Design of Fluorescent Substrates and Potent Inhibitors of CYP73As, P450s That Catalyze 4-Hydroxylation of Cinnamic Acid in Higher Plants†

Michel Schalk,‡ Yannick Batard,‡ André Seyer,§ Svetlana Nedelkina,|| Francis Durst,‡ and Danièle Werck-Reichhart*,‡

Département d'Enzymologie Cellulaire et Moléculaire, Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique UPR 406, 28 rue Goethe, 67000 Strasbourg, France, Laboratoire de Chimie Bio-organique, Faculté de Pharmacie, Centre National de la Recherche Scientifique URA 1386, 74 route du Rhin, 67048 Strasbourg Cedex, France, and Institute of Cytology and Genetics, 630090 Novosibirsk-90, Russia

Received June 30, 1997; Revised Manuscript Received September 29, 1997⊗

ABSTRACT: CYP73As are the major functional cytochromes P450 in higher plants. Several of them have been shown to catalyze the 4-hydroxylation of cinnamic acid, the first oxidative step in the synthesis of lignin, flavonoids, coumarins, and other phenylpropanoids. The coding sequence for CYP73A1, the enzyme from *Helianthus tuberosus*, has been isolated and expressed in yeast. Previous studies indicate that the yeast-expressed enzyme is capable of metabolizing cinnamic acid and several small, planar molecules but with low efficiency. Using this we further examined how CYP73A1 could bind and metabolize a set of possible alternate substrates. We show here that naphthalenes, quinolines, and indoles substituted with an aldehyde, a carboxylic, or a sulfonic acid group make good ligands and substrates for CYP73A1. The best ligands are hydroxynaphthoic acids, which show higher affinity than cinnamate. Naphthalene, 2-naphthol, and molecules with two-carbon side chains, such as natural and synthetic auxins, are not substrates of this enzyme. Methyl-2-naphthoate and 2-hydroxy-1-naphthoic acid are strong ligands of CYP73A1 but are not metabolized. Uncoupling and low spin conversion induced by these compounds suggest that their positioning in the heme pocket is inadequate for catalysis. These compounds can act as potent inhibitors of the second step of the phenylpropanoid pathway, the first described so far. The molecule which most closely mimics cinnamic acid, 2-naphthoic acid, is metabolized with a catalytic turnover and efficiency similar to those measured with the physiological substrate. Using this compound we designed a fluorometric assay to measure the catalytic activity of CYP73As. This assay was then used to monitor the CYP73As activity in microsomes from transgenic yeast and several plant species.

Cytochromes P450 from the CYP73A subfamily catalyze the 4-hydroxylation of cinnamic acid. This oxygenation reaction constitutes the second step in the plant-specific pathway of phenylpropanoid metabolism, that leads to the synthesis of lignin, anti-UV and insect attracting pigments, and several classes of defense-related molecules (*1*). The genes of at least 15 members of this P450 subfamily have now been isolated from different sources, including herbaceous and woody monocot and dicot plants. Theses genes usually share more than 85% identity at the amino acid level and possess long stretches of totally conserved sequences.

Interest in these P450s rests with their position upstream of the major pathway of plant secondary metabolism and its possible control. Better understanding of the roles of these enzymes holds significant physiological and economic importance. This is true with regard to lignin synthesis and pathogen resistance. The quantitative abundance of this enzyme in some plants suggests a crucial role, where CYP73A1 can represent up to 56% of the total P450 in wounded plant tissues (*2*). This may also be crucial for plants ability to process a significant proportion of their biomass, as lignin represents up to 35% of the dry weight of some woody plants (*3*). Finally, the possible control of the flux of metabolites between the main branch of the pathway and the synthesis of C6−C1 compounds, includes the biosynthesis of salicylic acid, a crucial signaling molecule that has been implicated in flowering, thermogenesis, and disease resistance (*4*). As a consequence, cinnamate hydroxylase may be an ideal target for manipulating lignification or defense-related reactions in plants. Moreover, if CYP73As are also capable of metabolizing xenobiotics, as suggested by previous studies, then their abundance in the plant kingdom may also confer resistance to environmental toxins. Plant are believed to act as metabolic sinks for environmentally harmful molecules (*5*). To assess these possible capabilities, we developed procedures to evaluate the metabolic capacity of these widespread and highly expressed oxygenases.

CYP73A1 is the cinnamate 4-hydroxylase (C4H)[1] from *Helianthus tuberosus*, a member of the Asteraceae family, closely related to sunflower. The coding sequence of the enzyme isolated from tuber tissues (*6*) was expressed in yeast (*7*). In microsomes from transgenic yeast, CYP73A1

†This work was supported by a MRES grant to M.S.

* Corresponding author. Fax: 33 3 88 35 84 84. E-mail: daniele.werck@ibmp-ulp.u-strasbg.fr.

‡ CNRS UPR 406.

§ CNRS URA 1386.

|| Institute of Cytology and Genetics.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1997.

[1] Abbreviations: C4H, *trans*-cinnamate 4-hydroxylase [NADPH: oxygen oxidoreductase (4-hydroxylating), EC1.14.13.11]; 2-NA, 2-naphthoic acid; NAH, 2-naphthoic acid 6-hydroxylase; HS, high spin; t_R, retention time.

S0006-2960(97)01575-4 CCC: $14.00

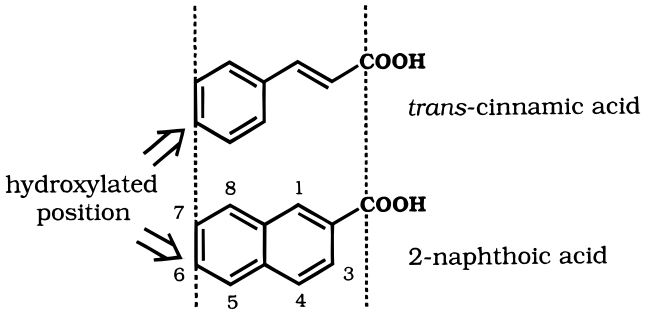


FIGURE 1: Strucural analogy between 2-naphthoic acid and *trans*-cinnamic acid.

represented up to 2% of the total protein. Its catalytic turnover was ten times higher than in membranes isolated from the original plant. Substrate specificity of the yeast-expressed enzyme was also investigated in the metabolism of 36 molecules (*8*). These were mostly secondary metabolites reported to be oxygenated by plant P450s and included other model substrates, drugs, or herbicides. That data showed that a number of small planar molecules with dipolar character were metabolized with low efficiency by CYP73A1. These alternate substrates included methoxycoumarin, ethoxycoumarin, *p*-chloro-*N*-methylaniline, and the herbicide chortoluron. However, the catalytic efficiency (V_{max}/K_m) of the dealkylation or hydroxylation of these molecules did not exceed 0.13 min^{-1} μM^{-1}. Based on this, we postulated that 2-naphthoic acid (2-NA) would be an ideal model substrate for the enzyme (Figure 1). Being planar and having the same size as cinnamic acid, its carboxylate moiety should ensure efficient access and/or positioning into the active site.

In this paper, we show that 2-NA is metabolized with the same efficiency as cinnamate, the natural substrate of CYP73A1. Several 2-NA analogs are also very good substrates of CYP73A1 while others behave as strong competitive inhibitors. This work provides a basis (i) for the design of a rapid fluorometric assays to measure CYP73As activity,[2] (ii) for an investigation of the active site topology, and (iii) for the design of new, specific inhibitors of this enzyme.

EXPERIMENTAL PROCEDURES

Chemicals. *trans*-Cinnamic acid, 2-naphthoic acid, benzoic acid, salicylic acid, and 2-naphthol were from Sigma. Methyl 2-naphthoate, 2-methylnaphthalene, 2-acetylnaphthalene, and 6-hydroxy-2-naphthoic acid were from Lancaster Synthesis (Morecambe, U.K.). 1-Naphthoic acid, 1-naphthalenesulfonic acid, and 2-naphthaldehyde were from Fluka. 2-Hydroxy-1-naphthoic acid, 1-hydroxy-2-naphthoic acid, 2-hydroxy-3-naphthoic acid, 2-naphthalenesulfonic acid, 1-hydroxy-2-naphthaldehyde, 4-hydroxy-1-naphthalenesulfonic acid, 2-naphthalenethiol, 2-aminonaphthalene, 1-chloronaphthalene, 2-naphthaleneacetonitrile, 1-naphthaleneacetic acid, 2-naphthaleneacetic acid, 6-hydroxy-2-naphthalenesulfonic acid, 2,6-dihydroxynaphthalene, quinoline-2-carboxylic acid, 5-isoquinolinesulfonic acid, indole-2-carboxylic acid, indole-3-carboxylic acid, and indole-3-acetic acid were from Aldrich. *trans*-[3-^{14}C]Cinnamic acid was from Isotopchim (Ganagobie, France). [1-^{14}C]Capric acid (C10:0) was from New England Nuclear. All chemicals were of the highest purity available from commercial sources and were used without further purification. The purity was 80%−98% depending on chemicals.

Yeast and Plant Microsomes. The *Saccharomyces cerevisae* strain W303-1B (*Mat* α; *ade2*-1; *his3*-11,-15; *leu2*-3,-112; *ura3*-1; *can*^{R}; *cyr*$^+$), also designated W(R), overexpressing its own NADPH-P450 reductase, was constructed by Truan *et al.* (*10*). Plasmid C4H/V60, yeast transformation and preparation of yeast microsomes were previously described (*7*). Microsomes from the W(R) yeast strain transformed with the V60 void plasmid were used as a negative control. Microsomes from *H. tuberosus* L. var. Blanc commun were prepared from the aminopyrine-induced tuber tissues as described previously (*11*). Extraction of *Vicia sativa* L. var. minor microsomes was as described (*12*). Maize (*Zea mays* L. var. LG11) embryo microsomes were prepared as described by Rahier and Taton (*13*).

Enzyme Assays. *trans*-Cinnamic acid hydroxylation was assayed as previously described (*14*). The NADPH-dependent metabolism of the other substrates was monitored by HPLC or fluorometry.

When possible, the metabolism of fluorescent molecules was recorded using a Shimadzu RF-5000 spectrofluorophotometer. The standard assay contained 25−75 pmol of P450, 4 mM glucose-6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase, and 0.1−1 mM potential substrate in 2 mL sodium phosphate (0.1 M, pH 7.4). The temperature of the assay medium was equilibrated at 30 °C before initiating the reaction by addition of 100 μM NADPH. Enzymatic activities were calculated from the time-dependent changes in fluorescence emission of the product. Excitation and emission wavelengths used in each case are given in tables and text.

The standard assay for HPLC analysis contained, in a final volume of 0.25 mL, 25−50 pmol of P450, 0.1 M sodium phosphate (pH 7.4), 500 μM NADPH, 4 mM glucose-6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase, and 0.1−1 mM substrate (10-fold the K_S or solubility limit if the K_S was too high). After 10−60 min at 30 °C, the incubation was stopped by addition of 20 μL of 4 N HCl and 1000 Bq of [1-^{14}C]capric acid as the internal standard. The reaction mixture was then extracted twice with 1 mL diethyl ether, and the concentrated extract was analyzed by HPLC as described below.

I_{50}, concentrations of inhibitor resulting in 50% inhibition of C4H activity when the substrate concentration is equal to K_m (i.e. 2 μM), were determined from a linear fit of (1/activity) versus inhibitor concentration. Duplicate incubations at six different concentrations of inhibitor (to give 0−90% inhibition) were used for each determination.

Kinetic data were fitted using the nonlinear regression program DNRPEASY derived by Duggleby and Leonard from DNRP53 (*15*).

Chromatographic Analysis. Substrates and products resulting from incubation of 2-NA or *trans*-cinnamic acid were separated by TLC on fluorescent silica plates (60 F254, Merck, Darmstadt), developed in toluene: acetic acid: water (6:7:1; v/v/v). Alternatively, and for other substrates, reactions products were analyzed by reverse-phase HPLC on a Waters model 510 instrument equipped with a Waters model 480 absorbance detector and a Raytest model Ramona-D solid scintillation counter. Separations were per-

[2] A brief description of the fluorometric NAH assay appeared in (*9*).

formed on a C18 5μ 100 × 4.6 mm Brownlee column using a mobile phase consisting of aqueous acetonitrile containing 0.1% acetic acid at a flow rate of 1 mL min^{-1}. The solvent composition was kept constant for 20 min at initial conditions which were adapted (from 5% to 25% CH_3CN) for each substrate and then modified as a linear gradient up to 80% CH_3CN over 10 min. The solvent composition was held at these final conditions for 10 min. The wavelength of UV detection was from 235 to 310 nm, depending on the substrate. Percentage of conversion was estimated from the diminution of substrate.

NMR and Mass Spectrometry Analysis. For NMR and mass spectrometry, the oxygenated metabolite of 2-NA was purified successively by TLC and HPLC. ^{1}H NMR spectra were recorded on a Brucker WP SY 200 (200 Mhz) instrument with CD_3OD as an internal reference. Chemical shifts are reported in δ (ppm). Before GC−MS analysis, the metabolite was silylated with a mixture of bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane and pyridine (1:1; v/v). The analysis was monitored on a 1% SE30 capillary column (30 m × 0.025 mm) programmed to rise from 80 to 250 °C at 3 °C min^{-1}, coupled to a LKB 900S mass spectrometer with an LKB 2130 computer on line.

Immunological Studies. A rabbit polyclonal serum, C4Hpa1 (*16*), raised against *H. tuberosus* purified C4H was used to check for CYP73 dependence of enzymatic activities in yeast and plant microsomes. The microsomes were incubated 5 min at room temperature with an equal volume of serum before measuring enzymatic activities.

Spectrophotometric Measurements. Spectrophotometric measurement of total P450 content, quantification of microsomal protein, and determination of the binding constants for the different substrates were performed as described previously (*8*). NADPH consumption was monitored by differential spectrophotometry as described (*8*).

RESULTS

Product of 2-NA Oxidation by CYP73A1. To test whether 2-NA was a good alternate substrate of CYP73As, 2-NA was incubated in the presence of NADPH and microsomes prepared from CYP73A1-transformed yeast. HPLC analysis (solvent:water:CH_3CN:CH_3COOH, 75:25:0.1, v/v/v) of an extract from the incubation medium showed the time-dependent formation of a single, more polar metabolite detected at 285 nm [t_R(substrate) = 4.1 min, t_R(product) = 20.3 min]. Formation of this metabolite was not observed in the absence of NADPH or after incubation with control yeast microsomes. Formation of this metabolite was completely inhibited by anti-(*H. tuberosus* C4H) antibodies. This polar metabolite was purified and concentrated by successive HPLC and TLC (R_f substrate = 0.55, R_f product = 0.68) and analyzed by GC−MS and NMR spectroscopy. The NMR spectrum of the product was ^{1}H NMR (200 Mhz, CD_3OD): δ, 7.14 (dd, J = 2.5 Hz, J = 9.6 Hz, 1H, C-7 H), 7.16 (d, J = 2.5 Hz, 1H, C-5 H), 7.68 (d, J = 8.7 Hz, 1H, C-4 H), 7.85 (d, J = 9.6 Hz, 1H, C-8 H), 7.93 (dd, J = 1.7 Hz, J = 8.7 Hz, 1H, C-3 H), 8.48 (d, J = 1.7 Hz, 1H, C-1 H). Major ion fragments being observed by GC−MS analysis of the same product after silylation were m/z 332 [M^+], 317 [$M^+ - 15$], [$M^+ - 59$], 243 [$M^+ - 86$], 151 [$M^+ - 181$], and 73 (base peak). Modeling (Figure 1) predicts the formation of 6-hydroxy-2-naphthoic acid. When spectra of this metabolite were compared to those obtained with commercial 6-hydroxy-2-naphthoic acid both mass and NMR spectra were identical to the authenticated sample.

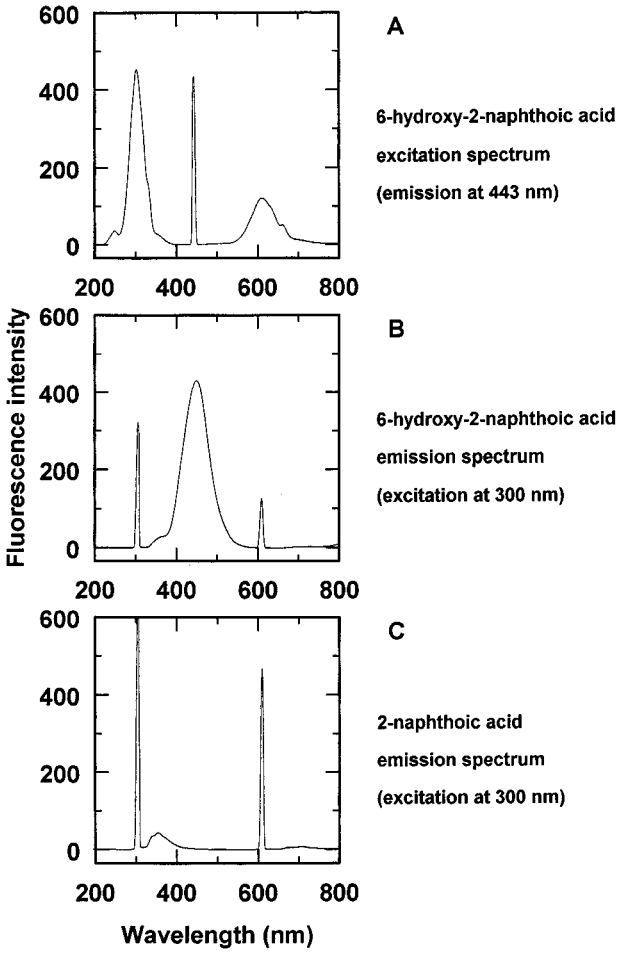


FIGURE 2: Fluorescence excitation and emission spectra of 2-naphthoic and 6-hydroxy-2-naphthoic acids. Spectra were recorded at 30 °C in 100 mM sodium phosphate pH 7.4, in the presence of 50 μM NADPH, 1 mM glucose-6-phosphate, 0.2 units of glucose-6-phosphate dehydrogenase, 0.1 mg of control-yeast microsomal protein, and 25 μM substrate or product. (A) Fluorescence excitation of the 6-hydroxy-2-naphthoic acid, emission measured at 443 nm. (B) Fluorescence emission of 6-hydroxy-2-naphthoic acid excited at 300 nm. (C) Fluorescence emission of 2-naphthoic acid excited at 300 nm.

Fluorescent Assay of 2-NA Hydroxylation. 6-Hydoxy-2-naphthoic acid, the product of the metabolism of 2-NA by CYP73A1, is a fluorescent molecule. This property was exploited to devise a rapid and sensitive assay of CYP73A catalytic activity as shown by the excitation and emission wavelengths of the substrate and product of the reaction (Figure 2). The optimal excitation wavelength of 6-hydroxy-2-naphthoic acid varies slightly with buffer composition and the presence of NADPH, substrate and microsomal protein. In 100 mM sodium phosphate pH 7.4 and in the presence of 50 μM NADPH, its maximum excitation is 300 nm (Figure 2A). Emission does not depend on the composition of the medium and is maximal at 443 nm (Figure 2B). 2-Naphthoic acid does not interfere with these measurements since, when excited at 300 nm, it does not emit at 443 nm (Figure 2C). Direct monitoring of the time-dependent change in fluorescence in the incubation medium is possible, as shown in Figure 3A.

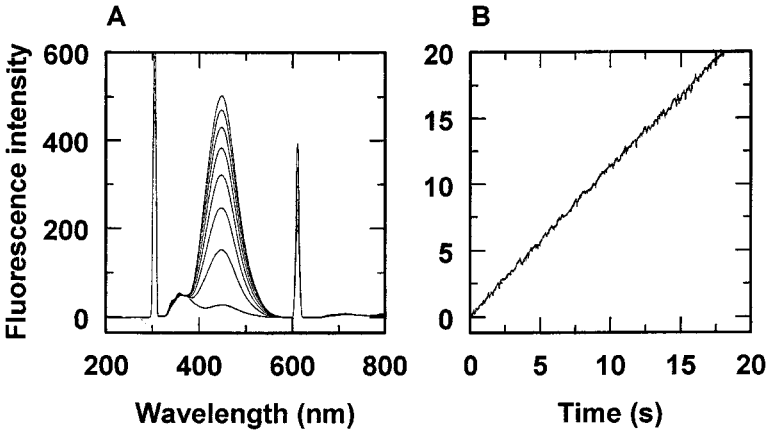


FIGURE 3: Formation of 6-hydroxy-2-naphthoic acid in the enzymatic assay. 2-Naphthoic acid was incubated at 30 °C in 100 mM sodium phosphate pH 7.4, in the presence of 50 μM NADPH, 1 mM glucose-6-phosphate, 0.2 units of glucose-6-phosphate dehydrogenase, and 15 nM CYP73A1. (A) Fluorescence emission (excitation at 300 nm) recorded every 5 min. (B) The variation of emission at 443 nm (excitation at 300 nm) was recorded as a function of time.

The standard assay, optimized for the fluorometric measurement of 6-hydroxy-2-naphthoate formation, is as follows: 100 μM 2-NA (stock solution in dimethyl sulfoxide), 50 μM NADPH, 0.2 units of glucose-6-phosphate dehydrogenase, and 1 mM glucose-6-phosphate in 100 mM sodium phosphate buffer, pH 7.4. The mix is equilibrated at 30 °C for 2 min in a thermostated cuvette before initiating the reaction by addition of 0.1−1 mg of microsomal protein. Fluorescence changes at 443 nm are directly recorded as a function of time, using an excitation wavelength of 300 nm (Figure 3B). The assay is calibrated by adding known amounts of 6-hydoxy-2-naphthoic acid to the incubation medium (250−1000 pmol). Under these conditions, the rates of 6-hydroxy-2-naphthoic acid formation measured with yeast microsomes are linear for 3−60 min, depending on substrate concentration.

Suitability for Measurement of CYP73A Activity in Plant Microsomes. The metabolism of 2-NA by microsomes from different plant species was assayed as described above in order to (i) confirm the suitability of the method for measurement of CYP73A1 activity in plant microsomes, (ii) test its efficiency to assay activity of other CYP73A enzymes, and (iii) check if naphthoic acid hydroxylase (NAH) activity is representative of CYP73As in plant extracts from different sources. Due to quenching of fluorescence the assay was not appropriate for measurements of CYP73A activity in microsomal fractions prepared from green plants contaminated with chlorophylls. NADPH-dependent metabolism of 2-NA was, however, fluorometrically detected in microsomes prepared from several non-green plant sources, such as tubers from *H. tuberosus* and etiolated shoots from *V. sativa* and maize. The measured activities varied from 0.8 to 5.7 pkat mg^{-1}, depending on the source of microsomes and were similar to those measured using other assays. Polyclonal antibodies raised against purified *H. tuberosus* C4H (*16*) were used to check if NAH activity measured in the plant membranes was exclusively dependent on CYP73As or if other enzymes were also involved. These antibodies were previously shown to be highly specific for C4H (*17*, *18*). In all plant microsomes tested inhibition was higher than 90% and was similar to that observed for yeast microsomes.

Binding of 2-NA to CYP73A1. Design of good substrates for a specific enzyme requires the optimization of shape complementarity between the molecules and the active site

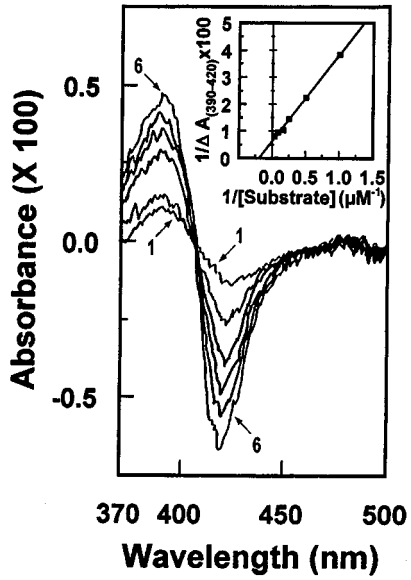


FIGURE 4: Binding of 2-naphthoic acid to CYP73A1. Difference spectra were recorded in 0.1 mM sodium phosphate, pH 7.4, containing CYP73A1 (0.15 μM) in transformed-yeast microsomes after addition of increasing amounts of 2-naphthoic acid to the sample cuvette. An equal volume of buffer was added to the reference. Curves 1−6 represent total 2-naphthoic acid concentrations of 1, 2, 4, 6, 12, and 27 μM. Inset: double-reciprocal plot of $\Delta A_{(390-420)}$ versus 2-naphthoic acid concentration.

of the protein. In the case of P450 this implies minimizing the degree of solvation of the heme pocket in order to avoid uncoupling and abortive catalytic cycles (*19*). The binding of substrates into the catalytic site of a P450 usually induces a shift of its absorbance maximum, redox potential, and iron spin state that results from the displacement of water bound as the sixth ligand to the heme iron. The efficiency of this displacement reflects the positioning and mobility of the ligand with respect to the catalytic Fe atom. The spectral shift is easily detected by differential spectrophotometry as a so-called type I spectrum with a peak at 390 nm and a trough at 420 nm (*20*). The variation of the differential absorbance $\Delta A_{(390-420)}$ versus substrate concentration allows the calculation of an apparent dissociation constant ($K_{s,app}$) (Figure 4). A high percentage of high-spin (HS) enzyme is an indication of low solvent accessibility and of a correct positioning of the molecule in the heme-pocket for catalysis (*21*, *22*). We previously showed that in the native yeast-

Table 1: Comparison of the Kinetic Parameters of CYP73A1 for 2-Naphthoic and *trans*-Cinnamic Acids[a]

kinetic constants	2-NAH	C4H
K_s (μM)	4.3 ± 0.2	5.3 ± 0.4
K_m (μM)	2.7 ± 0.3	2.2 ± 0.3
turnover (min^{-1})	73.1 ± 2.0	92.0 ± 2.9
V_{max}/K_m (min^{-1} μM^{-1})	27.4 ± 4.0	42.0 ± 6.7

[a] K_s were determined by difference spectrophotometry as shown in Figure 4. 2-NAH activity was assayed by fluorometry as described in Figure 3: assays were performed with 30 pmol of P450 in a total volume of 2 mL. For C4H activity, 3 pmol of P450 was incubated with 4 mM glucose-6-phosphate, 0.1 units of glucose-6-phosphate dehydrogenase, 100 μM NADPH, and 1−100 μM radiolabeled substrate in a total volume of 0.2 mL. Substrate and product were separated by TLC. The product was quantified by scintillation counting (14).

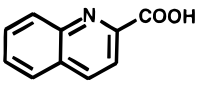
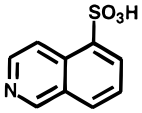
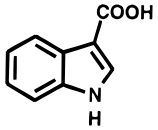
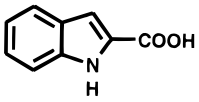
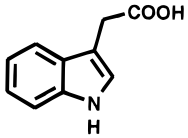
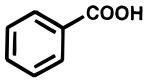
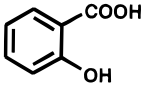
expressed CYP73A1, iron is totally low spin and that the binding of saturating concentrations of cinnamic acid results in a complete conversion into the high spin enzyme (*7*). We used a similar approach to test the positioning of alternate substrates. In the case of 2-NA, a typical type I binding spectrum was obtained: the apparent spectral dissociation constant and percentage of low spin to high spin conversion calculated from data in Figure 4 were $K_{s,app} = 4.3 \pm 0.2$ mM and % HS = 82.8 ± 4.7%, respectively. In the case of cinnamic acid, a $K_{s,app}$ of 5.3 ± 0.4 μM was determined with the same batch of yeast microsomes.

Determination of the Kinetic Constants of Hydroxylation of 2-NA. To test the efficiency of the metabolism of 2-NA by CYP73A1, the kinetic constants of the 6-hydroxylation reaction were determined using the fluorescent assay described above. Kinetics matched the Michaelis−Menten model. The apparent K_m was 2.7 ± 0.3 μM, and the catalytic turnover, calculated from the V_{max} and from the amounts of ferrous-CO enzyme, was 73.1 ± 2.1 min^{-1}. These values were very close to those observed for *trans*-cinnamic acid 4-hydroxylation from the same membrane preparation, indicating the efficient metabolism of 2-NA (Table 1).

Binding of Other Potential Alternate Substrates to CYP73A1. Many naphthalene derivatives are commercially available. 2-NA thus provided a good basis to begin investigating the substrate-specificity of CYP73As. A series of 2-NA analogs were tested for binding to the active site of the yeast-expressed CYP73A1. These include benzoate, salicylate, naphthalene derivatives differing in the position and nature of their substituents, and a few heterocyclic molecules of similar size and shape (i.e. substituted quinoline, isoquinoline, and indoles) listed in Table 2. For 1-hydroxy-2-naphthaldehyde, 4-hydroxy-1-naphthalenesulfonic acid, 2-naphthalenethiol, and 2-naphthaleneacetonitrile, which absorb strongly around 400 nm, the detection of binding spectra was impossible, despite the use of double cuvettes.

2-Acetylnaphthalene, 2-naphthol, indole-3-acetic acid, 1-naphthalene acetic acid, 2-aminonaphthalene, and salicylic acid induced no shift of CYP73A1 absorbance, even at concentrations up to the solubility limit. Other molecules, including naphthalene, 1-chloronaphthalene, 2-naphthaleneacetic acid, and benzoic acid, shifted the absorbance maximum of CYP73A1, but the amplitude of the difference spectra was too small for an accurate determination of binding characteristics. These molecules did not appear to have easy access to the heme-pocket or their position, orientation, or mobility in the active site was such that they did not displace the water molecule bound to heme iron in the resting enzyme.

Table 2: Structure of the Monocyclic and Heterocyclic Molecules Tested as Alternate Substrates and Inhibitors of CYP73A1

compounds	structures
Quinoline-2-carboxylic acid	
5-Isoquinolinesulfonic acid	
Indole-3-carboxylic acid	
Indole-2-carboxylic acid	
Indole-3-acetic acid	
Benzoic acid	
Salicylic acid	

5-Isoquinolinesulfonic acid induced a type II difference spectrum, with a peak at 425 nm and a trough at 400 nm, characteristic of compounds with a sp^2-hybridized nitrogen atom binding as proximal ligand to the ferric heme. Such type II ligands are usually poor substrates and inhibit the enzymatic activity (*20*). The amplitude of the difference spectrum obtained with this ligand was too small to determine binding constants. Addition of all other compounds to microsomes of yeast expressing CYP73A1 yielded saturable type I difference spectra. The value of $K_{s,app}$ and the % of HS form at saturating substrate concentrations calculated for each of them are given in Table 3. The whole set of molecules was also assayed for inhibiting CYP73A1-dependent *trans*-cinnamic acid hydroxylation. For competitive inhibitors, I_{50} values should have related to binding affinities, since $I_{50} = 2K_I$ when the substrate concentration equals the Michaelis constant. As shown by data in Table 3, the anticipated correlation between I_{50} and K_s was obtained in most cases. Some molecules, especially hydroxynaphthoic acids, behaved as strong ligands of CYP73A1, both by their dissociation constants and I_{50} indicating an affinity higher than for the physiological substrate. Other acid or aldehyde substituted planar molecules, such as indole-2-carboxylic, quinoline-2-carboxylic, 1- and 2-naphthalenesulfonic acids, 2-naphthaldehyde, and the methyl ester of 2-naphthoic acid also appeared to be good potential substrates of CYP73A1. Low binding, measured spectrophotometrically for other compounds, was confirmed by low inhibition. When binding measurements were not possible, inhibition provided useful information. For example, 1-hydroxy-2-naphthaldehyde (I_{50}

Table 3: Binding Parameters and Inhibition of CYP73A1 by the 2-NA Analogs[a]

compounds	K_S (μM)	HS (%)	I_{50} (μM)
2-hydroxy-1-naphthoic acid	0.28 ± 0.03	57.8 ± 3.2	0.89 ± 0.10
1-hydroxy-2-naphthoic acid	1.9 ± 0.3	78.9 ± 8.4	5.3 ± 0.5
3-hydroxy-2-naphthoic acid	3.6 ± 0.5	75.3 ± 4.9	10.4 ± 0.5
trans-cinnamic acid	5.1 ± 0.3	100	
2-naphthoic acid	4.3 ± 0.2	82.8 ± 4.7	13.7 ± 1.1
indole-2-carboxylic acid	12.0 ± 1.7	100 ± 11	29.7 ± 0.3
2-naphthalenesulfonic acid	19.4 ± 5.1	61.5 ± 9.3	37.7 ± 1.6
1-methyl-2-naphthoic acid	84.9 ± 3.7	22.5 ± 0.5	201 ± 18
1-naphthalenesulfonic acid	86.3 ± 17.5	55.6 ± 8.3	211 ± 15
2-naphthaldehyde	88.5 ± 31.3	25.2 ± 2.7	312 ± 28
1-naphthoic acid	125 ± 41	58.5 ± 12.6	228 ± 17
quinoline-2-carboxylic acid	217 ± 54	74.7 ± 6.6	423 ± 27
indole-3-carboxylic acid	398 ± 59	37.7 ± 2.4	751 ± 2.3
2-methylnaphthalene	547 ± 42	20.5 ± 6.9	> 1000
5-isoquinolinesulfonic acid	type II[b]		498 ± 9
1-hydroxy-2-naphthaldehyde	high absorbance[c]		11.0 ± 0.2
4-hydroxy-1-naphthalenesulfonic acid	high absorbance		733 ± 74
2-naphthalenethiol	high absorbance		706 ± 8
2-naphthaleneacetonitrile	high absorbance		>1000
naphthalene	small shift[d]		>1000
1-chloronaphthalene	small shift		>1000
2-naphthaleneacetic acid	small shift		nd
benzoic acid	small shift		> 1000
2-acetylnaphthalene	no shift[e]		319 ± 21
2-naphthol	no shift		748 ± 7
indole-3-acetic acid	no shift		970 ± 92
salicylic acid	no shift		>1000
1-naphthaleneacetic acid	no shift		>1000
2-aminonaphthalene	no shift		955 ± 81

[a] Difference spectra were recorded after addition of increasing concentrations of ligands to the sample cuvette containing 150 nM transformed-yeast microsomal protein. An equal volume of solvent (dimethyl sulfoxide) was added to the reference. K_s and percentage of high-spin enzyme at saturating ligand concentration were calculated from the double-reciprocal plots of $\Delta A_{(390-420nm)}$ versus substrate concentrations. Saturating *trans*-cinnamic acid was assumed to promote 100% low- to high-spin conversion. I_{50} values were obtained from a linear reciprocal fit of residual activity versus ligand concentrations giving 0−90% inhibition when cinnamate concentration is 2 μM. All values were calculated from duplicate experiments. [b] Type II, a type II spectral shift (peak at 425 nm and trough at 400 nm) was obtained with this compound; binding parameters were not determined. [c] High absorbance, high intrinsic absorbance of the compounds prevented the detection of ligand binding. [d] Small shift, low- to high-spin shifts were observed, but ΔA_{max} were too small for determination of binding parameters. [e] No shift, no spectral change was detected.

= 11 μM) was identified as a high-affinity ligand of the enzyme.

Metabolism of Alternate Substrates by CYP73A1. Several naphthalene derivatives have a high affinity for CYP73A1 and induce low- to high-spin transition of the oxidized enzyme. As a high percentage of low- to high-spin transition at saturating concentration usually reflects formation of a complex which favors oxygenation (*23*), the CYP73A1-dependent metabolism of the 2-NA analogs was investigated.

Metabolism was first assayed via substrate-dependent NADPH oxidation. Each ligand was added to CYP73A1-transformed yeast microsomes at a saturating concentration (10-fold the K_s) or at a solubility limit if K_s was too high. In most cases, the rates of substrate-induced NADPH oxidation (Table 4) were in agreement with the results from differential spectrophotometry and inhibition studies. For poor ligands (naphthalene, 2-methylnaphthalene, 1-naphthaleneacetic acid, and 2-naphthaleneacetic acid), NADPH consumption was low or undetectable. Most strong ligands induced rapid NADPH oxidation. A few triggered low rates of NADPH oxidation compared to those measured with other molecules for which the enzyme had a similar affinity. This was the case for methyl-2-naphthoate and 2-hydroxy-1-naphthoic acid, two molecules which did not effectively displace water from the heme iron, despite high affinity. Hydroxylation of *trans*-cinnamic acid was catalyzed by CYP73A1 with an excellent coupling of NADPH oxidation and product hydroxylation (*7*). With substrate analogs which fit less perfectly into the active site, uncoupling of the oxidation reaction is likely to occur. In this case, NADPH oxidation leads to the formation of superoxide, hydrogen peroxide, or water instead of the oxygenated product (*23*, *24*). Oxygenation of the C4H ligands was, therefore, confirmed by HPLC or fluorometry (Table 4). Besides *trans*-cinnamic acid and 2-NA, NADPH- and CYP73A1-dependent formation of polar metabolites was observed for a number of compounds: 1-hydroxy-2-naphthoic acid ($t_{R,S}$ = 29 min, $t_{R,P}$ = 10 min, mobile phase 20% CH_3CN), 3-hydroxy-2-naphthoic acid ($t_{R,S}$ = 26 min, $t_{R,P}$ = 6 min, mobile phase 20% CH_3CN), 2-naphthaldehyde ($t_{R,S}$ = 30 min, $t_{R,P}$ = 21 min, mobile phase 20% CH_3CN), 1-naphthoic acid ($t_{R,S}$ = 28 min, $t_{R,P}$ = 8 min, mobile phase 5% CH_3CN), quinoline-2-carboxylic acid ($t_{R,S}$ = 15 min, $t_{R,P}$ = 9 min, mobile phase 5% CH_3CN), indole-3-carboxylic acid ($t_{R,S}$ = 29 min, $t_{R,P}$ = 13 min, mobile phase 20% CH_3CN), and 1-hydroxy-2-naphthaldehyde acid ($t_{R,S}$ = 31 min, $t_{R,P}$ = 5 min, mobile phase 20% CH_3CN). Indole-2-carboxylic acid was the sole molecule leading to the formation of two metabolites ($t_{R,S}$ = 29 min, $t_{R,P1}$ = 4 min, $t_{R,P2}$ = 8 min, mobile phase 10% CH_3CN). The relative amounts of the two metabolites was constant and independent of incubation time. At present, no attempt has been made to further characterize these metabolites. For some very polar compounds, such as naphthalenesulfonic acids, metabolism was detected by fluorometry. NADPH- and CYP73A1-dependent conversion of 2- and 1-naphthalenesulfonic and of 4-hydroxy-1-naphthalenesulfonic acids was

Table 4: Metabolism of Alternative Substrates by CYP73A1[a]

compounds	NADPH oxidation (min^{-1})	metabolism (min^{-1})
2-hydroxy-1-naphthoic acid	29.7 ± 3.8	none
1-hydroxy-2-naphthoic acid	88.9 ± 38.5	75.6 ± 11.4
3-hydroxy-2-naphthoic acid	175 ± 62	85.0 ± 13.8
trans-cinnamic acid	98.7 ± 8.4	92.0 ± 2.9
2-naphthoic acid	97.3 ± 21.0	73.1 ± 2.0
indole-2-carboxylic acid	51.5 ± 1.0	77.2 ± 16.1
2-naphthalenesulfonic acid	84.0 ± 8.4	11.2 ± 0.3*
methyl-2-naphthoate	13.0 ± 0.8	none
1-naphthalenesulfonic acid	68.2 ± 4.8	fluorescent product*
2-naphthaldehyde	50.3 ± 1.8	46.8 ± 6.4
1-naphthoic acid	53.0 ± 7.9	5.9 ± 1.7
quinoline-2-carboxylic acid	85.4 ± 10.5	1.9 ± 0.2
indole-3-carboxylic acid	22.8 ± 5.1	4.9 ± 0.8
2-methylnaphthalene	0	none
isoquinoline-5-sulfonic acid	0	none
1-hydroxy-2-naphthaldehyde	60.1 ± 6.1	73.0 ± 31.8
4-hydroxy-1-naphthalenesulfonic acid	82.6 ± 11.9	fluorescent product*
2-naphthalenethiol	0	none
naphthalene-2-acetonitrile	0	none
naphthalene	0	none
1-chloronaphthalene	0	none
2-naphthaleneacetic acid	26.6 ± 5.5	none
2-acetylnaphthalene	0	none
2-naphthol	50.3 ± 5.6	none
indole-3-acetic acid	0	none
1-naphthaleneacetic acid	0	none
2-aminonaphthalene	0	none
benzoic acid	0	none
salicylic acid	0	none

[a] CYP73A1-dependent NADPH oxidation was monitored by difference spectrophotometry at 350 nm, after addition of a saturating concentration of substrate to the sample cuvette. CYP73A1 was 15 nM in the assay. Values are means ± SD of three determinations. The rate of metabolism of the different substrates was estimated, by HPLC, from the decrease in substrate peak height as described in text. Metabolism of the highly polar sulfonated derivatives (*) was assayed by fluorometry. For 1-naphthalenesulfonic and 4-hydroxy-1-naphthalenesulfonic acids metabolism was observed, but the rate was not estimated.

monitored following changes in fluorescence emission spectra using excitation wavelengths of 394, 296, and 358 nm, respectively. In the case of 2-naphthalenesulfonic acid, the reference metabolite (6-hydroxy-2-naphthalenesulfonic acid) was available and the K_m (14.2 ± 1.8 μM) and turnover (11.2 ± 0.3 min^{-1}) of the hydroxylation reaction could be determined. For 1-naphthalenesulfonic acid, no reference was available and only the Michaelis constant of the reaction (K_m = 44.4 ± 10.8 μM) was estimated. In the case of 4-hydroxy-1-naphthalenesulfonic acid, the variation in fluorescence was too low for such an estimation.

Competitive inhibitors of CYP73A1. For 2-hydroxy-1-naphthoic and methyl-2-naphthoate, despite low induced NADPH oxidation, no metabolism was observed either by HPLC or by fluorometry. These molecules, however, are good ligands of CYP73A1 and were therefore tested as inhibitors of CYP73A1. Kinetic analysis (Figure 5) confirmed that both molecules were competitive inhibitors of CYP73A1. The inhibition constants (K_I) were 48.4 ± 3.4 nM for 2-hydroxy-1-naphthoic acid and 98.3 ± 5.7 μM for methyl 2-naphthoate.

DISCUSSION

The currently accepted dogma is that plant P450s have greater substrate specificity than their mammalian liver

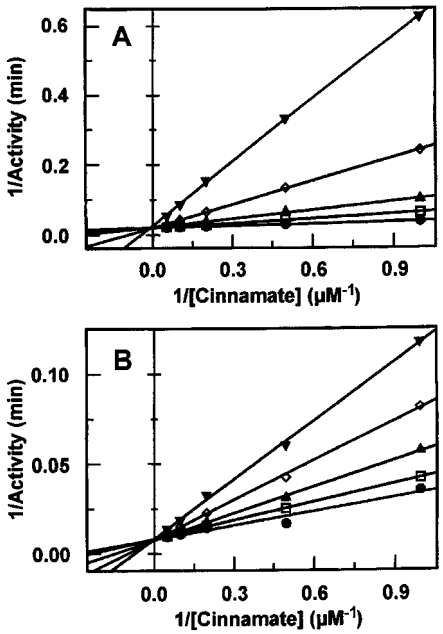


FIGURE 5: Lineweaver−Burk plots of the inhibition of *trans*-cinnamic acid hydroxylation by 2-hydroxy-1-naphthoic acid and methyl-2-naphthoate. Duplicate incubations were performed at 30 °C in 100 mM sodium phosphate pH 7.4, in the presence of 50 μM NADPH, 1 mM glucose-6-phosphate, 0.2 units of glucose-6-phosphate dehydrogenase, 5 nM CYP73A1, and various concentrations of substrate and inhibitor. The substrate concentrations used were 1, 2, 5, 10, and 20 μM. The inhibitor concentrations were (A) 0 (●), 0.06 (□), 0.2 (▲), 0.6 (◇), and 2 μM (▼) for 2-hydroxy-1-naphthoic and (B) 0 (●), 30 (□), 75 (▲), 150 (◇), and 300 μM (▼) for methyl 2-naphthoate.

counterparts. This idea stems from the fact that plants have to deal with a huge number of endogenous chemicals synthesized for defense against predators and pathogens, as allelochemicals, or as communication agents with other plants or organisms. However, a some data indicates that P450s involved in normal physiological processes can also participate in xenobiotic metabolism. For example, results from Zimmerlin and Durst (*25*) suggest that lauric acid subterminal hydroxylation and *ring*-hydroxylation of the herbicide diclofop by wheat microsomes are probably catalyzed by the same P450. Additionally, the avocado P450 CYP71A1, whose expression is related to fruit ripening, also is known to actively demethylate *p*-chloro-*N*-methylaniline (*26*). More recently, the yeast-expressed CYP73A1 was shown to oxidize several exogenous molecules with low efficiency (*8*). The oxidation of foreign compounds by plants is thus expected to result from the fortuitous overlap in substrate specificity by P450s involved in endogenous metabolism. If true, then the efficiency of xenobiotic metabolism will be determined by (i) the affinity of the exogenous molecule for a given P450 protein and by its concentration relative to that of the physiological substrate, (ii) its orientation relative to the ferryl oxygen and its mobility within the catalytic site, (iii) the intrinsic chemical reactivity of the oxidizable site(s) positioned within the reactivity sphere of the activated oxygen, and (iv) the presence and relative abundance of the concerned P450.

We show here that 2-NA almost perfectly mimics *trans*-cinnamic acid and is an excellent alternate substrate for C4H. Its binding parameters reflect both high affinity (K_s = 4.3 μM) for the enzyme and an adequate orientation within the catalytic site (83% of low- to high-spin transition). These

data are confirmed by the observed high turnover and catalytic efficiency compared to that measured for cinnamate. The size and planar character of the molecule and the presence and location of the negatively charged substituent thus seem to perfectly fit the active site. Slight steric hindrance, probably resulting from the bulk of the additional carboxylate bearing ring, prevents the total removal of solvent from the active site (less than 100% low- to high-spin transition). This hindrance slightly reduces the turnover of the reaction but does not seem to result in a significant twist of the molecule since a single product is obtained and the position of attack is conserved relative to cinnamic acid.

6-Hydroxy-2-naphthoic acid, the product of the reaction, is a strongly fluorescent molecule. Taking advantage of this fluorescence, we designed a new assay for measuring CYP73A1 activity. Compared to measurements of cinnamate hydroxylation, this assay is very rapid (i.e., performed in a few minutes), easy, and does not involve handling radiolabelled compounds. The test is representative for CYP73As from both transformed-yeast and plant microsomes from non-green, dicot or monocot tissues (including *H. tuberosus*, *V. sativa*, and maize). This is verified by the almost total inhibition 2-NAH activity by antibodies directed against purified *H. tuberosus* C4H protein. However, it does not preclude the possibility that other enzymes may contribute to 2-NA metabolism in other plant species.

Many 2-NA analogs are also good ligands and substrates of CYP73A1. The best ligands and substrates are the 1- and 3-hydroxy-2-naphthoic acids. The presence of an hydroxyl group adjacent the carboxylate increases the apparent affinity of these molecules for the active site. This indicates an improvement of the steric fit between the ligands and the protein, possibly resulting from an additional hydrogen bond with the basic amino acid anchoring the carboxylate or with another residue nearby, or from the displacement of solvent from the active site (*19*). However, the presence of the hydroxy substituent decreases the percentage of high spin enzyme compared to 2-NA. This is probably the result of a twisting or to a complete loss of mobility of the molecule within the heme-pocket. Metabolism of 1-hydroxy-2-naphthoic acid is less the same as 2-NA but increased in the case of the 3-hydroxylated molecule. For 2-hydroxy-1-naphthoic acid, by far the best ligand of CYP73A1, the orientation of the molecule seems to be modified further, so that carbon 6 is no longer in a position suitable for oxidative attack. 2-Hydroxy-1-naphthoic acid is not metabolized and behaves as a competitive inhibitor for the enzyme. The inhibition constant (K_I) was found to be 48.4 nM confirming the binding of this compound in the active site of the enzyme with very high affinity but in a manner which does not allow its hydroxylation. Low rates of NADPH oxidation are detected in the presence of this compound. Its binding in the heme pocket thus probably induces abortive catalytic cycles and generates superoxide, H_2O_2, or H_2O. A similar situation is observed with methyl ester of 2-NA that behaves also as competitive inhibitor of CYP73A1 but with less affinity (K_I = 98.3 μM). The methylation of the carboxylic function reduces the affinity, but also alters the orientation of the molecule in the active site so as to drastically decrease spin state transition and totally suppress catalytic activity. The low NADPH oxidation rate measured with this molecule is consistent with the inefficient displacement of the water ligand from the heme iron.

Other substrates of CYP73A1, such as 2- and 1-naphthalenesulfonic acids, 1-naphthoic acid, 2-naphthaldehyde, 3-indole- and 2-indolecaboxylic acids, and quinoline-2-carboxylic acid, but not naphthalene, show that the anchoring of substrate in the active site can be efficiently ensured by several negatively charged substituents positioned at 1 and 2 on a naphthalene or on other planar bicyclic structures equivalent in size. Monocyclic molecules, such as benzoic acid or the plant signaling molecule salicylic acid, are not substrates and are poor ligands and inhibitors of the enzyme. The size of the negatively charged substituent is apparently critical, as shorter (2-naphthol) or longer (2-naphthaleneacetic acid) side chains result in loss of binding and metabolism. This is particularly striking in the case of indole derivatives. Indole-3-carboxylic acid is quite a good ligand and substrate of CYP73A1, while its two-carbon side-chain homologue, the plant hormone indole-3-acetic acid (auxin), is only a poor inhibitor that does not produce a spin state transition and is not metabolized. For indole-2-carboxylic acid, a good substrate of CYP73A1, the positioning of the molecule in the catalytic site is optimal since this molecule induces a complete low- to high-spin transition of the enzyme. The production of two metabolites in constant proportions seems to indicate that an oxidative attack on both the 5 and 6 carbons is possible. This can be explained either by a rotation of the benzene cycle relative to naphthoate and a positioning of the 5−6 double-bond just perpendicular to the ferryl-oxo, or by two possible orientations of the substrate in the active site allowed by the almost symmetrical structure of the indole ring.

Naphthalenesulfonic acids are building blocks of azo dyes and are considered to be major nonbiodegradable contaminants of aqueous ecosystems (*27*). It is, therefore, noteworthy that CYP73A1 is capable of hydroxylating 1- and 2-naphthalenesulfonic acids. High rates of NADPH oxidation measured with these compounds may indicate rapid metabolism or induced uncoupling. Comparison of the kinetic parameters of 2-naphthalenesulfonic and *trans*-cinnamic acid hydroxylations suggests that a significant part of the NADPH oxidation observed in the presence of the sulfonic acids results from an uncoupling of the oxygenation reaction.

CONCLUSION

This is the first structure−activity study performed with a plant P450 enzyme. Our results indicate that naphthalenes substituted at position 1 or 2 with carboxyl, sulfonyl, or methanoyl groups, but not naphthalene itself, are substrates of CYP73A1. Moreover, methyl, hydroxyl, thiol, and amine-substituted molecules are not metabolized. Addition of an hydroxyl *ortho* to a 1- or 2-carboxylic acid leads to very high affinity binding. The size or planarity of the molecules appear to be critical since benzoic acids or acetate-substituted naphthalenes do not bind to the active site. Heterocyclic compounds, i.e. acid-substituted quinolines or indoles are also substrates of CYP73A1.

These new alternate substrates offer a set of fluorescent probes for rapid and sensitive detection of CYP73As enzymatic activity. They also provide a rational basis for designing specific inhibitors of CYP73As and for possibly

controlling the branch point between the C6-C1 derivation and other aspects of the phenypropanoid pathway. 2-Hydroxy-1-naphthoic acid is the first potent inhibitor of C4H described so far. Unlike α-amino-*β*-phenylpropionic acid, the widely used inhibitor of phenylalanine ammonia-lyase (*28*), 2-hydroxy-1-naphthoic acid now offers an alternative means of studying and regulating this important secondary pathway of plants.

ACKNOWLEDGMENT

The critical reading and linguistic improvement of the manuscript by R. Backhaus and F. Bernier is gratefully acknowledged. We thank Drs. D. Pompon and P. Urban for providing the W(R) yeast strain and the pYeV60 expression vector. We also thank M. F. Castaldi for her technical assistance.

REFERENCES

1. Werck-Reichhart, D. (1995) *Drug Metab. Drug Interact. 12*, 221−243.
2. Batard, Y., Schalk, M., Pierrel M. A., Zimmerlin, A., Durst, F., and Werck-Reichhart D. (1997) *Plant Physiol. 113*, 951−959.
3. Campbell, M. M., and Sederoff, R. R. (1996) *Plant Physiol*. 110, 3−13.
4. Klessig, D. F., and Malamy, J. (1993) *Plant Mol. Biol. 26*, 1439−1458.
5. Sandermann, H. Jr. (1992) *Trends Biochem. Sci. 17*, 82−84.
6. Teutsch, G. H., Hasenfratz, M. P., Lesot, A., Stoltz, C., Garnier, J. M., Jeltsch, J. M., Durst, F., and Werck-Reichhart, D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4102−4106.
7. Urban, P., Werck-Reichhart, D., Teutsch, H. G., Durst, F., Mignotte, C., Katzmaier, M., and Pompon, D. (1994) *Eur. J. Biochem. 222*, 843−850.
8. Pierrel, M. A., Batard, Y., Kazmaier, M., Mignotte-Vieux, C., Durst, F., and Werck-Reichhart, D. (1994) *Eur. J. Biochem. 224*, 835−844.
9. Durst, F., Benveniste, I., Schalk, M., and Werck-Reichhart D. (1996) *Methods Enzymol. 272*, 259−268.
10. Truan, G., Collin, C., Reisdorf, P., Urban, P., and Pompon, D. (1993) *Gene 125*, 49−55.
11. Werck-Reichhart, D., Gabriac, B., Teusch, H., and Durst, F. (1990) *Biochem. J. 270*, 719−735.
12. Weissbart, D., Salaün, J. P., Durst, F., Pflieger, P., and Mioskowski, C. (1992) *Biochim. Biophys. Acta 1124*, 135−142.
13. Rahier, A., and Taton, M. (1986) *Biochem. Biophys. Res. Commun. 140*, 1064−1072.
14. Reichhart, D., Salaün, J. P., Benveniste, I., and Durst, F. (1980) *Plant Physiol. 66*, 600−604.
15. Duggleby, R. G. (1984) *Comput. Biol. Med. 14*, 447−455.
16. Werck-Reichhart, D., Batard, Y., Kochs, G., Lesot, A., and Durst, F. (1993) *Plant Physiol. 102*, 1291−1298.
17. Gabriac, B., Werck-Reichhart, D., Teutsch, H., and Durst, F. (1991) *Arch. Biochem. Biophys. 288*, 302−309.
18. Kochs, G., Werck-Reichhart, D., and Grisebach, H. (1992) *Arch. Biophys. Biochem. 293*, 187−194.
19. Helms, V., Deprez, E., Gill, E., Barret, C., Hui Bon Hoa, G., and Wade, R. C. (1996) *Biochemistry 35*, 1485−1499.
20. Jefcoate, C. R. (1978) *Methods Enzymol. 52*, 258−279.
21. Fischer, M. T., and Sligar, S. G. (1987) *Biochemisty 26*, 4797−4803.
22. Raag, R., and Poulos, T. L. (1989). *Biochemistry 28*, 917−922.
23. Loida, P. J., and Sligar, S. G. (1993) *Biochemistry 32*, 11530−11538.
24. Raag, R., and Poulos, T. L. (1991) *Biochemistry* 30, 2674−2684.
25. Zimmerlin, A., and Durst, F. (1992) *Plant Physiol. 100*, 874−881.
26. Bozac, K. R., O'Keefe, D. P., and Christoffersen, R. E. (1992) *Plant Physiol. 100*, 1976−1981.
27. Bretscher, M. (1981) in *Microbial metabolism of xenobiotics and recalcitrant compounds* (Leisinger, T., Cook, A. M., Hütter, R., and Nüesch, J., Eds.) pp 65−74, Academic Press, London.
28. Amrhein, N., and Gödeke, K. H. (1977) *Plant Sci. Lett. 8*, 313−317.

BI971575K