Computer-Assisted, Structure-Based Prediction of Substrates for Cytochrome $P450_{cam}$

James J. De Voss and Paul R. Ortiz de Montellano*

Department of Pharmaceutical Chemistry
School of Pharmacy, University of California
San Francisco, California 94143-0446

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Cytochrome P450 enzymes catalyze an impressive array of oxidative transformations, but the factors that determine their substrate specificities are poorly understood.[1a] The ability to predict whether a molecule would be a substrate from knowledge of the active site structure and an understanding of the factors that determine substrate specificity are critical if new activities and specificities are to be engineered into P450 enzymes by mutagenesis. In addition, elucidation of the factors that determine substrate specificity is required to understand, predict, and attempt to modulate the physiological metabolism of pharmaceuticals and other xenobiotics. We report here successful use of a computer-based docking program to predict whether compounds entirely unrelated to camphor are substrates[2] for cytochrome $P450_{cam}$, a protein that normally oxidizes camphor and for which a crystal structure is available.[3]

The P450 catalytic cycle involves (a) binding of substrates to the ferric enzyme with a concomitant change in the oxidation potential from −300 to −170 mV; (b) reduction of the hemoprotein to the ferrous state by auxiliary electron transport proteins; (c) binding of oxygen to the ferrous iron; and (d) reduction of the dioxygen complex by a second electron to give a species equivalent to Fe^{V}=O and a molecule of water.[1] In $P450_{cam}$, the "Fe^{V}=O" species hydroxylates camphor (Table 1) to give 5-*exo*-hydroxycamphor. The trigger for catalytic turnover appears to be the redox potential change associated with substrate binding, as it is thought to be directly followed by electron transfer and oxygen binding. This suggests that any compound that binds in the active site of $P450_{cam}$ should alter the redox potential and initiate catalytic turnover of the enzyme.

The receptor constrained 3D screening program DOCK[4] was used to search a 20 000 compound subset of the Available Chemicals Directory (ACD) for "fit" within the $P450_{cam}$ active site. The top 500 compounds based on the contact score (a measure of extent of van der Waal's surface overlap between the protein and ligand) were saved. These compounds were then visually screened,[5] and 10 compounds were chosen as potential substrates for $P450_{cam}$ that (a) did not bear a formal charge at pH 7 (the $P450_{cam}$ active site is hydrophobic); (b) represented a variety of structural types unrelated to camphor; and (c) were expected to give metabolites that could be readily assayed by GCMS or HPLC.

A set of control compounds that were predicted not to be substrates was also selected. To do this, and to test the precision with which predictions could be made, a structural model was constructed in which Leu-244, a residue that interacts with camphor,[3] was replaced by an alanine (L244A "mutant"). This change increases the size of the active site by the volume of an isopropyl group. The 20 000 compound subset of the ACD was again searched with DOCK for compounds that fit into the enlarged active site, and six compounds were chosen that fit into the L244A but not the wild type active site.

The structures of all 16 compounds were minimized using the default molecular mechanics program within the SYBYL[6] modeling package to obtain better three-dimensional structures. These structures were redocked into the $P450_{cam}$ active site using DOCK in SINGLE mode, a procedure that results in a more intensive search for possible binding orientations. As a result, two compounds (**10** and **11**) migrated from the predicted "nonsubstrate" to the "substrate" list and one (**12**) in the opposite direction. Thus, compounds **1**−**11** in Table 1 were predicted to be substrates and **12**−**16** nonsubstrates for $P450_{cam}$.

Of the compounds (**1**−**11**) predicted to be substrates for $P450_{cam}$, seven (**1**−**7**) bind to the enzyme as judged by spin state changes and eight (**1**−**8**) are substrates as judged by NADH consumption (Table 1).[7,10,11] The magnitudes of the binding constants range from 4 μM to 4 mM and, as expected, are not related to the rank order assigned by DOCK. Some of the substrates are quite good (**2**−**4**, **8**), promoting enzyme turnover at 10−20% (0.5−2 s^{-1}) of the rate observed with camphor, while the turnover of others (**1**, **5**−**7**) is only a little above background. Compounds **12**−**16** were predicted not to be substrates for $P450_{cam}$ and, indeed, cause no detectable increase in NADH consumption.[12] Nevertheless, compounds **9**−**11** and **13**−**15** inhibit the binding of camphor. It is perhaps not surprising that some compounds bind nonproductively (i.e., not fully within the active site) to $P450_{cam}$ as there is an energetic

(1) Reviewed in the following: Sligar, S. G.; Murray, R. I. *Cytochrome P450: Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York; 1986; pp 429−503.

(2) Substrates are defined here as compounds that promote catalytic turnover of the enzyme to give organic metabolites as well as H_2O_2 or H_2O formed by uncoupled turnover.

(3) (a) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *J. Mol. Biol.* **1987**, *195*, 687. (b) Poulos, T. L.; Finzel, B. C.; Gunsalus, I. C.; Wagner, G. C.; Kraut, J. *J. Biol. Chem.* **1985**, *260*, 16122.

(4) (a) DesJarlais, R. L.; Sheridan, R. P.; Seibel, G. L.; Dixon, J. S.; Kuntz, I. D.; Venkataraghavan, R. *J. Med. Chem.* **1988**, *31*, 722. (b) DOCK has been used extensively to search for enzyme inhibitors, but this is the first use of this program for the more demanding task of identifying enzyme *substrates*.

(5) 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.; Langridge, R. *J. Mol. Biol.* **1988**, *6*, 13.

(6) SYBYL6.0 Tripos Inc., 1699 South Hanley Road, St. Louis, MO 63144-2913.

(7) Binding constants were determined by measuring the 417 → 392 nm shift due to the substrate-mediated low- to high-spin transition.[8] If a clear spin state change was not observed,[10] the ability of the compound to inhibit the chromophore change caused by camphor binding was determined. Some compounds alter not only the camphor affinity (K_s) but also the amplitude of the camphor-mediated chromophore change. The kinetic equations used to analyze these results are identical to those used for enzyme inhibitors and provide both a K_I value and an indication of the type of inhibition (Table 1).

(8) For $P450_{cam}$ ~92% of the substrate-free enzyme is low spin and 100% of the camphor-bound enzyme is high spin.[9a] A linear free energy relationship exists between the spin state and the oxidation potential,[9b] so the spectroscopic dissociation constant is thought to reflect productive binding.

(9) (a) Sligar, S. G. *Biochemistry* **1976**, *15*, 5399. (b) Fisher, M. T.; Sligar, S. G. *J. Am. Chem. Soc.* **1985**, *107*, 5018.

(10) Compounds **9**−**15** cause small (<6%), anomalous chromophore changes without the maximum at 392 nm characteristic of a low- to high-spin transition. In the case of **8** the chromophore change is small (<6%) and is difficult to interpret because of solubility limitations. The change observed with **7** is very small but is quantifiable and consistent with a normal spin state change.

(11) Catalytic turnover was assessed by measuring the increase in NADH consumption (Table 1). NADH consumption was monitored spectroscopically at 340 nm in incubations containing $P450_{cam}$ (1 μM), putidaredoxin reductase (2 μM), and putidaredoxin (8 μM) in 200 mM potassium phosphate buffer (pH 7.0). Substrates were added in ethanol (<1% final volume) to either their solubility limit or ≥10K_s. The background NADH consumption is 4−6 nmol min^{-1} (nmol of $P450_{cam}$)$^{-1}$. Oxygen consumption measurements (not shown) agree fully with the NADH data. Identification and quantitation of the organic metabolites and measurements of the fraction of uncoupled turnover to give H_2O_2 or H_2O are in progress and will be reported in the full paper. For **2**, the best substrate tested, uncoupling to give H_2O_2 accounts for less than 10% of the essentially equimolar oxygen and NADH consumed, a value similar to that measured for uncoupling during the turnover of camphor itself.

(12) The L244A mutant has been prepared and is inactive. It is therefore not possible to determine if the compounds predicted to be substrates for the mutant are indeed so.

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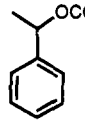
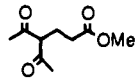
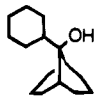
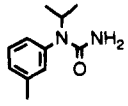
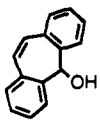
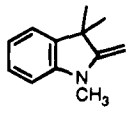
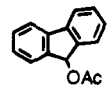
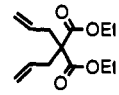
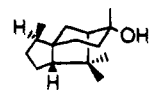
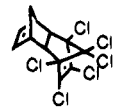
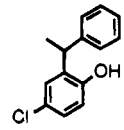
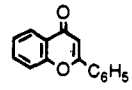
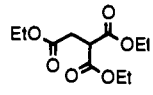
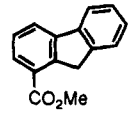
Table 1. Compound Binding to $P450_{cam}$ and Catalytic Turnover of the Enzyme

Compound		K_S[10] μM	K_I μM	Spin state change %	NADH nmol/min/ nmol P450
 camphor		2.0±0.1		100	530±10
	1	83±5		8	8±1
	2	4.3±0.3		91	110±5
	3	4400±600		13	41±3
	4	530±100		22	27±1
	5	27±4		30	6±1
	6	280±70		6	12±1
	7	228±32		<2	8±1
	8		1200 mixed		80±5

Compound		K_S[10] μM	K_I μM	Spin state change %	NADH nmol/min/ nmol P450
	9	>>300 inhibitor			5±1
	10	495 inhibitor			6±1
	11	73 compet			4±1
	12	-			4±1
	13	76 mixed			5±1
	14	71 mixed			6±1
	15	5000 compet			5±1
	16				5±1

preference for association of small hydrophobic compounds with hydrophobic protein sites.

Analysis of substrate docking with the program DOCK is shown here to be a promising method for the identification of potential P450 substrates. Thus, eight of 11 compounds were correctly predicted to promote catalytic turnover, and all five compounds predicted to be nonsubstrates were not substrates. Furthermore, the fact that the nonsubstrates were chosen by differential binding to the native and L244A active sites suggests that the method is sufficiently discriminating to facilitate the design of active sites with new specificities. The predictions made here are qualitative rather than quantitative, but the small molecule probes identified through this approach should lead to a better understanding of what makes a compound a *good* P450 substrate. For example, **3** binds poorly and causes a small spin state change and yet is a good substrate as judged by NADH consumption, whereas **5** binds well and causes a large spin state shift but is a very poor substrate. This contradicts the widely held view that the spin state shift directly triggers catalytic turnover. Preliminary correlation of substrate turnover with the total number of binding orientations predicted by DOCK suggests that a certain active site mobility is required of good substrates. Studies are underway to define this and other possible relationships between predicted fit and substrate activity.

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