarium oxysporum* at 2.2 Å resolution was solved [1; 2], providing the supporting evidence of a covalent N5–FAD adduct. The tetrameric structure (Figure 6.1) with asymmetric units was revealed to have three solvent-accessible channels. The biggest channel, which extends from the exterior of the protein, terminates at Asp402 and the N5 position on of the flavin at the side chain of Phe273.

![Figure 6.1. Crystal structure of nitroalkane oxidase from *Fusarium oxysporum*. Each subunit of the homotetramer is depicted as a differently colored Cα ribbon trace [2].](image)

Each subunit consists of a β-sheet domain in the central region and two α-helical domains, one at the C-terminal region and one at the N-terminal region. The FAD binds non-covalently in each subunit through several hydrogen bonds. The adenine moiety extends to the dimer–dimer interface where it interacts with His313 via π-stacking and hydrogen bonds with the side chains of Gln314 and Asn141. With the help of crystal structure important residues in the active site were identified to be Asp402, Arg409 and Ser276 (Figure 6.2).
Asp402 was identified to be in correct position to serve as the active-site base to abstract the α-proton from neutral nitroalkanes. The oxygen atoms on Asp402 form hydrogen bonds with the OH moiety of Ser276 and with the guanidino group of Arg409. Crystal structure data also provided clues on how the covalent flavin N(5)-substrate adduct is stabilized. Being excluded from the bulk solvent, the flavin adduct is sequestered from additional molecular influences. Hydrogen bonding of the isoalloxazine N1 and O2 atoms with amide protons and the hydroxyl group of Ser134 reduce the charge density on the N1 atom and consequently stabilize the adduct. The nitro group on the substrate as part of N(5)-substrate adduct interacts with the side chain of Asp402 to potentially reduce the capacity of NO₂⁻ to serve as a favorable leaving group.

The crystal structure of 2-nitropropane dioxygenase from *P. aeruginosa* at 2.0 Å resolution was solved as the model of the binary complex with FMN and the ternary complex with FMN and 2-nitropropane. The monomeric enzyme consists of two domains connected by two loops and has approximate dimensions of 40 Å x 40 Å x 60 Å (Figure 6.3).
A molecule of FMN is non-covalently bound in the deep active site pocket and is located near the interface between the two domains. The phosphate moiety of FMN is not solvent-accessible since it is buried completely inside the pocket. The FMN-binding pocket consists of ten residues Gly22, Gln24, Thr75, Lys124, Asp145, Ala150, Ser178, Gly180, Gly201, and Thr202 (Figure 6.4). The hydroxyl group of Thr75 is hydrogen-bonded to the N3 and O2 atoms of FMN. The side chain of Lys124 is hydrogen-bonded to the O2 atom on the isoalloxazine moiety, results consistent with positively charged group close to the N1-C2=O2 locus of flavin. The substrate molecule, on the si face of the isoalloxazine ring of FMN, is protected from the bulk solvent. His152 and Ser288 are located in proximity of 2-nitropropane in the active site. His152 is oriented in such a way that its unprotonated N1 atom interacts with the α-carbon of 2-nitropropane and is suitably positioned to abstract the α-proton of the neutral substrate. The side chain of Ser288 recognizes the nitro group of 2-nitropropane via a hydrogen bond. This hydrogen bond potentially increases the acidity of the adjacent carbon, facilitating its deprotonation by His152.
The focus of this chapter is on initial trials to obtain the crystals for 2-nitropropane dioxygenase from *Hansenula mrakii* and the attempt to optimize the conditions in which the crystals were found.
Experimental Procedures

**Materials and Methods.** All reagents were of the highest purity commercially available and were obtained from Hampton Research (Hampton Research, Aliso Viejo, CA, USA). 2-Nitropropane dioxygenase was prepared as described in Chapter 2 and stored in 50 mM potassium phosphate pH 7.4 at -20°C. Enzyme concentration was expressed per enzyme bound FMN content, using the experimentally determined $\varepsilon_{445\ nm}$ value of 13,130 M$^{-1}$ cm$^{-1}$. The concentration of purified 2-nitropropane dioxygenase was determined with the Bradford assay [4], using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

**Crystallization.** Crystals of recombinant 2-nitropropane dioxygenase from *Hansenula mrakii* were grown by the hanging drop vapor diffusion method, and initial crystallization conditions were obtained using commercially available sparse-matrix screening kits (Crystal Screen HR2-110 and Crystal Screen Lite HR2-128). The crystals were grown by mixing 2 µL of 2-nitropropane dioxygenase with 2 µL of reservoir solution. The enzyme drops were equilibrated over 1 mL of the reservoir solution at 23°C or 4°C. Prior to being flash-frozen by quick submersion into liquid nitrogen, single crystals were cryoprotected by being transferred into glycerol and allowed to incubate for 2 min at 23°C.
Results and discussion

No micro crystals were initially obtained from 2 µL of 2-nitropropane dioxygenase (2.3 mg/mL) mixed with 2 µL of reservoir solution containing reagents from Crystal Screen HR2-110 (Table 1). Growing crystals in second kit, Crystal Screen Lite HR2-128 (Table 2), yielded better results with 5.6 mg/mL of 2-nitropropane dioxygenase.

Colorless rod like crystals grew in 10 % (w/v) polyethylene glycol (PEG) 8000, 0.1 M sodium cacodylate trihydrate pH 6.5 and 0.2 M magnesium acetate (Crystal Screen Lite solution 18). To optimize the crystallization conditions, the concentration of PEG was varied from 12 to 17 % with 1 % increments. In addition, the size of PEG was also varied by using 3000, 5000, 6000 and 10,000 Da PEG. Crystals, obtained in polyethylene glycol, 0.1 M sodium cacodylate trihydrate pH 6.5 and 0.2 M magnesium acetate, did not appear to have yellow color so the further optimization was not performed.

Colorless fused crystals grew in 0.75 M lithium sulfate and 0.1 M sodium Hepes pH 7.5 (Crystal Screen Lite solution 16). To optimize the crystallization conditions, the concentration of lithium sulfate was varied from 0.5 to 1 M with 0.1 M increments. In addition, the concentration of Hepes was also varied from 0.05 to 0.20 M with increments of 0.05 M. Crystals, obtained in lithium sulfate and 0.1 M sodium Hepes pH 7.5, did not appear to have yellow color so the further optimization was not performed.

Needle-shaped yellow crystals initially grew in 15% (v/v) (+/-)-2-methyl-2,4-pentanediol (MPD), 0.02 M calcium chloride dehydrate and 0.1 M sodium acetate trihydrate pH 4.6 (Crystal Screen Lite solution 1). When needle-shaped crystals were re-seeded using same conditions, single bigger crystals grew. When mother liquor was made from commercially available compounds, as opposed to using the Crystal Screen Lite solution 1, no crystals were observed
even when concentration of enzyme was in range from 3.1 to 12.3 mg/mL or when 0.5 or 4 M nitroethane was used as ligand. Consequently, subsequent studies were carried out with the Crystal Screen Lite solution 1.

Heavy precipitation and subsequent formation of the skin on the surface of the vapor drop was observed when enzyme concentration was at least 4.5 mg/mL in the well. The formation of the skin was addressed by adding the glycerol to the mother liquor (5, 10 and 15%) or to the enzyme (10 and 20%). A lighter skin formed on the surface of the vapor drop when glycerol was added to crystallization conditions.

Future studies should focus on re-seeding the existing crystals into the wells containing the 15% (v/v) (+/-)-2-methyl-2,4-pentanediol (MPD), 0.02 M calcium chloride dehydrate and 0.1 M sodium acetate trihydrate pH 4.6 (Crystal Screen Lite solution 1). In addition, the heavy precipitation and formation of the skin on the surface of the vapor drop should be addressed by addition of another viscous compound. Alternatively growing the crystals in completely different conditions could provide large enough crystals for determination of the structure. For example, conditions tested with Crystal Screen HR2-110 could be re-tested with 4.5 mg/mL or larger final protein concentration in the well.
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References


Chapter VII

GENERAL DISCUSSION

The FMN-dependent enzyme 2-nitropropane dioxygenase from *Hansenula mrakii* catalyzes the oxidative denitrification of anionic nitroalkanes into corresponding carbonyl compounds and nitrite, with oxygen as electron acceptor. The biotechnological importance of this enzyme is found in the possible bioremediation applications for denitrification of harmful nitroalkanes that are used in chemical industry because they provide a quick and effective method of synthesizing common reagents [1; 2]. From a chemical standpoint, 2-nitropropane dioxygenase from *Hansenula mrakii* is an important new addition to a nitroalkane oxidizing family due to the enzyme preference for anionic nitroalkanes. In this study, the biophysical, biochemical, structural analysis of 2-nitropropane dioxygenase are presented, providing a solid background for future biochemical and mechanistic studies aimed at the elucidation of the chemical mechanism.

The recombinant 2-nitropropane dioxygenase from the yeast *H. mrakii* was expressed to high levels in *E. coli* cells. A fractionation step with 60% saturation of ammonium sulfate and a single chromatographic step using a DEAE column allowed for preparations of active and homogeneous enzyme. The biochemical characterization of 2-nitropropane dioxygenase has established that the enzyme requires FMN but not iron as cofactor for catalysis. Preference of the enzyme for unbranched anionic nitroalkanes or alkyl nitronates was confirmed by anaerobic substrate reduction studies as well as several kinetic studies with a number of different substrates. The enzyme shows minimal discrimination among unbranched alkyl nitronates with chain lengths ranging from 2 to 6 carbon atoms, as shown by the apparent $k_{cat}/K_m$ values between $1.1 \times 10^5$ M$^{-1}$s$^{-1}$ and $1.7 \times 10^5$ M$^{-1}$s$^{-1}$ for these substrates. The stabilization of flavosemiquinone
along with the formation of a flavin-N(5)-sulfi te adduct in 2-nitropropane dioxygenase was explained as a result of the stabilization of the negative charge that develops on N(1) locus of the flavin by a positive charge close to that region. A positive charge may be provided by either the electrical dipole of α-helix or a positively charged amino acid residue, which possibly is a lysine residue as indicated by the crystallographic structure of 2-nitropropane dioxygenase from *Pseudomonas aeruginosa* [3].

2-Nitropropane dioxygenase is inhibited by halide ions. In the presence of two to one ratio of chloride ions to flavin molecule inhibition of 40 % on 2-nitropropane dioxygenase activity is observed. While in presence of 1 mM potassium iodide, potassium bromide or potassium fluoride as determined in 50 mM potassium phosphate pH 6 with ethylnitronate as a substrate, enzyme activity is inhibited 18 %, 58 % or 19 %.

The pH dependence of the apparent second order rate constants \( (k_{cat}/K_m)^{app} \) of 2-nitropropane dioxygenase with ethylnitronate and butyl-1-nitronate as substrates is consistent with the requirement of a two ionizable group that must be protonated for catalysis with p\( K_a \) of 6.5 and 9.9. However, pH-dependent studies of the apparent second order rate constants \( (k_{cat}/K_m)^{app} \) with a mixture of anionic and neutral nitroalkanes with ethylnitronate/nitroethane and butyl-1-nitronate/nitrobutane as substrates, yielded bell-shaped pH profiles. A single ionizable group on the enzyme that must be protonated for catalysis was identified in both pH profiles correspond to the second p\( K_a \) of 9.9 in the pH profile with ethylnitronate. The ionizable group that must be unprotonated for catalysis corresponds to the p\( K_a \) value for the substrate.

As the first step in obtaining the crystal structure of 2-nitropropane dioxygenase crystals were successfully grown in wells containing the 15% (v/v) (+/-)-2-methyl-2,4-pentanediol (MPD), 0.02 M calcium chloride dehydrate and 0.1 M sodium acetate trihydrate pH 4.6.
In summary, the results obtained herein from the biochemical and kinetic characterization of a 2-nitropropane dioxygenase suggest that the enzyme: 1) was purified to high yields as recombinant enzyme; 2) has a preference for unbranched alkyl nitronates; 3) requires FMN as cofactor for catalysis; 4) has a negative charge that develops on the N(1)-C(2)=O locus of the bound flavin; 5) is inhibited by halide ions; 6) requires two unprotonated groups for catalysis with ethyl nitronate and butyl-1-nitronate as substrates. In addition, crystals obtained in this study provide first important step in obtaining the structural data for 2-nitropropane dioxygenase.

The calculated molecular weight of the 374-residue unit of 2-nitropropane dioxygenase (2NPD) is 41467 Da. The enzyme has a theoretical pI of 5.76. Amino acid sequence comparisons indicate that 2NPD is homologous to other nitroalkane oxidizing enzymes, with identities of 26.8 \% and 27.4 \% to nitroalkane oxidase and Neurospora crassa 2NPD, respectively (Appendix 1 and 2). Moreover, amino acid sequence comparison of H. mrakii 2NPD to another 2NPD from Burkholderia phymatum yields the highest percent identity of 34.7 \% in 340 residues overlap (Appendix 3).

In this study, biochemical characterization established that 2-nitropropane dioxygenase from Hansenula mrakii utilizes alkyl nitronates as substrates, and reacts poorly, if at all, with nitroalkanes. Preference of the H. mrakii enzyme for the anionic nitroalkanes makes this enzyme interesting addition to two nitroalkane-oxidizing flavoenzymes, nitroalkane oxidase [4] and N.cassa 2-nitropropane dioxygenase [5], which utilize neutral and both neutral and anionic nitroalkanes, respectively. Both enzymes initiate the oxidation of neutral substrates by abstraction of the proton from the α-carbon. His 196 was proposed to play a role of the base in active site of N. crassa enzyme [6], while Asp 402 has been shown to serve as the catalytic base in crystallographic and mutagenesis studies of nitroalkane oxidase [7; 8; 9]. His 197 in H. mrakii enzyme, which corresponds to the His 196 in N. crassa enzyme, is a conserved residue (Scheme
In this context, *H. mrakii* enzyme has a residue which can act as a base, but it does not show activity with the neutral nitroalkanes, can indicate that the His 197 is not in the right position to abstract the α-proton from the substrate and therefore initiate the catalysis with the neutral nitroalkanes.

**Scheme 7.1.** Amino acid sequence comparison of the conserved histidine residue.

The oxidation of nitroalkanes by flavin-dependent enzymes can occur via two catalytic strategies. First, as in case of nitroalkane oxidase with a formation of a flavin N(5)-substrate adduct [4]. Second, via single electron transfer reaction in which the transiently anionic semiquinone is observed for the *N. crassa* enzyme [6]. The catalytic strategy of *H. mrakii* enzyme is probably very similar to the *N. crassa* enzyme, since both enzymes have been shown to form anionic semiquinone upon incubation with organic substrates in absence of oxygen.

Nitroalkane oxidase (NAO) has a hydrophobic binding site sufficiently large enough to accommodate substrates with alkyl chain 4 carbons long, as indicated by substrate specificity constant with primary and secondary nitroalkanes as well as the nitro compounds which contain hydroxyl group [10]. A smaller binding pocket was also identified for the nitroalkane oxidase which can accommodate branched substrates [10]. In contrast to nitroalkane oxidase, 2-nitropropane dioxygenase from *Hansenula mrakii* does not follow any trend based on the length
of the alkyl chain, as the specificity constant of ethynitronate is similar to the specificity constant of the hexyl-1-nitronate. However, both enzymes have a preference for unbranched substrates. In this context, *H. mrakii* 2-nitropropane dioxygenase possibly has a cavity similar to nitroalkane oxidase, which can accommodate the branched part of the secondary substrate in addition to having a more flexible substrate binding site which can easily anchor any linear nitroalkanes.

The inability of nitroalkane oxidase to react with anionic nitroalkanes is due the tyrosine 398 residue, which must be protonated for binding of the nitro group of the substrate [4; 11]. Therefore, the ability of the 2NPD to bind and catalyze anionic nitroalkanes can indicate that the enzyme has a different binding strategy than hydrogen bonding to the nitro group, as it is case for NAO.

In conclusion, biochemical characterization of 2-nitropropnae dioxygenase from *Hansenula mrakii* in this study provides a solid background for future studies on the enzyme, which is important for both applied and fundamental reasons.

References


Appendix 1

26.8% identity in 41 residues overlap; Score: 34.0; Gap frequency: 0.0%

2NPDhm, 91 VNEEWLKKYDKIYKGKAGIEFDKKEKLLLYPSFRSIVDPQHP
NAO, 157 VGNEWVISGEKLWPSNGGWDYKGADLACVVCRVSDDPKP

25.9% identity in 27 residues overlap; Score: 32.0; Gap frequency: 0.0%

2NPDhm, 159 LQASDIKIFVTVTNLQEFQQAYESKLD
NAO, 327 LETSRLLVWKAVTLEDEALEWKVKLE

31.8% identity in 22 residues overlap; Score: 27.0; Gap frequency: 0.0%

2NPDhm, 332 ANDAKQSGKGPQYSAFLAGSNY
NAO, 140 ANWLQKGGPGLQTARSKVGNEW

33.3% identity in 15 residues overlap; Score: 27.0; Gap frequency: 0.0%

2NPDhm, 153 EAVIESLQASDIKIF
NAO, 368 ECVIDAMKAVGMKSY

44.4% identity in 9 residues overlap; Score: 26.0; Gap frequency: 0.0%

2NPDhm, 148 FGLPHEAVI
NAO, 250 FHVPHENLL

29.8% identity in 47 residues overlap; Score: 25.0; Gap frequency: 0.0%

2NPDhm, 21 APMAGASTLELAATVTLLGGIGSIPMGSLSEKCDIETQLNELFDELV
NAO, 288 ARAAFAEALVFAKSDDRGSGKHIEHQSVADKLIDCKIRLETSRLLL

29.6% identity in 27 residues overlap; Score: 24.0; Gap frequency: 0.0%
2NPDhm,       26 ASTLELAATVTRLGIGSIPMGSLSEK
NAO,          89 ATSITIVATALGLMPVILCDSPSLQEK
               *   **   *   ***

57.1% identity in 7 residues overlap; Score: 23.0; Gap frequency: 0.0%

2NPDhm,      150 LPHEAVI
NAO,         73 LVHESII
               *   ***

50.0% identity in 8 residues overlap; Score: 23.0; Gap frequency: 0.0%

2NPDhm,      311 QSSPLASI
NAO,         124 EGEPLASL
               ****

33.3% identity in 15 residues overlap; Score: 23.0; Gap frequency: 0.0%

2NPDhm,     79 FAHKEPRSGRADVNE
NAO,        298 FAKSDTRGGSKHIIE
               **   **   *

41.7% identity in 12 residues overlap; Score: 23.0; Gap frequency: 0.0%

2NPDhm,     158 SLQASDIKIFVT
NAO,         111 SLQEKFLKPFIS
               ***   **

50.0% identity in 10 residues overlap; Score: 22.0; Gap frequency: 0.0%

2NPDhm,     30 ELAATVTRLG
NAO,         233 ELAGHITTS
               ***   *   *

57.1% identity in 7 residues overlap; Score: 22.0; Gap frequency: 0.0%

2NPDhm,      6 QSFLKTF
NAO,         114 EKFLKPF
               ***   **
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NAO, 241 SGPHTFTEFHVPHEN
** ** *
Appendix 2

27.4% identity in 117 residues overlap; Score: 81.0; Gap frequency: 6.8%

2NP Dhm, 155
VIESLQASDIKIFVTVNLQEFQAYESKLDGVVLQGWEAGGHRGNFKANDVEDGQLKTL
2NP Dnc, 154
IIEALHASGFVVFQVGTVDARKAAADGADVIVAQGIDAGGHQLATGSGIVS-------

48.4% identity in 31 residues overlap; Score: 59.0; Gap frequency: 0.0%

2NP Dhm, 215
DLVSTIVDYIDSASISNPPFIIAAGGIHDDESIKELLQFNIAAVQLGTVWLPSQAT
2NP Dnc, 207-
LVPEVRDMLDFKEREVVVVAAGGVADGRGTVGGVGLGAEVUVVLGTRFTVAEAS

55.6% identity in 9 residues overlap; Score: 29.0; Gap frequency: 0.0%

2NP Dhm, 18 IIQAPMAGAStLEAATVTRLGGIGSIPMGs
2NP Dnc, 33 IISAPMYLIANGTLAAEVSAGGIGFVAGGS

50.0% identity in 14 residues overlap; Score: 29.0; Gap frequency: 0.0%

2NP Dhm, 26 ASTLELAATVTRLG
2NP Dnc, 74 ALSTELASARSRLG

23.5% identity in 34 residues overlap; Score: 29.0; Gap frequency: 0.0%

2NP Dhm, 188 VLQGWEAGGHRGNFKANDVEDGQLKTLDLVSTIV
2NP Dnc, 154 IIEALHASGFVVFQVGTVDARKAAADGADVIV

* * * * * *
54.5% identity in 11 residues overlap; Score: 28.0; Gap frequency: 0.0%

2NPDhm, 229 ISNPPFIIAAG
2NPDnc, 34 ISAPMYLIANG

*** *** *

50.0% identity in 12 residues overlap; Score: 27.0; Gap frequency: 0.0%

2NPDhm, 291 AAISGRNLRTIS
2NPDnc, 329 AASSGDNSRAVT

*** *** *

24.0% identity in 25 residues overlap; Score: 26.0; Gap frequency: 0.0%

2NPDhm, 225 DSASISNPPFIIAAGGIHDDESIKE
2NPDnc, 301 DGRAVRNASYDDHAAGVPFEENHKK

* * ** * *

40.0% identity in 10 residues overlap; Score: 25.0; Gap frequency: 0.0%

2NPDhm, 354 HKSWKDTRST
2NPDnc, 323 HKKFKEAASS

** * *

30.8% identity in 26 residues overlap; Score: 25.0; Gap frequency: 0.0%

2NPDhm, 65 ELVGDSGRIVNLNFHAKPEKPRSGRAD
2NPDnc, 156 EALHASGFVVFFQVGTVDARKAAAD

* ** * * * *

36.4% identity in 11 residues overlap; Score: 24.0; Gap frequency: 0.0%

2NPDhm, 106 AGIEFDKKEKL
2NPDnc, 315 AGVPFEENHKK

** * *

19.4% identity in 31 residues overlap; Score: 23.0; Gap frequency: 0.0%

2NPDhm, 244 DESIKELLQFNIAAVQLGTWLPSQATISP
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<td>15 QAALTKLNSWFPTTKNPVIIASAPMYLIANGT</td>
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40.0% identity in 10 residues overlap; Score: 22.0; Gap frequency: 0.0%

62.5% identity in 8 residues overlap; Score: 22.0; Gap frequency: 0.0%

30.0% identity in 10 residues overlap; Score: 22.0; Gap frequency: 0.0%

40.0% identity in 10 residues overlap; Score: 22.0; Gap frequency: 0.0%

44.4% identity in 9 residues overlap; Score: 21.0; Gap frequency: 0.0%

22.6% identity in 31 residues overlap; Score: 21.0; Gap frequency: 0.0%
22.7% identity in 22 residues overlap; Score: 21.0; Gap frequency: 0.0%

2NPDhm,   143 IVSFHFGLPHEAVIESLQASDI
2NPDnc,   164 VVFFQVGTVKDARKAAADGADV
         *    *    *    *    *

66.7% identity in 6 residues overlap; Score: 21.0; Gap frequency: 0.0%

2NPDhm,  191 GWEAGG
2NPDnc,  57 GFVAGG
         *    ***
Appendix 3

34.7% identity in 340 residues overlap; Score: 354.0; Gap frequency: 6.2%

2NPDhm, 2
RSQIQSLKTDFEVRYPIIQAPMAGASTLELAATVTRLGGISIPMGSLSEKCDAIETQUE

2NPDbp, 4
RSEGKPLLSSLGISKPIIQAPMAGVTTPALAAAVTNAGGLGSLGVGAM--

KAAARKTIR

**  *  *********  *  ***  ** **   *    *  *

2NPDhm, 62
NFDELVGSGRIVNLNFHAAHKEPGRSADVNEEKLKYYDKIYGKAGIEFDDKKEKLLLYPS

2NPDbp, 62
DTRALTSGP--FNINVFCHASA-
AANAKVEQEWLSLAPQFACKYGASAPEK-LSEIYTS

*  *  *  *  *  **  ***  **  **  **  ****

2NPDhm, 122
FRSIVDPQHTVRLKKNPLKIVSVFHPGLPEAVIESLQASDIFKVTVNLQEFQAYE

2NPDbp, 117
GDDQAMLDFLEEKPAIVSFHGFMPKETIKALDAGIVLLASATNLEEAQQVVD

*  *  *  **  **  **  **  ***  **  *****

2NPDhm, 182
SKLDGVLQGWAGHGRGKAHDVDQGLKTLDDLVTIVYIDASISNPPFIIAAGGI

2NPDbp, 173
AGVDALVAQGIEAGGHRGVFDPQVFDEG-LGTLALTRLvVEKFDL-----

--VIAAGGI

*  *  *  **  **  **  **  **  ***  ****  **

2NPDhm, 242
HDDESIHELQFNIAAVQLGTVWLPSQATISPEHLKMFQSPKS-

DTMMTAISGRNLRT

2NPDbp, 225
MDGAGIAAVLALGAQAAQLGTAFCPCPETSIDEYRRAILGDAALHTTLTSAISGRPARS

*  *  *  *  *  *  *  **  ***  ***  ****

2NPDhm, 301
ISTPFLRDLHQSSPLASIPDYPLPYDSFKSLANDAKQSGK

2NPDbp, 285
MVNRFT-ELGRASDRPATPDYPIAYDAGKALHAAAKAKGE

*  *  ****  **  **  **  **  **

50.0% identity in 10 residues overlap; Score: 26.0; Gap frequency: 0.0%

2NPDhm, 244
DESIEKLLQF

2NPDbp, 355
DEAVGRQRF

**  **
42.9% identity in 14 residues overlap; Score: 26.0; Gap frequency: 0.0%

2NPDhm, 26 ASTLELAATVTRLG
2NPDbp, 231 AAVLALGAQAAQLG

41.2% identity in 17 residues overlap; Score: 26.0; Gap frequency: 0.0%

2NPDhm, 23 MAGASTLELAATVTRLG
2NPDbp, 158 LASATNLEEAQQVVDAG

33.3% identity in 21 residues overlap; Score: 25.0; Gap frequency: 0.0%

2NPDhm, 299 RTISTPFLRDLHQSSPLASIP
2NPDbp, 4 RSEGKPLLSSLGISKPIIQAP

57.1% identity in 7 residues overlap; Score: 25.0; Gap frequency: 0.0%

2NPDhm, 123 RSIVDPQ
2NPDbp, 189 RGVFDPQ

30.4% identity in 23 residues overlap; Score: 24.0; Gap frequency: 0.0%

2NPDhm, 240 GIHDDESIKELLQFNIAAVQLGT
2NPDbp, 140 GMPSKETIKALKDAGIVLLASAT

36.4% identity in 11 residues overlap; Score: 24.0; Gap frequency: 0.0%

2NPDhm, 128 PQHPTVRLKLN
2NPDbp, 142 PSKETIKALKD

33.3% identity in 12 residues overlap; Score: 23.0; Gap frequency: 0.0%
2NPDhm, 225 DSASISNPPFII
2NPDbp, 62 DTRALTSGPFNI

55.6% identity in 9 residues overlap; Score: 22.0; Gap frequency: 0.0%

2NPDhm, 63 FDELVGDSG
2NPDbp, 192 FDPQVFDEG

50.0% identity in 8 residues overlap; Score: 22.0; Gap frequency: 0.0%

2NPDhm, 355 KSWKDTRS
2NPDbp, 58 KTIRDTRA

37.5% identity in 16 residues overlap; Score: 21.0; Gap frequency: 0.0%

2NPDhm, 60 LENFDELVGDSGRIVN
2NPDbp, 211 VEKFDLPVIAAGGIMD

29.4% identity in 17 residues overlap; Score: 21.0; Gap frequency: 0.0%

2NPDhm, 106 AGIEFDKKELKLLYPSF
2NPDbp, 83 ANAKVEQEWLWALPQF

23.5% identity in 17 residues overlap; Score: 21.0; Gap frequency: 0.0%

2NPDhm, 351 SNYHKSWKDTRSTEEIF
2NPDbp, 111 SEIYTSFQGDDQAMLDVF

46.2% identity in 13 residues overlap; Score: 20.0; Gap frequency: 0.0%

2NPDhm, 131 PTVRLLKNLKPKI
2NPDbp, 9 PLLSLLGISKPII

* ** ***

* ***

** * *

* ***
23.5% identity in 34 residues overlap; Score: 20.0; Gap frequency: 0.0%

2NPDhm,  34 TVTRLGIGSIPMGSLSEKCDAIETQLENFDELV  
2NPDbp,  146 TIKALKDAGIVLLASATNLEEAQQVVDAGVDALV  
   *   *   *     *      *        * **

29.4% identity in 17 residues overlap; Score: 20.0; Gap frequency: 0.0%

2NPDhm,  211 LKTLDLVSTIVDYIDSA  
2NPDbp,  341 LPAAELVARLEAELEDA  
      *    **       * *

66.7% identity in 6 residues overlap; Score: 20.0; Gap frequency: 0.0%

2NPDhm,  63 FDELVG  
2NPDbp,  197 FDEGLG  
        *** *

46.2% identity in 13 residues overlap; Score: 20.0; Gap frequency: 0.0%

2NPDhm,  26 ASTLELAATVTRL  
2NPDbp,  338 ARALPAAELVARL  
      * * * ***

80.0% identity in 5 residues overlap; Score: 20.0; Gap frequency: 0.0%

2NPDhm,  64 DELVG  
2NPDbp,  355 DEAVG  
      ***