Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1.
Koenig, S. H., Brown, R. D., & Brewer, C. F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 475.
Laidler, K. J. (1965) *Chemical Kinetics*, pp 15-17, McGraw-Hill, New York, NY.
Lis, H., & Sharon, N. (1973) *Annu. Rev. Biochem. 42*, 541.
Meirovitch, E., Lutz, Z., & Kalb, A. J. (1974) *J. Am. Chem. Soc. 96*, 7542.
Nicolau, C., Kalb, A. J., & Yariv, J. (1969) *Biochim. Biophys. Acta 194*, 71.
Poretz, R. D. (1968) Ph.D. Thesis, State University of New York at Buffalo, Buffalo, NY.
Reed, G. H., & Cohn, M. (1970) *J. Biol. Chem. 245*, 662.
Shephard, G. R., & Gurley, L. R. (1966) *Anal. Biochem. 14*, 356.
Shoham, M., Kalb, A. J., & Pecht, I. (1973) *Biochemistry 12*, 1914.
von Goldhammer, E., & Zorn, H. (1974) *Eur. J. Biochem. 44*, 195.
Wang, J. C., Cunningham, B. A., & Edelman, G. M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1130.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Yariv, J., Kalb, A. J., & Levitzki, A. (1968) *Biochim. Biophys. Acta 165*, 303.

Temperature-Dependent Spin Equilibrium of Microsomal and Solubilized Cytochrome P-450 from Rat Liver†

Dominick L. Cinti,* Stephen G. Sligar, G. Gordon Gibson, and John B. Schenkman

ABSTRACT: In the absence of exogenous substrate, temperature-induced type I and reverse type I (formerly called modified type II) spectral changes were observed with both rat liver microsomes and solubilized, partially purified cytochrome P-450. Similar changes were observed in the absolute spectrum of cytochrome P-450, i.e., increasing temperature reduced the absorption peak at 418 nm while simultaneously enhancing absorption in the 370–390-nm region in both liver preparations. An isosbestic point at 407 nm was noted and is indicative of a two-state equilibrium system. Spectral titration of type I and reverse type I spectral changes at 385 and 419 nm by dual wavelength spectrophotometry resulted in a slightly sigmoid-shaped curve with changes in temperature (3–40 °C). A graph of the log of the equilibrium constant vs. reciprocal temperature yielded a linear van't Hoff plot. From the slope and intercept an overall enthalpy change, $\Delta H = -14.66$ kcal/mol, and entropy change, $\Delta S = -49.88$ eu, in the reaction P-450$_{LS}$ ↔ P-450$_{HS}$ were obtained for hepatic microsomes. These thermodynamic parameters yield an equilibrium constant of 0.930 at 20 °C, indicating a significantly large amount (>48%) of high-spin cytochrome P-450 in the absence of added substrate, as corroborated by electron paramagnetic resonance spectroscopy. In contrast, the soluble cytochrome P-450 preparation yielded $\Delta H = -5.5$ kcal/mol and $\Delta S = -14.1$ eu. These state functions produced an equilibrium constant of 0.096 at 20 °C indicative of P-450 predominantly in the low-spin ferric form. These differences suggest that the microsomal environment of lipid and/or endogenous substrate has a dramatic effect on controlling the spin equilibrium. In addition to these thermodynamic parameters, the precise absolute extinction coefficients for pure high-spin and pure low-spin cytochrome were derived. These parameters for solubilized P-450 of $\epsilon_{HS} = 52$ mM^{-1} cm^{-1} and $\epsilon_{LS} = 126$ mM^{-1} cm^{-1} at 418 nm and the corresponding values for the membrane-bound protein indicate that only 54% of microsomal P-450 undergoes a temperature-dependent spin transition. For the liver microsomes, $\Delta\epsilon_{385-419nm}$ was found to be 126 mM^{-1} cm^{-1}.

Narasimhulu et al. (1965) observed the formation of a spectral transition in the Soret region of cytochrome P-450 upon addition of a steroid to a suspension of adrenal cortical microsomes. Less than 1 year later, two types of spectral changes were described when various drugs were added to liver microsomes (Remmer et al., 1966; Schenkman et al., 1967; Imai & Sato, 1966). One spectral change, termed "type I", was characterized by an absorption maximum at 385–390 nm and a minimum at 420 nm; a second spectral change, called "type II", was shown to possess absorption maxima at 425–435 nm and minima at 390–400 nm (Schenkman et al., 1967). A third spectral transition, initially called "modified type II", displayed an essentially mirror image of the type I spectral change and was subsequently described as a "reverse type I" spectral change (Schenkman et al., 1973). All three spectral transitions are induced following substrate addition to a liver microsomal suspension.

Mason and co-workers (1965a,b) described an electron paramagnetic resonance (EPR) spectrum in liver microsomes which resembled low-spin ferrihemoproteins and suggested that the EPR signal was related to cytochrome P-450. Cammer et al. (1966) reported similar findings and in addition showed that both type I and type II substrates affected the low-spin g_x and g_z components of the first-derivative EPR spectrum in opposite directions. For example, in the presence of aniline (type II substrate), the g_x signal was shifted to higher field while the g_z signal was shifted to lower field; the opposite occurred in the presence of hexobarbital, a type I substrate (Cammer et al., 1966). A conversion of the low-spin ferric hemoprotein to the high-spin form following addition of camphor was reported in the *Pseudomonas putida* cytochrome P-450 system by Tsai et al. (1970) and Peterson (1971). The spin state of ferric cytochrome P-450 can thus be modulated by substrate addition, shifting from low to high spin with type I compounds, vice versa with reverse type I compounds, or forming a new low-spin species with type II compounds. Similar spin-state changes occurred with adrenal cortex

† From the Department of Pharmacology (D.L.C., G.G.G., and J.B.S.), University of Connecticut Health Center, Farmington, Connecticut 06032, and the Departments of Molecular Biophysics and Biochemistry (S.G.S.), Yale University School of Medicine, New Haven, Connecticut 06510. *Received June 28, 1978.* This study was supported in part by U.S. Public Health Service Grants GM17021, GM24976, and RR07015 and a grant from the University of Connecticut Research Foundation.

0006-2960/79/0418-0036$01.00/0

mitochondrial P-450 (Whysner et al., 1970). In addition, both Hildebrandt et al. (1968) and Jefcoate & Gaylor (1969) reported in liver microsomes from 3-methylcholanthrene-treated animals an EPR signal at about $g = 6$ indicative of high-spin P-450. Although such reports were initially descriptive, Kumaki et al. (1978) attempted to correlate the observed spectral changes (type I, type II, and reverse type I) with the absolute spin state of the iron.

Recently, Sligar (1976) showed that cytochrome P-450$_{cam}$ in the presence or absence of substrate contained a mixture of high-spin and low-spin heme iron which was temperature sensitive. In related studies Rein et al. (1977) reported the presence of a substrate-induced high-spin/low-spin equilibrium for rabbit liver microsomal cytochrome P-450 and that this equilibrium was temperature dependent. At the same time Pierson & Cinti (1977) found that the microsomal type I and reverse type I spectral changes could be obtained without the addition of substrate simply by an alteration of temperature. Furthermore, it was shown by both Rein et al. (1977) and Pierson & Cinti (1977) that the substrate-induced types I and II spectral changes were affected by temperature.

In this paper we (1) compare the temperature-induced spectral changes in rat liver microsomes and partially purified cytochrome P-450, (2) correlate the temperature-induced spectral changes with the spin state of the ferrihemoprotein, (3) determine the molar extinction coefficient and the absolute content of high- and low-spin cytochrome P-450 iron in both microsomes and the partially purified preparation, and (4) determine the thermodynamic parameters (ΔH, ΔS, and ΔG) of the temperature-dependent spin equilibrium. It should be noted at the outset that all experiments described herein were performed in the absence of exogenous substrate. A thermodynamic analysis of the effect of temperature on substrate-induced spectral changes in rat liver microsomes and solubilized cytochrome P-450 will be the subject of a subsequent communication.

Methods

Preparation of Microsomes. Untreated male Sprague–Dawley rats (175–275 g), obtained from Charles River Breeding Labs (Wilmington, MA), were maintained on laboratory chow and water ad lib. Rats were decapitated, and livers were removed and perfused with 0.9% NaCl. Microsomes prepared by the calcium precipitation method (Cinti et al., 1972) were washed once with 0.15 M KCl and resuspended in 0.05 M Tris containing 0.15 M KCl, pH 7.4.

Isolation and Partial Purification of Cytochrome P-450. Cytochrome P-450 was solubilized and partially purified from liver microsomes of uninduced male Sprague–Dawley rats (230–290 g) by a modification of a previously described method (Gibson & Schenkman, 1978). The nonionic detergent Emulgen 911 was used for solubilization and cytochrome P-450 was partially purified on a lauric acid affinity column. The column was overloaded to yield the solubilized P-450 in one major pool which was freed of detergent with Bio-Beads and stored frozen at −20 °C in 0.05 M sodium phosphate, pH 7.25, containing 25% glycerol; the hemoprotein had a specific content of 4 nmol/mg of protein. The partially purified preparation was totally devoid of NADPH–cytochrome P-450 reductase activity and cytochrome b_5.

Recording of Absorption Spectra. All spectra were recorded on an Aminco DW-2 recording spectrophotometer with a temperature-controlled cuvette chamber, using split-beam and dual-wavelength modes. Changes in temperature were obtained with a Lauda K-2/R water bath that was attached to the cuvette chamber of the spectrophotometer and the temperature was monitored by a YSI temperature probe.

In some temperature studies employing the partially purified cytochrome P-450 preparations, a second cuvette chamber in line with but 10.5 cm from the end-on photomultiplier tube (PMT) was also used during the recording of difference spectra as the primary sample and reference cuvette holders are in thermal contact and hence cannot be temperature regulated separately. For these studies the sample chamber nearest the PMT was equilibrated at various temperatures while the reference cuvette in the secondary cuvette holder was maintained at room temperature (20 °C). Such experiments were not performed with microsomes because of the problem with light scattering. The absolute spectrum of liver microsomal cytochrome P-450 was recorded following the addition of an equimolar concentration of highly purified cytochrome b_5 (generously donated by Dr. Ingela Jansson) to a reference cuvette containing only buffer. The turbidity of the microsomal sample was offset in the reference cuvette by the addition of microliter amounts of milk; the 480-nm wavelength was selected to balance the turbidity.

Extraction and Analysis of Phospholipid. Residual phospholipid present in the solubilized, semipurified cytochrome P-450 was extracted with chloroform–methanol as described by Folch et al. (1959) after extensive dialysis against 0.01 M Tris buffer, pH 7.5, to remove the phosphate contribution by the buffer and was subsequently analyzed for organic phosphorus using the Fiske–Subarrow reducing agent (Dittmer & Wells, 1969). The phospholipid content of the semipurified cytochrome P-450 was 0.14 mg of phospholipid/nmol of P-450 compared to the microsomal value of 0.6 mg of phospholipid/nmol of P-450 (Nilsson & Dallner, 1975).

Electron Paramagnetic Resonance Spectra. EPR spectra were obtained at −160 °C using a Varian E-4 spectrometer equipped with a liquid nitrogen flow temperature controller. Spectra were recorded at a microwave frequency of 9.145 MHz, 10-G modulation amplitude, and a power of 50 mW.

The P-450 spin equilibrium constant was determined using the methods described by Sligar (1976). For the sake of clarity a more detailed description of the measurement and analysis methodology is presented.

In the simplest two-state model, at any physiological temperature, the ferric P-450 molecule was considered to exist as a mixture of high-spin (total spin, $S = {}^5/_2$) or low-spin ($S = {}^1/_2$) forms corresponding to the ordering of the five d-orbital electrons. To each of the high- and low-spin forms corresponds a unique optical spectrum. Thus, at any wavelength the total absorbance, A, = ϵ_{HS}[P-450$_{HS}$] + ϵ_{LS}[P-450$_{LS}$] where ϵ_{HS} and ϵ_{LS} refer to the extinction coefficients and [P-450$_{HS}$] and [P-450$_{LS}$] to the concentrations of high- and low-spin forms, respectively. In addition, at all wavelengths there exist a maximum, A_{max}, and minimum, A_{min}, value of absorbance which correspond to the limiting cases when the P-450 molecule is in either the complete low-spin or complete high-spin state. When P-450 exists as a mixture of spin states, the absorbance will fall between the limiting values, A_{max} and A_{min}, with the distance from the upper and lower limits being directly proportional to the high- and low-spin fractions. For example, at 418 nm the fraction of low-spin material is given by the ratio $(A - A_{min})/(A_{max} - A_{min})$ while the proportion of high spin is $(A_{max} - A)/(A_{max} - A_{min})$. Defining an equilibrium constant for the processes P-450$_{LS}$ ↔ P-450$_{HS}$ by K = [P-450$_{HS}$]/[P-450$_{LS}$] yields the following expression for K:

$$K = \frac{A_{max} - A}{A - A_{min}}$$

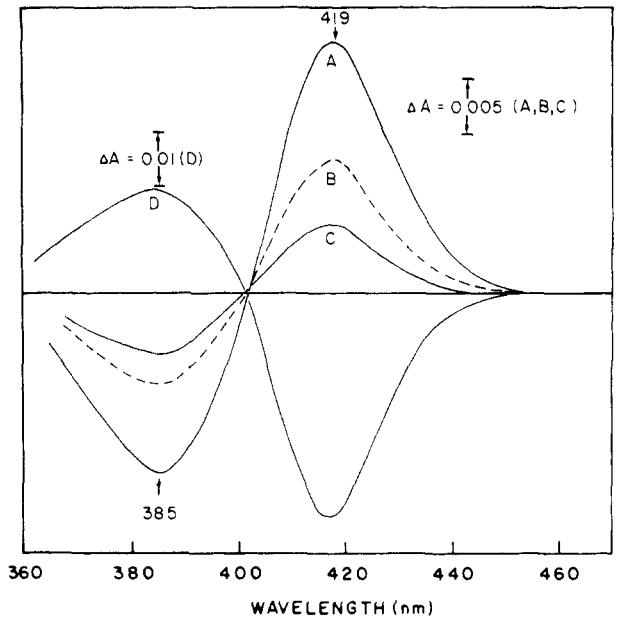

FIGURE 1: Temperature-induced type I and reverse type I spectral changes in rat liver microsomes. Microsomes suspended to a final protein concentration of 3 mg/mL with 0.1 M Tris buffer, pH 7.4, were divided into two cuvettes and a base line was recorded at 20 °C. The sample cuvette was then placed on ice until a temperature of 5 °C was attained; the cuvette was readmitted to the chamber and a difference spectrum was recorded immediately (curve A); curve B was obtained when the temperature in the sample cuvette reached approximately 10 °C; curve C was obtained when the temperature in the sample cuvette reached approximately 15 °C; curve D represents the sample warmed to 30 °C. Scan speed was 2 nm s^{-1}.

If this process is then assumed to follow standard van't Hoff behavior we can write

$$K = \frac{A_{max} - A}{A - A_{min}} = e^{\Delta G/RT} = e^{-\Delta S/R} e^{\Delta H/RT}$$

or

$$\ln K = \ln \frac{A_{max} - A}{A - A_{min}} = \frac{-\Delta S}{R} + \frac{\Delta H}{RT}$$

which completely describes the variation in the spin equilibrium constant with temperature. Since the values of A_{max} and A_{min} cannot be known a priori by any experimental means, they are used as parameters in a regression fit to the variation in the observed absorbance value as a function of temperature. Using the correlation coefficient as a convergence parameter for the least-squares straight line fit of $\ln [(A_{max} - A)/(A - A_{min})]$ vs. $1/RT$ one can determine the values A_{max} and A_{min} which give the best fit to the observed absorbances, as well as the slope ΔH and intercept $\Delta S/R$.

Results

Temperature-Induced Type I and Reverse Type I Spectral Changes in Rat Liver Microsomes and Solubilized Cytochrome P-450. Exposure of liver microsomes to a temperature differential results in a spectral change characteristic of that obtained in the presence of substrate. As seen in Figure 1, microsomes in the sample cuvette exposed to a lower temperature (5–10 °C) relative to microsomes in the reference cuvette which was maintained at room temperature (20 °C) exhibited a reverse type I spectral change with an absorption maximum at 419 nm and a minimum at 385 nm. The temperature-induced spectral changes also displayed an isosbestic point at about 405 nm, suggesting the conversion of a spectral species from one form to another. When the temperature of the microsomes in the sample cuvette was raised above that in the reference cuvette, a type I spectral change was produced. This is illustrated in Figure 1, curve D, in which the microsomes in the sample cuvette were warmed to 30 °C while the microsomes in the reference were maintained at 20 °C; the absorption maximum is at 385 nm while the minimum occurs at 419 nm. These observations indicate that the reverse type I spectral change is not a modified type II spectral change but is a mirror image of the type I spectral change. Similar observations were reported recently by Paul et al. (1976) with adrenal cortical mitochondria which have relatively large amounts of P-450. These temperature-induced spectral manifestations were not observed with the other liver subcellular fractions (nuclear, mitochondrial, or cytosolic).

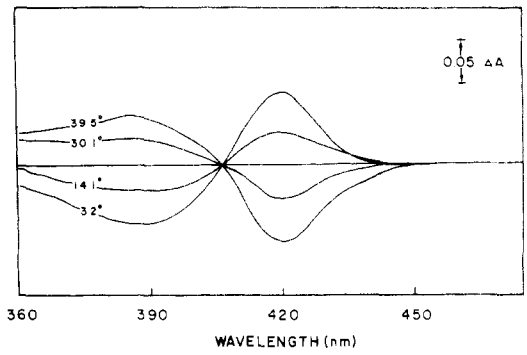

FIGURE 2: Temperature-induced type I and reverse type I spectral changes in partially purified cytochrome P-450 from rat liver microsomes. The cytochrome P-450 preparation was diluted to a final concentration of 1 μM with 0.05 M phosphate buffer, pH 7.4, containing 25% glycerol and divided into two cuvettes. The sample cuvette was placed in the primary holder adjacent to the PMT while the reference cuvette was placed in the secondary holder 10.5 cm from the PMT; a base line was recorded at 24.8 °C. As indicated in the figure, the temperature was varied from 3.2 to 39.5 °C. Scan speed was 2 nm s^{-1}.

The temperature-induced spectral changes were also observed with solubilized, partially purified cytochrome P-450 from rat liver microsomes (Figure 2). The nonparticulate, solubilized P-450 preparation allowed the use of two cuvette chambers as described under Methods, in which temperature-dependent difference spectra could be determined. As the temperature of the P-450 preparation in the sample cuvette was lowered relative to the reference cuvette, an absorption peak at 419–420 nm was obtained identical with that seen with microsomes; an absorption minimum was also formed at the same wavelength when the sample cuvette was warmed to a temperature higher than that of the reference (Figure 2). An isosbestic point was obtained at 406–407 nm, close to that observed with the microsomes.

The absolute absorption spectra of solubilized cytochrome P-450 were obtained by the use of only one cuvette holder immediately adjacent to the PMT; the reference cuvette contained 50 mM sodium phosphate buffer, pH 7.25, and 25% glycerol, while the sample cuvette contained the solubilized P-450 in the same buffer system. As the temperature of the sample cuvette was varied from 4 to 40 °C there resulted an increase in absorbance in the region spanning 360–390 nm and a concomitant loss of absorbance in the 410–420-nm region (Figure 3) analogous to that observed in difference spectra (Figure 2).

The microsomes behaved in a similar manner; the absolute absorption spectrum was obtained with microsomes in the sample cuvette and cytochrome b_5 and milk in the reference to offset the absorbance attributable to hemoprotein and turbidity. As seen in Figure 4, increasing the temperature

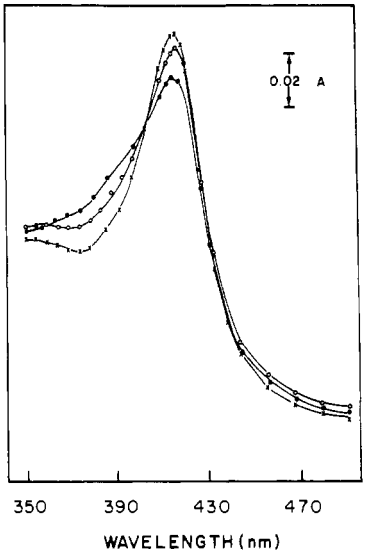

FIGURE 3: Effect of temperature on the absolute spectrum of partially purified cytochrome P-450. The preparation of cytochrome P-450 was diluted to a final concentration of 1 μM with 0.05 M phosphate buffer, pH 7.4, containing 25% glycerol and placed in the sample cuvette; to the reference cuvette was added an equivalent amount of buffer only. Spectra were recorded from 350 to 500 nm following variation in temperature from 4.5 to 40 °C. A base line was obtained with the buffer system in both sample and reference cuvettes; scan speed was 2 nm s^{-1} ×, 4.5 °C; O, 24.5 °C; ●, 40 °C.

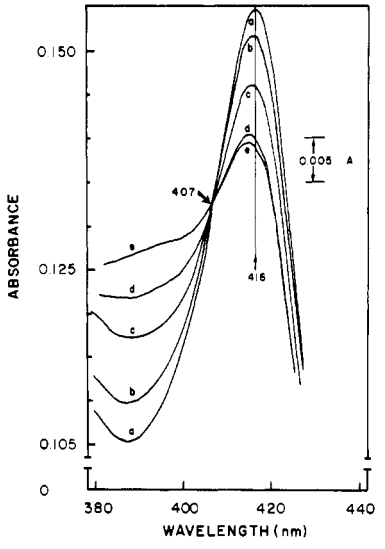

FIGURE 4: Temperature effect on the absolute spectrum of rat liver microsomal cytochrome P-450. Following the recording of the base line of equal light absorbance with 0.1 M Tris buffer, pH 7.4, microsomes in a final concentration of 1 mg/mL were added to the sample cuvette. An equivalent concentration of highly purified cytochrome b_5 was added to the reference cuvette. To offset the light scattering due to the turbidity, microliter amounts of milk were added to the reference cuvette, using 480-nm wavelength as a reference point. The absolute absorption spectra were then recorded as a function of temperature at a scanning speed of 2 nm s^{-1}: A, 6.5 °C; B, 16 °C; C, 26 °C; D, 37 °C; and E, 42 °C. Total absorbance at 416 nm was 0.153; the concentration of cytochrome P-450 was 0.669 nmol/mg of protein.

resulted in a large decrease in absorbance at 416 nm and a concomitant increase in absorbance at 385–390 nm. An isosbestic point was observed at 407 nm; in addition there was a slight but significant shift of 1.5 nm in the Soret band of cytochrome P-450 toward the blue region following an increase in temperature (Figure 4).

Spectral Titration of the Type I and Reverse Type I Spectral Changes as a Function of Temperature. When the

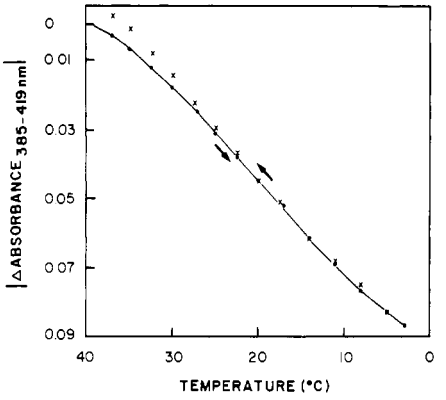

FIGURE 5: Temperature-dependent changes in the absolute value of absorbance (385–419 nm) of rat liver microsomes. Microsomes were suspended to a final concentration of 2 mg/mL with 0.1 M Tris buffer, pH 7.4, and warmed to 40 °C. Absorbance changes were then recorded using the dual wavelength mode of the DW-2 as the temperature was lowered in decrements of 2–3 °C (indicated by the arrow pointing downward to the right). The same suspension of microsomes was used to measure the absorbance change from 3 to 40 °C in increments of 2–3 °C (indicated by the arrow pointing upward to the left).

temperature-induced type I and reverse type I spectral changes were monitored at 385 and 419 nm by dual wavelength spectrophotometry, a slightly sigmoid-shaped curve was obtained with a change in temperature (Figure 5). The sample of microsomes in 0.1 M Tris buffer, pH 7.4, was warmed and the titration initiated at 40 °C. As the temperature was lowered from 40 to 3 °C in decrements of 2–3 °C, a corresponding decrease in absorbance occurred (loss of type I spectral change; Figure 5, arrow pointing in a downward direction). Increasing the temperature from 3 °C back to 40 °C resulted in an exact reversal of the absorbance change, i.e., an increased type I spectral change occurred (Figure 5, arrow pointing upward). This reversal was identical to approximately 25 °C; above this temperature, a greater absorbance change occurred with each 2–3 °C increment. Lowering the temperature from 40 to 25–30 °C caused an absorbance change which was always smaller than the absorbance change which occurred when the temperature was increased from 25 to 40 °C. This observation has been repeated several times and at present an explanation for this phenomenon is not known, although similar changes have been observed with the solubilized preparation. It should be noted that none of these effects can be attributed to baseline shifts caused by changes in turbidity since there were no spectral changes at the isosbestic points 405 and 490 nm with changes in temperature.

Spin Equilibrium Constant of Liver Microsomal and Solubilized Cytochrome P-450. It is now established that a type I substrate affects the heme environment of cytochrome P-450 by converting the iron(III) from a predominantly low-spin to a high-spin state, while a type II or reverse type I substrate affects the spin state in the opposite direction, i.e., from high- to low-spin conversion (Tsai et al., 1970; Peterson, 1971; Nebert & Kon, 1973; Nebert et al., 1977). In addition, Sligar (1976) showed that the equilibrium between the high- and low-spin states was shifted toward the low-spin form with decreasing temperature on purified cytochrome P-450_{cam}. When the temperature-induced absorbance changes, taken from Figure 5, were fit to an equilibrium constant following an Arrhenius temperature dependence according to the procedure described under Methods, a linear van't Hoff plot was obtained (Figure 6, top curve). From the slope of the linear plot and the y-intercept, the overall enthalpy, ΔH = −14.66 kcal/mol, and entropy, ΔS = −49.88 eu, in liver

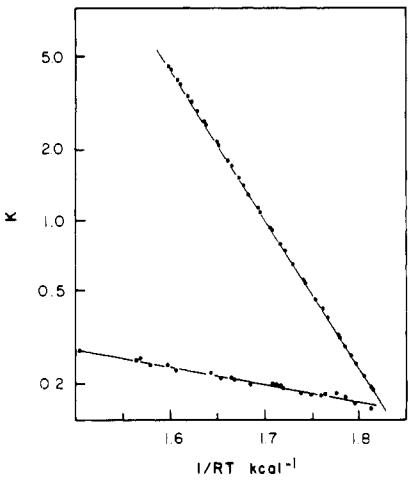

FIGURE 6: Equilibrium constant for the microsomal and partially purified cytochrome P-450 spin equilibrium, P-450$_{LS}$ ↔ P-450$_{HS}$, as a function of temperature. The temperature-induced difference spectra of liver microsomes and of partially purified P-450 were analyzed as described under Methods. The data represent two sets of points obtained from two rats on separate days. For the liver microsomes the correlation coefficient r, used as a convergence parameter in the fitting routine analyses, was 0.99998, and the enthalpy and entropy values describing the process were −14.66 kcal/mol and −49.88 eu, respectively (top curve). For the purified preparation the correlation coefficient was 0.998 and the thermodynamic parameters enthalpy and entropy were −5.5 kcal/mol and −14.1 eu, respectively (bottom curve).

Table I: Spin Content of Microsomal and Soluble Cytochrome P-450 in the Absence of Exogenous Substrate

	% high-spin P-450[a,b]	
temp (°C)	microsomes	soluble
4	17.9	5.6
20	48.2	9.7
37	78.5	16.2

[a] Based on the equilibrium constant K = (P-450$_{HS}$)/(P-450$_{LS}$) or simply HS/LS; HS = $K/(1 + K)$ and, conversely, LS = $1/(1 + K)$. [b] These values are the percentages of *only* that function of the total P-450 which undergoes a temperature-dependent change in spin state.

microsomes were obtained corresponding to the equilibrium reaction P-450$_{LS}$ ⇌ P-450$_{HS}$. These thermodynamic parameters yield a markedly temperature-dependent equilibrium constant, K, of 0.218, 0.930, and 3.750 at 4, 20, and 37 °C, respectively. These K values were obtained from

$$K = \frac{[\text{P-450}_{HS}]}{[\text{P-450}_{LS}]} = e^{-\Delta S/R} e^{\Delta H/RT}$$

Subsequent calculations indicate a very large amount of high-spin cytochrome P-450 in microsomes in the absence of added substrate (Table I). A linear van't Hoff plot was also obtained with the solubilized cytochrome P-450 preparation (Figure 6, bottom curve). However, the solubilized preparation yielded thermodynamic parameters which were significantly different from those determined with the microsomes: the ΔH value of −5.5 kcal/mol was 2.7 times lower than the value obtained with microsomes, while the ΔS value of −14.1 eu was 3.5 times lower. In addition, the equilibrium constant K for the solubilized preparation was 0.056, 0.096, and 0.163 at 4, 20, and 37 °C, respectively, values markedly different from that obtained with microsomes. Under these conditions, solubilized cytochrome P-450 contains the ferric protoporphyrin predominantly in the low-spin state (90%) whereas membrane-bound P-450 exists in almost equivalent amounts

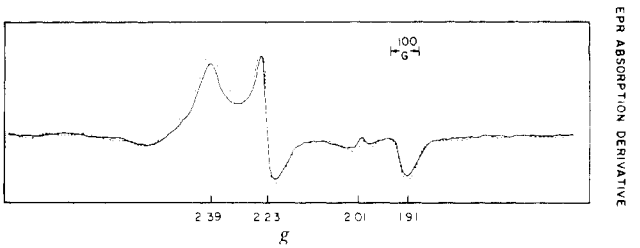

FIGURE 7: Electron paramagnetic spectrum of rat liver microsomal cytochrome P-450 following preincubation at 4 and 37 °C. Two samples of microsomes suspended in 0.1 M Tris buffer, pH 7.4, to a final concentration of 50 mg/mL were placed in EPR quartz columns and simultaneously preincubated at 4 and 37 °C, respectively, for 15 min. The samples were then rapidly frozen by submersion in stirred isopentane at −140 °C. The contents of the EPR tubes were frozen within a 1–2-s period and then stored at liquid nitrogen temperature. The EPR spectra were obtained at a microwave frequency of 9.145 GHz with a Varian E-4 spectrometer fitted for low-temperature operation; the microwave power was 50 mV; modulation frequency, 100 kHz; amplitude, 10 G; temperature, 77 K; (- - - -) 4 °C, (—) 37 °C.

of high and low spin (Table I). The total free energies for the spin equilibrium were calculated from $\Delta G = RT \ln K$, for both microsomes and the solubilized preparation, and found to be markedly different. For example, the ΔG value at 20 °C for the microsomes was −0.043 kcal/mol, in comparison to −1.373 kcal/mol for the soluble preparation. It should be noted that the thermodynamic parameters were derived from the initial temperature cycle in order to minimize high temperature induced degradation of P-450.

Inasmuch as our thermodynamic analysis of the microsomal P-450 spin equilibrium shows a large concentration of high-spin material, one would expect to see a significant contribution of 390-nm absorbance to the absolute spectra of whole microsomes. Such a contribution is apparent in Figure 4, seen as a significant increase and decrease in absorbance at 390 and 416 nm, respectively, with increases in temperature.

The Effect of Preincubation Temperature on the Electron Paramagnetic Resonance Spectra of Liver Microsomes. It is clear from the above that cytochrome P-450 exists in a temperature-dependent dynamic equilibrium between low- and high-spin forms. In order to correlate the temperature-dependent optical spectra with the actual spin state of the heme iron as monitored by electron paramagnetic resonance spectroscopy, two identical samples of whole microsomes were preincubated for 20 min at 4 and 37 °C, respectively, and rapidly frozen following the techniques outlined by Paul et al. (1976). Standard low-spin P-450 g values of 2.39, 2.23, and 1.91 were observed (Figure 7), with no dependence of resonance position on preincubation temperature. A small free-radical signal at 2.01 was seen which could be due to a flavin semiquinone or lipid radical. A distinct difference in the magnitude of the integrated low-spin resonances was seen, however, with the microsomes preincubated at 4 °C, showing a significantly larger amount of low-spin component. Double integration of the low-spin region yields a ratio [P-450$_{LS}$ (37 °C)]/[P-450$_{LS}$ (4 °C)] of 0.83, consistent with the optical data indicating a larger amount of hemoprotein present in the low-spin configuration as the preincubation temperature is lowered.

Discussion

The results presented here have indicated that hepatic cytochrome P-450, both in the membrane-bound and soluble forms, is capable of undergoing a spin transition of the heme iron from a predominantly low-spin to a high-spin configu-

ration as a function of increasing temperature. It is important to note that the experiments described have been carried out in the absence of exogenous substrate and reflect a temperature-dependent spin transition equilibrium between the states. This is substantiated by the fact that clear isosbestic points are observed in liver microsomes (Figure 1) and partially purified cytochrome P-450 (Figure 2) as a function of temperature, indicative of a two-state equilibrium between the low- and high-spin forms of the hemoprotein.

Using either intact microsomes or soluble cytochrome P-450, a typical reverse type I spectral change is observed by difference spectroscopy when the temperature in the sample cuvette is lowered relative to the reference. Thus, decreasing the temperature in the sample effectively shifts the spin equilibrium to the low-spin form resulting in a decreased absorbance of the 390-nm form and an increased absorbance of the 418-nm form of the hemoprotein, i.e., the observed reverse type I spectral change (Schenkman et al., 1972) with absorption maxima and minima at 420 and 385–390 nm, respectively. Conversely, increasing the temperature in the sample relative to the reference cuvette would result in a shift of the equilibrium to the high-spin form of the hemoprotein, resulting in the observed type I spectral change. Consistent with this analysis is the data presented on the effect of temperature on the absolute spectrum of soluble, partially purified cytochrome P-450 (Figure 3) and microsomal P-450 (Figure 4), where an increase in temperature resulted in an increase in absorbance at 370–390 nm (high-spin form).

Entropy and enthalpy values calculated from plots of ln K vs. $1/RT$ (Figure 6) showed a significant difference between liver microsomes and the solubilized, partially purified preparation. Thus in the soluble preparation, at any given temperature, the equilibrium was shifted in favor of the low-spin form, in contrast to microsomes which contained a greater amount of P-450$_{HS}$ (Table I). Hence, removal of cytochrome P-450 from the endoplasmic reticulum membrane by detergent and subsequent purification results in a hemoprotein which is predominantly in the low-spin form. As the soluble form of cytochrome P-450 is lipid depleted relative to microsomes, it is conceivable that lipid or an endogenous substrate can modulate the spin transition occurring with cytochrome P-450; in our soluble preparation more than 75% of the phospholipid had been removed during the purification procedure.

The data presented in this paper clearly illustrates a dynamic equilibrium between two spectral forms of cytocrome P-450 both in whole microsomes as well as in the partially purified enzyme. These temperature-induced difference spectra are analogous to those observed in the P-450 isolated from the bacterium *P. putida* (Sligar, 1976) and represent the interconversion of the heme iron between the low- and high-spin forms as verified by NMR susceptibility and Mössbauer spectroscopy. This change is found to be reversible and completely described by an equilibrium constant K = [P-450$_{HS}$]/[P-450$_{LS}$] which is governed by the standard-state enthalpy and entropy terms according to $K = \exp(-\Delta S/R + \Delta H/RT)$.

In order to extract the fundamental parameters of this transition it was absolutely necessary to employ a nonlinear regression procedure as described under Methods. From this analysis one obtains not only the enthalpy and entropy associated with the transition but also the maximal and minimal values of the absorbance change. If, for example, one is dealing with the absolute absorption at 416–418 nm, the derived A_{min} and A_{max} values correspond to the molecular extinction coefficients for pure high- and low-spin material. It should be noted that although A_{max} and A_{min} are determined to high precision in the analysis procedure, derivation of the corresponding extinction coefficients involves an accurate knowledge of the total cytochrome P-450 content. Nevertheless, the values of ϵ_{HS} and ϵ_{LS} for partially purified P-450 at 418 nm were found to be 52 mM^{-1} cm^{-1} (A_{min}/[P-450]$_{total}$) and 126 mM^{-1} cm^{-1} (A_{max}/[P-450]$_{total}$), respectively. In the case of microsomal cytochrome P-450 the analogous parameters derived from the absolute absorption spectrum (see Figure 4) also correspond to the molecular extinction coefficients for pure high- and low-spin hemoprotein. However, the absolute absorption at 416 nm is not solely attributed to cytochrome P-450; hemoproteins and flavins from mitochondrial contamination (although small, 1–3%) as well as microsomal flavins, cytochrome P-420, and other chromophores would contribute to the observed absorbance in this region. Nevertheless, the *difference* in derived maximum and minimum absorbance is directly proportional to the amount of P-450 undergoing the temperature-dependent spin transition. Hence, from the ΔA_{max} and ΔA_{min} at 416 nm and the concentration of microsomal P-450, one obtains a $\Delta\epsilon_{HS-LS}$ of 40 mM^{-1} cm^{-1}. The corresponding $\Delta\epsilon_{HS-LS}$ for the partially purified preparation was 74 mM^{-1} cm^{-1} (ϵ_{LS} = 126 − ϵ_{HS} = 52), suggesting that only 54% of the total microsomal cytochrome P-450 undergoes a temperature-induced spin transition, assuming that membrane components such as phospholipid do not alter the absolute extinctions. Conversely, all of the soluble cytochrome P-450 undergoes a temperature-induced spin transition.

Using microsomes one can also derive from the 385–419-nm difference spectrum the ϵ for the maximal absorbance change, ΔA_{max} (385–419), for the conversion of low-spin to high-spin material. Again, although the total absorbance change, ΔA_{max} (385–419), is known to both extremely high precision (high correlation coefficient in the regression analysis) *and* accuracy (reproducibility of the derived thermodynamic parameters using different microsomal preparations) the corresponding extinction accuracy is dependent on determination of total P-450. For the microsomal preparation the $\Delta\epsilon$ at 385–419 nm equals 126 mM^{-1} cm^{-1}, assuming that only 54% of the total microsomal cytochrome P-450 undergoes a temperature-induced spin transition.

With the fundamental parameters ΔH and ΔS describing the P-450 spin equilibrium quantitatively, one has a firm basis of comparison between partially purified and membrane-bound enzymes. Table I illustrates the dramatic effect the microsomal environment has on controlling the spin equilibrium. Isolation of the P-450 molecule from the lipid and/or possibly endogenous substrates clearly results in a shifting of the equilibrium substantially to the low-spin form of the cytochrome.

Since the interaction of ligands with cytochrome P-450 can be quantitatively understood in terms of a multidimensional free-energy coupling model (Sligar, 1976) and since the Gibbs free energy, enthalpy, and entropy are all state functions, the markedly different ΔH and ΔS values for partially purified P-450 and membrane-bound enzyme indicate an asymmetry in thermodynamic interaction between the high- and low-spin forms with a lipid and/or endogenous substrate.

One of the goals in the study of temperature-induced spectral changes indicative of an interconversion of high-spin heme iron to low spin was to relate these effects with the actual state of the d-orbital electrons as monitored by the conventional resonance spectroscopies. In the case of bacterial cytochrome P-450, the observed optical spectra were completely correlated

with the P-450 spin state as measured by NMR susceptibility and Mössbauer spectroscopy (Sligar, 1976). Inasmuch as the large quantities of recrystallized material required for these studies are not as yet available from mammalian sources, we are limited to making comparisons with low-temperature electron paramagnetic resonance studies. Since the $P\text{-}450_{LS} \leftrightarrow P\text{-}450_{HS}$ reaction is dynamic, ideally the equilibrium at any temperature from 0 to 40 °C could be quantitated if the sample could be frozen at a rate which is much faster than the forward and reverse rates for the conversion processes. The amount of low-spin cytochrome following the rapid freezing of identical microsomal samples was found to be greater at the lower preincubation temperature as predicted by the solution thermodynamic analysis (Figure 7). From the values of K determined by optical titration, a relative ratio of spin fractions was expected, where K (4 °C) = 0.218 and K (37 °C) = 3.75 are the equilibrium constants at the two preincubation temperatures. Double integration of the EPR spectra, however, yielded an f value much nearer unity, $f = 0.83$, indicating that substantial equilibration of the spin populations has occurred during the freezing process. Considering the rate constants for the spin interconversion of $P\text{-}450_{cam}$ (Sligar, 1976), which correspond to relaxation half-times of about 12 ms following a temperature jump, such an equilibration during freezing of the sample was not surprising. This does not negate the qualitative argument, however, that higher preincubation temperatures favor the high-spin form of the cytochrome.

$$f = \frac{1 + K\,(4\ ^\circ\mathrm{C})}{1 + K\,(37\ ^\circ\mathrm{C})} = 0.26$$

The equilibration of the spin states during the freezing process would also explain why such small high-spin values obtained with rats were recently reported by Kumaki et al. (1978); their data for control rat liver suggested only a 5–10% high-spin P-450 iron.

In summary we have shown that microsomal and solubilized cytochrome P-450 from rat liver exists as a mixture of two spectral forms which are interconvertible as the sample temperature is varied. The evidence of a clean isosbestic point in this temperature-induced spectral shift suggests a simple two-state model for the phenomenon. Similar spectral responses of cytochrome P-450 from the bacterium *P. putida* was correlated with a modulation of the quantum mechanical electron spin state of the cytochrome heme iron. Increasing the temperature of all P-450's examined converts the low-spin ($S = {}^1/_2$) ferric heme with a Soret maximum of 418 nm to the high-spin ($S = {}^5/_2$) form with a blue-shifted Soret (390-nm maximum). This two-state spin equilibrium is completely described thermodynamically by the Gibbs free energy, ΔG, enthalpy, ΔH, and entropy, ΔS. Extraction of these values from the absorbance vs. temperature data requires a nonlinear regression fit to the van't Hoff equation as described under Methods. This technique allows delineation of precise thermodynamic parameters which have well-defined and precise physicochemical meaning. We have shown unambiguously that intact microsomes contain roughly equal amounts of high- and low-spin cytochrome P-450 at 20 °C. When lipid and possible endogenous substrates are removed from P-450 by solubilization and purification, the molecule returns to a predominantly low-spin state which appears remarkably similar to substrate-free $P\text{-}450_{cam}$ from bacteria.

Acknowledgments

We wish to express our gratitude to Dr. Joseph Coleman for the use of the EPR facilities and to Lydia Polomski for her expert technical assistance.

References

Cammer, W., Schenkman, J. B., & Estabrook, R. W. (1966) *Biochem. Biophys. Res. Commun. 23*, 264.

Cinti, D. L., Moldeus, P., & Schenkman, J. B. (1972) *Biochem. Pharmacol. 21*, 3249.

Dittmer, J. C., & Wells, M. A. (1969) *Methods Enzymol. 24*, 482.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497.

Gibson, G. G., & Schenkman, J. B. (1978) *J. Biol. Chem. 253*, 5957.

Hildebrandt, A., Remmer, H., & Estabrook, R. W. (1968) *Biochem. Biophys. Res. Commun. 30*, 607.

Imai, Y., & Sato, R. (1966) *Biochem. Biophys. Res. Commun. 22*, 620.

Jefcoate, C. R. E., & Gaylor, J. L. (1969) *Biochemistry 8*, 3464.

Kumaki, K., Sato, M., Kon, H., & Nebert, D. (1978) *J. Biol. Chem. 253*, 1048.

Mason, H. S., Yamano, T., North, J. C., Hashimoto, Y., & Sakagishi (1965a) in *International Symposium on Oxidases* (King, T., Mason, H., & Morrison, M., Eds.) p 189, Wiley, New York.

Mason, H. S., North, J. C., & Vanneste, M. (1965b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 24*, 1172.

Narasimhulu, S., Cooper, D. Y., & Rosenthal, O. (1965) *Life Sci. 4*, 2101.

Nebert, D. W., & Kon, H. (1973) *J. Biol. Chem. 248*, 169.

Nebert, D. W., Kumaki, K., Sato, M., & Kon, H. (1977) *Microsomes Drug Oxid., Proc. Int. Symp., 3rd, 1976*, 224.

Nilsson, O., & Dallner, G. (1975) *FEBS Lett. 58*, 190.

Paul, D., Gallant, S., Orme-Johnson, N., Orme-Johnson, W., & Brownie, A. (1976) *J. Biol. Chem. 251*, 7120.

Peterson, J. (1971) *Arch. Biochem. Biophys. 144*, 678.

Pierson, W. C., & Cinti, D. L. (1977) *Biochem. Biophys. Res. Commun. 78*, 1139.

Rein, H., Ristau, O., Friedrich, J., Janig, G.-R., & Ruckpaul, K. (1977) *FEBS Lett. 75*, 19.

Remmer, H., Schenkman, J., Estabrook, R. W., Sasame, H., Gillette, J., Narasimhulu, S., Cooper, D. Y., & Rosenthal, D. (1966) *Mol. Pharmacol. 2*, 187.

Schenkman, J. B., Remmer, H., & Estabrook, R. W. (1967) *Mol. Pharmacol. 3*, 113.

Schenkman, J. B., Cinti, D. L., Orrenius, S., Moldeus, P., & Kaschnitz, R. (1972) *Biochemistry 11*, 4243.

Sligar, S. G. (1976) *Biochemistry 15*, 5399.

Tsai, R., Yu, C. A., Gunsalus, I. C., Peisach, J., Blumberg, W., Orme-Johnson, W. H., & Beinert, H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 1157.

Whysner, J. A., Ramseyer, J., & Harding, B. W. (1970) *J. Biol. Chem. 245*, 5441.