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The Substrate Specificity of Cytochrome $P450_{cam}$

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Abstract—The catalytic turnover of xenobiotics by cytochrome $P450_{cam}$ results in both the formation of organic metabolites and the uncoupled production of H_2O_2, and H_2O. Previous studies have shown that a receptor-constrained three-dimensional screening program (DOCK) can be used to identify potential ligands (*ergo* substrates) for the enzyme (De Voss, J. J.; Sibbesen, O.; Zhang, Z.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1997**, *119*, 5489). A new set of 10 compounds has now been examined to further test the substrate specificity of $P450_{cam}$ and the ability of DOCK to identify substrates for this enzyme. The results expand the known specificity of $P450_{cam}$ and define limitations in the use of DOCK to predict its substrate specificity.

Introduction

Cytochrome P450 enzymes catalyze a range of oxidative transformations, including carbon hydroxylation, heteroatom oxidation, π bond oxidation, and hydrocarbon desaturation.[1] Despite differences in the phylogenetic origin, cellular localization, electron donor partners, and substrate specificity of P450 enzymes, their active sites and catalytic mechanisms appear to be remarkably similar. The catalytic cycle of the known P450 enzymes consists of: (a) substrate binding with a concomitant decrease in the reduction potential of the heme iron atom; (b) reduction of the iron to the ferrous state; (c) formation of the ferrous dioxy (Fe^{+2}-O_2) complex; (d) reduction of the ferrous dioxy complex to an intermediate formally equivalent to a ferric peroxide (Fe^{+3} -OOH); (e) cleavage of the dioxygen bond to give a ferryl (formally $Fe^{+5}=O$) species; (f) oxidation of the substrate by the ferryl species, yielding an enzyme–product complex and (g) dissociation of the product (Fig. 1).[1] The primary function of the enzyme in this catalytic cycle is to promote formation of the ferryl species and to bind and orient the substrate in its proximity. The chemo- and regiospecificity of substrate oxidation, however, are largely determined by the intrinsic reactivities of the substrate sites that are accessible to the ferryl species in the enzyme–substrate complex. In addition to coupled turnover, which results in oxidation of the organic substrate, cytochrome P450 enzymes undergo uncoupled turnover to produce O_2-, H_2O_2, and H_2O.[2,3] The uncoupling of NAD(P)H consumption from organic substrate oxidation is due to dissociation of superoxide from the ferrous dioxy complex, dissociation of H_2O_2 from the ferric peroxide complex, and/or reduction of the final ferryl species to water by electrons provided by NAD(P)H (Fig. 1). A low degree of uncoupled turnover is normally observed with the resting enzyme, but the extent of uncoupled oxygen reduction is greatly increased by the binding of some ligands or substrates. Uncoupled turnover is thus a substrate-dependent catalytic process that produces reduced oxygen rather than organic metabolites.

The broad substrate specificity of individual P450 enzymes,[4] their preference for lipophilic substrates,[5,6] and the hydrophobic nature of the crystallographically-defined P450 active sites[7–10] suggest that the binding of P450 substrates is governed by hydrophobic and steric factors. Control of substrate binding by specific hydrogen bonding or ionic interactions is relatively infrequent. Little is known, however, about the detailed control of

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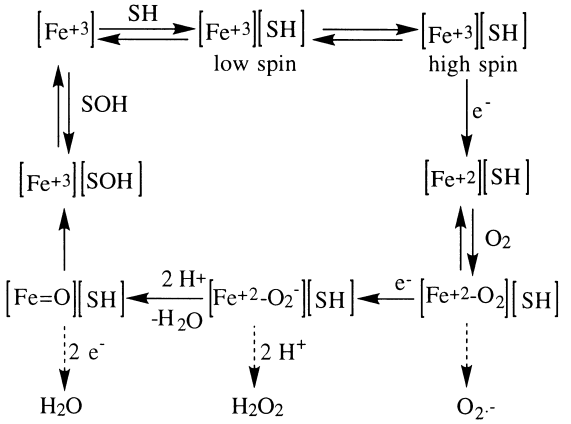

Figure 1. Catalytic cycle of cytochrome P450, showing the individual steps at which uncoupling can occur. The iron of the heme group is indicated as an iron atom in brackets.

substrate specificity even though a better understanding of P450 specificity would facilitate the identification of substrates for individual P450 enzymes, the design of P450 enzymes with tailored specificities, and the prediction of potentially toxic interactions.

Computational approaches to the identification of P450 substrates are attractive because the question of specificity can be dissected into distinct steps, nonpolar interactions primarily determine specificity, and the protein does not participate actively in the reaction of the ferryl species with the substrate. We recently described the use of a receptor-constrained docking program (DOCK) to identify potential substrates for $P450_{cam}$,[11] a soluble bacterial enzyme for which a crystal structure is available.[12–14] The study examined 16 compounds selected from a list of compounds identified by DOCK from a library of 20,000 compounds as either potential $P450_{cam}$ substrates or non-substrates. It was assumed in this study that binding of a compound, as predicted by the DOCK analysis of its ability to fit within the $P450_{cam}$ active site, is sufficient to trigger catalytic turnover of the enzyme. Catalytic turnover was defined as NADH- and substrate-dependent formation of either organic or reduced oxygen metabolites. In effect, after empirical parameter optimization, a correlation was found between the predicted ability of a compound to fit within the $P450_{cam}$ active site and whether it was a substrate or not. Some of the compounds identified as substrates were oxidized with moderate efficiency to organic products, but others primarily triggered uncoupled turnover.[12–14]

The set of compounds examined in the previous study was used to define the values for the variable parameters in DOCK, particularly the minimum contact distance allowed between substrate and active site atoms.[12–14] One important finding of the previous study was that a certain degree of mobility within the active site, or of active site deformability that allows substrate reorientation, is required for a compound to be a substrate. Varying the minimum allowed distance between substrate and protein atoms, which are artificially held in a rigid matrix, provides a formal construct for representing active site deformability. Decreasing the value of this parameter increases the extent to which substrate and protein atoms are allowed to overlap, which is formally equivalent to allowing the protein atoms a certain degree of freedom to move out of the way. To test the generality of the parameters derived earlier, we have used the same DOCK parameters to examine an independent library of compounds as potential substrates for $P450_{cam}$. The library consists of compounds previously investigated at Upjohn in studies of the fungal metabolism of organic compounds.[15–21] This relatively small library of compounds was chosen because; (a) it consists of small to medium size non-ionic compounds with a workable degree of water solubility; (b) it includes a related but diverse set of structural frameworks; and (c) metabolite identification is greatly simplified due to the availability of authentic standards for many of the potential metabolites.

Results

$P450_{cam}$ substrate predictions

The receptor-constrained docking program DOCK was recently used to identify potential cytochrome $P450_{cam}$ substrates from a data base.[12–14] To further test the validity of the system we have carried out a series of studies with a different library of potential substrate structures. For the present studies, the structure data base was assembled from compounds that had been previously investigated at Upjohn as substrates for microbial metabolism.[15–21] This approach was taken to obtain a data base of compounds that would be reasonably diverse, would have favorable physical properties (e.g. water solubility), and for which many of the metabolites would be available to simplify product identification. As reported earlier,[12–14] the three-dimensional structures of the compounds in the present library of structures were constructed using Sybyl energy minimization. The list of structures in the data base is available in a supplement to this manuscript.

Analysis of the influence of the variable parameters of DOCK on the accuracy of the predictions in the earlier study suggested that a key parameter was the minimum distance allowed between substrate and protein atoms. Empirically, the best predictions were obtained in the

earlier study with a value of 2.9 Å for this distance.[14] The value of this parameter that gives the best predictions has been similarly determined in the present study. Although the value of the parameter was initially set at different values for polar and nonpolar atoms, in the studies reported here a single value was used for both polar and nonpolar atoms. Thus, the binding of Sybyl-minimized structures to wild-type $P450_{cam}$ was examined by DOCK while gradually increasing the minimum contact distance from 2.6 to 3.2 Å (Fig. 2). Eight of the 10 structures were correctly predicted to be substrates for the $P450_{cam}$ model when the minimum contact distance was set to 2.7 Å for both polar and nonpolar atoms, but the two non-substrates were not correctly predicted.

Binding of compounds to $P450_{cam}$

The binding of a potential substrate to the cytochrome $P450_{cam}$ active site is a prerequisite for catalytic turnover. The spectroscopic dissociation constants (K_s), determined from the relationship between substrate concentrations and the spectroscopically measured low-to-high spin transitions for wild-type $P450_{cam}$, are given in Table 1. The K_s values, which vary from 0.4 μM for **6** to 16 μM for **2**, are to be compared with $K_s = 1.1$ μM for the natural substrate camphor.

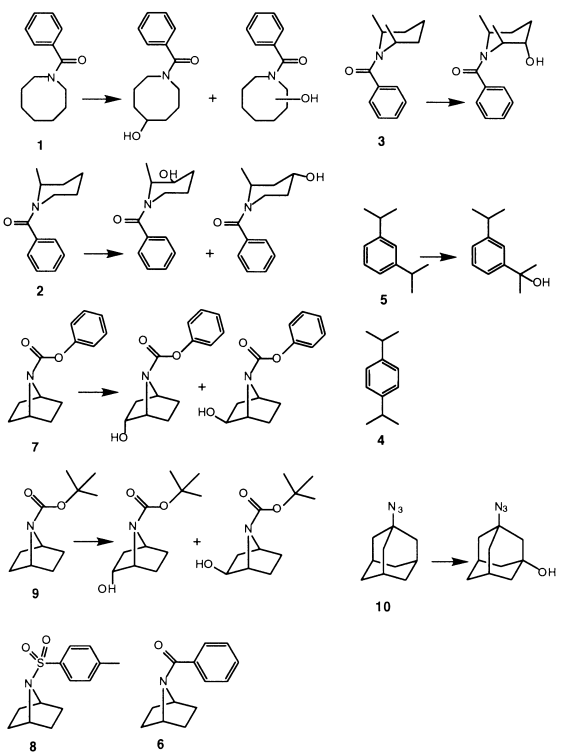

Figure 2. Structures of the compounds tested as potential substrates for $P450_{cam}$ and of the metabolites obtained from them.

Saturation of the $P450_{cam}$ active site with camphor results in complete conversion of the enzyme from the low to the high spin state. Of the new compounds examined, the two with the lowest K_s values, **6** ($K_s = 0.4$ μM) and **10** ($K_s = 0.6$ μM), convert 59% and 78%, respectively, of the enzyme to the high spin state. However, compound **2**, which has the highest K_s value (16 μM), causes a comparable maximum spin state change (69%). As reported earlier, there is little correlation between the binding affinity and the extent to which the enzyme is converted to the high spin state by a saturating concentration of the substrate.[14]

Oxygen consumption and uncoupling

NADH consumption provides a direct measure of the extent of catalytic turnover engendered by the binding of a substrate to $P450_{cam}$, with catalytic turnover encompassing both coupled turnover (substrate oxidation) and uncoupled turnover (reduction of O_2 to H_2O_2 and H_2O). In the presence of the natural substrate camphor, $P450_{cam}$ consumes NADH at the rate of 262 nmol·min^{-1} nmol^{-1}, ~98% of which is coupled to the 5-*exo*-hydroxylation of camphor. None of the unnatural substrates achieves a level of NADH consumption comparable to that of camphor (Table 1). The NADH consumption rates decrease in the order **10** > **2** > **3** > **7** > **6** > **1** > **9** > **5** > **4**. Compound **8** precipitates from the incubation solution under the experimental conditions, making its activity as a substrate difficult to evaluate. The proportion of the uncoupled formation of H_2O_2 increases in the order **1** < **10** < **3** < **7** < **6** < **2** < **9** < **4**, **5**.

$P450_{cam}$ metabolites

Most of the metabolites produced by $P450_{cam}$ were identified by gas–liquid chromatographic and mass spectrometric comparison with authentic standards. In the case of compound **3**, one of the metabolites was characterized by spectroscopic methods after isolation from a large scale incubation of the substrate with a PdR-Pd-$P450_{cam}$ fusion protein.[22] The metabolite formed from compound **5** was determined by comparison with an authentic standard synthesized by bromination of **5** followed by basic hydrolysis.

The oxidation of 1-benzoylcycloheptamethylenimine (**1**) by $P450_{cam}$ yields two hydroxylated metabolites in a 6:1 ratio. The major metabolite is identical to an authentic standard, *N*-benzoylhexahydro-5(2*H*)-azocin-5-ol, and the minor metabolite, for which no standard is available, has the mass spectrometric molecular ion expected

Table 1. Parameters for the interaction of DOCK-predicted substrates with wild-type $P450_{cam}$

Compd	K_s	Spin state change	NADH Used[a]	O_2 Used	H_2O_2 Formed	Organic products	Product formation[b]
	μM	%	$nmol \cdot min^{-1}\, nmol^{-1}$	$nmol \cdot min^{-1}\, nmol^{-1}$	%		%
Camphor	1.1	100	262	250	2	+	96
1	2.8	59	26	32	18	+	55
2	16.0	69	45	40	65	+	31
3	3.0	57	41	33	38	+	94
4	NA	< 4	6	9	100	–	< 1
5	1.4	32	8	8	100	+	1
6	0.4	59	32	28	58	+	37
7	2.1	36	37	26	39	+	4
8	0.9	24	ND[c]	ND	ND	ND	ND
9	4.5	33	14	8	72	+	1
10	0.6	78	49	31	25	+	20

[a]Background NADH consumption is 4–6 $nmol \cdot min^{-1}\, nmol^{-1}$.
[b]The turnover is the ratio of the area of the organic product peak divided by the area of the starting material plus organic product after both have been normalized versus the internal standard peak times ×100.
[c]Compound **8** precipitated at the substrate concentration (1 mM) utilized to measure catalytic turnover. No product was detected when lower concentrations were used.

for a monohydroxylated product. The chromatographic and mass spectrometric properties of the latter product suggest the ring system is intact, so the hydroxyl is at positions 3 or 4 rather than at position 2. The oxidation of compound **2** produces two monohydroxylated metabolites in a 3:1 ratio. The major product is identified by comparison with authentic standards as *N*-benzoyl-2-methylpiperidin-3-ol and the minor product as *N*-benzoyl-2-methylpiperidin-4-ol. Oxidation of *N*-benzoyl-2,6-dimethyl-piperidine (**3**) produces *N*-benzoyl-2,6-dimethylpiperidin-3-ol as the sole metabolite. α,α-Dimethyl-3-(1-methylethyl)benzene-methanol is the sole metabolite produced by $P450_{cam}$ from 1,3-diisopropylbenzene (**5**). The oxidation of compound **6** results, as indicated by the mass spectrometric and chromatographic properties of the single metabolite, in monohydroxylation of the bicyclic ring system. Although the exact position and stereochemistry of hydroxylation remain undefined, the site of the hydroxylation is not the bridgehead carbon as this would lead to formation of a ring opened structure. The metabolism of compound **7** by $P450_{cam}$ produces two products in a 2:1 ratio. The major metabolite is the *endo*-3-hydroxylated product, and the minor metabolite, which is shown by its mass spectrometric molecular ion at m/z 233.1 to be monohydroxylated, is the *exo*-3-hydroxylated product. Oxidation of compound **9** produces two metabolites in a 1:1 ratio. The two metabolites are the 3-*exo*- and 3-*endo*-hydroxyl derivatives of **9**. 1-Azidoadamantane (**10**) is cleanly oxidized to the bridgehead tertiary alcohol. Under the experimental conditions, compound **8** precipitates from the incubation solution and no products were detected even though **8** causes a 24% low to high spin shift with a binding constant (K_s) of 0.9 μM. Compound **4** is not a detectable $P450_{cam}$ substrate.

Discussion

Of the 10 compounds examined in this study as potential substrates for cytochrome $P450_{cam}$, eight are oxidized by the wild-type enzyme to organic products, in all cases with the concomitant formation of H_2O_2 (Table 1). The other two compounds (**8** and **4**) are not detectable substrates. None of the compounds is turned over at a rate comparable to that of camphor ($262\ nmol \cdot min^{-1}\, nmol^{-1}$) but six (**1**, **2**, **3**, **6**, **7**, and **10**) are oxidized at rates at least five times higher than the background level of NADH consumption. Among the compounds that give organic products, coupled turnover is best for compounds **1** and **10** and worst for compound **5**. In all cases, the organic products are obtained by monohydroxylation of a saturated carbon atom.

The hydroxylation of compound **10** occurs exclusively at a bridgehead position to give the tertiary alcohol (Fig. 2). The hydroxylation of **10** is thus similar to the previously reported hydroxylation of adamantane by $P450_{cam}$, which also occurs exclusively at the bridgehead carbon.[23] The crystal structure of adamantane bound in the active site of $P450_{cam}$ has been determined and evidence has been obtained that adamantane has a fair degree of mobility within the active site.[24] It is therefore not surprising that the oxidation occurs primarily at the carbon with the weakest C-H bond. The azido group in **10** is unlikely to suppress active site mobility and therefore also gives the product in which the weakest (bridgehead) C-H bond is oxidized.

The hydroxylation regiospecificities for the other substrates, most of which have several distinct but equally

reactive sites, are less readily rationalized and are likely to be influenced by protein-controlled orientation of the substrate within the active site. The oxidation regiospecificities in situations where protein interactions play a strong role are difficult to rationalize without recourse to molecular dynamics calculations. Nevertheless, two observations are warranted. Compounds **2** and **3** are closely related but compound **3** is both more symmetric and conformationally more rigid than **2**. These differences may explain the tighter binding of **3** than **2** ($K_s = 3$ and 16 μM, respectively) and the fact that **3** gives one whereas **2** gives two metabolites. A second notable point is that aromatic ring hydroxylation is not observed, either because the aromatic rings are bound away from the oxidizing species or because the intrinsic reactivity of the aromatic ring is lower than that of the secondary or tertiary carbons hydroxylated in the substrates.

The use of DOCK to distinguish between substrates and non-substrates was found in an earlier study of 16 compounds to be most accurate if the minimum distance allowed between substrate and active site atoms was set at 2.9 Å.[14] However, the activities as substrates of the present library of structures are most accurately rationalized when the DOCK minimum contact value is set at 2.7 rather than 2.9 Å (Fig. 3). This disparity confirms that the accuracy of DOCK in identifying $P450_{cam}$ substrates depends significantly on the choice of the minimum contact distance. The finding that an optimum value for the minimum contact distance may be difficult to define suggests that the primary utility of DOCK in the prediction of substrate specificity for $P450_{cam}$ may be in selecting substrate candidates from large data bases rather than in discriminating between closely related structures.

In sum, studies with a new library of compounds extend the range of known substrates for $P450_{cam}$ and confirm that the enzyme accepts a wide range of xenobiotics, although catalytic turnover is most highly coupled and efficient with the natural substrate camphor. The results also identify a limitation in the use of DOCK to predict substrates for $P450_{cam}$.

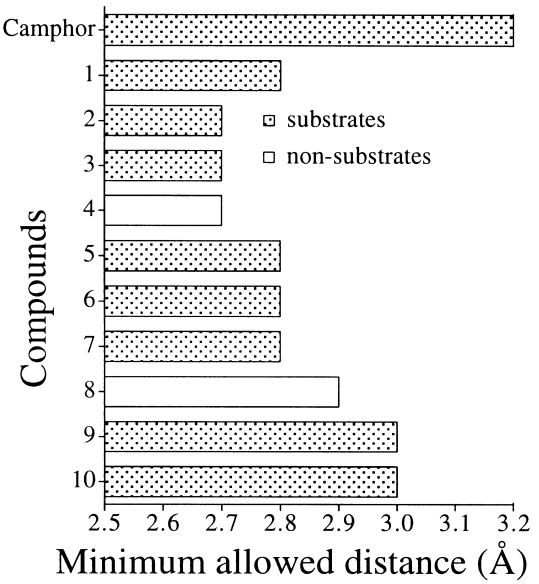

Figure 3. Relationship between the minimum distance allowed to separate a substrate and a protein atom and the prediction by DOCK that the compound is a substrate. The solid bars indicate compounds shown experimentally to be substrates, and the open bars compounds shown not to be substrates. The bars end at the minimum allowed distance beyond which the compounds are predicted by DOCK to not be substrates. The enzyme used in this study was wild-type ferric $P450_{cam}$.

Experimental

Materials and general methods

Cytochrome $P450_{cam}$, putidaredoxin reductase, and putidaredoxin were expressed heterologously in *Escherichia coli* and were purified as previously reported.[14] The substrates 1-benzoylheptamethylenimine (**1**), 1-benzoyl-2-methylpiperidine (**2**), 1-benzoyl-*cis*-2,6-dimethylpiperidine (**3**), benzoyl-7-azabicyclo[2.2.1]heptane (**6**), 7-phenyloxycarbonyl-7-azabicyclo[2.2.1]heptane (**7**), 7-*p*-toluenesulfonyl-7-azabicyclo[2.2.1]heptane (**8**), 7-*tert*-butyloxycarbonyl-7-azabicyclo[2.2.1]heptane (**9**), and 1-azidoadamantane (**10**) were obtained from Upjohn. 1,4-Diisopropylbenzene (**4**) and 1,3-diisopropylbenzene (**5**) were purchased from Aldrich. Of the standards used in metabolite identification, two were synthesized (see below) and the following were obtained from Upjohn: *N*-benzoylhexahydro-5(2H)-azocin-5-ol,[18] 1-benzoyl-2-methylpiperidin-3-ol,[19] 1-benzoyl-2-methylpiperidin-4-ol,[19] 2-*endo*-7-phenyloxycarbonyl-7-azabicyclo[2.2.1]-heptan-2-ol,[15] 2-*endo*-7-*tert*-butyl-oxycarbonyl-7-azabicyclo[2.2.1]heptan-2-ol,[15] 2-*exo*-7-*tert*-butyloxycarbonyl-7-azabicyclo-[2.2.1]heptane,[15] and 3-hydroxy-1-azidoadamantane.[16]

1-Benzoyl-*cis*-2,6-dimethylpiperidin-3-ol. The title compound was enzymatically produced by adding a 6 mg sample of 1-benzoyl-*cis*-2,6-dimethyl-piperidine (**3**) in 0.1 mL of ethanol to 200 mL of *E. coli* culture (OD_{600}~0.8) expressing the PdR-Pd-$P450_{cam}$ fusion proteind[22] (approximately 80 nmol). The mixture was aerated at 25 °C for 2 h. The cell culture was extracted with $CHCl_3$ (40 mL×3) and the combined extracts were then washed with water and dried over anhydrous Na_2SO_4. The residue obtained on solvent removal was chromatographed on a silica gel column with CH_2Cl_2/

MeOH (98:2) as the eluent. Solvent removal from the appropriate fractions gave 4.2 mg (68%) of the title compound: MS (EI): 233.2 (M^+); ^{1}H NMR (300 HZ, $CDCl_3$): δ 1.31 (d, 3H), 1.45 (d, 3H), 1.62 (m, 4H), 1.92 (m, 1H), 2.06 (m, 1H), 4.13 (m, 1H), and 7.28∼7.38 ppm (m, 5H). An attached proton test (APT) was performed to distinguish the different carbons: ($CDCl_3$,δ ppm): 22.1, 23.1, 36.6, 39.0, 47.7, 60.3, 64.4, 125.5, 128.4, 128.5, 136.8, 171.7.

α,α-Dimethyl-3-(1-methylethyl)benzenemethanol.[25]. The title compound was synthesized by slowly adding bromine (80 mg, 0.5 mmol) in CCl_4 (1 mL) to 1,3-diisopropylbenzene (96%, 423 mg, 2.5 mmol) at room temperature, followed by stirring of the reaction mixture for an additional 15 min. After removal of CCl_4 under reduced pressure, the residue was poured into 10 mL of 10% aqueous NaOH and the solution was refluxed for 12 h. The reaction mixture was extracted with ether (3×10 mL). The combined extract was washed with 10% $NaHCO_3$ solution (10 mL) and water (10 mL×2). The solvent was removed under reduced pressure and the residue was chromatographed through a small silica gel column with CH_2Cl_2 as the eluent. The appropriate fractions were collected and the solvent was removed in vacuo and a colorless oil was isolated (54 mg, 61%). EI-HRMS: *m/e* 178.1350 (178.1358 calcd for $C_{12}H_{18}O$); ^{1}H NMR (300 Hz, $CDCl_3$): δ 1.25 (d, 6H), 1.58 (s, 6H), 2.16 (s, 1H), 2.92 (m, 1H), 7.11∼7.37 (m, 4H).

Binding of compounds to $P450_{cam}$. The test compound in ethanol was added in increasing amounts to the sample cuvette, and an equal amount of ethanol was added to a matched reference cuvette. Both cuvettes contained 1 μM camphor-free $P450_{cam}$ in 500 μL of 200 mM potassium phosphate buffer (pH 7.4). The ethanol concentration did not exceed 1%. The solutions were mixed after each addition of substrate and/or ethanol and the difference spectrum was recorded. The value of K_s was determined by plotting $1/\Delta A$ versus $1/[S]$, the *x*-intercept of which yields $-1/K_s$ from the relationship $1/[S] = [E]\Delta\varepsilon/K_s\Delta A - 1/K_s$, where [S] is the concentration of the test compound, [E] is the concentration of $P450_{cam}$, Δε is the difference in molar absorptivity of the free and bound $P450_{cam}$ is the spectroscopic binding constant of the compound, and ΔA is the peak-to-trough absorbance in the difference spectrum.[26,27]

Oxygen consumption, NADH consumption, and H_2O_2 production

The reaction was carried out in a custom made quartz cell thermostated to 25 °C in which the O_2 concentration and the decrease in the NADH absorption maximum at 340 nm can be simultaneously monitored. A Hewlett–Packard 8452A diode array spectrophotometer and a Gilson Oxy5 Oxygraph electrode were connected to the cell. The reaction mixture contained, in a final volume of 1.1 mL, 200 mM potassium phosphate buffer (pH 7.4), 1 μM camphor-free $P450_{cam}$, 2 μM PdR, 4 μM Pd, 250 μM NADH, and the substrate added in 10 μL of ethanol. Substrates were present at a concentration 10-fold higher than their K_s values or just below their solubility limits. The NADH was added last to initiate the reaction. After 5 min incubation, a 0.3 mL aliquot of the incubation mixture was added to 0.2 mL of 0.3 M H_2SO_4 and the mixture was stored on ice until the H_2O_2 assay was carried out essentially as described previously (see below).[28] β-Mercaptoethanol was removed from the Pd prior to use by passage through a G-25 gel filtration column (100 mM TRIS, pH 7.4) because the thiol interferes with the peroxide assay and, at higher concentrations, raises the background NADH and O_2 consumption values. The kinetic rate for the NADH consumption was obtained from the resulting data by a zero-order calculation. The background NADH consumption values were recorded when ethanol (instead of substrate solution) was added to the mixture. The O_2 electrode was calibrated in distilled water at 25 °C, in which the O_2 concentration was taken to be 261 μM.

To quantitate H_2O_2, 0.125 mL of 6 mM ferrous ammonium sulfate and 0.125 mL of 6 M ammonium thiocyanate were added to the acidified aliquot of the reaction mixture taken above. The mixture was centrifuged at 12,000 RPM for 2 min to pellet precipitated protein and the absorbance was measured at 480 nm on a Hewlett–Packard 8452A diode array spectrophotometer. The amount of H_2O_2 formed was calculated from a standard curve made from a stock H_2O_2 solution.

Metabolite analysis and characterization

A 1 mL solution containing 1 μM camphor-free $P450_{cam}$, 2 μM putidaredoxin reductase, 8 μM putidaredoxin, 1 μM catalase (to remove any H_2O_2 formed in the reaction), 1 mM test compound (added in 10 μL ethanol), and 5 mM NADH was incubated at 25 °C for 1.5 h. A suitable amount of organic solvent (e.g. chloroform or diethyl ether) was added to the above reaction solution with 1-bromoadamantane as the internal standard. The extracted material was characterized by comparison with authentic standards by gas chromatography and tandem gas chromatography-mass spectrometry. Gas chromatography was performed on a Hewlett–Packard 5890 instrument equipped with a 30 m DB-17 capillary column under the following conditions: 60 °C for 1 min; gradient of 10 °C min^{-1} from 60–250 °C; finally 300 °C for 9 min. Tandem gas chromatography-mass spectrometry was carried out on a Hewlett–Packard model 5890 gas chromatograph interfaced with a VG-70 mass

spectrometer. The column and gas chromatographic program were as described above.

Molecular modeling

The coordinates of camphor-bound cytochrome $P450_{cam}$ were obtained from the Brookhaven National Laboratory's public data bank (PDB access code 2cpp) . The principles and algorithms that subserve the program DOCK 3.5 are described, and a manual can be found, online at http://www.cmpharm.ucsf.edu/kuntz/dock.html.[29] To use the program, it is necessary to: (a) generate a molecular surface of the enzyme active site; (b) generate a set of overlapping spheres of varying sizes that fill the cavity defined by the generated surface (the centers of the spheres serve as potential ligand atom positions); (c) generate a gridmap that allows calculation of the minimum distance between ligand and receptor (enzyme) atoms; (d) generate a 3-D ligand database in DOCK 3.5 readable format; and (e) run DOCK 3.5. We have chosen to run the program using contact (as opposed to force-field) scoring,[30] and to run the program repeatedly while varying the minimum allowed distance between ligand and enzyme atoms to find the optimal minimum distance to be used for distinguishing substrates from nonsubstrates. The minimum allowed distance was incrementally increased from 2.6 to 3.2 Å using the same distance for polar and nonpolar interactions. The 3-D structures of the test compounds were generated with the software package Sybyl 6.1 and were minimized by Sybyl energy minimization methods. Those structures were then written into a DOCK 3.5 readable database, which was searched by DOCK 3.5.

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