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Temperature Jump Relaxation Kinetics of the P-450$_{cam}$ Spin Equilibrium†

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ABSTRACT: The ferric spin-state equilibrium and relaxation rate of cytochrome P-450 has been examined with temperature jump spectroscopy using a number of camphor analogues known to induce different mixed spin states in the substrate-bound complexes [Gould, P., Gelb, M., & Sligar, S. G. (1981) *J. Biol. Chem. 256*, 6686]. All temperature-induced spectral changes were monophasic, and the spin-state relaxation rate reached a limiting value at high substrate concentrations. The ferric spin equilibrium constant, K_{spin}, is defined in terms of the rate constants k_1 and k_{-1} via $K_{spin} = k_1/k_{-1}$ = [P-450(HS)]/[P-450(LS)] where HS and LS represent high-spin ($S = {}^5/_2$) and low-spin ($S = {}^1/_2$) ferric iron, respectively, and the spectrally observed spin-state relaxation rate by $k_{obsd} = k_1 + k_{-1}$. A strong correlation between the fraction of high-spin species and the rate constant, k_{-1}, is observed. For a 3 °C temperature jump (from 10 to 13 °C), the 23% high-spin tetramethylcyclohexanone complex ($K_d = 45 \pm 20$ μM) is characterized by a ferric spin relaxation rate of $k_{obsd} = 1990$ s^{-1}, while the rates for the *d*-fenchone (41% high spin, $K_d = 42 \pm 10$ μM) and camphoroquinone (75% high spin, $K_d = 15 \pm 5$ μM) complexes are 1430 and 346 s^{-1}, respectively. The transition rates (k_{-1}) from high- to low-spin cytochrome with these substrates are 1380, 675, and 75 s^{-1}, respectively, suggesting that the decrease in k_{-1}, which correlates with the increasing fraction of high-spin species present at equilibrium, is due to a restricted access and recombination rate of the water axial ligand with the ferric iron.

The bacterium *Pseudomonas putida* has the ability to use the monoterpene camphor as its sole source of carbon and energy (Hedegaard & Gunsalus, 1965). The first step in the metabolism of camphor to isobutyrate and acetate involves a stereospecific hydroxylation at the 5-exo position of this substrate by the heme protein monooxygenase cytochrome P-450$_{cam}$. Cytochrome P-450$_{cam}$ is the ultimate electron acceptor of a soluble electron-transfer chain consisting of an NADH-coupled flavoprotein, putidaredoxin reductase, and an iron–sulfur protein, putidaredoxin. Upon binding the natural substrate camphor, the maximum of the Soret band for this cytochrome is significantly blue shifted, from 417 nm for the substrate-free cytochrome to 391 nm in the camphor-bound form (Tsai et al., 1972). This shift in the Soret band is due to a conversion of the porphyrin-chelated ferric iron from predominantly low spin ($S = {}^1/_2$) in the substrate-free form to predominantly high spin ($S = {}^5/_2$) in the camphor-bound form (Tsai et al., 1970; Philson, 1977; Sligar, 1976). The reduction potential for P-450$_{cam}$ becomes significantly more positive when the camphor substrate binds, shifting from −340 to −173 mV (Sligar & Gunsalus, 1976). This alteration in heme protein potential allows the first electron-transfer event from the iron–sulfur protein putidaredoxin to become thermodynamically facile, as putidaredoxin bound to P-450$_{cam}$ exhibits a reduction potential of about −196 mV. The first-order reduction rate for the P-450$_{cam}$ heme in the diprotein complex with putidaredoxin is likewise modulated by the presence of substrate (Pederson et al., 1976; Lange et al., 1977; Gould et al., 1981; Fisher & Sligar, 1985a).

Upon binding different camphor analogues, ferric P-450$_{cam}$ exists in varying proportions of high- and low-spin forms. Any conformational changes accompanying substrate binding appear to be minor local perturbations rather than large-scale global movements (Lewis & Sligar, 1983; Fisher et al., 1985; Poulos et al., 1985, 1986). Recently, the first electron-transfer rates between the iron–sulfur protein and various camphor and camphor analogue bound forms of cytochrome P-450$_{cam}$ were determined (Fisher & Sligar, 1985a). Linear free energy relationships were observed between the spin equilibrium and both the kinetically derived reduction potential and the activation energy for forward electron transfer. Thus, changes in the ferric spin state profoundly affect the initial reduction events in this cytochrome although the precise relationship between the substrate and protein structures in the modulation of the reduction potential and ferric spin state remains an open question.

Experimental evidence from X-ray structure analysis (Poulos et al., 1985, 1986) and NMR spectroscopy (Griffin & Peterson, 1975; Philson, 1975) indicates that water has easy access to the heme-containing active site of predominantly low-spin substrate-free cytochrome P-450$_{cam}$. Upon binding the substrate camphor, solvent water is excluded from the cytochrome P-450$_{cam}$ active site, and a predominantly high-spin heme iron is formed. A change in the heme spin state upon binding various camphor analogues is reflected in an alteration of the P-450$_{cam}$ active-site polarity (Fisher & Sligar, 1985b). These results indicate that there are significant reorganizational differences between the low- and high-spin forms of cytochrome P-450$_{cam}$.

To elucidate the possible origins of this structural interaction, static and dynamic measurements of the ferric spin equilibrium were monitored by temperature relaxation techniques. The thermodynamic parameters obtained by a van't Hoff analysis of the cytochrome P-450 spin equilibrium P-450·S_{hs} ↔ P-450·S_{ls}, where S represents the various camphor analogues, indicate that the magnitude of the enthalpy and entropy values increases as the fraction of low-spin species increases. This result is in agreement with the trends observed on the enthalpy and entropy values obtained for ferric spin equilibria for a number of heme proteins and inorganic model systems (Yonetani et al., 1972; George et al., 1964).

The dynamics of the ferric spin transition of various camphor or substrate analogue bound forms of cytochrome P-450$_{cam}$ were examined by temperature jump relaxation

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methods. A qualitative correlation between the fraction of high-spin species and the microscopic rate constant k_{-1} for the ferric spin equilibrium, $K_{spin} = k_1/k_{-1} = [P\text{-}450\text{-}S_{hs}]/[P\text{-}450\text{-}S_{ls}]$, was observed. These data suggest that the substrate molecules are intimately involved in controlling the macroscopic on rate of the water molecule that serves as the sixth axial ligand to the heme iron in the low-spin form of cytochrome $P\text{-}450_{cam}$.

Materials and Methods

Cytochrome $P\text{-}450_{cam}$ was purified by the method outlined by Gunsalus and Wagner (1979). Protein purity was monitored by the ratio of the absorbance values A_{391}/A_{280}, and only solutions with a ratio greater than 1.4 were used. *d*-Camphor, *d*-fenchone, 3,3,5,5-tetramethylcyclohexanone (TMCH), norcamphor, and camphoroquinone were purchased from Alrich Chemical Co. and used without further purification. Substrate-free cytochrome $P\text{-}450_{cam}$ was obtained by passage of the cytochrome over a Sephadex G-10 column equilibrated with 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, at 4 °C. The substrate-free eluant was then passed over a Sephadex G-25 column equilibrated with the final buffer (50 mM potassium phosphate/50 mM KCl, pH 7.0, unless specified otherwise). $P\text{-}450_{cam}$ was titrated by the method outlined by Gould et al. (1982) using a Hewlett-Packard 8450A rapid-scan diode array UV–visible spectrophotometer at 13 °C. This was the final temperature attained in the temperature jump experiments. Substrates were added as a methanolic solution with the total concentration of methanol not exceeding 0.5% final volume. The final protein concentration used for all the experiments was approximately 10 μM. Static temperature-dependent spin equilibria were determined by optical difference spectroscopy over a temperature range of 4–20 °C in increments of 3 °C. Substrate concentrations were at levels of 20–50-fold above the K_d value at 4 °C to ensure complete saturation of the heme-containing active site. The ferric spin equilibrium constant was determined at each temperature value by calculating the fraction of high-spin species present 20–50-fold above the K_d value using the ratio A_{417}/A_{391} outlined in the equation:

$$\frac{\epsilon_{417}A_{417} - \epsilon_{391}A_{391}}{A_{417}[\epsilon_{ls}(391) - \epsilon_{hs}(391)] + A_{391}[\epsilon_{hs}(417) - \epsilon_{ls}(417)]} \quad (1)$$

where $\epsilon_{ls}(391)$, $\epsilon_{hs}(391)$, $\epsilon_{ls}(417)$, and $\epsilon_{hs}(417)$ are the derived extinction coefficients for the pure low- and high-spin species: 47.9, 105.3, 119.0, and 60.1 mM^{-1} cm^{-1}, respectively (Sligar, 1976). Protein concentrations were determined by using the extinction coefficients for the reduced carbon monoxide spectrum at 446 nm (Gunsalus & Wagner, 1979). All temperature-induced spectral perturbations were completely reversible.

Temperature jump measurements were performed on a modified Messanlagen SBA7 transient spectrometer. Anode current from the phototube was converted to log voltage, and the trace was digitally recorded on a Nicolet 3091 digital oscilloscope. Data were transferred to a VAX 11/780 computer where the rate data were analyzed by the Guggenheim method. Temperature jumps were made from 10 to 13 °C, and 12 kinetic traces were recorded and averaged at 391 and 417 nm at each substrate concentration.

Results

Static Temperature-Dependent Ferric Spin Equilibrium Measurements. An optical difference spectrum was obtained when this cytochrome $P\text{-}450_{cam}$–substrate complex was subjected to varying temperature (Figure 1) (Cinti et al., 1979; Sligar, 1976), and the reference solution was maintained at 20 °C throughout. Observed temperature-dependent changes were completely reversible upon cyclic temperature variation. The existence of two isosbestic points at 406 and 460 nm indicates that the observed temperature effects can be described by a simple two-state system. The resulting absorbance changes were analyzed as defined under Materials and Methods, and the resulting equilibrium constant was plotted as a function of temperature using a standard van't Hoff analysis (Figure 2). In all cases, a linear relationship was observed with correlation coefficients greater than or equal to 0.996. The corresponding enthalpy and entropy changes for the high- to low-spin conversion for each substrate-bound cytochrome obtained from this analysis are presented in Table I. From these results, an obvious compensation exists for both enthalpy and entropy and the fraction of high-spin cytochrome initially present at 20 °C. The change in entropy favors the high-spin species for the ferric spin equilibrium in all camphor and camphor analogue bound forms of the cytochrome. Furthermore, this entropic advantage increases as the fraction

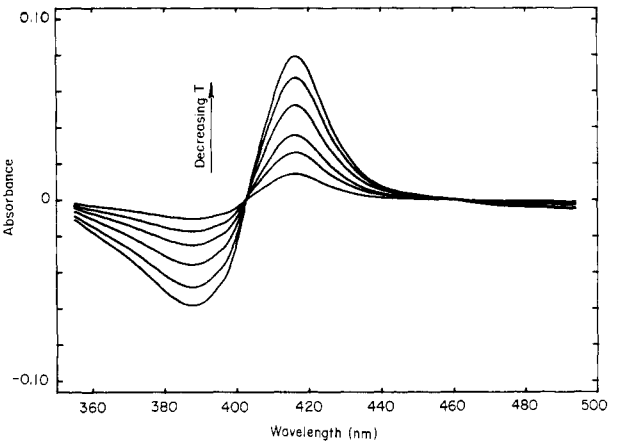

FIGURE 1: Typical temperature-induced difference spectra for tetramethylcyclohexanone-bound cytochrome $P\text{-}450_{cam}$. Traces were recorded at 17, 15, 13, 10, 7, and 4 °C against a reference sample at 20 °C. The temperature-dependent changes were completely reversible upon cyclic temperature variation. For the particular experiment illustrated, the $P\text{-}450_{cam}$ concentration was 10 μm in the presence of 2 mM TMCH. At this concentration of substrate, a 40-fold excess over the TMCH dissociation constant, saturation of the cytochrome $P\text{-}450_{cam}$ active site is ensured.

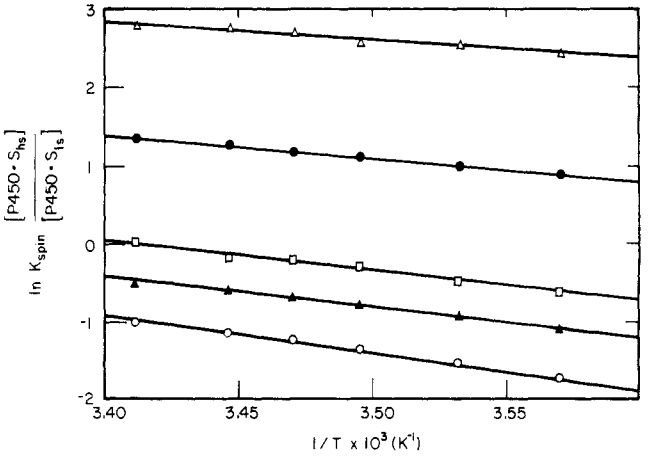

FIGURE 2: Equilibrium constant as a function of temperature for camphor (△), camphoroquinone (●), fenchone (□), norcamphor (▲), and tetramethylcyclohexanone (○) bound forms of cytochrome $P\text{-}450_{cam}$. The temperature-induced optical changes were analyzed by using the methods outlined under Materials and Methods.

Table I: Thermodynamic Parameters of the Cytochrome P-450$_{cam}$ Spin Equilibrium[a]

substrate/structure	K_{spin}[a] (20 °C)	% high spin (20 °C)	ΔH (kcal/mol)	ΔS (eu)
camphor	17.1	94	4.5	22.8
camphoroquinone	3.9	76	6.2	23.7
fenchone	1.1	52	7.6	26.0
norcamphor	0.7	42	7.9	26.2
tetramethylcyclohexanone	0.4	27	9.8	31.5

[a] $K_{spin} = [\text{P-450-S}_{hs}]/[\text{P-450-S}_{ls}] = \exp[\Delta S/R - \Delta H/RT]$.

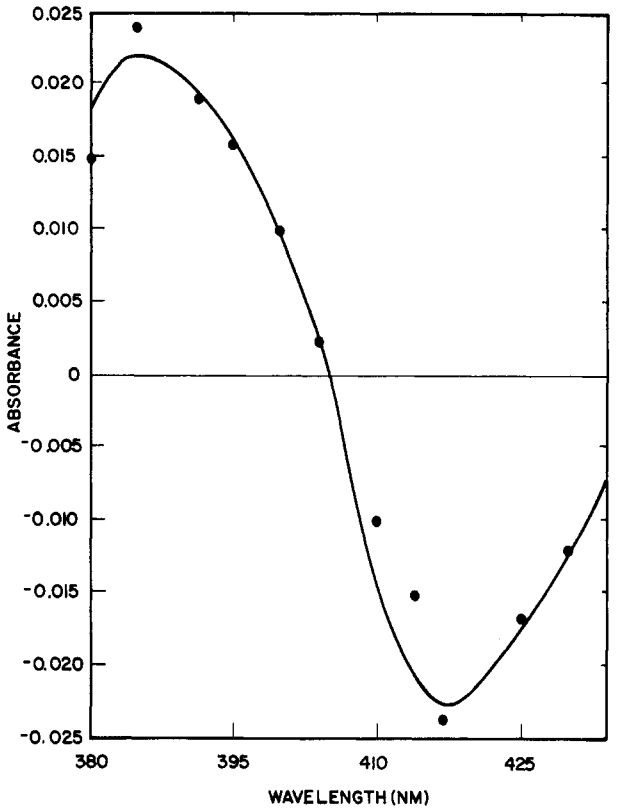

FIGURE 3: Temperature-induced difference spectrum constructed from kinetically observed spectral amplitudes. Each kinetically derived amplitude was collected for a 3 °C temperature jump of a 10 μM cytochrome P-450 solution in the presence of 3 mM norcamphor.

of low-spin species increases. Conversely, enthalpic contributions in the overall reaction P-450-S_{ls} ↔ P-450-S_{hs} are unfavorable, and the magnitude of this enthalpic change increases with the fraction of low-spin species present. Both the magnitude and sign of the entropy and enthalpy are consistent with the loss of the sixth axial ligand from the porphyrin-chelated ferric iron as the cytochrome shifts from a low-spin form ($S = {}^1/_2$) to a high-spin form ($S = {}^5/_2$). The larger magnitude of enthalpic and entropic changes observed with the substrates that induce more low-spin character in cytochrome P-450$_{cam}$ ferric spin equilibrium indicates that reorganization must accompany a spin transition in this cytochrome as the initial low-spin fraction increases.

Dynamic Temperature-Dependent Ferric Spin Equilibrium Measurements. The temperature-dependent absorbance changes with the various camphor analogue bound P-450 species were monitored kinetically at single wavelengths, and the maximal amplitudes of the observed changes at each wavelength were plotted in order to obtain a kinetic difference spectrum. The kinetically derived amplitudes for the norcamphor-bound form of P-450$_{cam}$ are shown in Figure 3. The magnitude of the observed Soret changes for a 3 °C temperature increase (i.e., increase at 391 nm, isosbestic point at

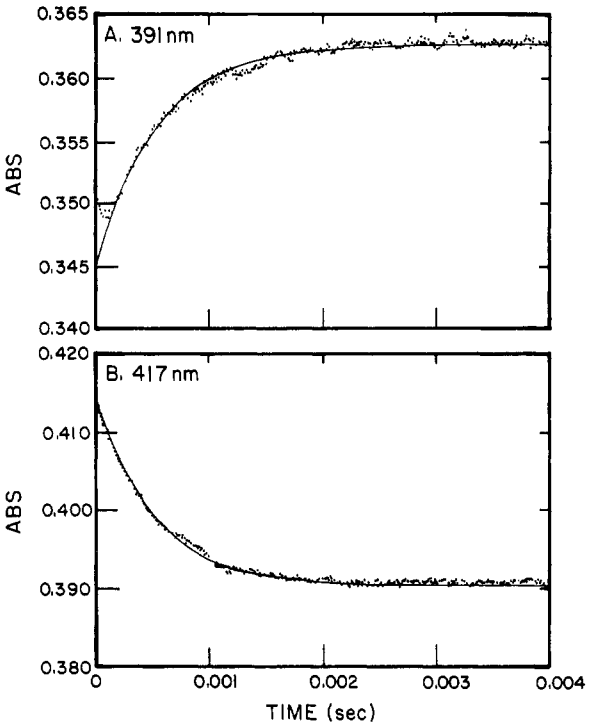

FIGURE 4: Representative kinetic traces for 10 to 13 °C temperature jump for cytochrome P-450$_{cam}$ in the presence of 1 mM norcamphor in a 50 mM potassium phosphate/50 mM Kcl buffer, pH 7.0. The solid trace represents a direct fit which follows first-order kinetics with a correlation coefficienct of 0.998. The observed rate constant calculated for each wavelength was 1890 s^{-1}.

Table II: Observed Relaxation Rates and Their Respective Microscopic Rate Constants for Substrate-Bound Forms of Cytochrome P-450$_{cam}$

substrate	k_{obsd} (s^{-1})	k_1 (s^{-1})	k_{-1} (s^{-1})
camphor	890 ± 200	860 ± 122	30 ± 12
camphoroquinone	346 ± 40	270 ± 31	75 ± 10
fenchone	1430 ± 130	880 ± 10	675 ± 113
norcamphor	2300 ± 200	1360 ± 277	980 ± 327
tetramethylcyclohexanone	1990 ± 202	560 ± 151	1380 ± 180

406 nm, decrease at 417 nm) is indicative of a ferric spin equilibrium and is completely identical with the temperature-induced difference spectrum obtained statically. All kinetic traces at all substrate concentrations exhibited simple first-order kinetics with one observed relaxation rate (Figure 4). First-order kinetics were previously observed in preliminary temperature jump studies with camphor-bound cytochrome P-450$_{cam}$ (Cole & Sligar, 1981). The observed relaxation rate exhibits saturation behavior at high substrate concentrations and indicates that the observed relaxation event cannot be explained by a simple bimolecular association mechanism (Eigen & de Maeyer, 1964). The temperature-induced absorption changes of cytochrome P-450$_{cam}$ were completely reversible. The observed relaxation rates were analyzed as a function of the free protein and substrate concentrations (derived from titration data at 13 °C) as illustrated in Figure 5 and Table II. The solid lines represent the expected relaxation rates derived from a function (eq 2b) that assumes the relaxation phenomenon is a simple two-step mechanism (eq 2a):

$$\text{P-450}_{ls} + \text{S} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{P-450}_{ls}\text{·S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{P-450}_{hs}\text{·S} \qquad (2a)$$

$$k_{obsd} = \frac{k_1 K_a[\text{P-450}_f + \text{S}_f]}{1 + K_a[\text{P-450}_f + \text{S}_f]} + k_{-1} \qquad (2b)$$

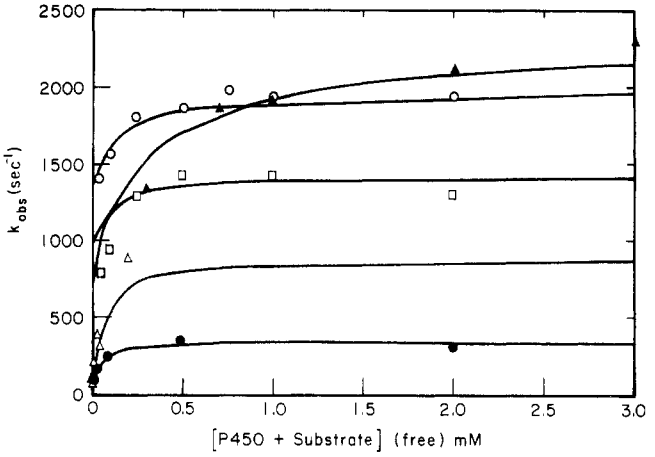

FIGURE 5: Relaxation rates as a function of the sum of the equilibrium substrate and protein concentrations. The solid lines are theoretical curves based on a simple two-step system assuming that the bimolecular association rate is fast compared to the slower conformational change accompanying the temperature-induced ferric spin transition (eq 2a,b, Table II). The representative substrates used were camphor (△), camphoroquinone (●), fenchone (□), norcamphor (▲), and tetramethylcyclohexanone (○). The macroscopic relaxation rate for the substrate-bound forms of the cytochrome and their corresponding microscopic rate constants are listed in Table II.

Table III: Comparison between Equilibrium and Kinetic Determinations of Spin Equilibrium and Substrate Dissociation Constants[a]

substrate	K_{spin}[b]		K_d[b] (μM)	
	static	kinetic	static	kinetic
camphor	13.3	30 ± 0.5	2.1 ± 0.9	3.5 ± 0.7
camphoroquinone	3.0	3.6 ± 0.1	16.0 ± 5.0	15.0 ± 5.0
fenchone	0.7	1.1 ± 0.4	42 ± 10	62 ± 40
norcamphor	0.5	1.6 ± 0.8	350 ± 50	211 ± 37
tetramethylcyclo-hexanone	0.3	0.4 ± 0.2	45 ± 20	95 ± 41

[a] All experiments were performed at 13 °C. [b] K_d and K_{spin} (kinetic) were determined as outlined under Results.

Here $K_a = k_{on}/k_{off}$, and $[P\text{-}450_f]$ and $[S_f]$ are the free protein and free substrate concentrations, respectively. Tsong and Yang (1978) have previously observed that a similar kinetic analysis performed on temperature jump data obtained for benzphetamine-bound mammalian cytochrome $P\text{-}450_{LM2}$ could adequately account for the observed plateau in the dependence of the observed relaxation rate on the free reactant concentration. In this analysis, the first step in this reaction sequence is a rapid equilibrium association reaction and defined by K_a. In this case, the observed dynamic absorbance change is due solely to the slower conformational changes occurring in the ferric spin transition. At high substrate concentrations, the relaxation rate approaches a maximum value, $k_{obsd} = k_1 + k_{-1}$, and the relaxation rate exhibits saturation behavior. At low substrate concentrations, the observed relaxation rate approaches the lower limit k_{-1}. The observed equilibrium constants $K_d = (1/K_a)/(1 + K_{spin})$ and $K_{spin} = k_1/k_{-1}$, derived by assuming a two-state mechanism, were compared to the equilibrium constants derived under static conditions and are listed in Table III. In most cases, there is good agreement between the statically derived constants and those obtained from the temperature jump experiments. Static and temperature jump experiments performed with the substrate-free form of cytochrome $P\text{-}450_{cam}$ revealed that the observed absorbance changes were not reversible. In the absence of substrate, there was a progressive formation of the denatured form of the cytochrome, P-420, evidenced by a reduced CO Soret maximum at 420 nm (Omura & Sato, 1964). No such

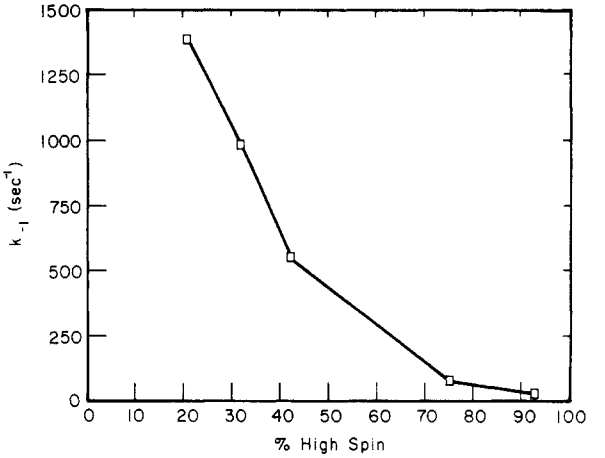

FIGURE 6: Correlation between the percent high-spin species and the microscopic rate k_{-1} for water binding to ferric $P\text{-}450_{cam}$.

formation of P-420 was observed with any of the substrate-bound cytochromes.

The variation in the microscopic rate constant, k_{-1}, determined with various substrates bound to $P\text{-}450_{cam}$ shows that a decrease in rate occurs as the fraction of high-spin species increases (Figure 6 and Table II). No such correlation is observed for the rate constant k_1. Since the ferric low-spin and high-spin species of cytochrome $P\text{-}450_{cam}$ are 6- and 5-coordinate, respectively (Poulos et al., 1985, 1986; Schiedt & Reed, 1981; White & Coon, 1982; LoBrutto et al., 1980; Koch et al., 1975; Griffin & Peterson, 1975), the increase in k_{-1} as the low-spin 6-coordinate fraction increases demonstrates that the recombination rate of the sixth ligand is increasing under these conditions. Therefore, the temperature jump kinetic results indicate that the recombination rate of the sixth ligand (either water or hydroxide ion) is ultimately controlled by the specific interaction of the substrate molecule and the protein.

DISCUSSION

The temperature-dependent ferric spin equilibrium of ferric cytochrome $P\text{-}450_{cam}$ was monitored through changes in the Soret region of the visible spectrum. In earlier studies, camphor and various camphor analogues were bound to cytochrome $P\text{-}450_{cam}$ (Gould et al., 1981; Fisher et al., 1985; Fisher & Sligar, 1985a,b; Atkins & Sligar, 1987) and used to produce various mixtures of high- and low-spin ferric species in order to examine the relationship between the spin equilibrium and various physical and chemical properties of this cytochrome. Static measurements of the temperature-dependent spin equilibrium indicate that there is an increase in entropy of the spin-state transition for those substrates which induce more initial low-spin character. In contrast, the enthalpy of the system decreases as the percentage of the high-spin ferric species increases. These results are in excellent agreement with the previously observed trends in enthalpy and entropy compensation (Sligar, 1976).

Numerous investigations have suggested that the low-spin ($S = {}^1/_2$) form of cytochrome $P\text{-}450_{cam}$ is 6-coordinate with the sixth axial ligand either water or hydroxide ion (Poulos et al., 1986; Schedit & Reed, 1981; LoBrutto et al., 1980; White & Coon, 1982; Griffin & Peterson, 1975). In contrast, the high-spin ($S = {}^5/_2$) form of cytochrome $P\text{-}450_{cam}$ has been shown to exist as a 5-coordinate complex (Poulos et al., 1985; LoBrutto et al., 1980; Koch et al., 1975). If the distribution of the high- and low-spin ferric cytochrome species induced in the presence of the various substrates is due to macroscopic populations of water-ligated (low-spin) and unligated (high-spin) ferric iron, a portion of the observed changes in the

enthalpy and entropy values for the ferric spin equilibrium of this cytochrome reflects a ligand change. The low-spin to high-spin transition is thus endothermic, and as a result, the high-spin form of the heme iron is favored at higher temperatures. By the same argument, the high-spin form would be favored by a positive entropy change due to the dissociation of the sixth axial ligand (H_2O/OH^-) and the release of ordered water molecules in the active site (Poulos et al., 1986b). Given this interpretation, we suggest that the larger distribution of high-spin character induced by a camphor analogue bound in the P-450$_{cam}$ active site is due to the greater exclusion of water from this pocket. Entropic contributions for the $S = {}^1/_2$ to $S = {}^5/_2$ transitions between the spin states with a multiplicity of $2S + 1$ can only contribute to an entropy of roughly 2 eu (George et al., 1964). Therefore, the larger entropic values observed for all the substrate-bound forms of the cytochrome P-450$_{cam}$ could reflect different amounts of water in the active site and a percentage of sixth axial ligation. A possible source of the entropy change decrease for the ferric spin-state transition of a high-spin species could reflect a decrease in the amount of bound water released as a result of an increase in the high-spin conformer. If this interpretation is correct, the low-spin species represents a more ordered system of protein and solvent due to the existence of ordered water structures within the protein active site. As a consequence, a decrease in the amount of bound water would lead to the observed decrease in entropy upon release of these water molecules.

In order to probe possible changes in the dynamics involved with the ferric spin equilibrium of various camphor and camphor analogue bound forms of cytochrome P-450$_{cam}$, temperature jump relaxation spectroscopy was used to determine the kinetics of the changes in the Soret region at 391 and 417 nm for a rapid 3 °C temperature jump. All camphor and camphor analgoue bound forms of the cytochrome exhibited monophasic relaxation rates and followed rigorous first-order kinetics. The perturbed system returned to its original absorbance value at the initial temperature (10 °C), and a clean isosbestic point was observed at 406 nm (Figure 3), indicating the existence of a simple two-step process. The relaxation rates approached a limiting value at high substrate concentrations (Figure 5), thus excluding the possibility that the observed kinetics were due to simple bimolecular association of the substrate with the cytochrome. A significant result obtained from these investigations is the correlation between the percentage of the low-spin character induced by the substrates and the microscopic rate constant, k_{-1}, describing the transition from high spin to low spin upon binding of a sixth heme ligand, (Figure 6 and Table II). If the ferric distribution is the result of different macroscopic populations of water ligation to the ferric iron, then k_{-1} represents the on rate of the ligation for the solvent-derived ligand (H_2O/OH^-). In this case, the increased ease of solvent entry into the cytochrome active site is a direct consequence of the given substrate–protein active-site topology.

Recent evidence obtained in our laboratory has shown that a lack of specificity in the hydroxylation event occurs when the substrate, norcamphor (which lacks three methyl groups), is present in the active site (Atkins & Sligar, 1987). With this substrate analogue, three hydroxylated products are obtained and are identified as 3-, 5-, and 6-*exo*-hydroxynorcamphor. The lack of specificity in the hydroxylation event is thought to arise from the elimination of several specific substrate–protein interactions (Poulos et al., 1985). When camphor is bound to cytochrome P-450$_{cam}$, several hydrophobic contacts between the protein and the bound substrate occur. Two specific hydrophobic interactions which occur between the protein active site and the camphor molecule are (1) the interactions between the 8,9-*gem*-dimethyl groups of camphor and the methyl moieties of valine-295 and (2) the hydrophobic interaction between the 10-methyl group of camphor and leucine-244 and isoleucine-247. It is possible that norcamphor, which lacks the 8,9-*gem*-dimethyl and 10-methyl groups of camphor, is less restrained in the active site, and as a result, the hydroxylation of this substrate is less specific. Also, less specific interaction between the protein contacts and the substrate can result in an increase in the dynamic protein fluctuations as the percentage of the low-spin form of the cytochrome increases. Thus, the alteration of specific interactions between the substrate and the protein can lead to an alteration in the accessibility of bulk solvent into the active site. This hypothesis could predict that the thermal factors derived from X-ray analysis for three separate segments of the protein structure (Poulos et al., 1986) should show a correlation to the percent low-spin character induced by a given camphor analogue.

The idea of an increased solvent accessibility into the cytochrome P-450$_{cam}$ active site as a function of the amount of low-spin character exhibited by this cytochrome is supported by a number of experimental observations. Pulsed NMR studies of ferric cytochrome P-450$_{cam}$ solutions (Griffin & Peterson, 1975; Philson, 1977) revealed that the rate of relaxation of solvent water protons was greater in the substrate-free form of the cytochrome than those rates seen for the cytochrome in the presence of either the nitrogen ligands metyrapone and 4-phenylimidazole or camphor. X-ray analysis of the camphor-free cytochrome P-450$_{cam}$ (Poulos et al., 1986) has shown that at least six water molecules (including the ligand) are present in the active site. In addition, second-derivative spectroscopy has shown that the microenvironment of approximately one tyrosine residue (tentatively identified as Tyr-96) in the cytochrome P-450$_{cam}$ active site becomes more apolar as the fraction of high-spin species increases in the presence of the various camphor and camphor analogues (Fisher & Sligar, 1985b). These results are further augmented by the temperature jump data presented herein.

The existence of an equilibrium between two macroscopic distributions involving the ligation state of the ferric iron has profound functional significance. One aspect of interest is the thermodynamic and kinetic regulation of the first electron-transfer rate between the cytochrome and its physiological electron-transfer partner, putidaredoxin. Thermodynamic control of the first electron-transfer event is achieved by gating of the reduction potential in cytochrome P-450$_{cam}$ (Sligar & Gunsalus, 1974; Sligar, 1976) via substrate and protein association. From the data discussed and presented herein, two major factors can be considered to be important in controlling the redox potential of cytochrome P-450$_{cam}$. These are (1) the nature of the heme axial ligands (Moore & Williams, 1977; Marchon et al., 1982) and (2) the effects of the surrounding protein environment (Falk & Perrin, 1961; Kassner, 1972; Stellwagen, 1978). The observed changes in the reduction potential in relation to the ferric spin state in cytochrome P-450$_{cam}$ depend on both the extent of ligation of the solvent-derived axial ligand and the degree of solvent exposure of the heme. While it is difficult to quantitate which factor is more important in determining the changes in the reduction potential, it is clear that the ultimate source responsible for determining both the extent of ligation of the sixth axial ligand and the changes in the polarity of the heme active site is related to the protein–substrate active-site topology. We have observed

correlations between the ferric spin equilibrium and the microenvironment of the heme, the reduction potential, and the recombination rate of the solvent ligation to the iron center. Only a portion of the total substrate binding free energy is used to modulate the ferric spin equilibrium, the rest being portioned between other protein modes.

One of the more interesting questions in cytochrome research is the role of the heme spin state in determining the rate of interprotein electron transfer. For the cytochrome P-450$_{cam}$ system, the initial electron-transfer event between putidaredoxin and the cytochrome exhibits an increase in rate with increasing fraction of initial ferric high-spin cytochrome species present (Fisher & Sligar, 1985a). In contrast, the solvent-derived ligand recombination rates documented in this paper become progressively lower as the high-spin character of cytochrome P-450$_{cam}$ increases. It is interesting to note that Mössbauer measurements of both the ferrous P-450$_{cam}$ and the ferrous myoglobin indicate that the metal center is in an $S = {}^5/_2$ high-spin state. If the reduction of cytochrome P-450$_{cam}$ resembles that of myoglobin, then the reduction of the 6-coordinate low-spin ferric state must result in a dissociation of the solvent-derived ligand from the iron center. Crutchley and co-workers (Crutchley et al., 1985) have proposed that the dissociation of a solvent-derived ligand must be the source of a large activation energy for the reduction of ruthenium-modified metmyoglobin. A derivatized pentaammineruthenium (histidine-48)–myoglobin complex was prepared to examine long-distance electron-transfer rates at defined distances. It was noted that the reduction of the high-spin metmyoglobin was significantly more temperature dependent than was the reduction of a low-spin pentaammineruthenium (histidine-33)–cytochrome *c* complex. Since the reduction of metmyoglobin to deoxymyoglobin results in the loss of an axial water molecule from the iron atom, it was concluded that the slow electron-transfer rate and large temperature dependence observed for the myoglobin system are due to the change in metal ligation. Similarly, the dependence of the initial electron-transfer rate between putidaredoxin and cytochrome P-450$_{cam}$ can depend on the amount of water ligated to the heme iron. Analysis of the electron-transfer rate data reveals a linear free energy relationship between the activation energy for the first electron-transfer event for the putidaredoxin–P-450 redox couple and the spin equilibrium free energy (Fisher & Sligar, 1985a). In this case, an increase in the activation energy for the initial electron transfer betwen putidaredoxin and an increasing fraction of low-spin cytochrome P-450$_{cam}$ is probably contributed by the reorganization of solvent dipoles accompanying the change in redox states of the heme iron (Warshel, 1982) and by dissociation of the solvent-derived sixth axial ligand from the heme iron.

In summary, the ferric spin equilibrium of cytochrome P-450$_{cam}$ exhibits a compensation between the enthalpy and entropy values as the system undergoes a spin transition. Both the enthalpy and entropy values increase as the low-spin character of the camphor analogue bound cytochrome increases. The ferric spin equilibrium appears to be the result of two distinct macroscopic populations of water-ligated 6-coordinate low-spin and unligated 5-coordinate high-spin ferric iron with the distribution of the low-spin and high-spin species determined by the active-site topology of the substrate–protein complex. We suggest that substrates which induce more high-spin character do so by effectively blocking easy access of water into the heme-containing active site as evidenced by the slower solvent–heme iron recombination rates. The functional consequence of the ferric spin equilibrium is reflected by alterations in the polarity of the active site. The increase in polarity and easy access of water at the heme-containing active site accompanying an increase in low-spin character represent a general mechanism of thermodynamic and kinetic control of the reduction potential and first electron-transfer rate for these heme systems.

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Registry No. Fe, 7439-89-6; P-450$_{cam}$, 9035-51-2; water, 7732-18-5; camphor, 76-22-2; camphoroquinone, 465-29-2; fenchone, 1195-79-5; norcamphor, 497-38-1; tetramethylcyclohexanone, 15189-14-7.

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Binding of a Nonionic Detergent to Membranes: Flip-Flop Rate and Location on the Bilayer

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ABSTRACT: The kinetic aspects of amphiphile interaction with intact membranes (unilamellar and multilamellar liposomes, sarcoplasmic reticulum vesicles) were studied, with the nonionic detergent octa(ethylene glycol) dodecyl monoether ($C_{12}E_8$) as a prototype. $C_{12}E_8$ was bound to these membranes noncooperatively and with a maximum of 0.6–0.8 mol per mole of phospholipid, before the onset of solubilization. Binding was not affected by ultrasonication to expose internal binding sites on the inner leaflet. All detergent could be removed from the membranes by treatment with hydrophobic beads. Furthermore, bound detergent, also from the inside of multilayered liposomes, comprising 10–20 bilayers, was quickly released by dilution of the membranes, followed by gel filtration. The time course of these processes was investigated with a rapid-filtration apparatus, using glass fiber filters to deposit membrane material. Both detergent binding and removal could be described by a monoexponential process with a half-time of approximately 350 ms for all types of membranes. Binding of detergent enhanced the intrinsic fluorescence of sarcoplasmic reticulum vesicles. This occurred in less than 100 ms, probably as the result of direct interaction of $C_{12}E_8$ with Ca^{2+}-ATPase at a few binding sites. The data show that flip-flop of $C_{12}E_8$ across lipid membranes is a rapid process that cannot account for incomplete detergent removal in reconstitution experiments [Ueno, M., Tanford, C., & Reynolds, J. A. (1984) *Biochemistry 23*, 3070–3076]. It is also suggested that other nonionized amphiphiles, including those with an anesthetic action, rapidly gain access to membrane proteins on the inside of the cell, even when used at low, clinical doses.

Nonionic detergents are widely used to purify and characterize membrane proteins [for a recent review see Møller et al. (1986)]. Like Triton X-100, the homogeneous detergent octa(ethylene glycol) dodecyl monoether ($C_{12}E_8$)[1] is an efficient solubilizer of membrane proteins. It was originally reported to be useful in the preparation of active Ca^{2+}-ATPase, first in an oligomeric state (le Maire et al., 1976) and subsequently in a monomeric state (le Maire et al., 1978; Dean & Tanford, 1978; Andersen et al., 1982). The detergent has also been used at subsolubilizing concentrations to perturb membrane structure and function, thereby providing a new approach to the characterization and understanding of the role of partial reactions during enzyme turnover. This line was followed, for example, for Ca^{2+}-ATPase (Lüdi et al., 1982; Andersen et al., 1983; McIntosh & Davidson, 1984; Champeil et al., 1986) and Na^+,K^+-ATPase (Huang et al., 1985).

Another promising application of nonionic detergents is their use in reconstitution studies after solubilization of membrane protein and lipid. Originally, octyl glucoside was used for this purpose (Helenius et al., 1981; Mimms et al., 1981; Jackson & Litman, 1982), due to its high cmc, which facilitates removal by dialysis or similar procedures. However, in many cases this carbohydrate detergent is too abrasive to maintain membrane proteins in an active state. Instead, successful attempts have been made to use $C_{12}E_8$ as a membrane protein and lipid solubilizing agent for reconstitution studies (Cornelius & Skou, 1984; Andoh & Yamamoto, 1985). In a comprehensive study of factors affecting vesicle size and other properties of reconstituted liposomes, Ueno et al. (1984) noted that it was difficult to remove a significant portion of $C_{12}E_8$ from the reconstituted vesicles. The authors suggested that

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[1] Abbreviations: $C_{12}E_8$, octa(ethylene glycol) dodecyl monoether; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EPC, egg phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; SR, sarcoplasmic reticulum; cmc, critical micelle concentration; Ca^{2+}-ATPase, calcium ion activated adenosinetriphosphatase; HPLC, high-performance liquid chromatography; ESR, electron spin resonance; DTT, dithiothreitol.

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