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## Tyrosine Motions in Relation to the Ferric Spin Equilibrium of Cytochrome P-450<sub>cam</sub><sup>†</sup>

Mark T. Fisher and Stephen G. Sligar\*

Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

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**ABSTRACT:** Second derivative spectroscopy was used to determine the percentage of tyrosine residues that are exposed to solvent in cytochrome P-450<sub>cam</sub> isolated from *Pseudomonas putida*. The ratio between two peak to trough second derivative absorbance differences has been shown to be dependent on the polarity of the microenvironment surrounding tyrosine residues [Ragone, R., Colonana, G., Balestrieri, C., Servillo, L., & Irace, G. (1984) *Biochemistry* 23, 1871]. With a number of camphor analogues that independently vary the spin equilibrium of the ferric cytochrome P-450<sub>cam</sub>, experiments have demonstrated that the percentage of tyrosine residues exposed to solvent is linearly dependent on the percentage of ferric high-spin species present. This is not simply a function of the extent of substrate binding since in all cases the substrate concentration was sufficient to ensure saturation of the cytochrome. The local microenvironment of approximately one tyrosine residue appears to be linearly correlated with the percentage of ferric high-spin cytochrome. Structural studies of cytochrome P-450<sub>cam</sub> using small-angle X-ray scattering [Lewis, B. A., & Sligar, S. G. (1983) *J. Biol. Chem.* 258, 3599] and high-pressure difference spectroscopy [Fisher, M. T., Scarlata, S. F., & Sligar, S. G. (1985) *Arch. Biochem. Biophys.* 240, 456] imply that global conformational changes linked to the spin equilibria are small. Together with the data reported herein, these results suggest that one tyrosine residue is involved in a conformational change that is directly linked with the spin equilibrium.

Cytochrome P-450<sub>cam</sub> is a heme monooxygenase from *Pseudomonas putida* that is the ultimate electron acceptor of a short electron-transfer chain consisting of the flavoprotein, putidaredoxin reductase, and an iron-sulfur protein, putidaredoxin. The role of this monooxygenase is to catalyze the specific hydroxylation of the monoterpene camphor in the first metabolic step by which this molecule can provide a sole carbon and energy source for this organism (Hegegaard & Gunsalus, 1965). To hydroxylate camphor, the porphyrin-chelated ferric iron must undergo an initial reduction by putidaredoxin (Peterson, 1971; Gunsalus & Lipscomb, 1972); dioxygen then binds to the ferrous cytochrome P-450<sub>cam</sub>, and a second electron is transferred to the dioxygen-bound ferrous iron, ultimately creating a reactive oxygen species that is responsible for the hydroxylation event. It is at the initial reduction step that the overall activity of this enzyme is thought to be regulated through a shift in the reduction potential of cytochrome P-450<sub>cam</sub>. The observed redox potential shifts from -340 mV in camphor-free cytochrome to -173 mV in the camphor-bound species (Sligar & Gunsalus, 1976). This shift in reduction potential is significant since putidaredoxin bound to cytochrome P-450<sub>cam</sub> has a redox potential near -196 mV. As a result, the reduction of camphor-bound cytochrome P-450<sub>cam</sub> is thermodynamically favorable and that of substrate-free protein is thermodynamically unfavorable (Sligar & Gunsalus, 1976). This potential change is reflected in an increase in the first electron-transfer rate from 0.22 s<sup>-1</sup> to 41 s<sup>-1</sup> in this dienzyme complex (Pederson et al., 1977; Fisher &

Sligar, 1985). The redox potential shift and increased electron-transfer rate provide both thermodynamic and kinetic control for the reduction of cytochrome P-450<sub>cam</sub>, thus preventing the deleterious waste of vital reducing equivalents and concomitant production of reduced oxygen products when substrate is not present (Sligar et al., 1974). This precise control of redox potential is thought to be dictated in part by a shift in the ferric spin equilibrium from a predominantly low-spin ( $S = 1/2$ ) form to a predominantly high-spin ( $S = 5/2$ ) form upon substrate binding (Tsai et al., 1970; Sligar, 1976). Accompanying this change in heme spin state is a blue shift in the wavelength maximum of the Soret region, which has been shown to be directly correlated with magnetic properties of the iron center (Philson, 1977). This spectrally observable change in the heme ligand field conformation provides a means to study the associated structure-function relationship of this cytochrome.

Investigating the source of the spin-state changes occurring in the P-450<sub>cam</sub> heme protein demands that the various discernible protein structural changes accompanying the two spin states be addressed. Small-angle X-ray scattering (Lewis & Sligar, 1983) and high-pressure UV-visible spectroscopy (Fisher et al., 1985) have been previously employed and strongly suggest that the conformational change involved with the spin equilibria of cytochrome P-450<sub>cam</sub> is a local rather than global protein conformational change.

Various analogues of the normally metabolized substrate camphor can give rise to varying proportions of low and high ferric spin equilibria upon binding to cytochrome P-450<sub>cam</sub> (Gould et al., 1981) (Figure 1). Through the use of these

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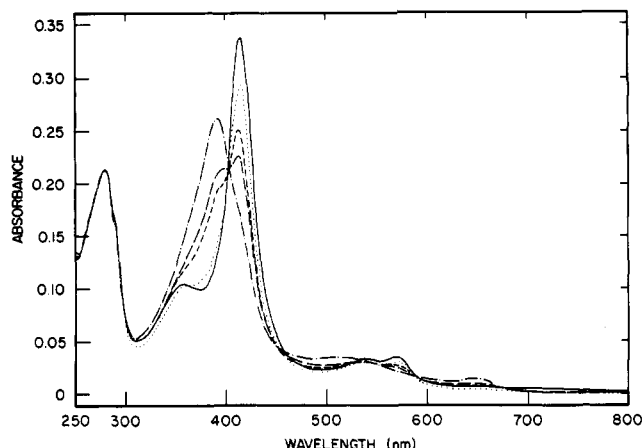


FIGURE 1: Absolute spectra of camphor (---), TMCH (---), norcamphor (---), camphenilone (---), and bound and substrate-free (—) cytochrome P-450<sub>cam</sub> at 23 °C. Each substrate-bound form shows a clean isosbestic point at 404 nm with varying degrees of initial conversion to the high-spin form.

camphor analogues, it is possible to independently vary and quantitate the spin, redox, and substrate binding equilibria (Murray et al., 1984). We have employed second derivative UV-visible spectroscopy (Ragone et al., 1984; Servillo et al., 1982) to determine whether there is any discernible change in the degree of tyrosine exposure to solvent when substrate-free and camphor or camphor analogue bound forms of the cytochrome are examined. Second derivative spectroscopy has been previously used to study conformational changes associated with tyrosine motions occurring in the interaction of hepatic cytochrome P-450<sub>cam</sub> reductase, P-450<sub>LM2</sub>,<sup>1</sup> and phospholipid (Ruckpaul et al., 1980), but these studies did not address a possible linkage of conformational changes with ferric heme spin state. This spectroscopic method provides another means of monitoring local conformational changes in cytochrome P-450<sub>cam</sub>. Results from our experiments indicate that the degree of tyrosine exposure is linearly dependent on the percentage of ferric high-spin P-450<sub>cam</sub> present. This observation provides the first documentation of a correlation between a functionally important protein motion and the regulatory role of the P-450<sub>cam</sub> spin equilibrium.

#### MATERIALS AND METHODS

The following compounds were purchased from Aldrich Chemical Co.: *d*-3-*endo*-bromocamphor, *d*-fenchone, camphenilone, adamantanone, adamantane, norcamphor, and 3,3,5,5-tetramethylcyclohexanone. *d*-5-*exo*-Hydroxycamphor and *d*-5-ketocamphor were generous gifts of Dr. I. C. Gunsalus (University of Illinois, Urbana) or were prepared by fermentation in our laboratory. *d*-5-*exo*-Bromocamphor was synthesized as previously described (Gould et al., 1981). *l*-Thiocamphor was prepared by treating *d*-camphor with H<sub>2</sub>S/HCl.

Cytochrome P-450<sub>cam</sub> was purified according to the method of Gunsalus & Wagner (1978). Protein purity determined by  $A_{391}/A_{280}$  was greater than 1.3. Substrate-free cytochrome P-450<sub>cam</sub> was obtained as outlined by Cole & Sligar (1981) and was prepared fresh. Buffer conditions were 50 mM potassium phosphate buffer, pH 7.0, at a temperature of 23 °C,

unless specified otherwise. Reduced cytochrome P-450<sub>cam</sub> was obtained by anaerobic photoreduction using 1  $\mu$ M proflavin as the reductant in the presence of 10 mM EDTA and 50 mM phosphate buffer, pH 7.0, and by exposing this solution to a 500-W heat-filtered xenon light source (Lipscomb et al., 1976). The O<sub>2</sub>-bound species was formed by admitting air into the Thunberg cuvette and shaking for 5 s to ensure O<sub>2</sub> saturation. The oxygen-bound species decays at a rate of 0.0077 s<sup>-1</sup> under experimental conditions and therefore requires the rapid acquisition of spectra before significant decay of the oxygen complex occurs. Ten spectra were taken within 30 s and stored on the HP8450A rapid-scan UV-visible spectrophotometer for second derivative analysis.

The fraction of high-spin cytochrome [HS] was obtained from optical spectra by using established extinction coefficients for pure high-spin and low-spin species at 391 and 417 nm. The observed differential absorption  $\Delta A$  is given by

$$\Delta A/[P-450_{cam}] = \{\epsilon_{HS(391)}[HS] + \epsilon_{LS(391)}(1 - [HS])\} - \{\epsilon_{LS(417)}(1 - [HS]) + \epsilon_{HS(417)}[HS]\} \quad (1)$$

where  $\epsilon_{HS(391)}$  and  $\epsilon_{LS(391)}$  are 105.3 and 47.9 nm<sup>-1</sup> cm<sup>-1</sup>, respectively, and  $\epsilon_{HS(417)}$  and  $\epsilon_{LS(417)}$  are 60.1 and 119.7 nm<sup>-1</sup> cm<sup>-1</sup>, respectively (Sligar, 1976).

Assays for second derivative spectroscopy were obtained with 5  $\mu$ M P-450<sub>cam</sub>. To ensure that the active site was saturated with substrate, substrate concentrations were used to saturate 95–99% of the cytochrome active sites according to independently derived dissociation constants (Murray et al., 1984; Fisher et al., 1985). All second derivative spectra were performed on a Hewlett-Packard 8450A rapid-scan UV-visible spectrophotometer. The two absorbance minima centered at 284 and 291 nm for native P-450<sub>cam</sub> and 283 and 290 nm for Gdn-HCl-denatured protein were used for analysis (Ragone et al., 1984; Servillo et al., 1982). The two maxima in the second derivative spectra were centered around 288 and 295 nm for native P-450<sub>cam</sub> and 287 and 294 nm for Gdn-HCl-denatured P-450<sub>cam</sub> (Figure 2). A value  $r$ , which is related to the degree of tyrosine exposure to solvent and is dependent on the tyrosine to tryptophan content of the protein, is obtained by using the formula (Ragone et al., 1984; Servillo et al., 1982):

$$r = \frac{\Delta A''_1}{\Delta A''_2} = \frac{A''_{288} - A''_{284}}{A''_{295} - A''_{291}} = \frac{Ax + B}{Cx + 1} \quad (2)$$

where  $\Delta A''_1$  and  $\Delta A''_2$  are second derivative differences at the two pairs of wavelengths mentioned in the above equation. The constants  $A$ ,  $B$ , and  $C$  correspond to

$$A = \frac{\Delta \epsilon''_1(\text{Tyr})}{\Delta \epsilon''_2(\text{Trp})} \quad B = \frac{\Delta \epsilon''_1(\text{Trp})}{\Delta \epsilon''_2(\text{Trp})} \quad C = \frac{\Delta \epsilon''_2(\text{Tyr})}{\Delta \epsilon''_2(\text{Trp})} \quad (3)$$

where  $\Delta \epsilon''$  is the difference between the second derivative of the molar extinction coefficients of the indicated pairs of fixed wavelengths (eq 1) and  $x$  is the molar ratio between tyrosine and tryptophan (Ragone et al., 1984; Servillo et al., 1982). Numerical values of the coefficients  $A$ ,  $B$ , and  $C$  in ethylene glycol (Table I) (Ragone et al., 1984) were used to calculate  $r_a$ , which represents the ratio if all tyrosine residues were embedded in the interior of the protein matrix.  $r_u$  is experimentally determined in the presence of 6 M Gdn-HCl (pH 6.5) and represents the corresponding value of  $r$  if all tyrosine residues were exposed to solvent (Ragone et al., 1984). The  $r_u$  value used to calculate the tyrosine/tryptophan ratio  $x$  was obtained from second derivative absorbance measurements on

<sup>1</sup> Abbreviations: P-450<sub>LM2</sub>, rabbit liver microsomal cytochrome P-450 inducible by in vivo pretreatment with phenobarbital; Gdn-HCl, guanidine hydrochloride; TMCH, 3,3,5,5-tetramethylcyclohexanone; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)amino-methane hydrochloride.

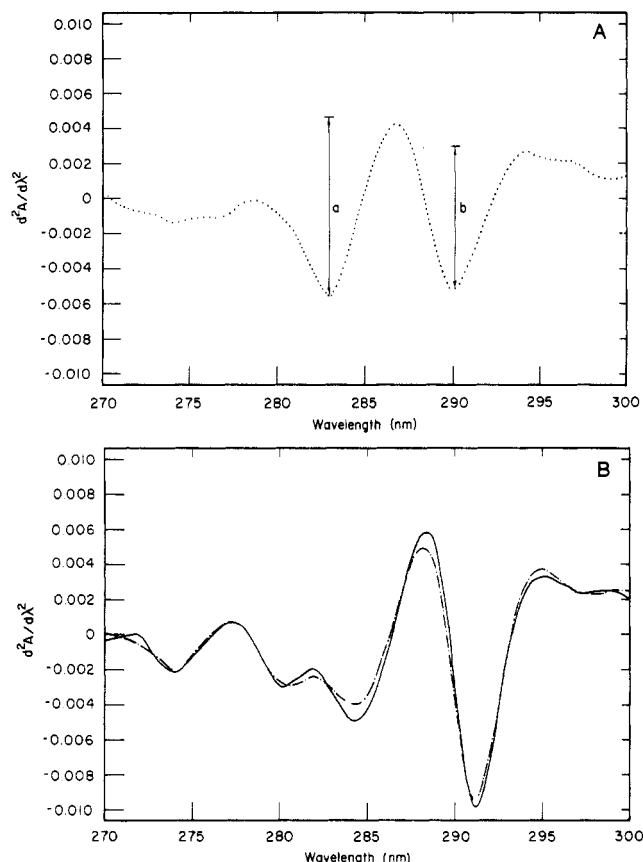


FIGURE 2: (A) Second derivative spectra of heme-extracted Gdn-HCl-denatured cytochrome P-450<sub>cam</sub>.  $r$  values ( $a/b$ ) are calculated from two peak to trough absorbances as indicated. The calculated  $r$  value (average of 30 spectra) is  $1.38 \pm 0.06$ . According to eq 2 (see text) the calculated tyrosine/tryptophan ratio is  $2.24 \pm 0.14$ , which agrees with previous protein sequencing data (Haniu et al., 1982). (B) Typical second derivative spectra of camphor-free (—) and camphor-bound forms (---) of cytochrome P-450<sub>cam</sub> in the region from 270 to 300 nm. The calculated  $r_n$  value is smaller for camphor-bound cytochrome P-450<sub>cam</sub>, which indicated that changes have occurred around the microenvironment of tyrosine residues.

Table I: Degree of Tyrosine Exposure and High-Spin Fraction

substrate	% high spin	$r_n$	no. of Tyr, $\alpha^b$	exposed Tyr
none	4.0	$0.789 \pm 0.015^a$	0.468	4.20
TMCH	14.0	$0.770 \pm 0.030$	0.451	4.06
norcamphor	44.5	$0.751 \pm 0.020$	0.435	3.92
fenchone	46.0	$0.756 \pm 0.020$	0.439	3.95
camphenilone	55.0	$0.752 \pm 0.020$	0.435	3.92
thiocamphor	60.5	$0.740 \pm 0.020$	0.425	3.83
5-ketocamphor	64.0	$0.741 \pm 0.030$	0.426	3.83
3-endo-bromo-camphor	67.0	$0.740 \pm 0.020$	0.425	3.83
adamantane	79.0	$0.732 \pm 0.020$	0.418	3.76
5-exo-bromo-camphor	81.0	$0.735 \pm 0.020$	0.42	3.78
camphor	95.0	$0.710 \pm 0.020$	0.398	3.58
adamantanone	99.0	$0.720 \pm 0.020$	0.407	3.66

<sup>a</sup>One standard deviation is indicated for an average of 30 spectra obtained for each  $r_n$  value. <sup>b</sup> $\alpha = (r_n - r_a)/(r_u - r_a)$ , where  $r_a = 0.260$  given  $A = -0.18$ ,  $B = 0.64$ , and  $C = -0.04$  (Ragone et al., 1984).  $r_u$  is experimentally determined to be  $1.38 \pm 0.06$ .

apocytochrome P-450<sub>cam</sub> prepared by extensive dialysis against 6 M Gdn-HCl (pH 5).  $r_n$  represents the ratio obtained when the protein is in its native state. The percentage of tyrosine residues that are exposed,  $\alpha$ , is calculated by

$$\alpha = (r_n - r_a)/(r_u - r_a) \quad (4)$$

Since these camphor analogues absorb in the UV region, the

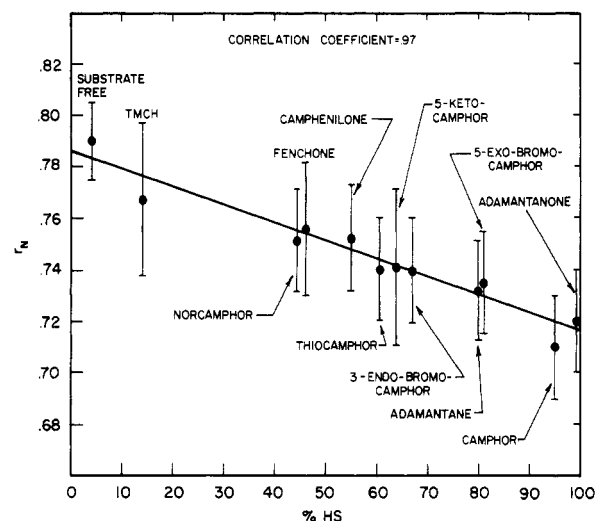


FIGURE 3: A linear correlation (correlation coefficient, 0.97) is obtained when  $r_n$  values are plotted against the percent high-spin fraction of each substrate-saturated cytochrome P-450<sub>cam</sub>. The camphor analogue adamantane, which lacks a ketone function, binds to cytochrome P-450<sub>cam</sub> yielding an  $r_n$  value that falls on the linear plot. This strongly suggests that H-bond formation between tyrosine and the substrate is not the origin of the spectral perturbation.

concentrations of these analogues were kept constant in both the reference and sample cuvettes. However, this is not a crucial precaution because an excess of substrate in the sample cuvette did not change the calculated  $r_n$  value.

## RESULTS

Figure 2A shows a typical second derivative spectrum recorded for heme-extracted Gdn-HCl-denatured cytochrome P-450<sub>cam</sub>. The  $r_u$  value is obtained from two peak to trough second derivative absorbance differences ( $a/b$ , Figure 2A) and is directly proportional to the tyrosine/tryptophan ratio as indicated by eq 2 (Servillo et al., 1982). The second derivative absorption spectra for native cytochrome P-450<sub>cam</sub> yields an  $r_n$  value that is related to the degree of tyrosine residues exposed to solvent (Ragone et al., 1984). The reported  $r_n$  value (Table I) for each camphor and camphor analogue bound P-450<sub>cam</sub> species was the mean value obtained from a total of 30 spectra. The corresponding changes in the peak and trough absorbances between the predominantly low-spin substrate-free form and the predominantly high-spin camphor-bound forms (Figure 2B) follow the same trend observed by Ragone et al. (1984) when model tyrosine compounds were placed in decreasingly polar environments (Figure 2). These changes in ultraviolet spectrum are not due to the heme center as no substantial absorbance changes accompanying spin-state and valence changes of the porphyrin-chelated iron in the region from 250 to 300 nm have been observed (Brill, 1977). For example, the UV difference spectrum between the inorganic metal complexes biscyanoferritroporphyrin (low spin) and hematin (high spin) showed a lack of both fine structure and substantial absorption bands in the 250–300-nm region (Brill & Sandberg, 1967).

The total degree of tyrosine exposure to solvent is observed to decrease as the percentage of high-spin cytochrome is increased (Figure 3) and is consistent with the equivalent of one tyrosine becoming embedded in the interior of the protein matrix (Table I) as the porphyrin-bound ferric iron shifts from a low-spin to a predominantly high-spin form. By use of the procedure described under Materials and Methods, an  $r_u$  value of  $1.38 \pm 0.06$  and hence a tyrosine to tryptophan ratio of  $2.24 \pm 0.14$  was calculated for cytochrome P-450<sub>cam</sub>, which is in

Table II: Dependence of Potassium Ion Concentration on Percent Ferric High Spin and Degree of Tyrosine Exposure<sup>a</sup>

% high spin	[K <sup>+</sup> ] (mM)	$r_n$
10	0	0.766
55	75	0.755
65	140	0.746
75	250	0.739

<sup>a</sup> Potassium ion from a 1 M stock solution (50 mM Tris-HCl, pH 7.0) was added to a sample cuvette containing 50 mM Tris-HCl, pH 7.0, 1.6 mM fenchone, and 5.3  $\mu$ M cytochrome P-450<sub>cam</sub>. Equal aliquots of the potassium ion stock solution were added to the reference solution to ensure that fenchone concentrations remain equal. A good correlation between the percent high spin and  $r_n$  value can be seen in relation to the linear plot in Figure 3.

excellent agreement with the expected ratio 2.25 derived from the amino acid sequence reported by Haniu et al. (1982).

In all but one camphor analogue (thiocamphor), a carbonyl function or related moiety is present. Adamantane lacks a carbonyl function and was used to observe the effects on the  $r_n$  value in relation to the percent high-spin species it induced. Although complete saturation could not be obtained with this camphor analogue due to the lack of adequate solubility, the  $r_n$  value obtained for this partially saturated cytochrome falls on the linear plot with the percent high-spin cytochrome P-450<sub>cam</sub> species it induces, indicating that a carbonyl function is not required in the substrate structure to induce this change in tyrosine microenvironment.

The ferric spin equilibrium of cytochrome P-450<sub>cam</sub> has been shown to be temperature-dependent (Sligar, 1976), where an increase in the low-spin species with the absorbance maximum at 417 nm increases with decreasing temperature. It must be noted however that the changes in low-spin content with decreasing temperatures are small (approximately 6%). No substantial correlation between the percent high-spin species present vs. the  $r_n$  values obtained for substrate-free cytochrome P-450<sub>cam</sub> was seen as the temperature was decreased from 27 to 4 °C (data not shown). Exposing camphor-free cytochrome P-450<sub>cam</sub> to higher temperatures (40–50 °C) irreversibly denatures the protein to give an absorption maximum at 378 nm for the oxidized ferric form and 420 nm for the ferrous CO-bound form. The corresponding  $r_n$  value is higher ( $0.92 \pm 0.03$ ) than that of the native substrate-free form, which suggests some unfolding of the native structure has taken place. This  $r_n$  value remains relatively the same when this denatured form of the protein is returned to 20 °C.

Increasing the potassium ion concentration in the presence of cytochrome and the camphor analogue fenchone results in an increase in the percentage of fenchone-bound high-spin cytochrome. The calculated  $r_n$  values in this case correlate very well with the percent high-spin species present (Table II). It is crucial to maintain the fenchone concentration at a level sufficient to ensure that the P-450<sub>cam</sub> active site remains saturated as the potassium ion concentration is decreased since it has been shown (Peterson, 1971) that an increase in potassium ion concentration will decrease the dissociation constant for substrate. This further demonstrates that it is the conformation of the high-spin cytochrome that is linked to the change in tyrosine microenvironment and not simply the presence of substrate at the active site.

The calculated  $r_n$  values between oxidized and reduced camphor-bound, fenchone-bound, and substrate-free cytochrome P-450<sub>cam</sub> were found to be very similar (Table III). No significant differences in either the shape or wavelength maximum are observed in the Soret region for the different camphor analogue bound forms of reduced cytochrome P-450<sub>cam</sub>. This observation provides further evidence that the

Table III: Comparison of  $r_n$  Values between Different Oxidation States of Cytochrome P-450<sup>a</sup>

substrate	% high spin Fe <sup>3+</sup>	$r_n$		
		Fe <sup>3+</sup> (oxidized)	Fe <sup>2+</sup> (reduced)	Fe <sup>2+</sup> -O <sub>2</sub> complex
camphor	95	0.709 $\pm$ 0.024	0.713 $\pm$ 0.024	0.706 $\pm$ 0.025
fenchone	46	0.742 $\pm$ 0.025	0.753 $\pm$ 0.025	
substrate free	4	0.79 $\pm$ 0.028	0.770 $\pm$ 0.024	

<sup>a</sup> The  $r_n$  values between oxidized and reduced forms of the cytochrome are similar and indicate conformational similarity between the oxidized and reduced proteins. Cytochrome P-450<sub>cam</sub> was photoreduced in the presence of 1  $\mu$ M proflavin, 10 mM EDTA, and 50 mM phosphate buffer at pH 7.0.

changes in the Soret region do not dictate the  $r_n$  values obtained from second derivative spectra in the 250–300-nm wavelength region. Furthermore, this result indicates that there is conformational similarity between the oxidized and reduced forms of cytochrome P-450<sub>cam</sub>. Also, no significant differences in  $r_n$  values between the reduced camphor-oxygen-bound cytochrome P-450<sub>cam</sub> ternary complex (average of 10 spectra) and the oxidized camphor-bound cytochrome P-450<sub>cam</sub> were observed (Table II), which suggests that the same conformational changes that exist in the ferric enzyme are reflected in the reduced and ferrous-oxygenated forms.

## DISCUSSION

By second derivative spectroscopy it was found that a ratio,  $r$ , between two peak to trough second derivative absorbance differences was particularly sensitive to both the total number of tyrosine residues present and the degree of their exposure to solvent (Ragone et al., 1984). Although the total absorption due to tryptophan residues is larger than that of tyrosine residues, tryptophan absorption spectra do not change significantly as their microenvironment changes polarity (Ragone et al., 1984). For various proteins the percentage of tyrosine exposed to solvent,  $\alpha$ , is in agreement with that derived from X-ray structures (Ragone et al., 1984). As outlined under Materials and Methods,  $\alpha$  is calculated from eq 4, where  $r_n$  and  $r_o$  represent a numerical ratio ( $a/b$ ) (Figure 2) for native and Gdn-HCl-denatured protein and  $r_a$  represents the ratio obtained where all residues would reside in the interior of the protein. The  $r_a$  value was obtained by knowing the tyrosine/tryptophan ratio,  $x$ , and the extinction coefficients were obtained by using model tyrosine and tryptophan compounds dissolved in ethylene glycol (Ragone et al., 1984).

Second derivative spectroscopy alone cannot determine whether the incorporation of the equivalent of one tyrosine residue into the interior of the protein is due to one tyrosine residue or a combination of all nine tyrosine residues. However, upon examination of results obtained from high-pressure studies (Fisher et al., 1985), the calculated volume changes accompanying camphor and camphor analogue pressure-induced dissociations suggested that global conformational changes are the same for camphor and camphor analogue binding. From these high-pressure investigations one might expect that the degree of exposure of the tyrosine residues would be the same for all substrates bound to the cytochrome. However, each of the substrates used induces a different change in the spin state of ferric heme when bound. Changes in the microenvironment of tyrosine residues appear to be directly correlated to the percentage of high-spin species present and hence to the ferric spin equilibrium of the substrate-bound cytochromes.

We suggest that a localized conformational change is directly involved in controlling the spin equilibrium, which also involves a change in the microenvironment of a single tyrosine residue. This must be a local rather than global change inasmuch as small-angle X-ray scattering has documented the same radius of gyration for the predominantly high-spin form ( $S = 5/2$ , camphor-bound cytochrome P-450<sub>cam</sub> (23.9 Å), and the predominantly low-spin form ( $S = 1/2$ ), substrate-free cytochrome P-450<sub>cam</sub> (23.7 Å) (Lewis & Sligar, 1983). The hypothesis that tyrosine motion or changes in the tyrosine microenvironment are intimately connected with the cytochrome P-450<sub>cam</sub> active site is supported by experimental results obtained by Ruckpaul and co-workers, who modified select tyrosine residues in hepatic cytochrome P-450<sub>LM2</sub> with tetranitromethane. In this case, changes in the P-450<sub>LM2</sub> absorption spectra in conjunction with tyrosine modification were prevented in the presence of an exogenous sixth ligand such as metyrapone. This suggests that the essential tyrosine is either close to the active site of P-450<sub>LM2</sub> or intimately involved in the conformational change accompanying the change in spin state (Janig et al., 1984).

Previous workers have documented that potassium ions can increase the fraction of ferric high-spin species present at a constant camphor concentration (Lang et al., 1977; Philson, 1977). It is clear from the second derivative absorbance measurements that the  $r_n$  values also decrease with increasing high-spin and potassium concentrations in the presence of a constant fenchone concentration. Here again the  $r_n$  value is shown to be directly dependent on the percent high-spin species present and independent of the amount of substrate present. Thus, the change in tyrosine microenvironment is linked to the conformational change monitored by the spin state of the heme protein.

According to the recently determined X-ray structure of camphor-bound cytochrome P-450<sub>cam</sub> (Poulos et al., 1985), one tyrosine residue (Tyr-98) seems to be an integral part of the active site of P-450<sub>cam</sub> and is therefore an excellent candidate for the manifestation of the observed changes in the second derivative spectrum. From this X-ray structural analysis, the tyrosine hydroxyl proton and the carbonyl oxygen of the substrate camphor are sufficiently close to form a hydrogen bond. In order to test whether this hydrogen bond is central to the observed changes in tyrosine microenvironment, the  $r_n$  value for the camphor analogue adamantane, which lacks a carbonyl function and yet is regioselectively hydroxylated (White et al., 1984), was studied. The data show that the  $r_n$  value falls on the same linear plot with the percentage of high-spin cytochrome P-450<sub>cam</sub> species induced by the binding of this substrate, suggesting that the proposed hydrogen bond between substrate carbonyl oxygen and tyrosine-98 is not required for the manifestation of the spin-state-linked second derivative absorbance changes. Therefore, the decrease in the  $r_n$  value with increasing high-spin content is most likely not the result of any electronic perturbation of the tyrosine-98 electronic structure due to alterations in hydrogen bond strength. The hydroxyl group of this tyrosine residue and the porphyrin-chelated iron moiety are separated by a distance of 9.5 Å. The angle between the aromatic plane of tyrosine-98 and the heme plane is approximately 76°. It is important to note, however, that tyrosine-98 is flanked by two phenylalanine residues (Phe-89 and Phe-100), and calculated distances from the X-ray analysis between the  $\gamma$ -carbon on tyrosine-98 and  $\gamma$ -carbon atoms on Phe-89 and -100 are 4.79 and 5.20 Å, respectively (Poulos et al., 1985). Although these two phenylalanine residues could provide the variability in the mi-

croenvironment of tyrosine-98, the exact position, as well as the movement and/or change in the microenvironment of the proposed essential tyrosine, is not known at present. The position of this proposed essential tyrosine may be revealed through X-ray analysis of the substrate-free form of cytochrome P-450<sub>cam</sub>.

Second derivative UV-visible spectroscopy performed with the reduced and oxidized forms of the cytochrome also reveals that these two macroscopic conformation states have very similar tyrosine microenvironments. Since the  $r_n$  value is actually a function of the absorbance of nine tyrosine residues, the fact that reduced and oxidized forms of the protein have similar  $r_n$  values indicates that the conformational changes induced by substrate binding in the oxidized form of cytochrome P-450<sub>cam</sub> are retained when the cytochrome undergoes a single-electron reduction. The effects of local and global structural variations in the cytochrome P-450<sub>cam</sub> macromolecule on the chemistry and second-electron-transfer events in the detailed reaction cycle are currently under investigation.

**Registry No.** K, 7440-09-7; Fe, 7439-89-6; P-450, 9035-51-2; TMCH, 14376-79-5; norcamphor, 497-38-1; *d*-fenchone, 7787-20-4; camphenilone, 13211-15-9; *l*-thiocamphor, 52078-93-0; *d*-5-ketocamphor, 13898-78-7; *d*-3-*endo*-bromocamphor, 10293-06-8; adamantane, 281-23-2; *d*-5-*exo*-bromocamphor, 10293-00-2; *d*-camphor, 464-49-3; adamantanone, 700-58-3; L-tyrosine, 60-18-4.

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## Temperature Dependence of Fibrin Polymerization: A Light Scattering Study<sup>†</sup>

Giovanni Dietler\* and Werner Känzig

*Laboratorium für Festkörperphysik, ETH-Hönggerberg, CH-8093 Zürich, Switzerland*

André Haeberli and P. Werner Straub

*Thromboselaboratorium, Medizinische Universitätsklinik, Inselspital, CH-3010 Bern, Switzerland*

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**ABSTRACT:** The aggregation of fibrin occurring in a fibrinogen solution upon addition of the enzyme thrombin has been studied prior to the sol-gel transition at different temperatures by means of dynamic light scattering and simultaneous measurement of the released fibrinopeptide A (FPA). The evolution of the polymer distribution with time was found to be independent of the temperature. The analysis of the experiments yields the explanation: with increasing temperature the rate of FPA release increases because it involves an activation energy, whereas the aggregation rate of the fibrin monomers decreases because it is exothermic. The light scattering experiments show that the state of aggregation is a chemical equilibrium that can be shifted by the addition of the tetrapeptide Gly-Pro-Arg-Pro. From dynamic light scattering data it is possible to derive the probability of bond formation between fibrin molecules and from it the aggregation enthalpy. For 30 °C a value of -19 kcal/mol was obtained.

The experimental work described and interpreted here was undertaken with the purpose to gain insight into the temperature dependence of the polymerization process going on in a fibrinogen solution after the addition of the enzyme thrombin. The attention was focussed on the following aspects: enzyme kinetics, evolution of the polymer distribution, polymerization enthalpy and the question of chemical equilibrium, and possible temperature dependence of the monomer conformation. As in previous work (Wiltzius et al., 1982b), the evolution of the polymer distribution in the pregelation phase is investigated by means of static and dynamic light scattering and simultaneous determination of the number of the enzymatically activated binding sites through measurements of the number of the released fibrinopeptides A (FPA).

The dependence of the enzymatic step upon temperature can be interpreted in terms of Michaelis-Menten kinetics. The temperature dependence of the spontaneous aggregation of the monomers is interpreted under the assumption of a chemical equilibrium. Although the reaction proceeds, no evidence was found for nonequilibrium states, at least in the early stages of the pregelation phase and for moderate thrombin concentrations. The chemical equilibrium can be shifted by variation of temperature, concentration, or pH (Scheraga, 1983) or by addition of the competitive peptides Gly-Pro-Arg or Gly-Pro-Arg-Pro (Laudano & Doolittle, 1978). A quantitative understanding of this equilibrium requires knowledge of the polymerization enthalpy. The derivation of this quantity from calorimetric measurements is insofar problematic as the cor-

rections that have to be made for chemical processes occurring together with the aggregation introduce a considerable uncertainty (Rozenfeld et al., 1976; Sturtevant et al., 1955). It will be shown that a combination of the light scattering experiments with measurements of the FPA release permits a specific determination of the polymerization enthalpy devoid of chemical interference with the aggregation process.

The investigation of monomer solutions by means of dynamic light scattering indicates a change of the conformation of the monomers with temperature. Since such a change might have an influence on the accessibility of the binding sites, it is likely to be a further temperature-dependent factor influencing the aggregation.

### EXPERIMENTAL PROCEDURES

**Sample Preparation.** A sample consisting of a solution of 2 mL of human fibrinogen (Imco, Stockholm, 2 mg/mL in 0.05 Tris-0.1 M NaCl buffer, pH 7.4) was rapidly thawed to the temperature desired for the experiment and transferred into a cylindrical quartz cell. For each experiment, 250  $\mu$ L of a thrombin solution with a concentration of 0.01 NIH unit/mL was freshly prepared from a stock solution with a concentration of 1 NIH unit/mL (bovine thrombin, Roche, Basel, Switzerland) and then added to the fibrinogen solution under gentle shaking of the cell.

Great care was taken to assure that the thrombin concentration was the same in each experiment: control runs have shown that the evolution of the scattering intensity with time is perfectly reproducible. Prior to the light scattering experiments, large contaminant particles were sedimented by centrifugation at 10000g during 120 s at the desired tem-

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