STEADY STATE KINETIC ANALYSES OF NITROALKANE OXIDASE MUTANTS

A Thesis

by

DRAGANA MILIVOJ BOZINOVSKI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2008

Major Subject: Biochemistry

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Committee Members,	Michael Polymenis
	J. Martin Scholtz
Head of Department,	Gregory D. Reinhart

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ABSTRACT

Steady State Kinetic Analyses of Nitroalkane Oxidase Mutants.

(May 2008)

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Chair of Advisory Committee: Dr. Paul F. Fitzpatrick

Nitroalkane oxidase (NAO) catalyzes the oxidation of neutral nitroalkanes to aldehydes and ketones with oxygen consumption and the production of hydrogen peroxide and nitrite. The enzyme is a flavoprotein from the fungus *Fusarium oxysporum*. The active site base, Asp402, abstracts one proton from the substrate to give a carbanion which then attacks the flavin adenine dinucleotide (FAD). The three dimensional crystal structure of NAO shows that Arg409 is 3.6 Å from Asp402. When Arg409 is mutated to Lys, the rate constant for proton abstraction decreases 100-fold. The three-dimensional structure of NAO also reveals the existence of a tunnel which extends from the protein exterior and terminates at the FAD N5 atom and the residues Asp402 and Phe401. We mutated amino acids in the tunnel into tryptophan, phenylalanine and leucine. The L99W, S276W and S276A enzymes showed the biggest decreases in both k_{cat} and k_{cat}/K_m; these amino acids are closest to the FAD molecule and the active site. Mutation of amino acids farther away from the active site showed very small changes in the kinetic parameters. Ser276 is hydrogen bonded to Asp402 in the wild-type enzyme. When this amino acid is mutated to alanine or tryptophan, k_3, the rate

constant for proton abstraction, decreases around 35 fold. Asp402, Arg409 and Ser276 constitute a catalytic triad in the active site of nitroalkane oxidase, and both Arg409 and Ser276 are important for positioning Asp402 and catalysis.

DEDICATION

This thesis is dedicated to my daughter Mia and husband Goran.

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I would like to thank my advisor, Dr. Paul F. Fitzpatrick, for his patience, guidance and support throughout my research, and to thank my committee members, Dr. Michael Polymenis and Dr. J. Martin Scholtz for their guidance throughout the course of this research.

Thanks also go to my friends and colleagues in the lab for making my time at Texas A&M University fun and a great experience.

Finally, I would like to thank my mother and father for their encouragement and support for all these years and thanks to my daughter Mia and my husband Goran for making me happy and for their love.

NOMENCLATURE

NAO	Nitroalkane Oxidase
ACAD	Acyl-CoA Dehydrogenase
FAD	Flavin Adenine Dinucleotide
NE	Nitroethane
NB	Nitrobutane
NH	Nitrohexane
NO	Nitrooctane
NCH	Nitrocyclohexane

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CHAPTER I

INTRODUCTION

Nitroalkane oxidase (NAO) from the fungus *Fusarium oxysporum* (ATCC 695) is a flavoenzyme that catalyzes the oxidation of nitroalkanes to the corresponding aldehydes or ketones, with the consumption of oxygen and the release of hydrogen peroxide and nitrite (Scheme 1) (1). The enzyme was first described in 1976 by Kido et al. (1), and it is induced in the fungus when F*usarium oxysporum* grows in the presence of nitroalkanes. The role of the enzyme is thought to be to protect the fungus from these toxic and carcinogenic compounds (2). Even though nitro compounds are carcinogenic and dangerous, they are widely distributed in our environment, and can be found in rocket fuels, wastes from the chemical industry, or even tobacco smoke. They are used in explosives, herbicides, pesticides, biocides, and drugs (3). So, having an enzyme that has the ability to transform these dangerous chemicals into less harmful compounds and restore the environment, altered by these contaminants, to its original condition is very important and interesting for investigation. Many organisms that produce nitroalkane compounds (such as *Penicillium atrovenetum* and *Hippocrepis comosa*) also produce enzymes that can transform these toxins into less harmful compounds, and in this way they protect themselves (4, 5, 6). On the other hand, there exist organisms targeted by nitroalkanes that have evolved strategies to detoxify these compounds. Some microbes

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This thesis follows the style of *Biochemistry*.

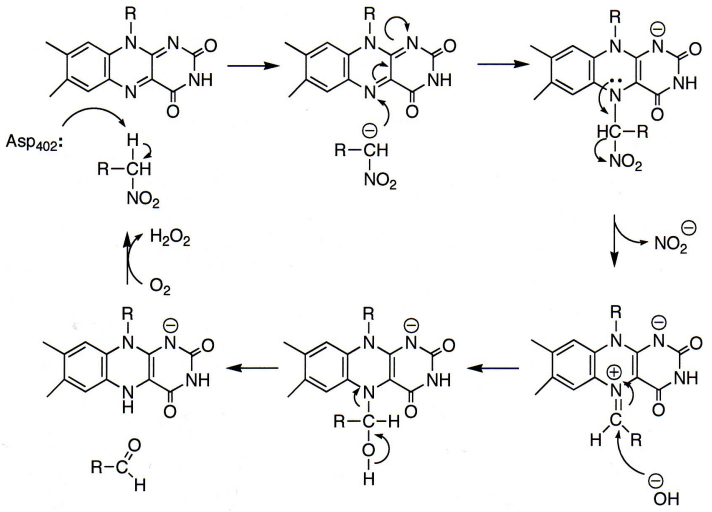
are able to exploit nitrochemicals so effectively that they can utilize these toxins as a sole source of nitrogen, carbon, and/or energy (7, 8). The soil fungus, *Fusarium oxysporum*, is an example of organism that can effectively metabolize a variety of nitroaliphatic compounds (9, 10, 11, 12).

Scheme 1

$$RCH_2NOO + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NO_2^- + H^+$$

The catalytic mechanism of NAO can be divided into two half reactions, the reductive and the oxidative half reactions (Scheme 2) (13, 14). In the reductive half reaction, a nitroalkane substrate reacts with the enzyme that contains an oxidized flavin to give a reduced flavin and an oxidized product. The active site base, Asp402, abstracts an α-proton from the neutral nitroalkane substrate to form a nitroalkane anion, which in the next step attacks the N5 position of the FAD cofactor and gives the initial adduct. This initial adduct then rearranges itself to release the nitrite and form the reactive cationic imine, which in the next step is attacked by hydroxide, and finally the reduced FAD is formed. This is the end of the reductive half reaction. In the oxidative half reaction, molecular oxygen reacts with the reduced FAD to form hydrogen peroxide and an oxidized FAD. At the end of the oxidative half reaction, the oxidized cofactor is restored and can react with another substrate.

Scheme 2



After cloning the gene for NAO from the fungus *Fusarium oxysporum* (15), the amino acid sequence of the cloned enzyme showed that NAO is homologous to the members of the acyl-CoA dehydrogenase family of flavoenzymes (15). The identities between these two families range from 23% to 27%, with similarities up to 46% (15), and the three-dimensional crystal structure also revealed that NAO and ACAD are homologous (16). With both families the initial step in the proposed mechanisms is the proton abstraction from the substrate by an active site base. In NAO this is followed by carbanion formation and an attack on the N5 position of FAD, while with the ACAD family the abstraction of α-proton allows the β-hydrogen to be transferred to the FAD as a hydride (17). NAO will not react with acyl-CoA substrates, and the ACAD family will not oxidize nitrochemicals (15). NAO also requires a neutral form of nitroalkane substrates for catalysis (14).

Two amino acids that are close to the active site base Asp402 are Arg409 and Ser276. Arg409 is electrostatically bonded to Asp402 in the wild-type enzyme, while Ser276 is hydrogen bonded to the active site base (18). These two amino acids are important for correct positioning of the active site base for catalysis. Ser276 is a part of the N5 channel which extends from the protein exterior and terminates at Asp402 and the FAD (16). In the thesis presented here we describe the effect of Arg409 replacement with the lysine on the enzyme mechanism, and evaluate the importance of the N5 tunnel for catalysis.

CHAPTER II

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich Chemical Corp. (Milwaukee, WI), unless otherwise specified. 1-[1,1-$^2H_2$]Nitrobutane was previously synthesized by Dr. Giovanni Gadda (19). Nitrooctane and nitrocyclohexane were synthesized with the help of Dr. Vijay Gawandi, as described in Ballini at al. (20) and Scheigetz et al. (21), respectively. Dr. Gawandi also helped with synthesizing 1-[1,1-$^2H_2$] nitrohexane, 1-[1,1-$^2H_2$]nitrooctane and 1-[1,1-$^2H_2$]nitrocyclohexane, as previously described in (19). The recombinant nitroalkane oxidase was expressed and purified as previously described in (15), and the mutations were generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The mutant enzymes were expressed and purified following the protocol for the wild type enzyme. DNA sequencing of the entire coding sequence of each mutant plasmid was performed at the Laboratory for Plant Genome Technologies of the Texas A&M University. Protein concentrations were determined using the ε_{446} value of 14.2 $mM^{-1}cm^{-1}$. Cyanoalkyl adducts were synthesized by incubating 10 μM wild-type or mutant NAO enzyme with 50 mM sodium cyanide in 100 mM HEPES, pH 8 at 4 °C. Spectra were measured using a Hewlett-Packard Model HP 8452A spectrophotometer equipped with a thermostated water bath.

Methods. Enzyme activity was measured in air-saturated 100 mM Hepes buffer and 0.1 mM FAD at pH 8.0 and 30 °C by monitoring oxygen consumption with a

computer-interfaced Hansatech Clark oxygen electrode (Hansatech Instruments, Pentney King's Lynn, U.K.). To prevent the formation of an anionic form of the substrate, stock solutions of nitroalkanes were prepared in DMSO and assays were initiated by the addition of substrate.

Data Analysis. Data were fit using the programs KaleidaGraph (Adelbeck Software, Reading, PA) and Igor Pro (WaveMetrics, Inc., Lake Oswego, OR). Steady state kinetic parameters were determined by fitting the data to equation 1. Here *v* is the initial velocity, k_{cat} is the maximal velocity, K_m is the Michaelis constant, S is the substrate concentration, and K_{ai} is the substrate inhibition constant. Steady state kinetic isotope effects were calculated from equation 2. Here F_i is the fraction of deuterium in the substrate, and $^{D}k_{cat}$ and $^{D}(k_{cat}/K_m)$ are the isotope effects for k_{cat} and k_{cat}/K_m, respectively.

$$v = k_{cat}\, S / (K_m + S + S^2 / K_{ai}) \qquad (1)$$

$$v = k_{cat}\, S/[K_m\, (1 + F_i\, (^{D}k_{cat}/K_m - 1) + S\, (1+ F_i\, (^{D}k_{cat} - 1)) + S^2/K_{ai}] \qquad (2)$$

CHAPTER III

THE ROLE OF ARG409 IN THE ACTIVE SITE OF NAO

Introduction. The ε nitrogen of Arg409 is 3.6 Å from the carboxylate oxygen of Asp402 in NAO (Figure 1) (18). For NAO, the primary nitroalkanes are the best substrates according to their k_{cat}/K_m values (10), and the k_{cat}/K_m value increases from nitromethane to nitrobutane, with nitrobutane being the best substrate for NAO. Nitroethane is a slow substrate and with this substrate the CH bond cleavage is rate limiting for the reductive half-reaction (19). Therefore, we used nitroethane to examine the effects of R409K mutation.

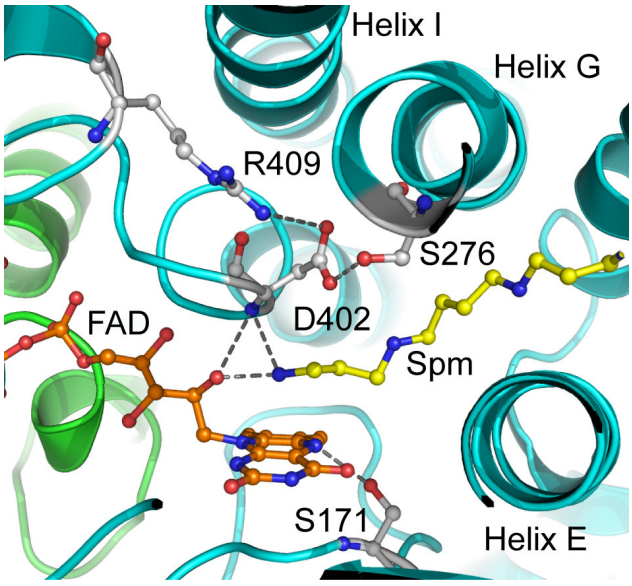
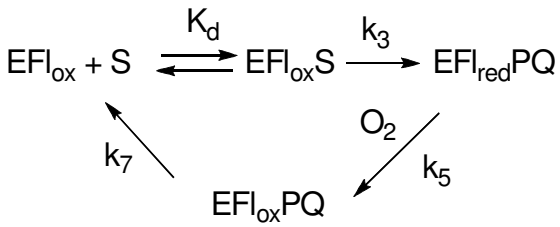


FIGURE 1: The active site of nitroalkane oxidase (18).

Results and Discussion. To examine the role of this amino acid in the catalytic activity of the enzyme, we mutated it to alanine, glutamine and lysine, but only the lysine mutation gave a soluble protein. The kinetic mechanism for the wild-type NAO is shown in Scheme 3, and this mechanism was used to analyze the kinetics of the NAO mutants (13). For this mechanism, by combining the steady state and the isotope effect data, we were able to obtain the rate constants for mutant enzymes, and to establish the effects of mutations on the single rate constants. In the mechanism presented in Scheme 3, K_d is the dissociation constant of the Michaelis complex ($EFl_{ox}S$), k_3 is the rate constant for proton abstraction, k_5 is the rate constant for reaction of the reduced enzyme with oxygen, and k_7 is the rate constant for the product release steps and any conformational changes that can happen while the oxidized enzyme product complex is converted to the active oxidized form of the enzyme. Equation 3 gives the relationship between the k_{cat} value and the rate constant for the chemical step (k_3) and product release (k_7). The relationship between the isotope effect on $^{D}k_{cat}$ and the intrinsic isotope effect on the CH bond cleavage step can be derived from equation 3 and is given by equation 4. Equation 5 presents the relationship between the k_{cat}/K_m (the second order rate constant for the reaction of a free enzyme and the substrate) and k_1, k_2 and k_3. The k_{cat}/K_m value is a combination of the binding steps (k_1 and k_2) and the catalytic step (k_3). Equation 6 is derived from equation 5 and represents the relationship between the isotope effect on k_{cat}/K_m and the intrinsic isotope effect on the substrate CH bond cleavage.

Scheme 3



$$k_{cat} = k_3k_7/(k_3+k_7) \quad (3)$$

$$^{D}k_{cat} = (^{D}k_3 + k_3/k_7)/(1 + k_3/k_7) \quad (4)$$

$$k_{cat}/K_m = k_1k_3/(k_2+k_3) \quad (5)$$

$$^{D}(k_{cat}/K_m) = (^{D}k_3 + k_3/k_2)/(1 + k_3/k_2) \quad (6)$$

The steady state kinetic parameters for the R409K enzyme with nitroethane as a substrate are shown in Table 1. The biggest effect was on the k_{cat}/K_m value, which decreased about 65 fold compared to the wild-type enzyme. The k_{cat} value only decreased six fold. We measured the isotope effects on both k_{cat} and k_{cat}/K_m using 1-[1,1-$^{2}H_2$]nitroethane, and found that the best fit was with identical isotope effects on k_{cat} and k_{cat}/K_m. The wild-type enzyme shows a small isotope effect on the k_{cat} value and a large isotope effect of 9.2 on the k_{cat}/K_m value. The equal isotope effect of 8.3 for R409K is not significantly different from the isotope effect on the k_{cat}/K_m value for the wild-type

enzyme. With nitroethane as a substrate, the deuterium isotope effect is 9.2, which indicates that with this substrate the rate limiting step in the reductive half reaction is the cleavage of the substrate CH bond (19). This means that the deuterium isotope effect on the k_{cat}/K_m value for the nitroethane is equal to the intrinsic isotope effect for this substrate (the value is 9.2).

Table 1: Steady State Kinetic Parameters for Wild Type and R409K NAO

enzyme	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat}/K_{O2} ($mM^{-1}s^{-1}$)[b]	$^{D}k_{cat}$	$^{D}(k_{cat}/K_m)$
wild-type[a]	15 ± 1.0	6.3 ± 0.4	2.3 ± 0.2	310 ± 30	1.4 ± 0.2	9.2 ± 1.1
R409K	2.6 ± 0.3	0.098 ± 0.006	13.4 ± 2.4	46 ± 12	8.3 ± 1.8	8.3 ± 1.8

[a] From reference 13. [b] From reference 18.

However, with this substrate the overall turnover is not limited by the chemical step; rather it is limited by product release from the oxidized enzyme (13). The small isotope effect on k_{cat} with nitroethane as a substrate for the wild-type enzyme is consistent with k_7 being much smaller than k_3. This step is 19-fold slower than reduction (13). In the case of R409K, the isotope effect on k_{cat} is much larger than for the wild type enzyme, so k_3 is much slower than k_7 ($k_7 >> k_3$); from equation 4 we have that $^{D}k_3$ is equal to $^{D}k_{cat}$, and from that we have that $k_{cat} = k_3$. The value of k_{cat} for R409K NAO enzyme is 2.6 s^{-1}, so k_3 is also 2.6 s^{-1} (Table 2). The value of k_3 for the wild-type enzyme was measured using the stopped-flow method and is 247 s^{-1} (13). We can see that k_3 decreases in the R409K mutant compared to the wild-type enzyme and by

disrupting this electrostatic interaction between Arg409 and Asp402, the rate constant for the CH bond cleavage decreases almost 100 fold.

Table 2: Intrinsic Kinetic Parameters for Wild Type and R409K NAO Mutants with Nitroethane as a Substrate

kinetic parameter	wild-type NAO[a]	R409K
K_d (mM)	14 ± 1	26 ± 5
k_3 (s^{-1})	247 ± 5	2.6 ± 0.3
k_5 ($mM^{-1}s^{-1}$)[c]	310 ± 30	46 ± 12
k_7 (s^{-1})	17 (16 ± 11)[b]	>13

[a] From reference 13. [b] Determined by using equations (3)-(6). [c] From reference 18.

In order to get more data and better understanding of the active site of NAO and catalysis, the three-dimensional structure of R409K was determined by X-ray crystallography (18). In the wild-type enzyme the ε nitrogen group of Arg409 is 3.6 Å apart from the carboxylate oxygen of Asp402, and there is a hydrogen bond (2.7 Å) between the hydroxyl group of Ser276 and the carboxylate OD2 of Asp402 (Figure 2A). When the arginine at position 409 is mutated to lysine, which is a shorter amino acid, this nitrogen is moved over 1 Å further away from Asp402 and the electrostatic bond that existed is disrupted (Figure 2B) (18).

This is consistent with loss of activity with R409K mutant, which indicates that this amino acid is important for positioning the active site base Asp402 for proton abstraction. By disrupting this bond the rate constant for CH bond cleavage decreases 100 fold. With the same mutant the hydrogen bond between Ser276 and Asp402 is unchanged. From Figure 2B we can see that Asp402 is moved further away from flavin in the R409K mutant, and the distance between nitrogen of Asp402 and OH flavin is increased from 3.11 to 3.37 Å. This increase in length between Asp402 and flavin is consistent with our data that this electrostatic interaction is important for properly positioning the active site base, and for the enzyme activity.

With the R409K enzyme there is no disruption of the protein structure and the decrease in activity and structural changes are related to the mutated residue. If we look at the structures of the mutant protein and wild-type enzyme we can see that both Arg409 and Ser276 are important, since they are critical active site residues involved in the proper positioning of Asp402, and it appears that these three amino acids constitute a catalytic triad in NAO (18).

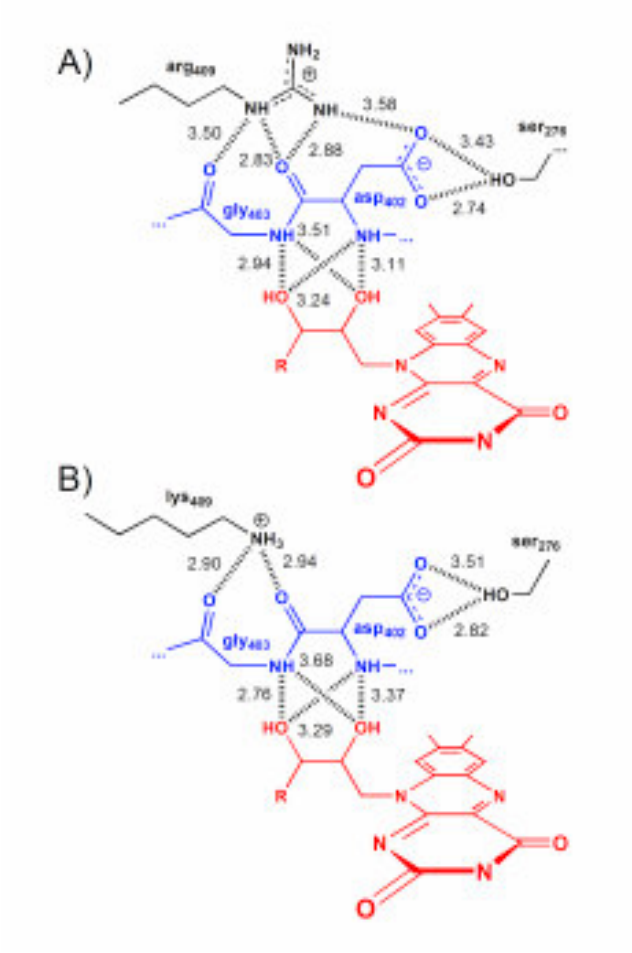


FIGURE 2: Hydrogen bonding interactions and distances deduced from the structures of A) wild-type, and B) R409K NAO (18).

CHAPTER IV

THE ROLE AND IMPORTANCE OF THE N5 CHANNEL IN NAO

Introduction. The three dimensional structure of NAO reveals that there are several channels from the protein surface to the FAD (16). The biggest one (the N1 channel) stretches from the exterior of the protein to the FAD N1 atom and the side chain of Phe273. Phe273 prevents the channel from extending to Asp402, which is the active site base (16). The acyl-CoA dehydrogenase family also has this channel, and for this family this is the substrate entrance channel. The second one is 26 Å long and is called the N5 channel. It also extends from the protein exterior and terminates at the FAD N5 atom and residues Asp402 and Phe401 (16). Figure 3 shows the N5 channel. The structure comes from the B subunit of an oxidized NAO, which contains a spermine molecule within the tunnel. The spermine molecule is an inhibitor of NAO and it occupies approximately 72% of the active site (16). A third channel is a solvent-excluded cavity, and it is located adjacent to the FAD *si* face.

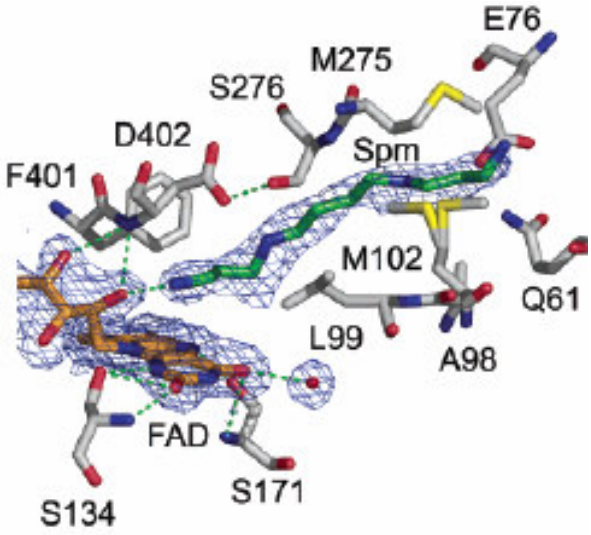


FIGURE 3: Subunit B of the oxidized NAO with the spermine molecule in the active site (16).

Results and Discussion. We were interested in establishing if the N5 tunnel is important for substrate entrance into and product release from the active site. To this end we made several mutations of amino acids that are located in the channel; the ones chosen had their side chains oriented toward the tunnel. Ala98, Leu99, Met102, Met275 and Ser276 were mutated to phenylalanine, leucine or tryptophan. To get the steady state data, we used five different nitroalkane substrates: four primary unbranched, with different chain lengths (nitroethane, nitrobutane, nitrohexane, nitrooctane), and nitrocyclohexane, a secondary nitroalkane.

Table 3: Steady State Kinetic Parameters for A98 NAO Mutants

	wild-type[a]	A98L	A98F	A98W
nitroethane				
k_{cat} (s^{-1})	15 ± 1.0	19 ± 3.0	15 ± 0.6	18 ± 0.8
k_{cat}/K_m ($mM^{-1}s^{-1}$)	6.3 ± 0.4	8.0 ± 3.0	11 ± 0.6	8.0 ± 0.8
K_m (mM)	2.3 ± 0.2	2.4 ± 0.6	1.3 ± 0.1	2.0 ± 0.2
K_{ai} (mM)	25 ± 3.0	22 ± 5.0	24 ± 2.0	18 ± 1.5
1-nitrobutane				
k_{cat} (s^{-1})	6.1 ± 0.4	4.2 ± 0.2	6.0 ± 0.2	3.0 ± 0.2
K_{cat}/K_m($mM^{-1}s^{-1}$)	220 ± 70	177 ± 73	121 ± 26	53 ± 11
K_m (mM)	0.03 ± 0.01	0.025 ± 0.005	0.05 ± 0.006	0.06 ± 0.01
K_{ai} (mM)	12 ± 2.0	49 ± 8.0	30 ± 2.8	27 ± 3.0
1-nitrohexane				
k_{cat} (s^{-1})	2.0 ± 0.07	2.7 ± 0.1	1.5 ± 0.1	2.4 ± 0.3
k_{cat}/K_m ($mM^{-1}s^{-1}$)	46.8 ± 9.8	95 ± 11	24 ± 3.0	16 ± 4.5
K_m (mM)	0.04 ± 0.01	0.03 ± 0.01	0.06 ± 0.03	0.2 ± 0.03
K_{ai} (mM)	-	-	-	2.0 ± 0.7
1-nitrooctane				
k_{cat} (s^{-1})	4.4 ± 0.4	5.1 ± 0.3	6.0 ± 0.3	2.1 ± 0.2
k_{cat}/K_m ($mM^{-1}s^{-1}$)	148 ± 46	121 ± 33	95 ± 12	52.4 ± 7.5
K_m (mM)	0.03 ± 0.01	0.04 ± 0.009	0.06 ± 0.02	0.04 ± 0.009
K_{ai} (mM)	8.3 ± 3.7	91 ± 54	19.5 ± 4.2	3.6 ± 1.8
1-nitrocyclohexane				
k_{cat} (s^{-1})	2.4 ± 0.09	2.8 ± 0.07	1.6 ± 0.07	1.04 ± 0.04
k_{cat}/K_m ($mM^{-1}s^{-1}$)	13 ± 1.6	24 ± 2.4	8.3 ± 2.0	1.8 ± 0.2
K_m (mM)	0.18 ± 0.05	0.12 ± 0.03	0.2 ± 0.04	0.5 ± 0.07
K_{ai} (mM)	208 ± 86	125 ± 26	195 ± 130	162 ± 47

[a] Steady state kinetic paramters for the wild-type enzyme with nitroethane and nitrobutane as substrates are from reference 13.

The results for the Ala98 mutants are given in Table 3. For these three mutants the biggest change was seen with A98W NAO, and this mutant shows decreases in both k_{cat} and k_{cat}/K_m with all substrates except nitroethane.

The steady state kinetic parameters for the Leu99 and Met102 NAO mutants are given in Table 4. With nitroethane as the substrate, the largest effect was on the k_{cat}/K_m with L99W, which was only 0.5% of the wild type, while k_{cat} decreased 10 fold compared to the wild-type enzyme. The large decrease in k_{cat} and k_{cat}/K_m for the L99W enzyme was also seen with other substrates. With M102W and nitroethane as the substrate there was a two-fold increase in both k_{cat} and k_{cat}/K_m compared to the wild-type value, while for the other substrates both parameters were very similar to the wild-type values. The M102F mutant shows big increases in k_{cat}/K_m with nitrobutane and nitrohexane as substrates. The k_{cat}/K_m is a combination of the binding steps (k_1 and k_2) and the catalytic step (k_3), so the big increase in k_{cat}/K_m with this mutation can be explained with increasing of k_1 step, binding of a substrate to the enzyme.

Table 4: Steady State Kinetic Parameters for L99 and M102 NAO Mutants

	wild-type[a]	L99F	L99W	M102F	M102W
nitroethane					
k_{cat}(s^{-1})	15 ± 1.0	14.5 ± 0.5	1.5 ± 0.1	52 ± 6.0	30.1 ± 2.6
k_{cat}/K_m ($mM^{-1}s^{-1}$)	6.3 ± 0.4	0.36 ± 0.02	0.03 ± 0.002	24.7 ± 3.8	12.6 ± 1.7
K_m (mM)	2.3 ± 0.2	39.5 ± 3.0	52 ± 6.4	2.1 ± 0.4	2.4 ± 0.4
K_{ai} (mM)	25 ± 3.0	-	-	12.0 ± 2.4	18.5 ± 3.1
1-nitrobutane					
k_{cat} (s^{-1})	6.1 ± 0.4	7.4 ± 0.7	2.5 ± 0.02	26.0 ± 1.4	6.8 ± 0.5
k_{cat}/K_m ($mM^{-1}s^{-1}$)	220 ± 70	95 ± 6.8	0.2 ± 0.018	730 ± 110	205± 65
K_m (mM)	0.03 ± 0.01	0.07 ± 0.03	12.3 ± 2.4	0.04 ± 0.005	0.03 ± 0.009
K_{ai} (mM)	2.0 ± 2.0	66 ± 24	-	3.6 ± 0.5	8.0 ± 1.8
1-nitrohexane					
k_{cat}(s^{-1})	2.0 ± 0.1	3.8 ± 0.09	0.3 ± 0.06	8.4 ± 0.7	1.9 ± 0.1
k_{cat}/K_m ($mM^{-1}s^{-1}$)	47 ± 10	172 ± 18	0.08 ± 0.02	288 ± 60	45 ± 7
K_m (mM)	0.04 ± 0.01	0.02 ± 0.005	3.7 ± 1.1	0.03 ± 0.008	0.04 ± 0.009
K_{ai} (mM)	-	-	-	1.9 ± 0.6	-
1-nitrooctane					
k_{cat} (s^{-1})	4.4 ± 0.4	4.2 ± 0.2	0.08 ± 0.005	23.1 ± 6.9	4.8 ± 0.6
K_{cat}/K_m ($mM^{-1}s^{-1}$)	148 ± 46	150 ± 25	0.1 ± 0.04	148 ± 20	68.5 ± 7.6
K_m (mM)	0.03 ± 0.01	0.03 ± 0.007	0.6 ± 0.2	0.2 ± 0.08	0.07 ± 0.01
K_{ai} (mM)	8.3 ± 3.7	16.2 ± 3.2	13.3 ± 4.3	0.4 ± 0.2	1.4 ± 0.6
1-nitrocyclohexane					
k_{cat} (s^{-1})	2.4 ± 0.09	4.8 ± 0.3	0.08 ± 0.005		2.3 ± 0.03
k_{cat}/K_m ($mM^{-1}s^{-1}$)	13 ± 1.6	4.2 ± 0.3	0.015 ± 0.0009		12.9 ± 0.7
K_m (mM)	0.18 ± 0.05	1.1 ± 0.1	3.8 ± 0.7		1.3 ± 0.013
K_{ai} (mM)	208 ± 86	22 ± 4	-		-

[a] Steady state kinetic paramters for the wild-type enzyme with nitroethane and nitrobutane as substrates are from reference 13.

The results for the Met275 and Ser276 mutants are shown in Table 5. The S276A mutation removes the hydrogen bond between the hydroxyl group of Ser276 and the carboxylate of Asp402. This hydrogen bond is also broken with S276W, but because of the size of tryptophan, there is a chance that this tryptophan is blocking the N5 tunnel. With S276A mutant the steady state kinetic analysis showed big decreases in both k_{cat} and k_{cat}/K_m with all nitroalkane substrates and the values for both went down several folds. The same decreasing tendency was seen with the S276W mutant on both k_{cat} and k_{cat}/K_m with nitroethane and nitrobuthane, but the steady state kinetic data were not obtained with the S276W enzyme and nitrooctane and nitrocyclohexane as substrates, because the reactions were too slow. M275W NAO showed two to four fold decreases with all the substrates.

We also wanted to see if there is any effect of the substrate size in the tunnel, so we looked at the substrate specificity. From Figure 4 we can see that there is no change in specificity when we go from nitroethane to nitrooctane as a substrate, so the size of a substrate does not affect the specificity of the enzymes.

Table 5: Steady State Kinetic Parameters for M275 and S276 NAO Mutants

	wild-type[a]	M275W	S276W	S276A
nitroethane				
k_{cat} (s^{-1})	15 ± 1.0	8.4 ± 0.9	7.8 ± 0.6	1.0 ± 0.01
k_{cat}/K_m ($mM^{-1}s^{-1}$)	6.3 ± 0.4	2.9 ± 0.3	0.4 ± 0.09	0.3 ± 0.02
K_m (mM)	2.3 ± 0.2	2.8 ± 0.6	19.8 ± 4.2	3.3 ± 0.2
K_{ai} (mM)	25 ± 3.0	21.6 ± 0.4	-	-
1-nitrobutane				
k_{cat} (s^{-1})	6.1 ± 0.4	2.9 ± 0.2	0.6 ± 0.02	0.3 ± 0.01
k_{cat}/K_m ($mM^{-1}s^{-1}$)	220 ± 70	49.7 ± 10.2	1.6 ± 0.1	4.1 ± 0.4
K_m (mM)	0.03 ± 0.01	0.05 ± 0.01	0.3 ± 0.04	0.09 ± 0.01
K_{ai} (mM)	12.0 ± 2.0	-	143 ± 36	173 ± 49
1-nitrohexane				
k_{cat} (s^{-1})	2.0 ± 0.1	0.97 ± 0.02	0.3 ± 0.02	0.3 ± 0.006
k_{cat}/K_m ($mM^{-1}s^{-1}$)	46.8 ± 9.8	43.6 ± 2.1	1.2 ± 0.2	1.5 ± 0.2
K_m (mM)	0.04 ± 0.01	0.02 ± 0.002	0.2 ± 0.02	0.2 ± 0.02
K_{ai} (mM)	-	-	147 ± 19	132 ± 25
1-nitrooctane				
k_{cat} (s^{-1})	4.4 ± 0.4	1.1 ± 0.08		0.2 ± 0.01
k_{cat}/K_m ($mM^{-1}s^{-1}$)	148 ± 46	16.9 ± 2.8		1.3 ± 0.2
K_m (mM)	0.03 ± 0.01	0.06 ± 0.01		0.3 ± 0.02
K_{ai} (mM)	8.3 ± 3.7	8.8 ± 2.8		26 ± 5
1-nitrocyclohexane				
k_{cat} (s^{-1})	2.4 ± 0.09	1.1 ± 0.02		0.4 ± 0.007
k_{cat}/K_m ($mM^{-1}s^{-1}$)	13 ± 1.6	5.5 ± 0.5		0.4 ± 0.01
K_m (mM)	0.18 ± 0.05	2.8 ± 0.6		0.2 ± 0.04
K_{ai} (mM)	208 ± 86	-		26 ± 4

[a] Steady state kinetic paramters for the wild-type enzyme with nitroethane and nitrobutane as substrates are from reference 13.

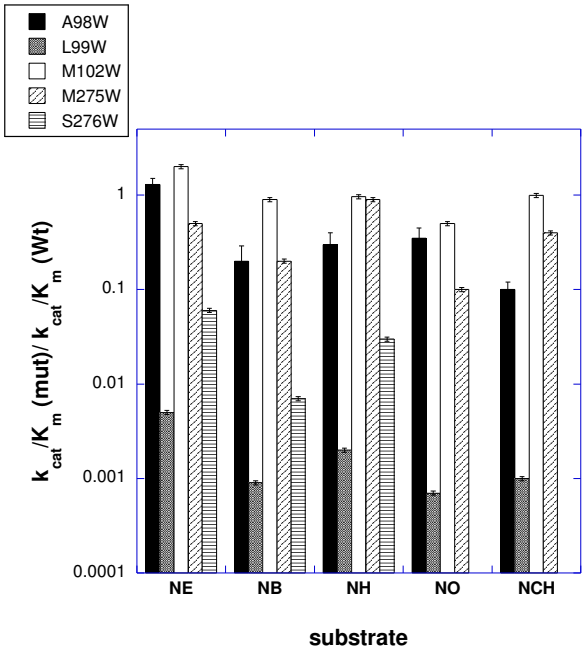


FIGURE 4: Substrate specificity of NAO mutants.

We also measured deuterium kinetic isotope effects with all the tryptophan mutants and all substrates, to see whether there are any effects of the mutation on the chemical steps. The wild-type enzyme exhibits a large isotope effect on the k_{cat}/K_m value for nitroethane and a relatively small effect on the k_{cat} value. With nitrobutane and nitrohexane the isotope effect on k_{cat} is close to 1 for the wild-type enzyme, while the isotope effect on k_{cat}/K_m is around 2. With nitrooctane the best fit of the data occurred with the identical isotope effects of 1.7 on the k_{cat} and k_{cat}/K_m values. The results are summarized in Table 6.

With the L99W enzyme and nitroethane as a substrate, the isotope effect on k_{cat} increased to 4.6, while the isotope effect on k_{cat}/K_m decreased to 5.4. With the same mutant and nitrobutane as a substrate the isotope effect increased on both k_{cat} and

k_{cat}/K_m, reaching 2.7 for k_{cat} and 8.7 for k_{cat}/K_m. With this enzyme, for nitrohexane and nitrooctane, the best fit for the data occurred with identical isotope effects of 3.1 and 6.9 on the k_{cat} and k_{cat}/K_m values respectively.

The S276W enzyme shows large and identical isotope effects on k_{cat} and k_{cat}/K_m of 9.9 with nitroethane as a substrate; with the same substrate for S276A NAO, the isotope effect on k_{cat} increased to 2.3 and for k_{cat}/K_m it is 12.8. The other tryptophan mutant enzymes have very similar isotope effects to the wild-type enzyme with all five nitroalkane substrates. With nitrocyclohexane as a substrate, the wild-type enzyme and all other mutants showed big isotope effect on k_{cat}/K_m (~20). A deuterium isotope effect bigger than 7 indicates a contribution from tunneling (22).

Table 6: Steady State Kinetic Isotope Effects for NAO Mutant Enzymes

	wild-type[a]		A98W		L99W		M102W	
	$^{D}(k_{cat})$	$^{D}(k_{cat}/K_m)$	$^{D}(k_{cat})$	$^{D}(k_{cat}/K_m)$	$^{D}(k_{cat})$	$^{D}(k_{cat}/K_m)$	$^{D}(k_{cat})$	$^{D}(k_{cat}/K_m)$
NE	1.4 ± 0.2	9.2 ± 1.1	1.6 ± 0.2	9.6 ± 1.0	4.6 ± 1.8	5.4 ± 1.7	2.1 ± 0.4	9.7 ± 2.0
NB	1.04 ± 0.09	1.7 ± 0.6	0.9 ± 0.05	1.4 ± 0.3	2.7 ± 0.9	8.7 ± 1.8	1.0 ± 0.09	1.6 ± 0.5
NH	1.2 ± 0.1	2.2 ± 0.6	1.0 ± 0.1	1.3 ± 0.2	3.1 ± 0.5	3.1± 0.5	1.1 ± 0.09	2.0 ± 0.5
NO	1.7 ± 0.1	1.7 ± 0.1	1.0 ± 0.08	2.5 ± 0.4	6.9 ± 2.0	6.9 ± 2.0	1.5 ± 0.08	1.5 ± 0.08
NCH	1.1 ± 0.04	20.5 ± 2.15	1.0 ± 0.1	31.0 ± 5.1	-	25.7 ± 4.5	1.3 ± 0.04	16.9 ± 2.0

	M275W		S276W		S276A	
	$^{D}(k_{cat})$	$^{D}(k_{cat}/K_m)$	$^{D}(k_{cat})$	$^{D}(k_{cat}/K_m)$	$^{D}(k_{cat})$	$^{D}(k_{cat}/K_m)$
NE	1.8 ± 0.4	12.7 ± 2.8	9.9 ± 3.0	9.9 ± 3.0	2.3 ± 0.2	12.8 ± 2.2
NB	0.97 ± 0.07	1.2 ± 0.3	1.2 ± 0.05	1.4 ± 0.3	1.4 ± 0.06	2.0 ± 0.5
NH	1.1 ± 0.04	3.1 ± 0.4	1.5 ± 0.1	2.1± 0.8	1.4 ± 0.04	1.5 ± 0.2
NO	1.9 ± 0.1	1.9 ± 0.1			1.8 ± 0.2	1.3 ± 0.4
NCH	1.2 ± 0.04	15.9 ± 2.05			1.1 ± 0.04	15.4 ± 1.0

[a] Steady state kinetic isotope effects for the wild-type enzyme with nitroethane and nitrobutane as substrates are from reference 13.

The L99W enzyme shows an increase in $^{D}k_{cat}$ and at the same time a decrease in $^{D}(k_{cat}/K_m)$ with nitroethane as a substrate. This allowed us to determine the rate constants for this mutant by combining the data from the steady state kinetics and the isotope effects, using equations (3) - (6). From Table 7 we can see that this mutation has decreased the rate constant for CH bond cleavage about 70-fold and the product release step around 6.5-fold. The product release step is still slower than chemistry, but the difference between them is less than for the wild-type enzyme.

Table 7: Intrinsic Kinetic Parameters for the Wild Type and NAO Mutants with Nitroethane as a Substrate

kinetic parameter	wild-type NAO[a]	L99W	L99F
K_d (mM)	14 ± 1	52 ± 6.0	26 ± 5
k_3 (s^{-1})	247 ± 5	3.4 ± 1.4	33 ± 12
k_7 (s^{-1})	17 (16 ± 11)[b]	2.6 ± 2.1	25.6 ± 18

[a] From reference 13. [b] Determined by using equations (3)-(6).

For the wild-type enzyme, nitrobutane shows much larger value for k_{cat}/K_m than the value for nitroethane. However, k_{cat} and the isotope effects are smaller, which indicates that the chemical step is faster with this substrate than with the nitroethane. A small isotope effect on k_{cat} is still consistent with k_7 being slower than k_3. From Table 8 we can see that k_3 for the wild-type enzyme is about 4000, determined by using the stopped-flow method, and k_3 is at least 200 times bigger than k_7 (13). The numbers in parentheses are determined by using equations (3) - (6) and they agree very well with the numbers determined by steady state kinetics and stopped flow. The errors for both k_3 and k_7 are big because the isotope effects on both k_{cat} and k_{cat}/K_m are small for the wild-type enzyme with nitrobutane as a substrate. The L99W mutation decreases the value of k_3 about 300-fold compared to the wild-type value, and the value of k_7 is similar to the wild-type value. A small increase in the isotope effect on k_{cat} is still consistent with k_7 being slower than k_3.

Table 8: Intrinsic Kinetic Parameters for Wild Type and L99W with Nitrobutane as a Substrate

kinetic parameter	wild-type NAO	L99W
K_d (mM)	nd	12.3 ± 2.4
k_3 (s^{-1})	~ 4000[a] (1200 ± 2800)[b]	12 ± 5.5
k_7 (s^{-1})	6.1 ± 0.4 (6.1 ± 20)[b]	3.2 ± 2.3

[a] Determined from the stopped flow method from reference 13.
[b] Determined by using equations (3)-(6).

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With nitrohexane and nitrooctane as substrates, the rate constants for wild-type enzyme and the L99W mutant were calculated using equations (3) - (6), combining steady state kinetic and the isotope effect data. From Table 9 we can see that with nitrohexane as a substrate both k_3 and k_7 are smaller, compared to the wild-type enzyme, and this mutation decreases the rate constant for CH bond cleavage about 35-fold and the product release step around 2.5-fold. The results show that with this substrate the product release step is still slower than the chemistry.

Table 9: Intrinsic Kinetic Parameters for Wild Type and L99W NAO with Nitrohexane as a Substrate

kinetic parameter	wild-type NAO	L99W
K_d (mM)	0.04 ± 0.09	4.9 ± 2.8
k_3 (s^{-1})	82 ± 9.3	2.3 ± 0.9
k_7 (s^{-1})	2.0 ± 0.3	0.8 ± 0.4

The same results are observed with nitrooctane, the longest linear substrate we used for the analysis. Table 10 shows that k_7 is also smaller than k_3 for both the wild-type enzyme and the L99W mutant. With the L99F mutation the isotope effect on k_{cat} increased to 4.6 with nitroethane as a substrate, while $^{D}(k_{cat}/K_m)$ is the same as the wild-type value, which indicates that this mutation decreased both the chemistry step and the product release step about 7-fold, compared to the wild type enzyme (Table 7).

Table 10: Intrinsic Kinetic Parameters for Wild Type and L99W NAO with Nitrooctane as a Substrate

kinetic parameter	wild-type NAO[a]	L99W
K_d (mM)	0.03 ± 0.01	0.5 ± 0.2
k_3 (s^{-1})	51.5 ± 10.8	0.14 ± 0.04
k_7 (s^{-1})	4.8 ± 1.4	0.4 ± 0.4

With both mutants, S276A and S276W NAO, k_{cat} and k_{cat}/K_m decreased with all five substrates. With S276A and nitroethane as a substrate there is an increase in the isotope effect on k_{cat} value from 1.4 to 2.3 and from Table 11 we can see that the S276A

mutation has decreased the rate constant for CH bond cleavage about 40-fold and the product release step around 14-fold, compared to the wild-type value. For the same substrate, nitroethane, S276W NAO shows an increase in the isotope effect on k_{cat}, and there is a large identical isotope effect of 9.9 on both k_{cat} and k_{cat}/K_m. Again, from equation 4 we have that $^{D}k_3$ is equal to $^{D}k_{cat}$ and from that that $k_{cat} = k_3$. So, k_3 for S276W NAO is 7.8 s^{-1} and it decreases in the S276W mutant compared to the wild-type enzyme 35-fold. By combining this data with equation 3, we get that the product release step k_7 is 1.1 s^{-1} (Table 11), and this step is ~ 15-fold slower than the wild-type value. With both S276A and S276W NAO, we obtained similar decreases in both k_{cat} and k_{cat}/K_m for all substrates. With both mutants the product release step is slower than the chemistry, and it is slower compared to the wild-type enzyme, so breaking the hydrogen bond is affecting the chemistry of the enzyme.

Table 11: Intrinsic Kinetic Parameters for the Wild Type NAO and S276 Mutants with Nitroethane as a Substrate

kinetic parameter	wild-type NAO[a]	S276A	S276W
K_d (mM)	14 ± 1	3.3 ± 0.2	19.8 ± 4.2
k_3 (s^{-1})	247 ± 5	6.3 ± 1.2	7.8 ± 0.6
k_7 (s^{-1})	17 (16 ± 11)[b]	1.2 ± 0.4	1.1 ± 0.1

[a] From reference 13. [b] Determined by using equations (3)-(6).

In the oxidative half-reaction, reduced NAO will react with oxygen to give oxidized FAD, which can now react with another substrate molecule. Table 12 gives values for K_{O2} for different NAO mutant enzymes. K_{O2} is equal to $k_3k_7/(k_3+k_7)k_5$, and here k_5 is a rate constant for the reaction of the reduced enzyme with oxygen. We can see that values for K_{O2} for different NAO mutants are very similar to the wild-type enzyme, so the mutations do not affect a reaction of the reduced enzyme with oxygen.

Table 12: K_m Values of Oxygen for NAO Mutants

Enzyme	wild-type	A98W	L99W	M102W	M275W	S276W	S276A
K_{O2} (μM)	82 ± 9	43 ± 6	56 ± 15	73 ± 18	84 ± 8	111 ± 19	72 ± 20

CHAPTER V

INACTIVATION OF NAO BY CYANIDE

Introduction. During the reductive half reaction of NAO, the substrate anion reacts with the FAD to form a flavin adduct which in the next step releases nitrite and generates a reactive cationic imine (Scheme 2). In the normal pathway this reactive imine will react with hydroxide and form a reduced FAD and products, but it can also be trapped with nitroalkane anion or cyanide (16, 23, 24). The first support for this reactive imine as a catalytic intermediate in the NAO pathway was the identification of the 5-nitrobutyl FAD. This adduct can also be formed *in vitro* when NAO that contains unmodified FAD is reacting with a combination of neutral and anionic nitroethane (24). This inactive form of FAD, which is also seen in the native enzyme from *Fusarium oxysporum*, is generated when the nitroethane anion attacks the reactive cationic imine during the turnover. Cyanide also has the ability to inactivate the enzyme, by binding to the same imine form and trapping this intermediate (23). With nitroethane as a substrate in the presence of cyanide the enzyme loses activity very fast, and the spectrum of flavin bleaches to a reduced form. These results confirm that the cyanide is binding during normal turnover, after the removal of the substrate proton, but before the formation of the fully reduced substrate.

Results and Discussion. Professor Allen M. Orville, who is collaborator with our lab and is working on crystallizing NAO, trapped the D402N enzyme with

nitrocyclohexane as a substrate and showed that the enzyme crystals exhibit an oxidized flavin spectrum (Figure 5).

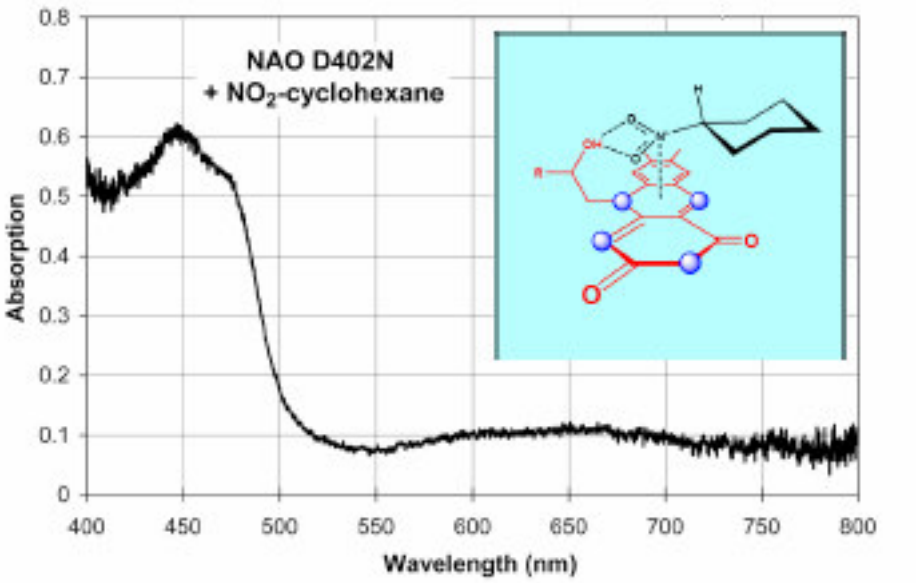

FIGURE 5: Single crystal microspectroscopy of D402N NAO with nitrocyclohexane as a substrate.

To confirm this result we repeated the same experiment as Dr. Orville did only using protein in solution. We did the experiments with both wild-type and the D402N enzyme, using cyanide as an inactivator with the longer substrates nitrohexane and nitrocyclohexane.

Cyanoalkyl adducts were synthesized by incubating wild-type or mutant NAO enzyme with sodium cyanide in 100 mM HEPES, pH 8 at 4 °C. Figure 6 A shows the spectral changes of wild-type NAO with nitrohexane as a substrate. Here we see a bleaching of the FAD to a reduced form after some time (18 min). Flavin is initially in the oxidized form, because oxidation is happening faster than the product release step. After some time, the concentration of oxygen decreases, and the rate of oxidation slows down, until all of the oxygen is used and the flavin is completely reduced. That is why in Figure 6 A after some time we see the flavin in the reduced form.

Figure 6 B shows the spectral changes during turnover of the wild-type NAO enzyme with nitrohexane as a substrate in the presence of 50 mM cyanide. Here we also see the bleaching of the oxidized form of FAD to a reduced form, but after a much longer time, because in the presence of cyanide a longer time is required to consume the same amount of oxygen. The same results were obtained using wild-type enzyme and cyanide with nitrocyclohexane as substrate (Figure 7).

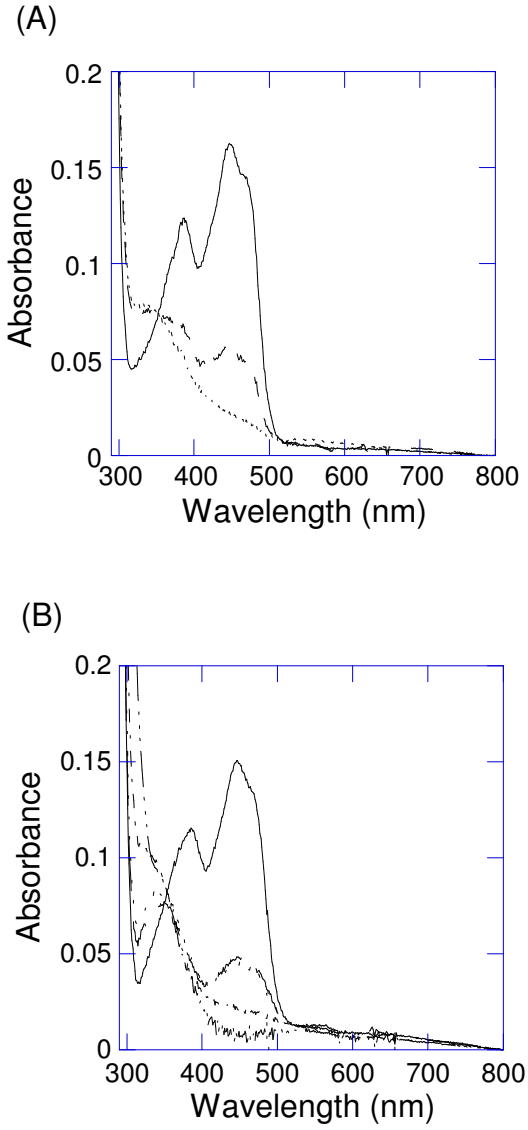

FIGURE 6: Spectral changes of wild-type nitroalkane oxidase during turnover with 1-nitrohexane without (A) and with (B) cyanide. (A) Wild-type NAO (10 µM) (——) was rapidly mixed with 1-nitrohexane (final concentration of 0.5 mM) and the spectrum was taken immediately (— —) and after 18 minutes (·····). (B) Wild-type NAO (10 µM) was rapidly mixed with CN (final concentration 50 mM) (——). Nitrohexane (final concentration of 0.5 mM) was then added and the reaction was monitored immediately (— —), after 21 minutes (·····), 20 hours (— · —), and a week (— ··· —).

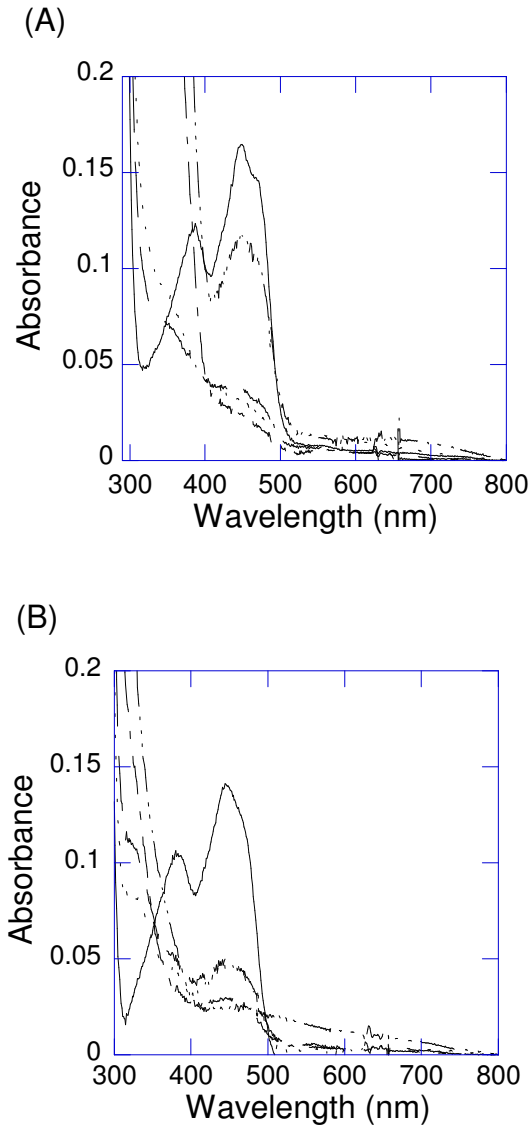

FIGURE 7: Spectral changes of the wild-type nitroalkane oxidase during turnover with 1-nitrocyclohexane without (A) and with (B) cyanide. (A) The wild-type NAO (10 µM) (——) was rapidly mixed with 1-nitrohexane (final concentration of 0.5 mM) and the spectrum was taken immediately (— —), after 15 minutes (·····), 20 hours (— · —) and a week (— ··· —). (B) Wild-type NAO (10 µM) was rapidly mixed with CN (final concentration 50 mM) (——). Nitrocyclohexane (final concentration of 0.5 mM) was then added and the reaction was monitored immediately (— —), after 30 minutes (·····) 20 hours (— · —), and a week (— ··· —).

With the D402N enzyme and nitrohexane as a substrate, the FAD stays in the oxidized form (Figure 8). The mutation of Asp402 to asparagine decreases the rate constant for CH bond cleavage about 140-fold, so enzyme stays in the oxidized form without or with cyanide (Figure 8). The same results were obtained with nitrocyclohexane as a substrate (Figure 9), and here we can also see that the enzyme stays in oxidized form with or without cyanide in solution. These data are in good agreement with the result obtained by Dr. Orville. He trapped D402N enzyme with nitrocyclohexane as a substrate and showed that the enzyme crystals exhibit an oxidized flavin spectrum. When we repeated the same experiments only using the enzyme in solution we obtain the same results, FAD stays in oxidized form. The results were consistent with both nitrohexane and nitrocyclohexane as substrates.

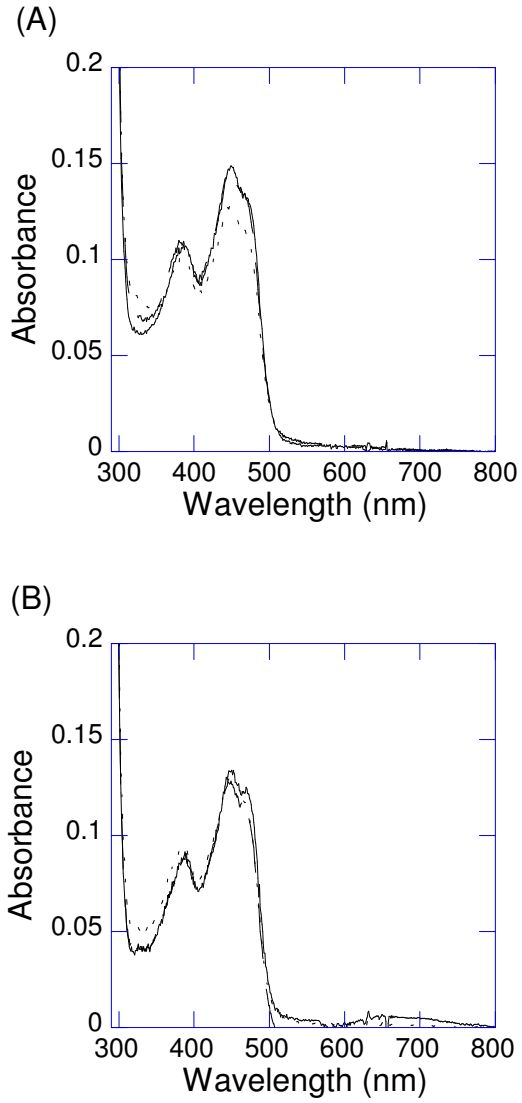

FIGURE 8: Spectral changes of D402N nitroalkane oxidase during turnover with 1-nitrohexane without (A) and with (B) cyanide. (A) D402N NAO (10 µM) (——) was rapidly mixed with 1-nitrohexane (final concentration of 0.5 mM) and the spectrum was taken immediately (— —) and after 15 minutes (·····). (B) D402N NAO (final concentration of 10 µM) was rapidly mixed with CN (final concentration 50 mM) and the spectral changes were measured at 446 nm (——). Nitrohexane (final concentration of 0.5 mM) was then added to mixture and the reaction was monitored at 446 nm (— —) and after 20 minutes (·····).

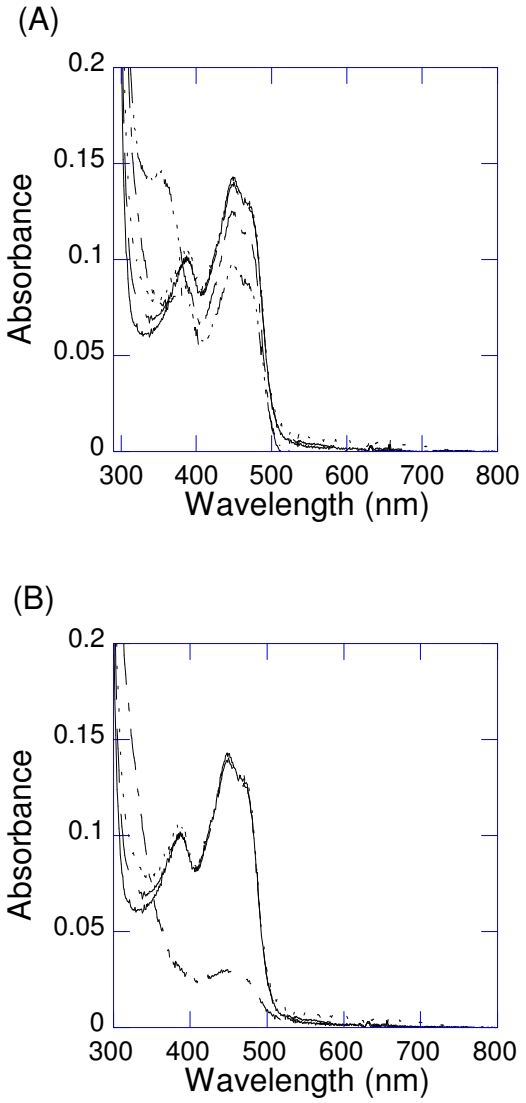

FIGURE 9: Spectral changes of D402N nitroalkane oxidase during turnover with 1-nitrocyclohexane without (A) and with (B) cyanide. (A) D402N NAO (10 μM) (——) was rapidly mixed with 1-nitrocyclohexane (final concentration of 0.5 mM) and was immediately monitored (— —), after 30 minutes (·····), 20 hours (— · —), and a week (— ··· —). (B) D402N NAO (10 μM) was rapidly mixed with CN (final concentration 50 mM) (——). Nitrohexane (final concentration of 0.5 mM) was then added to mixture and immediately monitored (— —), after 20 minutes (·····), and after 20 hours (— · —).

CHAPTER VI

SUMMARY

The studies presented here have focused on the role of the active site of NAO enzyme. In Chapter III the data showed that when arginine at position 409 in NAO enzyme is mutated to lysine, the rate constant for proton abstraction, k_3, decreases almost 100 fold. This decrease in activity in the R409K mutant compared to the wild-type enzyme indicates that this electrostatic interaction between Arg409 and Asp402 is important for properly positioning the active site base and for the enzyme activity, and by disrupting this electrostatic interaction, the rate constant for the CH bond cleavage decreases.

Chapter IV focused on the role and importance of the N5 channel in NAO enzyme. The results obtained with mutated amino acids from the channel indicate that the N5 channel is important for the activity of the enzyme; nitro substrates are entering the NAO active site through this channel and products are released through the same structure. By blocking this tunnel and the active site, both chemistry and product release steps are affected, and they decrease several fold. We saw this with both the Leu99 and Ser276 mutations, which are the ones closest to the active site. When Leu99 is mutated to tryptophan, a longer amino acid than the leucine, the tunnel is blocked and the access of the aliphatic substrate to the active site is affected. This is seen in decreases in both k_{cat} and k_{cat}/K_m with all five substrates that we used. With this mutant the biggest change happened on both k_3 and k_7, with product release step being still the slowest step in the

mechanism. On the other hand, Ser276 is hydrogen bonded to the active site base, is 2.7 Å from Asp402 in wild-type enzyme, and is also part of the N5 channel. So, we made two mutations, to alanine to break the hydrogen bond but not to block the tunnel, and to tryptophan to break the bond and at the same time to block the tunnel. The results showed us that with both S276A and S276W mutations, chemistry and product release steps decreased several fold, so the hydrogen bond between Ser276 and Asp402 is important for activity of the wild-type enzyme. This also shows that this amino acid is important for the interaction with Asp402 and for properly positioning of Asp402 for catalysis. So both Arg409 and S276 are in the active site of the enzyme, and with the Asp402 they constitute a catalytic triad in NAO. This triad is important for the activity of the enzyme, and when any of the amino acid is mutated or the bonds between them broken the activity is affected.

In Chapter V the inactivation of NAO enzyme is done by using cyanide, and the reaction were checked by using two substrates, nitrohexane and nitrocyclohexane. The results for wild-type NAO enzyme showed bleaching of the FAD to a reduced form with both substrates. Flavin is in a wild-type enzyme initially in the oxidized form and because oxidation is happening faster than the product release step, after some time the concentration of oxygen will decreases and the rate of oxidation will slow down until all of the oxygen is used and the flavin is completely reduced. In the presence of cyanide bleaching of the oxidized form of FAD to a reduced form in a wild-type enzyme will happened after a much longer time, because in the presence of cyanide a longer time is required to consume the same amount of oxygen. Data with the D402N enzyme and both

nitro substrates showed that in the mutated enzyme the FAD stays in the oxidized form. This mutation decreases the rate constant for CH bond cleavage about 140-fold, so enzyme stays in the oxidized form without or with cyanide. These results agree very well with the data obtained by Dr. Orville, so in the mutated enzyme FAD is in the oxidized form.

REFERENCES

1. Kido, T., Hashizume, K., and Soda, K. (1978) Purification and properties of nitroalkane oxidase from *Fusarium oxysporum*, *J. Bacteriol. 133*, 53-58.

2. R. A. Davis. In: G. D. Clayton and F. E. Clayton, Editors, *Patty's Industrial Hygiene and Toxicology*, Wiley, New York (1993), 599–662.

3. Venulet, J., and van Etten, R. L. (1970) *Biochemistry and Pharmacology of the Nitro and Nitroso Group*, Vol. 2, Interscience Press, New York.

4. Porter, D. J., and Bright, H. J. (1987) Propionate-3-nitronate oxidase from *Penicillium atrovenetum* is a flavoprotein which initiates the autoxidation of its substrate by O_2, *J. Biol. Chem. 262*, 14428-14434.

5. Hipkin, C. R., Salem, M. A., Simpson, D., and Wainwright, S. J. (1999) 3-Nitropropionic acid oxidase from horseshoe vetch (*Hippocrepis comosa*): a novel plant enzyme, *Biochem. J. 340*, 491-495.

6. Alston, T. A., Mela, L., and Bright, H. J. (1977) 3-Nitropropionate, the toxic substance of *Indigofera*, is a suicide inactivator of succinate dehydrogenase, *Proc. Natl. Acad. Sci. U.S.A. 74*, 3767-3771.

7. Spain, J. C. (1995) Biodegradation of nitroaromatic compounds, *Annu. Rev. Microbiol. 49*, 523-555.

8. Spain, J. C., Hughes, J. B., and Knackmuss, H. J. (2000) *Biodegradation of Nitroaromatic Compounds and Explosives*, CRC Press LLC, Boca Raton, FL.

9. Kido, T., Hashizume, K., and Soda, K. (1978) Purification and properties of nitroalkane oxidase from *Fusarium oxysporum*, *J. Bacteriol. 133*, 53-58.

10. Gadda, G., and Fitzpatrick, P. F. (1999) Substrate specificity of a nitroalkane oxidizing enzyme, *Arch. Biochem. Biophys. 363*, 309-313.

11. Singh, S. B., and Kulshrestha, G. (1991) Microbial degradation of pendimethalin, *J. Environ. Sci. Health B 26*, 309-321.

12. Zayed, S. M., Mostafa, I. Y., Farghaly, M. M., Attaby, H. S., Adam, Y. M., and Mahdy, F. M. (1983) Microbial degradation of trifluralin by *Aspergillus carneus*,

Fusarium oxysporum, and *Trichoderma viride*, *J. Environ. Sci. Health B 18*, 253-267.

13. Valley, M. P., and Fitzpatrick, P. F. (2003) Reductive half-reaction of nitroalkane oxidase: effect of mutation of the active site aspartate to glutamate, *Biochemistry 42*, 5850-6.

14. Heasley, C. J., and Fitzpatrick, P. F. (1996) Kinetic mechanism and substrate specificity of nitroalkane oxidase. *Biochem.Biophys.Res.Commun. 225*, 6-10.

15. Daubner, S. C., Gadda, G., Valley, M. P., and Fitzpatrick, P. F. (2002) Cloning of nitroalkane oxidase from *Fusarium oxysporum* identifies a new member of the acyl-CoA dehydrogenase superfamily. *Proc. Nat. Acad. Sci. USA 99*, 2702-2707.

16. Nagpal, A., Valley, M. P., Fitzpatrick, P. F., and Orville, A. M. (2006) Crystal structures of nitroalkane oxidase: insights into the reaction mechanism from a covalent complex of the flavoenzyme trapped during turnover. *Biochemistry 45*, 1138-1150.

17. Fitzpatrick, P. F., Orville, A. M., Nagpal, A., and Valley, M. P. (2005) Nitroalkane oxidase, a carbanion-forming flavoprotein homologous to Acyl-CoA dehydrogenase. *Arch. Biochem. Biophys. 433*, 157-165.

18. Fitzpatrick, P. F., Bozinovski, D. M., Héroux, A., Shaw, G. P., Valley, M. P., and Orville, A. M. (2007) Mechanistic and structural analyses of the roles of Arg409 and Asp402 in the reaction of the flavoprotein nitroalkane oxidase. *Biochemistry 46*, 13800-13806.

19. Gadda, G., Choe, D. Y., and Fitzpatrick, P. F. (2000) Use of pH and kinetic isotope effects to dissect the effects of substrate size on binding and catalysis by nitroalkane oxidase. *Arch. Biochem. Biophys*. *382*, 138-144.

20. Ballini, R., Barboni, L., and Giarlo, G. (2004) The first coversion of primary alkyl halides to nitroalkanes under aqueous medium. *J. Org. Chem. 69*, 6907-6908.

21. Scheigetz, J., Berthelette, C., Li, C., and Zamboni J. R. (2004) Base-catalyzed deuterium and tritium labeling of aryl methyl sulfones. *Journal of Labelled Compounds and Radiopharmaceuticals*, *47*, 881-889.

22. Kohen A. and Klinman J.P., (1999) Hydrogen tunneling in biology, *Chem. Biol. 6*, 191–198.

23. Valley, M. P., Tichy, S. E., and Fitzpatrick, P. F. (2005) Establishing the kinetic competency of the cationic imine intermediate in nitroalkane oxidase. *J. Am. Chem. Soc. 127*, 2062-2066.

24. Gadda, G., Edmondson, R. D., Russel, D. H., and Fitzpatrick, P. F. (1997) Identification of the naturally occurring flavin of nitroalkane oxidase from *Fusarium oxysporum* as a 5-nitrobutyl-FAD and conversion of the enzyme to the active FAD-containing form. *J. Biol. Chem. 272*, 5563-5570.

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