Mouse Pulmonary Cytochrome P-450 Naphthalene Hydroxylase: cDNA Cloning, Sequence, and Expression in *Saccharomyces cerevisiae*†

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ABSTRACT: We have isolated a cDNA clone, Nah-2, encoding the cytochrome P-450$_{Nah}$ (naphthalene hydroxylase) from a mouse lung λZAP cDNA library using anti-cytochrome P-450$_{Nah}$ IgG as a probe. This same antibody selectively blocked [Nagata, K., Martin, B. M., Gillette, J. R., & Sasame, H. A. (1990) *Drug Metab. Dispos. 18*, 557–564] the cytochrome P-450 in mouse lung microsomes that catalyzed the conversion of naphthalene to (1*R*,2*S*)-naphthalene 1,2-oxide, which has been postulated as a causative agent in the naphthalene-induced tissue-specific necrosis of Clara cells in mouse lung. The toxic effect is seen in mouse and not in rat. The cDNA encodes a polypeptide of 491 amino acids with a molecular mass of 50 kDa. Northern blot analysis with an Nah-2-specific probe revealed that the mRNA is expressed in a species-and tissue-specific manner, present only in mouse lung and liver and not in that of rat. The mRNA encoding Nah-2 is constitutively expressed and is not induced by either phenobarbital, pyrazole, pregnenolone 16α-carbonitrile, or 3-methylcholanthrene. Comparative amino acid sequence analyses with other documented members of the P-450 gene superfamily revealed that this encoded protein is in the IIF subfamily. To analyze its substrate specificity, the cDNA was inserted into the vector, pAAH5, and expressed in the *Saccharomyces cerevisiae* strain, AH$_{22}$. The presence of cytochrome P-450$_{Nah}$ in the microsomes isolated from transformed cells and analyzed by Western blot was confirmed by immunocomplexing product with anti-cytochrome P450$_{Nah}$ IgG. Furthermore, activity toward naphthalene in the microsomes from the transformed cells established that this clone encodes a naphthalene hydroxylase. Like lung microsomes and purified and reconstituted cytochrome P450$_{Nah}$, transformed yeast microsomes convert naphthalene primarily to the *trans*-(1*R*)-hydroxy-(2*R*)-glutathionyl-1,2-dihydronaphthalene conjugate, a stable form of the putative toxicant (1*R*,2*S*) oxide in the presence of glutathione and a mixture of glutathione S-transferases. Results of immunochemical studies support a role of this cytochrome P-450 in lung toxicity in mice exposed to high doses of naphthalene.

Naphthalene is a widespread environmental contaminant and is present in diverse sources such as ground water (Sitting, 1980) and cigarette smoke pyrolyzate (Schmelz et al., 1976). Industrial implications of naphthalene span from its use as a starting material in the syntheses of dyes and carbonyls to its elimination as a byproduct in the coke oven and smelting industry (Sitting, 1980). The finding that exposure of the mouse to high doses of naphthalene results in necrosis of the unciliated bronchiolar epithelial cell (Clara cell) of murine lung (Mahvi et al., 1977; Tong et al., 1982) has created concern over the possible health hazards of this agent. Furthermore, it has been shown that naphthalene is metabolized to a reactive and potentially toxic intermediate, naphthalene epoxide, by the cytochrome P-450 dependent monooxygenase system (Warren et al., 1982). Toxicants generated by the cytochrome P-450 isozymes in the respiratory system are of special interest because of easy access of environmentally borne substances to such organs and because of the inferior level of the detoxifying phase II enzymes in such tissues. Hence, it is critical to understand the metabolic potential of this system and, thus, the potential dangers of having certain chemicals reach the nasal and respiratory tissues.

Since recombinant DNA technology and its ancillary methods allow researchers to identify substrate specificity without the need to purify proteins, there has been a surge in the understanding of the role of cytochrome P-450 in biology. Several forms have been cloned, isolated, and characterized which may have been relatively unapproachable by classical biochemical means due to the extremely low levels of the cytochrome P-450s. At least four of the nine families (I–IV) of the cytochrome P-450 superfamily (Nebert et al., 1989) are involved in the metabolism of xenobiotics (Gonzalez, 1990) and include members found in the respiratory system. A cytochrome P-450 expressed in the olfactory neuroepithelium has been classified as a member of the II family (IIg) (Nef et al., 1989), although its substrate specificity is still unknown. Cytochrome P-450 p-2, prostaglandin ω-hydroxylase, has been cloned and isolated from a lung cDNA library using mRNA extracted from pregnant rabbit (Matsubara et al., 1987). This particular isoform belongs to the IV family and is designated IVA4. The isolation and purification of two other cytochrome

† The nucleotide sequence for Nah-2 reported in this paper has been submitted to GenBank under Accession Number J05349.

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P-450s from rabbit lung have led to the identification of two members which belong to the subfamily IIB4 (Gasser et al., 1988). Although the substrate preference of the various new isoforms has not been established, the possibility exists that expression of encoded forms in appropriate host cells will yield this critical information.

Since the low concentration of P-450 in the lung is such an obstacle to purifying important lung isoforms to homogeneity, we have circumvented this problem through the isolation of a mouse lung cDNA clone encoding Nah hydroxylase by immunocomplexing a fusion protein. Recently Nagata et al. (1990) succeeded in the purification of a cytochrome P-450[1] from untreated mouse liver which catalyzes the hydroxylation of naphthalene. Antibody prepared against this purified form completely blocked the NADPH-dependent formation of naphthalene–glutathione conjugate 2 catalyzed by mouse lung microsomes in the presence of glutathione and glutathione S-transferases. We have utilized this antibody to screen a mouse lung cDNA library for the clone reported in this study.

Experimental Procedures

Animals. Male NIH Swiss mice weighing 20–25 g were housed in plastic cages and allowed free access to standard chow and water. The mice were treated by intraperitoneal injection with phenobarbital (80 mg/kg in saline), pyrazole (150 mg/kg in saline), pregnenolone 16α-carbonitrile (75 mg/kg in corn oil), or 3-methylcholanthrene (80 mg/kg in corn oil) for three days and deprived of food overnight before sacrifice.

Materials. Naphthalene, 7-hydroxycoumarin, 7-ethoxycoumarin, and benzo[*a*]pyrene were from Aldrich (Milwaukee, WI) and 3-hydroxybenzo[*a*]pyrene was from the National Cancer Institute Chemical Repository. Deoxycytidine [α-^{32}P]triphosphate was supplied by Amersham (Arlington Heights, IL). Restriction enzymes and other reagents used in molecular biology techniques were from New England Biolabs (Beverly, NY), IBI Biochemical (New Haven, CT), Bethesda Research Labs (Bethesda, MD), or Boehringer-Mannheim (Indianapolis, IN). *Saccharomyces cerevisiae* strain AH_{22} was a gift from Dr. H. Ohkawa (Sumitomo Chemical Co., Hyogo, Japan). The vector pAAH5 was from Dr. B. Hall (University of Washington). Glutathione S-transferases were a gift from Dr. Allan Buckpitt (University of California, Davis, CA).

Construction of the Mouse Lung cDNA Library. Lungs were removed as quickly as possible from 10 DBA/2J male mice that were treated with pyrazole as described previously (Negishi et al., 1989) and immediately frozen by submersion in liquid nitrogen. The frozen tissues were pulverized in a dry ice mix in mortar with pestle and subjected to the isolation of total RNA according to the method of Chirgwin et al. (1979). Poly(A)+ RNA was affinity purified on an oligo(dT)–cellulose column. Double-stranded cDNA was synthesized from poly(A)+ RNA by the Okayama and Berg (1982) method and ligated to the bacteriophage vector λZAP (Stratagene, La Jolla, CA) with *Eco*RI linkers. Recombinant phage infected BB4 cells (Stratagene) were screened with anti-cytochrome $P\text{-}450_{Nah}$ IgG (Nagata et al., 1990) following the supplier's recommended protocol.

Nucleotide Sequence Determination of the Nah-2. cDNA-positive phages were plaque-purified by three rounds of plating, and the pBluescript SK$^-$ plasmid carrying the positive cDNA in the *Eco*RI site was rescued according to the in vivo excision protocol of Stratagene. Single colonies were cultured in LB broth containing ampicillin (100 μg/mL), and plasmid DNA was purified by the rapid PEG method of Mierendorf and Pfeffer (1989). After insert size estimation by *Eco*RI digestion, the clone bearing the longest insert, Nah-2, was selected for further characterization. For the sequence analysis of the plus and minus strands of Nah-2, two series of plasmids with nested deletions of the Nah-2 insert were constructed by first using the double restriction–digestion with the combinations *Apa*I–*Cla*I and *Sac*II–*Xba*I, respectively, and then using the Erase-A-Base kit (Promega). Direct dideoxy sequencing was carried out essentially as described before (Ritter et al., 1990), using highly purified and supercoiled double-stranded plasmid DNA as template and either the T7 (*Apa*I–*Cla*I series) or T3 (*Sac*II–*Xba*I series) primers (Stratagene).

Northern Blot Analysis of mRNA Isolated from Various Tissues of Rat and Mouse with ^{32}P-Labeled Nah-2 and Coh Probes. Lung, liver, kidney, and intestinal mRNA from the mouse and rat were isolated according to the guanidinium isothiocyanate method of Chirgwin et al. (1979) and affinity-purified by chromatography through oligo(dT)–cellulose (Collaborative Research Inc., Lexington, MA). For a comparative study of the effect of various drugs on mouse lung mRNA, the oligo(dT)–cellulose step was omitted. Poly(A)+ RNA (1–2 μg) or total RNA (20 μg) was isolated from intact lung tissue, electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, and transferred onto Zetabind membrane (AMF-CUNO, Meriden, CT) according to the manufacturer's instructions. The Northern blot was hybridized with ^{32}P-labeled probes for Nah-2 and Coh (coumarin hydroxylase), according to Church and Gilbert (1984). The probes were prepared by *Eco*RI digestion, followed by agarose gel separation and isolation by electroelution of the complete 1836-bp Nah-2 insert or 1400-bp 3′-*Eco*RI fragment of mouse coumarin 7-hydroxylase cDNA (Negishi et al., 1989). In one experiment, a 3′-specific probe was prepared which corresponded to the 3′-*Bam*HI fragment (360-bp) of Nah-2. The electroeluted DNA was then submitted to a second gel purification step using 1% low melting point agarose (Bethesda Research Labs) in 50 mM Tris–acetate buffer, pH 7.75. The respective bands were excised, melted, and diluted with 0.5× TE to a concentration of approximately 2 ng/μL. The inserts (50 ng each) were ^{32}P-labeled to a specific activity of greater than 1×10^9 dpm/μg using a random hexamer labeling kit (Pharmacia) and 6000 Ci/mmol ^{32}P-labeled deoxycytidine triphosphate (Amersham) in the presence of low melting point agarose (Struhl, 1985). Similarly, radiolabeled rat cyclophilin cDNA was used to normalize the amount of RNA added to the gel. Because cyclophilin is a highly conserved structure with a broad tissue and phylogenetic distribution (Koletsky et al., 1986), its cDNA has been adapted to establish mRNA levels (McKinnon et al., 1987).

Expression of Nah-2 in Yeast AH_{22} Cells. The full-length *Eco*RI insert, Nah-2, was polished with Klenow and ligated to the vector, pAAH5, for expression in yeast. pAAH5 contains ADH1 promoter and terminator sequences from the alcohol dehydrogenase (*ADH1*) gene and the *Leu2* gene from *S. cerevisiae*. This was accomplished by ligating polished Nah-2 (in 1% low melting point agarose) with pAAH5 that had been cleaved with *Hin*dIII, polished with Klenow, and dephosphorylated with calf intestinal alkaline phosphatase. The ligation was performed in solidified low melting point

[1] For the sake of clarity, cytochrome P-450m50b and its antibody, anti-P-450m50b IgG (11), will be henceforth referred to as cytochrome $P\text{-}450_{Nah}$ and anti-$P450_{Nah}$ IgG, respectively. This is to reflect its major substrate activity and identity with the cytochrome P-450 encoded by the cDNA clone, Nah-2.

agarose as described (Struhl, 1985). Products of the ligation reaction were used to transform frozen competent cells of the *Escherichia coli* strain, XLI-Blue (Stratagene), prepared using protocol 3 of Hanahan (1985). Isolates of the vector carrying the Nah-2 insert in the correct orientation with respect to the *ADH1* promoter, were used to transform cells of the yeast *S. cerevisiae* strain, AH_{22} [*MATa, leu2-3, 112 his4-519 canl* (*cir*$^+$)] (Oeda et al., 1985), by the lithium procedure described by Rothstein (1985). Wild-type AH_{22} is a *Leu2* auxotroph and was grown in enriched YPD medium. Yeast clones transformed with the expression unit, pNah-2, were selected by complementation for *Leu2* auxotrophy by growth in yeast minimal medium containing 0.01% of each amino acid (except leucine), 0.67% yeast nitrogen base (GIBCO Laboratories, Chagrin Falls, OH), and 1.0% dextrose. Microsomes were prepared as described (Oeda et al., 1985). For immunoblot analysis, microsomal protein was electrophoresed through sodium dodecyl sulfate containing polyacrylamide (Laemmli, 1970), electroblotted onto nitrocellulose, and immunoreacted with anti-cytochrome $P\text{-}450_{Nah}$ IgG (Nagata et al., 1990) followed by the goat anti-rabbit IgG–horseradish peroxidase conjugate as described (Towbin et al., 1979; Domin et al., 1984).

Biochemical Characterization of Nah-2-Encoded Monooxygenase Expressed in Yeast Microsomes. A total of 2 mg of microsomes from wild-type or transformed yeast was preincubated with cytochrome P-450 reductase (100 pmol) for 60 min on ice, followed by preincubation with either preimmune IgG or anti-cytochrome $P\text{-}450_{Nah}$ IgG (1 mg) for 15 min at room temperature. The treated microsomes were incubated in a 0.05 M phosphate buffer, pH 7.4, containing naphthalene (0.5 mM), glutathione (2 mM), glutathione S-transferases (5 units), $MgCl_2$ (5 mM), EDTA (1 mM), NADPH (1.5 mM 2×) for 160 min at 37 °C. Preliminary experiments established that the reaction was linear over 160 min in the generation of an optimum amount of product. The reaction was terminated by the addition of 2 volumes of ice-cold methanol. After the removal of protein by centrifugation, methanol was stripped from the mixture by bubbling a stream of nitrogen, and the residual aliquots were extracted with ethyl acetate three times to remove all nonpolar materials. The remaining aliquots were concentrated to approximately 200 μL. A total of 20 μL of each reaction was loaded onto a C^{18} Novapak column and chromatographed under a mobile phase which consisted of 5% acetonitrile, 1% acetic acid, and 94% water as described previously (Nagata et al., 1990). Assays of benzo[*a*]pyrene hydroxylase and ethoxycoumarin deethylase activity were performed by the fluorometric methods of Nebert and Gelboin (1968) and Greenlee and Poland (1978), respectively.

Results

Isolation of Mouse Lung Nah cDNA Clones. Complementary DNAs representing possible naphthalene hydroxylase clones were detected by screening a mouse lung λZAP cDNA library with anti-cytochrome $P\text{-}450_{Nah}$ IgG as described under Experimental Procedures. Ten positive pBluescript SK$^-$ clones possessing inserts of approximately equal length (1.5–1.8 kb) were isolated. Digestion of each with *Rsa*I and *Taq*I resulted in identical restriction patterns for all 10 clones. The longest of these, Nah-2, was chosen for sequence analysis.

Nucleotide Sequence of Nah-2. The sequence data show that the insert contains 1810 bp with an open reading frame of 1473 bp flanked by 61 and 276 bp of 5′ and 3′ untranslated sequences, respectively (Figure 1). The clone encodes a polypeptide containing 491 amino acids with the translation initiation codon indicated by nucleotide position +1. The translation stop codon, TGA, is located after deduced amino acid residue 491. A putative polyadenylation signal sequence is present in the cDNA at nucleotide 1719 with a short segment of poly(A)+ starting at nucleotide 1739.

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                                                    ACACAAGAACTTCAGA  -46
ACATTGCTGCAGAATACTGATACAGGCGCTGCAGCTGCCTTCACT                         -1
ATGGATGGTGTGAGCACAGCCATCTTGCTTCTCCTCCTGGCTGTC                         45
 Met Asp Gly Val Ser Thr Ala Ile Leu Leu Leu Leu Ala Val
ATCTCTCTGTCCCTGACCTTCAGCTCACGGGGCAAGGGCCAGCTG                         90
 Ile Ser Leu Ser Leu Thr Phe Ser Ser Arg Gly Lys Gly Gln Leu
CCTCCAGGACCCAAACCTCTCCCAATCCTGGGAAACCTGCTGCAG                        135
 Pro Pro Gly Pro Lys Pro Leu Pro Ile Leu Gly Asn Leu Leu Gln
CTTCGCTCCCAAGACTTGCTGACCTCCCTCACCAAGCTTAGCAAG                        180
 Leu Arg Ser Gln Asp Leu Leu Thr Ser Leu Thr Lys Leu Ser Lys
GAGTATGGGTCGGTGTTCACGGTGTACCTGGGGTCCAGGCCTGTG                        225
 Glu Tyr Gly Ser Val Phe Thr Val Tyr Leu Gly Ser Arg Pro Val
ATAGTCCTCAGCGGATACCAAACTGTGAAGGAGGCTCTTGTGGAC                        270
 Ile Val Leu Ser Gly Tyr Gln Thr Val Lys Glu Ala Leu Val Asp
AAAGGGGAGGAGTTCAGTGGCCGAGGCGCATACCCCGTCTTTTC                         315
 Lys Gly Glu Glu Phe Ser Gly Arg Gly Ala Tyr Pro Val Phe Phe
AACTTCACCAGGGGCAACGGCATCGCCTTCTCCGATGGAGAGCGC                        360
 Asn Phe Thr Arg Gly Asn Gly Ile Ala Phe Ser Asp Gly Glu Arg
TGGAAGATCCTCAGAAGGTTCTCTGTCCAAATCCTGCGGAACTTT                        405
 Trp Lys Ile Leu Arg Arg Phe Ser Val Gln Ile Leu Arg Asn Phe
GGCATGGGAAAAAGAAGCATCGAGGAGCGGATCCTGGAAGAAGGC                        450
 Gly Met Gly Lys Arg Ser Ile Glu Glu Arg Ile Leu Glu Glu Gly
AGCTTCCTGCTGGAGGTGCTGAGGAAAATGGAAGGCAAGCCCTTT                        495
 Ser Phe Leu Leu Glu Val Leu Arg Lys Met Glu Gly Lys Pro Phe
GACCCCGTGTTTATCCTGAGCCGCTCTGTGTCCAACATTATCTGC                        540
 Asp Pro Val Phe Ile Leu Ser Arg Ser Val Ser Asn Ile Ile Cys
TCTGTCGTCTTCGGAAGTCGCTTCGACTATGACGATGAGCGTCTG                        585
 Ser Val Val Phe Gly Ser Arg Phe Asp Tyr Asp Asp Glu Arg Leu
CTCACCATCATCCACTTTATCAATGACAACTTCAAGATTATGAGC                        630
 Leu Thr Ile Ile His Phe Ile Asn Asp Asn Phe Lys Ile Met Ser
AGCCCTTGGGGCGAGATGTACAACATCTTCCCAAGTGTCCTTGAT                        675
 Ser Pro Trp Gly Glu Met Tyr Asn Ile Phe Pro Ser Val Leu Asp
TGGATACCTGGGCCACACAAACGCTTGTTCCGGAACTTTGGAGGC                        720
 Trp Ile Pro Gly Pro His Lys Arg Leu Phe Arg Asn Phe Gly Gly
ATGAAAGATCTCATTGCCCGCAGCGTCCGCGAACACCAGGATTCC                        765
 Met Lys Asp Leu Ile Ala Arg Ser Val Arg Glu His Gln Asp Ser
CTGGACCCCAACTCTCCCCGGGACTTCATCGACTGCTTCCTCACA                        810
 Leu Asp Pro Asn Ser Pro Arg Asp Phe Ile Asp Cys Phe Leu Thr
AAGATGGCACAGGAGAAGCAAGACCCACTGAGCCACTTCAATATG                        855
 Lys Met Ala Gln Glu Lys Gln Asp Pro Leu Ser His Phe Asn Met
GATACCCTGCTGATGACCACACACAACCTGCTCTTCGGTGGCACA                        900
 Asp Thr Leu Leu Met Thr Thr His Asn Leu Leu Phe Gly Gly Thr
GAAACCGTGGGCACCACACTGCGTCACGCCTTCCTTATTCTTATG                        945
 Glu Thr Val Gly Thr Thr Leu Arg His Ala Phe Leu Ile Leu Met
AAATACCCCAAAGTGCAAGCCCGCGTGCAGGAAGAGATTGACCGT                        990
 Lys Tyr Pro Lys Val Gln Ala Arg Val Gln Glu Glu Ile Asp Arg
GTGGTGGGGCGCTCGCGGATGCCGACGCTGGAAGACCGTACATCC                       1035
 Val Val Gly Arg Ser Arg Met Pro Thr Leu Glu Asp Arg Thr Ser
ATGCCTTACACAGATGCAGTGATCCACGAAGTGCAACGCTTTGCT                       1080
 Met Pro Tyr Thr Asp Ala Val Ile His Glu Val Gln Arg Phe Ala
GACGTCATCCCCATGAACCTGCCTCACCGTGTCACTCGGGACACA                       1125
 Asp Val Ile Pro Met Asn Leu Pro His Arg Val Thr Arg Asp Thr
CCTTTCCGGGGCTTCCTGATACCCAAGGGCACAGATGTCATCACA                       1170
 Pro Phe Arg Gly Phe Leu Ile Pro Lys Gly Thr Asp Val Ile Thr
CTCCTTAACACTGTGCACTACGACTCGGACCAGTTCAAGACGCCT                       1215
 Leu Leu Asn Thr Val His Tyr Asp Ser Asp Gln Phe Lys Thr Pro
CAGGAGTTCAATCCTGAACATTTTCTGGACGACAATCATTCTTTC                       1260
 Gln Glu Phe Asn Pro Glu His Phe Leu Asp Asp Asn His Ser Phe
AAAAAGAGCCCCGCCTTCATGCCATTTTCGGCTGGACGTCGACTG                       1305
 Lys Lys Ser Pro Ala Phe Met Pro Phe Ser Ala Gly Arg Arg Leu
TGTCTGGGAGAGCCACTGGCGCGCATGGAGCTCTTCATATACTTC                       1350
 Cys Leu Gly Glu Pro Leu Ala Arg Met Glu Leu Phe Ile Tyr Phe
ACCTCCATTCTGCAGAACTTCACATTGCAGCCGCTGGTGGATCCT                       1395
 Thr Ser Ile Leu Gln Asn Phe Thr Leu Gln Pro Leu Val Asp Pro
GAGGACATCGACCTGACCCCGCTCAGCTCAGGGCTGGGCAATTTG                       1440
 Glu Asp Ile Asp Leu Thr Pro Leu Ser Ser Gly Leu Gly Asn Leu
CCAAGGCCTTTCCAGCTGTGTATGCACATTCGCTGAGTACTGCGC                       1485
 Pro Arg Pro Phe Gln Leu Cys Met His Ile Arg
CCAGGGACCCCTGTCCTTCTTCCAGTTGGGGTTCACTGTCATAGG                       1530
CCTCCATTGATATCTCTCTCACATGATCTTCCCTTAACCCTGGGC                        1575
CTGCCACGTATCAGTACTTTACCCCGCCTATCTTAAGCCCATCTT                        1620
CATGGAAAGAATGACGTGACAAAGGTGAAATACCCGTCTTATACG                        1665
CACAGAACCTATTCTATGATGCACCCTTTTCCTGTCTGTTTGTAT                        1710
CATTTCCTAGTAAATATCTTAATAACTGAAAAAAAAAAA                              1749
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FIGURE 1: Nucleotide and deduced amino acid sequence of the Nah-2 cDNA. The nucleotide sequence of the Nah-2 cDNA was formatted and translated using the DNAdraw program developed by Marvin Shapiro of the Division of Computer Research and Technology (NIH). Start and stop codons of the 1473-base open reading frame specifying the Nah protein are highlighted in reverse font. The putative polyadenylation signal is underlined.

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Nah MDGVSTAILLLLLAVISLSLTFSSRGKGQLPPGPKPLPILGNLLQLRSQD 50
IIF1   SI           LVC L  L   D  K     R  S      L C   50

Nah LLTSLTKLSKEYGSVFTVYLGSRPVIVLSGYQTVKEALVDKGEEFSGRGA 100
IIF1 M              MY  H  P R V       A        Q        D 100

Nah YPVFFNFTRGNGIAFSDGERWKILRRFSVQILRNFGMGKRSIEERILEEG 150
IIF1   A     K        S D   V  Q  I                      150

Nah SFLLEVLRKMEGKPFDPVFILSRSVSNIICSVVFGSRFDYDDERLLTIIH 200
IIF1      ADV  T  E    T V                L              R 200

Nah FINDNFKIMSSPWGEMYNI....FPSVLDWIPGPHKRLFRNFGGMKDLIA 246
IIF1 L      Q         L D LDPR   L   V    Q I Q  KCLR     250

Nah RSVREHQDSLDPNSPRDFIDCFLTKMAQEKQDPLSHFNMDTLLMTTHNLL 296
IIF1 H  HD  A ....       Q       E  E       H             296

Nah FGGTETVGTTLRHAFLILMKYPKVQARVQEEIDRVVGRSRMPTLEDRTSM 346
IIF1      K  S   H     A                L    A L A K  AA  346

Nah PYTDAVIHEVQRFADVIPMNLPHRVTRDTPFRGFLIPKGTDVITLLNTVH 396
IIF1                  I                 A                396

Nah YDSDQFKTPQEFNPEHFLDDNHSFKKSPAFMPFSAGRRLCLGEPLARMEL 446
IIF1   PS  L              A Q                    L       446

Nah FIYFTSILQNFTLQPLVDPEDIDLTPLSSGLGNLPRPFQLCMHIR 491
IIF1  L L A   S S     GA                    LRP   491
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FIGURE 2: Comparison of deduced amino acid sequences of the Nah-2 and IIF1 cDNAs. Computer alignment using the UWGCG program GAP shows the relative identity of the deduced amino acid sequences between the two from cDNA clones. Each box encloses a peptide sequence which represents a gap in the opposite protein.

Table I: Percent Homology of P-450$_{Nah}$ cDNA and Protein to Representative Members of Major P-450 Families and Subfamilies

cytochrome P-450	trivial name	species	nucleic acid % identity	amino acid % similarity[a]	amino acid % identity[a]
IA1	c	rat	46	57	33
IIA3	15α	mouse	59	73	53
IIB10	16α	mouse	57	71	49
IIC7	f	rat	56	71	52
IID1	db1	rat	52	61	40
IIE1	j	rat	56	71	48
IIF1		human	82	91	82
IIIA1	PCN	rat	42	50	25
IVA1	LAω	rat	39	48	23

[a] The deduced amino acid sequence of Nah-2 was aligned with other cytochrome P-450s and their percent similarity and identity were calculated with the UWGCG program GAP.

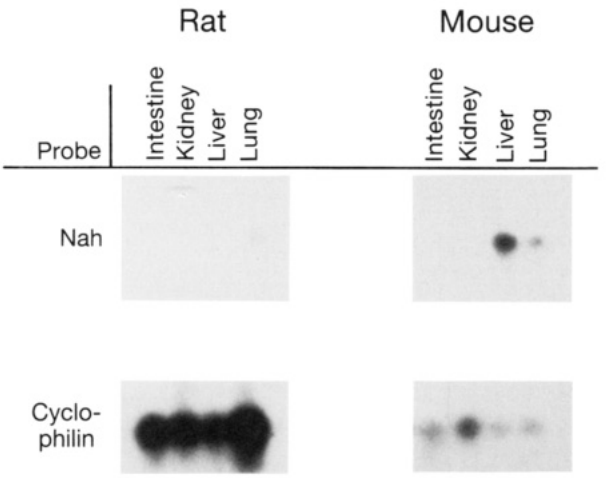

FIGURE 3: Distribution of Nah-2 mRNA in tissues from mouse and rat. Poly(A)+ RNA (1–2 μg) isolated from lung, liver, kidney, and intestine of rat and mouse were electrophoresed through formaldehyde–agarose, transferred onto a Zetabind membrane, and hybridized with ^{32}P-labeled random primed Nah-2 full-length cDNA as described under Experimental Procedures. After being washed, the blot was exposed to XAR-5 film to generate the autoradiogram shown.

Nucleotide alignment of Nah-2 against representative members of cytochrome P-450 families I–IV clearly classifies this clone to the IIF1 subfamily (Table I). Comparisons of the coding region of Nah-2 and the cytochrome P-450IIF1 cDNA revealed a high degree of sequence identity: 82% in nucleic acid and 82% in amino acid. Nevertheless, in the first nine N-terminal amino acids there are two mismatched amino acids (Figure 2), while there is none between the deduced N-terminal sequence of this cDNA and cytochrome P-450$_{Nah}$ purified from mouse liver microsomes (Nagata et al., 1990). In contrast, the nucleic acid and deduced amino acid sequences of Nah-2 are comparable in identity to other members of the II family, ranging from 52 to 59% and 40 to 53%, respectively.

Distribution of mRNA Coding for Cytochrome P-450$_{Nah}$. The ^{32}P-labeled Nah-2 cDNA was hybridized to mRNA from lung, liver, kidney, and small intestine of mouse and rat. The distribution of the mRNA was found to be species- and tissue-specific (Figure 3). There was no detectable Nah-2 mRNA in either kidney or small intestine of either species or in rat liver. After normalization of the Nah-2-specific mRNA hybridization signal to the level of cyclophilin mRNA present in each sample, the level of Nah-2 mRNA in rat lung was 50–100-fold less than that in the lungs of mice.

There was 5–10 times as much Nah-2 mRNA in mouse liver as in mouse lung. This ratio was not altered when a more specific 360-bp probe was used in the hybridization which corresponded to the last 85 bp of the coding region and the entire 3′-untranslated region (data not shown). In contrast, rat lung contains a higher level of mRNA that cross-hybridizes to the Nah-2 probe than does the liver.

Lung mRNA Levels after Treatment of Mice with Various Agents. In order to evaluate possible regulatory mechanisms associated with the Nah-2 gene, total RNA samples isolated from the lungs from mice pretreated with various inducing agents, including phenobarbital, pyrazole, pregnenolone 16α-carbonitrile, or 3-methylcholanthrene, were analyzed by Northern blot using ^{32}P-Nah-2 as probe. Unlike many other cytochrome P-450 mRNAs which are inducible by various xenobiotics, the level of Nah-2 mRNA in lung was not increased in response to treatment with any of the inducers tested, suggesting that this is a constitutively regulated form (Figure 4). These results are in agreement with the lack of inducibility of the lung microsomal naphthalene hydroxylase activity by these agents (H. Sasame, unpublished data). The validity of induction was documented by probing a duplicate

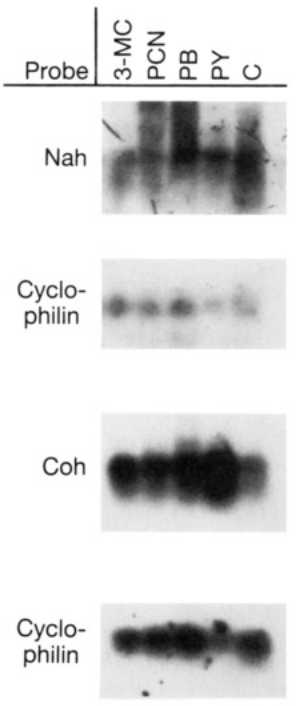

FIGURE 4: Effect of various inducing agents on the levels of Nah-2 mRNA in mouse lung. Total RNA (20 μg) from lung isolated from either untreated mice (C) or mice treated with pyrazole (PY), phenobarbital (PB), pregnenolone 16α-carbonitrile (PCN), or 3-methylcholanthrene (3MC) was subjected to Northern blot analysis as described in Figure 3. Separate filters were used in the analysis with the Nah and Coh probes.

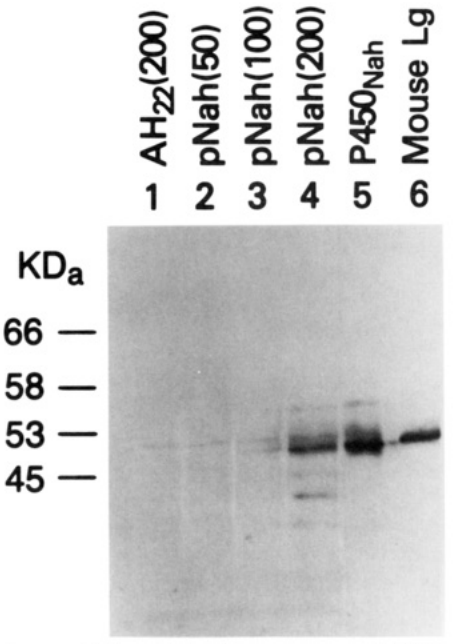

FIGURE 5: Immunoblot analysis of yeast-expressed P-450$_{Nah}$ protein. Microsomes from untreated mouse lung (30 μg, lane 6), wild-type AH_{22} cells (200 μg, lane 1), Nah-2-transformed AH_{22} cells (50 μg, 100 μg, and 200 μg, lanes 2–4, respectively), and P-450$_{Nah}$ (1 μg, lane 5) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The presence of Nah protein on the membrane was detected by immunostaining the protein with anti-cytochrome P-450$_{Nah}$ IgG followed by a goat anti-rabbit IgG–horseradish peroxidase conjugate color development. Molecular mass markers are egg albumin (45 kDa), glutamic dehydrogenase (53 kDa), catalase (58 kDa), and albumin (66 kDa). The identity of a cross-reacting 52-kDa protein in both wild-type and Nah-2-transformed yeast is currently unknown.

blot with ^{32}P-labeled Coh, a cDNA encoding coumarin hydroxylase (Negishi et al., 1989), which is known to respond to both phenobarbital and pyrazole. As shown in Figure 4, Coh mRNA was elevated in the lung of pyrazole-treated mice.

Western Immunoblot Analysis of Microsomal Protein from Yeast Expressing pNah-2. In order to determine whether the cells synthesized naphthalene hydroxylase protein, microsomes were isolated from yeast transformed with the recombinant plasmid, pNah-2, as well as from the wild-type cells. Only microsomal protein prepared from cells transformed with pNah-2 (Figure 5, lanes 3–4) exhibited an immunoreactive band with anti-cytochrome P-450$_{Nah}$ IgG at approximately M_r 50 000 by Western blot analysis. Furthermore, partially purified cytochrome P-450$_{Nah}$ from mouse liver resulted in a single positive band, indicating that the protein synthesized in yeast cells possesses the same molecular mass (Figure 5, lane 5). The microsomes isolated from untreated mouse lung showed a positive band at a slightly higher molecular weight than that of transformed yeast microsomes (Figure 5, lane 6). However, when these samples were combined and subjected to Western blot analysis, the combined sample migrated as a single positive band (data not shown).

Substrate Activity in Microsomes Isolated from pNah-2-Transformed Yeast. To establish the substrate specificity of the Nah-2-encoded cytochrome P-450, microsomes were prepared from yeast expressing Nah-2 and examined for monooxygenase activity toward three different substrates. An important feature of the encoded cytochrome P-450 was its selective regio- and stereoselective transformation of naphthalene to *trans*-(1*R*)-hydroxy-(2*R*)-glutathionyl-1,2-dihydronaphthalene (conjugate 2) when assayed in the presence of glutathione and glutathione S-transferases (Figure 6a). As has been previously shown for both mouse lung microsomes and the purified and reconstituted cytochrome P-450$_{Nah}$ from mouse liver, the ratio of conjugate 2 formed to that of either conjugate 1 or 3 was approximately 20–50-fold (Nagata et al., 1990). Accordingly, when the assay was performed with microsomes from yeast cells expressing pNah-2, the corresponding ratio was approximately 17–92-fold (Figure 6 legend).

The involvement of a monooxygenase system in the formation of naphthalene–glutathione conjugates was substantiated by the following observations (Figure 6): the formation of naphthalene–glutathione was completely blocked either (b) by the replacement of air with an atmosphere of CO/O_2 (80%:20%), (c) by the presence of anti-cytochrome P-450$_{Nah}$ IgG, (d) by the deletion of cofactor, NADPH, from the reaction, or (e) by the use of microsomes from wild-type AH_{22} cells.

Interestingly, the Nah-2-encoded cytochrome P-450 displayed a remarkably high degree of substrate specificity. There was no detectable formation of either 3-hydroxybenzo[*a*]pyrene from benzo[*a*]pyrene or 7-hydroxycoumarin from ethoxycoumarin in the microsomes (data not shown). In contrast, there was a very small but detectable level of ethoxycoumarin deethylase in the wild-type microsomes.

Discussion

In this report, we describe the isolation and characterization of a cDNA, Nah-2, which encodes an mRNA located specifically in the lung and liver of mouse and is responsible for the synthesis of a cytochrome P-450 dependent naphthalene hydroxylase. Similar to results obtained with a purified and reconstituted naphthalene hydroxylase from mouse liver, the encoded enzyme as expressed in yeast cells specifically catalyzes the metabolism of naphthalene to the (1*R*,2*S*) arene oxide intermediate. Due to its high reactivity, the product is

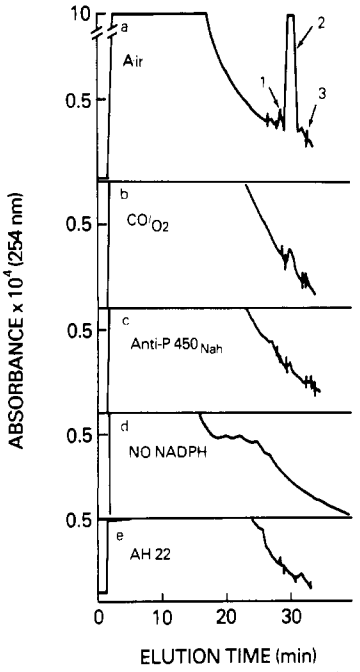

FIGURE 6: HPLC chromatogram of naphthalene–glutathione conjugates produced by Nah-2 expressed in yeast. Pretreated microsomes (2 mg) from wild-type AH_{22} and AH_{22} transformed with pAAH5 containing the Nah-2 cDNA in the correct orientation were incubated in 0.05 M phosphate buffer (pH 7.4) containing cytochrome P-450 reductase (100 pmol), glutathione (2 mM), purified glutathione S-transferases (5 units), $MgCl_2$ (5 mM), and NADPH (2 × 1.5 mM) at 37 °C for 160 for 160 min under the following conditions: (a) air, (b) CO/O_2 4:1, (c) anti-P-450_{Nah} IgG, (d) without NADPH, and (e), wild-type yeast microsomes (2 mg) with air. The procedure for the pretreatment of microsomes and assay of the formation of naphthalene–glutathione conjugates are described under Experimental Procedures. The arrows 1, 2, and 3 represent the three reaction products, naphthalene–glutathione conjugates 1, 2, and 3 with formation rates of 6, 102, and 1.1 pmol/(mg·min), respectively. Similarly, results from mouse lung microsomes were 1.1, 37, and 1.3 nmol/(mg·min), respectively.

conveniently trapped in vitro as glutathione conjugate 2 in the presence of glutathione and glutathione S-transferase. Buckpitt et al. (1982) demonstrated that cytochrome P-450 dependent naphthalene toxicity occurs in mouse lung and not in rat or hamster or other tissues and is due to the metabolism occurring in the Clara cells of the respiratory system. Three different sets of data strongly support the conclusion that the Nah-2-encoded monooxygenase is responsible for this toxicity: (a) immunochemically, anti-cytochrome P-450_{Nah} IgG specifically stained the Clara cells of the mouse lung (Chang et al., 1991); (b) the Nah-2 cytochrome P-450 converts naphthalene primarily to (1*R*,2*S*)-naphthalene 1,2-oxide (50:1 ratio versus (1*S*,2*R*)-naphthalene 1,2-oxide), as do target cells which are microdissected from the bronchiolar airways of mouse lung (72% Clara cells) (Plopper et al., 1991); (c) after mice had been allowed to develop tolerance to naphthalene following several days of administration of subtoxic doses of naphthalene, there was a significant protection from pulmonary necrosis upon challenging these mice with 300 mg/kg naphthalene (O'Brien et al., 1989). The degree of protection paralleled the decreased level of formation of naphthalene glutathione conjugate 2 in the microsomes isolated from the lungs of tolerant mice. It is noteworthy that there was no change in either the conjugate 1 or 3 in these microsomes.

The detection of Nah-2 mRNA in the lung and liver of mouse and not in those of rat supports the toxicity data (O'Brien et al., 1985) except for the high level in mouse liver. The cyclophilin-normalized ratio of Nah-2 mRNA liver to lung of mouse was 5–10-fold (Figure 3). The lack of toxicity in mouse liver following exposure to high doses of naphthalene most likely reflects the greater abundance of epoxide hydrases, glutathione, and glutathione S-transferases in liver than in lung (Boyd et al., 1980) and, thus, the capacity to protect the cells against the damage of the reactive intermediate formed from naphthalene in vivo. It should also be pointed out that the Clara cell which represents 2% of the cell population of the lung is the sole site of the Nah hydroxylase. The combination of a high cellular level of the toxifying enzyme with a low level of protective phase II enzymes most likely creates the conditions for damage to this population of cells when exposed to high doses of naphthalene. Hence, the relative amounts of the reactive intermediate formed specifically in the Clara cell to that in the liver cell has to be considered, and thus, the ratio of conjugate 2 formed by the in vitro microsomal systems isolated from the two tissues may be irrevelant in rationalizing the differences in in vivo toxicity.

One of the more intriguing observations resulting from investigations of the cytochrome P-450(s) responsible for generating naphthalene toxicity is that cytochrome P-450c (IA1), the major form of cytochrome P-450 induced by 3-methylcholanthrene treatment in the rat, has been shown to yield a similar metabolic profile of naphthalene–*N*-acetylcysteine conjugates (Van Bladeren et al., 1984). Because of the lower trapping efficiency estimated in their study (3–5%), we have repeated their experiment under our conditions which have been proven to trap over 90% of the reactive intermediate, naphthalene oxide (Buckpitt et al., 1987), and confirmed their finding. In spite of their similar stereospecific metabolism of naphthalene, a number of lines of evidence suggest that the mouse equivalent of this cytochrome P-450 is not involved in the cytochrome P-450 dependent naphthalene-derived lung injury. Recent immunocytochemical studies (Forkert et al., 1989) have shown that immunofluorescent labeling was not detected in the bronchiolar region of control mouse lung using monoclonal antibody against cytochrome P-450c. This is in agreement with earlier data which showed that anticytochrome P-450c IgG failed to block the formation of naphthalene glutathione conjugate 2 catalyzed by mouse lung system (Nagata et al., 1990). Furthermore, a published report has presented data (Chichester et al., 1990) showing that there is no benzo[*a*]pyrene hydroxylase activity (AHH) in the Clara cells of mouse lung, a known substrate of cytochrome P-450c (IA1) (Forkert et al., 1989). The Nah protein expressed in transformed yeast did not metabolize benzo[*a*]pyrene.

Another intriguing feature of the Nah-2 clone involves its relationship to that of the human lung P-450 IIF1 and a similar form in the rat. An mRNA(s) which hybridizes with the human IIF1 cDNA was shown to be expressed in rat tissues, highest in lung with trace amounts in liver and kidney and not detectable in intestine (Nhamburo et al., 1990). Similarly, the Nah-2 probe detected low levels of RNA in rat lung but not in liver, kidney, and intestine (Figure 3). Although the rat lung contains cross-hybridizing RNA, rat lung microsomes generate the three naphthalene glutathionyl conjugates in approximately equal amounts (Buckpitt et al., 1987). In mouse the ratio of Nah-2 mRNA is oppositive from rat, higher in liver than lung (Figure 3). The presence of a similar form in rat lung concomitant with the rat showing resistance to naphthalene Clara cell toxicity further indicates that the rat and mouse forms generate apparently different metabolites of naphthalene.

On the basis of the rather high percentage of identical amino acid residues shared between Nah-2 and the human lung IIF1

isozyme (Nhamburo et al., 1990), it was of interest to compare their substrate specificities. Vaccinia virus mediated expression of the human IIF1 cDNA in HepG2 cells possessed a clear ethoxycoumarin deethylase activity. In contrast, the Nah-2 protein expressed in yeast exhibited no detectable activity for this substrate. In order to clarify this further, the formation of naphthalene–glutathione conjugate 2 from naphthalene by mouse lung microsomes was subjected to Michealis–Menten kinetic analysis. The presence of ethoxycoumarin at a 1- or 4-fold excess relative to naphthalene (used at the K_m concentration) failed to inhibit the formation of conjugates, thus negating ethoxycoumarin as a competitive substrate (data not shown). Preliminary data indicate that vaccinia-expressed IIF1 in HepG2 cells also catalyzes the hydroxylation of naphthalene but produces a marked lower ratio of conjugate 2:conjugate 1 or 3 in the presence of glutathione and glutathione S-transferases (Kenneth Korzekwa and Frank Gonzalez, personal communication). These data suggest that, in spite of their high overall deduced amino acid sequence identity, the forms have different substrate specificities. This is not an unusual observation. Published reports (Lindberg & Negishi, 1989; Uno et al., 1990) have shown that a single amino acid change can dramatically convert substrate specificity.

It is also interesting to note that in the alignment of protein sequences deduced from the IIF1 and Nah-2 cDNAs, two gaps exist, one at positions 221–224 in the Nah isoform and the other at 260–263 in the IIF1 protein (Figure 2, boxes). In view of the fact that the gaps involve related amino acid residues, they may exert an important influence on the tertiary structure of the proteins and possibly contribute to the observed disparity in substrate specificity.

In this study, we have utilized an antibody developed against a purified mouse liver naphthalene hydroxylase cytochrome P-450 to isolate a form which is implicated in the lung-specific necrosis observed in mice, but not in rats or hamsters, following naphthalene injection. While it is well recognized that differences in species and organ metabolism exist and are related to specific chemical toxicities, the molecular bases for this variation leading to different toxicological outcomes in response to chemicals are complex and often difficult to rationalize. In the case of naphthalene, our data likely explain the basis for the species difference in toxicity between mouse and rat. The results suggest that the presence of the naphthalene-bioactivating enzyme, Nah, in the environment of mouse lung is sufficient to confer susceptibility to naphthalene toxicity and that organ-specific, protective factors or detoxifying pathways must exist which account for the different toxicological responses of the mouse lung and liver to this important environmental contaminant.

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Electron Spin Echo Envelope Modulation Studies of the Cu(II)-Substituted Derivative of Isopenicillin N Synthase: A Structural and Spectroscopic Model†

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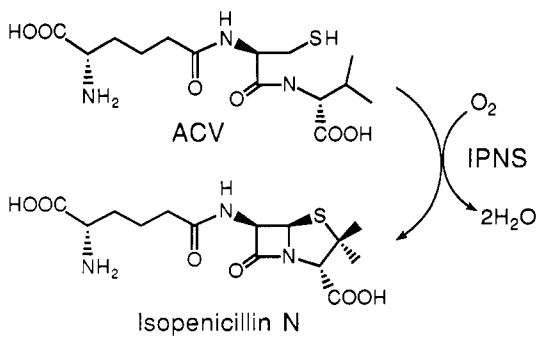
ABSTRACT: Electron spin echo evelope modulation spectroscopy (ESEEM) was used to study the active site structure of isopenicillin N synthase (IPNS) from *Cephalosporium acremonium* with Cu(II) as a spectroscopic probe. Fourier transform of the stimulated electron spin-echo envelope for the Cu(II)-substituted enzyme, Cu(II)IPNS, revealed two nearly magnetically equivalent, equatorially coordinated His imidazoles. The superhyperfine coupling constant, A_{iso}, for the remote ^{14}N of each imidazole was 1.65 MHz. The binding of substrate to the enzyme altered the magnetic coupling so that A_{iso} is 1.30 MHz for one nitrogen and 2.16 MHz for the other. From a comparison of the ESEEM of Cu(II)IPNS in D_2O and H_2O, it is suggested that water is a ligand of Cu(II) and this is displaced upon the addition of substrate.

Isopenicillin N synthase (IPNS) is a non-heme iron(II)-containing enzyme found in penicillin- and cephalosporin-producing microorganisms (Baldwin & Abraham, 1988; Robinson, 1988; Baldwin, 1989; Chen et al., 1989; Baldwin & Bradley, 1990). This enzyme catalyzes the oxidative ring closure reactions of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) to form isopenicillin N, the precursor of all other penicillins, with concomitant four-electron reduction of a single equivalent of dioxygen to form 2 equiv of H_2O. The oxidation of ACV to form the β-lactam and thiazolidine rings was found to be catalyzed by IPNS with complete retention of configuration.



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Insights into the mechanism of IPNS catalysis have been obtained from kinetic studies using different substrate analogues (Baldwin, 1989; Baldwin & Abraham, 1988; Robinson, 1988; Baldwin & Bradley, 1990); however, much less has been learned about the active site structure of the enzyme and the configuration of the enzyme–substrate complex. In our previous communication (Ming et al., 1990), we have shown that the Cu(II)-substituted IPNS [Cu(II)IPNS] could be used for spectroscopic studies of the active site structure of the enzyme. A direct interaction of the Cys-S moiety of the substrate with Cu(II) in the active site was clearly demonstrated by an intense S-to-Cu charge-transfer band at 385 nm. Both EPR and electronic spectra of Cu(II)IPNS showed a tetragonally distorted type 2 copper site. The presence of endogenous His residues has also been suggested by NMR studies of Fe(II)-IPNS and Co(II)IPNS.

Pulsed EPR spectroscopy is a powerful technique for the study of the active site structure of copper enzymes (Mims & Peisach, 1981; McCracken et al., 1987; Mims et al., 1984; Zweier et al., 1982). By measuring the electron spin echo envelope modulation (ESEEM) arising from the superhyperfine interaction between Cu(II) and nearby weakly coupled nuclei, we can identify the type and the number of Cu(II) ligands. We report here our ESEEM studies of Cu(II)IPNS which show the presence of two equatorially coordinated His residues and also provide insights into the substrate interaction and solvent accessibility at the active site of this biomedically significant metalloenzyme.

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