MODIFICATION OF THE NUCLEOTIDE COFACTOR-BINDING SITE OF CYTOCHROME P450 REDUCTASE TO ENHANCE TURNOVER WITH NADH IN VIVO

Calvin Lee Elmore
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ABSTRACT OF DISSERTATION

Calvin Lee Elmore

The Graduate School
University of Kentucky
2003
MODIFICATION OF THE NUCLEOTIDE COFACTOR-BINDING SITE
OF CYTOCHROME P450 REDUCTASE TO ENHANCE
TURNOVER WITH NADH IN VIVO

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements of the degree of Doctor of Philosophy in
The Graduate School
at the University of Kentucky

By
Calvin Lee Elmore
Lexington, Kentucky

Director: Dr. Todd D. Porter, Associate Professor, Pharmaceutical Sciences
2003
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ABSTRACT OF DISSERTATION

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NADPH-cytochrome P450 reductase is the electron transfer partner for the cytochromes P450, heme oxygenase, and squalene monooxygenase, and is a component of the nitric oxide synthases and methionine synthase reductase. P450 reductase shows very high selectivity for NADPH and uses NADH only poorly. Substitution of tryptophan 677 with alanine (W677A) has been shown by others to yield a 3-fold increase in turnover with NADH, but profound inhibition by NADP+ makes the enzyme unsuitable for in vivo applications. In the present study site-directed mutagenesis of amino acids in the 2'-phosphate-binding site of the NADPH domain, coupled with the W677A substitution, was used to generate a reductase that was able to use NADH efficiently in vivo without inhibition by NADP+. Of 11 single, double, and triple mutant proteins, two (R597M/W677A and R597M/K602W/W677A) showed up to a 500-fold increase in catalytic efficiency ($k_{cat}/K_m$) with NADH. Inhibition by NADP+ was reduced by up to four orders of magnitude relative to the W677A protein and was equal to or less than that of the wild-type reductase. Both proteins were 2- to 3-fold more active than wild-type reductase with NADH in reconstitution assays with cytochrome P450 1A2 and with squalene monooxygenase. In a recombinant cytochrome P450 2E1 Ames bacterial mutagenicity assay the R597M/W677A protein increased the sensitivity to dimethylnitrosamine by approximately 2-fold, suggesting that the ability to use NADH
afforded a significant advantage in this \textit{in vivo} assay. In addition to providing a valuable tool for understanding the determinants of nucleotide cofactor specificity in this and related enzymes, these mutants might also lend themselves to creation of bioremediation schemes with increased enzymatic activity and robustness \textit{in situ}, as well as cost-effective reconstitution of enzyme systems \textit{in vitro} that do not require the use of expensive reducing equivalents from NADPH.

C. Lee Elmore

April 25, 2003

Key words: NADPH-Cytochrome P450 reductase, structure-function studies, cofactor-swapping, bioremediation, Ames assay
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April 25, 2003
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For Eugenia Fox Yates
ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor, Dr. Todd D. Porter, for his guidance and friendship during my dissertation research. I would not have been able to complete this project without his support, encouragement and enthusiasm.

I would also like to thank the rest of my dissertation committee, Dr. Daret St. Clair, Dr. Bernard Hennig, Dr. Joseph Chappell, and Dr. Daniel Tai for their contributions during committee meetings and qualifying examination. I would also like to thank Dr. Louis Hersh for agreeing to serve as outside examiner for my dissertation defense.

My parents, Randy and Martha Elmore, deserve a great deal of credit for any success I have achieved. They raised me to understand that nothing in life that is truly fulfilling is easy.

Lastly, I would like thank my wife and best friend, Molly Elmore, for her unwavering faith in me.
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CHAPTER ONE
INTRODUCTION

Cytochrome P450 Reductase, a Unique Flavoprotein

NADPH-cytochrome P450 reductase (EC 1.6.2.4) is the electron-donating partner for the cytochromes P450, squalene monooxygenase, and heme oxygenase, and is a component of the nitric oxide synthases and methionine synthase reductase. P450 reductase is essential to cholesterol and steroid synthesis, and its importance is underscored by the developmental abnormalities and embryonic lethality observed in P450 reductase-null mice (Shen, O'Leary et al. 2002). P450 reductase contains one mol of flavin adenine dinucleotide (FAD) and one mol of flavin mononucleotide (FMN) per mol of enzyme. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) transfers two electrons as a hydride ion to the FAD of the enzyme, and FMN then accepts single electrons from the FAD and acts as the exit point to the protein acceptor such as the cytochromes P450.

Structure of Cytochrome P450 Reductase

P450 reductase is a multi-domain protein with the FAD and NADP(H) domains homologous with ferredoxin-NADP⁺ reductase and the FMN domain homologous with flavodoxin, suggesting that P450 reductase was formed from an ancestral gene fusion event (Porter and Kasper 1986; Porter 1991). A fourth connecting domain acts as a hinge to orient the two flavin-containing domains for electron transfer, and an NH₂-terminal membrane-binding domain anchors the protein to the cytosolic side of the endoplasmic reticulum and the nuclear membrane. The three-dimensional structure was determined by X-ray crystallography, and superbly illustrates the modular structure of this unique flavoprotein (Wang, Roberts et al. 1997). Figure 1.1 illustrates, in cartoon format, the multi-domain structure of the enzyme. The NADP(H) domain adopts the typical dinucleotide fold structure consisting of alternating α–helices and β–strands, with the nicotinamide cofactor binding in a cleft between the FAD and NADP(H) domains.
While the adenine portion of the dinucleotide cofactor was tightly bound in crystallographic preparations, the nicotinamide portion was found to adopt multiple conformations. However, none of the nicotinamide conformations were representative of the catalytic form of the enzyme. First, the distances between nucleotide and FAD were too great and more interestingly, access to the flavin was blocked by the penultimate C-terminal residue, a tryptophan (Wang, Roberts et al. 1997). Several drawings based on the three-dimensional structure of P450 reductase are included in Figs. 1.2-1.4 and illustrate the nucleotide binding pocket of the protein as well as the key residues which are likely to determine nucleotide cofactor specificity.
FIGURE 1.1 Cartoon illustrating the multi-domain structure of NADPH-cytochrome P450 reductase.
FIGURE 1.2 Space-filling drawing of NADPH-cytochrome P450 reductase with FAD and FMN domains noted. The NADPH-binding region is outlined with the adenine portion of the dinucleotide partially filling the pocket. The nicotinamide portion of NADPH adopted multiple conformations in the crystal structure and is not shown. The figure is adapted from Wang et al (Wang, Roberts et al. 1997).
FIGURE 1.3 Ribbons diagram representation of NADPH-cytochrome P450 reductase with bound FAD, FMN, and the adenine portion of NADPH. This figure was adapted from Wang et al. (Wang, Roberts et al. 1997).
FIGURE 1.4 Diagram of the NADPH-binding domain of P450 reductase. Residues mutated in these studies are labeled. Notice that serine 596, arginine 597, and lysine 602 form a binding pocket for the 2'-phosphate of NADPH. Also, tryptophan 677 is shown in a conformation that would block productive binding of the nicotinamide portion of NADPH. The figure is adapted from Wang et al (Wang, Roberts et al. 1997).

Enzymology of Cytochrome P450 Reductase

Cytochrome P450 reductase donates reducing equivalents from NADPH to redox partners such as the cytochromes P450. Two electrons are transferred from NADPH
via hydride transfer to the FAD; FAD transfers single electrons to FMN. The acceptor protein accepts two electrons one-at-a-time in a two electron reduction. Human P450 reductase cycles from the three electron reduced state to the one electron reduced state (Figure 1.5), highlighting the importance of redox potentials of the flavins for electron transfer. The reaction was determined by Sem and Kasper to be two-site ping-pong with cytochrome c (Sem and Kasper 1994).

FIGURE 1.5 Electrons flow into NADPH-cytochrome P450 reductase from NADPH by hydride transfer. The reductase cycles between a three and one electron reduced state. FAD accepts two electrons from NADPH, but FMN acts as a one-electron donor/acceptor and shuttles single electrons to the target molecule.

In the case of the cytochromes P450, in general, molecular oxygen is split: one oxygen atom is reduced to water, the other used for carbon oxidation. The general P450 reaction scheme is presented in Fig. 1.6, adapted from a review by F. P. Guengerich (Guengerich 2001). The iron containing heme of P450 is bound to the enzyme through four heme nitrogens, and also a cysteine, which causes the characteristic absorbance maximum at 450 nm. Histidine ligand-heme proteins (e.g. hemoglobin) have an absorbance maximum of 420 nm. In the absence of substrate, the heme iron is in the ferric state. Substrate binding triggers a one electron reduction from P450 reductase, leading to the formation of ferrous iron. Molecular oxygen then becomes bound to the ferrous iron center. A second, one electron reduction from P450 reductase leads to splitting of the oxygen-oxygen bond, with the release of one atom as water. The remaining oxygen-iron complex forms the reactive FeO$_{3+}$ which is able to oxidize substrate.
FIGURE 1.6 The reaction cycle of the typical cytochrome P450 cycle. NADPH-cytochrome P450 reductase donates electrons in a two electron reduction of the heme of P450. Molecular oxygen is split, one atom is utilized to oxidize substrate, and the other atom is reduced to water. Figure adapted from an excellent review of cytochrome P450 metabolism by F. P. Guengerich. (Guengerich 2001)

The physiologically relevant nucleotide cofactor for cytochrome P450 reductase is NADPH. The reductase can be driven by non-physiological concentrations of NADH in the millimolar range, while NADPH facilitates catalysis at low micromolar levels. The ratio between the $K_m$ with NADPH versus the $K_m$ with NADH is higher for P450 reductase than that of other NADPH specific enzymes, see Fig. 1.7 (Sem and Kasper 1993). Understanding the nature of P450 reductase’s high selectivity for NADPH is one of the primary aims of this research. The only chemical difference between NADPH and NADH is the presence of a 2'-phosphate on the adenine ribose, as shown in Figs. 1.8 and 1.9. Therefore, this phosphate must be a key factor in determining specificity.
between the two nucleotides. This must also be true for other enzymes which are specific for the nicotinamide adenine dinucleotide cofactors. These nucleotides are generally utilized differentially by cells; the NADH pool is important for catabolism and the NADPH pool for anabolic reactions.

FIGURE 1.7 NADPH-cytochrome P450 reductase maintains high selectivity for NADPH over NADH (Sem and Kasper 1993). Even among similar NADPH-utilizing enzymes (Arnon 1965; Cove 1967; Huang, Appleman et al. 1990; Scrutton, Berry et al. 1990), P450 reductase has unusually high specificity for the phosphate-containing nucleotide.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Km NAD(H) (μM)</th>
<th>Km NADP(H) (μM)</th>
<th>Ratio</th>
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<td>2.7</td>
<td>4300</td>
</tr>
<tr>
<td>GR</td>
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<td>21.7</td>
<td>90</td>
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<tr>
<td>DHFR</td>
<td>320</td>
<td>6.5</td>
<td>49</td>
</tr>
<tr>
<td>FNR</td>
<td>3770</td>
<td>7.2</td>
<td>520</td>
</tr>
<tr>
<td>NR</td>
<td>3000</td>
<td>13</td>
<td>200</td>
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</tbody>
</table>

CPR, P450 reductase; GR, glutathione reductase; DHFR, dihydrofolate reductase; FNR, ferredoxin-NADP+ reductase; NR, nitrate reductase
FIGURE 1.8 NADPH and NADH differ only in the presence of a phosphate on the number two carbon of the adenine ribose. In NADH this moiety is a hydroxyl group.
Regulation of Cytochrome P450 Reductase

While there are many isoforms of cytochrome P450 enzymes in mammals, there is only one protein responsible for donating electrons, NADPH-cytochrome P450 reductase. P450 reductase is universally expressed, though the highest levels are found in the liver. While many of the cytochromes P450 are highly inducible, P450 reductase is independently regulated and is present at basal levels. The single reductase gene is localized on human chromosome 7, and, like other house-keeping genes, the promoter region lacks TATA and CCAAT boxes (O'Leary, Beck et al. 1994). The GC-rich promoter region does contain nine consensus sequences for the SP1 transcription factor, however it appears that the proximal two sites are most important for transcription, but are not absolutely required (O'Leary, McQuiddy et al. 1996). Furthermore, a thyroid response element was discovered by O'Leary and others (O'Leary, Li et al. 1997). Indeed, hypophysectomy of adult male rats produces an 80-85% decrease in liver P450 reductase protein levels, causing a drop of up to 85% in the
level of P450 reductase activity, likely due to a decrease in observed P450 reductase mRNA levels (Ram and Waxman 1992).

**Knock-out Studies**

Basic questions regarding the importance of functional P450 reductase were addressed in recent reports that describe both the complete and conditional-hepatic knock-out of the P450 reductase gene in mouse. Complete loss of the P450 reductase gene results in embryonic lethality at day 11 of gestation, further characterized by developmental abnormalities in the neural tube, cardiac, eye and limb (Shen, O'Leary et al. 2002). These results emphasize the important role P450 reductase plays in providing electrons to enzymes important for development, including many involved in cholesterol synthesis and breakdown, retinoic acid metabolism, and heme catabolism. Conditional deletion of the P450 reductase gene in the liver of adult mice by the Cre/lox system leads to an impaired ability to both generate cholesterol and to eliminate it through bile acid synthesis, and is characterized by elevated hepatic lipids, reduced serum cholesterol levels and reduced bile flow. Furthermore, hepatic-null reductase mice completely lost microsomal P450 function in the liver, resulting in a five-fold induction of P450 enzymes with essentially no activity. Phenobarbital administration in hepatic reductase-null mice induced prolonged sleep at a concentration that produced no sleep in wild-type animals. Dosing of acetaminophen led to a 90% drop in hepatic glutathione levels and a rise in serum indicators of liver failure in wild-type animals, whereas glutathione levels and biomarkers of liver health remained stable in P450 reductase-null animals. Surprisingly, hepatic-null mice were able to survive and breed, emphasizing the predominant role of the P450 system in xenobiotic metabolism in adult liver (Henderson, Otto et al. 2003).

**Bioremediation**

A primary goal of this research is to develop mammalian enzyme systems with improved activity in bacteria, which could lead to more effective bioremediation
schemes. Modification of P450 reductase to use both NADH and NADPH could contribute to the effort to develop recombinant P450 systems in bacteria for chemical degradation. Bioremediation is the use of biological agents to break down xenobiotics in the environment. Important factors affecting the potential for successful bioremediation include the bioavailability of the target toxicant, subsequent transport into cells with access to the appropriate metabolizing enzymes, or, alternatively, the secretion of appropriate biodegradation enzymes, and the ability of cells to function under \textit{in situ} environmental conditions (Pieper and Reineke 2000). Intrinsic chemical properties, such as stability of target compounds also play a role (Dua, Singh et al. 2002). Nature has provided an enormous number of bacterial species capable of degrading waste chemicals. However, limitations include toxicity to biodegrading organisms, low nutrient and oxygen levels \textit{in situ}, and the lack of appropriate metabolizing enzymes for complete degradation. The latter limitation can be addressed with the use of genetically engineered microorganisms.

Over the last decade, many gene sequences for isoforms of the cytochromes P450 have been discovered and published. Furthermore, many have been expressed in bacteria along with P450 reductase to produce enzymatic activity comparable to that seen in liver microsomes, see Guengerich et al. for a list (Guengerich, Parikh et al. 1997). In addition to bioremediation, applications of these systems include cytochrome P450 structure-function analysis, screening of P450 substrates to generate profiles, and bacterial mutagenicity assays (Guengerich and Parikh 1997).

If naturally occurring enzymes fail to provide the necessary enzymatic activity for bioremediation, several techniques for improving enzyme turnover and/or chemical specificity may be attempted. These methods include rational redesign by site-directed mutagenesis based on genetic sequence alignments with similar enzymes and/or structure data. Random mutagenesis and gene shuffling, a.k.a. \textit{in vitro} gene recombination, approaches compliment rational approaches, as long as there is an effective screening technique available for sifting through the large numbers of proteins generated (Guengerich 1995).
Ethical issues regarding the introduction of genetically modified microorganisms into the environment cloud the future of such tools for in situ bioremediation (Guengerich 1995). However, only the creation of useful remediation tools including genetically engineered bacteria will allow these ethical problems to be addressed. Additional limitations to the use of genetically altered bacterial strains include the expense of monitoring enzyme activity and engineered bacterial growth in situ and the possibility of reduced survival rates among genetically modified bacteria (Sayler and Ripp 2000). Monitoring introduced strains can be accomplished by PCR of recombinant DNA or the mRNA transcripts (Pieper and Reineke 2000) or through bioluminescence approaches (Dua, Singh et al. 2002).

**Recombinant Cytochrome P450/Cytochrome P450 Reductase Systems**

While expression of mammalian P450/reductase systems in bacteria has been shown to be a valuable research tool and has promise as a tool for biodegradation (Guengerich 1995), enzyme activity in recombinant bacterial systems appears to be limited by glucose availability (Shet, Fisher et al. 1997). One explanation for this might be that the generation of NADPH is largely dependent on the glucose shunt pathway, which is operative primarily in high glucose environments. Supporting this hypothesis are data from Bochner and Ames (Bochner and Ames 1982) that indicate that bacterial NAD(H) levels exceed NADP(H) levels by approximately 4:1. To address the hypothesis that heterologously expressed P450 systems in bacterial cells are limited by the availability of NADPH, I constructed a series of reductase proteins designed to use NADH more efficiently. Specific mutations in the nicotinamide cofactor 2'-phosphate-binding site were generated based on the crystal structure of the reductase from rat (Djordjevic, Roberts et al. 1995; Wang, Roberts et al. 1997), and from sequence alignments of rat cytochrome P450 reductase (Porter and Kasper 1985) with other NADPH- and NADH-specific reductases including spinach ferredoxin-NADP⁺ reductase (Karplus, Daniels et al. 1991), nitrate reductases from Neurospora crassa (NADPH)
(Okamoto, Fu et al. 1991), and corn (NADH) (Hyde and Campbell 1990), and NADH-cytochrome b₅ reductase (Yubisui, Naitoh et al. 1987), as shown in Fig. 1.10.

**FIGURE 1.10** A sequence alignment between NADPH-cytochrome P450 reductase and two related NADPH-specific enzymes along with two related NADH-specific enzymes.

<table>
<thead>
<tr>
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</tbody>
</table>

The functions of these amino acids have been studied previously in cytochrome P450 reductase, as well as in the cytochrome P450 reductase homologues nitrate reductase and ferredoxin-NADP⁺ reductase. For example, studies by Sem and Kasper (Sem and Kasper 1993) have shown that arginine 597 of P450 reductase from rat plays a critical role in binding the 2’-phosphate of NADPH, and thereby discriminating between NADPH and NADH; substitution of this residue with methionine produced a reductase with a greater than 100-fold higher \( K_m \) value for NADPH, while the \( k_{\text{cat}} \) for NADPH remained at a level comparable to wild-type. The \( k_{\text{cat}} \) and \( K_m \) values for NADH were similar between wild-type and the Arg597Met substitution (Sem and Kasper 1993). However, substitution of serine or threonine for arginine 922 of NADPH-nitrate reductase from *Neurospora crassa*, which corresponds to arginine 597 of P450 reductase, was shown by Shiraishi et al. to be unable to significantly alter the specific activity (\( \mu \text{mol/min} \)) with either NADPH or NADH (Shiraishi, Croy et al. 1998). This indicates a fundamental difference in the amino acid residues that these two proteins utilize to bind nucleotide cofactor. Mutation of arginine 931 to glutamine in nitrate reductase, which corresponds to lysine 602 of P450 reductase, led to a decrease in NADPH specific activity (\( \mu \text{mol/min} \)) and an increase in NADH specific activity.
Substitution of aspartic acid for serine 921 in nitrate reductase had a large effect on cofactor specificity, leading to 2% of wild-type specific activity (µmol/min) with NADPH, but 15-fold greater specific activity (µmol/min) with NADH versus wild-type (Shiraishi, Croy et al. 1998). These changes in specific activity were correlated with the decrease in the catalytic efficiency ($k_{cat}/K_m$) for NADPH and an increase for NADH for that protein. Double substitution of S921D/R931S in nitrate reductase led to the greatest switch in cofactor specificity, greater than 73,000-fold compared to wild type ($k_{cat}/K_m$; NADPH/NADH; wild-type/mutant), but improved the catalytic efficiency ($k_{cat}/K_m$) versus wild-type with NADH by only 2.5-fold (Shiraishi, Croy et al. 1998).

Similar studies have been carried out in ferredoxin-NADP⁺ reductase from spinach, a closely related flavoprotein involved in NADPH production. Lysine 244 of ferredoxin-NADP⁺ reductase, which corresponds to lysine 602 in P450 reductase, was mutated to glutamine, increasing both the $K_m$ and the $k_{cat}$ with NADPH (Aliverti, Lubberstedt et al. 1991), while NADH activity was not studied in this protein. These comparisons indicate that although high sequence homology exists in the 2'-phosphate binding regions of these closely related proteins, it is likely that three-dimensional amino acid arrangements ultimately determine the function these residues serve.

Substitutions of the C-terminal tyrosine in ferredoxin-NADP⁺ reductase from pea produced similar results to the W677A substitution with cytochrome P450 reductase presented by Döhr et al. (Dohr, Paine et al. 2001), i.e. a decrease in $K_m$ and increase in $k_{cat}$ for NADH (Orellano, Calceterra et al. 1993), but lower $K_m$ and $k_{cat}$ for NADPH (Orellano, Calceterra et al. 1993; Piubelli, Aliverti et al. 2000). Thus, although the phosphate-binding amino acids differ between these proteins, aromatic C-terminal residues probably perform a similar function in both enzymes in order to maintain selectivity for NADPH over NADH. Döhr et al (Dohr, Paine et al. 2001) demonstrated that tryptophan 677 appears to block access of the nicotinamide portion of the NADPH cofactor to the flavin, and proposed that this amino acid plays a role in removal of NADP⁺ after hydride transfer. This amino acid is suggested to contribute to cofactor
specificity by blocking the nucleotide-binding site from both NADPH and NADH: NADPH, with higher binding affinity due to a 2'-phosphate-binding pocket, is evidently able to displace the tryptophan and form a productive electron-transfer complex, whereas NADH cannot bind tightly enough to displace the tryptophan. Consistent with this hypothesis, removal or substitution of W677 greatly increases turnover with NADH, but leads to inhibition by NADP⁺. I hypothesized that mutations in the 2'-phosphate-binding site might reduce the affinity of the enzyme for NADP(H) and reduce or prevent the inhibition by NADP⁺. Several highly conserved residues make up the 2'-phosphate-binding motif, including serine 596, arginine 597, and lysine 602; the position of these amino acids in the NADP(H)-binding domain are shown in Fig. 1.4. The present studies reveal that the combination of 2'-phosphate-binding site mutations with the W677A substitution yields a cytochrome P450 reductase that uses NADH without inhibition by NADP⁺. As any enzyme to be used in in vivo applications such as bioremediation must retain activity in a mixed nucleotide environment, this reductase may prove useful in such applications.
CHAPTER TWO
GENERATION OF CYTOCHROME P450 REDUCTASE COFACTOR MUTANTS

Introduction to Site-Directed Mutagenesis

Site-directed mutagenesis is an important technique for protein structure-function studies. It allows selected amino acids in a protein to be replaced with either conservative or nonconservative substitutions. Effects of amino acid substitutions can be analyzed after subsequent expression and purification of these altered proteins or after expression in intact cells. Several other tools make site-directed mutagenesis studies more relevant and conclusive, including sequence alignments of closely-related proteins, computer programs that predict structural motifs and techniques which directly determine three-dimensional structure such as X-ray crystallography and NMR.

To test the hypothesis that NADPH might be limiting in bacterial cells expressing a recombinant P450 system in vivo, I needed P450 reductase(s) that use NADH under physiologically relevant conditions. Targeting amino acids outlined in Chapter One, I used site-directed mutagenesis to substitute amino acids believed to be important for nucleotide cofactor binding and recognition.

Generation of P450 Reductase Mutants

Site-directed mutagenesis was carried out in either pIN3OR6XHis, a P450 reductase bacterial expression plasmid in which a 6xhistidine tag was placed at the N-terminus of the reductase, or in pIN3ER plasmid coexpression vector containing the cDNAs for rat P450 reductase and human P450 2E1 (Porter and Chang 1999). Whole plasmid amplification (see Fig. 2.1) was performed as described in the QuickChange™ site-directed mutagenesis kit (Stratagene) with changes noted. Primers were designed that contained the desired base changes necessary for the desired amino acid substitution (Fig. 2.1; Step 1). Primers were designed to have a melting temperature greater than or equal to 78°C. Alternatively, the set of primers from above were
combined with other primers and amplified by PCR to generate a longer primer for whole plasmid amplification. These extended primers generally had a length between 100 and 500 base pairs and contained the desired base changes. These longer primers were generally more efficient in producing whole plasmid amplification. PCR reactions contained 5-50 ng of plasmid template DNA, 50 pmols of each primer or 2 µL of PCR product incorporating mutations, deoxynucleotide triphosphates (dNTPs) (200 µM), 2.5 units of Pfu Turbo Polymerase (Stratagene), Pfu Turbo Polymerase buffer, and double deionized water (ddH₂O) to 50 µL total volume. Ninety-five degrees Celsius was used to melt template DNA, ~55°C was used as the annealing temperature, and ~68°C was used for extension. Pfu polymerase generally incorporates dNTPs at a rate of 1-2 minutes/kilobase of template. Therefore, for pIN3OR6xHis (~9,500 bp) and pIN3ER (~11,000 bp) extension times of 19 and 22 min were used. Eighteen cycles were carried out in a Perkin-Elmer 2400 thermocycler. Products of amplification were visualized on a 1% agarose gel with ethidium bromide. Ten units of Dpn I were added to digest methylated parental template DNA (Step 2). One to five µL of the reaction mixture was transformed into chemically competent E. coli (Step 3). Clones were screened by sequencing.
Subcloning is a method of moving sections of double-stranded DNA from one vector to another, as depicted in Fig. 2.2. I used it rarely because of the ease of whole plasmid amplification, but occasionally when I wanted to introduce a mutation into a plasmid vector or, more commonly, to combine mutations that were far enough apart on their plasmid vector that a unique restriction site was located between the mutations. This allowed one mutation to be excised, purified, and ligated into a plasmid vector containing the second mutation. Generally, between 100-1,000 ng of plasmid DNA was digested with restriction endonucleases (2-10 U) (Step 1). These restriction enzymes were both compatible in the same buffer, or the DNA was purified to remove buffer and a second buffer was added. Five-prime phosphates were removed from vector DNA to prevent religation of incompletely digested plasmid DNA, as follows: Alkaline phosphatase (10 U) was added directly to the original restriction digest and incubated for 1 hour at 37°C. Phosphatase activity was terminated by addition of 2 µL 0.5 M
EDTA followed by incubation at 65°C for 20 min. Insert DNA was separated by 1% agarose gel, and the band was excised with a razor blade (Step 2). Gel pieces were spun in a 0.45 µm nylon membrane centrifugal spin tube (Costar) and collected in 1x TAE. DNA was visualized on a 1% agarose gel to determine quantitation. Dephosphorylated vector was then added to insert at a 1:3 molar ratio with ligase (1U) and incubated overnight a 4°C (Step 3). Two µL of the ligation reaction was transformed into chemically competent *E. coli* and positive clones were identified by DNA sequencing.

**FIGURE 2.2 Mutagenesis by sub-cloning.**

Mutagenesis by overlap-extension (Fig. 2.3) allows multiple changes to primary genetic sequences or the creation of chimeric constructs by targeted splicing of two unique DNA sequences to create proteins with new and interesting activities. Primers are designed that incorporate the desired mutations (or the novel DNA to be joined) at the five prime ends. These “inside” primers are combined with “outside” primers to
create templates that overlap at positions found in the five prime ends of the “inside” primers. An eight-amino acid substitution was introduced by overlap-extension polymerase chain amplification as described (Slack 1998) to generate a reductase with the following substitutions: S596D, R597R, E598A, Q599P, A600E, H601A, K602W, and V603D. Two chimeric proteins were designed, containing the NADH-binding region of b5 reductase in place of the NADPH-binding region of P450 reductase. One chimera contained the “linker-arm” of P450 reductase, and was named “OR/b5R-short”. The other chimera contained the connecting region of b5 reductase, and was named “OR/b5R-long”. OR/b5R-short was cloned and confirmed by DNA sequencing, yet failed to yield protein sensitive to P450 reductase antibody. The OR/b5R-long construct was designed, but was never completed for expression experiments. All mutations were confirmed by sequencing.
Purification and Quantitation of Proteins

With all constructs, a fresh colony was selected for protein expression and grown overnight in LB media at 37°C. Overnight culture was used to inoculate up to 6 liters of Terrific Broth at 37°C followed by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the O.D.600 reached 0.8; culture was continued
overnight at 30°C with shaking (125 rpm). Cells were harvested by centrifugation and
the membrane fraction was isolated and solubilized as described (Porter and Chang
1999), omitting the dithiothreitol and 4-methylpyrazole. Cells were lysed by French
press in buffer consisting of 20 mM KPO₄ (pH 8.0) and 50 mM KCl. The lysate was
clarified at 10,000 x g, and a second supernatant fraction was obtained by centrifugation
at 100,000 x g. The membrane pellet was resuspended in a glass dounce homogenizer
with a type B pestle in 5 mL of 10 mM Tris-Acetate (pH 8.0) and 20% glycerol.
Solubilization of membranes was carried out on ice with stirring by addition of Triton X-
100 dropwise to 1%. Solubized membranes were then clarified at 100,000 x g and the
supernatant used for affinity purification.

Wild-type P450 reductase and mutants were purified by metal affinity
chromatography using TALON IMAC metal affinity resin (Clontech) as shown
schematically in Fig. 2.4. Typically 3 mL of metal affinity resin (1.5 mL column volume)
in ethanol was added to a 10 mL plastic column (Biorad). The column was equilibrated
in twenty bed volumes of column buffer: 300 mM NaCl, 50 mM NaPO₄ (pH 8.0), 1%
Triton X-100, and 10% glycerol. Solubilized membranes were added to the column and
recycled several times for maximum binding. Twenty column volumes of column buffer
were used to wash away unbound material. Proteins were eluted in column buffer plus
150 mM imidazole (pH 8.0), and stored in the same buffer at -20°C.
Results of Protein Expression/Purification Experiments

The purified proteins were largely homogeneous, and migrated with a molecular weight of 78.5 kDa, as did wild-type reductase (Fig. 2.5); immunoblotting with antibody to P450 reductase indicated that the lower molecular weight contaminants were predominantly reductase degradation products (Fig. 2.6). Reductase was quantified by boiling to release the flavins and measuring the absorbance at 450 nm using an extinction coefficient of 23.5 mM$^{-1}$ (Vermilion and Coon 1978), see Fig. 2.7.

Purification of Squalene Monooxygenase

Purification of squalene monooxygenase was performed as described by Laden et al. (Laden, Tang et al. 2000). The cDNA encoding squalene monooxygenase, lacking the first 330 base pairs, from human was cloned previously (Laden, Tang et al. 2000) into the pTYB4 expression vector, part of the IMPACT T7 System (Clontech) which expresses the enzyme along with an intein protein splicing element from yeast.
and a chitin binding domain. Bacterial cells were lysed by French press in buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 0.1 mM EDTA, and 0.1% Triton X-100, and centrifuged at 12,000 x g. The supernatant was loaded on a chitin column equilibrated in the same buffer. Following washing by 20 bed volumes of buffer, the column was incubated with buffer containing 30 mM β-mercaptoethanol overnight at 4°C. Squalene monooxygenase was eluted and buffer changed to 20 mM Tris-HCl (pH 7.4), 0.1% Triton X-100 by centrifugal dialysis and stored at -80°C. Enzyme was quantified with the Coomassie Plus Protein Assay Reagent Kit (Pierce).

FIGURE 2.5 Sodium dodecyl sulfate - polyacrylamide gel electrophoresis of P450 reductase mutants. Affinity-purified reductase proteins were fractionated on an 8.75% polyacrylamide gel and stained with Coomassie blue. Lane 1, molecular mass standards at 221, 133, 93, 67, 56, 42, and 28 kDa; 2, wild-type reductase; 3, S596D; 4, R597M; 5, K602W; 6, SD/WA; 7, SD/KW; 8, W677A; 9, RM/WA; 10, KW/WA; 11, 8 amino acid substitution; 12, RM/KW; 13, RM/KW/WA; and 14, SD/WA.
FIGURE 2.6 Western blots of P450 reductase mutants. Lane 1, molecular mass standards at 221, 133, 93, 67, 56, 42, and 28 kDa; 2, wild-type reductase; 3, S596D; 4, R597M; 5, E598A; 6, K602W; 7, molecular mass standards; 8, SD/RM; 9, SD/KW; 10, W677A; 11, RM/WA; 12, KW/WA.
FIGURE 2.7 Flavin spectra of wild-type P450 reductase and mutant proteins.

A Comparison between Native Cytochrome P450 Reductase and the 6x-histidine Tagged P450 Reductase Construct

A 6x-histidine tag allows easy, often single-step, column-affinity purification of proteins. However, the addition of six additional histidine residues at the N- or C-terminus may alter protein function. Many newer 6x-histidine tag vectors allow the removal of the 6x-histidine tag after affinity purification using a thrombin-cleavage site. However, this technique invariably yields proteins with additional non-native amino acids at the cleavage site. In order to address this concern with 6x-histidine tagged P450 reductase clones, aniline hydroxylation experiments were carried out with wild-type and 6x-histidine tagged P450 reductases with cytochrome P450 2E1. Reductase concentrations were varied, with all other variables held constant, in order to generate $K_m$ and $V_{max}$ numbers for the two proteins. Results of these experiments are shown in Table 2.1.

Cytochrome P450 reductase was purified by 2'5'-adenosine diphosphate agarose affinity chromatography. Wild-type proteins were eluted in 50 mM Tris (pH 7.7), containing 0.1 mM EDTA, 10% glycerol and 2 mM 2'-adenosine monophosphate. Wild-type reductase was quantified by boiling an aliquot and measuring the flavin absorbance at 450 nm. 6x-histidine tagged reductase protein was purified as described in detail earlier in this chapter. Cytochrome P450 2E1 protein from rabbit was expressed in E. coli. Membranes were solubilized in octyl-glucoside detergent. Proteins were changed into phosphate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol. Cytochrome P450 2E1 was quantified by carbon monoxide difference spectra measuring heme at 450 nm. Aniline hydroxylation assays contained 10-150 pmols reductase, 50 pmols P450 2E1, 1 mM NADPH, and 2.5 mM aniline in the phosphate buffer described above. Reactions were initiated by addition of NADPH and were incubated at 37°C for 0, 15 and 30 minutes to determine reaction rates. para-Aminophenol production was measured as an increase in absorbance at 630 nm using
an HP 8453 spectrophotometer. Michaelis-Menten kinetic constants were determined using GraphPad Prism 3.

These enzyme assays show that wild-type cytochrome P450 reductase and the 6x-histidine tagged reductase are not statistically different in their ability to stimulate the hydroxylation of aniline to para-aminophenol. Additionally, during purification, both wild-type and 6x-histidine tagged reductase were found to be inserted into bacterial membranes, suggesting the N-terminal 6x-histidine tag did not alter the enzyme conformation significantly enough to alter the ability of the N-terminal membrane binding domain to function correctly. Therefore, 6x-histidine tagged P450 reductase is considered to be functionally the same as wild-type for these experiments.

TABLE 2.1 A comparison between wild-type and 6x-histidine tagged reductases. Kinetic constants derived from reductase-supported hydroxylation of aniline by P450 2E1, in vitro. Values represent best fits ± S.D.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_m$ (nM reductase)</th>
<th>$V_{max}$ (nmols p-AP/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>22.6 ± 3.0</td>
<td>17.4 ± 0.8</td>
</tr>
<tr>
<td>6x-histidine Tag</td>
<td>14.9 ± 3.8</td>
<td>15.2 ± 1.0</td>
</tr>
</tbody>
</table>
CHAPTER THREE
ACTIVITY ANALYSIS OF CYTOCHROME P450 REDUCTASE
MUTANTS WITH CYTOCHROME C

Introduction to Cytochrome c Reductase Activity Assays

P450 reductase proteins with altered cofactor specificity were evaluated with the non-physiological electron acceptor cytochrome c. Michaelis-Menten kinetic constants were determined with each enzyme. Comparisons between \( K_m \), \( k_{cat} \) and catalytic efficiency (\( k_{cat}/K_m \)) allowed a means of objectively evaluating the effects of amino acid substitutions. Cytochrome c is inexpensive, stable and readily accepts electrons from P450 reductase. The rate of reduction of the bound heme of cytochrome c can be measured at 550 nm.

Steady State Kinetics with Cytochrome c

\( K_m \) and \( k_{cat} \) determinations were carried out with 60 \( \mu \)M cytochrome c from horse heart (Sigma) in 100 mM Tris, pH 8.0 with an ionic strength of 0.75 M with KCl. Reactions were started by the addition of nucleotide cofactor (NADPH or NADH) and were monitored for 40 seconds at room temperature on an HP 8453 spectrophotometer (Hewlett-Packard), following the absorbance change at 550 nm. An extinction coefficient of 21.1 \( mM^{-1} \) was used to quantitate cytochrome c reduction (Vermilion and Coon 1978). Catalytic constants were estimated by non-linear regression using Prism (GraphPad Prism 3).

Results of Cytochrome c Reductase Assays

I generated mutants of cytochrome P450 reductase that have decreased specificity for the physiological cofactor NADPH. These mutants fall into three categories: those containing mutations in the 2'-phosphate-binding region of NADPH; a single mutant in which tryptophan 677, which is positioned directly opposite of the FAD
isoalloxazine ring, is replaced with alanine; and double and triple combinations of these two. As expected, single and double substitutions in the 2'-phosphate-binding region yielded proteins with greatly reduced catalytic efficiency ($k_{cat}/K_m$) with NADPH (Table 3.1). A comparison of catalytic constants of P450 reductase proteins is shown in Figs. 3.1-3.4. The R597M, S596D, K602W, and R597M/K602W proteins showed a 40- to 13,000-fold increase in the $K_m$ for NADPH as compared to wild-type with cytochrome c as the electron acceptor, with the K602W substitution having the least effect on $K_m$ and the S596D mutation producing the highest $K_m$. Of these mutants, the S596D showed the most profound decrease in the $k_{cat}$ with both NADPH and NADH, indicating an overall detrimental effect on electron transfer; all of the mutants containing S596D had little or no activity with either cofactor. Proteins with the R597M substitution (R597M and R597M/K602W) maintained turnover numbers with NADPH and NADH that were comparable to that of the wild-type enzyme, whereas the K602W mutation decreased turnover with both cofactors in the absence of the R597M substitution. As an alternative to individual substitutions, the segment from S596 to V603 (8 amino acids) was replaced with the corresponding segment from NADH-cytochrome b$_5$ reductase, a closely related NADH-dependent flavoprotein (Yubisui, Naitoh et al. 1987). The resulting mutant protein bound flavin but had no observable cytochrome c reductase activity. Considering that this protein bound flavin, the lack of activity was most likely due to alterations of the secondary structure of the 2'-phosphate binding pocket, such that productive nucleotide binding was lost.

The W677A mutant retained only 1.2% of wild-type activity ($k_{cat}$) with NADPH, while the $K_m$ for NADPH remained similar to wild-type when assayed with cytochrome c. In contrast, the W677A mutant had a $k_{cat}$ with NADH that was approximately 3-fold higher than that of wild-type with NADH, while the $K_m$ of NADH decreased by approximately 250-fold. The W677A enzyme also showed the most profound change in NADH catalytic efficiency with a greater than 700-fold increase versus wild-type. Coupled with a decrease in catalytic efficiency with NADPH of 74-fold, it is apparent that this single amino acid contributes remarkably to cofactor specificity for NADPH versus NADH in wild-type P450 reductase.
Proteins containing altered 2'-phosphate-binding domains and W677A substitution showed kinetics resembling the W677A mutation alone. However, the $K_m$ for NADPH was significantly increased compared to wild-type in the K602W/W677A, R597M/W677A, and R597M/K602W/W677A proteins by 1.5, 2.4, and 54-fold, respectively. With NADH, conversely, the $K_m$ values for these proteins remain similar to the W677A alone. Turnover numbers with the combined substitutions with NADH are lower than the W677A enzyme. However with the R597M/W677A and R597M/K602W/W677A proteins, $k_{cat}$ values remain equal to or greater than wild-type with NADH. With NADPH, a 4-, 7-, and 43-fold decrease in $k_{cat}$ values is seen with the R597M/K602W/W677A, R597M/W677A, and K602W/W677A proteins, respectively.

FIGURE 3.1 Comparison of $K_m$ values for 2'-phosphate-binding mutant reductase. ND indicates no detectable activity. WT, wild-type P450 reductase; SD, Ser596Asp; RM, Arg597Met; KW, Lys602Trp.
FIGURE 3.2 Comparison of $K_m$ values for mutant reductases containing the Trp677Ala substitution. ND indicates no detectable activity. WT, wild-type P450 reductase; SD, Ser596Asp; RM, Arg597Met; KW, Lys602Trp; WA, Trp677Ala; R/K/W, Arg597Met/Lys602Trp/Trp677Ala.

![Figure 3.2](image)

FIGURE 3.3 Comparison of $k_{cat}$ values for 2'-phosphate-binding mutant reductases. ND indicates no detectable activity. WT, wild-type P450 reductase; SD, Ser596Asp; RM, Arg597Met; KW, Lys602Trp.

![Figure 3.3](image)
FIGURE 3.4 Comparison of $k_{cat}$ values for mutant reductases containing the Trp677Ala substitution. ND indicates no detectable activity. WT, wild-type P450 reductase; SD, Ser596Asp; RM, Arg597Met; KW, Lys602Trp; WA, Trp677Ala; R/K/W, Arg597Met/Lys602Trp/Trp677Ala.
TABLE 3.1 Catalytic activities of P450 reductase mutants with NADPH and NADH. The values represent best fits ± S.D. — indicates lack of measurable activity.

| Mutant            | NADPH | | | NADH | | | | NADPH/NADH | | |
|------------------|-------|--------|--------|-------|--------|--------|--------|------------|--------|
|                  | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | | | $k_{cat}/K_m/k_{cat}/K_m$ | |
|                  | (min$^{-1}$) | (µM) | (min$^{-1}$*µM$^{-1}$) | (min$^{-1}$) | (µM) | (min$^{-1}$*µM$^{-1}$) | | | | |
| WT               | 3,280 ± 50 | 1.6 ± 0.1 | 2,000 | 1,210 ± 140 | 27,600 ± 4,600 | 0.044 | 45,000 | |
| S596D            | 140 ± 9 | 20,600 ± 2,000 | 0.007 | 101 ± 6 | 10,100 ± 1,300 | 0.010 | 0.7 | |
| R597M            | 2,980 ± 70 | 156 ± 10 | 19 | 1,350 ± 110 | 14,100 ± 2,200 | 0.10 | 200 | |
| E598A            | 2,390 ± 90 | 0.8 ± 0.2 | 3,000 | 1,250 ± 110 | 13,200 ± 2,300 | 0.09 | 30,000 | |
| K602W            | 1,110 ± 20 | 64.0 ± 3.2 | 17 | 101 ± 4 | 5,330 ± 630 | 0.019 | 900 | |
| W677A            | 38 ± 3 | 1.4 ± 0.4 | 27 | 3,220 ± 70 | 103 ± 8 | 31.2 | 0.9 | |
| S596D/R597M      | — | — | — | 199 ± 40 | 12,300 ± 5,000 | 0.02 | — | |
| S596D/K602W      | — | — | — | — | — | — | — | |
| R597M/K602W      | 2,830 ± 140 | 3,900 ± 440 | 0.73 | 819 ± 129 | 18,500 ± 4,900 | 0.044 | 16 | |
| S596D/W677A      | — | — | — | — | — | — | — | |
| R597M/W677A      | 468 ± 13 | 3.8 ± 0.4 | 120 | 2,040 ± 80 | 99 ± 14 | 21 | 5.7 | |
| K602W/W677A      | 77 ± 3 | 2.5 ± 0.6 | 31 | 370 ± 16 | 168 ± 22 | 2.2 | 14 | |
| R597M/K602W/W677A| 920 ± 22 | 87 ± 9 | 11 | 1,190 ± 30 | 162 ± 12 | 7.3 | 1.5 | |
| 8 Amino Acid Sub.| — | — | — | — | — | — | — | |
Several reductase clones were generated that did not warrant extensive study or discussion. These include a P450 reductase clone containing alanine substituted for glutamic acid at position 598, the importance of which was suggested by its semiconserved appearance at this position in sequence alignments with similar NADPH-specific enzymes (Fig. 1.10). This mutation lowered the $K_m$ by approximately one-half with both NADPH and NADH in cytochrome c reductase assays (Table 3.2) when compared to wild-type P450 reductase. When combined with the Ser596Asp mutation a similar decrease in $K_m$ with both nucleotides was seen compared to the Ser596Asp alone. The $k_{cat}$ values of Glu598Ala and the Ser596Asp/Glu598Ala reductases were not considered statistically different from wild-type or Ser598Asp, respectively, with either nucleotide. The modest overall degree of change seen with the $K_m$ values and lack of a significant effect on $k_{cat}$ values, coupled with the fact that similar effects were seen with both NADPH and NADH, suggested a limited role for the glutamic acid 598 in influencing cofactor specificity. A triple mutant reductase containing Ser596Asp, Arg597Met and Trp677Ala substitutions was also generated. However, consistent with other reductases containing the Ser596Asp substitution, this protein had negligible ability to reduce cytochrome c with either nucleotide cofactor.

**TABLE 3.2 Catalytic activities of P450 reductases containing the Glu598Ala substitution. The values represent best fits ± S.D.**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.6 ± 0.1</td>
<td>3,280 ± 50</td>
<td>27,600 ± 4,600</td>
<td>1,210 ± 140</td>
</tr>
<tr>
<td>E598A</td>
<td>0.8 ± 0.2</td>
<td>2,390 ± 90</td>
<td>13,200 ± 2,200</td>
<td>1,250 ± 110</td>
</tr>
<tr>
<td>S596D</td>
<td>20,600 ± 2,000</td>
<td>140 ± 9</td>
<td>10,100 ± 1,300</td>
<td>101 ± 6</td>
</tr>
<tr>
<td>S596D/E598A</td>
<td>9,300 ± 1,800</td>
<td>176 ± 16</td>
<td>5,900 ± 2300</td>
<td>62 ± 9</td>
</tr>
</tbody>
</table>
**CHAPTER FOUR**  
**INHIBITION OF P450 REDUCTASE MUTANTS BY NADP⁺**

*NADP⁺ as an Inhibitor of Cytochrome c Reductase Activity*

As demonstrated by Döhr et al. (Dohr, Paine et al. 2001), substitution of the penultimate amino acid in P450 reductase with alanine (W677A) produces a reductase with 1000-fold higher specificity for NADH than NADPH. Despite this remarkable increase in turnover with NADH, the most striking feature of the W677A mutant is that it is highly inhibited by NADP⁺ (Dohr, Paine et al. 2001). This inhibition is illustrated in Figure 4.1 with a W677A mutant I constructed. The following experiments were designed to evaluate the effect of combining 2'-phosphate binding mutations with the W677A enzyme on inhibition by NADP⁺. Results of these experiments are shown in Fig. 4.2.
FIGURE 4.1 Inhibition of W677A Reductase by NADPH. Graph indicates enzymatic activity of wild-type and W677A reductases with cytochrome c.

**NADP⁺ Inhibition Experiments**

Assays were carried out in 100 mM Tris (pH 8.0), 0.75 M ionic strength with KCl, 60 µM cytochrome c, and varying concentrations of NADP⁺. Reactions were carried out for 90 seconds and were monitored on a Hewlett Packard 8453 diode array spectrophotometer. Reduction of cytochrome c was monitored at 550 nm using a millimolar extinction coefficient of 21.1 mM⁻¹.

**NADP⁺ Inhibition Results**

The mutations in the phosphate-binding region described in Chapter 3 were combined with the W677A substitution in order to overcome the profound inhibition by NADP⁺. I combined the S596D, R597M, and K602W mutants, as well as the R597M/K602W double mutant, with W677A. Consistent with the other S596D mutants, S596D/W677A produced a reductase with little or no activity with either cofactor.
However, the R597M/W677A, K602W/W677A, and R597M/K602W/W677A proteins were active and displayed reduced catalytic efficiency with NADPH as compared to wild-type and increased catalytic efficiency with NADH (Table 3.1). Of these three proteins, the triple mutant (R597M/K602W/W677A) showed a 30,000-fold change in cofactor specificity (catalytic efficiency). Inhibition of cytochrome c reduction by NADP$^+$ was determined with these proteins and compared to that of wild-type reductase (Fig. 4.2). With the W677A protein, inhibition by NADP$^+$ increased approximately 125-fold, from an IC$_{50}$ of 17.4 µM (wild-type) to an IC$_{50}$ of 138.3 nM. Addition of the R597M mutation to the W677A protein returned inhibition by NADP$^+$ to a level comparable to wild-type reductase. Inhibition by NADP$^+$ was greatly decreased in the R597M/K602W/W677A protein to an IC$_{50}$ of 870 µM, 50-fold higher than the IC$_{50}$ for wild-type reductase.
The results of experiments outlined here characterize the inhibition by NADP⁺, a product of wild-type P450 reductase catalysis. A reductase useful for in vivo applications should retain maximal activity in a mixed-nucleotide environment (NADPH and NADH). The W677A substitution creates a reductase strongly activated by NADH, but inhibited by NADP⁺ at a concentration two orders of magnitude lower than the wild-type reductase. The additional substitution of arginine to methionine at position 597 lowers inhibition to that seen with the wild-type reductase, indicating that the binding of the 2'-phosphate of NADPH is an important component for NADP⁺-mediated inhibition. The double substitution protein (R597M and K602W) is inhibited by millimolar
concentrations of NADP⁺, a level much greater than wild-type, further emphasizing the importance of phosphate-binding residues in cofactor specificity.
CHAPTER FIVE

ACTIVITY ANALYSIS OF CYTOCHROME P450 REDUCTASE MUTANT PROTEINS
WITH SQUALENE MONOOXYGENASE AND P450 1A2

Activity of P450 Reductase Proteins with Physiological Redox Partners

Cytochrome c is an ideal substrate, as discussed in Chapter Two, for monitoring the kinetic activity of P450 reductase in vitro. However, cytochrome c is not a physiological redox partner for the reductase. In fact, cytochrome c is proposed to have a different binding site on P450 reductase than the cytochrome P450s (Shen and Kasper 1995). Assays utilizing physiological redox partners were needed to show that activities of cofactor substitution mutants characterized by cytochrome c are not unique to that substrate.

Squalene monooxygenase is an FAD-containing protein that catalyzes the addition of an oxygen atom across the 2,3-double bond of squalene, a linear 30-carbon intermediate in the cholesterol biosynthesis pathway, and is dependent on P450 reductase for reducing equivalents. The product of the reaction is 2,3-oxidosqualene, which can be separated from squalene by thin layer chromatography and radiolabel visualized by electronic autoradiography. Cytochrome P450 1A2, with reducing equivalents from P450 reductase, catalyzes the O-deethylation of ethoxyresorufin to resorufin. Resorufin is a highly fluorescent molecule and its formation can be monitored by fluorescence spectroscopy.

Squalene Monooxygenase Assays

Squalene monooxygenase assays were carried out as described (Laden, Tang et al. 2000) based on the method of Bai and Prestwich (Bai and Prestwich 1992). Reactions contained 20 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 30 µM FAD, 1mM NADPH, 28 pmols of cytochrome P450 reductase, and 19 pmols of squalene monooxygenase in 200 µL total volume. Incubations were started by the addition of 8
nmols of C\textsuperscript{14}-labeled squalene and were carried out for 30 minutes at 37°C. Reactions were stopped by extraction into methylene chloride and separated by thin layer chromatography on silica plates (Whatman) in 5% ethyl acetate in hexane. Plates were counted by electronic autoradiography on a Packard Instant Imager.

**Cytochrome P450 1A2 Assay: Ethoxyresorufin O-deethylase Activity (EROD)**

Purified human P450 1A2 was obtained from Oxford Biomedical and activity was determined by measuring deethylation of ethoxyresorufin (Sigma) to resorufin, as described (Burke and Mayer 1974). Incubation mixtures (2 ml) contained 2 µM ethoxyresorufin, 42 pmols of P450 1A2, 40 pmols reductase, 1 mM NADPH and/or NADH, and 0.1 µg/µl of phosphatidylcholine in 100 mM potassium phosphate buffer, pH 7.7. Following a 5-min preincubation at 37°C, reactions were started by the addition of cofactor and resorufin fluorescence was recorded for 15 minutes with an excitation wavelength of 510 nm and an emission wavelength of 586 nm. Turnover rates were calculated from linear time points.

**P450 Reductase Mutants Activate Physiological Redox Partners**

I determined the ability of the wild-type and three mutant proteins to support squalene monooxygenase activity with both NADPH and NADH (Fig. 5.1). Consistent with the results obtained with cytochrome c, the W677A protein showed limited activity with NADPH, having only ~13% of the activity of wild-type reductase. The addition of the R597M substitution to the W677A protein restored full activity with NADPH, while the further addition of the K602W substitution (R597M/K602W/W677A) reduced activity with NADPH to approximately 28% of wild-type activity. With NADH, the wild-type reductase supported squalene monooxygenase only poorly, and the W677A protein was only slightly more active. However, the double and triple mutants were 2.7- and 2.3-fold more active with NADH than the W677A mutant.
Cytochrome P450 1A2 catalyzes the O-deethylation of ethoxyresorufin, and the sensitivity of this assay made it ideal for further evaluation of reductase mutations. The results of these experiments are shown in Fig. 5.2. All mutant proteins showed significantly lower deethylase activity with NADPH than wild-type. With NADH all the mutants showed similar activity, roughly twice the activity of wild-type with NADH. To explore how these mutants might fare in a mixed cofactor environment, we determined EROD activity with equal concentrations of NADPH and NADH (1 mM). The W677A reductase exhibited only 3.5% of wild-type activity, despite the presence of ample NADH, indicating a marked inhibition by NADP⁺. In contrast, the R597M/W677A and R597M/K602W/W677A proteins showed activity levels approaching those seen with NADPH or NADH alone, indication that inhibition by NADP⁺ was not a factor.
FIGURE 5.2 O-deethylation of ethoxyresorufin by CYP 1A2 and P450 reductase mutants.
CHAPTER SIX
ACTIVITY ANALYSIS OF CYTOCHROME P450 REDUCTASE MUTANTS IN A RECOMBINANT AMES TEST

The Recombinant Ames Assay

The traditional Ames assay, depicted in Fig. 6.1, is a valuable tool for screening compounds which might be capable of causing mutations to DNA. The assay utilizes Salmonella typhimurium strains that are incapable of growth in media lacking histidine. This is due to a mutation in several genes necessary for the biosynthesis of histidine (Venitt and Parry 1984). These mutations are, depending on the strain, frameshifts or base pair substitutions. Reversion mutations which restore the ability to synthesize histidine are caused by DNA damaging chemicals. Because many compounds must be metabolized to their mutagenic form, mammalian enzymes must be added to the Ames test. The traditional Ames assay typically utilizes a rat liver homogenate (S9 fraction) for bioactivation of compounds. The make-up of this homogenate is typically poorly characterized and contains a mixture of enzymes normally present in rat liver. One limitation of the traditional Ames test is false negative results; e.g. when a chemical such as dimethylnitrosamine, which is a known human carcinogen, fails to generate a positive Ames test. This may be due to either to lack of the appropriate activating enzyme in the S9 or to DNA repair in tester bacteria. To address the first possibility recombinant plasmids which carry the cDNAs for expression of mammalian metabolizing enzymes (CYP 2E1 and P450 reductase) were constructed (Porter and Chang 1999), see Fig. 6.2; CYP 2E1 is believed to be the primary activator of dimethylnitrosamine, in vivo. However, cells transformed with a plasmid coexpressing CYP2E1 and P450 reductase failed to generate a positive Ames test (Cooper and Porter 2000). To address the possibility that the dimethylnitrosamine-induced mutations were being repaired, further studies were carried out in strains of S. typhimurium which lack, due to chromosomal deletion, one or both of the $O^6$-methylguanine DNA methyltransferase genes $ogt_{ST}$ and $ada_{ST}$ (YG7104 $ogt$- and YG7108 $ogt$-, $ada$-) (Yamada, Matsui et al. 1997). With these strains and expression plasmid, a
recombinant Ames test which is sensitive to dimethylnitrosamine was developed (Cooper and Porter 2000). This system allowed me to explore properties of P450 reductases that are able to utilize NADH in an “in vivo” setting. Furthermore, I could test the hypothesis that NADPH levels limit turnover of P450 reductase in vivo.

FIGURE 6.1 Traditional Ames assay.

The Recombinant Ames Assay: Materials and Procedures

The activation of dimethylnitrosamine in *Salmonella typhimurium* strain YG7108 was carried out as described (Cooper and Porter 2000) with pIN3ER plasmids expressing human cytochrome P450 2E1 and either wild-type rat cytochrome P450 reductase or the R597M/W677A, or R597M/K602W/W677A mutant proteins. Single colonies obtained from electroporation of pIN3ER WT and mutant plasmids were used to inoculate overnight cultures in LB media 100 µg/mL ampicillin with IPTG-mediated induction of protein expression. Cells were mixed with increasing concentrations of
dimethylnitrosamine in top agar and plated onto histidine-deficient Vogel-Bonner plates. After incubation for 48 hours at 37°C, visible colonies were counted.

**FIGURE 6.2 Recombinant Ames assay.**

*P450 Reductase Proteins Activate P450 2E1 in vivo*

To evaluate the effectiveness of the mutant reductases *in vivo*, each was coexpressed with cytochrome P450 2E1 in *Salmonella typhimurium* mutagenicity tester strains. The number of revertants generated with dimethylnitrosamine, a mutagenic substrate activated by P450 2E1, was determined with each reductase protein (Fig. 6.3). Bacteria containing the R597M/W677A reductase yielded nearly twice as many revertants as wild-type, suggesting that the improved ability to use NADH increased turnover and mutagen activation. Bacteria containing the R597M/K602W/W677A reductase showed very little mutagen activation, and this was shown to be due to very low protein expression (Fig. 6.4).
FIGURE 6.3 Activation of dimethylnitrosamine by CYP 2E1 and reductase proteins, *in vivo*. WT, wild-type; RM/WA, Arg597Met/Trp677Ala; RM/KW/WA, Arg597Met/Lys602Trp/Trp677Ala.
FIGURE 6.4 Western blot of P450 reductase WT, RM/WA and RM/KW/WA expression in YG7108 *Salmonella typhimurium* used in a recombinant Ames test. Lane 1, affinity purified wild-type reductase; 2, pIN3ER WT whole cell lysate; 3, pIN3ER RM/WA; 4, pIN3ER RM/KW/WA.

*NADH-utilization May Offer an Advantage in the Recombinant Ames Assay*

The Ames tests data presented above suggest that NADPH might be limiting under standard laboratory growth conditions, as suggested previously (Shet, Fisher et al. 1997). As shown, the R597M/W677A reductase mutant, which is able to utilize NADH in addition to NADPH, roughly doubled the number of revertants compared with wild-type. To further examine this possibility, mechanisms by which bacteria regulate oxidized and reduced pools of NADPH and NADH were studied, with the purpose of developing ways to modulate levels of reduced nucleotides in bacteria *in vivo*. By artificially raising or lowering the concentration of reduced NADPH or NADH, the question of rate-limitations due to P450 reductase could be studied by further Ames tests.

Living organisms, including bacteria, require reducing equivalents for respiration and subsequent production of ATP. These catabolic reducing equivalents are provided primarily by NADH (and FADH$_2$). Cells also require reducing power for biosynthetic reactions. Electrons for these anabolic reactions are supplied by NADPH. Bacteria, as
with other organisms, have control mechanisms which maintain proper redox balance between oxidized and reduced NADPH and NADH pools. In *Salmonella typhimurium*, redox status is maintained by regulation of input of carbohydrates into glycolysis, which ultimately generates NADH, and the pentose phosphate and Entner-Doudoroff pathways which generate NADPH.

The primary source of the hydride for NADPH in mammals comes from the pentose phosphate pathway, also known as the hexose monophosphate shunt. In this process, glucose-6-phosphate is converted to ribulose 5-phosphate, producing two NADPH for each molecule converted. Ribulose-5-phosphate is then converted to pyruvate and enters the glycolytic (NADH) pathway. While believed to be an important pathway in *E. coli*, the pentose phosphate pathway probably supplies less than 50% of NADPH needs (Csonka and Fraenkel 1977); Orthner and Pizer similarly found that the pentose phosphate pathway is a major user of glucose (10 - 30%) in *E. coli* (Orthner and Pizer 1974). While roughly half of necessary NADPH is produced via the pentose phosphate pathway, *E. coli* contain a transhydrogenase capable of transferring electrons directly from NADH to NADPH which makes a minor contribution to NADPH levels; the remainder of NADPH is produced through an unidentified pathway (Csonka and Fraenkel 1977). The Entner-Doudoroff pathway is present in *Salmonella typhimurium*, as well as *E. coli*, and is a second pathway from glucose to pyruvate during glycolysis that does not generate fructose-6-phosphate, but instead produces 2-keto-3-deoxy-6-phosphogluconate (KDPG) (Peekhaus and Conway 1998). The Entner-Doudoroff pathway shares its first two steps, oxidation of glucose to gluconate and phosphorylation to 6-phosphogluconate, with the pentose phosphate pathway in bacteria in a process that produces NADPH and is believed to provide an advantage during growth in gluconate-rich environments. Inhibition or upregulation of one of these three pathways might provide a suitable mechanism by which the ratio of reduced NADPH to NADH could be altered in bacterial cells.

As my data (Fig. 6.3) suggested that the availability of reduced NADPH might be limiting to the wild-type P450 reductase/P450 2E1 system in *Salmonella typhimurium*
under standard laboratory conditions when grown on glucose, means by which the oxidized versus reduced NADPH and NADH ratios could be modulated in vivo would be valuable tools to evaluate the significance of such a limitation. The pentose phosphate pathway, Entner-Doudoroff pathway, and the NADPH/NADH transhydrogenase are contributors to the NADPH pool in Salmonella typhimurium. 6-Amino-nicotinamide is a known inhibitor of NAD(P)H dependent enzymes, including what appears to be a pseudo-specific block of the oxidative steps of the hexose monophosphate shunt in mammals (Kohler, Barrach et al. 1970). Though this compound would inhibit the pentose phosphate pathway and would thereby lower NADPH levels, it would also be likely to inhibit all nicotinamide coenzyme utilizing enzymes, including the mutant proteins being evaluated, as well as those enzymes necessary for production of the nucleotides themselves, and was therefore not used. KDPG, mentioned above as an intermediate of the Entner-Doudoroff pathway, is a bacteriostatic metabolite that can inhibit the pentose phosphate pathway at the 6-phosphogluconate dehydrogenase step (Fuhrman, Wanken et al. 1998; Peekhaus and Conway 1998) and would also presumably lower NADPH levels. This compound, however, is not readily available commercially. Finally, it was noted that transhydrogenase activity was low in complex media, but high on glucose media (Houghton, Fisher et al. 1976). Gerolimatos and Hanson reported that the inhibitor in complex media was likely to be the amino acid leucine, which is able to inhibit the transhydrogenase reaction by up to 80% (Gerolimatos and Hanson 1978). Leucine is readily available and can be easily added to culture media. Inhibition of transhydrogenase activity might be expected to lower NADPH levels by inhibiting its synthesis from NADH during exponential growth. On the other hand, the use of gluconate in place of glucose as the primary carbohydrate source might be expected to increase the NADPH concentration by stimulating the pentose phosphate shunt below the rate limiting step of the pentose phosphate pathway (Fig. 6.5). In the following experiments, gluconate was added to stimulate the Entner-Doudoroff/pentose phosphate pathways (Pathways B & C). Leucine was added as an inhibitor of transhydrogenase (Pathway D).
FIGURE 6.5 Major pathways of production of NADP(H) reduction in *Escherichia coli* and *Salmonella typhimurium* (Csonka and Fraenkel 1977; Gerolimatos and Hanson 1978; Peekhaus and Conway 1998).

![Diagram of metabolic pathways]

**Experimental Design**

Ames tests were carried out as described above (Cooper and Porter 2000). The activation of dimethylnitrosamine in *Salmonella typhimurium* strain YG7108 was carried out with pIN3ER plasmids expressing human cytochrome P450 2E1 and either wild-type cytochrome P450 reductase or the R597M/W677A protein. Single colonies obtained from electroporation of pIN3ER and mutant plasmid were used to inoculate overnight cultures in LB media containing 100 μg/mL ampicillin and IPTG-mediated induction of protein expression. Cells were mixed with increasing concentrations of
dimethylnitrosamine in top agar and plated onto histidine-deficient Vogel-Bonner plates. Vogel-Bonner plates contained 1% glucose, 1% gluconate, or 1% glucose with 2 mM leucine. After incubation for 48 hours at 37°C, visible colonies were counted.

**Results of Reduced Nucleotide Pool Manipulation**

Ames assays were designed to analyze the effects of additions or changes to the culture media on mutagen activation by cells containing the mutant reductases. These culture media changes were intended to alter NADH/NADPH cofactor ratios in the cells. Gluconate was expected to increase NADPH levels relative to cells grown in glucose, whereas leucine was expected to decrease NADPH levels by blocking the transhydrogenase reaction. Media favoring an increase in NADPH over NADH should favor the wild-type reductase. Conversely, media designed to increase the NADH/NADPH ratio should favor the R597M/W677A protein versus wild-type, although my data with the *in vitro* experiments suggests that the R597M/W677A mutant should be rather insensitive to variation in nucleotide levels, assuming they remain at roughly the same total concentration [NADPH+NADH], because this mutant is able to utilize both cofactors at roughly the same efficiency. Consistent with expectations, with wild-type reductase gluconate slightly increased mutagenicity of DMN, whereas the addition of leucine to the medium slightly decreased mutagenicity (Fig. 6.6). However, the extent of the increase or decrease in mutagenicity was very small and not statistically significant, as indicated by the overlapping error bars. These results indicate that the effects of these media modifications on cofactor levels are likely to be small and easily compensated in the cells.
Unexpectedly, cells containing the R597M/W677A reductase mutant maintained a higher rate of mutagen activation in the presence of gluconate, despite the expectation that increased NADPH levels would not favor this enzyme. Indeed, DMN activation in gluconate media was greater than that obtained in glucose media. These results suggest that gluconate may simply increase reduced cofactor levels available to the enzyme, and thereby increase overall activity. DMN activation in the presence of leucine was essentially unchanged from that obtained in glucose media, arguing that preventing NADPH generation by the transhydrogenase pathway does not impair the activity of the R597M/W677A reductase mutant (Fig. 6.7). This would further suggest that the elevated activation of DMN in these cells, relative to cells containing wild-type reductase, can be attributed to the greater ability of the R597M/W677A reductase to use the more abundant NADH cofactor. It should be noted, however, that cofactor levels/ratios were not measured in these cells.
Western blotting of P450 reductase shows qualitatively that the wild-type reductase is expressed at a slightly higher level than the R597M/W677A protein (Fig. 6.8); sodium dodecyl sulfate-polyacrylamide gel electrophoresis shows equal protein loading. As all recombinant Ames assays show revertants with the RM/WA protein at a level roughly double that seen with the wild-type enzyme, this increase in DMN activation with the R597M/W677A reductase cannot be attributed to higher expression levels of this mutant protein, but rather must be an intrinsic characteristic of this protein. This is even more surprising given the generally lower activity of this protein when purified and assayed in vitro, as noted in the earlier studies (Chapters 3 and 5).
FIGURE 6.8 Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (Lanes 1 & 2) and Western blot (Lanes 3 & 4) of YG7108 Salmonella typhimurium tester strains expressing reductases proteins. Lane 1 & 3, wild-type reductase; 2 & 4, R597M/W677A.

In agreement with this blot data, cytochrome c reductase activities were measured with whole cell lysates of YG7108 Salmonella tester cells containing wild-type or the R597M/W677A reductase and indicate lower activity with the R597M/W677A reductase than with wild-type (Fig. 6.9). Assays were carried out in 100 mM Tris (pH 8.0), 0.75 mM ionic strength with KCl, 30 µM cytochrome c and 45 µg of total whole cell lysate protein. Reactions were started by addition of 1 mM nucleotide or a combination of 1mM for both nucleotides, and were carried out for 60 seconds and were monitored on a Hewlett Packard 8453 diode array spectrophotometer. The total reduction of cytochrome c was monitored at 550 nm using a millimolar extinction coefficient of 21.1 mM$^{-1}$ for one minute at room temperature.
FIGURE 6.9 Cytochrome c reductase activities of YG7108 *Salmonella typhimurium* cell lysates coexpressing cytochrome P450 2E1 and wild-type or mutant reductases.

As with the blotting data (Fig. 6.8), the lower cytochrome c reductase activity of the R597M/W677A reductase mutant relative to wild-type reductase *in vitro* is in marked contrast to the mutagenicity data obtained *in vivo*. Although various explanations are possible, one possibility consistent with all available data is that NADH is significantly more abundant than NADPH in bacterial cells, and thus the R597M/W677A reductase, with a 2-fold greater *k*\(_{\text{cat}}\) and 500-fold greater catalytic efficiency with NADH than the wild-type enzyme, has significantly greater activity in this *in vivo* setting.
Cytochrome P450 reductase has a 45,000-fold preference for NADPH over NADH, although the two cofactors differ only in the presence of a 2'-phosphate group on the adenine-ribose of NADPH. The three-dimensional structure of P450 reductase revealed three amino acids that interact with this phosphate: serine 596, arginine 597, and lysine 602 (Wang, Roberts et al. 1997) and it is assumed that these amino acids dictate the specificity for NADPH. Consistent with this prediction, Sem and Kasper (Sem and Kasper 1993) showed that substitution of a methionine for the positively charged arginine at position 597 produces a reductase with impaired ability to differentiate between the two cofactors. The studies presented here support this role for arginine 597, and demonstrate that substitution of lysine 602 leads to a further loss in specificity for NADPH. Of the four amino acids examined in this study, these two have the greatest influence on NADPH specificity: when combined these substitutions increase the $K_m$ for NADPH by 2,400-fold. The effect of these two substitutions on the $K_m$ for NADH was less predictable: although both decreased the $K_m$ individually (R597M 2-fold and K602W 5-fold), when combined the decrease was only about 1.5-fold. Moreover, the K602W mutation greatly decreased turnover with NADH. It is unclear why the substitution of tryptophan at this position should have such a detrimental effect on NADH-dependent activity, as sequence alignments suggest that a tryptophan is found at this position in NADH-specific enzymes (Fig. 1.10). Given the ambiguity in the alignments of the NADH-specific proteins in this region, it is possible that substitutions other than tryptophan at this position might more readily accommodate NADH binding and turnover.

Work by Scrutton and Perham (Scrutton, Berry et al. 1990; Bocanegra, Scrutton et al. 1993; Mittl, Berry et al. 1993) on the conversion of glutathione reductase to an NADH-specific enzyme suggested that an acidic residue opposite the adenine-ribose
hydroxyls should enhance the binding of NADH. This position is occupied by serine 596 in P450 reductase (Fig. 1.10). Consistent with this hypothesis, several NADH-specific reductases, including cytochrome b$_5$ reductase, contain an aspartate at this position. We generated the corresponding mutant in P450 reductase (S596D) but the resulting protein exhibited poor turnover with both NADH and NADPH. Although it was anticipated that a negatively charged amino acid at this position would strongly oppose NADPH binding by repelling the 2'-phosphate (and indeed, the $K_m$ for NADPH increased by over 20,000-fold), the marked decrease in $k_{cat}$ with NADH was not expected. Although the $K_m$ for NADH decreased by greater than half, it was not enough to overcome the 90% decrease in catalytic activity. The incorporation of additional mutations in the 2'-phosphate-binding site (R597M, K602W) or the W677A mutation did not restore activity with either cofactor, suggesting that the S596D substitution disrupted the structure of the cofactor-binding pocket. Thus, like the K602W mutation, the substitution of an “NADH-specific” amino acid at this position had the expected effect on activity with NADPH but did not enhance activity with NADH.

Gutierrez et al. (Gutierrez, Doehr et al. 2000) reported that, in contrast to native P450 reductase, a stable enzyme-NADP$^+$ complex could be formed with a reductase in which histidine is substituted for tryptophan 677. This tryptophan lies directly opposite the FAD isoalloxazine ring and is suggested to serve as a gating mechanism for cofactor binding. In support of this idea, movement of the tryptophan is seen during NADP(H) binding and release (Gutierrez, Paine et al. 2002). Loss or substitution of this tryptophan should enhance the binding of both NADH and NADPH, and indeed a remarkable increase in turnover with NADH is seen in W677 mutants (Dohr, Paine et al. 2001). However, turnover with NADPH is greatly decreased, apparently due to formation of this stable enzyme-NADP$^+$ complex. Second-site mutations that decrease the affinity of the enzyme for NADP(H) would be expected to decrease the stability of the enzyme-NADP$^+$ complex and decrease the inhibition by NADP$^+$. Consistent with this expectation, the R597M/W677A and R597M/K602W/W677A proteins were inhibited only at high NADP$^+$ concentrations, and retained good activity in the presence of both cofactors in an in vitro P450 assay.
Wild-type P450 reductase is evolutionarily designed to exclusively bind NADPH. Gluconate was added as a substrate to increase the NADPH/NADH ratio in *Salmonella typhimurium* during Ames tests. Wild-type reductase showed higher activation of DMN on gluconate in agreement with an increase in reduced NADPH levels versus glucose. The amino acid leucine was added to decrease the ratio of NADPH to NADH through inhibition of the transhydrogenase. Consistent with this hypothesis, wild-type P450 reductase showed reduced ability to activate DMN in the presence of leucine. The R597M/W677A protein, which showed strong NADH-dependent activity *in vitro*, produced a more sensitive Ames test, presumably due to a greater ability to utilize NADH for cytochrome P450 2E1-mediated activation of dimethylnitrosamine. However, the R597M/W677A protein also showed high activity with gluconate, a media designed to increase the NADPH/NADH ratio. Though unexpected, these data do not exclude the possibility that NADH utilization provides the R597M/W677A proteins superior activity *in vivo*.

**Future Studies with P450 Reductase Proteins**

Although I have identified mutations that enhanced the ability of P450 reductase to use NADH in the presence of NADPH, I did not create a reductase that is more efficient with NADH than NADPH. Even with the best mutants (R597M/W677A and R597M/K602W/W677A), the catalytic efficiency values \( (k_{cat}/K_m) \) for NADPH and NADH were, at best, equivalent. Thus it remains unclear how to engineer a P450 reductase to preferentially use NADH. Notably, a number of NADH-utilizing enzymes exhibit a preference for NADH over NADPH; for example, turnover with NADPH in NADH-dependent E. coli dihydrolipoamide dehydrogenase is undetectable (Bocanegra, Scrutton et al. 1993). Sequence comparisons of the cofactor-binding region of this protein to P450 reductase do not reveal obvious differences that might explain the greater selectivity of the dehydrogenase for NADH. Further biochemical and perhaps biophysical studies on P450 reductase will be needed to identify the additional changes needed to enhance selectivity for NADH. “Second-generation” mutants, based on the results of present studies, might be more effective. For example, substitution of aspartic
or glutamic acid for arginine 597, rather than methionine as was done in the present studies, might promote binding of NADH; the uncharged methionine residue reduced the catalytic efficiency with NADPH by 100-fold, but only increased the efficiency with NADH by 2-fold. The alignment data with NADH-specific enzymes suggests that an acidic amino acid in this segment is necessary for efficient NADH binding, but the exact location is unclear. In my studies, an acidic amino acid was incorporated at the position immediately preceding Arg597, but this substitution (Ser596Asp) yielded a protein with little activity with either nucleotide cofactor. Perhaps the location in 3-dimensional space of this acidic residue is critical to function. A second possible “second-generation” mutant, replacement of lysine 602 with alanine, rather than tryptophan as chosen from sequence alignment, might decrease NADPH-binding without negatively influencing interactions with NADH due to a bulky hydrophobic side-chain. Substitution of an acidic side-chain at the 602 position might also improve binding of NADH by providing an additional hydrogen bonding residue for the adenine hydroxyl of NADH.

Site-directed mutagenesis, while a hypothesis-driven technique, limits the number of substitutions that may be analyzed. A technique, such as random mutagenesis or gene shuffling, which maximizes the number and types of enzyme alterations, might produce a P450 reductase that shows a more profound switch in cofactor specificity than those mutants presented here. Random mutagenesis has been used to generate P450 enzymes with altered substrate specificity. In studies by Guengerich and colleagues, indole was shown to be metabolized by P450 1A2 and randomly generated 1A2 mutants coexpressed with P450 reductase in E. coli, to a variety of pigment compounds, including indigo (Nakamura, Martin et al. 2001). Such a screening technique might provide a way to analyze large numbers of randomly generated P450 reductase mutants by their ability to stimulate the P450 1A2-mediated conversion of indole to indigo. Perhaps the Ames test might provide an adequate screen for sorting the large number of enzymes produced. One limitation of this screen is that it might be difficult to distinguish between NADH-specific and reversion to wild-type NADPH activity. Additionally, an Ames assay screen would be expensive due to
the large numbers of plates that would be needed to analyze the many mutant proteins generated.

The studies reported here do indicate that it is possible to engineer P450 reductase for use *in vivo* by rational design and subsequent evaluation of enzymes *in vitro*. In addition to providing a valuable tool for understanding the determinants of nucleotide cofactor specificity, these mutants might also lend themselves to creation of bioremediation with increased enzymatic activity, as well as cost-effective reconstitution of enzyme systems that do not require the use of expensive reducing equivalents from NADPH.
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PERSONAL INFORMATION

Name: Calvin Lee Elmore
Date of Birth: September 24, 1972
Place of Birth: Athens, Georgia

EDUCATION

September 1998 – December 2002
Graduate Center for Toxicology
University of Kentucky
Lexington, Kentucky

Bachelor of Science in Environmental Health Science
Environmental Health Science Program
The University of Georgia
Athens, Georgia

HONORS AND AWARDS

2002 Graduate Center for Toxicology, Curriculum Committee
2001 Graduate Center for Toxicology, Admissions Committee,
2001 Graduate Center for Toxicology, Program Review Committee
2000 – 2001 Toxicology Student Forum, President
1999 – 2002 National Institutes of Environmental Health Training Grant Support
1999 – 2000 Toxicology Student Forum, Secretary
1995 Epsilon Nu Eta, Environmental Health Sciences Department University of Georgia

PRESENTATIONS

2002 Seminar, Department of Biochemistry, University of Texas Health Science Center. “Building cofactor specificity into cytochrome P450 reductase”

2002 Lecture, TOX 509, Graduate Center for Toxicology, University of Kentucky. “Definitions, Concepts, and Principles of Toxicology”

2001 Seminar, Graduate Center for Toxicology, University of Kentucky. “Building a Better Reductase: Can We Improve on Nature?”

PUBLICATIONS


ABSTRACTS
