Cytochrome P450cin (CYP176A), Isolation, Expression, and Characterization*

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The cytochrome P450s (P450s)1 are a superfamily of oxidative hemoproteins (1, 2) that carry out an enormous variety of oxidative transformations. These range from simple alkene epoxidation and heteroatom oxidation all the way through oxygen insertion into unactivated C–H bonds and C–C bond cleavage (3). P450s are broadly categorized as either soluble, e.g. most bacterial P450s, or as membrane bound/microsomal monooxygenases, e.g. most eukaryotic P450s. All P450s, however, contain a prosthetic heme group that is ligated to the protein backbone by a cysteinyl sulfur coordinated to the heme iron. It is this heme-thiolate moiety that is responsible for much of the chemistry carried out by these enzymes. The typical reaction catalyzed by a P450 is given by Reaction 1,

\[
\text{RH} + \text{NAD}^+ + \text{H}^+ + \text{O}_2 \rightarrow \text{ROH} + \text{NADH} + \text{H}_2\text{O}
\]

**REACTION 1**

The exact mechanism of the oxidation reaction is still the subject of much debate, despite an enormous amount of work (3, 4). The electrons derived from the nicotinamide cofactors are generally transferred to the P450 one at a time by auxiliary reductin protein(s) that are characteristic of the origin of the enzymes. Thus, bacterial P450s generally employ an FAD-dependent ferrodoxin reductase and an iron-sulfur protein to mediate this transfer, whereas microsomal P450s employ a single protein that contains both an FAD and an FMN domain.

Investigations of bacterial P450s have provided much of our current understanding of P450 mechanism and structure. This is particularly true of P450 cam isolated from Pseudomonas putida, in part because it was the first P450 available in relatively large amounts (5). However, some features of P450 cam appear to be unique to this enzyme, e.g. a potassium-binding site (6), and not typical of this enzyme class. P450 cam still remains the most studied of all P450s and one of the few bacterial enzymes of this family that has been heterologously expressed with its redox partners as a catalytically active system (7). It is a biodegradative enzyme that catalyzes the conversion of camphor to 5-exo-hydroxycamphor, which initiates a cascade of biodegradative reactions that allow P. putida to live on camphor as its sole source of carbon and energy (8). P450 cam and others involved in such pathways, e.g. P450 lin (9) and P450 terp (10), are generally characterized by high substrate specificity and high rates of turnover. As such, they make attractive starting points for attempting to design biocatalysts

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1 The abbreviations used are: P450, cytochrome P450; CPR, cytochrome P450 reductase; P450cam, CYP176A1; P450lin, CYP101; P450terp, CYP108; P450bus, CYP107H; RZ, (A9)-trans/(A9)-cineole used when determining the purity of P450 samples (> is better); orf, open reading frame; IPTG, isopropyl-β-d-thiogalactopyranoside; Amp, ampicillin; TB, Terrific broth; cineole, 1,8-cineole.

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that can carry out synthetically useful oxidative transformations. We wished to isolate a new bacterial biodegradative P450 system that could be (i) used for comparison with P450<sub>cam</sub>, (ii) heterologously expressed as a catalytically active system, and (iii) used as the basis for constructing libraries of P450s that would be explored for their ability to catalyze useful oxidative transformations. Such a P450 system was implicated in the bacterial utilization of 1,8-cineole (cineole, 1, Scheme 1).

Eucalypt trees produce a mixture of nearly 50 hydrocarbons termed the "essential oil" which contains extremely high concentrations of the monoterpene cineole 1. The role of cineole 1 is somewhat unclear, although it is believed to be involved in a number of functions including defense against herbivores (11, 12) and pathogens (13), attracting pollinators and fruit-dispersing animals (14), and/oas an allelopathic agent (15, 16). The Australian eucalypt population collectively produces and releases an estimated 500,000 tons of cineole 1 into the environment annually (17). Studies indicate that the major part of the released cineole 1 is rapidly removed by microbial oxidation (18), with lesser quantities consumed by native wildlife (19) and bushfires.

In 1979 MacRae et al. (18) isolated a soil microorganism identified as <i>Pseudomonas flava</i>, which could utilize cineole 1 as its sole source of carbon and energy, and oxidized derivatives of cineole 1 were identified in the growth medium. Subsequently, Trudgill and co-workers (20) identified a <i>Rhodococcus</i> species also capable of using cineole 1 as its sole carbon source and similarly identified oxygenated cineole derivatives. Interestingly, it was found that the initial oxidation of cineole 1 by the two organisms produced enantiomeric forms of 2-hydroxycineole 2. A metabolic pathway for the catabolism of cineole 1 in <i>Rhodococcus</i> sp. C1 was proposed, based on the isolated metabolites (Scheme 1). Thus, cineole 1 is initially oxidized to 2-hydroxycineole 2 by a monoxygenase, and further transformations lead to a highly functionalized hydroxyketo acid 3 that may spontaneously form the isolated dihydrofuranone 4 or be incorporated into central metabolic pathways.

Despite the relatively large amount of information concerning the chemistry of the cineole 1 biodegradative pathway, nothing was known about the enzymes involved. Early attempts at isolating the proposed monoxygenase that catalyzes cineole 1 oxidation failed (21). Several lines of evidence suggested, however, that the monoxygenase might be a P450. The biodegradative utilization's of a number of monoterpens, including camphor, linalool, and terpineol, have all been shown to be initiated by oxidations catalyzed by P450s (21). Additionally, CYP3A4 was recently identified as being the main P450 isoform responsible for cineole 1 metabolism in humans, with the primary product, 2-hydroxycineole 2, being the same as the one implicated in bacterial biodegradation (22). Finally, the relative scarcity of other enzymes, e.g. non-heme iron oxygenases capable of carrying out such an oxidation, also suggested a P450 would be involved.

We thus set out to isolate and characterize a monoxygenase system involved in the bacterial utilization of cineole 1. Reported here is the isolation, overexpression, and purification of a P450, P450<sub>cin</sub>, believed to be responsible for cineole 1 oxidation. In addition, the sequences of two genes that may encode the required reduct partners for P450<sub>cin</sub> are described along with preliminary characterization of the P450 that supports its involvement in the biodegradation of cineole 1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Yeast extract and Bacto-tryptone were obtained from BD PharMingen. Reagent grade 1,8-cineole was obtained from Aldrich. Sodium dithionite (sodium hydrosulfite) was purchased from Aldrich. Nylon hybridization transfer membranes (GeneScreen Plus) were obtained from PerkinElmer Life Sciences. All other chemicals were purchased from commercial sources and were of the highest grade generally available. The restriction enzymes and polynucleotide kinase were obtained from New England Biolabs, Inc. (Beverly, MA), and Roche Molecular Biochemicals based on availability. Terminal deoxynucleotidyltransferase was obtained from Promega Corp. (Madison, WI). Escherichia coli strain, DH5αF' s80dlacZAM15 Δ(uacZYA-argF) U169 deoR recA1 endA1 hsdR17(r<sup>R</sup> m<sup>K</sup> ) strain, was obtained as frozen competent cells from Invitrogen. Sonication was achieved using a Branson Sonicator set to 50% duty cycle.

**Isolation of the Microorganism**—Soil samples were collected from several sites beneath eucalypt trees within a small eucalypt forest at the University of California, San Francisco. A dirt/water slurry prepared from each sample was transferred to minimal medium (23), and bacteria were selected for the ability to use 1,8-cineole as the sole carbon source. Following several rounds of serial culturing (1:50 dilution) at 30 °C, the cell culture was spread onto minimal medium plates (1.5% agar plates) with 0.5 g of cineole in the lid, yielding a single pure strain according to the protocol of Peterson and Lu (23). The strain was propagated in 10–15 ml of minimal medium with 20 μl of 1,8-cineole at 30 °C.

**Tryptic Digest of P450<sub>cin</sub>**—Internal amino acid sequence for P450<sub>cin</sub> was obtained from tryptic peptides of P450<sub>cin</sub>. These were purified by microbore high pressure liquid chromatography and sequenced using automated Edman degradation protein sequencer. These data were used in concert with that obtained via matrix-assisted laser desorption ionization-mass spectrometry to yield the sequence of three fragments (Table 1).

| Fragment | Sequence | M<sup>+</sup> | Technique |
|----------|----------|-------------|------------|
| 1        | WAITHVENPFEAGAEFAELVAHAR | 2690.4 | Auto/MS* |
| 2        | WAITHVENPFEAGAEFAELVAHAR | 2788.8 | Auto/MS |
| 3        | GEGDAATWLANIFAR | ND* | Auto |

* Auto, automated Edman sequencing; MS, mass spectrometry; ND, not determined.

**Preparation of Whole Cell DNA**—Cells were grown on minimal medium (23) with 1,8-cineole as the sole carbon source at 30 °C. Whole cell DNA was prepared according to Peterson et al. (24).

**Generation of an Oligonucleotide Probe to Cytochrome P450<sub>cin</sub>**—A 92-bp PCR product was amplified from whole cell DNA (Citrobacter braakii) using degenerate primers (5'-ATAT GAA TUC TGG CCG ATM CAN CAH GT-3' and 5'-TATC GGATCC CCG ATG GCC RGG NAC-3') designed based upon five amino acids at either end of fragment 1 (Fig. 1). PCR was performed according to the following protocol: 94 °C for 3 min (94 °C for 1 min, ramp to 37 °C over 2 min, 72 °C for 1 min) for 5 cycles; (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min) for 25 cycles; 72 °C for 10 min; 4 °C. The product was treated with BamHI and EcoRI and cloned into similarly cut pCRII<sup>®</sup> (Invitrogen). The insert was sequenced using an automated sequencer using M13 forward and reverse primers, confirming the central non-degenerate 42 bp coded for the

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2 The Degenerate DNA code used is as follows: R = A or G; K = G or T; H = A, C, or T; M = A or C; N = A, T, C, or G.
same 14 amino acids seen in fragment 1 (Table I). The 92-bp insert was labeled with α-[32P]dCTP using terminal deoxynucleotidyltransferase (25) and denatured at 100 °C prior to use in subsequent hybridizations.

**Library Construction and Screening**—DNA isolated from C. braakii was digested with a bank of restriction enzymes (KpnI, PstI, SalI, PstII/HindIII, SalI/HindIII, and XbaI) and ligated and self-ligated on an agarose gel (0.7% w/v, 0.5× TBE). The DNA was transferred to a nylon membrane (Gensecreen Plus PerkinElmer Life Sciences) by capillary transfer, dried, and probed with labeled oligonucleotide (26).

 Autoradiography revealed bands of suitable size in PstI (4 kb) and SalI (3 kb) digests. These latter digests were repeated, separated by electrophoresis, and the bands of appropriate size recovered by gel extraction.

The recovered DNA fragments were ligated into PstI- or SalI-digested pUC19 and transformed individually into library efficiency DH5α cells producing PstI and SalI libraries. Approximately 4,000–5,000 recombinant cells for each fragment were plated onto 2× YT plates containing ampicillin (50 μg/ml) and incubated overnight. Colonies were lifted onto nylon membranes, and their DNA was fixed by heat. For the identification of fragments, the DNA was digested with restriction enzymes (BamHI, EcoRI, and HindIII) and XbaI and probed with the labeled oligonucleotide. The identities of the clones were confirmed by restriction analysis and Southern hybridization. A positive PstI (4 kb) clone (designated pc1) was identified. Restriction analysis of pc1 revealed the SalI (3 kb) fragment (cloned as pc2) was contained entirely within the PstI (4 kb) fragment and so its isolation was not pursued further. To gain further sequence information, probes generated by the random primer method (27) using the PstI insert as template were used to probe an EcoRI/SphI digest of C. braakii DNA. An SphI (2.9 kb) and EcoRI/SphI (5.7 kb) fragment were isolated and cloned into pUC19 to give pC3 and pC4, respectively (Fig. 2).

**Sequence**—Careful restriction analysis of clones pC1 and pC4 identified a series of small internal fragments that were subcloned and sequenced using the M13 forward and reverse primers. Gaps remaining in the sequence were filled by employing suitable synthetic primers. Sequencing data were analyzed using the BLAST algorithm (NCBI Database), and three open reading frames (cinA, cinB, and cinC) were identified which were sequenced completely in both directions.

**Cloning of P450cin**—Primer sets were designed to either end of cinA containing suitable terminal restriction sites (EcoRI and NotI for N-terminal primer 5′-CC GATT CATAGT CATG TGG CAG AGT CGC-3′ and XbaI and EcoRI for the C-terminal primer 5′-CC GAA TCTTAGA GGA GTC TGTC TCA TTC G3′). A PCR experiment was performed with pC1 as template DNA according to the following parameters: 94 °C for 3 min; 30 cycles of 94 °C for 45 s, 52 °C for 1 min, 72 °C for 1 min 30 s. The single 1.2 kb product was digested with EcoRI and inserted into pUCP855 (28). The combined recombinant extracts were digested over magnesium sulfate and concentrated in vacuo, and the residue was analyzed by gas chromatography-mass spectrometry (BP-5 column (30 m × 0.25 mm) as follows: temperature program 60 °C to 250 °C at 4 °C/minute). A peak was observed with a mass spectrum consistent with that reported (29) for 2-hydroxyceineol (2) (gas chromatography-mass spectrometry m/z 170 (M⁺; 6%); 155 (M-C₆H₅), 126 (37), 106 (70), 93 (34), 71 (59), 69 (37), 43 (100)).

**Results**

**Isolation of C. braakii**—A microorganism capable of utilizing 1,8-cineole 1 as its sole carbon source was isolated through culture enrichment (23) of bacteria from the cineole 1-enriched soils beneath eucalypt trees. The procedure yielded a pure strain identified using the Biolog Identification System (Biolog, Inc., Hayward, CA) (30) as C. braakii. This strain grew best at room temperature on a variety of nutrient-rich media. However, growth of such media resulted in loss of the ability to grow on cineole 1 as a sole carbon source, and thus the organism was generally maintained on minimal medium (23) in the presence of cineole 1.

**Examination of C. braakii**—A dilute solution of P450cin was prepared and characterized by UV in the presence of cineole. The protein content of the solution was determined (24) and compared with the value obtained after the addition of 180 mM KH₂PO₄, pH 7.2. A phosphate gradient (5–300 mM KH₂PO₄) was run (0.5 ml/min). P450cin eluted at 120 mM KH₂PO₄. Fractions with an RZ of >1.2 were combined. The cytochrome P450 content was determined from the difference spectrum between samples that had been reduced with sodium dithionite and carbon monoxide and saturated versus a sodium dithionite-treated reference sample using an ε₃₄₅ of 125 cm⁻¹ M⁻¹. Purified protein was snap-frozen in liquid nitrogen and stored at −80 °C. The procedure produced 42 mg of P450cin per liter of culture.

**Calculation of Molar Extinction Coefficients**—A dilute solution of P450cin was prepared and characterized by UV in the presence of cineole. The protein content of the solution was determined (24) and compared with the value obtained after the addition of 180 mM KH₂PO₄, pH 7.2. A phosphate gradient (5–300 mM KH₂PO₄) was run (0.5 ml/min). P450cin eluted at 120 mM KH₂PO₄. Fractions with an RZ of >1.2 were combined. The cytochrome P450 content was determined from the difference spectrum between samples that had been reduced with sodium dithionite and carbon monoxide and saturated versus a sodium dithionite-treated reference sample using an ε₃₄₅ of 125 cm⁻¹ M⁻¹. Purified protein was snap-frozen in liquid nitrogen and stored at −80 °C. The procedure produced 42 mg of P450cin per liter of culture.

**Purification of Recombinant Cytochrome P450cin**—A dilute solution of P450cin was prepared and characterized by UV in the presence of cineole. The protein content of the solution was determined (24) and compared with the value obtained after the addition of 180 mM KH₂PO₄, pH 7.2. A phosphate gradient (5–300 mM KH₂PO₄) was run (0.5 ml/min). P450cin eluted at 120 mM KH₂PO₄. Fractions with an RZ of >1.2 were combined. The cytochrome P450 content was determined from the difference spectrum between samples that had been reduced with sodium dithionite and carbon monoxide and saturated versus a sodium dithionite-treated reference sample using an ε₃₄₅ of 125 cm⁻¹ M⁻¹. Purified protein was snap-frozen in liquid nitrogen and stored at −80 °C. The procedure produced 42 mg of P450cin per liter of culture.

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**Purification of P450cin from C. braakii**—A P450cin was purified from a large scale culture of C. braakii grown on minimal medium in the presence of cineole 1. The purification protocol used was based on those published for P450am54 P450long, and P450Blut (9, 10, 31). A refined version of this protocol is currently used for purification of recombinant P450cin (see below).
The procedure yielded purified protein with a peak fraction possessing an RZ (A415/A280) of \(-1.28\). The protein isolated was clearly a P450 by CO difference spectroscopy and had a molecular mass of \(\sim 45\) kDa by SDS-PAGE analysis. Importantly, the heme Soret absorbance at 415 nm shifted to 392 nm in the presence of cineole 1. This is characteristic of the spin state change of the heme iron (low to high spin) seen upon binding of substrate to a P450 and strongly suggested that the isolated P450 was involved in cineole 1 metabolism.

**Tryptic Digestion and Sequencing of Protein Fragments**—N-terminal sequencing of P450cin (purified from *C. braakii*) using automated Edman degradation failed to produce useful data, presumably due to N-terminal blocking. However, tryptic digestion of the protein followed by high pressure liquid chromatography purification of the resultant fragments gave several peptides amenable to Edman degradation. A combination of automated Edman degradation sequencing and mass spectrometry yielded the sequences of three fragments that are shown in Table I. Fragments 1 and 2 are clearly related, differing only in the identity of the first amino acid. However, as their sequences were independently obtained, we were confident of their reliability, and this sequence was chosen as the basis upon which to design oligonucleotides for use in isolation of the gene encoding P450cin. Fragment 3 was not useful for probe design due to the preponderance of amino acids with highly degenerate codons. We chose to amplify a larger segment of the P450 gene by PCR in order to generate a better probe, rather than simply employ short degenerate oligonucleotides. Thus, degenerate primers were designed to either end of the DNA fragments isolated via Southern hybridization experiments and cloned as pC1–pC4, such that the insert was employed to isolate two further genomic fragments that were cloned into pUC19 as pC3 and pC4, such that the entire operon containing P450cin could be sequenced (Fig 2).

**Operon Analysis**—Sequencing of the above DNA fragments revealed a putative operon consisting of three open reading frames with overlapping stop and start codons (Fig 2). All ORFs were analyzed using the BLAST algorithm (32) and the GenBank data base. The first ORF (cinA) encoded a 405-amino-acid protein with a predicted molecular mass of 45 kDa. Homology analysis indicated that it was clearly a P450, being similar to a number of bacterial P450s (Table II), including P450cam (27% identity and 46% similarity) but different enough to be classified as the sole member of a new P450 family CYP176A1 (2). All of the peptide fragments that had been characterized from the tryptic digest of the isolated P450 were encoded by this gene.

The two other ORFs (cinB and cinC) appeared to encode the expected redox partners for a catalytically functional P450 system. The second ORF (cinB) encoded a 49-kDa, 452-amino-acid protein that was apparently an NADPH-dependent ferredoxin reductase on the basis of sequence homology (Table II). The final gene (cinC) encoded a protein (16 kDa and 155 amino acids) that was most similar to the FMN-containing domain of cytochrome P450 reductase (Table II). The participation of a dedicated flavodoxin as a P450 redox partner has not been described previously, but *E. coli* flavodoxin has been reported to provide an unnatural redox partner for a variety of heterologously expressed cytochromes (33).

**Subcloning and Expression of P450cin**—A PCR-based approach was used to clone the cinA gene into the expression vector pCWori (34). Expression of the encoded protein after induction with IPTG was monitored by SDS-PAGE and CO difference spectroscopy of cell lysates. It was found that although polypeptide production occurred at temperatures from 25 to 37°C, P450 holoprotein was only observed when the growth temperature was below 28°C. CO difference spectroscopy indicated that P450 was produced at concentrations of \(-200\) nmol/liter of original culture. Characterization of the purified protein by N-terminal sequencing and electrospray mass spectrometry gave similar results. Both techniques clearly identified the protein as P450cin, and indicated that some degree of N-terminal processing of the protein had occurred. The degree of processing varied between different preparations, with some having the major species present derived...
from loss of the N-terminal methionine, whereas others had the predominant protein derived from loss of the first six amino acids of the predicted sequence.

In Vivo Oxidation of Cineole by P450_cin—When P450_cin was expressed in E. coli in the presence of cineole 1, a metabolite was produced with mass spectral characteristics consistent with that of 2-hydroxycineole 2. This compound was not produced when E. coli not expressing P450_cin was grown in the presence of cineole 1. This result strongly supports the hypothesis that P450_cin catalyzes the first monooxygenation in the biodegradation of cineole 1.

Spectral Characteristics of Cytochrome P450_cin—The protein was first examined by UV-visible spectroscopy (Fig. 3) that revealed the typical characteristics of a bacterial P450, a heme Soret at 415 nm in the absence of substrate which shifted to 392 nm upon the addition of cineole 1. This spectral change is correlated with a change in the heme iron from low to high spin and is associated with the binding of substrate to bacterial P450s and as such was the first evidence that cineole 1 was indeed the natural substrate of this enzyme. Reduction of the protein with dithionite and treatment with CO resulted in the dissociation constant of ligand-P450 complex; \( K_d \) when cultured on a large scale, and although it grew well on nutrient rich-media, such growth resulted in loss of its ability to utilize cineole 1. This latter fact perhaps suggests that the

\[
[S]_{TOT} = (K_d \Delta A) / (\Delta A_{max} - \Delta A) + \Delta A / \epsilon
\]

where \([S]_{TOT}\) is the concentration of added ligand; \(K_d\) is the dissociation constant of ligand-P450 complex; \(\Delta A\) is the measured peak to trough difference in absorbance between 415 and 392 nm; \(\Delta A_{max}\) is the maximal \(\Delta A\) value; and \(\epsilon\) is the molar absorptivity constant for the difference in absorbance at 415 and 392 nm of the high (HS) and low spin (LS) forms of the P450, i.e. \((\epsilon_{415HS} - \epsilon_{392HS}) + (\epsilon_{392LS} - \epsilon_{415LS})\). This calculation was made with the following assumptions: 1) \([S]_{TOT} = [S]_{FREE} + [ES]\); and 2) \([E]_{TOT} = [E]_{FREE} + [ES]\), where [ES] is the concentration of the P450-ligand complex; \([S]_{TOT}\) and \([E]_{TOT}\) are the total and free ligand concentration, respectively; and \([E]_{TOT} \text{and} [E]_{FREE}\) are the total and free P450 concentration, respectively. Because of the high affinity of the enzyme for its ligand, the assumption that \([ES] < [S]_{TOT}\), which would greatly simplify the calculation, is not valid.

### DISCUSSION

Prior to the beginning of this work, much was known about the chemistry of cineole 1 biodegradation. Two organisms had been independently isolated previously that could live on cineole 1 as their sole source of carbon and energy (18, 20). From an investigation of the oxidized cineole metabolites isolated from growth media, a consensus biodegradative pathway was proposed (Scheme 1), although proceeding via intermediates with different absolute stereochemistry in each of the two organisms. Nothing was known about the enzymes involved in the proposed pathway, despite some early investigations (21). Our results support the hypothesis that a P450 is responsible for the initial step in the biodegradation of cineole 1.

We isolated from the soil of a eucalypt forest a strain of *C. braakii* that could utilize cineole 1 for growth and energy. *C. braakii* is a Gram-negative motile rod that is a common environmental organism and that has recently been reclassified from *Citrobacter freundii* (36). *P. flava* and *Rhodococcus*, in which cineole 1 utilization had been found previously, are also common Gram-negative organisms. Given the quantity of cineole 1 that is believed to be released into the environment by eucalyptus, we were not surprised to find that a third genus of bacteria has developed the ability to grow on this compound. The organism grew poorly on minimal medium in our hands when cultured on a large scale, and although it grew well on nutrient rich-media, such growth resulted in loss of its ability to utilize cineole 1. This latter fact perhaps suggests that the
genes responsible for cineole 1 metabolism are plasmid-borne as has been shown for the P450s involved in camphor and terpineol utilisation (10, 37). The difficulty in growing the organism clearly meant that isolation of the gene encoding the cineole monooxygenase would be essential for its characterization.

Initial approaches to isolating the P450 gene focused on its expected homology with other P450s, because this seemed to offer the most direct route to P450cin. Homology-based methodology in bacterial P450 systems is particularly difficult, however, because most of these proteins reside in sparsely populated families with only limited regional homology to one another. However, with this in mind, a degenerate oligonucleotide was constructed based upon the conserved region that includes the cysteine that ligates the heme iron and used to probe genomic DNA from C. braakii. A second protocol used this oligonucleotide in a genomic PCR with another degenerate primer based upon a conserved region seen in the redoxin family of P450s. Although most of the conserved residues found in P450s were encoded in the P450cin gene, as seen by its strong similarity to a homologue in C. braakii genomic DNA failed to yield usable results. Believing that sensitivity was the source of our problems, we chose to label the probe with [α-32P]dCTP in the presence of terminal deoxynucleotidyltransferase (25). TdT is known to transfer up to 30 nucleotides to the 3’ end of single-stranded DNA, which results in an order of magnitude increase in the specific activity and thus sensitivity of the probe. Utilization of [α-32P]dCTP-labeled oligonucleotide resulted in the eventual isolation of DNA fragments containing the P450cin gene.

Sequencing of the isolated DNA revealed a putative P450cin operon that contained three open reading frames with overlapping start and stop codons (Fig. 2). The first of these genes clearly encoded a P450, as seen by its strong similarity to a number of other bacterial P450s (Table II). The encoded protein was classified as CYP176A1 and trivially denoted as P450cin. Although most of the conserved residues found in P450s were also seen in P450cin, there was one striking exception (Fig. 5). Threonine 252 in P450cam is located on the I helix, a conserved structural feature of P450s, and is believed to hydrogen-bond to a water molecule during the oxygen activation process and thus direct delivery of a proton to the distal oxygen in the iron-dioxygen complex (38, 39). This specific delivery is believed to result in cleavage of the O–O bond to yield the active ferryl species; delivery of a proton to the proximal oxygen is thought to result in the dissociation of hydrogen peroxide (so-called uncoupling). Given the essential role of this residue in catalysis it is not surprising that a threonine or serine is seen in this position in essentially all known P450s. An exception occurs in P450eryF (CYP107A1), in which it is replaced by an alanine; in this enzyme a hydroxyl group located on the substrate is believed responsible for the positioning of the required water molecule. In P450cin, however, sequence alignments suggest that the threonine has been replaced with an asparagine. Clearly, the amide functionality of the asparagine could serve to hydrogen-bond to a water molecule and thus direct proton delivery and cleavage of the dioxygen.
bond. However, this would be the first time that a hydroxyl group has not filled this role, and further experimentation will be required to validate this hypothesis.

The two further genes discovered during the sequencing of the putative operon appear to encode potential redox partners for P450cam. Although this arrangement is identical to that seen in the P450cam and P450terp operons, the identity of these redox partners is quite different. In general there are two types of P450, as classified by their auxiliary electron transport partners. In bacteria and mitochondria (class I P450s) there are two proteins that mediate the transfer of electrons from the pyridine nucleotide to the P450; one is a FAD-containing ferredoxin reductase, and the other is an iron-sulfur protein, either 2Fe-2S or 3Fe-4S. In eukaryotes (class II P450s), there is generally a single flavoprotein, cytochrome P450 reductase that contains both an FAD and an FMN cofactor, which mediates P450 reduction. Other less common arrangements also exist for electron transfer. P450cam (40) accepts electrons directly from NADPH, whereas P450cam, P450terp (41), a bacterial P450, incorporates both a class II-like reductase and a P450 domain into a single polypeptide. Class III P450s, e.g. CYP74A, do not require exogenous redox partners because they act upon peroxide-containing substrates (42–44). Analysis of the second gene in the P450cam operon revealed that it had strong homology with adenoredoxin reductase, the ferredoxin reductase that functions in the adrenal mitochondrial system (Table II). However, the third gene of the operon appeared to be a flavodoxin and not an iron-sulfur-containing protein, because a BLAST search indicated that it was most homologous to the FMN domain of CPR (Table II). This suggests that P450cam utilizes a novel, natural redox system for electron transfer, which consists of a FAD-dependent flavodoxin reductase and an FMN-containing flavodoxin. This system is intermediate between the classical class I bacterial P450 that utilizes two proteins, but which includes one with an Fe-S cluster, and the class II P450 that only employs a single protein but utilizes FAD and FMN cofactors. Although there is no precedent for this type of system occurring naturally, it has been reported that flavodoxins can act as “unnatural” redox partners for P450s. Waterman and co-workers (33, 45) have shown that the flavodoxin reductase and flavodoxin systems from both E. coli and Anaabaena will serve as functional redox partners for a variety of (heterologous) mitochondrial P450s. It should be noted that there is as yet no firm experimental evidence that links P450cam with the two proteins as its redox partners. However, as mentioned, the arrangement of the genes within the operon is identical to that seen with both P450cam and P450terp and their redox partners.

It is interesting to note that although the biodegradative P450s such as P450cam, P450terp, and P450cin occur in an operon closely associated with their redox partners, this is not the case for many other bacterial P450s uncovered by genome sequencing projects. For example, in Mycobacterium tuberculosis of the ~20 P450s identified, at least 14 have no obvious operon-associated redox partners. Perhaps this implies that biodegradative P450s are necessarily organized into operons with their electron transfer proteins to ensure coordinated expression and efficient functioning. Other bacterial P450s, which may not be required to operate as efficiently as biodegradative ones that supply the cell carbon and energy, may employ general redox partners such as cellular flavodoxins or ferredoxins.

P450cin was heterologously expressed in E. coli at a level of ~2000 nmol/liter of culture. Purification, including a hydroxyapatite column (46) as a final polishing step, gave essentially a homogeneous protein (~1000 nmol/liter) after three steps. Its identity was confirmed by N-terminal sequencing and electrospray mass spectrometry, which also indicated that some N-terminal processing (truncation of up to six amino acids) had occurred in a batch-dependent manner. However, spectral analysis of the protein (see below) revealed no effect of this inhomogeneity of the protein. Although the catalytic activity remains to be explored, it is not expected that this variation will significantly alter its efficiency, given that the N terminus is not implicated in either the mechanism of oxygen activation or the interaction with redox partners in any other bacterial P450 (47).

We postulated that as P450cin was believed to have a flavodoxin as a redox partner, it might utilize the endogenous E. coli flavodoxin/flavodoxin reductase system. Waterman and co-workers (33, 45) had reported that this system was responsible...
sible for the observed catalytic activity in a variety of heterolo-
guously expressed P450s. Thus, we expressed P450\textsubscript{cin} in the presence of cineole 1, and careful gas chromatography-mass spectrometry analysis of the organic extracts of the growth medium revealed the presence of a metabolite tentatively identi-
fied as 2-hydroxycineole 2. This compound was not produced when cineole 1 was added to the growth medium of \textit{E. coli} not expressing P450\textsubscript{cin}. While not conclusive, this result clearly demonstra-
tes that P450\textsubscript{cin} is capable of cineole 1 hydroxylation and strongly suggests that it is involved in its biodegradation.

UV-visible analysis of the heterologously expressed P450\textsubscript{cin} revealed the typical characteristics of a bacterial P450 (Fig. 3). Significantly, a spin state change of the heme iron from low spin to high spin was observed in the presence of cineole 1, typical of a bacterial P450 in the presence of its substrate. This and the tight binding of cineole 1 to P450\textsubscript{cin} ($K_d = 0.7 \mu M$) strongly support our hypothesis that the natural substrate for this enzyme is cineole 1, because it mirrors exactly the behavior of other terpene-degrading P450s with their substrates, e.g., P450\textsubscript{cam} (5), P450\textsubscript{org} (10), and P450\textsubscript{cin} (9). These observations combined with our \textit{in vivo} turnover rates also provide the first real evidence that a P450 is involved in the biodegradation of cineole 1. It will be of interest to determine the way in which this strong, specific binding of cineole 1 is achieved because it presents little, if any, opportunity for H-bonding. Such inter-
actions are known to play an important role in the specific number of unique features, such as an unusual asparagine

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