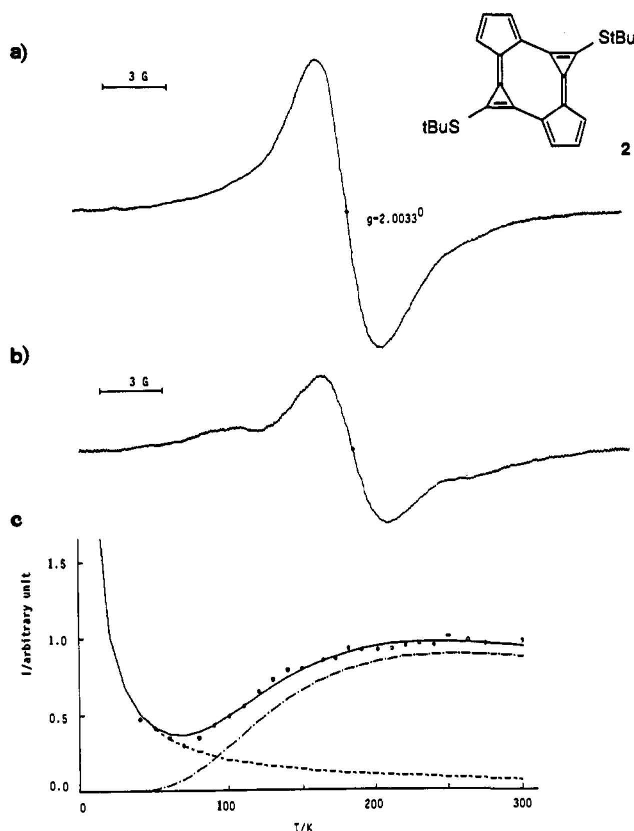

Figure 2. The ESR spectra of the microcrystalline solids of **2** measured at (a) 299 K and (b) 99 K, respectively. (c) Plot of experimental intensity (O) versus temperature and decomposition into triplet (---) and impurity signal (– – –) signals. Sum of calculated intensities (—).

inated with some monoradial impurities.[12] By using the equation

$$I = (N_m \mu_B^2 g^2)[\tfrac{1}{2}(\tfrac{1}{2} + 1)/T] + (N_\alpha \mu_B^2 g^2)[1(1 + 1)]/[T\{1 + \tfrac{1}{3} \exp(\Delta E/kT)\}] \quad (4)$$

where I is the signal intensity calibrated with a reference TANOL, N_m and N_α are the respective amounts of monoradical and diradical, and ΔE is the energy gap between the singlet and the thermally excited triplet, N_m, N_α, and ΔE were determined: $N_m = 2.0 \times 10^{17}$ molecules/mol, $N_\alpha = 2.3 \times 10^{18}$ molecules/mol, and $\Delta E = 0.81$ kcal/mol. The excited diradical contribution to the ground state of **1** is remarkably small (10^{-4}–10^{-3}%).

The present finding provides a first instance of excited diradical contribution to the ground state of a closed-shell nonalternant hydrocarbon system, although this has already been known for quinodimethane and the quinone derivatives, Chichibabin's hydrocarbon,[13] and 2,5-bis(3,5-di-*tert*-butyl-4-oxocyclohexadienylidene)thieno[3,2-*b*]thiophene.[14] Such a contribution should increase in the excited state; some useful applications of this unique physical property may be expected in the near future.

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(12) The amount of monoradical was much less for **2** freshly obtained by recrystallization. However, on exposing the sample to air, the monoradical concentration gradually increased. For **1** even the recrystallized sample contained a large amount of monoradical impurity because of its oxidation susceptibility. Accordingly, the temperature change of the ESR signal intensity was not investigated in detail.

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Theoretical and Experimental Analysis of the Absolute Stereochemistry of *cis*-β-Methylstyrene Epoxidation by Cytochrome P450$_{cam}$

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Members of the cytochrome P450 family of enzymes can generally be divided into two classes based on the stringency of their substrate specificity: one for enzymes evolutionarily optimized to oxidize specific physiological substrates (e.g., aromatase, lanosterol 14-demethylase) and a second for enzymes that primarily oxidize xenobiotics (e.g., most hepatic cytochrome P450 enzymes). Understanding the binding of substrates to both classes of cytochrome P450 enzymes, but particularly to the low-specificity forms, is a challenging task because substrate binding is frequently determined in these enzymes by relatively nonspecific lipophilic interactions. Despite the difficulties, clarification of this problem is important for efforts to predict the roles of individual isozymes in drug and xenobiotic metabolism, to develop therapeutically useful, isozyme-specific inhibitors, and to tailor the substrate specificity of cytochrome P450 enzymes for biotechnological purposes.

Cytochrome P450$_{cam}$, a cytosolic enzyme expressed by *Pseudomonas putida*, catalyzes the first step in the degradation of camphor under conditions where camphor is the sole carbon source for the organism.[1] As the only cytochrome P450 enzyme for which a crystal structure is available,[2] it has become the template for all efforts to model the active sites of the membrane-bound enzymes. The crystal structure of cytochrome P450$_{cam}$ suggests the existence of three relatively specific interactions between the substrate and the enzyme: hydrogen bonding of the camphor oxygen to Tyr 96 and interaction of the substrate methyl groups with Val 295 and Val 247.[2,3] Experimental support for these interactions is provided by the fact that site-specific replacement of the tyrosine by a phenylalanine or the valines by isoleucines decreases the regio- and stereospecificity of the oxidation reaction.[4,5] Cytochrome P450$_{cam}$ is generally considered to be a camphor-specific enzyme, but it has been shown to oxidize cam-

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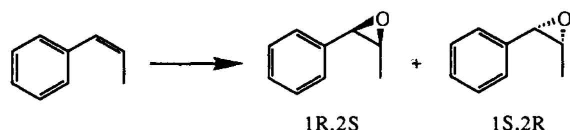
phor analogues such as norcamphor, 5,5-difluorocamphor, pericyclocamphanone, adamantanone, adamantane, and 5,6-dehydrocamphor.[6,7] Force field and heat of formation calculations have predicted oxidation results for some of these analogues in good agreement with the experimental data.[8] We report here that cytochrome P450$_{cam}$ oxidizes a simple olefin unrelated to camphor with high stereoselectivity and independently predict the stereoselectivity of the reaction by energy minimization and molecular dynamics (MD) calculations. The remarkable agreement found between the experimental and calculated enantiomeric ratios demonstrates the potential utility of computational methods in characterizing and predicting the binding of lipophilic substrates to cytochrome P450 enzymes.

Incubation of cell-free cytochrome P450$_{cam}$ with *cis*-β-methylstyrene followed by gas-liquid chromatography (GLC) of the products[9] shows that the epoxide is formed, without loss of the cis olefin stereochemistry, at the rate of 1.3 nmol/nmol of P450 per min.[10,12] Stereochemical analysis of the epoxide metabolites was accomplished by using a chiral capillary GLC column.[13] The epoxide metabolite gives two GLC peaks which coelute with the peaks of the epoxide obtained by reaction of *cis*-β-methylstyrene and *m*-chloroperbenzoic acid. Assignment of chirality to the components of the individual peaks is based on literature data[14] and on chiral GLC correlation with a known, unequal mixture of the epoxide enantiomers provided by Dr. Thomas Kodadek.[15] Analysis of the epoxide of *cis*-β-methylstyrene produced by cytochrome P450$_{cam}$ shows that it consists of an 89:11 (±2) mixture of the 1*S*,2*R* and 1*R*,2*S* enantiomers, respectively. Cytochrome P450$_{cam}$ thus not only oxidizes *cis*-β-methylstyrene but does so with remarkable stereoselectivity.



Theoretical studies using AMBER[16] to minimize enzyme-substrate orientations and molecular dynamics simulations were carried out in parallel with experimentation. The initial conformation of *cis*-β-methylstyrene, a structure with the methyl group out of the plane of the aromatic ring by about 40°, was optimized by using AMI.[17] Two separate MD simulations of 125 ps each were run for four different minimized orientations of the *cis*-β-methylstyrene docked into an extended binding site of cytochrome P450$_{cam}$.[18] Coordinates were saved every 0.2 ps, and the relative orientation of the olefin π system to the ferryl oxygen was monitored to determine the preferred face for each of these 5000 MD snapshots. Snapshots with the methyl-substituted C_β atom of the substrate farther than 4 Å from the ferryl oxygen were not counted since these distances were considered to be unreactive toward oxygen addition. The results of these simulations yield a product ratio (1*S*,2*R*/1*R*,2*S*) of 84/16, based upon the orientations of the olefin π system with respect to the putative heme-bound ferryl oxygen atom. These results lead to the prediction that the 1*S*,2*R* enantiomer should be formed in approximately 70% enantiomeric excess, a value very close to that found experimentally.

The present results indicate that cytochrome P450$_{cam}$, despite its evolutionary specificity for camphor, readily oxidizes unrelated substrates. The only real limitation on whether a compound is a substrate for cytochrome P450$_{cam}$ appears to be its size (unpublished work). The high stereospecificity of the oxidation of *cis*-β-methylstyrene, a compound with no hydrogen bonding or polar functions, must be determined primarily by contact or dispersion forces. As shown here, successful theoretical analysis of the binding of this olefin to the active site of the enzyme requires molecular dynamics simulations. The agreement between the resulting stereochemical prediction and the experimental result provides both strong support for the validity of the model and the methods used and insight into the origin of the stereoselective control of product formation.

Acknowledgment. This work was supported by National Institutes of Health Grants GM 25515 (P.O.M.) and GM 29743 (G.H.L.). Calculations were carried out at the Pittsburgh Supercomputer Center sponsored by the National Science Foundation.

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(9) GLC was done on a 0.5 mm × 30 m DB-5 column programmed to run at 35 °C for 1 min, then to rise at 70 deg/min to 80 °C, to hold at 80 °C for 3 min, and finally to rise at 2 deg/min to 150 °C. The retention times for *cis*-β-methylstyrene and its epoxide are 13.7 and 20.3 min, respectively.

(10) Incubations were carried out with cytochrome P450$_{cam}$ purified from *P. putida* essentially as reported in the literature.[11] Typical incubations (30 min at 25 °C) contained 1 μM P450$_{cam}$, 8 μM putidaredoxin, 2 μM putidaredoxin reductase, 1 mM *cis*-β-methylstyrene, and 1 mM NADH in 50 mM potassium phosphate buffer (pH 7.0).

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(13) Following extraction of the incubation mixture with 0.5 mL of hexane, the epoxide metabolite was purified prior to chiral GLC analysis by normal-phase HPLC (Alltech Partisil silica 5 μm column eluted isocratically at 1 mL/min with 2.5% tetrahydrofuran in hexane: detector at 260 nm): epoxide retention time, 8.0 min. Chiral GLC analysis was carried out on a 0.25 mm × 30 m Chiraldex G-TA capillary column (Advanced Separation Technologies, Inc.) at 120 °C. The retention times for the 1*S*,2*R* and 1*R*,2*S* *cis*-β-methylstyrene epoxide enantiomers were 9.7 and 11.9 min, respectively.

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(18) The binding site consisted of 87 amino acids within ~12 Å of the Fe of the heme unit. The backbone atoms were constrained by a harmonic potential of 100 kcal/Å^2.

Structures of Proteins in Solution Derived from Homonuclear Three-Dimensional NOE-NOE Nuclear Magnetic Resonance Spectroscopy. High-Resolution Structure of Squash Trypsin Inhibitor

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The nuclear Overhauser effect (NOE) is the main source of distance constraints used in the calculation of biomacromolecular structures from NMR data.[1-3] To date, distance constraints have been derived primarily from two-dimensional NOE measurements. Recently the potential of homonuclear three-dimensional NOE-NOE experiment for obtaining new types of protein connectivities has been demonstrated.[4,5] The homonuclear 3D NOE spectra should contain more information relating to distance criteria than

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