CYTOCHROME P450 LIMONENE HYDROXYLASES OF *MENTHA* SPECIES

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SUMMARY

The oxygenation pattern of the monoterpenoids of mint (*Mentha*) species is determined by regiospecific cytochrome P450-catalyzed hydroxylation of the common olefinic precursor (-)-limonene. In peppermint, C3-allylic hydroxylation leads to (-)-*trans*-isopiperitenol that ultimately is converted to (-)-menthol, whereas in spearmint, C6-allylic hydroxylation leads to (-)-*trans*-carveol that is oxidized to (-)-carvone. The limonene-6-hydroxylase and the cytochrome P450 reductase were purified from the oil glands of spearmint, and the system was reconstituted. Amino acid sequences from the purified hydroxylase were utilized to design primers with which a large, non-degenerate PCR product was prepared. This probe was employed to screen a spearmint oil gland cDNA library from which the corresponding full-length cDNA was isolated. This clone provides the tool for isolating the homologous cDNA species from peppermint and related *Mentha* species.

KEY WORDS

limonene hydroxylase, monoterpene hydroxylase, carveol, isopiperitenol, *Mentha* spp.

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INTRODUCTION

Terpenoids represent the largest family of plant natural products, exceeding 20,000 individual compounds /1/ and significantly outnumbering the alkaloids and phenylpropanoids combined /2/. P450 cytochromes play an essential role in the metabolism of terpenoids in reactions ranging from the oxygenation of carotenes to xanthophylls /3/ (tetraterpenoid photosynthetic accessory pigments), the demethylation of phytosterols /4/ (triterpenoid membrane structural components), the biosynthesis of gibberellins /5/ (diterpenoid plant hormones), and the production of certain phytoalexins /6/ (sesquiterpenoid defensive agents). In the monoterpene series, cytochrome P450 hydroxylases are responsible for establishing the oxygenation pattern of the various skeletal types /7,8/. For example, in the pinane (Fig. 1) and thujane (Fig. 2) monoterpenes, P450 catalyzed allylic hydroxylation of the parent olefins sets in motion a series of enzymatic reactions involving subsequent oxidation to the corresponding conjugated carbonyl compound, reduction of the now-activated α-β-double and, ultimately, reduction of the carbonyl to give a spectrum of stereochemically related products /9-11/. Such oxygenated monoterpene derivatives are largely responsible for the characteristic odors and flavors of the essential oils /12/ and often fulfill communication and defensive functions in the plants that produce them /2/.

Much of our recent research has been focused on a set of genetically very closely related cytochrome P450 hydroxylases of *Mentha* (mint) species that are involved in the metabolism of the simple *p*-menthane monoterpene olefin (-)4*S*-hmonene. This focus derives from the consideration that these enzymes are very similar in all features but the regiochemistry of oxygen insertion, and that detailed study of their properties could reveal fundamental structure-function relationships in cytochrome P450 catalysis. In this brief review, we provide an update of our research with these limonene hydroxylases.

THE LIMONENE HYDROXYLASES OF *MENTHA*

The monoterpene constituents of the essential oils of the genus *Mentha* (family Lamiaceae) are distinguished by the position of oxygenation on the *p*-menthane ring /13/. Of the agronomically significant mints /14/, peppermint [*M.* x *piperita* L., a sterile cross of *M. aquatica* L. x *M. spicata* L. /15,16/] and related species (*M.*

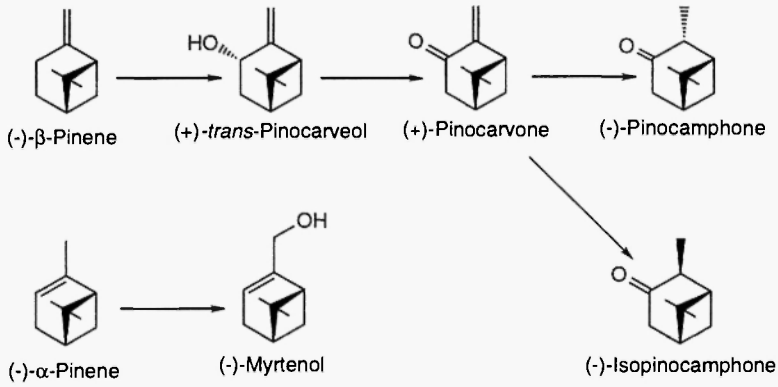

Fig. 1: Allylic oxidation-conjugate reduction pathway for the metabolism of (-)-pinenes in hyssop (*Hyssopus officinalis*).

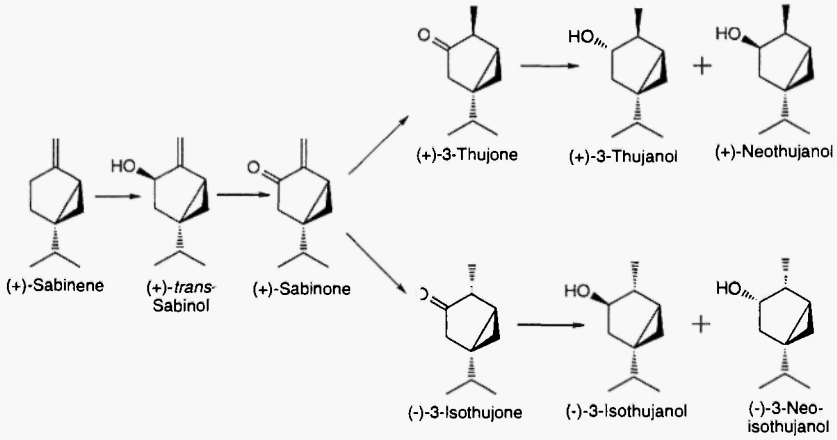

Fig. 2: Allylic oxidation-conjugate reduction pathway for the metabolism of (+)-sabinene in *Salvia*, *Tanacetum* and *Artemisia* species.

aquatica, *M. arvensis* and *M. pulegium*) produce almost exclusively monoterpenes bearing an oxygen function at C3 such as menthol (responsible for the cooling sensation of peppermint), whereas spearmint types [native spearmint (*M. spicata* L.), Scotch spearmint (*M.* x *gracilis* Sole, a cross of *M. arvensis* L. x *M. spicata* /17,18/) and *M. crispa*] produce almost exclusively monoterpenes bearing an oxygen function at C6, typified by carvone (responsible for the typical spearmint note) (Fig. 3).

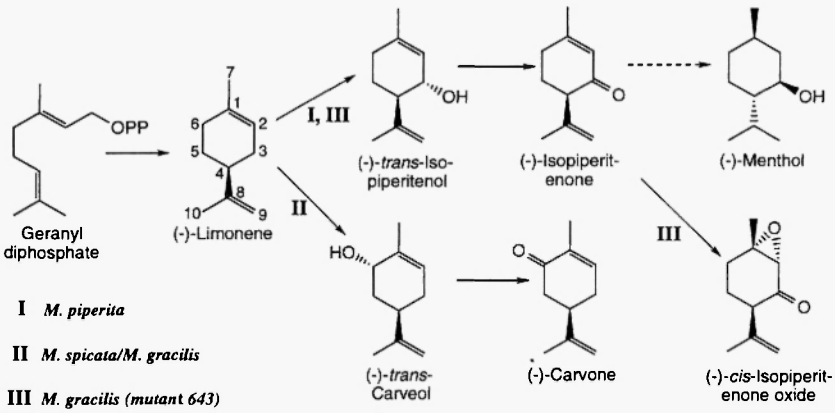

Fig. 3: Pathway for monoterpene biosynthesis in *Mentha* species involving regiospecific cytochrome P450-catalyzed hydroxylation of (-)-limonene as the essential determinant of the C3- or C6-oxygenation pattern of the resulting *p*-menthane metabolites.

The genetic basis of the C3- and C6-oxygenation patterns was the target of early studies /19,20/ and over the years has been examined in a lengthy series of hybridization experiments in which strains or varieties that contained a particular compound were crossed with strains lacking that compound, followed by numerous back-crosses between F_1 hybrids and either parent /21/. The *p*-menthane oxygenation pattern was established to be under the control of two closely linked diallelic loci designated ***Lm*** and ***C***; the dominant ***Lm*** allele is postulated to prevent C3-oxygenation whereas the recessive ***lm*** allele is thought to allow this conversion. In contrast, the dominant ***C*** allele is proposed to stimulate C6-oxygenation while its recessive counterpart *c* does not promote this conversion /21/. The two dominant alleles are believed to be tightly linked, accounting for the observation that the C3- and C6-oxygenation patterns are mutually exclusive and that C6-oxidation is dominant to C3-oxidation /22-24/. It has been suggested that ***Lm*** represents a regulatory gene that controls the expression of C3-hydroxylation and ***C*** is a structural or regulatory gene that controls C6-hydroxylation /25/. However, it is unlikely that the roles of ***Lm*** and ***C*** in *Mentha* can be clarified until the structures of the hydroxylase genes, and the ***Lm*** and ***C*** genes and gene products, are known and the molecular level interactions between these entities deciphered.

Biosynthetic investigations have demonstrated that the regiospecificity of oxygenation is established very early in the monoterpene biosynthetic sequence in which (-)-4*S*-limonene, the first cyclic olefin to arise from the common isoprenoid intermediate geranyl pyrophosphate and the precursor of *both* oxygenated series /26/, is hydroxylated at C3 to yield (-)-*trans*-isopiperitenol (in peppermint-type species) or at C6 to afford (-)-*trans*-carveol (in spearmint-type species) /27/. The remaining enzymatic machinery responsible for the subsequent redox transformations of isopiperitenol to menthol is present in both peppermint and spearmint species; however, carveol is a poor substrate for these reactions, with the result that only the dehydrogenation product, carvone, accumulates in spearmint types /28/.

Since the monoterpenes of *Mentha* and other members of the Lamiaceae are produced and accumulated in glandular trichomes /29,30/, highly specialized secretory structures found on the surfaces of the leaves, the hydroxylase systems were examined in cell-free extracts isolated specifically from these structures /31,32/ by procedures established for mixed-function oxidases /33/. Microsomal preparations from the epidermal oil glands of peppermint, native spearmint and Scotch spearmint catalyze the NADPH- and O_2-dependent allylic hydroxylation of (-)-limonene /27,28/ and the responsible enzymes meet most of the established criteria for cytochrome P450-dependent mixed function oxidases (including inhibition by CO and blue light reversal) /5,33/. The reactions catalyzed are completely regiospecific. In Scotch and native spearmint, the cytochrome P450-dependent hydroxylases give rise exclusively to (-)-*trans*-carveol. (-)-*trans*-Isopiperitenol cannot be detected as a product, indicating the presence of only (-)-limonene-6-hydroxylase /27,28/ (Fig. 3). Conversely, microsomes obtained from peppermint glands produce only (-)-*trans*-isopiperitenol when incubated with (-)-limonene; (-)-*trans*-carveol is not detected as a product, indicating the exclusive presence of (-)-limonene-3-hydroxylase /27/. These results are entirely consistent with the accumulation of C6-oxygenated *p*-menthane derivatives in spearmint and of C3-oxygenated *p*-menthane compounds in peppermint, and they establish these cytochrome P450 hydroxylases as the key enzymes that determine the type of monoterpenes formed in most *Mentha* species.

An interesting result, that bears on the genetic control of monoterpene hydroxylation in mints, has been obtained from the biochemical evaluation of a γ-radiation-induced mutant of Scotch

spearmint (*M.* x *gracilis*) that produces a peppermint-type oil (C3-oxygenation pattern), unlike the spearmint-type oil (C6-oxygenation) found in the wild type /28/. Assay of all the enzymes responsible for the production of both the C3-oxygenated and C6-oxygenated families of monoterpenes from (-)-limonene indicated that both the mutant and wild type possess a virtually identical complement of catalysts, with the exception of the microsomal, cytochrome P450-dependent (-)-limonene hydroxylase; the C6-hydroxylase producing (-)-*trans*-carveol in the wild type has been entirely replaced by a C3-hydroxylase producing (-)-*trans*-isopiperitenol in the mutant. In addition, the mutant, but not the wild type, catalyzes the cytochrome P450-dependent, stereospecific (1*S*,2*S*)-epoxidation of the α,β-double bond of the ketones formed via C3-hydroxylation to produce the corresponding oxides, such as (-)-*cis*-isopiperitone oxide /28/ (Fig. 3). All the evidence is consistent with the same protein carrying out both oxygenase activities (i.e., C3-hydroxylation and 3-keto-C1,C2-epoxidation); there is precedent for such bifunctional P450 cytochromes in other systems /34,35/. These results suggest that irradiation resulted in either mutation of the C6-hydroxylase structural gene, converting it to a 3-hydroxylase-1,2-epoxidase, or mutation of a regulatory gene (perhaps ***Lm***) to a form which suppresses 6-hydroxylation and activates a nascent 3-hydroxylase-1,2-epoxidase. The coupling of 3-hydroxylase and 1,2-epoxidase activity in a single gene product could explain the origin of a number of *Mentha* hybrids in which monogenic differences result in the production of 1,2-epoxides as well as the expected C3-ketones /36/. This bifunctional cytochrome P450 is probably related to, but distinct from, the C3- and C6-limonene hydroxylases of peppermint and spearmint, respectively, neither of which possesses epoxidase activity /28/.

All of the *Mentha* hydroxylases thus far examined appear to be highly specific for limonene as substrate, although they exhibit only a modest degree of enantioselectivity in this regard. The utilization, at respectable rates, of (+)-4*R*-limonene as a hydroxylation substrate (25-50% of V_{rel} for the (-)-4*S*-enantiomer at saturation /27/) indicates that the axial or equitorial orientation of the C4-isopropenyl substituent with respect to the cyclohexenyl π-system is a minor determinant of reaction competence. On the other hand, of numerous monoterpene olefins tested, including several positional isomers of limonene, only the 8,9-dihydro analog serves as an alternate substrate for ring (C3 or C6) hydroxylation. The high degree of substrate specificity, and the

regioselectivity of allylic oxidation observed with limonene as substrate in these glandular-derived systems of plant origin, is in marked contrast to limonene-oxygenating systems in other phyla which tend to be considerably less discriminating. In addition to the strict regiochemistry of the *Mentha* C3- and C6-hydroxylases, these enzymes are also readily distinguishable based on differential inhibition by substituted azoles /27,28/. In all other properties, the peppermint, spearmint, scotchmint and mutant hydroxylases are very similar, perhaps not surprising in view of the fact that they are genetically so closely related in sharing native spearmint (*M. spicata*) as a parent. Thus, in K_m value (19 ± 1 μM), V_{rel} (62.5 ± 1.5 nmol/h•mg microsomal protein), pH optimum at 7.4 and inhibition kinetics with CO and cytochrome *c*, they are essentially identical /27,28/.

PURIFICATION AND RECONSTITUTION

The limonene hydroxylases of *Mentha* oil glands are constitutive biosynthetic enzymes and, unlike many cytochrome P450-dependent systems thus far isolated from plants, they do not appear to be inducible to higher levels of activity. This disadvantage, however, is balanced by the fact that the secretory cells of the glands can be isolated from the leaf surfaces to provide a highly enriched starting material for enzyme isolation. The minute yellow oil glands are the sites of both monoterpene biosynthesis and storage, and their removal from the upper and lower leaf surfaces is accomplished by controlled agitation in a viscous buffer containing reducing agents, antioxidants, polymeric adsorbents and glass beads /32,37/. During the leaf surface abrasion process, the gland subcuticular oil reservoir is ruptured and the supporting stalk cell is fractured to provide a disc of eight secretory cells. These secretory cell clusters are purified by sequential filtration through nylon mesh of progressively smaller pore size; typically, 40 g of immature leaves will yield about 7×10^7 gland cell clusters.

The secretory cells of the clusters are disrupted by sonication in a buffer containing polystyrene beads and vinylpyrrolidone polymers to adsorb deleterious lipophilic substances and phenolic materials during liberation of the soluble and membranous enzymes. In spite of these precautions, considerable terpenoid and flavonoid materials partition into the membranes during cell disruption, as evidenced by both spectroscopic evaluation and GLC-MS analysis of membrane extracts. This feature, in addition to the unleashing of active oxygen species on

cell disruption (a problem only partially alleviated by antioxidants or addition of superoxide dismutase, peroxidase or catalase), contributes to the rapid loss of P450 measured spectrally or by catalysis in these crude preparations.

Microsomes prepared from the sonicate by differential centrifugation are the starting material for purification of the limonene hydroxylases. The yields of microsomal protein are quite low (1 mg of oil gland microsomal protein from 10 g of leaves, with specific content of cytochrome P450 of ~1 nmol/mg); however, the specific hydroxylation activities observed, in the range of 4 nmol/min•mg, are typical of other plant P450 systems /38-45/. Over 97% of the hydroxylase activity of the intact oil glands can be recovered in the microsomal pellet, although the proportion of "operationally-solubilized" activity generated during tissue disruption varies significantly. This effect may be related to environmental and developmental influences on the levels of endogenous, detergent-like, small molecules that assist in membrane solubilization during cell breakage; the solubilized limonene hydroxylase remains as P450, with minimal conversion to the P420 form, suggesting that "solubilization" does not involve phospholipase activity. Sucrose density gradient centrifugation shows the microsomal limonene hydroxylase activity to migrate at a density of 1.12 g/cm^3 (little purification is achieved by this procedure), and SDS-PAGE of total microsomal protein demonstrates this material to be dominated by a very prominent 55-57 kDa protein band in which at least two cytochrome P450 species can be discerned by immunoblotting using several polyclonal antibody preparations (see below).

A wide range of ionic, non-ionic and zwitterionic detergents were tested for solubilizing limonene hydroxylase from the microsomes and, while recoveries of cytochrome P450 were generally high, severe losses of hydroxylase activity were often encountered. Solubilization with octyl glucoside and Emulgen 911 gave the best results and the course of the subsequent purification was monitored in several ways: (a) Spectroscopically by CO-difference spectra or substrate-binding spectra. Typically, spearmint limonene-6-hydroxylase is isolated in the high spin or mixed spin state, presumably due to interaction with endogenous substrate(s). As purification progresses, a peak at 420 nm emerges in the direct spectrum, which partially reverts to the high spin form when (-)-limonene is added. (b) By antibody binding assay (dot blotting) with rabbit anti-avocado P450 /42/ or anti-P450$_{cam}$ (Oxygene, Dallas, TX). The former polyclonal antibody preparation recognizes at

least two proteins in the 55-57 kDa region on Western blotting of spearmint, scotchmint and peppermint microsomal extracts. (c) By reconstitution of NADPH-supported hydroxylation with purified cytochrome P450-reductase from mung bean /46/ or mint. This was only practical at the final stages of the work when the mint reductase was routinely available. (d) By SDS-PAGE. As indicated previously, microsomal preparations are dominated by proteins of mass 55 to 57 kDa.

A range of methods was evaluated for the purification of spearmint hmonene-6-hydroxylase. Hydrophobic interaction chromatography (affinity elution with (-)-limonene plus detergent) yielded sufficient pure protein for amino-terminal and internal (V8-proteolysis) microsequencing, whereas preparative PAGE provided antigen suitable for polyclonal antibody production in rabbits /47/ (the purified IgG recognizes a 57 kDa microsomal protein from all of the mint species). However, these protocols were not reproducible and, thus, were unsuitable for routine use with large-scale preparations in which the hydroxylase and cytochrome P450 reductase could be concurrently fractionated.

A superior method for cytochrome P450 purification, that promoted heme preservation and the removal of small lipophilic contaminants, was developed that involves microsome solubilization with Emulgen 911 followed by protein precipitation with 30% polyethylene glycol (M_r ~4,000). The suspended protein is first fractionated on DEAE-Sepharose (KCl elution), a step which also separates NADPH-dependent cytochrome P450 (cytochrome *c*) reductase. The highly enriched cytochrome P450 fractions (specific content up to 9 nmol/mg) are concentrated by ultrafiltration and applied to a second DEAE-Sepharose column, this time in 10 mM CHAPS, with KCl elution as before, to give the apparently homogeneous hydroxylase (a single band at 57 kDa by SDS-PAGE) in 8% overall yield from microsomes. The isolated protein exhibits an absorbance maximum at 420 nm in the Soret region. Upon reduction with dithionite, the spectrum is slightly red-shifted, and the CO-bound ferrocytochrome shows a maximum at 448 nm.

The cytochrome P450 reductase from spearmint (measured as cytochrome *c* reductase) was isolated by Emulgen 911 detergent solubilization of microsomes and 30% polyethylene glycol precipitation, as above. The reductase is then separated from the cytochrome by anion exchange chromatography on DEAE-Sepharose, and purified

by combination of dye-ligand interaction chromatography on Matrex Gel Red A and affinity chromatography on 2,5-ADP Sepharose (elution with NADPH). The reductase can be obtained in about 20% yield after this 200-fold purification, and exhibits a single major band at ~80 kDa on SDS-PAGE that cross-reacts on immunoblotting with polyclonal antibodies raised against Jerusalem artichoke /48/ and mung bean /46/ cytochrome P450 reductases.

For reconstitution, the purified reductase (110-150 mU) from spearmint or mung bean /46/ and the cytochrome P450 limonene-6-hydroxylase (200 pmol) are combined on ice, followed by the addition of flavins and dilauryl phosphatidyl choline and raising the temperature to 32°C. Dilution of the mixture with buffer, and the addition of saturating concentrations of (-)-4*S*-limonene and NADPH, affords levels of *trans*-carveol production corresponding to turnovers in the range of 0.02-0.2/sec. Rates for reconstituted cytochrome P450 systems of plant origin are typically 1% or less of those observed in normal microsomal catalysis /49/.

cDNA ISOLATION

The glandular trichomes are the principal site of monoterpene biosynthesis in *Mentha*, and the secretory cells of these structures are thus highly enriched in the relevant enzymes and their corresponding messages. To avoid the complications of screening a whole leaf cDNA library, an oil gland library was therefore constructed. The previously developed methods /32,37/ for the isolation of oil gland secretory cell clusters were employed with some modification. The nature of the leaf surface abrasion protocol limited the use of strong denaturants that are typically added to inactive endogenous nucleases during RNA isolation. As an effective alternative, the low molecular weight RNase inhibitor aurintricarboxylic acid /50/ was added at all stages of gland cell isolation. The resulting secretory cells were frozen in liquid N_2, powdered with a mortar and pestle, and extracted with 6M guanidine-HCl and then organic solvents. RNA was either specifically precipitated or pelleted through a CsCl gradient. mRNA was isolated by oligo(dT)-cellulose column chromatography, and the quality was assessed by *in vitro* translation and SDS-PAGE of the translated proteins. Typically, 1 g of gland cells yields 200-500 μg of total RNA from which roughly 1% poly(A)$^+$ RNA could be isolated. cDNA synthesis from mRNA and construction of the λ ZAP cDNA expression library were carried out with a commercial kit (Stratagene).

The spearmint limonene-6-hydroxylase, purified by hydrophobic interaction chromatography and SDS-PAGE, was employed for microsequencing of the amino-terminus and of internal fragments generated bv "in gel" V8 proteolysis /51/. The enzymatic proteolysis procedure yielded three major peptides on SDS-PAGE whose combined mass equaled approximately 60 kDa; one partially sequenced peptide represented the amino-terminus, a second originated from near the carboxy-terminus (based on homology to an avocado P450 sequence /52/), and the third was presumed to originate from the central portion of the protein. Degeneracy considerations prevented direct use of the amino acid sequence data for oligonucleotide probe construction.

Short degenerate primers were designed to prime the termini of each peptide sequence for PCR amplification of the corresponding nucleotide sequences using spearmint oil gland-derived cDNA as target. The PCR primers bordering the central peptide amplified a DNA fragment of appropriate size that was verified by cloning and sequencing. A non-degenerate forward primer was then designed from the internal nucleotide sequence which, when combined with a reverse primer designed to the original carboxy-terminal peptide, permitted the amplification of a highly specific 700 bp DNA fragment. This probe was next used to screen the spearmint oil gland cDNA library by plaque hybridization, from which a full length cDNA was isolated and sequenced. This cDNA clone encoded all of the amino acid micro-sequence data at the expected positions as well as the amino acid motifs conserved among known P450 proteins, including the heme binding site. The deduced amino acid sequence also showed significant homology to other cytochrome P450 sequences of plant origin /52-57/.

Sequence information from the full length spearmint hydroxylase cDNA was utilized to construct a selective probe for the isolation of the corresponding (-)-limonene hydroxylase genes from peppermint and other *Mentha* species. Specifically, PCR methods were employed to generate a 500 bp probe designed to recognize the 5′-DNA sequence encoding the hydroxylase amino-terminus. This probe insures the isolation of only full length cDNA clones and is highly specific for the *Mentha* (-)-limonene hydroxylase P450 cytochromes.

PROSPECTS

The limonene hydroxylases of *Mentha* provide a unique opportunity to examine active site structure-function relationships in a closely

related set of P450 cytochromes. Sequence comparison between these very similar catalysts, along with the use of photoprobes to localize the substrate binding sites /58/, should target small domains and, potentially, individual residues responsible for differences in regiochemistry and as candidates for mutagenesis. Functional overexpression /59/ of recombinant native and mutant forms, and evaluation of kinetics and product outcomes coupled to the use of fluorinated substrate analogs and NMR-based methods /60/, should permit the determination of substrate binding topography with respect to the heme-iron. With advances in the X-ray crystallographic analysis of P450 cytochromes /61,62/ it may ultimately be possible to physically model the subtle active site structural features that underly the strict regiochemistry of oxygen insertion by these limonene hydroxylases.

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