Cytochrome P450–Benzphetamine Interactions in the Endoplasmic Reticulum: Studies Using a Monoclonal Antibody to P450b

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ABSTRACT: A monoclonal antibody (MAb) to phenobarbital-induced rat cytochrome P450b was used to study the interaction of the substrate benzphetamine (Bz) with cytochromes P450 in liver microsomes. Binding of Bz to liver microsomes from phenobarbital-treated rats was monitored by the substrate-induced type I spectral change. The MAb maximally inhibited this spectral change by 49%, providing a probe to distinguish MAb-specific P450b from other Bz-binding P450s. Thermodynamic parameters of the interaction were determined in the absence and presence of MAb. The MAb did not influence the spin-state equilibrium of substrate-free P450b, but it increased the low spin content of substrate-bound P450b. The MAb also decreased the affinity of both high and low spin P450b for Bz. The temperature dependence of the Bz-binding interactions revealed a transition near 20 °C. Fluorescence polarization measurements of the membrane probe 1,6-diphenyl-1,3,5-hexatriene also revealed a transition at this temperature. The MAb comparably inhibited Bz binding to high spin P450b in the low and high temperature regions, whereas MAb inhibition of Bz binding to low spin P450b was greater in the low temperature region than in the high temperature region. These results indicate temperature-dependent changes in membrane structure that modulate both Bz binding to P450b and MAb–P450b–Bz interactions. These results also demonstrate the utility of MAbs for evaluating P450–substrate binding microequilibria of MAb-specific P450s in the presence of other P450s while in the natural membrane environment of the endoplasmic reticulum.

The cytochromes P450 metabolize a wide variety of xenobiotics such as drugs and carcinogens, as well as endobiotics such as steroids and prostaglandins (Lu & West, 1980; Ortiz de Montellano et al., 1986; Ryan & Levin, 1990). The different forms of P450[1] exhibit unique catalytic activity profiles toward various substrates. A single P450 generally acts on several substrates, and a given substrate can often be metabolized by several P450s. The ultimate fate of a given substrate thus depends on the type and amounts of P450s present in a tissue and the interactions of these P450s with the substrate.

Studies of P450–substrate interactions fall into two general categories. First, one can examine a single purified P450 in a reconstituted system; this approach has the advantage of defining all components and the disadvantage that the functional characteristics of P450 do not necessarily represent those in the endoplasmic reticulum. A second approach is to study the interaction of substrate with microsomes that contain a multiplicity of P450s; the major advantage is that it utilizes a native system, but data interpretation is difficult because several P450s may possibly bind and/or metabolize the substrate. A major advance in overcoming this difficulty has been the advent of MAbs to different P450s (Gelboin & Friedman, 1985; Friedman et al., 1985a). Various studies have demonstrated the utility of MAbs for immunoassay of specific P450s (Song et al., 1984; Cheng et al., 1984; Song et al., 1985; Friedman et al., 1985b; Robinson et al., 1989; Lorr et al., 1989) and inhibition of P450-catalyzed microsomal activities (Fujino et al., 1984; Pelkonen et al., 1986; Pasanen et al., 1987). In such studies, the contribution of a given MAb-specific P450 to the parameter of interest (e.g., enzymatic activity) can be gauged in the presence of a microsomal mixture of P450s, by simply performing measurements in the absence and presence of the MAb.

MAb 4-7-1 to the PB-inducible rat P450b form has been prepared (Park et al., 1984), and it was found to completely inhibit the demethylation of benzphetamine by purified P450b in a reconstituted system (Fujino et al., 1990). We now utilize this MAb to examine the behavior of P450b and define the various interaction parameters of the P450b–Bz couple in microsomes.

EXPERIMENTAL PROCEDURES

Preparation of Rat Liver Microsomes. Male Sprague-Dawley rats (6 weeks old) were treated with phenobarbital (80 mg/kg) for three days by daily intraperitoneal injection, and microsomes were prepared as described (Park et al., 1984). The concentration of cytochrome P450 was determined by the method of Omura and Sato (1964) using a molar extinction coefficient of 91 mM^{-1} cm^{-1}. The protein concentration was assayed by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Monoclonal Antibody. Monoclonal antibody 4-7-1 to P450b was prepared as previously described (Park et al., 1984), obtained from Dr. S. S. Park (National Cancer Institute) and purified from ascites fluid (Stanker et al., 1985).

Benzphetamine Demethylation Assay. N-Demethylation of benzphetamine was assayed at 37 °C by measuring formaldehyde formation. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 0.2 mg/mL microsomes, 1 mM Bz, 0.6 mM NADPH, and 0.6 mM NADH in 1 mL. The reaction was initiated with the addition of NADPH and NADH. After 10 min, the reaction was stopped by addition of an equal volume of 10% trichloroacetic acid, followed by centrifugation to remove the

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[1] Abbreviations: P450, rat liver microsomal cytochrome P450; Bz, benzphetamine; PB, phenobarbital; PB-microsomes, liver microsomes from PB-treated rats; MAb, monoclonal antibody; DPH, 1,6-diphenyl-1,3,5-hexatriene.

precipitate. The amount of formaldehyde in the supernatant was determined by the method of Nash (1953), using a standard curve established with 5,5-dimethyl-1-(hydroxymethyl)hydantoin (Sigma, St. Louis, MO). To measure the inhibition of activity by MAb 4-7-1, microsomes and excess MAb were preincubated at room temperature for 15 min prior to the start of the reaction.

Substrate-Binding Difference Spectra. Type I substrate-binding spectra (Schenkman et al., 1967) were obtained using an Aminco DW2000 spectrophotometer. PB-microsomes (0.36 mg/mL) suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol were added to the sample cuvette, and the buffer solution was added to the reference cuvette. When present, 0.1 mg/mL MAb was preincubated with microsomes for 15 min. After a baseline was recorded, a small volume of Bz (in water) was added to both cuvettes. The spectrum was monitored until there was no further change and the substrate induced difference spectrum was calculated by subtraction of the baseline with a minor correction for volume change. From titration experiments with Bz, the spectral dissociation constant K_s (Schenkman et al., 1967) and maximum absorbance change ΔA_{max} were calculated by least squares curve fitting of the absorbance changes (390 nm–420 nm) versus the concentration of Bz. Temperature was maintained with an external water circulator bath with constant stirring of microsomal samples; temperature was measured with a probe (Yellow Springs Instrument Co., Yellow Springs, OH) inserted in the cuvette.

The microequilibria for substrate (S) binding and for conversions between P450 in the low spin ($P450_l$) and high spin ($P450_h$) states are

$$K_3 = \frac{[P450_l][S]}{[P450_l\text{-}S]}$$

$$P450_l \rightleftharpoons P450_l\text{-}S \quad (\pm S)$$

$$K_1 = \frac{[P450_h]}{[P450_l]} \qquad K_2 = \frac{[P450_h\text{-}S]}{[P450_l\text{-}S]}$$

$$P450_h \rightleftharpoons P450_h\text{-}S \quad (\pm S)$$

$$K_4 = \frac{[P450_h][S]}{[P450_h\text{-}S]}$$

The constants were calculated from difference spectral measurements at different temperatures. Using the measurements in the absence of Bz, the low–high spin equilibrium constant K_1 can be determined from a least-squares fit of the absorbance difference A (absorbance at 390 nm minus that at 420 nm) obtained at temperature T according to (Cinti et al., 1979; Tamburini & Gibson, 1983)

$$\ln K_1 = \ln \frac{A - A_l}{A_h - A} = \frac{\Delta S}{R} - \frac{\Delta H}{RT}$$

where A_h and A_l are the absorbance differences occurring with 100% high and 100% low spin P450, respectively.

After K_1 was obtained, the values of K_2 and K_3 were determined from the relationships (Tamburini & Gibson, 1983)

$$\Delta A_{max} = \frac{(K_2 - K_1)\Delta\epsilon P450_t}{(K_1 + 1)(K_2 + 1)}$$

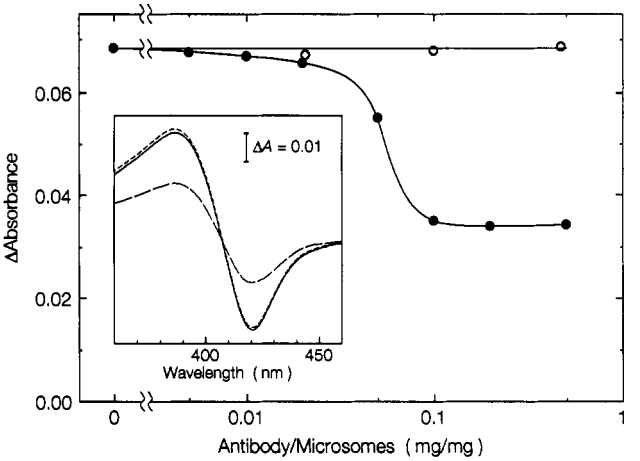

FIGURE 1: Effect of MAb on the substrate-binding difference spectrum of PB-microsomes. PB-microsomes were suspended in 0.1 M potassium phosphate buffer (pH 7.4) including 20% glycerol at 0.5 mg/mL. The concentration of benzphetamine was 1.5 mM. Absorbance differences between 390 nm and 420 nm at 23.7 °C in the presence of MAb 4-7-1 (●) or control MAb HyHel-9 (○) to lysozyme are shown. Inset: benzphetamine-binding difference spectra in the absence of MAb (—) and in the presence of 0.25 mg/mL MAb 4-7-1 (– –) or MAb HyHel-9 (- - -).

and

$$K_s = \frac{K_3(K_1 + 1)}{(K_2 + 1)}$$

where $\Delta\epsilon$ is the difference extinction coefficient for the low–high spin couple and has a value of 126 mM^{-1} cm^{-1}, and $P450_t$ is the total concentration of P450. The remaining constant K_4 was then readily obtained from the relationship $K_1K_3 = K_2K_4$. The enthalpy and entropy changes, ΔH and ΔS, respectively, were calculated for each reaction from the slopes and intercepts of van't Hoff plots of the equilibrium constants. When the plots exhibited a break, the slopes of the linear segments were estimated using data points at the temperature extremes: lines were calculated using three, four, or five points, and the line with the best least-squares fit was selected.

Membrane Microviscosity Measurement. These were determined from fluorescence polarization measurements of the DPH fluorophore. This probe was added (0.001 vol of a 0.15 mM solution in tetrahydrofuran) to the microsomal suspension (0.1 mg/mL) in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, and polarization was measured on a SLM Model 8000c photon counting spectrofluorometer, using a water circulator bath to maintain the temperature. Microviscosities were calculated from polarization data as described (Shinitzky & Barenholz, 1978).

Chemicals. Benzphetamine hydrochloride and DPH were obtained from Sigma. Other chemicals were the highest grade commercially available.

RESULTS

We utilized the substrate-induced type I difference spectrum, which reflects the low to high shift in the P450 spin state, as a spectral probe for binding of Bz to microsomal P450s. Figure 1 shows the type I substrate induced difference spectrum and that addition of MAb 4-7-1 maximally decreased its magnitude by 49%. The effect of the MAb 4-7-1 on microsomal Bz-demethylase activity was also assessed (Table I) and was found to decrease the activity by 39%. This MAb thus inhibited both the spectral change and activity of microsomal P450s. A control MAb (HyHel-9 to lysozyme) affected neither the spectrum nor the activity.

Table I: Inhibition of Benzphetamine-Demethylase Activity of PB-Microsomes by MAb 4-7-1 to P450b[a]

MAb/ microsomes (mg/mg)	activity (%) (nmol/ (mg·min))	MAb/ microsomes (mg/mg)	activity (%) (nmol/ (mg·min))
0	46.8 ± 0.4 (100)	0.10	31.4 ± 0.1 (67)
0.02	44.5 ± 0.2 (95)	0.20	28.3 ± 0.2 (61)
0.05	38.9 ± 0.4 (83)	0.50	28.5 ± 0.3 (61)

[a] Activities of PB-microsomes (0.2 mg/mL) were measured in the presence of different amounts of MAb 4-7-1 to P450b. Values indicate mean ± SD of three experiments.

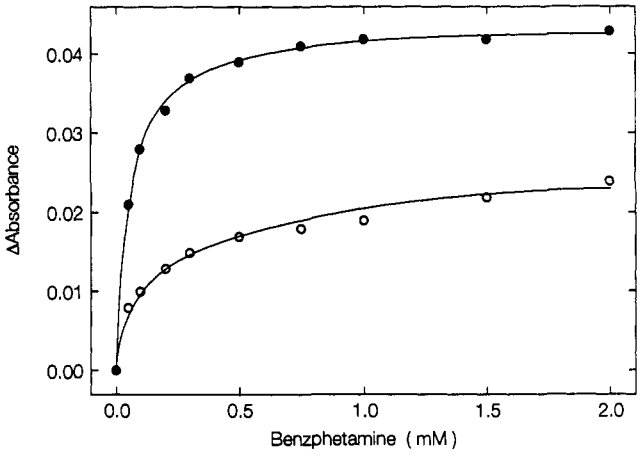

FIGURE 2: Absorbance change upon titration of PB-microsomes with benzphetamine. PB-microsomes (0.36 mg/mL) were suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol with (○) or without (●) 0.1 mg/mL MAb 4-7-1, at 19.6 °C. Aliquots from a stock solution of benzphetamine were added, and spectra were taken. The absorbance changes between 390 nm and 420 nm were plotted against the concentration of benzphetamine.

Figure 2 shows the absorbance change of the 390–420-nm couple upon titrating PB-microsomes with Bz in the absence and presence of MAb 4-7-1. We performed these measurements over the temperature range from –0.4 °C to 36.9 °C and determined the spectral dissociation constant K_s (Schenkman et al., 1967) and maximum absorbance change ΔA_{max} at each temperature. ΔA_{max} increased with temperature both in the absence and in the presence of MAb (Figure 3A). The MAb depressed ΔA_{max} at all temperatures by about 50%, which indicates that the MAb inhibited the extent of the Bz-induced low to high spin shift of microsomal P450s. The temperature dependence of K_s is shown in the van't Hoff plots in Figure 3B. The MAb increased K_s and thus decreased the effectiveness of Bz in inducing the low–high spin shift. The data for ΔA_{max} and K_s, both in the absence and in the presence of MAb, could be represented by two lines with a break corresponding to 20 °C.

The data in Figure 3 thus reveal (1) that binding of MAb 4-7-1 alters the spectral binding parameters and can thus be used to distinguish the properties of MAb-specific P450 from other microsomal P450s that bind Bz and (2) a break in the temperature dependence of these parameters, which may correspond to a membrane phase transition (Jain, 1988). In order to obtain more detailed information on the system, we determined the microequilibrium constants (Tamburini & Gibson, 1983) defined in the Experimental Procedures section.

Figure 4 shows the values of the four derived microequilibrium constants (defined in the Experimental Procedures section) as van't Hoff plots. The thermodynamic parameters ΔH and ΔS obtained from the plots, as well as representative

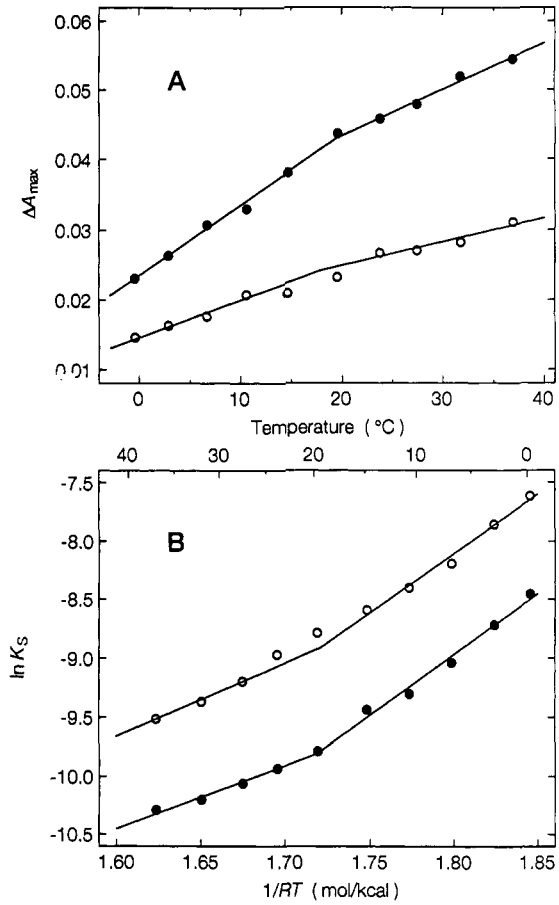

FIGURE 3: Temperature dependence of binding of benzphetamine to PB-microsomes. (A) The variation in the maximum absorbance change (ΔA_{max}) with temperature. (B) van't Hoff plot of the spectral dissociation constant (K_s). These parameters were calculated from titration curves obtained at different temperatures in the presence (○) or absence (●) of MAb 4-7-1.

equilibrium constants obtained at a single temperature (19.6 °C), are presented in Table II.

The plots for K_1 (Figure 4A) appear linear and are the same in the absence and in the presence of MAb, which indicates that the MAb has no effect on the low–high spin equilibrium of substrate-free microsomal P450s. The plots for K_2 (Figure 4B) are linear and show that addition of MAb decreased K_2 by half through a reduction in both enthalpy and entropy changes (Table II). In contrast to the case with substrate-free P450s, the MAb thus increased the low spin content of substrate-bound P450s.

The plots of K_3 (Figure 4C) and K_4 (Figure 4D) first show that the binding affinity of microsomal P450s for Bz increases with temperature. The addition of MAb results in higher values and thus reduces the affinity of both low and high spin P450s for Bz. The MAb lowered the affinity of high spin P450s (K_4) to a greater extent than that of low spin P450s (K_3). For K_3 this effect was not uniform with temperature since the MAb had a lesser effect at higher temperatures than at lower temperatures. In addition, the data both in the absence and in the presence of MAb were nonlinear but could be represented by two straight lines with breakds at 18–20 °C. The thermodynamic parameters ΔH_1 and ΔS_1 were thus determined for the lower temperature range, and ΔH_2 and ΔS_2 were likewise determined for the higher temperatures. These parameters are shown in Table II. While at lower temperatures the MAb did not significantly affect the ΔH_1 or ΔS_1 of the K_3 and K_4 equilibria, at higher temperatures it resulted in more negative values for both ΔH_2 and ΔS_2. Since

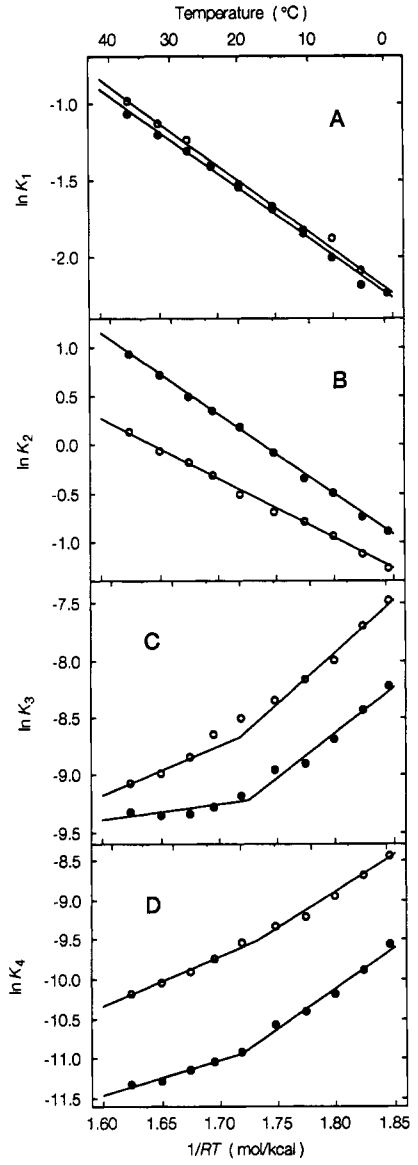

FIGURE 4: Temperature dependence of the microequilibrium constants for the interaction of PB-microsomes with benzphetamine. The microequilibrium constants K_1, K_2, K_3, and K_4 were calculated as described in the text and are shown in panels A, B, C, and D in the presence (O) and absence (●) of MAb 4-7-1.

these parameters are for the Bz dissociation reaction, the MAb thus decreased the enthalpic and increased the entropic favorability for Bz binding in the higher temperature range.

Since several parameters exhibited biphasic behavior with temperature, we investigated membrane fluidity to determine whether a membrane phase transition occurred. We thus measured DPH fluorescence polarization as a probe of membrane microviscosity (Shinitzky & Barenholz, 1978). The microviscosity data (Figure 5) revealed a distinct break corresponding to a temperature of 20 °C, both in the absence and in the presence of MAb, that was similar to that observed for the Bz binding data in Figure 4C,D. The MAb also increased membrane microviscosity, an effect which may arise from its complexation with membrane bound P450s to reduce the rotational diffusion of the complex and influence the ordering of neighboring lipids.

DISCUSSION

We utilized two independent probes, substrate binding and MAb recognition, to focus on substrate binding by a single

Table II: Thermodynamic Parameters of the Interaction of PB-Microsomes with Benzphetamine[a]

	MAb	K_{eq}[b]	ΔH_1 (kcal mol^{-1})	ΔS_1 (eu)	ΔH_2 (kcal mol^{-1})	ΔS_2 (eu)
K_1	–	0.21	5.4 ± 0.1	15.5 ± 0.4		
K_1	+	0.22	5.5 ± 0.1	15.9 ± 0.5		
K_2	–	1.21	8.3 ± 0.1	28.6 ± 0.4		
K_2	+	0.61	6.2 ± 0.1[c]	20.2 ± 0.4[c]		
K_3	–	103 μM	–7.9 ± 0.9	–45.5 ± 3.4	–1.4 ± 0.6	–23.0 ± 2.1
K_3	+	202 μM	–9.1 ± 0.6	–48.4 ± 2.3	–4.3 ± 0.8[c]	–31.9 ± 2.7[c]
K_4	–	18.1 μM	–10.3 ± 1.0	–57.0 ± 3.6	–4.3 ± 0.4	–36.6 ± 1.3
K_4	+	72.9 μM	–9.2 ± 0.5	–50.6 ± 1.9	–6.3 ± 0.4[c]	–40.7 ± 1.3[c]

[a] The microequilibrium constants K_1, K_2, K_3, and K_4 were determined in the absence (–) and presence (+) of MAb 4-7-1. Enthalpy and entropy changes were determined from the van't Hoff plots in Figure 4: the equilibria corresponding to K_1 and K_2 were monophasic throughout the temperature range and yielded ΔH_1 and ΔS_1; the biphasic equilibria characterized by K_3 and K_4 yielded ΔH_1 and ΔS_1 for the lower temperature region and ΔH_2 and ΔS_2 for higher temperatures. [b] K_{eq} represents the value of the corresponding microequilibrium constant at 19.6 °C. [c] The value in the presence of MAb is significantly different ($P < 0.01$) than the value in the absence of MAb.

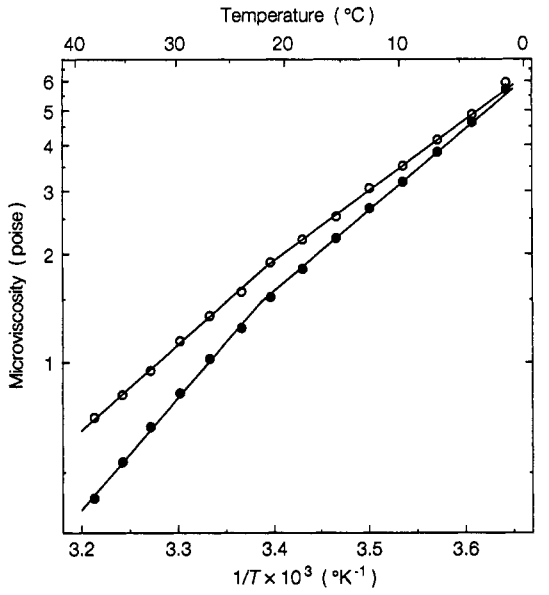

FIGURE 5: Temperature dependence of microviscosity of PB-microsomes. The microviscosities of PB-microsomes (0.1 mg/mL) with (O) or without (●) 0.02 mg/mL MAb 4-7-1 were determined at various temperatures from DPH fluorescence polarization measurements.

P450 in the presence of a microsomal mixture of P450s. Microsomes contain multiple P450s with different Bz-binding properties, and MAb 4-7-1 to P450b was used to distinguish the Bz-binding behavior of this P450 from that of other P450s. This MAb has been shown by sensitive immunoassays to react with P450b as well as the related P450e and P450a forms (Waxman et al., 1987). When this MAb was used in immunoaffinity purification using PB-microsomes, a single band on an SDS gel was obtained whose amino-terminal sequence corresponds to P450b/e (unpublished data, Friedman et al.); these forms are indistinguishable since they are 97% identical and have the same N-terminal sequence. Although the MAb thus potentially recognizes three P450s, P450a and P450e exhibit negligible Bz-demethylase activity relative to P450b, on the basis of the activities and levels of these P450s in PB-microsomes (Guengerich et al., 1982). Upon addition of MAb, changes in the binding parameters of PB-microsomes are therefore essentially entirely derived from P450b, since this is the only P450 that both binds Bz and is recognized by the MAb.

Although we observed some interesting behavior for K_s and ΔA_{max} upon varying temperature or adding MAb, there is no simple correspondence between substrate binding and these spectral parameters. These commonly determined parameters for P450–substrate binding are not easily interpretable since they are complex functions of several microequilibrium constants (Tamburini & Gibson, 1983; Sligar, 1976; Gibson & Tamburini, 1984) that relate P450 spin and substrate-binding equilibria. In order to interpret our data in terms of these elementary reactions, we calculated the various microequilibrium constants defined therein (Tamburini & Gibson, 1983).

Since K_1 was the same in the absence and presence of MAb (Figure 4A), the MAb has no effect on the low–high spin equilibrium of substrate-free P450b. The spin state is controlled by binding of an aqua ligand to heme iron: the ligand is present in the low spin but absent in the high spin state (Poulos et al., 1987; Raag & Poulos, 1989). The MAb thus does not affect the heme–aqua ligand binding in the absence of substrate. This finding does not rule out a possible MAb-induced change in the conformation or dynamics of other regions of the P450b.

K_2 was greater than K_1, reflecting the substrate stabilization of the high spin P450s and displacement of aqua ligand by substrate. However, in contrast to that observed with substrate-free P450b, the MAb halved K_2 and thus increased the low spin content of the Bz-bound P450b. The MAb thus partially inhibits aqua ligand displacement by Bz and thereby reduces ΔS, which corresponds to decreased entropic favorability of the low to high spin conversion. Thus, when substrate occupies its binding site, binding of MAb to its P450b epitope transmits an effect to the heme, which enhances binding of the aqua ligand to the heme iron. One possible mechanism for the effect of MAb on K_2 is that of modifying the dynamics and/or conformation of substrate-binding amino acids such that the substrate orientation with respect to the heme iron is altered and the substrate does not displace the aqua ligand from the actve site; retention of this ligand would then favor a low spin state. Another possibility is that for Bz-bound but not substrate-free P450b the MAb alters dynamics and/or conformation of a solvent channel to increase solvent accessibility and facilitate aqua ligand binding to heme.

The data for K_3 and K_4 show that high spin microsomal P450s have a higher affinity for Bz than the low spin forms, reflecting substrate stabilization of high spin P450s. Bz binding is an entropy-driven reaction (negative ΔS for dissociation) and is more entropically favorable in the lower than the higher temperature region (compare ΔS_1 to ΔS_2 in Table II). The MAb did not affect the thermodynamic parameters of Bz binding to either low or high spin P450b at lower temperatures. At higher temperatures, the MAb resulted in a more favorable ΔS and a less favorable ΔH for binding. The resulting entropy–enthalpy compensation yielded ΔG values such that the MAb inhibited Bz binding to high spin P450b more than to low spin P450b.

It is instructive to examine the energetics of the spin state and substrate binding equilibria via a free energy diagram. Figure 6 is based on the free energies calculated from ΔH and ΔS (Table II) at two temperatures (37 °C and 10 °C) representing the higher and lower temperature regions, respectively. At 37 °C, the inversion of relative stabilities of the spin states upon the addition of Bz is evident: this effect arises from a greater free energy decrease for high spin than low spin P450s (top left panel). In the presence of MAb (top right panel), there is a smaller decrease in free energy upon

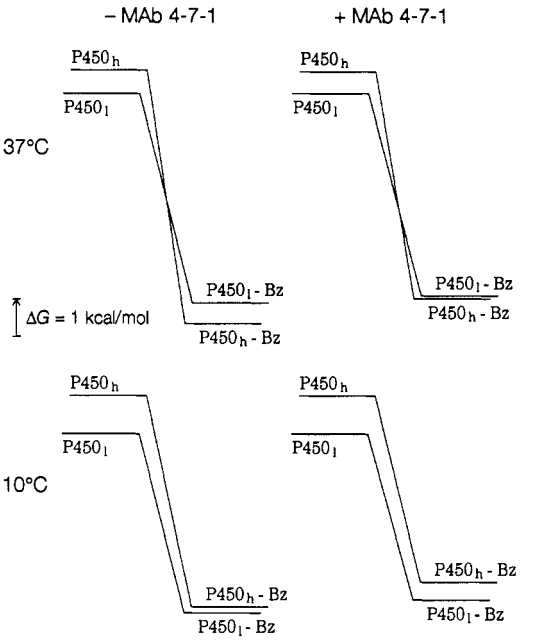

FIGURE 6: Free energy diagram of spin state and substrate binding equilibria. Free energy values were determined as described in the text at both 37 °C (top row) and 10 °C (bottom row), in the absence (left column) and presence (right column) of MAb 4-7-1. $P450_l$ and $P450_h$ represent low and high spin P450.

Bz binding to the high spin P450s, resulting in nearly identical energies for both spin states. In the lower temperature region, both in the absence and in the presence of MAb (lower panels), the free energy decrease upon Bz binding is such that the free energies of the spin states retain their relative order.

The biphasic nature of the Bz binding parameters suggests its association with a membrane phase transition (Shinitzky & Barenholz, 1978). Similar biphasic plots with breaks around 20 °C have also been observed for Bz demethylation by liver microsomes (Yang et al., 1977) and by a reconstituted P450 system (Tsong & Yang, 1978; Taniguchi et al., 1987), for microsomal oxidation of ethanol (Puntarulo & Cederbaum, 1989), and for reduction kinetics of P450 in microsomal (Peterson, et al., 1976; Tamburini et al., 1984) and reconstituted (Tamburini et al., 1984; Smettan et al., 1984) systems. Although a membrane phase change can explain the biphasic behavior of microsomes, alternative explanations are that the substrate binding or catalytic parameters derive from several P450s with different temperature dependencies and/or that the parameters for individual P450s vary with temperature. We therefore carried out measurements of DPH fluorescence polarization as an independent measure of membrane fluidity. The coincidence of breakpoint temperatures for both membrane microviscosity and the van't Hoff plots suggests that a mechanism related to membrane structure/dynamics is most likely responsible for the biphasic behavior of Bz binding.

Our results thus support a hypothesis whereby the changes in the Bz binding parameters arise from a membrane phase transition. Many hydrophobic P450 substrates such as Bz are thought to partition predominantly into the membrane, from which they can more easily diffuse to a P450 binding site near the membrane (Kominami et al., 1986; Kuhn-Velten et al., 1989). The increase in Bz binding to P450s with temperature may arise from increased partitioning of Bz to the membrane. Different solubilities of substrate in two membrane phases with different structures/dynamics can thus account for the observed breaks in the Bz-binding parameters. Another factor to consider is that changes in membrane structure may alter P450 conformation such as to increase

affinity for Bz; this is less likely, however, since only a small amino-terminal segment of P450 is though to be embedded in the membrane, with the bulk of the molecule exposed to solvent (Vergères et al., 1989; Sakaguchi et al., 1987; De Lemos-Chiarandini et al., 1987; Nelson et al., 1988).

In a previous report, the van't Hoff plot for the interaction of Bz with PB-induced rabbit P450 in dimyristoylphosphatidylcholine liposomes exhibited a breakpoint (Taniguchi et al., 1984). However, the plot using liver microsomes from PB-treated rabbits was linear, an observation not consistent with our findings. The discrepancy may arise from differences in the microsomal source (rabbit vs rat in our study), which may differ in lipid and/or protein composition and phase transition behavior. In addition, since the data of this previous study covered a narrower temperature range (8–30 °C) than the range of 0–37 °C that we examined, nonlinearity would not be as readily detectable.

Monoclonal antibodies to P450s have been used to distinguish specific P450s within microsomes from total P450s by methods such as immunoassay, immunopurification, and enzyme inhibition (Gelboin & Friedman, 1985; Friedman et al., 1985a). We now have used an MAb as a probe for substrate binding in the native microsomes, rather than in the purified state. Thus, MAbs provide us a new parameter of analysis beyond measurement of P450 content or catalytic activity to provide information on substrate binding by MAb-specific P450s.

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