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Tyrosine-96 as a Natural Spectroscopic Probe of the Cytochrome P-450$_{cam}$ Active Site

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ABSTRACT: The previously described correlation between the ferric spin equilibrium of cytochrome P-450$_{cam}$ and the environmental polarity of tyrosine residues (Fisher et al., 1986) has been further examined with the use of site-directed mutagenesis and active-site affinity reagents. Whereas the wild-type demonstrates an increase in environmental polarity of approximately one tyrosine residue, the mutant protein Y96F, in which Tyr-96 has been changed to Phe-96, demonstrates a lack of spin-state-dependent change in the second-derivative ultraviolet absorption spectrum. This suggests that the active-site Tyr-96 serves as a ultraviolet spectroscopic probe which can be utilized to determine the relative degree of water access to the active site for various substrate/protein complexes. The affinity reagent isobornyl mercaptan has been used to demonstrate the utility of this probe in determining the active-site polarity when substrate analogues are bound at the active site. In addition, the sensitivity of Tyr-96 to environmental polarity has been used to demonstrate that the product/enzyme complex, formed with 5-*exo*-hydroxycamphor, may be associated with increased water access to the heme iron. This may provide a means for turning off electron transfer when the product, instead of the substrate, is bound at the active site.

Cytochrome P-450$_{cam}$, isolated from *Pseudomonas putida*, catalyzes the regiospecific hydroxylation of the monoterpene camphor in the first committed step of the complex catabolism of this compound, thus allowing the organism to utilize camphor as a sole carbon source. Cytochrome P-450$_{cam}$ has served as the prototypical model for the chemical and physical characterization of the various oxidized, reduced, and oxygenated states of the heme center for the entire family of P-450 monooxygenases (Murray et al., 1986). This is largely due to its soluble nature, its availability in gram quantities, and the recently published set of X-ray crystal structures for the camphor-bound, substrate-free, and metyrapone-inhibited forms of the enzyme (Poulos et al., 1985, 1986, 1987, 1988).

It is well documented that camphor binding is associated with a change from the low-spin, aquo-liganded ferric enzyme to a high-spin, five-coordinate form. This change in spin state, which can be monitored spectrally by a change in the visible region of the absorbance spectrum, is accompanied by a change in reduction potential from −300 to −170 mV, allowing for electron transfer from the physiological electron-transfer partner putidaredoxin (Sligar, 1976). The observed change in reduction potential is of central importance in the regulation of catalysis by this enzyme, and structural differences between the low-spin and high-spin forms of the enzyme must be understood if the complete reaction cycle and mechanisms of substrate oxidation are to be completely documented.

The relationship between the active-site structure of P-450$_{cam}$ and the regulation of the spin state is being aided through the use of site-directed mutagenesis (Atkins & Sligar, 1988, 1989), rapid reaction kinetics (Fisher & Sligar, 1987) and equilibrium potential measurements (Fisher & Sligar, 1988), and insight gleaned from the available X-ray structures. Recently, second-derivative ultraviolet (UV)[1] spectroscopy was used as probe of the P-450$_{cam}$ active site, and it was demonstrated that the ferric spin equilibrium is coupled to the relative environmental polarity or degree of solvent exposure of approximately one tyrosine residue (Fisher et al., 1985). A linear correlation was observed between the fraction of high-spin enzyme ob-

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[1] Abbreviations: Y96F, site-directed mutant in which Tyr-96 has been changed to Phe-96; IBM, isobornyl mercaptan; UV, ultraviolet.

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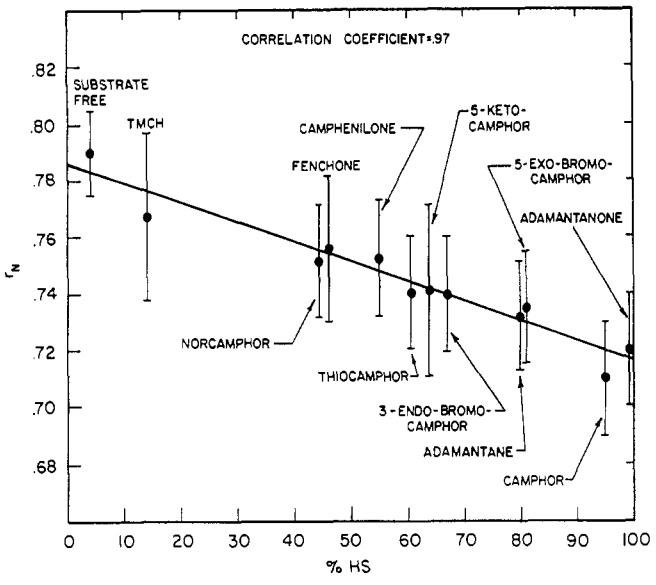

FIGURE 1: Linear correlation is obtained when r_n values from the second-derivative UV spectra are plotted against percent high-spin P-450 elicited by each substrate. The change in r_n value observed for substrate-free vs camphor-bound forms of the enzyme correspond to a change in the solvent exposure of approximately one tyrosine residue.

tained with various substrates and the spectral peak-to-trough ratio, r_n, calculated from the second-derivative spectrum, which reflects the local environment of tyrosine residues (Ragone et al., 1984). This relationship is demonstrated in Figure 1. This correlation may arise from protein conformational changes associated with binding of various substrate analogues which elicit different spin-state equilibria. However, a comparison of the substrate-free and camphor-bound crystal structures indicates no significant protein conformational change upon camphor binding (Poulos et al., 1985, 1986). There is, instead, a camphor-induced expulsion of six water molecules from the active site (Figure 2). One tyrosine residue, Tyr-96, hydrogen bonds to the carbonyl moiety of camphor, yet is left unshielded from the cluster of water molecules in the absence of camphor. It had been proposed that the relative degree of solvent exposure of this tyrosine-96 residue, as a function of spin-state equilibrium, is responsible for the differences in the second-derivative UV spectra of various substrate-bound forms of P-450$_{cam}$ (Fisher et al., 1985). This hypothesis is further supported by the observation that the on-rate of solvent water to the heme iron is correlated with the percentage high-spin P-450 elicited by a series of substrate analogues (Fisher et al., 1987), such that substrates which induce a greater fraction of high-spin form of the enzyme result in more restricted access of the heme pocket to solvent. A recent X-ray structure of a norcamphor-P-450$_{cam}$ complex supports this model (Raag & Poulos, 1989). Here we report an extension of the second-derivative spectroscopy studies, utilizing site-directed mutagenesis and additional affinity reagents, to conclusively demonstrate that Tyr-96 is indeed the source of the spectroscopic differences and that it is sensitive to the local environment in addition to the degree of solvation by water. The utility of second-derivative UV spectroscopy as an extremely sensitive, noninvasive, probe of the active site of cytochrome P-450$_{cam}$ is herein demonstrated.

Materials and Methods

Cytochrome P-450$_{cam}$ was purified from *Escherichia coli* as previously described (Gunsalus et al., 1978). The site-directed mutant Y96F, where Tyr-96 is replaced by phenylalanine, was prepared as described elsewhere (Atkins & Sligar,

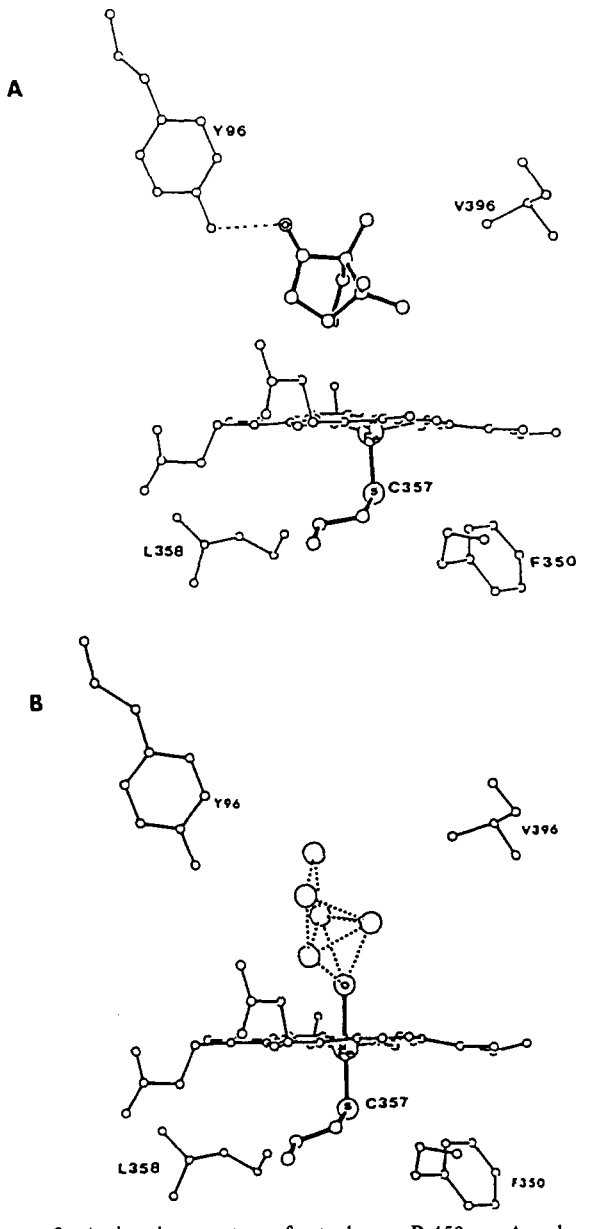

FIGURE 2: Active-site structure of cytochrome P-450$_{cam}$. An edge-on view of the camphor-bound and substrate-free forms of the enzyme is shown. Tyrosine-96 hydrogen bonds to the substrate camphor in the high-spin complex. In the absence of camphor, a cluster of water molecules occupies the heme pocket and affords a low-spin complex.

1988) utilizing the methods established by Taylor et al. (1985) and an in vitro mutagenesis kit purchased from Amersham. Camphor was purchased from Aldrich and used without further purification. The 5-*exo*-hydroxycamphor was generously provided by Dr. I. C. Gunsalus, University of Illinois. Isobornyl mercaptan was synthesized as previously described (Dus et al., 1980; Subluskey et al., 1951). Second-derivative spectra were obtained with 4 μM P-450$_{cam}$, in 50 mM Tris (pH 7.4) in the presence of 200 mM KCl on a Hewlett-Packard 8450 rapid-scan UV–visible spectrophotometer. Samples contained either zero or 200 μM samples of the appropriate substrates. The reported r values represent the average of 10 spectra where the r values are obtained from the relation:

$$r = \frac{\Delta A''_1}{\Delta A''_2} = \frac{A''_{288} - A''_{284}}{A''_{295} - A''_{291}} = \frac{Ax + B}{Cx + 1}$$

where $\Delta A''_1$ and dA''_2 are second-derivative differences at the two pairs of wavelengths specified in the equation and which correspond to peaks and troughs characteristic of the tyrosine

environment (Ragone et al., 1984; Servillo et al., 1984). First, the tryptophan to tyrosine ratio is obtained from the above relation where the parameters A, B, and C correspond to 0.20, 0.66, and −0.09, respectively, for the denatured protein in guanidinium chloride (r_u) and

$$A = \frac{\Delta\epsilon''_1(\text{Tyr})}{\Delta\epsilon''_2(\text{Tyr})} \qquad B = \frac{\Delta\epsilon''_1(\text{Trp})}{\Delta\epsilon''_2(\text{Trp})} \qquad C = \frac{\Delta\epsilon''_2(\text{Tyr})}{\Delta\epsilon''_2(\text{Trp})}$$

and where $\Delta\epsilon''$ is the difference between the molar extinction coefficients for the second-derivative spectra, and x is the molar ratio between tyrosine and tryptophan (Ragone et al., 1984). The r_u ratio reflects the relative tyrosine exposure when all of the tyrosine residues are exposed to solvent in the denatured state of the protein (Ragone et al., 1984). Additionally, a value r_a is calculated by using values for A, B, and C which have been determined in ethylene glycol. The resulting r_a (0.260) affords an estimate of the peak-to-trough ratio if all tyrosine residues were buried within the interior of the protein matrix (Ragone et al., 1984). The values used for A, B, and C were obtained from the same study and are $A = -0.18$, $B = -0.64$, and $C = -0.04$ in ethylene glycol. The r_n ratio reflects the relative tyrosine exposure to solvent in the native conformation of the protein. The relative fraction of tyrosine exposure in the native conformation can be calculated as $(r_n - r_a)/(r_u - r_a)$.

Results

The site-directed mutant Y96F, in which tyrosine-96 has been changed to a phenylalanine, has previously been demonstrated to have altered binding affinity for camphor, altered spin-state regulation, and a loss of the complete regiospecificity of hydroxylation (Atkins & Sligar, 1988). Comparison of the second-derivative UV spectra for the wild-type and mutant enzymes reveals further structural insight. The denaturated forms, obtained in 6 N guanidinium chloride, clearly demonstrate a difference in the tyrosine:tryptophan ratio, as expected for removal of tyrosine-96. The spectra (not shown) afford r_u values of 1.37 and 1.29 for the wild-type and mutant, respectively. These values can be used to calculate the tyrosine:tryptophan ratio (Ragone et al., 1984; Servillo et al., 1984). The ratios obtained from these r_u values are 2.22 and 1.99, in good agreement with the difference in ratios obtained from the actual values of 1.80 (9 Tyr/5 Trp) and 1.60 (8 Try/5 Trp). Thus, replacement of one tyrosine residue with a phenylalanine is easily detected by this method.

Furthermore, the change in net tyrosine exposure, when considered as function of the ferric spin equilibrium, is altered in the Y96F mutant. Previously, it has been shown that the change in ferric spin state accompanying substrate binding is characterized by a change in the degree of solvent exposure of one tyrosine residue (Fisher et al., 1985). Typical second-derivative spectra are shown in Figure 3. The small changes in the peak to trough ratio, A/B, are completely reproducible and essentially identical with the changes observed previously for camphor binding to the wild-type enzyme. Here, A and B refer to the difference in spectral maxima and minima as shown in Figure 3, and are *not* related to the coefficients found in the equations defining r_a and r_u. When the Y96F mutant is titrated with camphor, however, there is almost a complete lack of change in the second-derivative spectra, so the substrate-free and camphor-bound forms of Y96F are nearly identical (Figure 3). It is important to point out that the Y96F enzyme is only driven to a 59% high-spin configuration upon camphor binding at room temperature. It is, therefore, necessary to establish that the decrease in the magnitude of the change in r_n for the substrate-free and

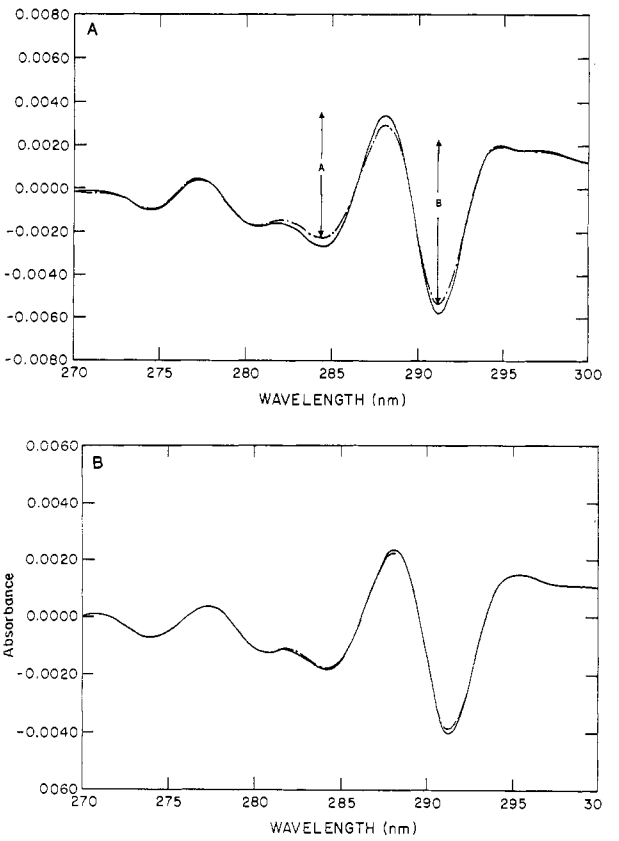

FIGURE 3: Second-derivative spectra of the camphor-bound and substrate-free enzymes. (A) Wild-type P-450$_{cam}$. (B) Y96F. The solid lines are spectra obtained with substrate-free protein. Dashed spectra are obtained with camphor-bound protein.

camphor-bound forms of Y96F is not simply due to the incomplete conversion of spin state. From the previously established correlation between spin state and r_n values, a 59% high-spin configuration would be expected to result in a change in r_n values of approximately 0.05, where the complete change from 5% high spin to 95% high-spin associated with camphor binding is accompanied by a change in r_n values of 0.07. With Y96F, camphor binding results in a change in r_n values of 0.011. From the r_n and r_u values, an estimate of the relative number of tyrosines exposed to solvent can be calculated (Ragone et al., 1984). For the wild-type, the change in the number of tyrosines exposed is approximately 0.6 ± 0.03 upon camphor binding. For Y96F, the same analysis yields a value of 0.09 ± 0.02 for the change in the number of tyrosines exposed. This drastic reduction in the magnitude of the changes associated with camphor binding is well outside of the range of reduction which could be explained on the basis of the change in spin state alone. A 59% high-spin species would be expected to afford a change in tyrosine exposure equivalent to 0.38 Tyr residue. These results unequivocally demonstrate that the conclusions offered previously (Fisher et al., 1985) are correct: The spectroscopically observable correlation between the ferric spin equilibrium and the UV second-derivative r_n values is predominantly due to contributions from tyrosine-96. This dramatically emphasizes the utility of tyrosine-96 as a sensitive, noninvasive spectroscopic probe of the active site of cytochrome P-450$_{cam}$. It is interesting to note that the Y96F mutant exhibits a second-derivative spectrum closely analogous to the substrate-free, low-spin form of the wild-type enzyme. Perhaps this reflects the relative hydrophilic environment of the remaining tyrosine residues, regardless of spin state. These results are summarized in Table I.

Table I

protein/substrate	% high spin	r_u	r_n	α,[a] fraction of total Tyr exposed	no. of Tyr exposed
wild-type/−camphor	5	1.37 ± 0.05	0.774 ± 0.015	0.459	4.13
wild-type/+camphor	95		0.700 ± 0.016	0.393	3.53
Y96F/−camphor	5	1.29 ± 0.05	0.780 ± 0.018	0.505	4.04
Y96F/+camphor	59		0.769 ± 0.010	0.494	3.95
wild-type/+IBM	*b*		0.690 ± 0.017	0.385	3.47

[a] $\alpha = (r_n - r_a)/(r_u - r_a)$. [b] Split Soret.

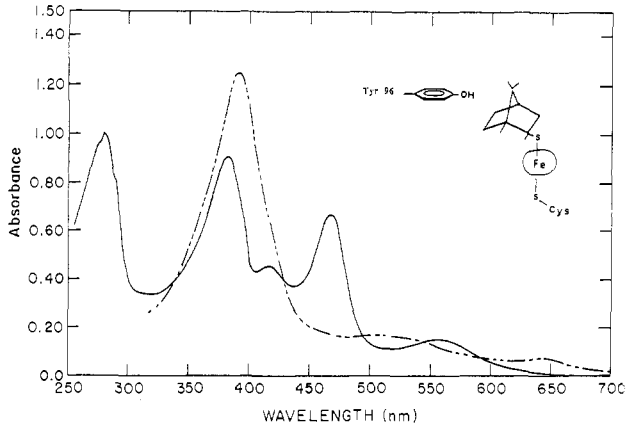

FIGURE 4: Optical spectrum of the P-450$_{cam}$/IBM complex, demonstrating the "split Soret". The small peak at 391 nm results from a small amount of high-spin species with camphor bound. From the visible spectrum shown here, it would be impossible to estimate the degree of hydration of the active site. The second-derivative UV spectrum indicates, however, that no water is present in the active site when IBM is bound. (—) IBM-bound P-450$_{cam}$. (---) Camphor-free P-450$_{cam}$.

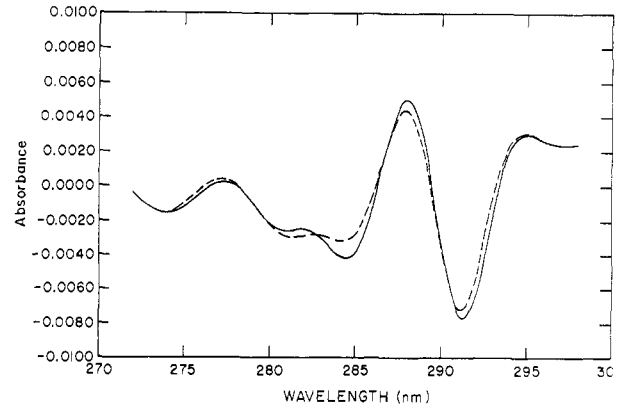

FIGURE 5: Second-derivative spectra of the P-450$_{cam}$/IBM complex. (—) Camphor-free P-450$_{cam}$. (---) IBM-bound P-450$_{cam}$.

Having established that tyrosine-96 is the residue contributing to the observed spin state dependence of the second-derivative spectrum, the local environment of the active site can be probed in the presence of various inhibitors and affinity reagents. Of particular interest are compounds which elicit unique visible spectra, where a simple spin-state equilibrium does not exist and cannot be used to predict the relative degree of hydration of the active site. One such compound is isobornyl mercaptan (IBM), an affinity reagent previously used with P-450$_{cam}$ (Dus et al., 1980) and affording a visible spectrum that is suggestive of a bisthiolate-ligated heme (Dus et al., 1980; Dawson et al., 1987). Obviously, the unique "split Soret" obtained with the IBM/P-450$_{cam}$ complex (Figure 4) precludes an estimate of the water accessibility to the active site when this compound is bound. The second-derivative UV spectra (Figure 5) suggests, however, that tyrosine-96 is completely shielded from solvent with IBM bound and that no water is present at the active site. This conclusion, although expected, could not be made from the visible spectrum alone. The r_n value obtained in the presence of IBM even suggests that tyrosine-96 experiences a slightly less polar environment than with camphor bound. This may be partially due to a lack of the hydrogen bond to tyrosine-96 and to the irreversible nature of the IBM complex which provides a strong metal ligand, and therefore completely abolishes water accessibility to the heme iron. With camphor bound, a small amount of total enzyme still exists as the low-spin form (5%). The r_n values and relative tyrosine exposure values for the wild-type, Y96F, and the IBM/wild-type complexes are summarized in Table I.

In addition to the demonstration that the second-derivative spectrum can be used as a probe of the active site, the results obtained with IBM further indicate that the observed spectral changes in the UV region, which may occur with changing spin state, may be considered independently of the changes which occur in the visible/Soret region. The optical spectrum obtained in the presence of IBM is drastically different from the normal high-spin spectrum, yet affords an r_n value exactly what would be expected for a tight-binding affinity reagent expected to displace water from the active site.

The available X-ray crystal structures for cytochrome P-450$_{cam}$ offer immensely valuable models for predicting interactions between protein residues and various substrates and inhibitors (Poulos et al., 1985, 1986, 1987, 1989). The crystal structure for the mixed-spin norcamphor/P-450$_{cam}$ complex shows partial water occupancy at the heme (Raag & Poulos, 1989). The second-derivative spectroscopic technique also provides considerable structural information on substrate complexes. One such complex, for which very little structural data are available, is the P-450$_{cam}$/5-*exo*-hydroxycamphor complex. The 5-*exo*-alcohol is the sole product obtained from NADH-supported camphor metabolism, and the relative binding specificity between camphor and hydroxylated product must be understood to completely define the energetics of catalysis and the role of any protein structural changes which might mediate progress through the enzymatic reaction cycle. Therefore, we have utilized second-derivative UV spectroscopy with 5-*exo*-hydroxycamphor bound to P-450$_{cam}$. The product alcohol binds to the resting ferric enzyme with a 10-fold greater equilibrium dissociation constant (10 μM) than the substrate does in the presence of 200 mM KCl (Atkins & Sligar, 1988). The visible spectrum of the complex indicates a high-spin population of 26% at room temperature (data not shown). The second-derivative UV spectrum affords an r_n value of 0.760 ± 0.011. From consideration of the correlation between spin state and r_n values described above, these values for 5-*exo*-hydroxycamphor would place it precisely on the curve depicting this relationship, suggestive of a conserved binding site structure for the product of the reaction.

Discussion

The identification of tyrosine-96 as the residue responsible for the spin-state-dependent changes in the second-derivative UV spectrum of cytochrome P-450$_{cam}$ has been accomplished

by site-directed mutagenesis. When this amino acid is replaced with phenylalanine, substrate-free and camphor-bound proteins are characterized by nearly identical second-derivative spectra. This contrasts what is observed with the wild-type enzyme, where camphor binding is easily monitored by differences in the r_n values obtained from such spectra. As discussed by others, tyrosine residues exhibit greater sensitivity to changes in the local dielectric constant than tryptophan or phenylalanine (Ragone et al., 1984; Servillo et al., 1984). The fact that the effect on the r_n values is not completely lost in the Y96F mutant may be a result of some contribution of phenylalanine residues. The small change in r_n value observed with Y96F may reflect the phenylalanine-96 sensitivity to solvent exposure. In addition, other aromatic amino acids including Phe-87 and Phe-98 are situated in direct contact with the camphor carbonyl moiety, and would be expected to undergo changes in solvent exposure with camphor-dependent expulsion of water from the active site (Poulos et al., 1985, 1986, 1989). The small contribution of these individual phenylalanines may, additively, be sufficient to afford small spin-state-dependent changes in the UV spectra.

With the strong experimental support that tyrosine-96 provides a relatively specific probe of the active-site polarity, the previously reported results with various intermediate forms of P-450$_{cam}$ become worthy of reiteration (Fisher et al., 1985). In this previous study, it was shown that the camphor-bound forms of the oxidized, reduced, and dioxyferrous states of P-450$_{cam}$ experienced no significant changes in the degree of active-site solvent exposure. To the extent that protein conformation at the active site might alter relative solvent accessibility, these results suggest that no protein residues which are distant from the active site in the available crystal structures are brought to the substrate binding and oxygen binding pocket as the enzyme proceeds through these various states of catalysis. If there is significant rearrangement of the active-site structure, it is not accompanied by the opening of any channels which allow for solvent entry.

The results obtained with the product of the P-450$_{cam}$ reaction, 5-*exo*-hydrocamphor, are noteworthy and require interpretation. The second-derivative spectrum of the P-450$_{cam}$/product complex affords an r_n value and high-spin population (26%) which would place it exactly on the previously defined curve depicting the relationship between these parameters. It is not immediately obvious how the addition of a hydroxyl group to the substrate, without subsequent conformational change of the active-site structure, would result in increased solvent exposure of the heme environment. From the second-derivative spectral results, it appears that the small fraction of high-spin enzyme present when the hydroxycamphor is bound does not result from a completely "dry" active site, with the hydroxyl group of the product providing a weak ligand to the iron and producing a predominantly low-spin complex. Instead, the large difference in spin state between substrate and product is, on the basis of the second-derivative results, due to an actual change in solvent accessibility. Either the hydroxycamphor does not bind in the same orientation or location as camphor does, or the added polarity of the bound molecule is sufficient to attract water molecules to the active site. Alternatively, it is conceivable that the hydroxyl group, in the absence of water molecules, is sufficiently polar to affect the UV spectrum of nearby tyrosine residues although it would be expected to be orientated at a considerable distance from tyrosine-96. The spin state of the product complex is an essential feature of the mechanisms of P-450 catalysis since a high-spin product complex would result in further electron transfer and the continuation of the catalytic cycle before product exit and substrate entry. It appears that P-450$_{cam}$ "switches off" electron transfer to the product complex by maintenance of a predominantly low-spin configuration when hydroxycamphor is bound and that this is accomplished by allowing greater solvent access to this form of the enzyme active site. This polarity change may be directly responsible for the change in protein redox potential (Fisher & Sligar, 1988; Poulos et al., 1989). The X-ray crystal structure of the hydroxycamphor/P-450$_{cam}$ complex may explain how this increased water accessibility is achieved, or if the hydroxyl group on the camphor skeleton is sufficient to affect the second-derivative spectrum.

Registry No. IBM, 6704-56-9; P-450, 9035-51-2; L-Tyr, 60-18-4; camphor, 76-22-2; 5-*exo*-hydroxycamphor, 13948-58-8.

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