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SPIN STATE CONTROL OF THE HEPATIC CYTOCHROME P450 REDOX POTENTIAL

S. G. Sligar*, D. L. Cinti+, G. G. Gibson+ and J. B. Schenkman+

* Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520 and
+ Department of Pharmacology, The University of Connecticut Health Science Center, Farmington, CT 06032

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SUMMARY - We have measured the oxidation-reduction potential of isolated and partially purified cytochrome P450 from uninduced rat liver, both in the presence and absence of Type I substrates. Native P450 is found to have a potential of -300 mV with respect to the standard hydrogen electrode, while the addition of benzphetamine or hexabarbital increases the redox potential to -237 mV and -225 mV respectively. Quantitation of the thermally induced S = 1/2 to S = 5/2 spin transition of the ferric heme iron for this P450 preparation both substrate free as well as with bound benzphetamine and hexabarbital indicate an increase in the high spin (S = 5/2) fraction on the binding of Type I substrates. The relevant oxidation-reduction equilibria of the heme chromaphore are presented in terms of a thermodynamic model for the control of the observed cytochrome P450 redox potential through this modulation of the spin configuration of the five d-electrons of the ferric heme iron induced by the binding of Type I substrates.

Mammalian metabolism of drugs, carcinogens, and environmental toxins often involves substrate oxidation catalyzed by the P450 mixed function oxidase of the hepatic endoplasmic reticulum. The chemistry of hydroxylation by the P450 system involves the two electron reductive cleavage of atmospheric oxygen with one atom incorporated into the substrate and the other reduced to water (1-4). The two electrons required for this process are supplied by pyridine nucleotide (NADPH) dehydrogenation through the action of an FAD-FMN flavoprotein NADPH-cytochrome P450 reductase (5-7).

Investigation of the P450 monoxygenase heme protein from the soil bacterium Psudeomonas putida using various magnetic and optical techniques (8-17) has shown that the binding of the substrate, camphor, alters the configuration of the iron d-orbital electrons, changing from predominantly low spin configuration (S = 1/2) to a high spin (S = 5/2) state at room temperature. Similar behavior is obtained with the P450 monoxygenase system isolated from mammalian sources (18-27). Additionally, it was demonstrated that the absolute spin state of both the Pseudeomonas (13,14,17) and hepatic P450 systems (23-25) is strongly temperature dependent. A description of spin state regulation through association of Type I substrates to purified hepatic P450 has been beautifully presented by Ruckpaul and coworkers (24,26) who carefully documented substrate binding and spin state equilibrium constants. A thermodynamic linkage model provided the vehicle for describing the regulation of the microbial P450

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oxidation-reduction potential via alterations in the spin state of the heme iron (17,26-28). In this communication we present measurements of the redox potential of partially purified hepatic microsomal P450 from untreated rat both in the absence of exogenous substrates or when saturated with added benzphetamine or hexobarbital. These oxidation-reduction potentials can be correlated with the spin equilibrium constant of the ferric hemoprotein as well as with the analogous data from the bacterial hemoprotein component of the camphor hydroxylase system. Emerging is a model for the detailed regulation of cytochrome P450 redox potential through the strength and symmetry of the heme iron ligand field in the ferric protein.

MATERIALS AND METHODS

Cytochrome P450 was purified from the hepatic microsomes of uninduced Sprague-Dawley rats as previously described (25,29). Specific content of P450 used in all experiments described herein is 9.0 nmole P450/mg protein with no detectable amount of cytochrome P420, NADPH-cytochrome P450 reductase, NADH-cytochrome b_5 reductase, or cytochrome b_5 in the preparation.

All redox potentials were quantitated with the dye photoreduction technique as previously used (30) with Safranine T with a midpoint potential of -289 mV (31,32) as indicator and mediator. Potentials were quantitated at 25°C in 1 ml of a 50 mm sodium phosphate buffer, pH 7.25, containing 10 mM EDTA and 25% v/v glycerol. Ten nmoles each of dye and protein were rendered anaerobic by repeated evacuation and flushing of a Thunberg cell with argon that had been freed of oxygen by passage over heated BASF catalyst (Kontes Glass Co.). The system potential (E_D) was set by irradiation for varying lengths of time with a heat filtered 500 watt Xenon lamp, and the fraction of hemoprotein and dye reduced determined by the optical density at 420 nm and 518 nm respectively (17). Safranine T was negligible absorbance at 420 nm and P450 is isosbestic (oxidized-reduced) at 518 nm rendering deconvolution of the P450 and dye absorbances at these wavelengths unnecessary. Data are displayed as standard Nernst plots. No evidence for dye association to P450 was obtained by either absorption or fluoresence spectroscopy. Spin state equilibrium constants under identical experimental conditions were determined as previously reported (17,24,25,27).

Concentrations of substrate used for the redox titrations are sufficient to guarantee complete saturation of the P450, as easily determined spectrophotometrically for the ferric protein. From the results presented and analogy with the bacterial camphor hydroxylase, both substrates are seen to drastically raise the midpoint potential of cytochrome P450 when bound to the hemoprotein. Free energy conservation demands that if substrate binding shifts E_m to more positive values then substrate must bind tighter to the ferrous form (17,30). Thus substrate remains bound to the cytochrome throughout the redox titration and the substrate bound and free proteins may be described with the same free energy coupling model described in the next section.

RESULTS AND DISCUSSION

The oxidation-reduction equilibrium of isolated, substrate-free cytochrome P450 is shown in Figure 1 as a standard plot of the Nernst equation

$$E_D = E_m(\text{dye}) - \frac{RT}{F} \ln \left[\frac{f_D^r}{f_D^o}\right] = E_m(\text{protein}) = \frac{RT}{F} \ln \left[\frac{f_p^r}{f_p^o}\right] \qquad (1)$$

where f_p^r, f_p^o, f_D^o are the fractions (f) of protein (P) and dye (D) reduced (r)

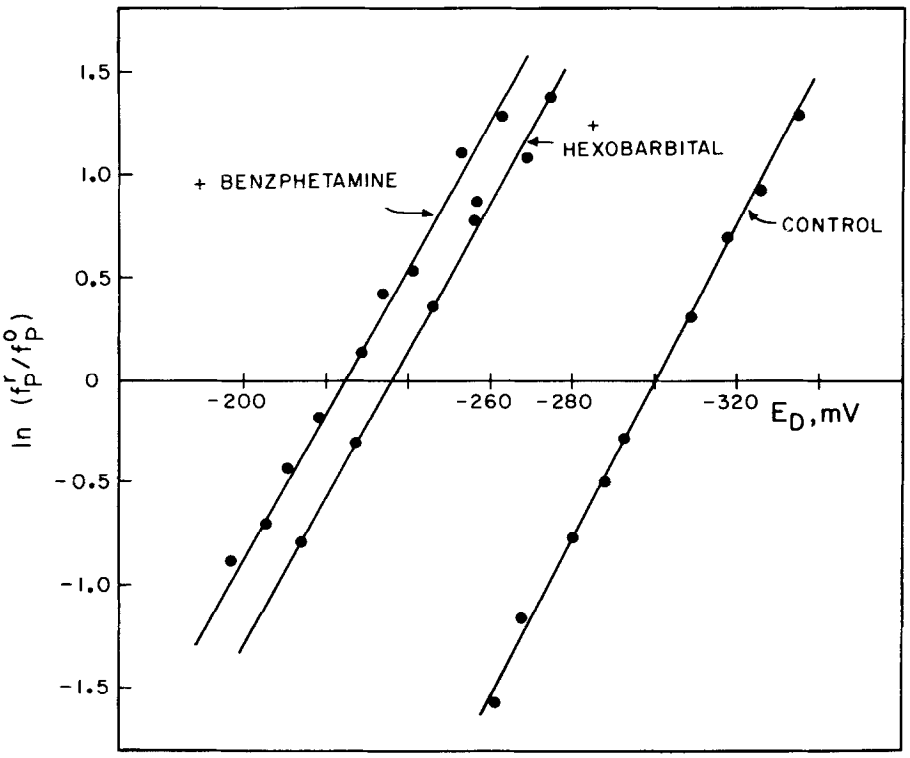

Figure 1. Redox potential titration of purified hepatic cytochrome P450. A standard plot of the Nernst equation is presented with E_D the total system potential which is monitored as described in Methods. Data for both substrate free P450 as well as bound with benzphetamine (1 mM) and hexobarbital (1 mM) are presented. Buffer conditions and methodologies are as described in Methods with a total P450 concentration of 9 μM, 10 μM Safranine T, and 10 mM EDTA.

and oxidized (o) respectively. E_D is the resulting system potential, and E_m the midpoint potentials. R is the gas constant, T the absolute temperature, and F the Faraday constant. From the intersection at equal concentrations of oxidized and reduced P450 a midpoint potential of E_m = -300 mV was obtained for the substrate free cytochrome. Figure 1 also shows analogous data obtained in the presence of the Type I substrates (33) benzphetamine and hexobarbital as described in the legends. Both substrates are seen to shift the oxidation-reduction potential of the protein to less negative values; -225 mV for benzphetamine and -237 mV for hexobarbital (Table I).

The effect of substrate on the P450 redox potential must be separated from effects due to the documented modulation of the ferric P450 spin state. Figure 2 shows the temperature variation of the ferric spin equilibrium constant K both for native P450 and when saturated with benzphetamine and

TABLE I

SPIN AND REDOX EQUILIBRIUM CONSTANTS OF CYTOCHROME P450

System	spin state		redox state	
	$K_a(25°)$	%HS	K_{obs}	E_{obs}(mV)
Bacterial P450[a]				
- camphor	0.089	8	1.2×10^5	-303
+ camphor	14.4	94	8.0×10^2	-173
Hepatic P450				
- substrate[b]	0.113	10	1.2×10^5	-300
+ benzphetamine	0.607	38	5.9×10^3	-225
+ hexobarbital	0.549	35	9.4×10^3	-237

(a) Reference 17

(b) Reference 28

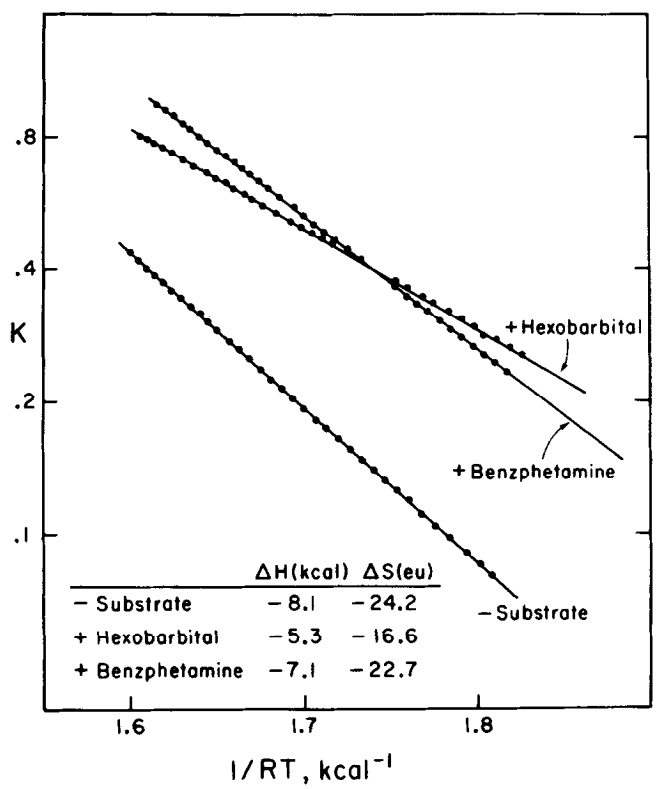

Figure 2. Thermally induced spin state transition in hepatic cytochrome P450. Thermodynamic data for P450 both sbustrate free as well as saturated with benzyphetamine or hexobarbital are presented. Spin equilibrium constants are fit by the procedure described in Methods (17,24,25) using a two parameter convergence routine.

hexobarbital. Table I summarizes the values of K and the corresponding percentages of high spin cytochrome at 25°C for this partially purified preparation. Also presented in Table I are the values for the Pseudomonas camphor hydroxylase (17).

Detailed Mossbauer (8) and susceptability (34,35) measurements on bacterial cytochrome P450 have shown this protein to exist in a totally high spin (S = 2) state even at cryogenic temperature (4°K). Magnetic circular dichroism spectra of both microbial and hepatic P450 at room temperature are quite similar and indicate a complete S = 2 state for the mammalian protein (36). Hence the thermodynamic "cube" model describing the linkage between spin, redox, and substrate binding equilibria (17) can be simplified to a planar scheme (26) with only the ferric spin state energy and the microscopic high spin oxidation-reduction potential with the absolute spin configuration of ferric hepatic cytochrome P450 controlling the observed oxidation-reduction potential of the protein. This interaction of spin and redox equilibria in the P450 systems can be described by a simplified energy coupling model:

$$P450(Fe^{3+})_{LS} \xrightleftharpoons{K_a} P450(Fe^{3+})_{HS} \xrightleftharpoons[\pm e^-]{K_b} P450(Fe^{2+})_{HS} \qquad (2)$$

Here K_a is the ferric spin equilibrium constant presented in Table I. K_b represents the microscopic redox equilibrium constant for reducing high spin P450 heme. Defining K_a and K_b as:

$$K_a = \frac{[P450(Fe^{3+})_{HS}]}{[P450(Fe^{3+})_{LS}]} \qquad K_b = \frac{[P450(Fe^{2+})_{HS}]}{[P450(Fe^{3+})_{HS}]}$$

it can easily be shown that the observed redox potential of the cytochrome, E_m, can be expressed in terms of the microscopic constants K_a and K_b:

$$E_m = \frac{RT}{F} \ln(K_b) + \frac{RT}{F} \ln \frac{1 + K_a}{K_a} \qquad (3)$$

The above equation predicts a linear relationship between observed potential and $\ln[(1 + K_a)/K_a)]$ for both substrate free and substrate bound P450 if all substrate effects are manifested in the constant K_a. In this case the substrate binding energy does not enter into the equation as long as the cytochrome is always saturated (see Methods). Figure 3 correlates the observed redox potential for hepatic and bacterial P450 as suggested by equation (3). The excellent linear relationship clearly demonstrates the regulation of cytochrome P450 redox equilibria via the spin state of the heme iron. Both mammalian and microbial protein are described by the same high spin redox equilibrium constant, K_b, or potential E_b = -175 mV (ordinate intercept Figure 3),

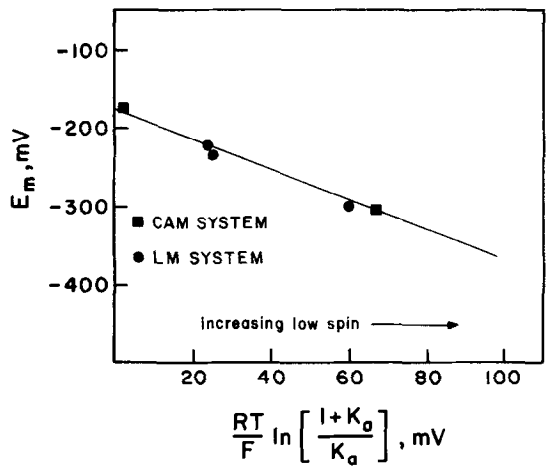

Figure 3. Spin state control of the observed cytochrome P450 oxidation-reduction potential. The three spin and redox equilibria derived from Figure 1 and Table I are plotted according to the model described by equation (3) together with the analogous data obtained for the bacterial camphor P450 monoxygenase (25). The excellent agreement between microbial and hepatic P450 indicates a universality of spin state control of redox equilibria in the P450 oxygenases.

characteristic for adding an electron to the ground orbital of the S = 5/2 manifold and hence catalytic regulation of the observed P450 oxidation-reduction potential is dictated primarily by the energy level structure of the ferric iron 3d electrons as modulated by temperature and substrate association.

An observed potential of -300 mV for partially purified substrate free P450 from untreated rat supports a similar value reported by Coon and coworkers (37) for highly purified P450 from phenobarbital induced rabbits. Earlier titrations using a microsomal preparation (38) reported somewhat lower values, although the fractions oxidized and reduced were quantitated at cryogenic temperatures. When the temperature dependence of the ferric spin equilibrium constant is considered, the lower potential observed by Waterman and Mason is seen to be in close agreement with the theory reported herein since at cryogenic temperatures the added free energy of the ferric spin equilibrium serves to shift the observed potential to more negative values. Similar spin-state modulation of the adrenal cytochrome P450 redox potential has been described for purified adrenal mitochondrial cytochrome by N. Orme-Johnson and coworkers (39). Elegant and exciting experiments by Rein and coworkers (26,27) using highly purified P450 from phenobarbital induced rabbits also illustrate a substrate induced spin-state change and indicate the possibility for such effects to control the observed oxidation-reduction equilibria.

The substrate induced change in redox potential of bacterial cytochrome P450 is clearly reflected primarily in a modulation of the forward rate constant for electron transfer into the hemoprotein (40,41). It is thus suggested from the present studies that the binding of Type I substrates to hepatic cytochrome P450 could increase the rate of electron flow from NADPH-reductase to P450 by modulating the spin state equilibrium of the electron accepting cytochrome. As this redox transfer process is rate limiting for some substrates in hepatocyte metabolism, the heme iron spin equilibrium could be of prime importance in the regulation of monoxygenase activity. Investigations are currently in progress in our laboratories to determine the method of control of electron flow in oxidative disposition in hepatocytes.

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