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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Hermitt Lyell Jarraway

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IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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THERMODYNAMIC CHARACTERIZATION
OF THE LINKED S1 IN STATE AND
BINDING EQUILIBRIA OF FIVE
CYTOCHROME P-450 
SUBSTRATE ANALOGS

BY

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THESIS

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INTRODUCTION

MATERIALS AND METHODS

RESULTS

Spin state thermodynamics of substrate free cytochrome :+50
Spin state thermodynamics of substrate bound cytochrome :±50
Substrate binding to cytochrome :±50

DISCUSSION

FIGURES

1. Catalytic cycle of :±50
2. Origin of protein spin state
3. Thermodynamic square linking spin and binding
4. Absolute spectra of analog saturated :±50
5. Effect of substrate concentration on spin state
6. Temperature dependence of spin equilibrium
7. Temperature difference spectra of spin equilibrium
8. Temperature dependence of spin equilibrium
9. Titration curve of :±50 with norcamphor
10. Temperature dependence of norcamphor dissociation
11. Some substrate analogs
12. Tanabe-Sugano diagram
13. Thermodynamic cube linking spin, binding and redox
The soil bacterium *Pseudomonas putida* has the ability to metabolize the monoterpene camphor, a volatile molecule of carbon and oxygen, converting this monoterpene to acetate and isobutyrate. The first reaction of the pathway is carried out by cytochrome P-450, a mixed function oxidase which catalyzes the 5-hydroxylation of camphor. The enzyme (abbreviated cyt P-450 or m, for monoxygenase) utilizes electron equivalents from NADH, through a flavoprotein dehydrogenase and an iron-sulfur redoxin called putidaredoxin, to cleave molecular oxygen. One oxygen atom is inserted into the hydrocarbon chain while the other is reduced to water (figure 1). The catalytic cycle occurs as previously described (1) by binding of substrate to the oxidized heme protein; reduction of the heme iron by the input of one electron; binding of atmospheric dioxygen; input of the second electron creating a reactive oxygen species; and the decay of the oxygenated intermediate to the 5-exo alcohol, water and oxidized enzyme. The bacterial P-450 monoxygenase system, easily obtained and purified, has provided valuable insight into the mechanisms of molecular oxygen cleavage and substrate oxidation catalyzed by adrenal and hepatic P-450 oxygenase systems.

Heme-proteins such as P-450, undergo spectrophotometrically observable transitions upon redistribution of electrons in their porphyrin iron atom. These spectroscopic changes reflect energy differences in the distribution of electrons over the non-degenerate 3d orbitals, which arise from the strength and symmetry of the ligand field about the
Figure 1- catalytic cycle of H-450. (1) oxidized H-450 (m^2)
binds substrate S. (2) Substrate bound cytochrome
picks up an electron, reducing the heme iron. (3)
Reduced H-450 binds atmospheric dioxygen. (4) The
second electron activates the oxygen, and the hydroxy-
ylation reaction is catalyzed.
Charges on ligands interact with the d-shell electron density causing a splitting of the energies of the five 3d electronic orbitals. Obviously, the amount of charge on the ligand and its proximity relative to the heme iron dictate the extent of the observed orbital splitting. In heme proteins the ligand is at a locus of the octahedral iron atom toward which two orbitals of the metal point. Therefore, the energies of these two orbitals are increased relative to the other three (see figure 2A). Electrons may be distributed over these orbitals in any scheme consistent with the Pauli exclusion principle. The possibilities for the five electrons of oxidized iron are depicted in figure 2B. It should be noted that an octahedral ligand symmetry is not entirely correct, but this model serves very well to provide a basic understanding of heme spin state (2). In cytochrome c-450, the electrons may be distributed such that the total spin angular momentum is \( S = \frac{1}{2} \) or \( S = \frac{5}{2} \) for the oxidized enzyme. The low spin \( (S=1/2) \) form of the protein is thought to correspond to a hexacoordinate ferric ion with cysteiny1 thiolate and solvent axial ligands, while the high spin form is associated with a 5-coordinate iron atom out of the porphyrin plane with only the thiolate axial ligand. Since the electron distribution is governed by the coordination chemistry of the heme iron, it is clear that changes in spin state must be linked to conformational changes within the protein itself.

In solution the low and high spin states of the protein exist in an equilibrium dependent on substrate concentration, salt concentration, temperature and other factors. Upon binding of substrate, for example, the oxidized c-450 enzyme undergoes
Figure 2A- 3d orbital splitting by the ligand field about a heme iron. Orbitals pointed toward a charged ligand are elevated in energy with respect to remaining orbitals due to repulsions between metal and ligand electrons.

Figure 2B- Possible electronic configuration of an iron ion in oxidized cytochrome: $\pm 3/2 \rightarrow \pm 5/2$ and the two main states of oxidized cytochrome: $-450 \text{ cm}^{-1}$ is the predominant state in state of reduced cytochrome.
a blue shift in the absorption maximum of the Soret band, corresponding to a shift from an equilibrium dominated by the low spin protein to one in which the high spin form is prevalent. The low spin protein is characterized by an absorption band at 416 nm while the high spin absorbs at 391 nm. It is clear that these are the only spin states occupied by the ferric protein, evidenced by an isosbestic point at 406 nm. In addition to substrate binding, the spin equilibrium of Fe(III)C6H6 may be altered by changes in temperature of the system, both in the presence and absence of substrate. Independent variation of these spin state variables provides a means of detailed thermodynamic analysis and leads to the four state model proposed by Gillard (3) describing the relationship between spin equilibrium and substrate binding for cytochrome c (Figure 1). The model predicts that by each μH, 1, 2, 3 to be free in spin form of the enzyme, and those μH, 4, 5, the substrate bound high spin form.

In that study the thermodynamic parameters of enthalpy change and entropy change for the equilibria $K_1 = [H^{O}] [S] / [H] [S]^{O}$ and $K_2 = [H] [S]^{O} / [H] [S]^{O}$ for camphor were obtained through Van't Hoff analysis, and the free energy changes of each interaction at 20°C were calculated. It should be emphasized that since modulation of the spin equilibria results at least in part from protein conformational change, the thermodynamic parameters defining the $K_1$ and $K_2$ equilibria describe not only the redistribution of d-shell electrons but these internal protein motions as well. In fact, the enthalpy and entropy changes for the electron redistribution are comparatively negligible. The free energy changes at 20°C for $K_1 = [H] [S] / [H] [S]^{O}$ and $K_2 = [H] [S]^{O} / [H] [S]^{O}$
Figure 3- four state thermodynamic model linking substrate binding and spin state of oxidized $\text{Fe}^{3+} \text{FeS}_{50}\text{Cu}_{24}$. This model predicts two paths by which the substrate free, low spin form may become substrate bound, high spin protein. Both paths are energetically equal.
LIGAND AND SPIN STATES
OF FERRIC CYT c

\[ m_{ls}^0 \xrightarrow{\Delta G_1} m_{ls}^{os} \xrightarrow{\pm S} m_{ls}^{os} \xrightarrow{\Delta G_2} m_{ls}^{ic} \]

\[ m_{ls}^0 \xrightarrow{\pm S, \Delta G_3} m_{ls}^{ic} \xrightarrow{\Delta G_1} m_{ls}^{os} \]
were calculated from the dissociation constant of camphor binding (see results section). Thus the free energy thermodynamic square was solved at 20°C for camphor substrate. It will be noted, however, that values for enthalpy and entropy changes were not obtained for the $k_3$ and $k_n$ equilibria, binding of substrate to the low spin and high spin protein respectively.

It was the purpose of this study to thoroughly complete the thermodynamic data by obtaining $\Delta H$ and $\Delta S$ for all four equilibria using Van't Hoff analysis of dissociation constants obtained by spectral titration of the enzyme with substrate. This was done not only for the normal substrate camphor, but certain camphor analogs as well. These analogs induce varying degrees of the high spin form upon binding to $\epsilon$-450. For example, camphor induces 96% of the protein to be in the high spin state at saturating concentrations while tetramethylcyclohexanone (TMC) induces only 25% high spin (see figure 4). Thus, using substrate analogs, it is possible to independently vary the spin equilibrium, substrate concentration and temperature of the system. This allows for the determination of thermodynamic parameters of the four equilibria as a function of spin state position. Three interesting observations may be made from this study. First, in accord with earlier observations (5), it was noticed that the affinity of substrate binding to the $\epsilon$-450 enzyme is entirely independent of the spin equilibrium on the concomitant shift in wavelength of the Soret maximum; that is, the dissociation constant is indepen
Figure 4 - absolute spectra of cytochrome f 450_Cam saturated with various substrate analogs. The peak at 416 nm is the absorption due to low spin protein in solution and the peak at 391 nm corresponds to absorption of high spin i-450. The spectra of adamantanone and camphor are very similar to that of camphoroquinone and were omitted for clarity.
SUBSTRATE ANALOG SATURATED P-450

- Substrate Free
- TMCH
- Norcamphor
- Camphorquinone
dent of maximum percent high spin. The second observation was that over the temperature range examined, 5°C to 17°C, no break in the dissociation constant occurred for any substrate, in contrast with kinetic results previously reported (6). These data had shown the dissociation constant of camphor to be temperature dependent over the range from 4°C to 13°C but temperature independent (constant) above 13°C. The final and most significant observation concerns the thermodynamics of the spin equilibrium of substrate bound cytochrome (h₂ equilibrium). It can be seen from figure 8 that the slopes of the Van't Hoff plots are the same for all substrates; that is, the enthalpy change for this interaction is constant for all spin equilibrium positions and the driving force is dictated solely by the change in entropy. A similar observation was made for highly purified rat liver 1-450 saturated with various drugs, however this was not analyzed in detail thermodynamically (7).
MATERIALS AND METHODS

Cytochrome i-450 was purified according to the method of Gunsalus and Wagner (8) and substrate free forms were obtained as outlined by Cole and Sligar (9). All buffers, reagents and substrates used were purchased pure. Absorbance spectra were obtained with a Hewlett-Packard 8450a UV-visible spectrophotometer fitted with a two-station Hewlett-Packard 89100A temperature control accessory, temperatures were accurate to within 0.2°C of the recorded temperature.

All studies were conducted in 100 mM potassium phosphate buffer, 75 mM potassium chloride, pH 7.0. Ionic strength has a profound effect on spin equilibrium, so measures were taken to keep the potassium ion concentration rigidly constant. When percent high spin vs. substrate concentration is plotted for a titration of i-450 with substrate, a curve similar to that exemplified by norcamphor binding is obtained (see figure 5). The decrease in high spin at very high substrate concentrations is due to binding of substrate to a second site. Increasing potassium ion concentration tends to increase the maximum high spin obtained upon titration and broadens the plateau region of the curve (for norcamphor, 4 to 6 mM). Studies of spin state change at saturating substrate concentrations were conducted in this plateau region to avoid the observation of competing spin and binding equilibria. The buffer conditions chosen provide a sufficiently broad plateau region for binding of all substrates examined so that only the spin equilibrium is observed. It should be pointed out that a direct comparison of the data presented here and those presented in the Sligar
Figure 5- the effect of substrate concentration on I-450, spin equilibrium. Binding of substrate pushes the protein to a high spin state. The reduction in percent high spin at very high substrate concentrations is due to binding of substrate to a second site, possibly releasing substrate from the first site. The maximum percent high spin and the breadth of the plateau region increase as ionic strength is increased.
paper is not possible because of differing buffer conditions.

The equilibrium between high and low spin states may be generally analyzed spectrophotometrically at a given wavelength with the equation $K = (a-a_{ls})/(a_{hs}-a) = a_{hs}/a_{ls}$, where $a$ is the measured absorbance, and $a_{ls}$ and $a_{hs}$ are the absorbances of pure low and high spin protein respectively. The spin equilibria of substrate saturated protein were obtained through temperature difference spectroscopy, where the reference cuvette was incubated at $27^\circ C$ while an identical cuvette was varied over a $27^\circ C$ to $2^\circ C$ range. Difference spectra were measured at seven temperatures within this range (see norcamphor example, figure 7), and absorbance values at 406 nm and 416 nm recorded. To correct for spectral drift, the isosbestic point at 406 nm was established at zero for all spectra. The fraction high spin was calculated by:

$$f_{hs} = \left[1 - \frac{(a_{416} - a_{450,hs})}{(a_{416} - a_{450,ls})}\right] / \left(\frac{a_{416} - a_{416}}{a_{ls} - a_{hs}}\right)$$

where $a_{416}$ and $a_{416}$ are the established extinction coefficients of pure low spin and pure high spin forms, 119.7 m$^2$cm$^{-1}$ and 60.1 m$^2$cm$^{-1}$ respectively (10). From the fraction high spin, the equilibrium constant is easily calculated:

$$K = f_{hs}/(1-f_{hs}).$$

The spin equilibrium constants for substrate free protein were obtained from absolute spectra at temperatures ranging from $-5^\circ C$ to $15^\circ C$, and analysis with the same equations.

Dissociation constants were obtained from absolute spectral titrations of $m^0$ at various temperatures. Fraction high spin in this case is obtained from the ratio of absorbance at 416 nm to that at 391 nm for a given free substrate concentration.
Rearrangement of equation (1) yields:

$$A_{416} / [C_{-450}] = (1 - f_{hs})(a_{416} - a_{n416}) + a_{n416}. \quad (3)$$

Similar analysis at 4416 nm yields:

$$A_{391} / [C_{-450}] = f_{hs}(a_{391} + a_{n391}) + a_{n391}. \quad (4)$$

Dividing equation (3) by equation (4) and rearranging yields finally:

$$f_{hs} = \frac{a_{416} - a_{391}(A_{416} / A_{391})}{a_{416} - a_{n416}(a_{416} / a_{391})(A_{416} / A_{391})} \quad (5)$$

where $a_{n416}$ and $a_{416}$ are given above, and $a_{n391}$ and $a_{391}$ are 105.3 meV cm$^{-1}$ and 47.9 meV cm$^{-1}$ respectively. This equation conveniently allows the protein concentration term to drop out of consideration. The extent of saturation upon substrate titration of cytochrome $-450_{Cam}$ is just the ratio of the observed fraction high spin at the free substrate concentration of interest to the fraction high spin of fully saturated protein.

Original attempts to solve the thermodynamic square for different substrates involved the Van't Hoff analysis of the protein-substrate complex at various substrate concentrations, usually non-saturating. These Van't Hoff plots did not yield straight lines, indicative of the competing spin and binding equilibria. As expected, the deviation from linearity decreased as plateau concentrations were approached, indicated by the correlation coefficient approaching unity. The goal was to deconvolute these data into separate spin and binding equilibria. These efforts were abandoned, however, owing to mathematical complexities and potential for inaccuracy.
RESULTS

Interactions between the four states of oxidized P-450_Carb. depicted in figure 3 may be described by the following equations:

\[
\begin{align*}
\Delta G_1 &= -RT \ln \frac{[m_{hs}^0]}{[m_{ls}^0]} \\
\Delta G_2 &= -RT \ln \frac{[m_{hs}^0]}{[m_{ls}^0]} \\
\Delta G_3 &= -RT \ln \frac{[m_{hs}^0]}{[m_{ls}^0]} \\
\Delta G_4 &= -RT \ln \frac{[m_{hs}^0]}{[m_{ls}^0]} \\
\end{align*}
\]

Since \( \Delta G \) is a state function, the sums of the free energies for all pathways between any two states must be equal. Hence, \( \Delta G_1 + \Delta G_2 = \Delta G_3 + \Delta G_4 \) and \( K_1 K_3 = K_2 K_4 \). Each free energy change is associated with a change in enthalpy and a change in entropy. These relationships allow the thermodynamic square to be completely solved; thus the driving force for any interaction of interest may be calculated by \( \Delta G = \Delta H - T \Delta S \).

Spin state thermodynamics of substrate free cytochrome P-450. As mentioned previously, spin equilibrium constants for substrate free protein (\( K_i = \frac{[m_{hs}^0]}{[m_{ls}^0]} \)) were obtained from the absolute absorbance values at 416 nm. Decreasing the temperature of cytochrome P-450 induces a shift to the low spin form, decreasing the value of \( K_i \). A plot of \( \ln K_i \) vs. \( 1/RT \) results in a straight line (see figure 6) which yields \( \Delta H = 9.3 \) kcal/mole and \( \Delta S = 26.1 \) e.u. These values are very close to those previously obtained by Sligar but under somewhat different buffer conditions (50 mM potassium phosphate buffer containing 160 mM potassium chloride).

It should be noted that temperature difference spectroscopy was not employed in this particular study because of the
Figure 6- Van't Hoff plot of the spin equilibrium of substrate-free cytochrome $-450_{\text{C.W.}}$. Changes in enthalpy and entropy are similar in magnitude to those obtained by oligar (3).
Temperature Dependence of $m^o$ Spin Equilibrium

$\Delta H = 9.3 \text{ kcal}$

$\Delta S = 26.1 \text{ e.u.}$

$r = 0.9970$
instability of substrate free protein at higher temperatures. Above about 20°C, it was noticed that the spin equilibrium was constantly changing over time, varying by as much as five percent in either direction of the true position. The variation in spin state of the protein in both the reference and sample cuvettes made good difference spectra impossible to obtain. For this same reason, relatively low temperatures (-5°C to 15°C) were employed for the study and the average of fifteen absorbance values used for calculations. It was also noticed that addition of extremely small amounts of substrate, concentrations for below the dissociation constant, were sufficient to stabilize spin state drift.

In state thermodynamics of substrate bound cytochrome c-550. Again, lower temperatures correspond to a lower fraction high spin, and a lower value of $K_2$. Figure 7 shows the increase in absorbance at 416 nm and the decrease at 391 nm as the temperature is decreased relative to an identical reference sample for c-550, saturated with norcamphor. Similar spectra were obtained for camphor, adamantone, cresphoroquinone and T Ach bound protein. Van't Hoff analysis (figure 6) revealed that $\Delta H$ is the same for all interactions, within experimental error, and $\Delta S$ varied with the maximum fraction high spin induced by substrate binding. This observation suggests that the driving force of the conversion of the high spin form to the low spin form is dictated exclusively by entropy $S$. The enthalpy $\Delta H$ and its change in terms of cytochrome c-550 structure and function are discussed further in a subsequent section.
Figure 7- temperature difference spectrum of norcamphor bound cytochrome P-450. Temperatures represented are 27°C (baseline), 22°C, 17°C, 14.5°C, 9.5°C, 4.5°C and 2°C. Similar spectra were obtained for enzyme interaction with each substrate studied.
Figure 8- Van't Hoff plot of the spin equilibrium of cytochrome i/.50 saturated with various substrate analogs. The similar slopes indicate that the driving force of spin interconversion is the change in entropy from one state to the other.
substrate binding to cytochrome i-450. Substrate binding in the four state model has been shown to follow simple stoichiometry (3). The observed saturation upon titration with substrate is:

$$\sum = (A_2 - A_0)/(A_1 - A_0) = \left[\frac{[2]}{[2]} + K_3(1 + K_1)/(1 + K_2)\right]$$

(7)

where \(A_2\) is the absorbance at any ligand concentration, \(A_0\) is the absorbance of substrate free \(-450_{Camph}\), and \(A_1\) is the absorbance corresponding to completely saturated protein. The dissociation constant is therefore given by

$$K_d = K_3(1 + K_1)/(1 - K_2).$$

(8)

Plots of \(\sum\) vs. log free substrate concentration followed the expected sigmoidal form (see norcamphor example, figure 9) for all substrates at all temperatures; thus, the free substrate concentration at \(\sum = 0.5\) is numerically equal to the dissociation constant. Dissociation constants were obtained in this manner for each substrate at 17°C, 13°C, 9°C, and 5°C (see norcamphor example, figure 10). These were then used to calculate dissociation constants for binding to the low spin form alone, \(K_3\), from equation (8), and the high spin form alone, from the relationship \(K_4 = K_1K_3/\lambda_2\). These calculated dissociation constants for each form were analyzed with a Van't Hoff plot, and the thermodynamic parameters of \(\Delta H\) and \(\Delta S\) were obtained for each interaction. The results of these analyses are shown in tables I through IX.

Some general observations may be made from these data. First, from table 9, it will be noted that the maximum fraction high spin induced by a substrate and the dissociation constant of that substrate from the protein show absolutely no correlation.
Figure 9- titration curve for norcarboxin at 17°C. Titration of
1.000 with all substrates at all temperatures studied
followed similar curves.

Figure 10- blower of the center of the titration curves for
norcarboxin at 17°C, 13°C, 9°C and 5°C. Dissociation
constants for each substrate at each temperature are
numerically equal to the substrate concentration at
\[ \Sigma = 0.5. \]
A comparison of the dissociation constants of camphoroquinone, norcamphor, and T. Ch demonstrates this quite clearly. One may be surprised that the dissociation constant of norcamphor is better than an order of magnitude greater than those of other substrates, indicating much weaker binding. However, simple models of the substrates show that norcamphor is considerably smaller and probably has fewer contact points than the others, and is therefore expected to bind much less tightly. A second observation, which may be made from Tables VII and VII, concerns the large negative entropy changes relative to enthalpy changes observed upon dissociation of substrate from either the low or high spin forms ($K_L$ and $K_H$). This is to be expected, since in most cases the predominant component of the free energy of substrate binding to protein is an increase in entropy. This corresponds to a hydrophobic interaction in which the substrate is pushed out of the water solvent so that water molecules may be involved in maximal hydrogen bonding interactions with each other.

A number of significant observations may be made from Table VIII alone. First, it is noticed that for each substrate $K_H$ is greater than $K_L$, that is, the free energy of substrate dissociation from the high spin form of the protein is more unfavorable than dissociation from the low spin form. Thus, binding to the high spin form is more favorable than binding to the low spin form, in accord with previous predictions (3). In general, dissociation free energies $\Delta G_L$ and $\Delta G_H$ are dependent on the maximum fraction high spin induced by substrate substrates that induce a lower fraction high spin have been.
The binding energies to both low and high spin protein. The overall free energy change in going from substrate free, low spin, to substrate bound, high spin protein (ΔGp) is also less favorable for substrates that induce lower fractions of high spin, as should be expected. The only deviation from this observation is that of D.C.I., which because of its significant structural differences from other substrates, may behave in an unusual manner. This point is discussed in a later section.

One final observation, interesting but not critical, concerns the effect of temperature on the various states of the protein for different substrates. Since the enthalpies of substrate dissociation vary considerably from one substrate to another, the equilibrium of each species in solution does not vary with temperature the same with all substrates. In other words, a Van't Hoff plot of substrate binding to either low or high spin protein for two substrates will show a crossover point, at which temperature the equilibrium constants for the two substrate interactions will be the same. Obviously, these differences in enthalpies will affect the distribution of species over the entire square, including spin state equilibria. For this reason, adamantanone induces a higher fraction high spin at temperatures below 24°C than does camphor. This is the only crossover point observed for the different substrates over the examined temperature range.
### TABLE I: CALCIUM

<table>
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<tr>
<th>K</th>
<th>290 K</th>
<th>286 K</th>
<th>282 K</th>
<th>278 K</th>
<th>$\Delta H (\text{kcal/mole})$</th>
<th>$\Delta S (\text{eu})$</th>
<th>r</th>
</tr>
</thead>
<tbody>
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<td>$k_1$</td>
<td>0.04999</td>
<td>0.03988</td>
<td>0.03164</td>
<td>0.02492</td>
<td>9.29</td>
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*Values for $k_D$ are best-fit values for $k_D$. These were used to calculate $k_3$ and $k_4$. Values for $k_3$, $k_4$, $k_5$, and $k_6$ expressed in terms of micromolar concentrations.*
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<th>$K$</th>
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### Table III: Calculated Values

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<th>p</th>
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<td>5.766</td>
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<td>24.6</td>
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Thermodynamics is limited in the sense that while it predicts the propensity for a given process to occur, it cannot predict any mechanistic details of the reaction. Because of this, it would seem thermodynamics is of little value in obtaining a clear picture of enzyme action. However, combining the thermodynamics of the interaction with various substrates can yield some information about substrate binding, enzyme intermediates and even electron transfer processes, especially if something is known about both substrate and protein structure. The goal of this section is to relate experimental observation to actual physical processes which may occur within the protein.

Since only five of the fairly broad range of possible substrate analogs were employed in this study (see figure 11), conclusions may be drawn only in very general terms.

The first observation concerns the independence of induced high spin and dissociation constant for a given substrate. This suggests that any substrate-protein contact points responsible for the change in spin state are not the most part independent of contact points responsible for locking the substrate into the active site. Although very interesting, the problem of relating fraction high spin to protein structure is still complicated with generalization. However, suggests a link between the basis of the substrate bridge and fraction high spin. This can be seen from the data presented here. Camphor and camphoroquione, which have two bridge methyl groups, and adamantane, which has a very bulky bridge structure, all induce better than 90% high spin protein. On the other hand, norcamphor,
with no bridge methyls, induces only 55% high spin while TcCH, with no bridge group at all induces only 24%. This argument alone might suggest that protein contacts with the substrate bridge group are not as important in substrate binding as other possible substrate-protein contact points. Again, it should be emphasized that this is but a crude approximation; further studies with other substrates are necessary to determine the extent to which this observation holds true.

Tetramethylcyclohexanone is an unusual substrate compared to other analogs in terms of its structure and its binding to 1-450. Not only does TcCH lack the bridge group common to most substrates, it contains bulky methyl groups in positions where none are found on other substrates. Assuming the ketone oxygen is hydrogen bonded to the tyrosine 98 residue on the protein (11), these methyl groups could be in a position to interact sterically with the home group. Another interesting observation concerning TcCH structure is that it can exist in two conformations in solution where bridged substrates are locked into one conformation. These TcCH conformations correspond to the chair and boat configurations typical of cyclohexane derivatives; thus there are four possible ways TcCH can sit in the active site, corresponding to quite a difference in the positioning of the methyl groups and the ketone oxygen. TcCH also seems to exhibit some unusual behavior upon binding to cytochrome. It induces a fairly high proportion of the inactive R-428 form of the enzyme; substantially more than that is seen with other substrates. An interesting question is whether a correlation exists between the number of contacts the substrate makes with the protein and its ability to induce the inactive form.
pected binding thermodynamics mentioned in the previous section, the difference in structure relative to other substrates, and the formation of excessive $\alpha$-420. If in fact the structure of TmCH somehow induces $\alpha$-420 formation, the deviation from the expected thermodynamics could result from substrate dissociation from both the $\alpha$-420 and $\alpha$-450 forms. Further characterization of $\alpha$-450-TmCH interaction could yield some insight into the binding mechanisms of cytochrome $\alpha$-450.

As mentioned previously, kinetic data had shown a break in the temperature dependence of the dissociation constant for camphor at $17^\circ$C. Data presented here from the spectral titration of $\alpha$-450_Cam with camphor and four other substrate analogs suggest no such break, at least over a 5°C to 17°C temperature range. A few comments should be made regarding these experiments. First, the Van't Hoff plot for the dissociation of camphor did not strictly obey linearity, as indicated by the comparatively low correlation coefficient (0.9895). Van't Hoff plots for other substrates followed linearity much more closely, indicating that a break in temperature dependence of dissociation constants for these is much less likely. Since only four data points were obtained for the spectral titration Van't Hoff analysis, there is considerable room for experimental error. A more complete study would involve the titration of $\alpha$-450 with substrate at several more temperatures, particularly above $17^\circ$C. It was noticed, however, that the thermodynamic parameters obtained by spectral titration with camphor (from the least-fit line through the data points) are similar in those predicted by the temperature dependence studies of the complex with camphor directly. This supports independent evidence that
in the temperature dependence of dissociation constants is unclear. If in fact there is a break in the Van't Hoff plot for camphor, it is yet more unclear why substrate analogs should show no break over the same temperature range.

The fact that the spin equilibria of cytochrome P-450Cam is governed by entropy may be rationalized in two ways, by either focusing on possible protein conformational changes which lead to spin state changes or by focusing on the actual heme iron ligand symmetry in relation to substrate binding. First, since entropy reflects the degree of equilibration of systems, the differential degree of spin equilibria induced by various substrates could reflect a differential degree of tightening of protein structure (12). This model implies a relatively "floppy" substrate free form of the protein which tightens upon binding of substrate, the extent of tightening depending on specific bulk properties of the bound substrate. The tightening then corresponds to the conformational changes needed to shift the protein from the low to the high spin state. This model would explain the thermal spin state drift of substrate free P-450 described previously: conformational changes within the floppy substrate free protein could induce random high spin formation; the slow spin relaxation times (msec) observed previously (9) upon thermal perturbation correspond to these thermal motions within the protein in this model.

The other way of explaining entropic spin state control is through probabilistic arguments concerning ligand field strength. As mentioned the basic premises of this treatment, an analysis of a Boltzmann curve in 10 moles (Figure 12), this entails...
This plot non-quantitatively depicts the stability of the high and low spin protein forms as a function of ligand field strength. Higher ligand field strengths correspond to low spin, while low ligand field strengths are associated with the high spin form. It has been suggested that only two ligand field energies exist, corresponding to the low and high spin forms. Thus, no bending of the H₂O-Fe⁺ bonding interaction occurs.
Theoretical diagram shows the stability of the high and low spin forms of the protein as the ligand field strength is increased. Obviously, the high spin state is more stable (lower energy) at weaker ligand field strengths than the low spin state; the converse is true with stronger ligand fields. It has been suggested (13) that the shift in spin state upon thermal perturbation is due to the population of one state over the other by the addition or subtraction of the solvent ligand. In terms of the diagram, only two ligand strength energies exist, corresponding to the low and high spin forms of the protein; no ligand field energies between these (corresponding to differential lengths of the H-bond bending interaction) may exist. The entropic control of spin state in this argument relates to the probability that a certain substrate will displace the solvent molecule. Again, this is related to the bulk of the substrate molecule involved in the binding process. The slow spin relaxation times in this case correspond to the rate constant of the solvent molecule breaking its interaction with the heme iron and escaping the protein.

The control of spin state through entropic factors could have been predicted: in light of the control of the redox potential of cytochrome b-550 by spin state. It was noticed (3) that there exists a difference of 150 mV in redox potential between the low and high spin forms of the protein. The redox potential of cytochrome c in ferricytochrome c-oxidase is positioned such that it will readily give up electrons to high spin cytochrome b but not the low spin form. The linkage between spin and redox allows for the expansion of the spin-state thermodynamic couple of figure 7 into the redox-driven redox couple of
of figure 13. From the conservation of thermodynamic state functions (in this case the state functions associated with the L2, L5, L9, and L9 equilibria) and the dominant entropy factors associated with the adiabatic electron transfer process, it is clear that the control of protein spin state is necessarily a result of changes in entropy. These arguments would suggest a linear relationship between the free energies of these redox potentials and ferric spin state, and indeed such a correlation is observed (14).
Figure 13: the thermodynamic cube describing the relationships between spin, binding, and redox states of the cytochrome c-450 protein. This model results from the fact that both the binding of substrate and the redox potential are regulated by the spin state of the protein.
REDOX, SPIN, AND LIGAND STATES OF CYT P


