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The Structural Basis for Substrate-Induced Changes in Redox Potential and Spin Equilibrium in Cytochrome P-450$_{CAM}$[†]

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ABSTRACT: The crystal structures of cytochrome P-450$_{CAM}$ complexed with the alternative substrates norcamphor and adamantanone have been refined at 2.0-Å resolution and compared with the native, camphor-bound form of the enzyme. Norcamphor lacks the 8-, 9-, and 10-methyl groups of camphor. Thus, specific interactions between these groups and phenylalanine 87 and valines 247 and 295 are missing in the norcamphor complex. As a result, norcamphor binds about 0.9 Å further from the oxygen-binding site than does camphor, which allows sufficient room for a water molecule or hydroxide ion to remain coordinated with the heme iron atom. The larger adamantanone occupies a position closer to that of camphor and, as in the camphor-bound enzyme, the heme iron remains pentacoordinate with no solvent molecule coordinated as a sixth ligand. A comparison of crystallographic temperature factors indicates that norcamphor is more "loosely" bound than are either camphor or adamantanone, as might be expected from the relative sizes of the different substrates. The looser fit of norcamphor in the active-site pocket results in a less specific pattern of hydroxylation. The presence of an aqua ligand is the likely structural basis for the norcamphor–P-450$_{CAM}$ complex having both a lower redox potential and higher percentage of low-spin heme than do either the camphor–P-450$_{CAM}$ or adamantanone–P-450$_{CAM}$ complexes.

Cytochromes P-450 are a group of *b*-type heme proteins that catalyze the hydroxylation of aromatic and aliphatic substrates in a variety of metabolic processes. The most extensively studied P-450 is the camphor hydroxylase from *Pseudomonas putida*, or P-450$_{CAM}$ (Wagner & Gunsalus, 1982; Gunsalus et al., 1974; Debrunner et al., 1978; Gunsalus & Sligar, 1978). P-450$_{CAM}$ is a 45 000-Da polypeptide containing a single ferric protoporphyrin IX. As with many *b*-type heme proteins, P-450$_{CAM}$ equilibrates between low-spin, $S = {}^1/_2$, and high-spin, $S = {}^5/_2$, states (Sharrock et al., 1976; Tsai et al., 1970). The binding of the substrate, camphor, shifts the spin equilibrium toward the high-spin form and also shifts the redox potential from about −300 to −170 mV (Philson, 1976; Sligar, 1976; Sligar & Gunsalus, 1976; Fisher & Sligar, 1985). A structural basis for these changes has been provided by a comparison between the substrate-free and -bound crystal structures (Poulos et al., 1985, 1986, 1987). In the substrate-free structure, the camphor pocket is filled with solvent molecules, and one of these, a water molecule or hydroxide ion, coordinates with the heme iron atom. However, in the camphor-bound structure, the heme iron is pentacoordinate and no ordered solvent molecules are observed at the active site. It is likely that the lower redox potential of the substrate-free structure is due to additional stabilization of the ferric (Fe^{3+}) state as a result of the higher dielectric environment provided by the active-site solvent molecules. Since the low-spin state is favored by the substrate-free enzyme, we believe that the strong-field hydroxide ligand, OH^-, is axially coordinated with the heme iron atom, rather than the weak-field ligand, H_2O.

Fisher and Sligar (1985) have further analyzed the relationship between redox potential and spin equilibrium by measuring both the redox potentials and the spin equilibria of various substrate–P-450$_{CAM}$ complexes and have found a linear free energy relationship between the two. The substrate norcamphor is metabolized with relatively low regiospecificity, giving at least three detectable hydroxylated products (Atkins & Sligar, 1988). Binding of this substrate results in an enzyme with a low redox potential (−206 mV) and with a significant population fraction (54%) of the heme iron in the low-spin state. In contrast, substrates such as adamantanone and camphor, from each of which a single hydroxylated product is formed, shift the heme iron spin equilibrium almost entirely (>95%) to high spin and exhibit significantly higher redox potentials, −175 and −170 mV, respectively. Interestingly, there is no readily discernible relationship between binding constant and spin state (Fisher & Sligar, 1985). We have postulated (Poulos et al., 1986) that these substrate-associated shifts in redox potential and spin equilibrium are controlled by the degree of hydration at the active site and by water

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Table I: Summary of Data Collection

	norcamphor	adamantanone
max resolution (Å)	1.71	2.08
total observations	125 392	54 729
R_{sym}[a]	0.078	0.061
data collected (%)	to 3.11 Å, 93	to 3.77 Å, 100
	to 2.47 Å, 91	to 2.99 Å, 100
	to 2.16 Å, 85	to 2.62 Å, 99
	to 1.96 Å, 79	to 2.38 Å, 97
	to 1.82 Å, 73	to 2.21 Å, 90
	to 1.71 Å, 28	to 2.08 Å, 38
$I/\sigma I$	at 2.01 Å, 1.90	at 2.12 Å, 1.88
	at 1.81 Å, 1.32	at 2.10 Å, 1.15
	at 1.71 Å, 1.38	at 2.08 Å, 0.63

[a] $R_{sym} = \sum|I_i - \langle I_i\rangle|/\sum I_i$, where I_i is the intensity of the ith observation and $\langle I_i\rangle$ is the mean intensity.

coordination with the heme iron atom. To test this hypothesis, we chose to determine the crystal structures of the norcamphor and adamantanone complexes of P-450$_{CAM}$ since these are the best characterized of the alternative substrates (Atkins & Sligar, 1988; White et al., 1984), both with respect to their specificity as substrates and as to their effects on redox potential and spin equilibrium of P-450$_{CAM}$.

Materials and Methods

P-450$_{CAM}$ was crystallized according to our earlier procedure (Poulos et al., 1982). To prepare the substrate complexes, crystals were soaked for 2 days in an artificial mother liquor consisting of 40% saturated ammonium sulfate, 0.05 M potassium phosphate, and 0.25 M KCl adjusted to pH 7.0, saturated with either norcamphor or adamantanone. Data were collected from a single crystal of each complex by using a Nicolet area detector and a Rigaku rotating anode. Although nominally the diffraction data extend to approximately 1.7 Å and to 2.0 Å for the norcamphor– and adamantanone–P-450$_{CAM}$ complexes, they are relatively complete only to about 1.8 and 2.2 Å, respectively. A summary of data collection statistics is presented in Table I.

Crystallographic refinement was carried out by using the restrained parameters–least-squares package of programs (Hendrickson & Konnert, 1980). Briefly, initial $F_o - F_c$[1] difference Fourier maps were based on structure factor calculations using coordinates from the refined 1.63-Å camphor–P-450$_{CAM}$ structure and diffraction data obtained from the norcamphor–P-450$_{CAM}$ or adamantanone–P-450$_{CAM}$ complexes. Camphor coordinates were not included in the structure factor calculations. Substrates were modeled by using coordinates for these molecules with optimized geometry, provided by Dr. Gilda Loew (Stanford Research Institute), were positioned into the $F_o - F_c$ maps, and were refined together with the protein. The structures of the three substrates are shown in Figure 1. As in the camphor–P-450$_{CAM}$ complex, hydrogen bonds between the norcamphor and adamantanone carbonyl oxygen atoms and Tyr-96 OH were clearly evident in the electron density maps of both of these substrates complexed with P-450$_{CAM}$. Positioning of norcamphor was straightforward, but the more symmetrical adamantanone proved more difficult. A trial-and-error approach was employed to fit the adamantanone, using $F_o - F_c$ maps as guides to the correct orientation. The substrate orientation was judged to be correct when $F_o - F_c$ maps, contoured at 3σ (where σ is the standard deviation of the difference electron density calculated over the entire asymmetric unit), showed little or no density in the vicinity of the substrate. A final summary of the refinements is provided in Table II. Comparison of both coordinate and temperature factor shifts were carried out as described in an earlier study (Poulos & Howard, 1987).

[1] Abbreviations: F_o, observed structure factors; F_c, calculated structure factors.

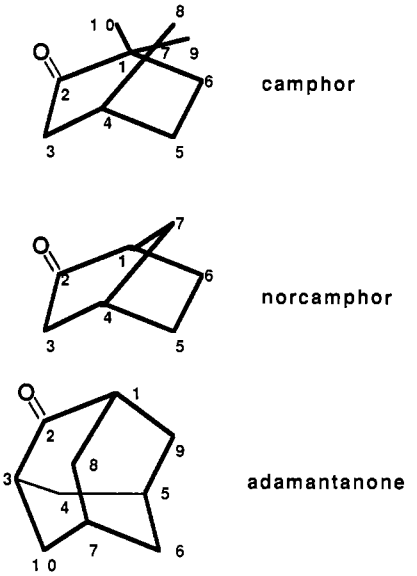

FIGURE 1: Structures of camphor, norcamphor, and adamantanone, numbered as in the text.

Table II: Summary of Crystallographic Refinement

	norcamphor	adamantanone
resolution range (Å)	10.0–2.0	10.0–2.08
reflections measured	24 922	22 149
reflections used, $I > 2\sigma(I)$	21 278	18 379
R factor[a]	0.18	0.19
rms deviation of bond distances (Å)	0.018	0.028
rms deviation of bond angles (Å)	0.030	0.037
rms deviation of dihedral angles (Å)	0.035	0.040

[a] $R = \sum|F_o - F_c|/\sum F_o$.

Results and Discussion

Parts a and b of Figure 2 show the initial $F_o - F_c$ difference Fourier maps for both the norcamphor and adamantanone complexes of P-450$_{CAM}$. The main difference between the two is that a coordinated water molecule or hydroxide ion is clearly evident ($>10\sigma$ positive difference density) in the norcamphor complex but not in the adamantanone complex. After a few cycles of refinement with only the norcamphor coordinates added to the set of P-450$_{CAM}$ coordinates, $F_o - F_c$ and $2F_o - F_c$ maps continued to show the presence of the axial aqua ligand, which was therefore included in further refinement. The final refined $2F_o - F_c$ maps are shown in parts c and d of Figure 2.

A comparison between refined sets of coordinates indicated that there were no large shifts in the positions of protein atoms. However, even though the carbonyl oxygen atom of all three substrates (camphor, adamantanone, and norcamphor) hydrogen bonds with the OH group of Tyr-96, other corresponding atoms of the three substrates occupy rather different positions in the active site (Figure 3). The average shift of norcamphor carbon atoms from their respective camphor positions is 0.9 Å, the minimum shift being 0.78 Å and the maximum, 1.04 Å. The direction of the shift is *away* from the axial coordination site and *toward* Val-295 (Figure 3a).

Both its smaller size and lack of the specific contacts with Val-247 and Val-295, contacts made by camphor, suggest that

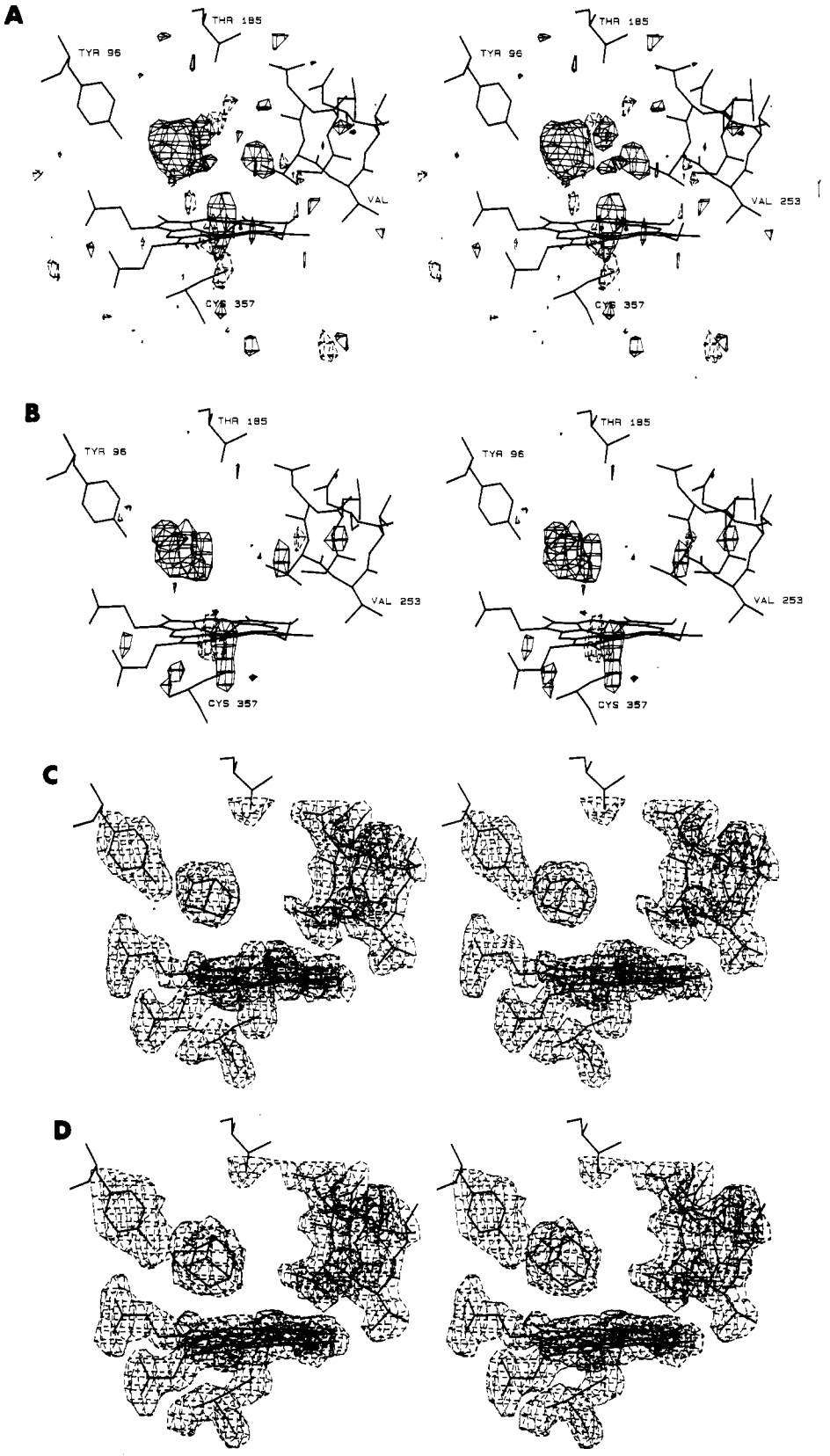

FIGURE 2: Stereoscopic views of the initial $F_o - F_c$ and final $2F_o - F_c$ electron density maps for the norcamphor and adamantanone complexes of P-450$_{CAM}$. In panels a (norcamphor) and b (adamantanone), the $F_o - F_c$ maps are contoured at $\pm 4\sigma$ with the negative contours shown as dashed lines. These maps are superimposed on the model for the camphor–P-450$_{CAM}$ complex (camphor coordinates have been removed for clarity). Notice the large lobe of positive density of the distal axial coordination position (above the heme plane in this view) present in the norcamphor difference map (panel a) but absent in the adamantanone difference map (panel b). In panels c and d are shown the final $2F_o - F_c$ electron density maps, contoured at $+1\sigma$, for the norcamphor– and adamantanone–P-450$_{CAM}$ complexes, respectively, superimposed on their final refined coordinates.

norcamphor should be more loosely bound by P-450$_{CAM}$. Crystallographic temperature factors are often good indicators of atomic mobility, and one would expect norcamphor atoms to exhibit higher temperature factors than do the atoms of the

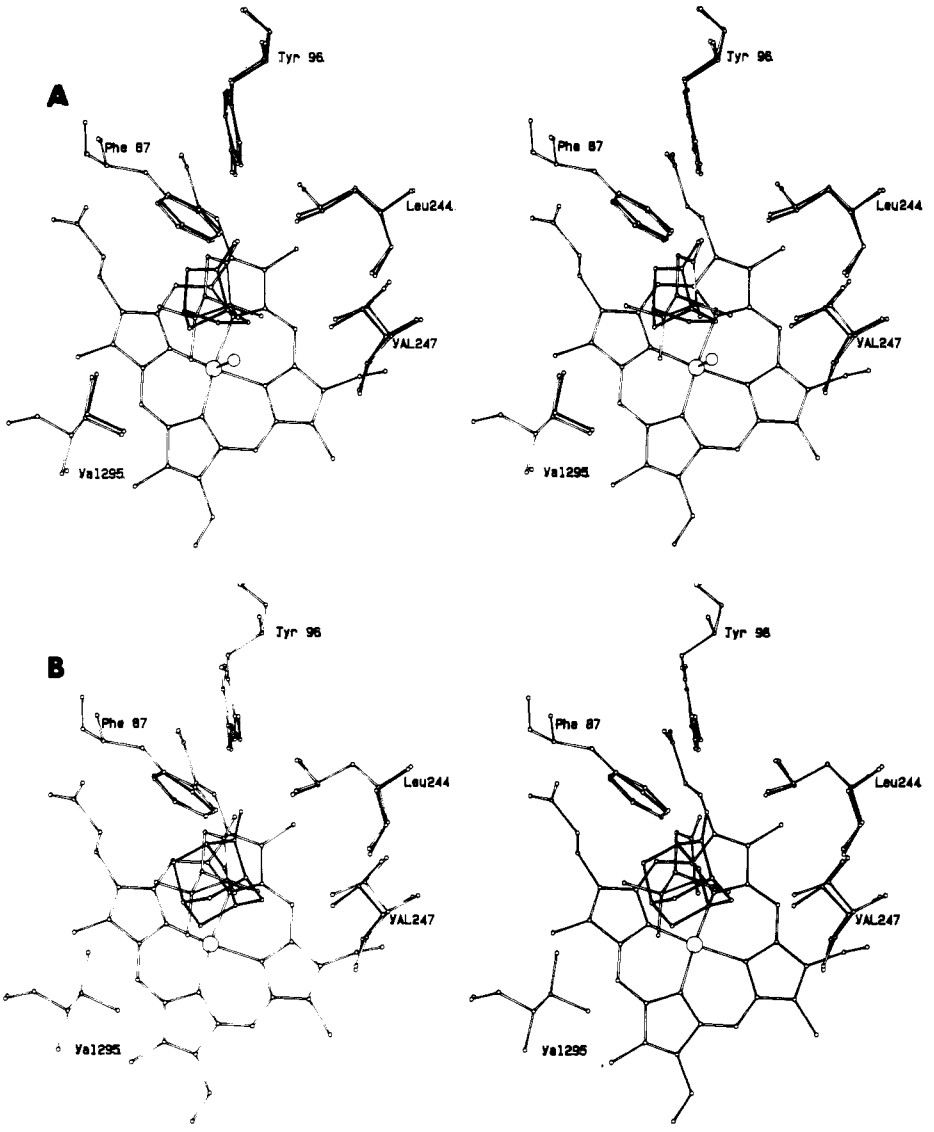

FIGURE 3: Stereoscopic models of the refined norcamphor– (panel a) and adamantanone–P-450$_{CAM}$ (panel b) complexes superimposed on the model of the camphor–P-450$_{CAM}$ complex. The camphor–P-450$_{CAM}$ complex is shown with transparent bonds, and the norcamphor– and adamantanone–P-450$_{CAM}$ complexes are shown with solid bonds. The axial water ligand also is shown in the norcamphor–P-450$_{CAM}$ complex (see panel a).

larger camphor or adamantanone molecules. Shifts in temperature factors between the different substrates alone, which were larger than the root-mean-square (rms) difference in protein and heme atom temperature factors between the different enzyme-substrate complexes, were considered significant. The rms difference in temperature factors between the norcamphor–P-450$_{CAM}$ and camphor–P-450$_{CAM}$ structures is 2.33 Å^2, while this value is 2.38 Å^2 for the adamantanone–P-450$_{CAM}$/camphor–P-450$_{CAM}$ comparison. The average temperature factors for camphor, adamantanone, and norcamphor atoms are 16.20, 16.49, and 33.53 Å^2. The 17.33-Å^2 average temperature factor increase of norcamphor atoms with respect to camphor atoms is well above the rms difference of all protein and heme atom temperature factors between these two structures (2.33 Å^2), thus demonstrating that norcamphor is more loosely bound than either camphor or adamantanone, as expected.

This looser fit of the norcamphor provides an explanation for why norcamphor is metabolized less specifically than camphor. Norcamphor is primarily hydroxylated by P-450$_{CAM}$ at the 5-exo and 6-exo positions with about 8% hydroxylation at the 3-exo position (Atkins & Sligar, 1988). In contrast, camphor and adamantanone are metabolized only to 5-*exo*-hydroxycamphor and 5-hydroxyadamantanone, respectively, by P-450$_{CAM}$ (Gelb et al., 1982; White et al., 1984). Interestingly, a recent theoretical treatment of norcamphor binding to P-450$_{CAM}$ correctly predicted that both 5-exo and 6-exo hydroxylation should occur (Collins & Loew, 1988) when norcamphor binds to the P-450$_{CAM}$ active site in an orientation most closely resembling that of camphor. These authors also correctly predicted the small amount of 3-*exo*-norcamphor produced on the premise that the missing interactions with Phe-87, Val-295, and Val-247 would allow norcamphor to rotate by approximately 130° about its carbonyl group into an orientation where hydroxylation at the 3-exo position would be possible. Though our electron density maps indicate only one orientation of norcamphor that appears to favor 5-exo hydroxylation, the possibility of a second orientation for norcamphor in the active site cannot be ruled out. It is often possible to distinguish two orientations that are approximately equally favored in electron density maps of refined crystal structures. However, in a case where one possible orientation represents such a small proportion of the population (8%), it is likely to go undetected. The fact that norcamphor also appears to be relatively mobile, as judged by the temperature factors of its atoms, may also serve to obscure a possible second

orientation of low occupancy.

Due to its symmetry, there are only five unique types of carbon atoms in the adamantanone molecule, equivalent to carbons 1 (=3), 2, 4 (=8, 9, 10), 5 (=7), and 6 (Figure 1). Only carbons 2 (the carbonyl carbon) and 6 (the carbon diametrically opposed to the carbonyl group) have no symmetry equivalents. Whether the adamantanone molecule is held fixed within the active site of P-450$_{CAM}$ and hydroxylated at a single 5-carbon or whether it is free to rotate and can be hydroxylated at either of the type-5 carbons cannot be determined from the isolated product, 5-hydroxyadamantanone. Examination of adamantanone in the active site of P-450$_{CAM}$ leads us to conclude that rotation about the carbonyl bond of adamantanone probably occurs. Although the fit of adamantanone into the active site appears rather snug, its van der Waals envelope is symmetric, and there are no large protrusions that would obviously hinder rotation about its carbonyl bond.

From the refined coordinates of the adamantanone–P-450$_{CAM}$ complex, it is apparent that adamantanone carbons of types 4 and 5 are the closest to, and about equidistant from, the heme iron atom. It is also possible, by a rotation of approximately 90° about the carbonyl bond, to produce an orientation for adamantanone consistent with the $2F_o - F_c$ electron density map, where C6 is closer to the iron atom than are any of the type-4 or –5 carbons. We believe this latter orientation is incorrect on the basis of several positive and negative features that result in the $F_o - F_c$ difference electron density map.

Although at least three types of adamantanone carbon atoms may approach the iron atom, two factors appear to favor C5 hydroxylation. First, regardless of which orientation consistent with the $2F_o - F_c$ electron density map the adamantanone molecule adopts in the active site, a type-5 carbon is always the closest substrate carbon atom to the expected location of the iron-linked oxygen atom thought to be involved in substrate hydroxylation. Second, type-5 carbons are the only tertiary carbons accessible to the iron-linked oxygen atom. Since a tertiary carbon radical is expected to be more stable than either a secondary or primary carbon radical (White et al., 1984), hydroxylation of tertiary type-5 carbons will be thermodynamically favored over hydroxylation of any of the secondary type-4 or -6 adamantanone carbons.

The most important result from this study is that the heme iron atom remains hexacoordinate in the norcamphor complex but becomes pentacoordinate in both the camphor and adamantanone complexes. This difference suggests that the linear free energy relationship between redox potential and spin-state equilibrium, noted by Fisher and Sligar (1985), is controlled by the aqua ligand. The presence or absence of the aqua ligand at the heme coordination site is itself controlled by steric crowding. Norcamphor, at closest approach, is 3.0 Å from the aqua ligand, whereas the camphor molecule is only 2.61 Å from the aqua ligand site, with the result that camphor displaces the aqua ligand upon binding to P-450$_{CAM}$.

We envision two mechanisms whereby the presence of substrate near the aqua ligand could control the redox potential and spin-state equilibrium of P-450$_{CAM}$. The first is a two-state, or all-or-none, mechanism in which the redox potential and spin equilibrium are rigorously coupled to the equilibrium between a hexacoordinate and a pentacoordinate heme. In this case, the heme is either fully low-spin and hexacoordinate or fully high-spin and pentacoordinate, and the substrate, by displacing the heme iron axial aqua ligand, shifts the equilibrium between the two extremes. Different ratios of low-spin/hexacoordinate and high-spin/pentacoordinate enzyme–substrate complexes in the population result in the observed fraction of each spin component with each different substrate.

In the second mechanism, a nearby substrate molecule shifts the redox potential and spin equilibrium of the enzyme without affecting the state of heme iron coordination. In this case, spin state and axial coordination of the heme iron are not tightly coupled, and the heme iron can exhibit a significant fraction of the high-spin component and yet remain hexacoordinate. The reason we consider this mechanism is that in the norcamphor–P-450$_{CAM}$ complex the aqua ligand exhibits full occupancy and a very low temperature factor (4 Å^2), yet this complex is only 54% low spin (Fisher & Sligar, 1985).

One way that a change in spin state could be achieved while a hexacoordinate heme is maintained is by shifting the pK of the aqua ligand. The proximal axial Cys ligand to the heme iron is actually a thiolate anion [S^-, references in Dawson et al. (1982)], so that the net charge on the heme in the ferric (Fe^{3+}) state would be –1 if the distal axial ligand is OH^- and 0 if it is H_2O (there is a formal charge of –2 on the porphyrin ring system). Moreover, the relatively high dielectric milieu in the substrate-free enzyme due to the active-site water molecules should provide additional stabilization of the net charge of –1 on the thiolate–heme–OH^- system. When any substrate or inhibitor enters the active site, some or all of the active-site water molecules are displaced, resulting in a local decrease in the dielectric environment. Such a decrease should favor protonation of the aqua ligand to give a neutral thiolate–heme–H_2O system. Indeed, Sligar and Gunsalus (1979) have found that a proton preferentially binds to the high-spin form of P450$_{CAM}$ and have tentatively assigned the distal heme ligand as the site of protonation. We now known this ligand to be a water molecule, and presumably the basic form is an OH^- ion. Moreover, it was found that the pK of protonation of the low-spin enzyme is much lower than that of the high-spin enzyme (Sligar & Gunsalus, 1979). That is, axial coordination of OH^- is preferred in the low-spin state, and protonation of the strong-field ligand, OH^-, to generate the weak-field ligand, H_2O, is favored in the high-spin state. In addition, by removing a negative charge from the heme, protonation will also favor an increase in redox potential.

Relevant to the present study, we end with a brief discussion of our recent paper on the structures of various P-450$_{CAM}$–inhibitor complexes (Poulos & Howard, 1987). 1- and 4-phenylimidazoles and metyrapone each form an axial N–Fe bond with the heme iron atom, and each of these complexes is low spin (Dawson et al., 1982), although redox potentials have not been reported. In contrast, 2-phenylimidazole is prevented from forming a bond with iron due to steric constraints and instead binds at the camphor site in such a way as to leave even more room for water coordination than was found in the norcamphor complex. 2-Phenylimidazole bears no resemblance to any of the known P-450$_{CAM}$ substrates, yet it binds quite well (K_D = 0.007 mM; Lipscomb, 1980), giving a hexacoordinate, low-spin complex. The overall impression from these various substrate- and inhibitor-binding studies is that the P-450$_{CAM}$ active-site pocket is rather unspecific and should be able to accommodate a variety of aromatic or aliphatic molecules within a certain size range.

The key question then is, given that P-450$_{CAM}$ will bind a variety of molecules, what are the factors that control whether any individual molecule will be efficiently metabolized? Orientation of the substrate with respect to the iron-linked activated oxygen atom, substrate mobility, and chemical reactivities of individual substrate atoms are three important

factors. The present study adds substrate-dependent aqua ligand displacement and modulation by the substrate of aqua ligand protonation as two additional factors that should be considered.

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