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The Cytochrome P450 family is a class of enzymes responsible for the breakdown of about 75% of the drugs that are administered (18). The 2E1 isoform of this family is known as the "ethanol-inducible" member, because ethanol has demonstrated the capacity to affect the metabolism of other 2E1 substrates (15, 16). It has been proposed that elevated concentrations of ethanol in the liver are responsible for the activation of acetaminophen and nitrosamine constituents found in cigarette smoke into hepatotoxic byproducts, and that an increase in polarity in a very hydrophobic active site may have a significant role in perpetuating these toxic effects (2-3, 23). In the present study, the objective was to probe the influence of ethanol and subsequently, increased active site polarity, on the catalytic scheme of other $P450_{2E1}$ substrates. Additionally, due to the fact that many other P450 isoforms contain hydrophobic active sites and therefore, may also be affected by changing polarity in the active site environment, the implications of these studies were evaluated in the context of the P450 family as a whole.

Potential evidence regarding the existence of an effector site adjacent to the catalytic site, and its role in 2E1 catalysis, was also probed. Designed to examine the effect of ethanol on the previously observed substrate inhibition pattern in p-nitrophenol oxidation, results from current studies indicated that ethanol potentially disrupts ligand-ligand interactions between the catalytic and effector sites. The ability of ethanol to potentially alter rate-limiting steps in 2E1-mediated benzyl alcohol oxidation was explored through intrinsic isotope effect studies. Despite being limited by the purity of

the benzyl alcohol substrate, an apparent shift or “unmasking” of the isotope effect was observed when ethanol was added. Finally, reversibility studies were carried out to evaluate the capacity of ethanol to interfere with 2E1 inactivation by 4-nitrobenzaldehyde, a potent irreversible inhibitor of the 2B4 isoform (7). When ethanol was present in low concentrations, a synergistic effect was exhibited, where the activity of 2E1 was reduced to an even greater degree than reactions containing the aldehyde alone. An increase in enzyme activity was observed in reactions containing the aldehyde and higher concentrations of ethanol, relative to those containing only the aldehyde, suggesting that ethanol exerted a pseudo-protective effective in this case.

THE ADAPTIVE NATURE OF CYTOCHROME $P450_{2E1}$-MEDIATED ETHANOL OXIDATION: IMPLICATIONS ON THE OVERALL CATALYTIC SCHEME

by

Katherine Grace Ryan

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Approved by

_____
Committee Chair

To my family and friends,

Your unconditional love and support has carried me

Whenever I felt I could no longer stand on my own,

And to my Papa Joe and Grandpa Ryan,

Though you are no longer with me,

Your spirit has never stopped guiding me.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at the University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

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Date of Acceptance by Committee

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Date of Final Oral Examination

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CHAPTER I

INTRODUCTION

Cytochrome P450 enzymes, a class of monooxygenases that play an important role in the metabolism of various xenobiotic compounds, have been studied at length in order to obtain more detailed models of the mechanisms involved in catalysis. Different isoforms of the enzyme are identified by their substrate specificity and mechanism of catalysis, and are of high interest in the study of drug-drug interactions in the human body (1-3). Details concerning oxidative mechanisms and substrate selectivity for the 2E1 isoform have been elusive, and a lack of knowledge in this area has limited the overall potential of the isoform as a therapeutic target. Our investigation focused on elucidating further mechanistic details in 2E1 catalysis, particularly the effects of ethanol on catalysis.

To extract further mechanistic details, studies were carried out to observe the effect of ethanol on the substrate inhibition pattern previously seen with *para*-nitrophenol (PNP). The role of a recently proposed "effector site" existing distal to the active site, was also probed in these studies (4). Assuming that ethanol competes for binding within the active site, a shift in substrate inhibition pattern for PNP should be evident. We were able to observe this shift at different concentrations of ethanol, and the implications of this observation will be discussed.

In addition, the ability of ethanol to influence observed isotope effects in the 2E1-mediated oxidation of benzyl alcohol, by changing active site polarity, was evaluated. Because the active site of $P450_{2E1}$ is very hydrophobic in nature, we proposed that an increased concentration of ethanol may lead to alterations in the precise mechanism for benzyl alcohol oxidation by this isoform. The C-H bond-breaking step is associated with a substantial activation barrier, and large isotope effects (KIE > 7) have been observed when this step is rate-limiting (5, 6). However, increases in active site polarity may change the energetics of this process, whereby O-H bond activation becomes the favored process. Thus, we have investigated the effect of increasing ethanol concentration and active site polarity on the intrinsic isotope effect of benzyl alcohol, which serves to probe the critical bond-breaking step in the overall mechanism.

Finally, ethanol exhibited a protective effect against the potent irreversible inhibitor 4-nitrobenzaldehyde, at high concentrations. At low concentrations of ethanol, however, ethanol appeared to enhance the inhibitory effects of the aldehyde. Recent literature has suggested that 4-nitrobenzaldehyde binds irreversibly to the heme group, creating an adduct that produces an inactive form of the enzyme (7). These studies appear to further support the notion that ethanol has a dramatic effect on the 2E1 metabolic profile. We have assessed the results of these experiments in relation to the broad scheme of $P450_{2E1}$ catalysis.

I. A. Cytochrome P450 Role in Drug-Drug Interactions

The Cytochrome P450 family is responsible for metabolizing a very large variety of substrates, including many of the drugs on the current market, as well as components of the human diet. Several cases of liver toxicity have been documented as a result of multiple xenobiotic compounds (that are metabolized by the same P450 isoform) being present at a given point in time. Whether through competitive or non-competitive mechanisms, complete inhibition or slight alterations of the normal catalytic pathway produces these toxic effects. Two of the more commonly known drug interactions associated with P450 enzymes are grapefruit juice and Taxol® (3A4), and ethanol and acetaminophen (2E1) (9-14). Elucidating the mechanisms behind toxic effects that can arise from concurrent administration of certain substances is essential to ensure the efficacy and safety of treatment regimens.

Taxol® is used primarily as an anti-cancer treatment, and is metabolized by the $P450_{3A4}$ isoform. Studies have been done to investigate the role of grapefruit juice and its respective components as potential inhibitors of 3A4 (9-12). In vitro experiments have indicated that a class of compounds known as flavonoids (more specifically, furanocoumarins) may be responsible for the inhibitory effects, through enzyme inactivation (12). A reduction in the active form of 3A4 by these compounds resulted in increased systemic concentrations of Taxol®, stimulating a drug overdose type of response.

The specific mechanism behind the effect of ethanol on 2E1-mediated acetaminophen metabolism has not been as straightforward. It is thought that ethanol

"induces" the expression of the enzyme, due to the increased protein levels that have been documented upon ethanol treatment in rats (13, 14). This increase in 2E1 levels, and subsequently, an increase in the amount of acetaminophen metabolized via 2E1, is one possible explanation for acetaminophen toxicity when ethanol is present. Toxicity is thought to occur via the generation of highly reactive byproducts in the presence of ethanol, such as N-acetyl-p-quinolone imine (NAPQI), during acetaminophen breakdown (15, 16). Whether there is a single pathway by which damage occurs, or if multiple events contribute simultaneously, is still under great debate. It was postulated that when ethanol is present in the liver, it competes with acetaminophen for 2E1, but that the majority of the NAPQI is produced after ethanol has been cleared from the system (15). Due to an increase in the half-life of the 2E1 isoform after exposure to ethanol (from 7 hours to 37 hours), as well as a decrease in enzyme degradation, the metabolism of acetaminophen is accelerated approximately 24 hours after ethanol consumption. Consequently, to rationalize the alterations in normal xenobiotic metabolism as a result of ethanol consumption, a more thorough understanding of the unique catalytic profile of 2E1, as well as the adaptive nature of ethanol oxidation is necessary.

1. B. Cytochrome $P450_{2E1}$

In recent studies, Cytochrome $P450_{2E1}$ enzymes have been implicated in oxidative stress brought on by alcohol consumption and a number of other environmental factors by producing reactive oxygen species in the liver **(**2, 3**)**. This enzyme may be responsible for generating hepatotoxic and carcinogenic intermediates in the course of metabolizing acetaminophen and N,N-dimethylnitrosamine (a constituent of cigarette smoke). Crystal

structures of the $P450_{2E1}$ active site have recently been released, and have provided valuable information on the conformational features of the isoform (17). **Figure I. 1** shows the active site cavity, as well as a smaller distal cavity (gray mesh).

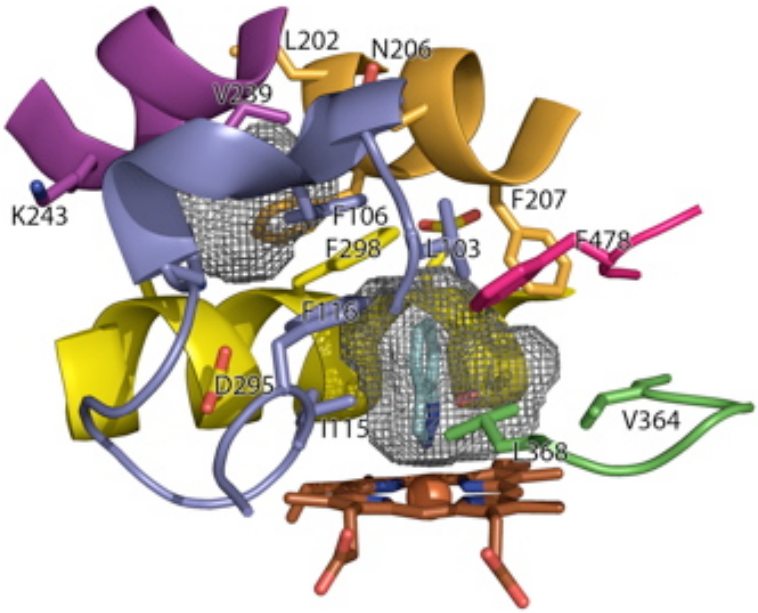

Figure I. 1: Crystal structure of the $P450_{2E1}$ active site, showing the active site (190 $Å^3$) and adjacent smaller cavity (77 $Å^3$). (reproduced with permission from (17))

The 2E1 active site is one of the smallest of all the isoforms, which is consistent with the enzyme's affinity for smaller substrates (18). The interior is also very hydrophobic in nature, so it is curious that ethanol is a substrate for the enzyme at all. The mere fact that 2E1 plays a major role in the metabolism of such a polar molecule, despite the hydrophobicity of the active site environment, suggests that possible adaptations in catalysis may be in response to the increase in polarity.

Although 2E1 has the inherent ability to activate various substrates into highly reactive products, this is even more pronounced at elevated levels of ethanol concentration. Induction of the enzyme and the subsequent generation of reactive species have been connected with ethanol-induced liver injury (15). Our working hypothesis was that there is a connection between the induction and subsequent elevated expression of 2E1 and the change in catalytic mechanism, to overcome the highly polar conditions of any one particular active site. Therefore, an attempt was made to establish experimental evidence regarding this adaptation in metabolism. The information from these studies may be valuable as a toxicological model for further in vivo studies, in an effort to reduce drug-drug interactions related to ethanol consumption and subsequent catalysis by the $P450_{2E1}$ isoform.

I. B. i. Role of Effector Site in Catalysis

In the broader spectrum of $P450_{2E1}$ catalysis, details concerning substrate specificity and oxidative mechanisms have been limited, and a lack of knowledge in theses areas has limited the overall potential of the enzyme as a therapeutic target. In light of this, studies were done by Collom et al. (2008) to discern the mechanism of substrate inhibition previously demonstrated by this isoform, though relatively unexplored (4). The studies suggest the existence of a site distal to the active site above the heme; an "effector" site that does not participate in catalysis, but instead has an allosteric effect on catalysis. This hypothesis was initially supported through steady state analysis with p-nitrophenol, shown in **Figure I. 2.**

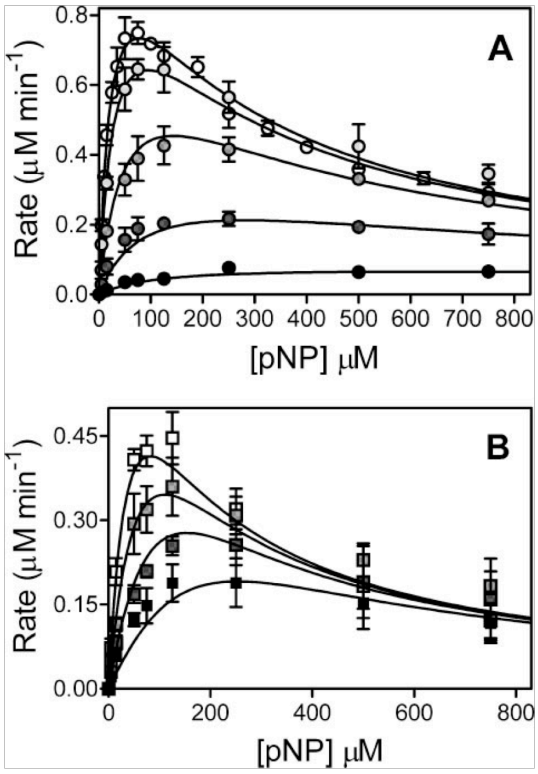

Figure I. 2: Michaelis Menten analysis of PNP oxidation in the presence of inhibitors 4-methyl pyrazole (A) and indole (B), at increasing concentrations of each inhibitor. Kinetic parameters were calculated based on a single site competitive model (reproduced from with permission from (4)).

The decrease in activity following the maximum velocity is not consistent with the traditional model for single substrate kinetics. Rather, it suggests that a second substrate binds to an effector site at higher concentrations, and inhibits catalysis within the active site. The significance of the effector site was further supported by the fact that despite an observed V_{max} of 0.76 μM/min, traditional Michaelis-Menten calculations indicated that V_{max} should have been 1.2 μM/min. Thus, only 64% of the maximum rate was actually reached, and it was concluded that certain catalytic properties are masked by the use of the traditional single substrate Michaelis-Menten kinetic model for the 2E1 isoform (4).

I. C. Influence of Ethanol on $P450_{2E1}$ Catalytic Scheme

Studies regarding the role of the ethanol in oxidative stress and subsequent liver damage have been ongoing for several decades (1-3, 13-16). Multiple theories regarding the specific pathway by which ethanol causes oxidative damage have been proposed in the literature. **Figure I. 3** shows two of the more widely accepted pathways.

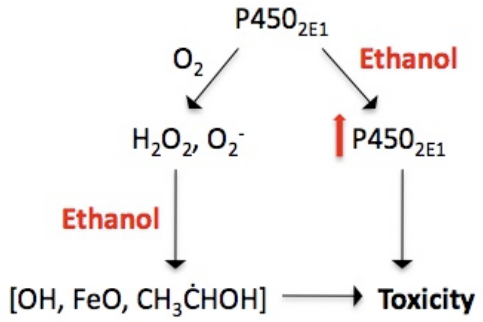

Figure I. 3: Role of Cytochrome $P450_{2E1}$ in oxidative stress after ethanol treatment.

It has been reported that ethanol consumption resulted in increased levels of $P450_{2E1}$, leading to the assumption that ethanol somehow "induces" the isoform by up-regulation of protein expression (13). However, the results of these studies showed little or no increase in 2E1 mRNA levels, suggesting that the effect was not at the level of transcriptional activation. Instead, it was postulated that the increase in 2E1 levels was due to ethanol-mediated stabilization of the protein, and that this stabilization rendered the enzyme resistant to degradation (14).

In addition to possible up-regulation of 2E1 expression, another potential explanation for ethanol-mediated toxicity is the generation of highly reactive species that have the capacity to alter other substrates. In particular, the 1-hydroxyethyl radical is assumed to activate acetaminophen into N-acetyl-p-benzoquinone imine (NAPQI), which contributes significantly to acetaminophen toxicity **(**15**)**. Despite the amount of experimental evidence regarding the subsequent generation of reactive species, the catalytic details that lead to their production are not well understood (16). This may be due, in part, to the transient nature of the metabolic intermediates. Thus, in order to further characterize any possible interactions between ethanol and other substrates during metabolism, a more thorough profile of ethanol oxidation is necessary.

I. C. i. Mechanism of 2E1-Mediated Ethanol Oxidation

Previous mechanistic studies done by Guengerich et al. (19, 20) have indicated that the oxidation of ethanol proceeds through a "dual hydrogen abstraction pathway", where abstraction from the alpha carbon was assumed to be the initial step, followed by oxygen rebound from the heme group to form a gem-diol intermediate, and then elimination of water to form the acetaldehyde product (**Figure I. 4**). A similar mechanism was proposed by Vaz et al. for the oxidation of benzyl alcohol to benzaldehyde by the 2E1 isoform (21).

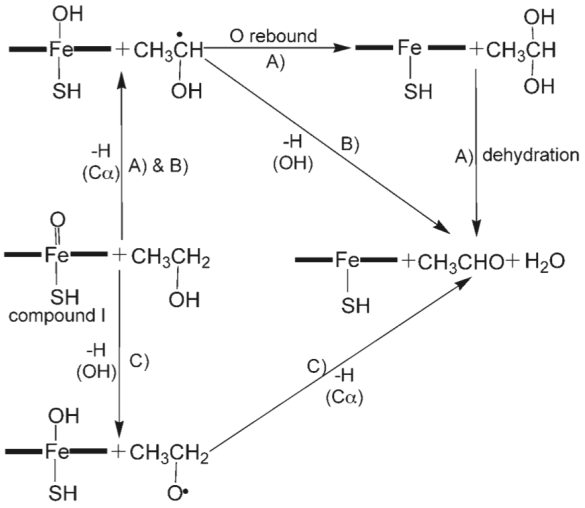

Figure I. 4: Three proposed mechanisms for ethanol oxidation catalyzed by $P450_{2E1}$ (reproduced with permission from (22)).

It was also suggested in the study that the active site was sterically unhindered for about 10 angstroms above the heme, allowing for several ethanol molecules to be present at any given time. This suggests that the active site may exist in conditions of both low and high polarity, and that polarity may cause a change in the catalytic scheme. Using energetic models, recent studies have proposed a new mechanism for oxidation in the presence of a highly polar active site, a "reverse dual hydrogen abstraction", where abstraction occurs initially at the hydroxyl group, with no oxygen rebound, in order to counteract increasing polarity in an environment that is very hydrophobic in nature (22).

Energy profiles were calculated for conditions of varying polarity, to determine the favored pathway in each case. **Figures I. 5 and I. 6** illustrate the energy profiles from the gem-diol and reverse dual hydrogen abstraction mechanisms, respectively, for both low and high active site polarity conditions.

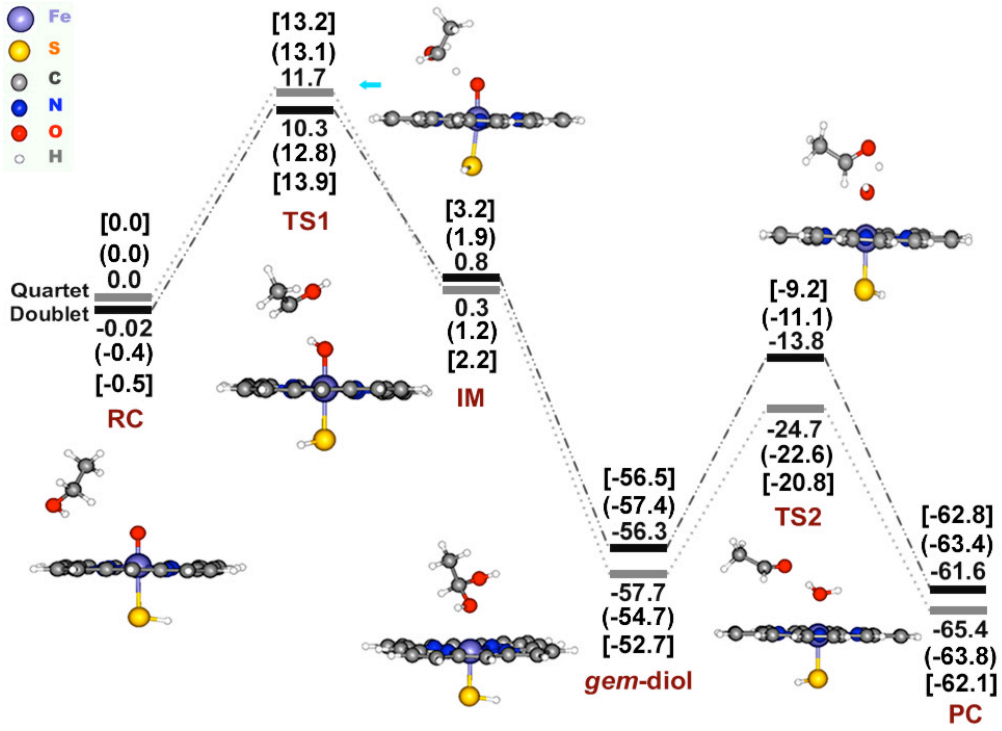

Figure I. 5: Energy profile for ethanol oxidation via gem diol formation. Gas phase (no parentheses), weak polar medium (parentheses), and polar medium (square brackets) energies are shown (reproduced with permission from (22)).

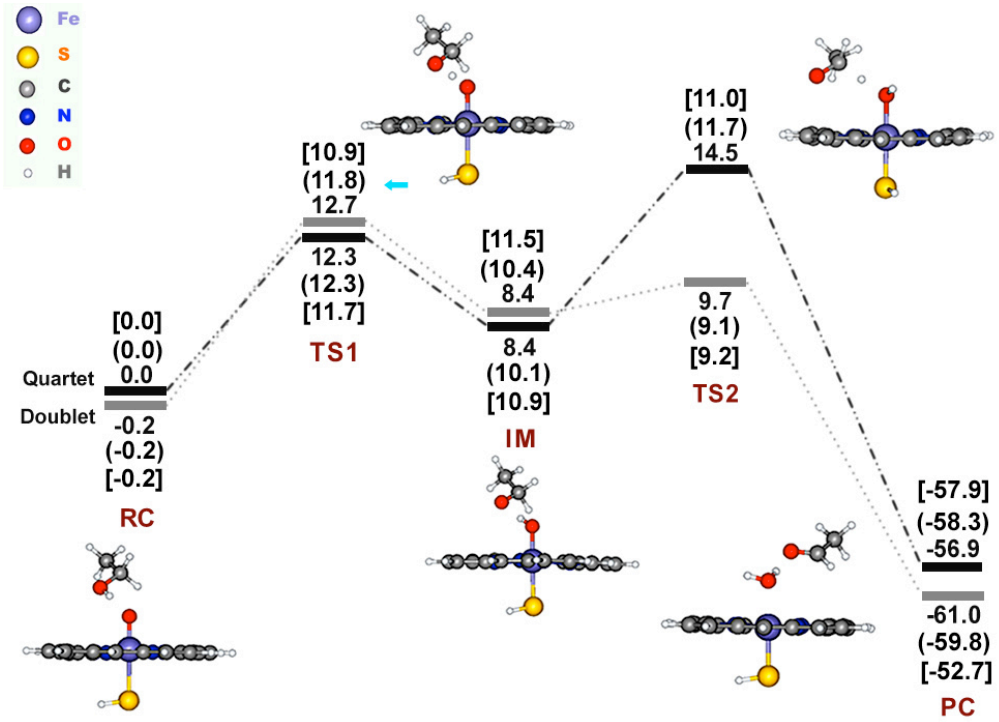

Figure I. 6: Energy profile for ethanol oxidation via reverse dual hydrogen abstraction mechanism. Gas phase (no parentheses), weak polar medium (parentheses), and polar medium (square brackets) energies are shown (reproduced with permission from (22)).

A rate-limiting step of hydrogen abstraction from the alpha carbon is assumed for the gem-diol mechanism, and **Figure I. 5** shows that the energy barrier for this step increases when corrections are done for both low and high polarity environments. By contrast, hydrogen abstraction from the hydroxyl group is assumed to be the rate-limiting step for the reverse dual hydrogen abstraction pathway, and as **Figure I. 6** illustrates, a decrease in the energy barrier for this step is seen for each environmental case. In summary, initial gas phase calculations result in a 2.0 kcal/mol lower energy barrier for the gem-diol mechanism, however, corrections for each polar environment raise this barrier to 1.3 kcal/mol higher than the proposed reverse dual hydrogen abstraction

mechanism. These observations have lead to the conclusion that the switch in metabolic pathway can explain the adaptive response of $P450_{2E1}$ to high blood ethanol concentration, which results in an acceleration of metabolism.

I. C. ii. Metabolic Adaption to Increasing Active Site Polarity

Studies previously done by Raner et al. (7) evaluated possible intermediates formed during $P450_{2E1}$ oxidation of aldehyde substrates. The traditional pathway for P450 proceeds through an intermediate known as Compound I, an iron-oxo intermediate that initiates nucleophilic attack on the substrate. However, through an examination of the inactivation of P450 by aldehydes, an alternate pathway through a peroxo-iron intermediate has been proposed. The inactivation of the enzyme occurs when a radical species, produced as the peroxo- intermediate decomposes, reacts with the heme to form an adduct (**Figure I. 7)**.

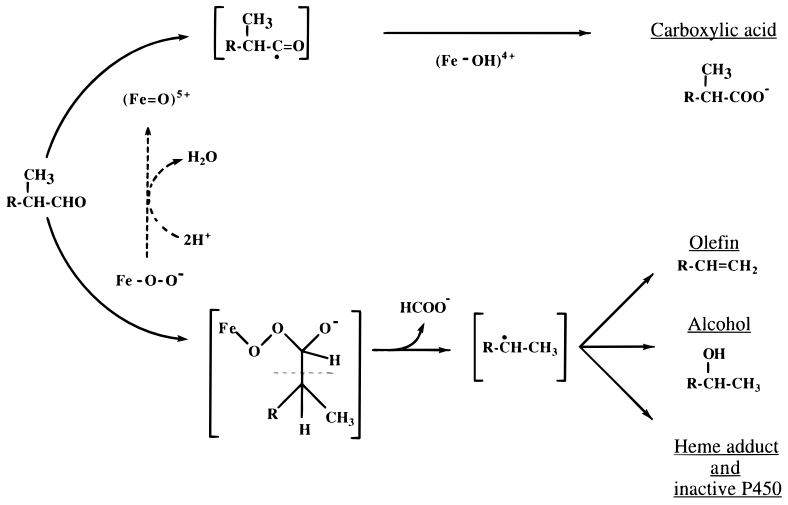

Figure I. 7: Proposed pathways for P450-mediated conversion of aldehyde substrates to carboxylic acid, olefin and alcohol products. (reproduced with permission from (7))

Catalysis resulted in the formation of two distinct products from the oxidation of the aldehyde substrate. The first product, formed through the traditional pathway involving Compound I, was the corresponding carboxylic acid. The second product was an n-1 alcohol that could be quantified by HPLC. This product is thought to form via a peroxo pathway within the P450 catalytic cycle. The results from this study further support the notion that P450 enzymes may have multiple pathways for the metabolism of a given substrate. Regarding the current study, it is uncertain whether a change in active site polarity would influence the partitioning between these pathways; however, it is reasonable to expect that it would.

In order to consider possible causes for adaptations in catalysis, certain details regarding the active site structure should be noted. **Figure I. 8** gives an overhead view of the substrate access channel, the catalytic site, and the effector site of $P450_{2E1}$.

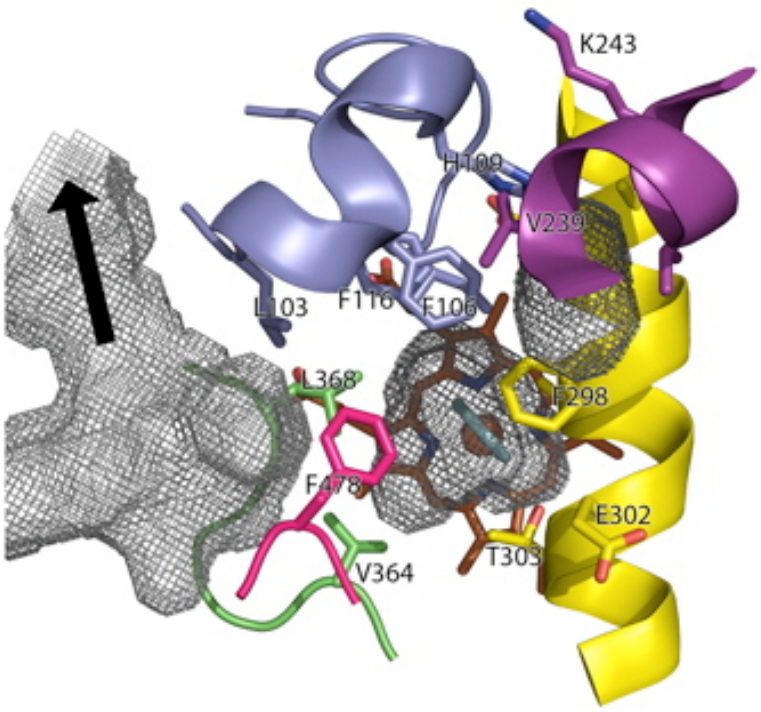

Figure I. 8: Overhead depiction of the $P450_{2E1}$ active site. Substrate access channel is indicated by the directional arrow. (Reproduced with permission from (17))

Two phenylalanine residues separate the catalytic and effector sites, which re-emphasizes the hydrophobic nature of the environment. It may be reasonable to conclude, therefore, that the presence of ethanol in high concentrations initiates the rotation of those two residues (in order to minimize unfavorable interactions) and subsequently "opens the gate" to the distal effector site. This change in conformation would theoretically allow for

greater substrate flexibility, which may lead to changes in bond cleavage and the formation of atypical products. Therefore, with the assumption that 2E1 is capable of adapting its metabolic profile under conditions of varying polarity, the objective of these studies was to characterize any interactions between ethanol and several different substrates. Because the active site is very hydrophobic in nature, an increased concentration of ethanol may lead to alterations in the metabolism of other substrates of this isoform. The implications of this research may also be applied to other Cytochrome P450 isoforms, as most P450 active sites are hydrophobic and, therefore, may also be capable of adapting their catalytic schemes under the stress of very polar conditions. With this in mind, our ultimate goal is not only to characterize this unique metabolic phenomenon in the 2E1 isoform, but also to expand and apply these results to the broader scheme of the Cytochrome P450 enzymatic profile.

CHAPTER II

EXPERIMENTAL METHODS

II. A. *Para*-nitrophenol Substrate Inhibition Assays

Rabbit liver microsomes served as a source of $P450_{2E1}$, and stock solutions of 1 mM PNP and 10% ethanol (v/v) were used for each analysis. Typical reaction mixtures contained 5 μL of microsomes, 50-700 μM PNP, 34 or 51 mM ethanol, and NADPH-regenerating system (2 microunits/μL glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate, 1.0 M potassium phosphate buffer {pH 7.4} containing 33 mM MgCl2, and 500 μM NADP+) in a final reaction volume of 500 μL. Reaction mixtures were initiated upon addition of NADPH regenerating system, and incubated at 37^{o}C for 30 minutes. Reactions were quenched with 200 μL of 6% perchloric acid, placed on ice for 10 minutes, and centrifuged for 10 minutes at 3500 rpm. The supernatant was extracted for analysis by HPLC. The reaction catalyzed by 2E1 is shown in **Figure II. 1**, where quantification of the p-nitrocatechol product by peak integration was used to determine reaction velocity. A standard curve was generated using stock solutions of pure p-nitrocatechol (5-25 μM), where peak integration was plotted as a function of concentration (**Figure II. 2)**. The resulting linear regression was used to determine the concentration of product formed in each reaction. Reaction velocity (in μM PNC formed

per minute) was subsequently calculated by dividing the total amount of product formed by the assay duration time of 30 minutes.

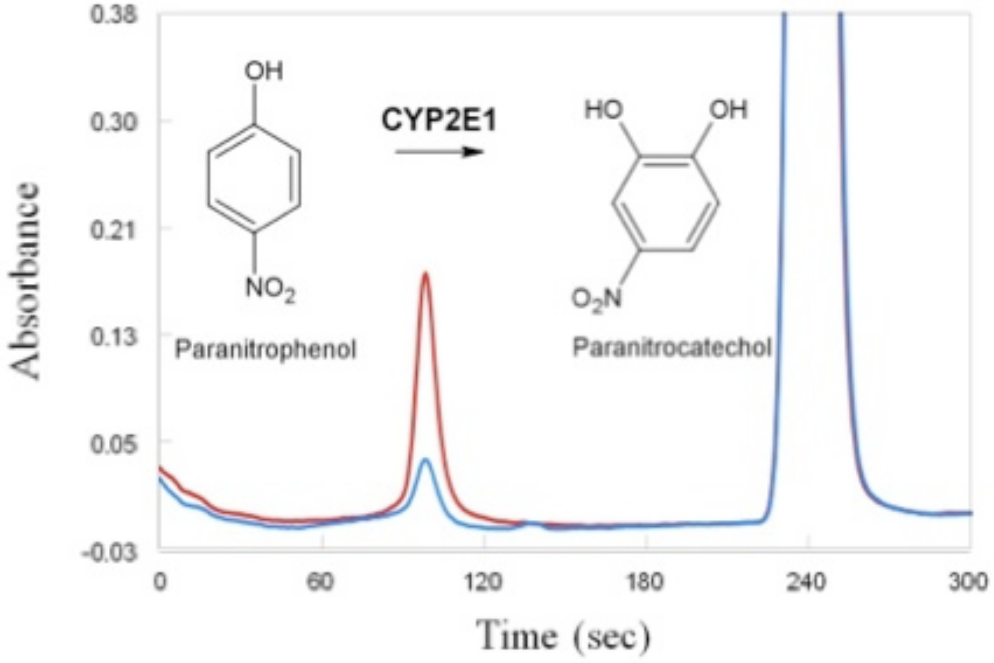

Figure II. 1: Representative HPLC chromatogram of $P450_{2E1}$-mediated conversion of p-nitrophenol (R_t 240 seconds) to p-nitrocatechol (R_t 100 seconds).

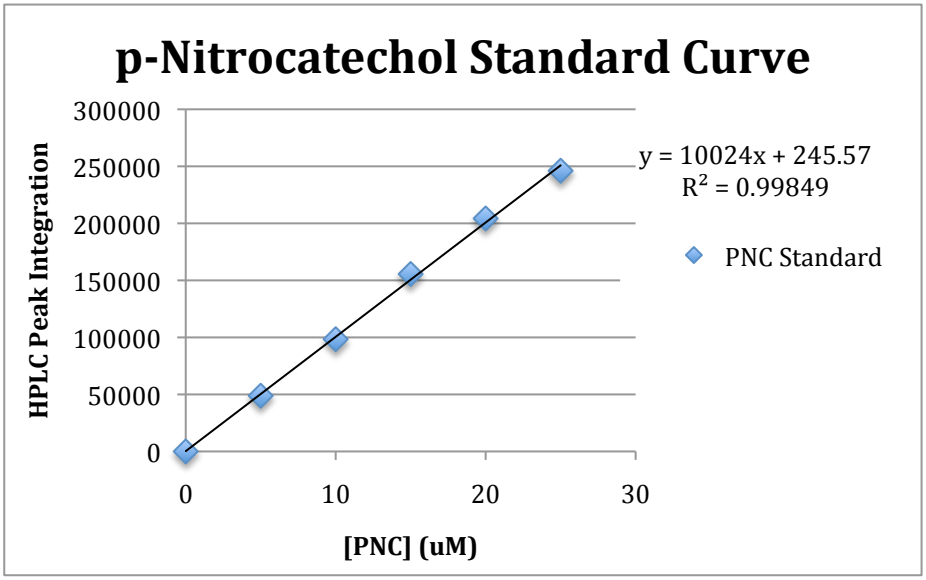

Figure II. 2: Standard curve of HPLC peak integration versus p-nitrocatechol (PNC) concentration for conversion of peak area to reaction velocity in studies where p-nitrophenol was the probe substrate. Reaction velocities were calculated by dividing the total amount of PNC by 30 minutes (total reaction run time), to give units of μM PNC formed per minute. Standard solution concentrations ranged from 5-25 μM PNC. HPLC conditions were identical to those used in PNP assays.

II. A. i. HPLC Analysis and Determination of Michaelis-Menten Constants.

Product formation was quantified by HPLC, (Shimadzu LC-20AT/ Prominence Liquid Chromatography system) using a SPC-20A/ Prominence UV/Vis Detector at a wavelength of 340 nm. Samples were injected in 40 μL aliquots onto an Agilent C8 reverse phase column, with a mobile phase consisting of 25% acetonitrile and 74.5% water (acetic acid present at 0.5% in both solutions). The flow rate was set to 0.600 mL/minute, with a total run time of 5 minutes per sample. Reaction velocity was plotted as a function of substrate concentration, and kinetic constants were determined using

nonlinear regression analysis (GraphPad Prism, GraphPad Software, Inc.). The Michaelis Menten equation was used to determine the K_m and V_{max} values for the control reaction (**Equation. II. 1**).

$$v_0 = \frac{V_{max}[S]}{K_m + [S]}$$

Equation II. 1.

For reactions containing ethanol as an inhibitor (at increasing concentrations), Km_{app} and K_i were all determined using the following equation:

$$Km_{app} = K_m * (1 + [I]/K_i)$$

Equation II. 2.

The concentration of the ethanol inhibitor is represented by [I], and Km_{app} is the observed K_m value upon the addition of inhibitor to the reaction mixture.

II. B. Deuterium Isotope Studies.

Reaction conditions were identical to the PNP studies except for the following: PNP was replaced with 1.5 μmol [1-$^{2}H_{1}$]-benzyl alcohol (purified by HPLC), and 0, 17, 34, or 51 mM ethanol. After incubation, equal volumes of CH_2Cl_2 were added to quench each reaction, and the CH_2Cl_2 extract was analyzed by GC-MS. GC-MS temperature program conditions were 70^{o}C for 2 min, followed by 8^{o}C/min increase to a final temperature of 250^{o}C, which was held for 5 min. Under these conditions, the benzaldehyde product eluted at 6.3 minutes. Peak ratios at 106 m/z and 107 m/z, corresponding to benzaldehyde and [^{2}H]-benzaldehyde, respectively, were used to determine the intrinsic isotope effect.

II. B. i. Synthesis and Purification of 1-[$^{2}H_{1}$]-Benzyl Alcohol

Synthesis protocol was adapted from the procedure used by Vaz et al. (21). A 25 mL reaction mixture of 5.0 M benzaldehyde in 20% ethanol and 250 mmol of sodium borodeuteride (98% isotopic purity, Aldrich) was stirred overnight at room temperature. The solution was subsequently diluted with 25 mL of water, and extracted twice with 10 mL portions of CH_2Cl_2. The desired product was isolated by fractionation with HPLC (**Figures II. 3 and II. 4**).

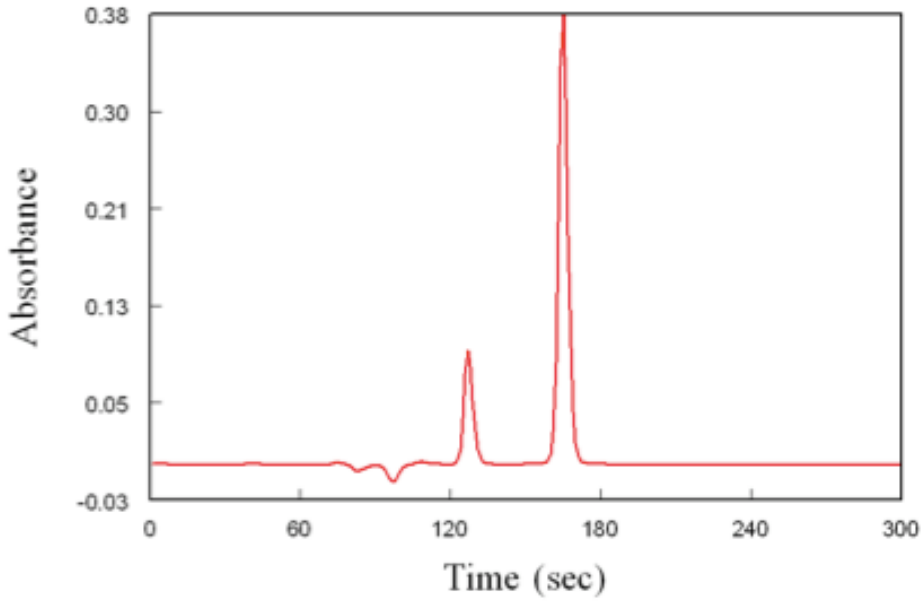

Figure II. 3: HPLC chromatogram of benzaldehyde (R_t 2.7 min) and 1-[$^2H_1$]-benzyl alcohol product (R_t 2.1 min), from synthetic reaction mixture. Separation was achieved by injecting 100 µL of sample onto an Agilent C8 column (4.6 x 150 mm), with a mobile phase of consisting of 60% acetonitrile, 39.5% H_2O, 0.5% acetic acid. Flow rate was 1 mL/min, and the total run time was 5 minutes. Absorbance detection was set to 254 nm.

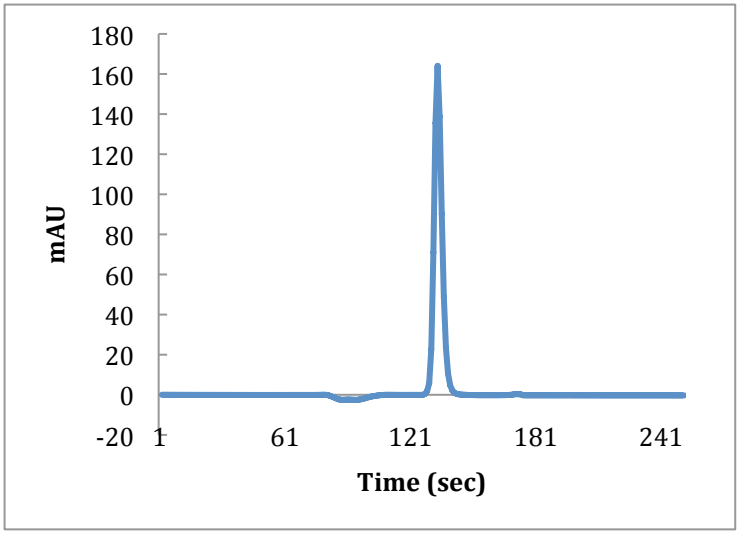

Figure II. 4: HPLC chromatogram of fraction containing 1-[$^{2}H_{1}$]-benzyl alcohol product purified from initial synthetic reaction mixture. HPLC conditions were identical to those used for the reaction mixture.

II. B. ii. GC-MS Analysis of Purified 1-[$^{2}H_{1}$]-Benzyl Alcohol

After HPLC purification from the synthetic reaction mixture, incorporation of deuterium into the benzyl alcohol product was confirmed by GC-MS (m/z peak 109), as shown in **Figure II. 5**. The purity of the product was assessed by comparing the peak intensity of the deuterated form of the alcohol to the undeuterated form (m/z peak 108).

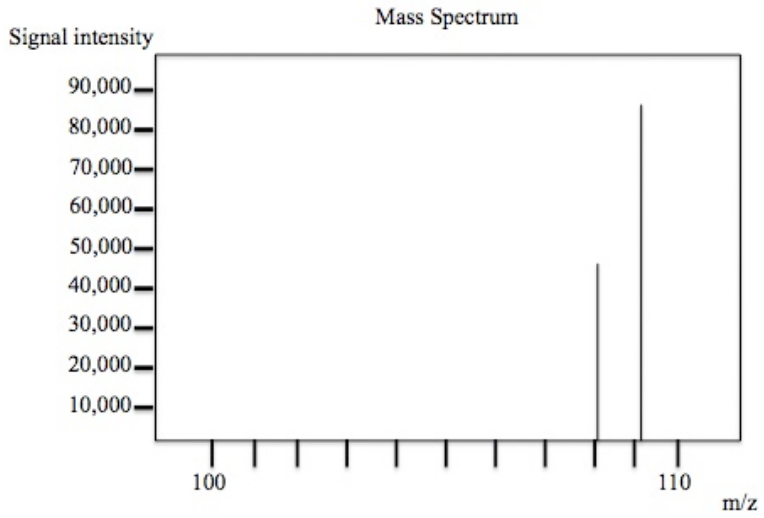

Figure II. 5: Mass spectrum of purified 1-[$^{2}H_{1}$]-benzyl alcohol. GC-MS temperature program conditions were 70^{o}C for 2 min, followed by 8^{o}C/min increase to a final temperature of 250^{o}C, which was held for 5 min. A volume of 1 µL was injected via split less injection, and the injection and ion source temperatures were set to 250^{o}C. Under these conditions, benzyl alcohol eluted with a retention time of 7.6 minutes. The presence of 1-[$^{2}H_{1}$]-benzyl alcohol was confirmed by a peak at 109 m/z, corresponding to the mono-deuterated form of benzyl alcohol (108 m/z).

The peak intensity of the deuterated product was approximately double that of the undeuterated form. However, the calculation of a true intrinsic isotope effect is dependent upon the use of a highly pure solution of deuterated compound, to ensure that any change in label incorporation within the product is truly the result of a change in bond cleavage. Therefore, interpretation of the preference for bond cleavage, as determined by incorporation of the deuterium label into the aldehyde product, would be dramatically altered by the presence of the undeuterated alcohol in the reaction mixture. In light of this, calculated intrinsic isotope effect values from our studies were solely used to analyze a general shift in label incorporation, rather than true intrinsic isotope effects.

II. C. 4-Nitrobenzaldehyde Reversibility Studies

The reversibility assay was used to evaluate whether ethanol could disrupt the 4-nitrobenzaldehyde-mediated inactivation of $P450_{2E1}$. By incubating the enzyme mixtures under different experimental conditions prior to the addition of the probe substrate, it was possible to draw conclusions about the mode of interaction between ethanol and 4-nitrobenzaldehyde.

II. C. i. Reversibility Assay Protocol

Four separate enzyme samples were prepared side-by-side, each with a total volume of 100 μL. A schematic of the experimental setup for each sample is shown in **Figure II. 6.** Following a pre-incubation for 15 min at 37 ^{o}C, the activity of $P450_{2E1}$ was assayed by diluting each sample 1:20 in final reaction mixtures containing 1.0 mM NADPH, 50 μM p-nitrophenol and 100 mM phosphate buffer (pH 7.4), and subsequently assayed as described previously in PNP substrate inhibition studies. Each experiment was carried out in duplicate.

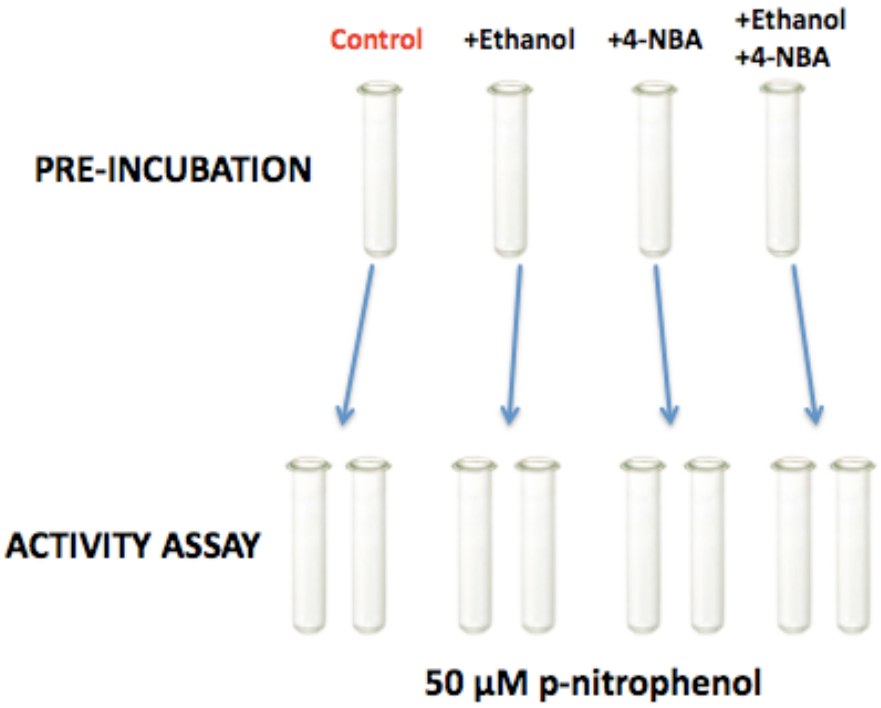

Figure II. 6: Experimental setup for 4-nitrobenzaldehyde reversibility assays. Each pre-incubation mixture contained 50 µL of a 100 mM phosphate buffer (pH 7.4) solution, 20 µL of rabbit liver microsomes, 5 µL of a 10 mM NADPH solution, and the specified substrates. Fifteen microliters of a saturated solution of 4-nitrobenzahyde was added to samples labeled +NBA. Reaction samples labeled +Ethanol contained 17, 34, or 51 mM ethanol. Following pre-incubation, 25 µL was added to each of the activity assay mixtures (carried out in duplicate). Each activity assay mixture contained 100 mM phosphate buffer (pH 7.4), 1.0 mM NADPH, and 50 µM p-nitrophenol (probe substrate).

CHAPTER III

RESULTS AND DISCUSSION

III. A. PNP Substrate Inhibition Studies

In order to assess the nature of a recently proposed "effector" site that exists distal to the heme active site, the present study was employed to investigate the role of the site in the substrate inhibition pattern observed in PNP oxidation, and the influence of ethanol on this phenomenon. **Figure III. 1** is an illustration of the potential ligand-ligand interactions between the two sites.

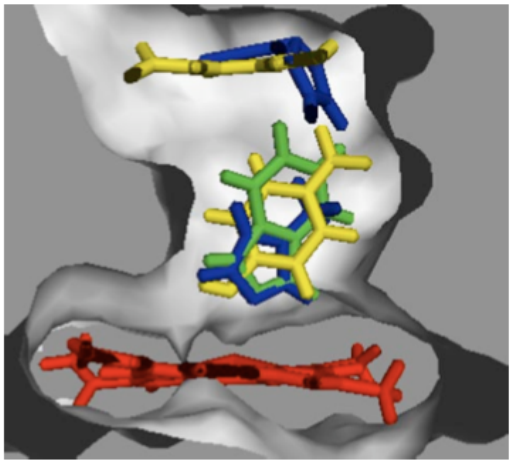

Figure III. 1: Surface depiction of catalytic and effector binding sites for $P450_{2E1}$ (reproduced with permission from (4)).

Interactions between molecules occupying the catalytic and effector sites may have the potential to alter catalysis, either through the generation of unexpected products, or by altering the rate-limiting step in reactions. In light of the proposed rate-limiting step of product release in ethanol oxidation (19, 20), it may be reasonable to conclude that interactions between p-nitrophenol molecules occupying both sites slows the process of product formation and release, which results in the observed decrease in activity at high concentrations of substrate.

Because the substrate inhibition phenomenon is observed at higher PNP concentrations, it was postulated that this was the result of PNP molecules binding to an effector site and inhibiting catalysis. In the current study, ethanol was added to the reaction mixture at increasing concentrations to observe any influence on PNP oxidation kinetics. Our hypothesis is that the capacity of ethanol to influence PNP substrate inhibition, by disrupting interactions between sites, should result in a loss of substrate inhibition. If a shift is observed, it may be postulated that ethanol competes with subsequent PNP molecules for binding to the active site, resulting in the change in the observed substrate inhibition pattern. Thus, in vitro evidence concerning the site's apparent role in catalysis was ascertained through analysis of kinetic constants.

III. A. i. Michaelis-Menten Analysis

As illustrated in **Figure III. 2**, a decrease in activity for the control (containing only PNP) was seen at concentrations of 150 μM and above, which agrees with recent studies by Collom et al. (4) where, in the absence of inhibitor, a decrease in activity above 100 μM PNP was also observed (**Figure I. 2)**, and such observations were

reproduced in the current study. **Figure III. 3** shows the results from steady state oxidation assays in control reactions. Also included are the reaction velocities beyond a substrate concentration of 150 μM (truncated from experimental data, as calculated via nonlinear regression, GraphPad Prism) to provide a fit to the traditional Michaelis-Menten kinetic model, where saturation of the enzyme at or around the maximum velocity of the reaction is demonstrated by a plateau in the curve. This allowed for comparison between expected Michaelis-Menten behavior and the apparent substrate inhibition phenomenon demonstrated previously in the literature (4), as well as in the present study.

Based on the Michaelis-Menten model calculations, the velocity for the control reaction should have reached a maximum of approximately 0.179 μM/minute. However, at a substrate concentration of 150 μM, the velocity only reached 0.149 μM/minute before a decrease was observed. Thus, only 83% of the V_{max} predicted by the traditional Michaelis Menten kinetic model was actually reached. The difference between constants calculated from traditional single-site kinetic models and those determined experimentally indicated that a more complicated kinetic model may be necessary to accurately analyze the 2E1 catalytic scheme. A similar conclusion was reached in the prior studies done by Collom et al. (4), where only 64% of the predicted maximum velocity was observed. Such conclusions have prompted further characterization of the substrate inhibition phenomenon using inhibitors, to characterize the unique kinetic profile of the 2E1 isoform. More specifically, the role of the proposed "effector site" in the observed decline in activity at higher substrate concentrations was assessed.

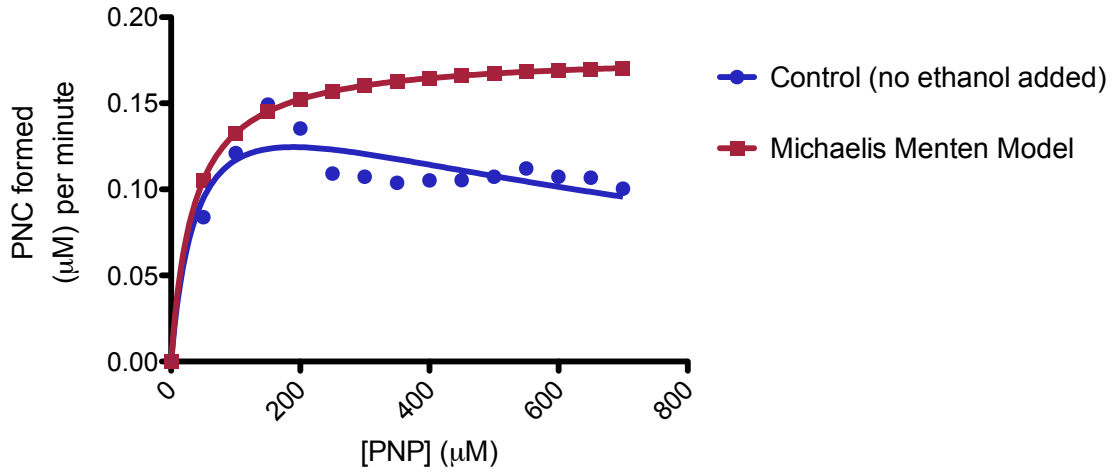

Figure III. 2: Plot of reaction velocity versus substrate concentration, for the oxidation of p-nitrophenol to p-nitrocatechol by $P450_{2E1}$. Michaelis Menten Model data (red) is truncated from experimental data (blue) at 150 μM PNP, and the remaining velocities calculated using GraphPad Prism (nonlinear regression, Michaelis-Menten equation) to provide a basis for comparison between trends in experimental velocities and typical Michaelis-Menten kinetic behavior. Reaction velocities for the control data were calculated from p-nitrocatechol peak integration, detected at 340 nm, using the standard curve shown in **Figure II. 2**.

III. A. ii. Effect of Ethanol on PNP Substrate Inhibition

To probe further details regarding the role of the proposed effector site in the 2E1 catalytic profile, as well as the effect of ethanol on the kinetic behavior of the isoform, varying concentrations of ethanol were added to the reaction mixture. The results of assays containing 34 mM and 51 mM ethanol are shown in **Figure III. 3**.

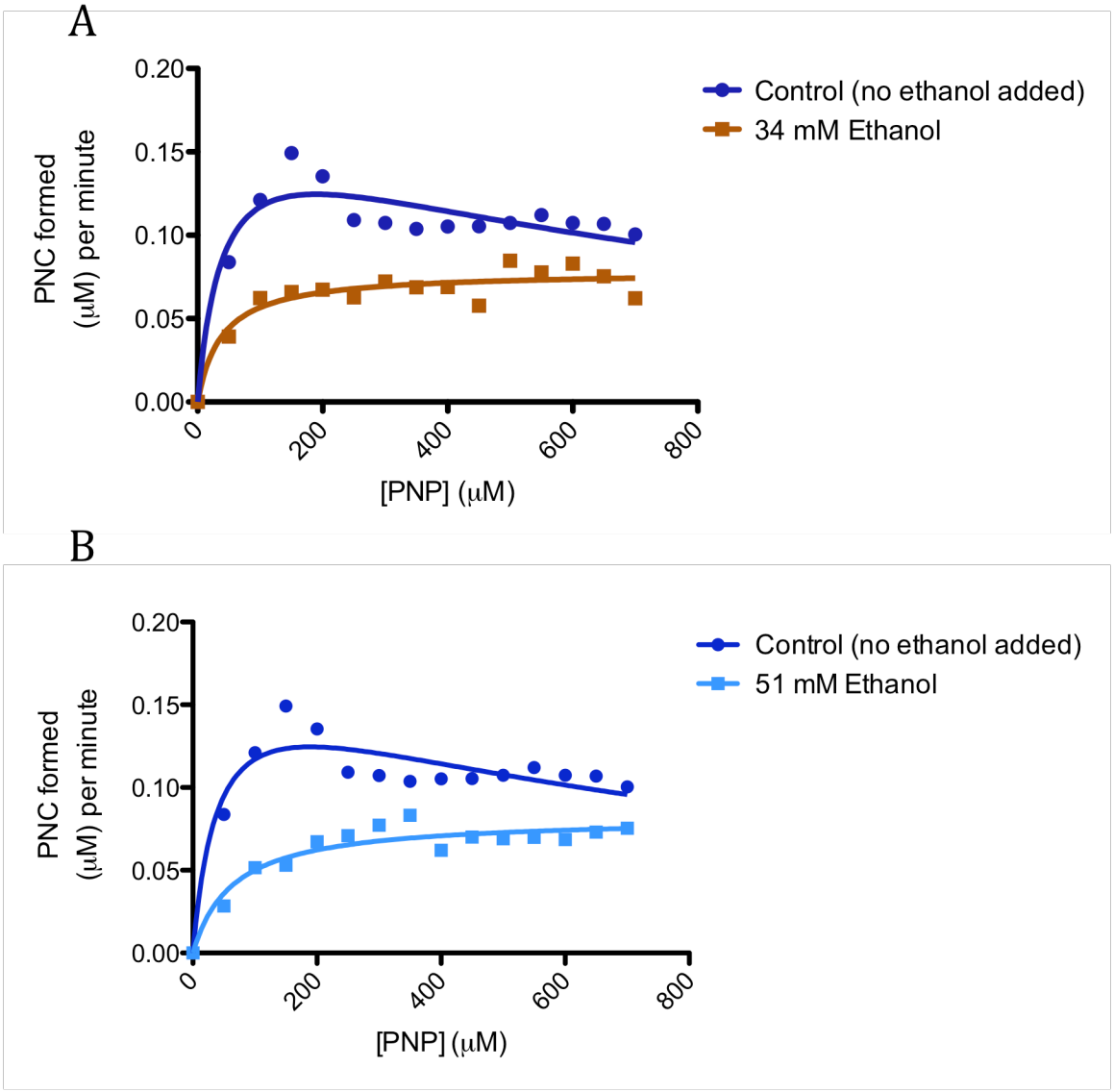

Figure III. 3: Plot of reaction velocity versus substrate concentration, for the oxidation of p-nitrophenol to p-nitrocatechol by $P450_{2E1}$, for reactions containing no inhibitor, 34 mM ethanol (A) or 51 mM ethanol (B). Reaction velocity was calculated from p-nitrocatechol peak integrations, detected at 340 nm, using the standard curve in **Figure II. 2**.

Upon the addition of ethanol, the oxidation of p-nitrophenol appeared to demonstrate more Michaelis-Menten like behavior, showing saturation rather than a substrate inhibition pattern. Interestingly, the maximum velocity for reactions containing ethanol did not approach the V_{max} of 0.179 μM/minute predicted by the Michaelis-Menten model for the control; rather, they appeared to approach the plateau seen after the observed decrease in activity, at a reaction velocity of approximately 0.080 μM/minute. We concluded that this plateau represented the saturation of the effector site by PNP in the control reaction, based on the assumption that product release was rate-limiting in the overall reaction. This conclusion was further supported by the observed kinetic trends after ethanol was added to the reaction mixture, where the maximum velocity appeared to converge at approximately the same value. In order to provide a more substantial basis for this conclusion, it was necessary to assess the mode of action by which ethanol interfered with catalysis.

To determine the mode of inhibition, the apparent V_{max} and K_m values calculated from reactions containing ethanol were compared to those calculated for the Michaelis-Menten model curve (**Table III. 1)**. Comparison with the model data allowed for more straightforward analysis of the influence of ethanol in determining a potential mechanism for inhibition, given that a definitive two-site substrate inhibition model to fit this particular case has not yet been established **(**23).

Table III. 1: Summary of kinetic constants from PNP Substrate Inhibition studies.

	Km (μM)	Vmax (μM/min)	Km_{app}	$Vmax_{app}$
Michaelis Menten Model	34	0.179	N/A	N/A
Experimental Control	29	0.149	N/A	N/A
34 mM Ethanol	N/A	N/A	40	0.079
51 mM Ethanol	N/A	N/A	64	0.082

Comparison of the control and apparent maximum velocities, as well as the enzyme's affinity for PNP (represented by K_m values, which indicate the substrate concentration needed to reach half the maximal velocity) formed the basis for assessing mode of inhibition demonstrated by ethanol **(**24). Upon adding 34 mM and 51 mM ethanol, the apparent K_m values were 40 μM and 64 μM, respectively, and the increase in value was an indication that ethanol was competing with PNP for binding at the active site. In general, an increase in K_m when a potential inhibitor is added to the reaction mixture suggests that a higher concentration of the substrate is necessary to reach the maximum velocity of the reaction (24). A change in the V_{max} value was also observed when ethanol was present, where reactions containing ethanol appeared to converge at a maximum of approximately 0.080 μM PNC formed per minute. The difference between the observed value from the Michaelis-Menten model and control reactions, and those containing ethanol suggests that ethanol also interferes with a particular step in catalysis. In light of the fact that the addition of ethanol resulted in a change in both K_m and V_{max} values, we concluded that the mode of inhibition was mixed in nature, where ethanol potentially demonstrated the capacity to interfere with substrate binding and catalytic

conversion to product. More specifically, we concluded that ethanol inhibits the binding of PNP to the active site in a competitive nature (based on the increase in K_m), but also has an effect on the rate-limiting step of product release (as indicated by the difference in maximum velocity). The existence of the effector site, and its potential role in this scheme was ultimately supported by the mixed mode of inhibition by ethanol in these studies and the fact that the maximum velocity of the reactions containing inhibitor converged at the same value observed in control reactions when saturation of the second site occurred.

III. B. Isotope Effect Studies with 1-[$^2H_1$]-Benzyl Alcohol

Similar to the mechanism proposed by Wang et al. (21) for ethanol oxidation, the oxidation benzyl alcohol by $P450_{2E1}$ is traditionally assumed to proceed through a dual hydrogen abstraction pathway, where abstraction from the alpha carbon was found to be partially rate-limiting. In the reverse dual-hydrogen abstraction pathway, hydrogen abstraction from the hydroxyl group would be rate-limiting, and the change the in rate-limiting step may occur as a result of increasing polarity in the 2E1 active site (22). In light of this, we attempted to evaluate the effect of ethanol, and the subsequent change in active site polarity on the partitioning between pathways and alterations in the rate-limiting step in catalysis.

In the current study, experiments probing the effect of ethanol on the intrinsic isotope effect of benzyl alcohol appeared to provide support regarding the influence of ethanol on a wide range of 2E1 metabolic schemes. Deuteration at a single position on carbon one of benzyl alcohol would traditionally allow for evaluation of a particular

bond-breaking step critical to catalysis. The method is based on the idea that differences in the vibrational energy of a C-H versus a C-D bond vary due to a significant mass difference between the hydrogen and deuterium atoms (24, 25). The vibrational energy of the C-D is much lower due to the increase in mass of deuterium, which results in greater difficulty breaking that particular bond. Such a scenario manifests itself by decreasing the observed reaction rate with respect to the rate seen when a hydrogen is abstracted (24-26). By comparing the two experimental reaction rates, the importance of the bond in question in the context of the overall reaction can be evaluated (27).

The possible mechanistic routes are shown in **Figure III. 4**, where the conventional intrinsic isotope effect is calculated based on the ratio of peaks at the m/z value for both possible products. However, the purity of the benzyl alcohol substrate limited our ability to determine absolute isotope effects. As a result, signal intensities for the deuterated and undeuterated product formed in each experiment was used to observe a general shift in the preference for bond cleavage, not for the determination of true isotope effects. The results for control reactions, and reactions containing 17, 34, or 51 mM ethanol are shown in **Figures III. 5 -III. 8.**

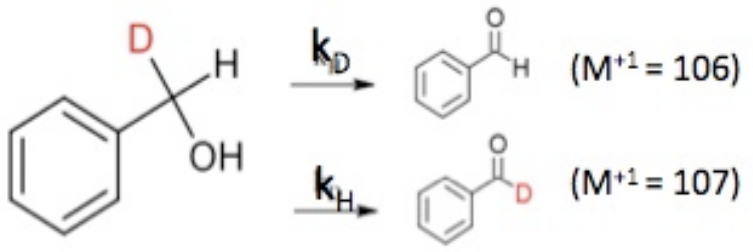

Figure III. 4: $P450_{2E1}$ oxidation of 1-[$^2H_1$]-benzyl alcohol.

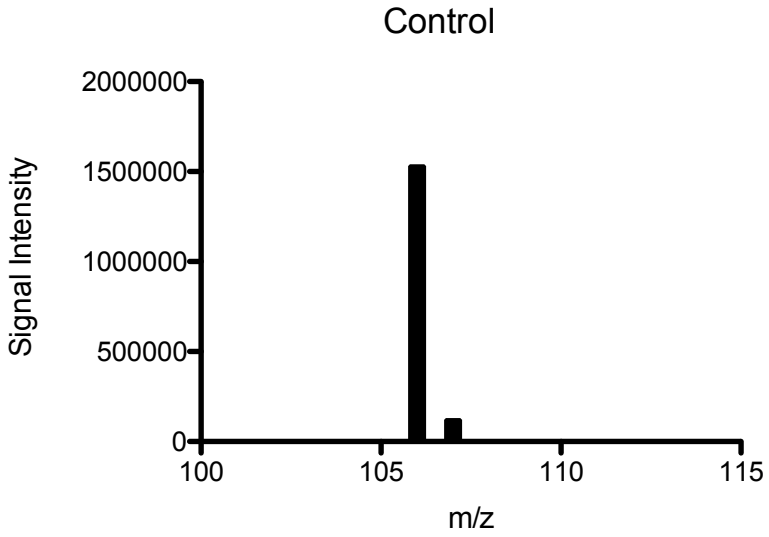

Figure III. 5: GC-MS fragmentation pattern for benzaldehyde formed from 1-[$^2H_1$]-benzyl alcohol in the control reaction (containing no ethanol). GC-MS conditions were identical to those used in the purification of 1-[$^2H_1$]-benzyl alcohol.

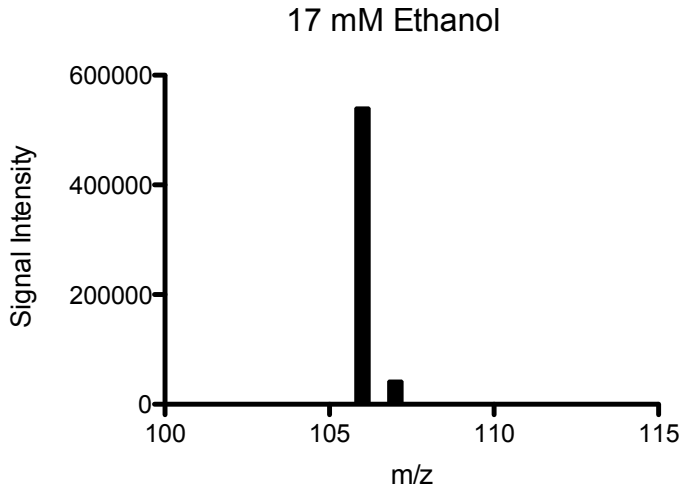

Figure III. 6: GC-MS fragmentation pattern for benzaldehyde formed from 1-[$^2H_1$]-benzyl alcohol in the reaction containing 17 mM ethanol. GC-MS conditions were identical to those used in the purification of 1-[$^2H_1$]-benzyl alcohol.

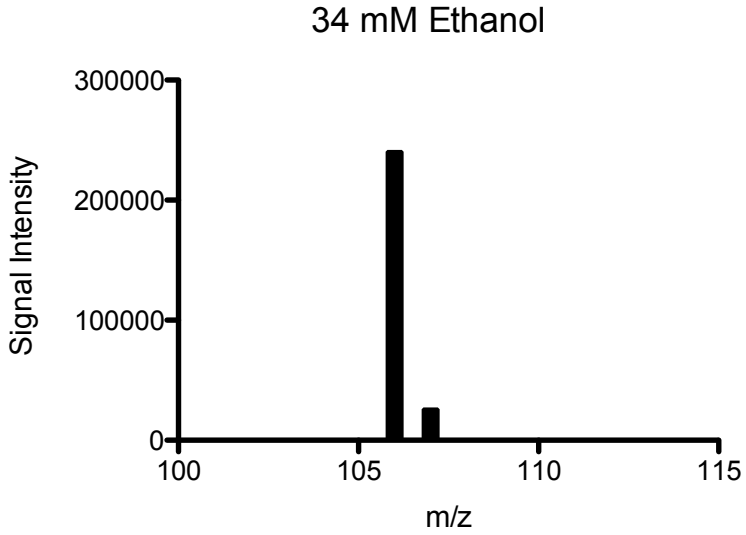

Figure III. 7: GC-MS fragmentation pattern for benzaldehyde formed from 1-[$^2H_1$]-benzyl alcohol in the reaction containing 34 mM ethanol. GC-MS conditions were identical to those used in the purification of 1-[$^2H_1$]-benzyl alcohol.

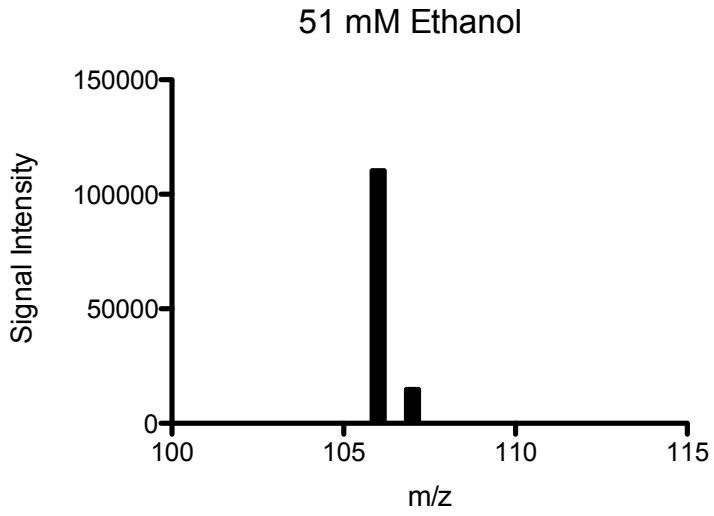

Figure III. 8: GC-MS fragmentation pattern for benzaldehyde formed from 1-[$^{2}H_{1}$]-benzyl alcohol in the reaction containing 51 mM ethanol. GC-MS conditions were identical to those used in the purification of 1-[$^{2}H_{1}$]-benzyl alcohol.

III. B. i. Determination of Intrinsic Isotope Effect

Assuming that pure deuterated benzyl alcohol was used, the intrinsic isotope effect could be quantified by the ratio of product m/z peak intensities, which serves to represent the rate of deuterated versus non-deuterated product formation, and was calculated according to **Equation III.1**:

$$^{K}D = \frac{k_H}{k_D}$$

Equation. III.1.

Due to the lack of pure substrate, theoretical isotope effect values were calculated to observe any overall shift in bond cleavage upon the addition of ethanol. The dominating peak in each case (106 m/z) represented the un-deuterated product, with a small percentage of products retaining the deuterium label. **Table III. 2** lists the GC-MS signal

intensities and theoretical ^{k}D values for each experimental condition, as determined through analysis of the GC-MS chromatograms in **Figures III. 5 – III. 8**.

Table III. 2: Signal intensities for conversion of 1-[$^{2}H_{1}$]-benzyl alcohol to benzaldehyde or ^{2}H-benzaldehyde, at different concentrations of ethanol.

	Control	17 mM Ethanol	34 mM Ethanol	51 mM Ethanol
Signal Intensity (107 m/z)	120,677	42,490	25,880	15,207
Signal Intensity (106 m/z)	1,531,000	540,123	240,400	110,670
K_H/K_D	**0.0788**	**0.0786**	**0.108**	**0.137**

The magnitude of the intrinsic isotope effect values appeared to suggest that cleavage of the deuterium label was more favorable than hydrogen abstraction, despite the lower vibrational energy associated with the increase in mass. It was concluded that the use of impure substrate was responsible for the observed isotope effect values. Even so, the apparent increase in isotope effect magnitude for reactions containing higher concentrations of ethanol indicated that there may still be some influence exerted on the preferred metabolic pathway. The rate of the undeuterated product formation was significantly higher than that of deuterated product formation in the control, as well as reactions containing 17 mM ethanol. The change in label incorporation was observed beyond an ethanol concentration of 17 mM, which is below the reported K_m of 20 mM for this substrate (19, 20). At concentrations above the reported K_m value, the amount of

deuterated product formed began to decrease, indicating that an "unmasking" of the isotope effect may be taking place, where hydrogen abstraction was more efficient than deuterium abstraction in the presence of ethanol.

III. C. 4-Nitrobenzaldehyde Reversibility Studies

In order to further evaluate the potential influence of ethanol on $P450_{2E1}$ mechanism, the ability of ethanol to interfere with the inactivation of rabbit Cytochrome P450s by the aldehyde substrate was probed in these experiments. **Figure III. 9** shows the amount of product formed of these experiments for control reactions, reactions containing 4-nitrobenzaldehyde or ethanol, and those containing both compounds (where the conversion of p-nitrophenol to p-nitrocatechol was used to determine the level of 2E1 activity). The activity of 2E1 was significantly reduced upon the addition of 4-nitrobenzaldehyde to the reaction mixture, and was reduced to a lesser degree when ethanol was added.

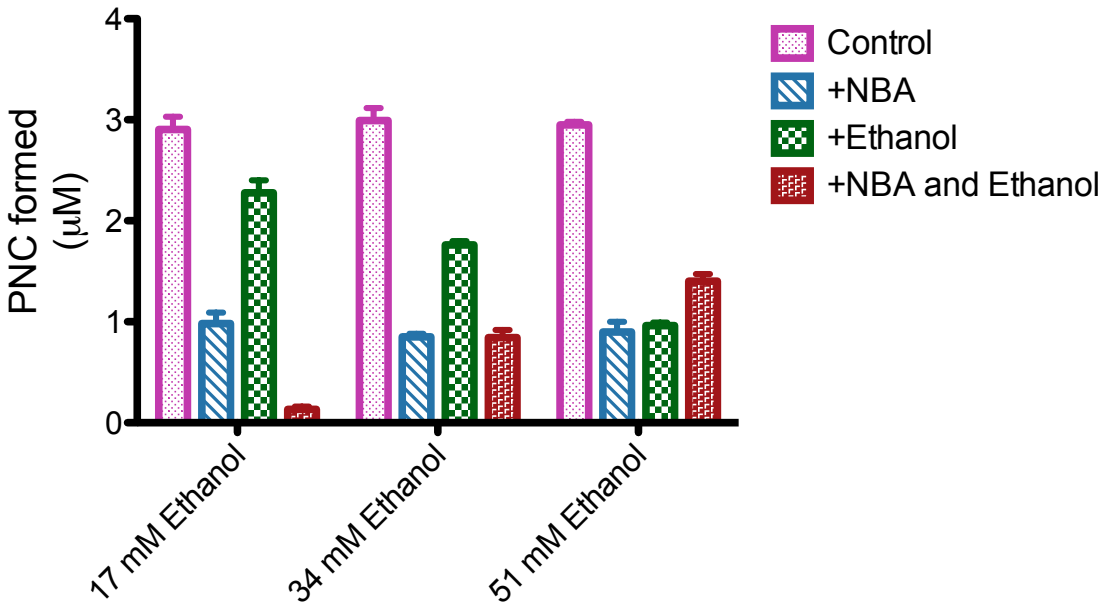

Figure III. 9: Plot of $P450_{2E1}$ activity in control reactions, and in the presence of 4-nitrobenzaldehyde, ethanol, or both. The concentration of ethanol was varied to evaluate the effect on activity, as measured by the conversion of p-nitrophenol to p-nitrocatechol.

This is consistent with the idea that both compounds can act as inhibitors, but 4-nitrobenzaldehyde is much more potent, as it has been documented as one of the most potent irreversible inhibitors of the 2B4 isoform (7). Also shown in **Figure III. 10**, the percent activity of the enzyme increased when incubated with higher concentrations of ethanol and 4-nitrobenzaldehyde, relative to reactions containing only the aldehyde. The degree of increase was dependent on the amount of ethanol present, where the activity was even further restored as the concentration of ethanol increased.

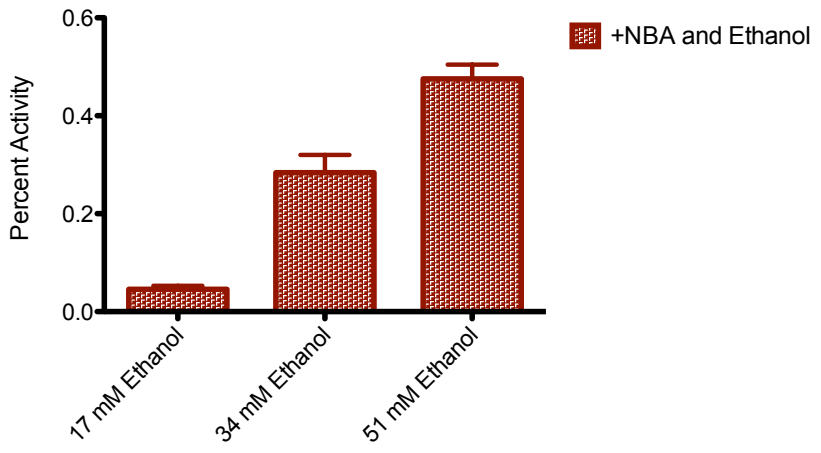

Figure III. 10: Plot of percent activity of $P450_{2E1}$, as measured by the conversion of p-nitrophenol to p-nitrocatechol, in the presence of 4-nitrobenzyaldehyde (saturated) and17 mM, 34 mM or 51 mM ethanol.

At the lowest concentration of ethanol, the inhibitory effects appear to be additive, as the activity when both compounds are present was lower than when either compound was present independently. However, as the concentration of ethanol increased, the activity when both compounds were present was greater than what was observed independently, indicating that ethanol may have been exhibiting a pseudo-protective effect at this stage. Because 4-nitrobenzaldehyde may be an irreversible inhibitor of this isoform, it may be postulated that the restoration of activity in the presence of higher concentrations of ethanol may also be connected to the increased polarity of the active site.

To rationalize the conclusion that active site polarity also played a role in the protective effect of ethanol observed in these studies, the results of these studies were evaluated in the context of the conventional mechanism of P450 inactivation by aldehyde

substrates, as proposed by Raner et al. (7). The mechanism is shown in **Figure III. 11,** where the peroxo intermediate at the active site attacks the aldehyde via nucleophilic attack, creating a free radical that eventually covalently attacks the enzyme and renders it inactive. The synergistic effect that was observed in reactions containing 17 mM ethanol seems to suggest that the formation of the peroxo intermediate and subsequent inactivation is favored under conditions of low active site polarity. The restored activity that was observed in reactions containing 34 and 51 mM ethanol was also rationalized mechanistically. As shown in **Figure I. 7**, proton donation is required for the conversion of the peroxo to the iron-oxo heme intermediate, and this results in the formation of the carboxylic acid by the active form of the enzyme. Based on the assumption that as active site polarity increases, the amount of available proton donors also increases, we proposed that a shift in the intermediate formed during catalysis may be responsible for the restoration in activity. This notion was supported in these studies, where activity of the enzyme increased as the ethanol concentration increased. Because the protective effect was observed at higher concentrations of ethanol, and consequently, increased active site polarity, it was concluded that ethanol may cause a shift in the partitioning of the inactivation pathway, through a change in the particular heme intermediate formed during metabolism.

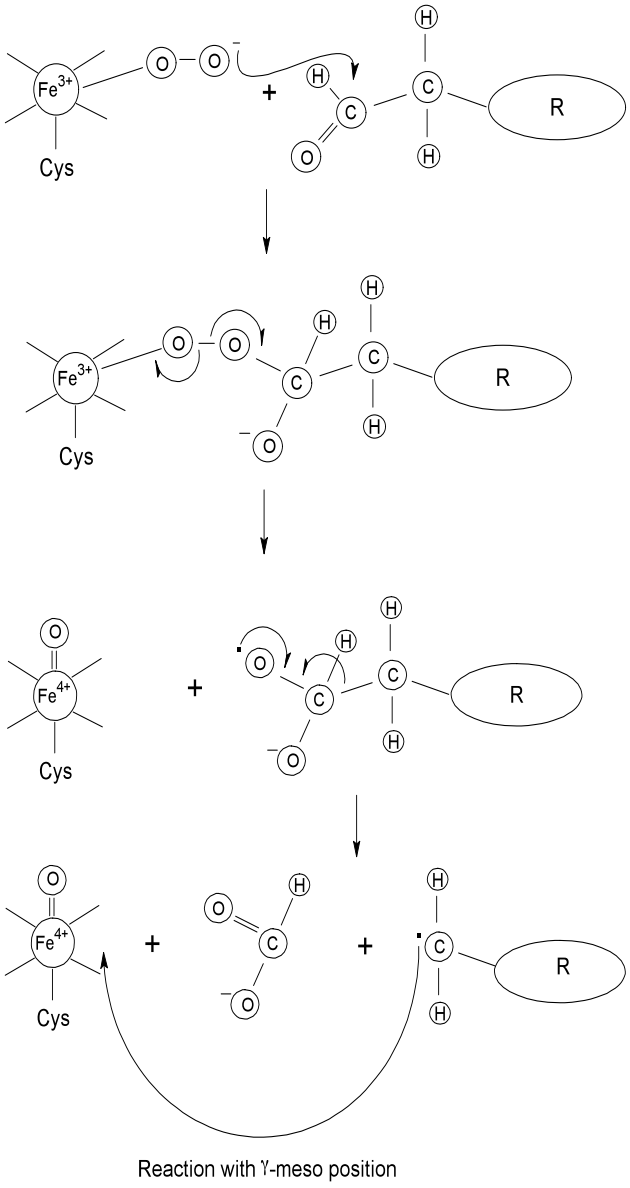

Figure III. 11: Proposed mechanism for inactivation of $P450_{2E1}$ by aldehydes, through adduct formation at the meso- position of the heme group.

CHAPTER IV

CONCLUSIONS

Ethanol has demonstrated the ability to influence a number of Cytochrome $P450_{2E1}$ catalytic schemes. The previously documented event of substrate inhibition that occurs with p-nitrophenol was thought to occur as a result of ligand-ligand interactions between one substrate bound at the active site, and another in a distal cavity described as an "effector site" (4). A decrease in activity was observed at higher concentrations of PNP, but the addition of ethanol restored the kinetic profile to Michaelis-Menten model behavior. Acting through mixed type inhibition, it was assumed that ethanol disrupted the interaction of PNP molecules between the catalytic and effector sites.

Given the fact that the active site of the 2E1 isoform is very hydrophobic, the addition of a polar molecule causes a great deal of stress. In the case of these studies, the stress of increasing polarity caused a dramatic change in the metabolic scheme of benzyl alcohol and 4-nitrobenzaldehyde**.** Although the purity of the deuterated benzyl alcohol substrate limited our ability to analyze true isotope effects, we were able to observe an "unmasking" of the isotope effect when ethanol was added to the reaction mixture. More specifically, it appeared that as the concentration of ethanol increased, hydrogen abstraction became more favorable. Furthermore, the ability of ethanol to influence the partitioning of the 4-nitrobenzaldehyde pathway, potentially through a shift in heme

intermediate formation, also supports the notion that increased active site polarity alters the traditional catalytic scheme.

Based on these studies, the influence of ethanol appeared to be quite substantial, with the effects being more pronounced at elevated concentrations. This simulates conditions *in vivo* where humans consume larger amounts of ethanol, and the risk for toxic effects is increased. However, further studies to connect these findings to mechanisms behind specific drug-drug interactions associated with $P450_{2E1}$ would be even more beneficial to the toxicological profile of this isoform.

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