Substrate Docking Algorithms and Prediction of the Substrate Specificity of Cytochrome $P450_{cam}$ and Its L244A Mutant

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Abstract: The substrate specificity of cytochrome P450, defined as the ability of a compound to promote NAD(P)H and O_2 utilization in the production of either organic or reduced oxygen metabolites, is largely determined by steric and hydrophobic interactions. P450 specificity may therefore be determined by the "fit" of a compound within the active site. A receptor-constrained three-dimensional screening program (DOCK) has been used to select 11 compounds predicted to fit within the $P450_{cam}$ active site and 5 compounds predicted to fit within the L244A $P450_{cam}$ but not wild-type active site. The 16 compounds were evaluated as $P450_{cam}$ substrates by measuring (a) binding to the enzyme, (b) stimulation of NADH and O_2 consumption, (c) enhancement of H_2O_2 production, and (d) formation of organic metabolites. Seven of the compounds predicted to fit in the active site, and none of the compounds predicted not to fit, were found to be substrates. Compounds predicted to fit very tightly within the active site were poor or non-substrates. The L244A $P450_{cam}$ mutant was constructed, expressed, purified, and shown to readily oxidize some of the larger compounds incorrectly predicted to be substrates for the wild-type enzyme. The 5 ligands selected to fit the L244A but not wild-type sites were not detectable substrates, presumably because they fit too tightly into the active site. Retroactive adjustments of the docking program based on an analysis of the docking parameters, particularly variation of the minimum distance allowed between ligand and protein atoms, allow correct predictions for the activity of 15 of the 16 compounds with wild-type $P450_{cam}$. The DOCK predictions for the L244A mutant were also improved by changing the minimum contact distances to disfavor the larger compounds. The results indicate that ligands that fill the active site are marginal or non-substrates. A degree of freedom of motion is required for substrate positioning and catalytic function. If parameters are chosen to allow for this requirement, $P450_{cam}$ substrate predictions based on ligand docking in the active site can be reasonably accurate.

The cytochrome P450 enzymes are members of a large family of hemoproteins[1] that catalyze a wide range of oxidative and reductive transformations, including carbon hydroxylation, π-bond oxidation, heteroatom oxidation, hydrocarbon desaturation, and halocarbon dehalogenation.[2] The enzymes of the cytochrome P450 family can be usefully classified in a variety of ways. Classification of the enzymes according to their phylogenetic origin shows that the best characterized enzymes are of mammalian or bacterial origin, although knowledge of the enzymes from plants and insects is rapidly growing.[3] The enzymes can also be classified according to whether a protein with two flavin prosthetic groups or a binary system consisting of a flavoprotein and an iron−sulfur protein transfers electrons from NAD(P)H to the hemoprotein.[4] The enzymes can also be grouped according to their cytosolic or membrane location: as a rule, eukaryotic P450 enzymes are membrane bound and prokaryotic enzymes are soluble.[4b,5] One consequence of this difference in cellular location is that crystal structures are available for a growing number of soluble bacterial P450 enzymes but none is yet available for a membrane-bound P450.[6]

Despite the categorical differences among P450 enzymes in terms of origin, electron transfer partner(s), cellular localization, substrate specificity, and reactivity, the available evidence suggests that the active sites and catalytic mechanisms of P450 enzymes are remarkably similar. Catalytic turnover involves (a) substrate binding, usually with a concomitant change in redox potential that enables the heme to accept electrons from its electron transfer partner, (b) reduction of the iron to the ferrous state, (c) binding of molecular oxygen to give a ferrous dioxy complex ($Fe^{2+}-O_2$), (d) reduction of the ferrous dioxy complex to give a ferric peroxide (formally $Fe^{3+}-OOH$) intermediate, (e) conversion of the ferric peroxide to what is formally a ferryl ($Fe^{5+}=O$) species, (f) reaction of the ferryl species with the substrate to give the enzyme−product complex, and (g) dissociation of the product to regenerate the resting enzyme (Scheme 1).[2] The primary catalytic role of the enzyme is to produce the ferryl species under controlled conditions: the role of the enzyme in the reaction of the ferryl species with the substrate appears to be limited to juxtaposing the substrate and the ferryl species and restricting the sites on the substrate

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(1) Nelson, D.; Koymans, L.; Kamataki, T.; Stegeman, J.; Feyereisen, R.; Waxman, D.; Waterman, M.; Gotoh, O.; Coon, M.; Estabrook, R.; Gunsalus, I.; Nebert, D. *Pharmacogenetics* **1996**, *6*, 1−42.

(2) Ortiz de Montellano, P. R. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd ed.; Ortiz de Montellano, Ed.; Plenum Press: New York, 1995; pp 245−304.

(3) Schuler, M. *Crit. Rev. Plant Sci.* **1996**, *15*, 235−284.

(4) (a) Strobel, H. W.; Hodgson, A. V.; Shen, S. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd ed.; ortiz de Montellano, Ed.; Plenum Press: New York, 1995; pp 225−245. (b) Mueller, E. J.; Loida, P. J.; Sligar, S. G. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd ed.; Ortiz de Montellano, Ed.; Plenum Press: New York, 1995; pp 83−124.

(5) von Wachgenfeldt, C.; Johnson, E. F. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd ed.; Ortiz de Montellano, Ed., Plenum Press: New York, 1995; pp 183−224.

(6) (a) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *J. Mol. Biol.* **1987**, *195*, 687−700; (b) Ravichandran, K. G.; Boddupalli, S. S.; Hasemann, C. A.; Peterson, J. A.; Deisenhofer, J. *Science* **1993**, *261*, 731−736. (c) Hasemann, C. A.; Ravichandran, K. G.; Peterson, J. A.; Deisenhofer, J. *J. Mol. Biol.* **1994**, ***236***, 1169−1185. (d) Cupp-Vickery, J. R.; Poulos, T. L. *Struct. Biol.* **1995**, *2*, 144−153.

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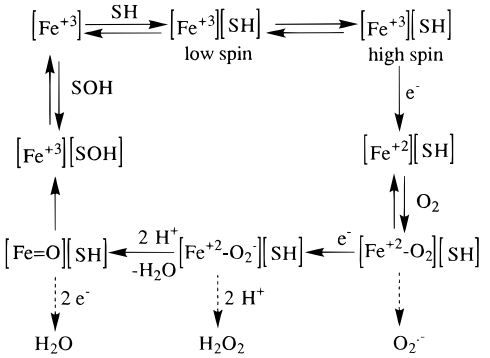
Scheme 1. Catalytic Cycle of Cytochrome P450, Showing the Individual Steps at which Uncoupling Can Occur[a]



[a] The iron of the heme group is indicated as an iron atom in brackets.

accessible to the ferryl species. In view of the diversity of P450 enzymes, the multiplicity of their substrates, and the breadth of the reactions they catalyze, it is not surprising that the catalytic cycle can be subverted. One example of such a diversion is reductive metabolism, a reaction that competes with the binding of oxygen to the ferrous intermediate.[7] A more general phenomenon is uncoupling of the consumption of oxygen and NAD(P)H from oxidation of the organic substrate.[7,8] This uncoupling can stem from (a) dissociation of superoxide from the ferrous dioxy complex, (b) dissociation of H_2O_2 from the ferric peroxide complex, or (c) reduction of the final ferryl species to water by electrons derived from NAD(P)H (Scheme 1). Uncoupled turnover is thus a catalytic process that produces reduced oxygen metabolites rather than organic products.

The factors that control the substrate specificity of P450 enzymes are not well understood. In general, the binding and orientation of potential substrates appears to be determined by hydrophobic and steric interactions and only infrequently by specific hydrogen bonding or ionic interactions. This conclusion derives from the preference of the enzymes for lipophilic substrates,[9] the nature of the active sites in the four bacterial enzymes for which crystal structures are available,[6] and inferences made from sequence alignments of P450 enzymes of known and unknown tertiary structure.[10] The specific site that is oxidized, once the molecule is bound within the active site, is largely determined by the intrinsic reactivity of the sites on the molecule that are accessible to the iron-bound oxidizing species.

A more refined understanding of P450 substrate specificity is highly desirable for practical reasons. For example, it is critical to the prediction of whether a specific compound will be a substrate for a given P450 enzyme, for the design and construction of P450 enzymes with tailored substrate specificities, and for the prediction of potential toxic interactions. Three features make the application of computer algorithms to the elucidation and prediction of P450 substrate specificity particularly attractive: (a) specificity is primarily determined by nonpolar interactions, (b) the enzyme has little active role in the reaction of the ferryl species with the substrate, and (c) the question of specificity can be dissected into distinct steps. The premise tested in this work is whether binding of a compound to $P450_{cam}$ without coordination to the iron atom, as judged by a simple analysis of its ability to fit within the active site, is sufficient to trigger catalytic turnover of the enzyme. According to the conventional definition, a good substrate is one that is efficiently converted to organic metabolites. However, the formation of reduced oxygen metabolites is also a measure of catalytic turnover because the partitioning between organic versus oxygen metabolites occurs at a branch point distal to the initiation of catalytic turnover. The ratio of organic to reduced oxygen products is thus a measure of the quality rather than the extent of catalysis. In elementary terms, a P450 substrate is a compound that initiates catalytic turnover regardless of the nature of the products. The intent of this study was therefore to develop a practical approach for evaluating the ability of potential substrates to bind within the $P450_{cam}$ active site, to determine the extent to which predictions of fit correlate with experimentally measured substrate activities, and to identify and optimize parameters that influence this correlation. Parts of the results of these studies have been communicated in preliminary form.[11]

Results

Prediction of $P450_{cam}$ Substrates. Cytochrome $P450_{cam}$ (CYP101), a monooxygenase required by *Pseudomonas putida* for growth on camphor as its sole carbon source, is the best characterized P450 enzyme.[4b] Crystal structures are available of $P450_{cam}$ in a variety of states, including as the camphor-bound and camphor-free protein and as the ferrous−CO complex.[6a,12] The change in oxidation potential from −300 to −170 mV associated with the binding of camphor to $P450_{cam}$ makes possible its reduction by putidaredoxin, for which the oxidation potential is −196 mV.[13] The enzyme normally catalyzes the 5-*exo*-hydroxylation of camphor, but recent work has demonstrated that $P450_{cam}$ also oxidizes compounds other than camphor, albeit with a higher degree of uncoupling of substrate oxidation from NADH and O_2 consumption.[11,14] $P450_{cam}$ has been chosen for the present studies because (a) it is a well-characterized system of known structure, (b) substrate binding does not significantly alter the active site geometry,[6a,12a] and (c) the system is experimentally versatile.

We have employed DOCK,[15] a receptor-constrained three-dimensional screening program, to evaluate the fit of potential substrates within the $P450_{cam}$ active site. DOCK has been successfully used to identify lead molecules for the development of enzyme inhibitors and receptor ligands.[15,16] The three steps involved in the use of the program are the following: (a) construction of a negative image of the molecular surface of the active site,[17,19a] (b) screening a data base of three dimensional structures to determine their fit within the active site volume

(7) Goeptar, A. R.; Scheerens, H.; Vermeulen, N. P. E. *Crit. Rev. Toxicol.* **1995**, *25*, 25−65.

(8) Archakov, A. I.; Bachmanova, G. I. *Cytochrome P-450 and Active Oxygen*; Taylor & Francis: New York, 1990; pp 129−138.

(9) (a) White, R. E.; McCarthy, M. *Arch. Biochem. Biophys.* **1986**, *246*, 19−32. (b) Duquette, P. H.; Erickson, R. R.; Holtzman, J. L. *J. Med. Chem.* **1983**, *26*, 1343−1348.

(10) Gotoh, O. *J. Biol. Chem.* **1992**, *267*, 83−90.

(11) (a) De Voss, J. J.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 4185−4186. (b) De Voss, J. J.; Ortiz de Montellano, P. R. *Methods Enzymol.* **1996**, *272*, 336−347.

(12) (a) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *Biochemistry* **1986**, *25*, 5314−5322. (b) Raag, R.; Poulos, T. L. *Biochemistry* **1989**, *28*, 7586−7592.

(13) (a) Sligar, S. G.; Gunsalus, I. C. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1078−1082. (b) Sligar, S. G. *Biochemistry* **1976**, *15*, 5399−5406.

(14) (a) Fruetel, J. A.; Collins, J. R.; Camper, D. L.; Loew, G. H.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1992**, *114*, 6987−6993. (b) Jones, J. P.; Trager, W. F.; Carlson, T. J. *J. Am. Chem. Soc.* **1992**, *115*, 381−387. (c) Fruetel, J.; Chang, Y. T.; Collins, J.; Loew, G.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1994**, *116*, 11643−11648. (d) Filipovic, D.; Paulsen, M. D.; Loida, P. J.; Sligar, S. G.; Ornstein, R. L. *Biochem. Biophys. Res. Commun.* **1992**, *189*, 488−495. (e) Loida, P. J.; Sligar, S. G.; Paulsen, M. D.; Arnold, G. E.; Ornstein, R. L. *J. Biol. Chem.* **1995**, *270*, 5326−5330. (f) Gelb, M. H.; Malkonen, P.; Sligar, S. G. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 853−858. (g) Stevenson, J.-A.; Westlake, A. C. G.; Whittock, C.; Wong, L.-L. *J. Am. Chem. Soc.* **1996**, *118*, 12846−12847.

(15) DesJarlais R. L.; Sheridan, R. P.; Seibel G. L.; Dixon J. S.; Kuntz I. D.; Venkataraghavan, R. *J. Med. Chem.* **1988**, *31*, 722−729.

and assigning a score to the fit of each compound, and (c) selection of compounds to be tested from the resulting list based on additional criteria (see below). In effect, a 20 000 compound subset of the Available Chemical Directory (ACD)[19b] was screened with DOCK. Contact scoring, which assigns the highest scores to compounds that maximize nonpenetrating contacts between the substrate and the active site, was used to evaluate the goodness of fit.[18] High contact scores are considered desirable for the identification of potential inhibitors, but the suitability of high contact scores for the evaluation of potential substrates had not been examined prior to this study. Of the 500 compounds with the highest contact scores, a small group of structurally diverse compounds was selected (Table 1) that did not have functionalities ionized at physiological pH, were relatively simple structures, and represented a diversity of structural types.[11,19]

To test the ability of this computer-assisted approach to discriminate between substrates and nonsubstrates, a group of compounds predicted not to fit within the P450$_{cam}$ active site was also identified (Table 2). In order to make this a rigorous test, the P450$_{cam}$ active site model based on the crystal coordinates was modified by replacing the side chain of Leu-244 with that of an alanine (i.e., by removing three carbons from the crystal coordinates) while holding the rest of the atoms fixed in their original positions. Dock was then run on the "mutated" active site exactly as done for the native enzyme, and a difference list was made of the compounds that fit within the model L244A but not wild-type active site. This approach, which requires that DOCK differentiate between ligands that bind to sites that differ by only a small volume, generated a list of predicted nonsubstrates closely related to the predicted substrates (Table 2).[11]

CONCORD Versus SYBYL Generated Structures. Three-dimensional structures of the compounds in the Available Chemicals Directory were generated with the program CONCORD,[19c] which uses a set of empirical bond length and bond angle rules to convert two-dimensional to three-dimensional structures. After DOCK analysis of a 20 000 compound subset of the ACD, the 500 compounds with the highest contact scores were saved and visually screened. Ten compounds (**1**−**9**, **12**) were chosen as potential substrates from the wild-type P450$_{cam}$ screen, and six compounds (**10**, **11**, **13**−**16**) were chosen as probable nonsubstrates from the L244A P450$_{cam}$ search. Since better three-dimensional structures than those provided by CONCORD can be generated by using the molecular mechanics functions in the SYBYL modeling package,[19d] the molecules selected from the initial DOCK analysis of the ACD were minimized by using the default values in SYBYL and were reDOCKed into the active site. Optimization of the structures with the SYBYL molecular mechanics programs resulted in two compounds (**10**, **11**) migrating from the nonsubstrate to the substrate list and one (**12**) migrating in the opposite direction.

Dependence of Prediction on DOCK Parameters. Initial efforts to predict P450$_{cam}$ substrates were carried out with the default settings for the adjustable parameters in DOCK. However, as these default settings are not necessarily optimal for the identification of substrates, we have examined the effects of varying some of these parameters. Studies of the minimum distance allowed between an atom of the substrate and an atom of the enzyme before the contact is classified as prohibitive have been particularly fruitful. Two such distances are defined in DOCK, one for polar and one for nonpolar interactions: the default values for these distances are 2.3 and 2.8 Å, respectively. These distances have been found to be critical determinants of the substrate predictions. The results in Tables 1 and 2 are based on the default values, but increasing the distances to 2.4 and 2.9 Å for polar and nonpolar contacts, respectively, shifts compounds **8**−**10** from the predicted substrate to the nonsubstrate column. The two contact distances have also been set to the same value, and the DOCKing of the compounds, using both the CONCORD and SYBYL-minimized structures, has been reexamined as a function of this single minimum contact distance. As shown in Figure 1, if the minimal contact distance is set at 2.9 Å, all but one of the 16 compounds are correctly predicted to be either substrates or nonsubstrates of P450$_{cam}$. The exceptions are compound **3**, using the CONCORD structures, and compound **6**, using the SYBYL structures.

The best active site model to be used for the substrate docking studies is also of concern. The ferric, camphor-bound enzyme after subtraction of the camphor coordinates was used for the above studies, but docking to the ferrous, ferrous dioxy, or putative ferryl states of the protein could also be envisaged. However, of these enzyme forms, coordinates are only available for the ferrous state. Fortunately, although NMR and X-ray crystallography indicate that ferric P450$_{BM-3}$ undergoes a major conformational change when the enzyme is reduced to the ferrous state,[21] the data on P450$_{cam}$ indicate that its active site is little altered by changes in the redox and ligation states of the protein.[6a,12a] The ferrous−CO complex, for which a crystal structure is available,[20] can therefore be used with some confidence as a model for the ferrous dioxy complex, although the CO and O_2 ligands differ in that the first is essentially linear and the second bent. As shown in Figure 1, correct predictions can be made for 15 of the 16 compounds if the minimum contact distance for binding to the Fe^{2+}−CO complex is set to 2.7 Å rather than the 2.9 Å found for docking to the ferric protein. However, from the results, there is no obvious advantage to selecting the ferrous−CO complex over the better defined ferric site as the docking target.

Binding of Compounds to P450$_{cam}$. The binding of compounds to P450$_{cam}$ was evaluated spectroscopically by quantitating the low to high spin transition evidenced by a decrease in the absorption maximum of the resting enzyme at 417 nm and the corresponding increase in the enzyme−substrate

(16) (a) DesJarlais, R. L.; Seibel, G. L.; Kuntz, I. D.; Furth, P. S.; Alvarez, J. C.; Ortiz de Montellano, P. R.; DeCamp, D. L.; Babé, L. M.; Craik, C. S. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 6644−6648. (b) Shoichet, B. K.; Stroud, R. M.; Santi, D. V.; Kuntz, I. D.; Perry, K. M. *Science* **1993**, *259*, 1445−1450. (c) Ring, C. S.; Sun, E.; McKerrow, J. H.; Lee, G. K.; Rosenthal, P. J.; Kuntz, I. D.; Cohen, F. E. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3583−3587. (d) Bodian, D. L.; Yamasaki, R. B.; Buswell, R. L.; Stearns, J. F.; White, J. M.; Kuntz, I. D. *Biochemistry* **1993**, *32*, 2967−2978.

(17) (a) Richards, F. M. *Annu. Rev. Biophys. Bioeng.* **1977**, *6*, 151−176. (b) Connolly, M. L. *Science* **1983**, *221*, 709−713.

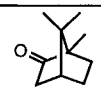
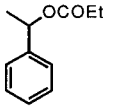
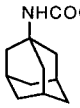
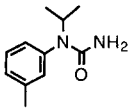
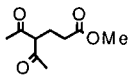
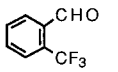
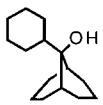
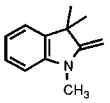
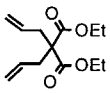
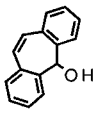
(18) Kuntz, I. D.; Meng, E. C.; Shoichet, B. K. *Acc. Chem. Res.* **1994**, *27*, 117−123.

(19) (a) Molecular graphics visualization and molecular surface generation were carried out with the MidasPlus program from the Computer Graphics Laboratory, University of California, San Francisco (supported by NIH RR-01081): Ferrin, T. E.; Huang, C. C.; Jarvis, L. E.; Langridge, R. *J. Mol. Graphics* **1988**, *6*, 13−27. (b) MDL Information Systems, Inc., San Leandro, CA, USA. (c) Rusinko, A.; Sheridan, R. P.; Nilakatan, R.; Haraki, K. S.; Bauman, N.; Venkataraghavan, R. *J. Chem. Inf. Comput. Sci.* **1989**, *29*, 251. (d) SYBYL 6.0 Tripos Inc., 1699 South Hanley Road, St. Louis, MO 63144−2913 USA; (e) Molecular graphics images were produced using the MidasPlus program from the Computer Graphics Laboratory, University of California, San Francisco (supported by NIH RR-01081): Ferrin, T. E., Huang, C. C., Jarvis, L. E., and Langridge, R., *J. Mol. Graphics* **6**, 13 (1988); (f) SPHGEN: Kuntz, I. D., Blaney, J. M., Oatley, S. L., Langridge, R., and Ferrin, T. E. (1982) *J. Mol. Biol.* **161**, 269−288.

(20) Raag, R.; Poulos, T. L. *Biochemistry* **1989**, *28*, 7586−7592.

(21) (a) Modi, S.; Sutcliffe, M. J.; Primrose, W. U.; Lian, L.; Roberts, G. C. K. *Nature Struct. Biol.* **1996**, *3*, 414−417. (b) Li, H., and Poulos, T. L. *Biochimie* **1996**, *78*, 695−699.

Table 1. Active Site Docking Parameters and Experimental Activities of Potential $P450_{cam}$ Substrates

Entry	Compound	DOCK[a] Max. Score (Number of orientations) Rank	Volume Å3	K_S or K_I μM	High Spin %	NADH[b] nmol min^{-1} $nmol^{-1}$	% H_2O_2 from O_2
Camphor		126 (5000)	160	1.1 ± 0.1	100	530 ± 10	0-8
1		141 (84) 267	194	83 ± 5	8	8 ± 1	100
2		129 (56) 143	205	4.3±0.3	91	110 ± 5	5-10
3		127 (1) 233	210	280 ± 70	6	12 ± 1	100
4		123 (28) 414	189	4400 ± 600	13	41 ± 3	31
5		138 (430) 60	141	530 ± 100	22	27 ± 1	50
6		129 (2) 120	249	27 ± 4	30	6 ± 1[f]	100
7		132 (368) 52	199	1200 ± 200[c]	ND[d]	80 ± 5	11
8		125 (2) 39	248	74 ± 25[c]	<2	4 ± 1	100
9		146 (1) 164	232	>>300[c]	<1	5 ± 1	100
10		159 (1) ND[e]	258	500 ± 260[c]	<1	6 ± 1	100
11		125 (3) ND[e]	221	228 ± 32	<2	8 ± 1	100

[a] DOCK results given as (i) the maximum binding energy score in "DOCK kcals" from DOCKing of each compound in SINGLE mode; (ii) the number of orientations generated in the SINGLE DOCK run, and (iii) the DOCK rank of each compound after the SEARCH run of the ACD subset. [b] Background NADH consumption is 4−6 nmol min^{-1} $nmol^{-1}$, but the values have not been corrected for this background because it may differ in the presence of substrates. [c] K_I, determined by inhibition of camphor binding. [d] Not determined because the UV−visible absorption of compound interfered with spectrophotometric assay. [e] These compounds were not ranked as they were not identified as substrates in the original DOCK search. [f] An organic product is formed even though there is little stimulation of NADH consumption.

complex absorption maximum at 392 nm (a Type I spectroscopic change).[22] The results are expressed as the spectroscopic binding constant K_s (Table 1) determined by plotting the peak to trough amplitude of the difference spectrum versus the reciprocal of the substrate concentration. The K_s values thus obtained for compounds **1**−**6** and **11** ranged from 4 μM for **2** to 4400 μM for **4**. These values are to be compared with K_s = 1.1 μM under similar conditions for the natural substrate camphor.

If a normal spectroscopic change was not observed, the ability of the test compound to inhibit the low to high spin transition produced by camphor was examined and the data were used to

(22) Petersen, J. A.; Lorence, M. C.; Amarneh, B. *J. Biol. Chem.* **1990**, *265*, 6066−6073.

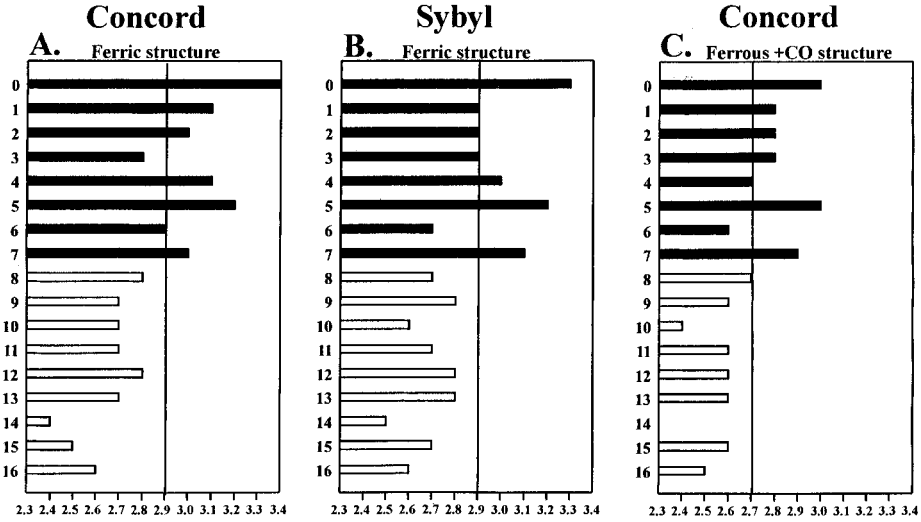
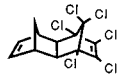
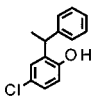
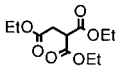
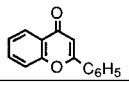


Figure 1. Correlation of the activity of compounds **1**−**16** as P450$_{cam}$ substrates with the value of the minimum distance allowed between substrate and active site atoms. (A) DOCKing of Concord generated substrate structures within the ferric P450$_{cam}$ active site model, (B) DOCKing of Sybyl minimized substrate structures within the ferric P450$_{cam}$ active site model, and (C) DOCKing of Concord generated substrate structures within the Fe^{+2}−CO P450$_{cam}$ complex as a model of the ferrous dioxygen intermediate. Solid bars indicate experimental substrates and open bars experimental non-substrates. The vertical lines indicate the minimum interaction distance that gives the best fit of the computational predictions with the experimental results.

Table 2. Active Site Docking Parameters and Experimental Activities of Compounds Predicted Not To Be Substrates for P450$_{cam}$

Entry	Compound	Volume Å^3	K_S or K_I μM	High Spin %	NADH[a] nmol min^{-1} nmol^{-1}	% H_2O_2 from O_2
12		289	Not Detected	>8	4±1	100
13		238	75±23	>6	5±1	100
14		244	4300±420	>1	5±1	100
15		231	Not Detected	>1	5±1	100
16		226	72±26	>1[b]	6±1	100

[a] Background NADH consumption is 4−6 nmol min^{-1} nmol^{-1}, but the values in the table are uncorrected because the background may differ in the presence of substrates. [b] A decrease is seen in the Soret at 417 nm with a corresponding increase at 430 nm.

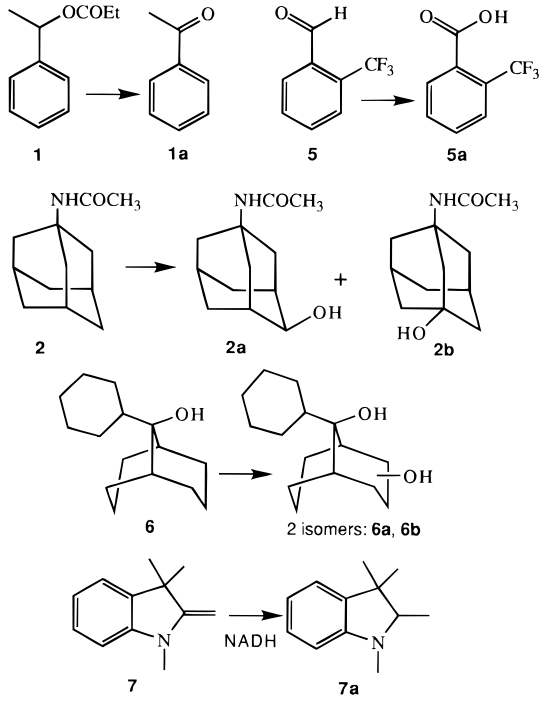
calculate a K_I. Compounds **7**−**10**, **13**, **14**, and **16** significantly inhibited the spectroscopically-measured binding of camphor. The best, and nearly equipotent, inhibitors of the binding of camphor were **8**, **13**, and **16**.

Oxygen Consumption and Uncoupling. The activity of compounds as P450$_{cam}$ substrates has been evaluated by measuring their ability to stimulate NADH consumption. A comparable but not necessarily identical estimate of substrate activity is provided by the ability of the compounds to stimulate O_2 consumption. The extent of uncoupled turnover can be partially evaluated by comparing the increase in NADH and O_2 consumption with the increase in the formation of H_2O_2. In the absence of highly precise O_2 consumption measurements, it has not been possible to estimate the uncoupled production of water. Water is formed by reduction of the ferryl species in a reaction that competes with actual oxidation of the organic substrate (Scheme 1). The uncoupling estimated in these studies (Tables 1−3) is thus a maximum, as formation of water by reduction of the ferryl species consumes two NADH molecules rather than the single NADH used when the substrate is hydroxylated or oxygen is reduced to H_2O_2.

The catalytic oxidation of camphor is both rapid and tightly coupled, less (possibly much less) than 8% of the O_2 consumed by the enzyme being funneled into reactions other than substrate hydroxylation (Table 1). Of the unnatural substrates, **2** is turned over the most effectively and its oxidation is only slightly less well coupled than that of camphor. For the other compounds, the consumption of NADH (and oxygen) decreases in the following order: **7** > **4** > **5** > **3** > **1** > **6**. The coupling within this series of compounds with regard to H_2O_2 formation decreases in essentially the same order (**7** > **4** > **5** > **1**, **3**, **6**), with the caveat that the degree of uncoupling for the last three very poor substrates cannot be differentiated. The finding that substrates at the lower end of the O_2 consumption scale appear as the most uncoupled is to some extent artifactual because P450$_{cam}$ maintains a low residual turnover rate in the absence of a substrate, all of which results in uncoupled product formation. If the substrate-mediated increase in O_2 consumption is small, a large error is introduced by the background reduction of O_2 to H_2O_2. It is difficult to correct for the background O_2 consumption and H_2O_2 production because the values for these parameters probably decrease in an unpredictable manner in the presence of a substrate. The experimental values in Tables 1−3 have therefore not been corrected for the background values.

P450$_{cam}$ Metabolites. Organic products have been identified in the reactions of the P450$_{cam}$ system with substrates **1**, **2**, **5**, **6**, and **7** (Scheme 2). The products have been identified by tandem gas−liquid chromatography−mass spectrometry, comparison with authentic standards, and in some instances, NMR characterization. Comparison with authentic material shows that benzylic hydroxylation of **1** by P450$_{cam}$ is followed by elimination of the propionate moiety to give acetophenone (**1a**). Gas chromatography−mass spectrometry shows that oxidation of the adamantylamine derivative **2** produces two monohydroxylated metabolites. Comparison with authentic standards identifies the two products as **2a** and **2b**. Comparison of the product derived

Scheme 2. Structures of Products Formed in Cytochrome $P450_{cam}$ Oxidation Reactions



from **5** with authentic materials identifies the metabolite as 2-(trifluoromethyl)benzoic acid (**5a**). Compound **6** is poorly metabolized but gives a low yield of two products (**6a**, **6b**) shown by mass spectrometric analysis to be monohydroxylated derivatives. The exact structures of these products have not been established, although the mass spectrometric fragmentation patterns indicate that the hydroxyl is not in the cyclohexane ring (i.e., the mass increase is found in the fragment from which the cyclohexyl ring has been eliminated).

The metabolism of **7** is unusual in that comparison with authentic material identifies the product as the reduced compound **7a**. Due to the unusual nature of the product, control incubations were carried out in the absence of the individual components of the reaction mixture. In fact, the reductive reaction takes place when **7** is incubated with NADH alone. $P450_{cam}$ therefore does not appear to catalyze this transformation of **7**, although oxygen consumption experiments indicate that **7** triggers extensive coupled and uncoupled turnover of the enzyme (see below). However, no other organic product has been detected in the reaction mixture.

No organic products have been isolated from the incubations with compounds **3**, **4**, or **8**−**11** even though compounds **3** and particularly **4** consume a significant amount of NADH above the background level.

L244A $P450_{cam}$ Mutant. Several compounds were predicted to be unacceptable as substrates for $P450_{cam}$ based on differential screening of their fit within the active site of $P450_{cam}$ and the L244A model active site obtained by replacing the Leu by an Ala side chain. To examine the predictive utility of identifying compounds that fit within the L244A but not wild-type active site, we constructed a cDNA coding for the L244A $P450_{cam}$ mutant, expressed it in *Escherichia coli*, and purified the protein. The L244A expressed at 20 °C is catalytically active and has spectra identical with those of the wild-type enzyme (not shown). The K_s value in 200 mM potassium phosphate buffer for the binding of camphor to the mutant is 4.9 ± 1 μM, a value approximately 4-fold higher than that for binding of camphor to the wild-type enzyme (K_s = 1.1 μM). Spectroscopic studies indicate that saturation of the active site with camphor converts 85% of the protein to the high-spin form, assuming that the absorption coefficient for the spin state change is the same for the wild-type and mutant enzymes. The L244A mutant hydroxylates camphor to the normal 5-*exo*-hydroxycamphor metabolite without the detectable formation of alternative products (not shown). Measurements of the production of H_2O_2 indicate that the oxidation of camphor by the L244A mutant is tightly coupled and produces no more H_2O_2 than turnover by the wild-type enzyme. The rate of camphor hydroxylation is 150-250 nmol min^{-1} $nmol^{-1}$, a value that is 2−3-fold lower than that obtained for the wild-type enzyme under comparable conditions. The L244A mutation thus appears to have only minor consequences for the structure of the protein or its ability to oxidize camphor.

Table 3. Parameters for the Interaction of Compounds with the L244A $P450_{cam}$ Mutant

compd no.	K_s, μM	spin change, %	NADH,[a] nmol min^{-1} $nmol^{-1}$	% H_2O_2 from O_2	metabolite detection
Compounds Predicted To Be Substrates For Both Wild-Type and L244A $P450_{cam}$					
camphor	4.9	85[b]	86 ± 3	7 ± 1	yes
8	3.3	31	24 ± 2	66 ± 4	yes
9	N/A[c]	2	6 ± 1	100	no
10	N/A[c]	<2	5 ± 1	100	no
11	0.8	16	23 ± 3	61 ± 4	yes
Compounds predicted to be substrates for L244A but not wild-type $P450_{cam}$					
12[d]	N/A[c]	<2	6 ± 2	100	no
13	1.1	36	<4	98 ± 2	no
14	N/A[c]	<4	6 ± 1	92 ± 4	no
15[d]	N/A[c]	<2	4 ± 1	100	no
16[d]	N/A[c]	<2	6 ± 1	100	no

[a] These values have not been corrected for a background value of 6 nmol min^{-1} $nmol^{-1}$ because it is likely to be lower in the presence of substrates. [b] The maximum absorbance change in the L244A spectrum caused by camphor binding is based on the assumption that the molar absorbance coefficients for the spin state changes in the L244A and wild-type proteins are the same. [c] N/A: no detectable spectroscopic perturbation. [d] Compound precipitated at the usual 1 mM concentration, so data were obtained at 0.15 mM substrate concentration.

Binding of Compounds to L244A Mutant. Table 3 provides a summary of the binding of various compounds to L244A $P450_{cam}$. Two compounds (**8** and **11**) predicted to be substrates for the wild-type enzyme, but which were not detectable substrates, are shown by their K_s values to bind with much higher affinity to the L244A mutant than to the wild-type enzyme (Tables 1 and 3). The enhanced binding affinity is paralleled by significant substrate-binding-mediated conversion of the protein from the low to the high spin state (31 and 16%, respectively, for **8** and **11**) even though neither compound causes a detectable spin state change in the wild-type protein. Furthermore, **8** and **11** cause substantial catalytic turnover, as judged by NADH consumption of 23−24 nmol min^{-1} $nmol^{-1}$ (Table 3), in contrast to the background level of NADH consumption observed with the wild-type protein (Table 1). Of the NADH consumed, 61−66% is used to reduce O_2 to H_2O_2. However, in the oxidation of **8**, a part of the reducing equivalents is used to hydroxylate the substrate to a product that migrates as a single peak by GLC. The structure of this product has not been determined. We have not succeeded in detecting an organic product in incubations with **11**, although the coupling data suggest that one is formed. In contrast to the results with compounds **8** and **11**, compounds **9** and **10**, which were not substrates for the wild-type enzyme, are also not detectable substrates for the L244A mutant (Table 3).

Of the five compounds predicted with the DOCK default parameters to bind to L244A but not wild-type P450$_{cam}$ (**12**−**16**), only **13** gave a good binding spectrum (K_s = 1.1 μM), causing a 36% conversion from the low to the high spin state (Table 3). However, NADH consumption remained low for this compound and for the other four compounds predicted to be substrates for the L244A mutant, and no organic products were detected for any of the five compounds. Three of the compounds (**12**, **15**, **16**) were relatively insoluble under the reaction conditions and precipitated, so data for these compounds were obtained with a lower (0.15 mM) substrate concentration than that (1 mM) used for the other compounds.

Discussion

A major question examined here is whether the ability to fit within the P450$_{cam}$ active site, as determined by computer-assisted docking of rigid 3-dimensional structures into a rigid, crystallographically determined P450$_{cam}$ active site model, can be used to identify compounds that are substrates for the enzyme. In addition to fit, the predictions are subject to the additional constraints that the compounds must be largely unionized and soluble under the assay conditions. Furthermore, for the purpose of these studies, substrates are defined in their broadest sense as compounds that trigger catalytic turnover and lead to the production of either organic or reduced oxygen metabolite(s).

It is important to understand the limitations of a rigid-body docking approach whether one uses DOCK, as done here, or an alternative docking algorithm. The most significant limitation is that single energy-minimized conformers of potential substrates are docked as rigid structures into an inflexible active site model. No allowance is made for the existence of other energy-accessible conformations or for conformational alterations mediated by interactions with the protein. The same is true of the active site into which the substrates are docked, as it is derived from the enzyme crystal structure and is held invariant during the docking studies. No allowance is made for the adjustment of active site residues to accommodate individual substrates. Furthermore, it is not clear which P450$_{cam}$ active site structure is the most appropriate for docking predictions. Substrates bind initially to the ferric state of the protein, but catalysis requires reduction to the ferrous state, oxygen binding to give the ferrous dioxy complex, and formation of a putative ferryl complex (Scheme 1). The ferric enzyme has been primarily used for the present studies. Although docking to other enzyme intermediates must be considered, docking to the $Fe^{2+}-CO$ complex as a model of the $Fe^{2+}-O_2$ complex did not improve the predictions, suggesting that for P450$_{cam}$ the ferric state is an adequate docking target. Finally, in the case of the L244A mutant, the docking studies were carried out with a model of the enzyme derived from the crystal structure of the native rather than mutant protein.

Additional limitations are provided by (a) differences between the true active site volume and the negative image of it created by SPHGEN (a DOCK subroutine)[19f] and (b) the fact that we have chosen to use DOCK 3.0 without considering electrostatic or hydrogen bonding interactions. This is a serious limitation in some contexts, but appears not to be critical here because the binding of most substrates to P450 enzymes is controlled by nonpolar interactions. Nevertheless, binding of camphor, the natural substrate, to P450$_{cam}$ involves a hydrogen bond between Tyr-96 and the camphor ketone group. This hydrogen bond contributes to the fact that camphor is an excellent substrate and is converted to a single product despite the fact that it was ranked by DOCK below the top 500 ligands for the enzyme. The rank the DOCK contact score assigns to a ligand is based exclusively on steric fit and is highest when the substrate−enzyme surface contact is highest without intrusion of the substrate into the protein structure. This contact scoring system is designed for the evaluation of potential inhibitors, for which maximum surface contact is desirable, so that the ranking of a potential inhibitor increases in proportion to its ability to fill the site. The present results suggest that filling the site as tightly as possible is not a good indicator of activity as a substrate (see below), so the DOCK rankings in Table 1 do not correlate with activity.

In view of the limitations, it is surprising that the DOCK-based predictions are as good as they are. Of eleven compounds identified with the DOCK default parameters as potential P450$_{cam}$ ligands, seven either stimulate the consumption of NADH and the production of either H_2O_2 or organic metabolites or lead to the formation of organic products without a detectable change in total consumption of NADH (Table 1). The seven can therefore be classified as substrates. Impressively, none of the five compounds identified as ligands for the L244A but not wild-type enzyme are substrates for the wild-type enzyme (Table 2). Thus, the initial DOCK-based evaluation of the 16 compounds as potential ligands for wild-type P450$_{cam}$ correctly predicted the behavior of the compounds as P450 substrates 75% of the time. Retrospective analysis indicates that even better results are possible if the value for the minimum contact distance between the protein and substrate atoms is optimized. By using a single value of 2.9 Å for this minimum contact distance, all but one of the compounds (94%) are correctly accounted for as substrates or nonsubstrates (Figure 1).

The predictions for the L244A P450$_{cam}$ model obtained with the default DOCK parameters are less accurate. Of the five compounds predicted to be substrates for the L244A mutant, based on docking to a structure model created by replacing the Leu-244 side chain with an alanine side chain (Table 3), only one (**14**) is possibly a marginal substrate. The other four (**12**, **13**, **15**, **16**), as judged by the failure to enhance NADH consumption and the absence of detectable organic products, are not substrates for the L244A mutant. One factor that must be considered in this context is that the docking studies were done with a model of the L244A structure derived from the crystal coordinates of the wild-type enzyme rather than of the mutant itself. The model assumes that the L244A mutation decreases the volume of the residue at position 244 without causing significant reorganization of the active site. This assumption is valid for some but not all mutations, and the accuracy of the results would clearly be improved if a model based on crystal coordinates of the mutant itself were used for the docking studies.

Important information with respect to the L244A mutant is provided by the compounds that were predicted to be, but were not, detectable substrates for the wild-type enzyme. Two of these compounds (**8**, **11**) are good substrates for the L244A mutant (Table 3). Both compounds are predicted with the default DOCK parameters to bind to the L244A mutant site, but to do so without filling the cavity as completely as they fill that of wild-type P450$_{cam}$. As already noted, comparison of the DOCK predictions with the experimental results reveals that compounds that completely fill the active site are either very poor or nonsubstrates. An imperfect measure of the tightness of fit is provided by the number of orientations which DOCK finds for a given molecule within the active site (Table 1). Compound **2**, the best substrate other than camphor, can be docked in 56 orientations by using the default DOCK param-

eters. Of the other six compounds judged to be substrates, all but two have many docking modes. The two exceptions are **3** and **6**, neither of which is more than a marginal substrate. On the other hand, the four compounds predicted to be ligands but not found to be detectable substrates (**8**−**11**) can only be fit by DOCK into the active site in one or two orientations. This relationship between tightness of fit and substrate activity is reinforced by the finding that compounds **8** and **11**, which are not detectable substrates for the wild-type enzyme, are good substrates for the L244A mutant.

Although the volume of a compound does not correlate in a simple manner with its activity as a substrate, it is clear from the data in Tables 1 and 2 that compounds with volumes much larger than ∼210 Å^3 are unlikely to be substrates. This holds for compounds (**8**−**11**) predicted by DOCK to be P450$_{cam}$ substrates but which are not, as well as compounds actually predicted to be nonsubstrates (**12**−**16**). The only exception is compound **6**, with a volume of 246 Å^3, but it is a very poor substrate. Although poor substrates can be found among the smaller compounds (i.e., compound **1**, volume 194 Å^3), the best indicator of a poor or nonsubstrate is a size that barely allows the compound to fit into the active site. Retrospectively, this observation rationalizes both the activity of **8** and **11** with the L244A mutant and the inactivity of compounds **12**−**16** as substrates for the L244A mutant. Compounds **8** and **11** fill the P450$_{cam}$ active site too tightly to be more than trace substrates, but are sufficiently well accommodated in the larger L244A site to be acceptable substrates for that protein. Compounds **12**−**16**, chosen to be too large for the wild-type site, by definition barely fit into the L244A site. It is therefore NOT surprising that they are not acceptable substrates for the mutant enzyme. One approach to the selection of substrates for a mutant enzyme suggested by these results is to employ a differential DOCK procedure using a relatively long minimum contact distance between the protein and the substrate atoms, as this would bias the selection of substrates toward compounds that do not absolutely fill the active site cavity. Retrospective docking of the 16 CONCORD structures in the model of the L244A active site, using a minimum contact distance of 2.9 Å, correctly predicts that compounds **9** and **13**−**16** are substrates (Figure 2). The two incorrect predictions are compounds **10** and **12**, which are predicted to be substrates. Thus, 14 of the 16 predictions are correct. Approximately the same accuracy is achieved with the Sybyl minimized structures (Figure 2).

The DOCK approach predicts fairly well whether or not a compound will initiate catalytic turnover but, not unexpectedly, it is not a good predictor of the extent to which catalytic turnover results in oxidation of the substrate rather than uncoupled reduction of molecular oxygen. Minimal uncoupling, as with camphor, is observed with compounds **2** and **7**, intermediate uncoupling with **4** and **5**, and essentially complete uncoupling with compounds **1** and **3** (Table 1). Compound **6** is a very poor substrate but gives both H_2O_2 and hydroxylated products. Likewise, the products formed from the organic substrates cannot be predicted by the present approach, as it requires a sophisticated analysis of both the intrinsic reactivity of the substrate sites and the time averaged location and orientation of the substrate within the active site. Predictions of this nature have been made with the help of molecular dynamics simulations for the epoxidation of substituted styrenes, sulfoxidation of substituted thioanisoles, oxidation of ethylbenzene, and other reactions.[14] All the isolated and characterized products (**1a**, **5a**, **2a**,**b**, **6a**,**b**) in the present studies are the result of straightforward carbon hydroxylation reactions with the exception of **7a**, a minor product that is an artifact due to direct reduction of the substrate

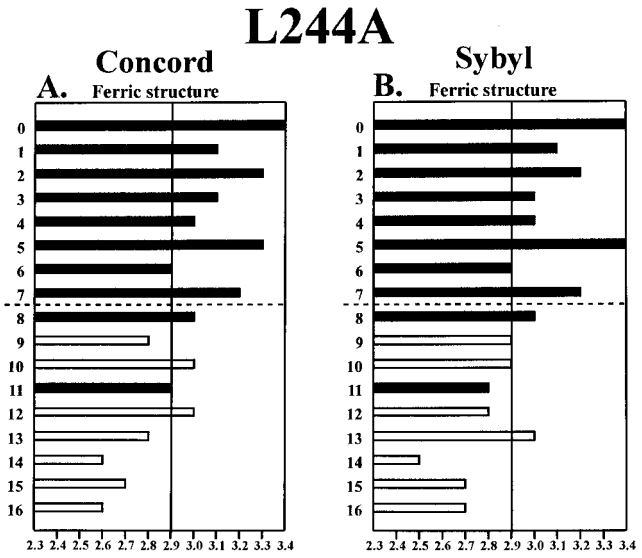


Figure 2. Prediction of the activity of compounds **1**−**16** as substrates for L244A P450$_{cam}$ as a function of the minimum distance allowed between substrate and active site atoms. (A) DOCKing of Concord generated substrate structures within the ferric L244A P450$_{cam}$ active site model and (B) DOCKing of Sybyl minimized substrate structures within the ferric L244A P450$_{cam}$ active site model. Solid bars indicate experimental substrates and open bars experimental non-substrates. The vertical lines are drawn at a value of 2.9 Å for the minimum interaction distance.

by NADH. We have not been able to isolate a major product from the oxidation of compound **7**, which is shown by the NADH consumption studies to be a good, well-coupled substrate (Table 1). The oxidation of **7** apparently produces a polymeric or water-soluble product that is not readily extracted and/or characterized by the methods used in this study.

Conclusions

In the structure-based design of enzyme inhibitors, efforts are usually made to fill the active site and to maximize inhibitor−protein contacts. The present results indicate that the same criteria are not appropriate for the selection of P450 substrates. Allowance must be made for a degree of active site plasticity to allow (i) optimal positioning of the substrate within the site and (ii) the active site and substrate motions required for catalytic turnover. The present rigid body docking approach makes no allowance for either global or localized conformational flexibility, but an averaged degree of freedom can be introduced into the DOCK predictions by varying the minimum distance allowed between the substrate and the protein atoms. A short value for this distance increases the acceptance of substrates that are tightly wedged into the active site, whereas a long value allows for an averaged looseness of fit and therefore, indirectly, for a small degree of conformational and translational freedom. Retrospective analysis of the data in Tables 1 and 2 suggests that a single contact distance of 2.9 Å correctly rationalizes the activity of 15 of the 16 compounds with P450$_{cam}$ (Figure 1). A modestly lower accuracy is obtained in rationalizing the data for the L244A mutant (Figure 2).

Experimental Section

Materials. The cytochrome P450$_{cam}$, putidaredoxin, and putidaredoxin reductase clones in pIB125 were kindly provided by Dr. Julian Peterson (University of Texas Southwestern Medical Center). Camphor, **1** ((±)-1-phenyl ethylpropionate), **2** (*N*-(1-adamantyl)acetamide), **3** (1-isopropyl-1-(*m*-tolyl)urea), **4** (methyl 4-acetyl-5-oxohexanoate), **7** (2-methylene-1,3,3-trimethylindoline), **10** (diethyl diallylmalonate),

11 (dibenzosuberenol), **12** (isodrin), **13** (DL-4-chloro-2-(α-methylbenzyl)phenol), **14** (triethyl 1,1,2-ethanetricarboxylate), **15** (methyl 1-fluorenecarboxylate), **16** (flavone), and H_2O_2 were purchased from Aldrich. Compound **5** (2-(trifluoromethyl)benzaldehyde) was purchased from Lancaster, **6** (9-cyclohexylbicyclo[3.3.1]nonan-9-ol) from Janssen, and **8** (cedrol) from K&K Laboratories. Compound **9** was prepared by acetylation of 9-fluorenol (from Lancaster) with acetic anhydride. Putidaredoxin reductase was heterologously expressed in *E. coli* and purified as reported in the literature.[22]

Analytical Methods. Absorption measurements were made on an SLM-Aminco DW-2000 or a Hewlett-Packard Model 8452A diode array UV−vis spectrophotometer. Gas−liquid chromatography was performed on a Hewlett-Packard Model 5890 instrument equipped with a flame ionization detector and interfaced to a Hewlett-Packard 3365 ChemStation. Tandem gas chromatography−mass spectrometry was performed on a Hewlett-Packard Model 5890 gas chromatograph interfaced with a VG-70 mass spectrometer.

Construction of a P450$_{cam}$ pCW Vector and Expression and Purification of the Protein. An *NdeI* site was introduced by polymerase chain reaction (PCR) at the 5′ end of the P450$_{cam}$ gene in the previously prepared pBScam plasmid.[23] The 5′ PCR primer contained a *SacI* sequence and an *NdeI* site that spanned the ATG start codon of the gene. The 3′ primer was complementary to a region of the P450$_{cam}$ gene approximately 450 bp from the 5′ end of the gene. The PCR product was ligated directly into the PCRII vector (In Vitrogen, San Diego, CA) according to the manufacturer's instructions. The 5′ end of the gene was then isolated from a *SacI*/*SphI* digest of the pCRII vector and ligated into similarly cut pBScam to give a new plasmid pBScam1. This was sequenced to ensure that the wild-type sequence was maintained. Digestion of pBScam1 with *NdeI*/*XhoI* gave the gene which was ligated into *NdeI*/*SaII* cut pBACE. The gene was then excised from pBACE as an *NdeI*/*XbaI* fragment and ligated into a similarly cut pCW vector to give pCWcam. The expression system yielded approximately 1500 nmol/L of P450$_{cam}$.

Expression and purification were carried out essentially as described for the P450$_{cam}$−putidaredoxin−putidaredoxin reductase fusion protein.[24] Bacterial cells collected by centrifugation were resuspended in 50 mL of cold (4 °C) buffer (pH 7.5) containing 50 mM Tris/HCl, 50 mM KCl, 1 mM ethyldiaminetetraacetic acid, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, 100 μM camphor, and 10 mg of lysozyme. After the mixture was stirred at 4 °C for 2 h, the suspension was frozen in liquid nitrogen, thawed at 4 °C, and sonicated with a Branson sonicator (medium power output) until the viscous consistency due to the nucleic acids was disrupted (∼2 min). The soluble fraction after centrifugation at 100 000*g* for 25 min was loaded onto an anion exchange column (DEAE Fast Flow) equilibrated in buffer A (50 mM KP_i, 50 mM KCl, 100 μM camphor, and 10 mM β-mercaptoethanol, pH 7.5), washed extensively with buffer A, and eluted with a 0−500 mM KCl gradient in buffer A. The fractions containing the P450$_{cam}$, identified by absorption at 418 nm, were combined and concentrated in an Amicon ultrafiltration cell equipped with a YM-30 membrane prior to gel filtration chromatography (S-200 Sepharose) in buffer A plus 200 mM KCl (but no camphor). Residual camphor was removed by passage through a PD-10 (Pharmacia) column. The purified proteins were stored at −70 °C.

Construction and Expression of the L244A P450$_{cam}$ Mutant. In order to carry out the site specific mutation by a standard procedure,[25] pBScam was digested with *SphI* and *SaII*, and the resulting 650 base-pair bp fragment was isolated from low melting temperature agarose and ligated into M13mp19, yielding the vector M13ps. The following complementary 23 base-pair oligomer with the appropriate sequence mismatch (CAG → GGC) was synthesized by the Biomolecular Resource Center of the University of California, San Francisco: CGACCAGTAAGGCGCCACACATC. The oligomer was used to prime the polymerization reaction, using as a template single-stranded DNA isolated from phage particles present in the media of M13ps-infected DH5αF′ *E. coli* cells. The double-stranded DNA was transfected into DH5αF′ cells and plated with lawn cells in 0.6% 2× YT top agar on 2× YT plates. The plates were incubated overnight at 37 °C.

Plaques were lifted onto nitrocellulose disks (BioRad Laboratories), air dried, and baked for 3 h at 80 °C in a vacuum oven. After the disks were rinsed in 2 × SSC, they were hybridized overnight at 40 °C in 5 × Denhardt's solution, 5 × SSPE, 0.1% SDS, and 50 μg/mL of tRNA with the ^{32}P-kinase-labeled mutagenic oligomer.[26] The filters were then washed in 6 × SSC twice at 4 °C for 20 min each, followed by washes at 65 °C in a tetramethylammonium chloride buffer to detect one base-pair mismatches.[27] X-ray film was exposed to the washed filters and the plaques with the mismatch were isolated and re-screened in a second round of plaque purification. The positive plaques from the second round were isolated and amplified, and the DNA was isolated and sequenced. A *SphI*/*BstEII* fragment was cleaved out of the replicative form, recloned into the expression vector to give pBScamL244A, and transformed into DH5α cells for expression. Individual colonies were picked and sequenced.

A *BspEI*/*HindIII* fragment from digestion of pBScamL244A was purified and ligated into a similarly cut pCWcam vector. Expression yielded approximately 1000 nmol/L of the mutant protein, which was purified as described for wild-type P450$_{cam}$.

Construction of a Putidaredoxin pCW Vector and Expression and Purification of the Protein. To place the Pd gene provided in the pIBI25 vector by J. Peterson into the pCW expression vector, it was necessary to introduce an *NdeI* site at the 5′ end of the gene and an *XbaI* site at the 3′ end. This was done by PCR, employing primers that matched either the 3′ or 5′ end of the gene and also carried the appropriate restriction sites. The PCR product was ligated directly into the PCRII vector according to the manufacturer's instructions and the construct was sequenced. The gene was then removed as an *NdeI*/*XbaI* fragment and was ligated into similarly cut pCW. The yield of the protein expressed at 30 °C and purified as reported in the literature[28] was approximately 2500 nmol/L.

Binding of Compounds to P450$_{cam}$. Increasing amounts of the test compound in ethanol were added to the sample cuvette, and an equal amount of ethanol was added to a matched reference cuvette, both of which contained 1 μM camphor-free P450$_{cam}$ in 400 μL of 0.2 M potassium phosphate buffer (pH 7.0). The ethanol concentration did not exceed 1%. Dimethyl sulfoxide was used instead of ethanol with compound **12**. The solutions were mixed after each aliquot and the difference spectrum recorded. The value of K_s was determined by plotting $1/\Delta A$ vs $1/[S]$, the x-intercept of which yields $-1/K_s$ from the relationship $1/[S] = [E]\Delta\epsilon/(K_s\Delta A) - 1/K_s$, where [S] is the concentration of the test compound, [E] is the concentration of P450$_{cam}$, $\Delta\epsilon$ is the difference in molar absorptivity of the free and bound P450$_{cam}$, K_s is the spectroscopic binding constant of the compound, and ΔA is the peak-to-trough absorbance in the difference spectrum.[29,30]

K_I Measurement. K_I values were obtained by determining the K_s value of camphor as described above in the absence and in the presence of at least three concentrations of the inhibitor. The four or more points thus obtained were plotted against the inhibitor concentration, and the K_I value was determined from the graph.

Oxygen Consumption, NADH Consumption, and H_2O_2 Production. The reaction was carried out in a custom made quartz cell in which the O_2 concentration and the decrease in the NADH absorption maximum at 340 nm can be simultaneously monitored. The NADH consumption rate was obtained from the resulting data by a zero-order calculation. The reaction mixture contained, in a final volume of 1.1 mL, 200 mM potassium phosphate buffer (pH 7.0), 1 μM P450$_{cam}$, 2

(23) Tuck, S. F.; Graham-Lorence, S.; Peterson, J. A.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1993**, *268*, 269−275.

(24) Sibbesen, O.; De Voss, J. J.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1996**, *271*, 22462−22469.

(25) Zoller, M. J.; Smith, M. *Methods Enzymol.* **1983**, *100*, 468−500.

(26) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 1989; p 1.98−1.99.

(27) Wood, W. I.; Gitschier, J.; Lasky, L. A.; Lawn, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1585−1588.

(28) Gunsalus, I. C.; Wagner, G. C. In *Methods in Enzymology*; Fleischer, S.; Packer, L., Eds.; Academic Press: New York, 1978; Vol. 52, Part C, pp 166−188.

(29) Estabrook, R. W.; Peterson, J.; Baron, J.; Hildebrandt, A. In *Methods in Pharmacology*; Chignell, C. F., Ed.; Appleton-Century-Crofts: New York, 1972; Vol. 2, pp 303−350.

(30) Rao, S.; Wilks, A.; Hamberg, M.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1994**, *269*, 7210−7216.

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 of 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).[14c] 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. To determine the background consumption values, ethanol alone was added to the reaction 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. 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 of 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 gas chromatography, gas chromatography−mass spectrometry, and nuclear magnetic resonance spectroscopy.

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