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Controlling the regiospecificity and coupling of cytochrome P450cam: T185F mutant increases coupling and abolishes 3-hydroxynorcamphor product

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Abstract

Cytochrome P450cam (P450CIA1) catalyzes the hydroxylation of camphor and several substrate analogues such as norcamphor and 1-methyl-norcamphor. Hydroxylation was found experimentally at the 3, 5, and 6 positions of norcamphor, but only at the 5 and 6 positions of 1-methyl-norcamphor. In the catalytic cycle, the hydroxylation of substrate is coupled to the consumption of NADH. For camphor, the degree of coupling is 100%, but for both norcamphor and 1-methyl-norcamphor, the efficiency is dramatically lowered to 12% and 50%, respectively. Based on an examination of the active site of P450cam, it appeared that mutating position 185 might dramatically alter the product specificity and coupling of hydroxylation of norcamphor by P450cam. Analysis of molecular dynamics trajectories of norcamphor bound to the T185F mutant of cytochrome P450cam predicted that hydroxylation at the 3 position should be abolished and that the coupling should be dramatically increased. This mutant was constructed and the product profile and coupling experimentally determined. The coupling was doubled, and hydroxylation at the 3 position was essentially abolished. Both of these results are in agreement with the prediction.

Keywords: molecular dynamics simulations; protein engineering; rational enzyme redesign

The cytochromes P450 are a superfamily of heme enzymes that activate molecular oxygen and subsequently catalyze a wide variety of reactions, including hydroxylation of carbon and heteroatoms, dealkylation of amines and ethers, epoxidation of olefins, and reductive dehalogenation (Porter & Coon, 1991). They are involved in steroid biosynthesis, fatty acid metabolism, and detoxification of a wide variety of xenobiotics. The understanding, prediction, and control of P450–substrate interactions is therefore of paramount interest for engineering cytochromes P450 as biotechnological tools or for design of therapeutical inhibitors.

The most well-studied isozyme of the P450 superfamily is cytochrome P450cam from the soil bacterium *Pseudomonas putida* (Gunsalus & Sligar, 1978; Murray et al., 1985; Sligar & Murray, 1986), which catalyzes the hydroxylation of camphor as a first step in the utilization of camphor as a carbon and energy source. The native substrate, camphor, is hydroxylated by P450cam with 100% regiospecificity at the C5 position and with 100% coupling between NADH consumption and alcohol formation. However with several alternate substrates, including norcamphor and 1-methyl-norcamphor, the reaction proceeds with a loss of regiospecificity and lower coupling to NADH consumption. For example when norcamphor is the substrate only 12% of the electrons consumed in this reaction can be accounted for by substrate hydroxylation (Atkins & Sligar, 1987). The remainder are lost to hydrogen peroxide and water formation. Accessibility of the iron-bound dioxygen or activated oxygen to solvent, and hence protons, could promote electron flow from the thiolate-heme system to the oxygen, allowing water and/or hydrogen peroxide formation while compromising sub-

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strate hydroxylation (Atkins & Sligar, 1987). Uncoupling is presumably caused by high mobility of the substrate leading to unfavorable orientations relative to the activated oxygen or promoting the presence of water molecules. Reducing the mobility of the substrate while maintaining a reactive geometry by appropriate changes in the active-site residues should increase the coupling and regiospecificity of alcohol formation.

Structures have been determined for both camphor-bound and camphor-free P450cam as well as numerous alternate substrates and inhibitors (Kinemage 1; reviewed in Raag & Poulos, 1991; Poulos & Raag, 1992). The product specificity of the hydroxylation reaction has been experimentally determined both for the native substrate, camphor, and for a number of substrate analogs including norcamphor and 1-methyl-norcamphor (White et al., 1984; Atkins & Sligar, 1988, 1989). This wealth of experimental and structural data make P450cam an excellent target for rational redesign efforts. Norcamphor is observed to be highly mobile within the completely buried active site, as reflected by its high temperature factor relative to that determined for camphor (Raag & Poulos, 1989). This is consistent with recent theoretical studies that show that norcamphor has at least two distinct conformational minima within a fixed representation of the active site, whereas camphor had only a single minima (Collins & Loew, 1988). Recent molecular dynamics simulations on the binary, norcamphor–P450cam complex (Bass et al., 1992b) showed multiple reorientations of the substrate consistent with the experimentally observed high mobility. In contrast, simulations on the camphor-bound complex showed that camphor has no tendency to rotate in the active site (Paulsen et al., 1991; Paulsen & Ornstein, 1992). Furthermore, in the norcamphor-bound trajectory the substrate was shown to move away from the heme iron consistent with low coupling of electrons to norcamphor hydroxylation; while camphor showed no such tendency, consistent with its high degree of coupling.

The effect of several different active-site mutations, including Y96F, V247A, and V295I, on the hydroxylation of camphor, norcamphor, and 1-methyl-norcamphor has been examined (Atkins & Sligar, 1989). The loss of the hydrogen bond donor in the Y96F mutant has a profound effect on the spin conversion when camphor is the substrate, but a much smaller effect on the regiospecificity (92% of the product is still 5-exo-hydroxycamphor), and no effect on the coupling of NADH consumption to product formation. The effects of the V247A and V295I mutants on either the regiospecificity or efficiency of camphor hydroxylation are also quite small. However, for the alternate substrates norcamphor or 1-methyl-norcamphor, the effects of these single site mutants on regiospecificity or coupling are much more pronounced. For this reason, we decided to focus on the effect of mutations on norcamphor hydroxylation. In addition to the three mutants described above, a recent combination computational/experimental study examined the effect of a Phe to Trp mutation at position 87. It was found that this mutant had increased coupling when norcamphor was the substrate, but almost no change in the hydroxylation pattern was seen (Bass et al., 1993).

In the work presented here, we have focused on yet another region of the active site. Experimentally, it has been observed that the additional methyl group in 1-methyl-norcamphor increases both the regiospecificity and coupling of the hydroxylation reaction relative to that seen for norcamphor (Atkins & Sligar, 1989). In the crystal structure of camphor-bound P450, the 1-methyl group is in van der Waals contact with the side chain of T185. We therefore asked whether increasing the bulk of this side chain could compensate for the absence of the methyl group in norcamphor and thus lower substrate mobility and thereby increase regiospecificity and coupling. We describe theoretical and experimental results for the hydroxylation of norcamphor by the T185F mutant. Norcamphor bound to the mutant enzyme is predicted to have restricted translational and rotational freedom compared with the wild-type simulation. The number of conformations in the molecular dynamics simulations of the T185F mutant consistent with hydrogen abstraction nearly doubled (from 21% to 39%), and no conformations consistent with formation of the 3-alcohol were observed. We report the experimental findings that the coupling between NADH consumption and norcamphor hydroxylation has doubled and that virtually no 3-alcohol production was observed. Both of these results are in agreement with the analysis of the molecular dynamics trajectories.

Results

Simulation results

Two trajectories were calculated for norcamphor bound to the T185F mutant. The trajectories started from the same minimized structure but had different initial velocities assigned to the atoms. The active site of the minimized starting structure for the T185F simulations is shown in Figure 1 superimposed over the active site of the wild-type enzyme (see also Kinemage 2). Previous calculations have shown that for smaller more mobile substrates the calculated product profile and degree of coupling can vary significantly between trajectories (Bass & Ornstein, 1993; unpubl.). The global properties of the enzyme in the two trajectories were, however, quite similar. The average root mean square (rms) deviation in heavy atom positions over the final 100 ps was 1.69 Å and 1.79 Å for the two T185F trajectories. If only the main-chain atoms are considered, these values drop to 1.38 Å and 1.54 Å, respectively. These values are consistent with those seen previously for norcamphor-bound wild-type P450cam (Bass et al., 1992b).

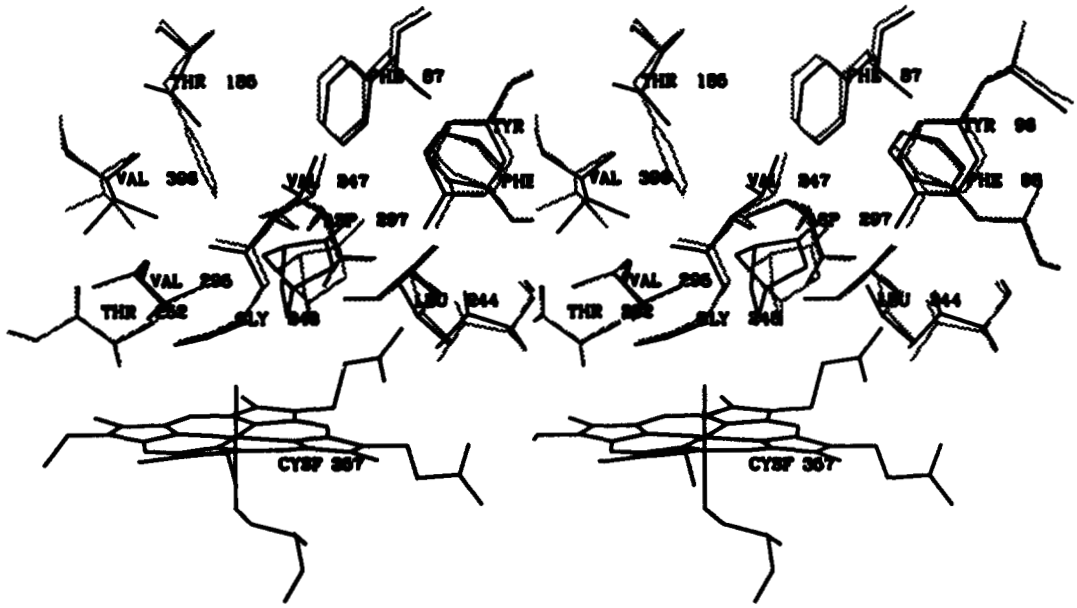

Fig. 1. Superimposed stereo views of the active site of norcamphor-bound cytochrome P450cam. The energy minimized wild-type active site is in black and the T185F mutant is in gray. Only the heavy atoms of the enzyme and substrate are shown.

One of the key interactions responsible for providing specificity to the hydroxylation of norcamphor is the hydrogen bond between the side chain of Y96 and the substrate carbonyl. The removal of this interaction in the Y96F mutant results in a dramatic increase in the production of the 3-alcohol from 8% to 23%. In simulations of norcamphor bound to the wild-type enzyme, although substrate mobility is relatively high, this hydrogen bond is quite strong with an average donor-hydrogen to acceptor-oxygen distance of 1.68 Å (Bass et al., 1992b). In the two trajectories of the T185F mutant, this hydrogen bond was present in over 95% of the structures examined and the mean hydrogen–oxygen distance was 1.71 Å. The introduction of the bulkier phenyl group at position 185 did not result in a weakening of this hydrogen bond.

One indicator of the degree of coupling between NADH consumption and product formation is the distance between the ferryl oxygen and the center of mass of the substrate. The mean value of this distance in the T185F trajectories was 4.8 ± 0.2 Å compared with 5.2 ± 0.3 Å in the wild-type enzyme. The slightly shorter distance in the mutant is suggestive of an increase in coupling. A more quantitative prediction of the degree of coupling can be obtained from a detailed examination of the substrate orientation relative to the ferryl oxygen. From each of the 150-ps trajectories, structures were saved every 0.5 ps, giving a total of 602 conformations. Each conformation was classified on the basis of geometric criteria as either favorable or unfavorable for hydrogen abstraction. The criteria used were the substrate hydrogen to ferryl oxygen distance and the ferryl oxygen-substrate-hydrogen-substrate carbon angle. All conformations in which a hydrogen was less than 3.25 Å from the ferryl oxygen and the corresponding oxygen–hydrogen–carbon angle was within 45° of linearity were counted as favorable conformations for abstraction to occur. Only hydrogens on the secondary carbons, C3, C5, and C6, were considered as potential abstraction sites based on mechanistic considerations and computed heats of formation of the various radicals (see Discussion) (Collins & Loew, 1988; Bass & Ornstein, 1993). Based on these criteria, there were 235 favorable conformations (39%) in the pair of trajectories. We have found in previous simulations of either camphor, thiocamphor, or norcamphor bound to the wild-type enzyme that this percentage is correlated with the experimentally observed coupling (Paulsen & Ornstein, 1992). The predicted coupling value of 39% in the T185F simulations compares with a value of 21% previously calculated for the wild-type enzyme using the same computational procedure (Paulsen & Ornstein, 1992). Thus on the basis of our simulations, we predict that there should be an approximate doubling of the coupling in the T185F mutant relative to the wild-type enzyme.

In Figure 2, the distance from the ferryl oxygen to the three known hydroxylation sites is plotted as a function of the time course of the simulation. Figure 2A is from a trajectory of norcamphor-bound wild-type enzyme (Paulsen & Ornstein, 1992). The substrate undergoes five distinct rotations during the time course as compared to the complete lack of rotations seen in Figure 2B for the T185F mutant. The behavior of norcamphor bound to the wild-type enzyme shown in Figure 2A is typical of that seen in several previous simulations of the binary complex (Bass & Ornstein, 1993; Bass et al., 1992b, 1993). On the

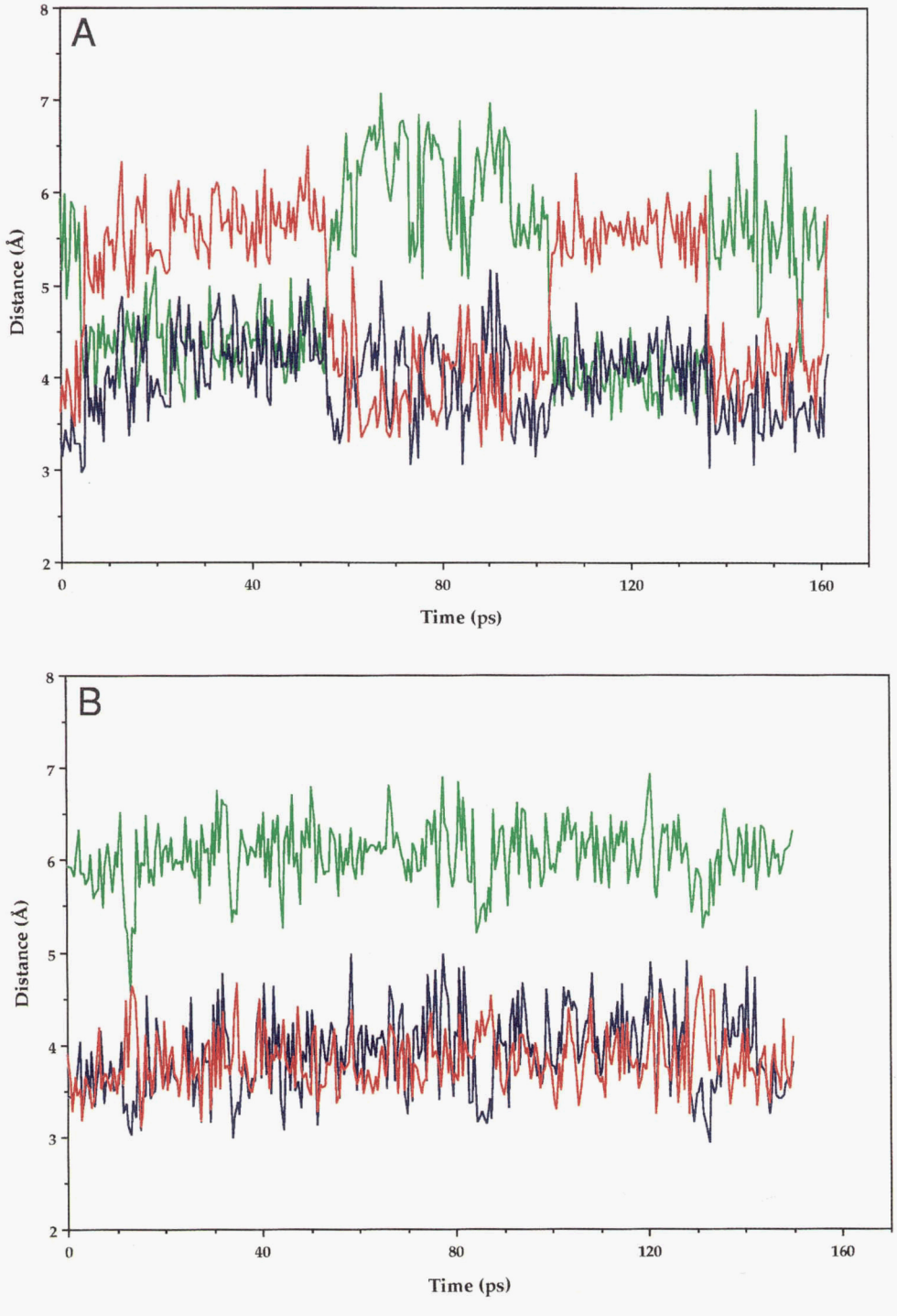

Fig. 2. Distance between C3, C6, and C5 of norcamphor and the oxygen atom bound to the heme group as a function of the time course of the simulations of the ferryl oxygen intermediate starting from (**A**) the wild-type enzyme and (**B**) the T185F mutant. The curves in green, red, and blue monitor the distances involving C3, C6, and C5, respectively.

other hand, the lack of mobility seen for norcamphor bound to the T185F mutant is similar to what is seen for camphor in the wild-type enzyme (Paulsen et al., 1991; Paulsen & Ornstein, 1992). In the second mutant trajectory, there was a single rotation at 62 ps into the 150-ps trajectory, which brought C7, the bridgehead carbon, into a relatively favorable abstraction geometry but still did not result in any favorable C3 conformations.

Previous simulations of norcamphor-bound wild-type enzyme suggested that a single active-site side chain, that of Phe 87, had unusually high mobility (Bass et al., 1992b). During the time course simulated, this side chain was seen to make multiple transitions between its X-ray conformation and an alternate conformation that results in a larger more open substrate pocket. The movement of the Phe 87 side chain to its alternate conformation per-

mits the rotation of a smaller substrate like norcamphor to occur more easily. One question concerning the T185F mutant is whether the mutation affects the mobility of the substrate directly or indirectly by altering the dynamics of Phe 87. The evidence from the pair of trajectories is inconclusive. In the first trajectory, the calculated rms fluctuation in the side-chain dihedral angle chi 1 of Phe 87 was 10.5°, which is only slightly higher than that of the neighboring aromatic groups and significantly lower than the value calculated from wild-type trajectories (Bass et al., 1992b). In the second trajectory, the calculated rms fluctuation of the same dihedral angle increased dramatically to 37.7°, which is similar to the value determined for several simulations of the wild-type enzyme.

In addition to predicting coupling, the geometric criteria described above can be used to predict product distributions. Of the 235 active conformations in the pair of T185F trajectories, 86 favored hydroxylation at C5, whereas 149 favored hydroxylation at C6 resulting in a predicted product ratio of 64:36 in favor of the 6-alcohol. No conformations were observed that were consistent with the formation of the 3-alcohol.

Experimental results

Both the T185F mutant and wild-type enzyme were overexpressed in *Escherichia coli*, and their product profiles were determined. The results are summarized in Table 1. The product distribution and coupling determined for the wild-type enzyme are in reasonable agreement with previously determined values (Atkins & Sligar, 1987). The product distribution of the T185F mutant with 55% 5-, and 45% 6-hydroxynorcamphors differs significantly from the wild-type product profile. The production of the 3-alcohol has been essentially abolished and the amount of 5-hydroxynorcamphor produced has been slightly increased. In addition, the coupling between NADH consumption and product formation increased from 12% in the wild-type enzyme to 25% in the T185F mutant.

Table 1. *Coupling and product specificity of norcamphor hydroxylation*

	Wild type[a]	T185F
Theoretical results		
Coupling[b] (%)	21	39
Products[c]		
3-OH (%)	0–1	0
5-OH	63	36
6-OH	37	64
Experimental results		
Coupling (%)	12[d]	25[d]
Products		
3-OH (%)	8	0–1
5-OH	47	55
6-OH	45	45

[a] The theoretical values for the wild-type enzyme are taken from Paulsen and Ornstein (1992).

[b] Determined from the number of conformations seen in which a hydrogen on a secondary carbon of the substrate was less than 3.25 Å from the position of the ferryl oxygen and in which the oxygen-hydrogen-carbon angle was within 45° of linear. Based on the variability between trajectories, the error associated with each value is about 10%.

[c] Ratio determined by comparing the total number of conformations that are consistent with hydrogen abstraction from a particular carbon to the total number of configurations that meet the criteria stated in footnote b.

[d] The errors associated with each of the experimental values in this column were less than 5% of the measured values.

Discussion

Experimentally, the addition of a single methyl group at the 1- position of norcamphor is found to have a dramatic effect on the hydroxylation of this substrate by wild-type P450cam. The interaction of this methyl group with the side chain of T185 restricts substrate mobility sufficiently to increase the coupling of the reaction from 12% for norcamphor to 50% for 1-methyl-norcamphor (Atkins & Sligar, 1989). In addition, the restricted motion results in greatly enhanced regiospecificity. The production of the 3-alcohol is lowered and the ratio of 5-alcohol to 6-alcohol shifts from nearly equimolar to 80:20 in favor of 5-hydroxy product. We wished to determine if we could mimic the effect of this methyl group with an appropriate active-site mutant. We focused on increasing the bulk of the side chain of residue 185. This residue is the closest to the 1-methyl group in the X-ray structure. In addition, the effect of mutations in this region of the active site has not been examined in previous studies. We chose to study the T185F mutant in hopes that this rather large mutation would have a significant effect on regiospecificity and coupling. A second goal of this research was to test our ability to predict in advance the effect of mutations on these two properties by analyzing molecular dynamics trajectories. In previous work by our group and others, some success had been seen in predicting the regiospecificity or stereospecificity of P450cam for novel substrates (Collins et al., 1991; Ortiz de Montellano et al., 1991; Filipovic et al., 1992) and predicting the effect of active-site mutations (Bass et al., 1993).

Analysis of the trajectories of norcamphor-bound T185F results in a predicted product profile that is different from that predicted for the wild-type enzyme in several important ways. Most importantly, the bulkier phenyl group has restricted the rotational mobility of the substrate. In simulations of the wild-type enzyme, multiple rotations of norcamphor are usually observed on the timescale of the simulation (Bass et al., 1992b; Paulsen & Ornstein, 1992). This rotational mobility is apparently responsible for the lack of regiospecificity in the hydroxylation of norcamphor and also contributes to the low de-

gree of coupling between NADH consumption and product formation. In contrast, in the two T185F simulations, only a single reorientation in one trajectory was observed. This lack of rotational mobility means that no conformations are observed in which C3 is close to the ferryl oxygen leading to a prediction of no 3-alcohol product, which is in reasonable agreement with the experimental observation. In addition, the substrate spends less time in what are presumed to be unreactive conformations, which should improve the coupling between NADH consumption and product formation. Furthermore, the bulkier side chain at position 185 will result in less void volume in the active site, which should make the entry of excess water into the active site more difficult and also improve coupling.

In addition to the direct effect of the phenylalanine at position 185 on the mobility of norcamphor, the T185F mutant may have an indirect effect as well. Several previous simulations of either camphor or norcamphor bound P450cam showed that only one side chain lining the active site (F87) underwent any dihedral angle transitions during the time course simulated (Paulsen et al., 1991; Bass et al., 1992b). The motions of this side chain seem to permit increased mobility of smaller substrates like norcamphor. Mutation of this residue to a tryptophan resulted in decreased mobility for the side chain and a concomitant increase in coupling (Bass et al., 1993). In one of the two T185F trajectories, this side chain showed essentially wild-type behavior, while in the other trajectory, the mobility of F87 was significantly lowered from that seen for the wild-type enzyme. Both simulations predicted a decrease in 3-alcohol and an increase in coupling suggesting that the direct effect of T185F is crucial. However, the trajectory in which F87 was quiet showed a significantly larger increase in coupling than the trajectory in which F87 was mobile suggesting that the indirect effect is also important.

One potentially troubling aspect of the T185F simulations is that, as in previous simulations of the wild-type enzyme and the F87W mutant, a large number of conformations occur in which the bridgehead carbon C7 is in an apparently favorable position for hydrogen abstraction by the ferryl oxygen. In camphor, this position is blocked by two methyl groups and so is not a candidate for hydrogen abstraction. However, in norcamphor, it is a methylene carbon, as are C3, C5, and C6, all of which are hydroxylated. No measurable 7-alcohol has been observed for either the wild-type enzyme or any mutant examined to date. The molecular dynamics prediction is based on the assumption that geometrical factors alone are responsible for the observed product ratios. The C7 results clearly indicate that geometry is only one of several factors. Semiempirical calculations of the heats of formation of the various norcamphor radicals using either MNDO, AM1, or PM3 suggest that the C7 radical is less stable than the C3, C5, and C6 radicals by 2–4 kcal/mol (Collins & Loew, 1988; Bass & Ornstein, 1993). This difference is significantly smaller than that seen between C5 and the tertiary carbons C1 and C4 but may contribute to the lack of 7-alcohol. The calculated difference in heats of formation could account for a ratio of between 7:1 and 50:1 in the amount of bridgehead hydroxylation and hydroxylation at the other sites. On the assumption that no 7-alcohol is produced for a combination of geometric, energetic, and kinetic reasons, we calculated product ratios and coupling excluding conformations favorable for C7 as unreactive in the same way that we excluded the tertiary carbons C1 and C4.

Although there was qualitative agreement between the experiments and the molecular dynamics simulations in that significant amounts of both the 5- and 6-alcohol should be produced by the T185F mutant while the production of the 3-alcohol should be essentially abolished, the prediction missed the ratio of 5- and 6-alcohols. The pair of simulations predicted a 3:2 ratio in favor of the 6-alcohol, while experimentally the ratio was 4:3 in favor of hydroxylation at C5. There are several possible reasons for this difference. One possibility is a lack of sufficient sampling in the simulations. The predicted product ratios can vary substantially from trajectory to trajectory, and it is possible that the addition of more trajectories could result in a prediction in better quantitative agreement with the experimental results. However, there is possibly a more fundamental problem that affects both the experiment and the calculations. On the one hand, all of the experiments were performed using a racemic mixture of the L and D isomers of norcamphor. To date, these isomers have not been successfully separated. On the other hand, only the D isomer of norcamphor was modeled in the present simulations. It is possible that the D and L isomers may be preferentially hydroxylated at different sites and so the experimental product profile is the average of the profiles for the D and L isomers, while the calculations are for the D isomer only. Recent calculations of the binary complex of norcamphor and wild-type P450cam have shown distinctly different product profiles for the L and D isomers (Bass & Ornstein, 1993).

There was also qualitative agreement between the experimentally observed coupling and product profile for our T185F mutant catalyzing the hydroxylation of norcamphor and the model on which we based our hypothesis: the hydroxylation of 1-methyl-norcamphor by wild-type P450cam. However, there were still quantitative differences. The active-site mutation did increase coupling dramatically but still not to the level seen for 1-methyl-norcamphor (50%). In the same way, the percentage of 5-alcohol was measurably increased in the T185F mutant and yet still fell far short of the 80% yield seen for 1-methyl-norcamphor or the absolute regiospecificity seen for the native substrate, camphor. Thus the mutation at position 185 could not mimic the exact effect of adding an additional methyl group to the substrate.

Materials and methods

Molecular dynamics simulations

The system for which trajectories were calculated consisted of residues 10–414 of cytochrome P450cam, the heme moiety at the active site with atomic oxygen as a distal ligand, a bound substrate (norcamphor), and 240 crystallographic waters. Water 515, thought to be the cation that activates this enzyme (Poulos et al., 1987), was modeled as a sodium ion. The crystallographic structure of norcamphor-bound P450cam, determined by Poulos and coworkers (Raag & Poulos, 1989) and obtained from the Protein Data Bank, served as the starting point for the simulations (Bernstein et al., 1977).

The simulations were done using an all hydrogen model. Side-chain polar hydrogens were positioned so as to maximize intramolecular hydrogen bonding, using a previously described automated method (Bass et al., 1992a). In order to compensate for the absence of the shielding influence of the bulk solvent, all potentially charged amino acid side chains were made net-neutral and a distance-dependent dielectric was used. This approach has been successfully used in several recent studies (Kruger et al., 1985; Makinen et al., 1989; Åqvist & Tapia, 1990; Mehler & Solmajer, 1991; Solmajer & Mehler, 1991; Swaminathan et al., 1991; Braatz et al., 1992; Gu & Brady, 1992). Although only a small number of explicit waters were included, this should have a small effect on the predictions made because we are focusing on the dynamics of the buried active site. The dynamic behavior of surface residues in the protein will be most susceptible to the method of modeling bulk solvent, while the interior of the protein will be much less affected by the method of modeling bulk solvent. Placing full charges on the physiologically charged residues in simulations on camphor-bound cytochrome P450cam did not significantly change the mobility of the substrate or its preferred orientations (Paulsen & Ornstein, unpubl.). The Discover simulation package (Biosym Technologies Version 2.7) with the consistent valence forcefield of Hagler and coworkers was used (Hagler, 1985; Dauber-Osguthorpe et al., 1988). The parameters for the heme were identical to those described previously (Paulsen & Ornstein, 1991). The valence and nonbonded parameters for the ferryl oxygen were the same as previously used (Paulsen & Ornstein, 1992).

For each simulation, the system was first minimized with all heavy atoms fixed to relax the added hydrogens, and then 500 steps each of steepest descents and conjugate gradients were performed with all atoms free to move to allow any remaining hotspots to relax. The atomic velocities were initialized using a Maxwellian distribution at 50 K, and the system was gradually warmed to 300 K over a period of 10 ps. After this, the simulations were continued at 300 K for 140 ps using a 1.0-fs time step. Nonbonded interactions were evaluated using a group-based switching function between 9.5 and 11.5 Å and the nonbonded pair list was updated every 20 time steps.

Site-directed mutagenesis

An application of the polymerase chain reaction (PCR) technique (Sligar et al., 1991) was used to produce site-directed mutants of cytochrome P450cam (Koga et al., 1985; Unger et al., 1986). This procedure utilized two consecutive rounds of amplification. In the first step, the sequence between the mutagenic primer and a universal pUC primer was amplified using a double-stranded wild-type plasmid as a template. The resulting DNA was then used in the second PCR as one primer along with the other universal primer to generate full-length mutant construction that was subcloned back into pUC for expression.

TAQ polymerase was purchased from Promega (Madison, Wisconsin), and the restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories (Bethesda, Maryland). All enzymes were used according to manufacturers' specifications. DNA sequencing was performed by the dideoxy method (Sanger & Coulson, 1975) using the Sequenase Version 2.0 kit from United States Biochemicals (Cleveland, Ohio). Other standard DNA manipulations were performed according to established protocols (Sambrook et al., 1989).

Protein purification

Wild-type and mutant cytochrome P450cam were overexpressed in *E. coli* (Unger et al., 1986) and purified by standard procedures (Stayton & Sligar, 1991). Putidaredoxin and putidaredoxin reductase were overexpressed in *E. coli* (Koga et al., 1989; Davies et al., 1990) and purified as previously described for purification from *Pseudomonas putida* (Gunsalus & Wagner, 1978) with minor modifications (Davies et al., 1990).

Norcamphor metabolism

Enzyme reactions were performed in 50 mM Tris, pH 7.4, 1 mM norcamphor, 200 mM KCl, and 250 μM NADH with 0.5 μM cytochrome P450cam, 10 μM putidaredoxin, and 5 μM putidaredoxin reductase. Product distribution and coupling of the reaction were determined in triplicate for both the T185F mutant and the wild-type enzyme. The product distribution was determined using gas chromatography and mass spectral analysis as described previously (Atkins & Sligar, 1987). The error in the experimental results was always smaller than 5% of the measured value.

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