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Binding of Benzo[*a*]pyrene by Purified Cytochrome P-450$_c$[†]

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ABSTRACT: Benzo[*a*]pyrene (BP) fluorescence-emission intensities in phospholipid micelles are quantitatively described over a broad range of lipid and BP concentrations by excitation that is linearly dependent upon BP concentration and an offsetting excimer quenching that is dependent upon the square of the BP concentration. The fluorescence of BP is quenched by the presence of cytochrome P-450$_c$ in proportion to the concentration of the cytochrome in the micelles and in accord with stoichiometric complex formation. Parallel optical titrations indicate a change in spin state of P-450$_c$ to a predominantly high-spin state that correlates directly with the percentage fluorescence quenching of complexed BP. Neither change occurs with five other purified forms of rat liver P-450 that have low activity in BP metabolism. *N*-Octylamine, a ligand that binds to the heme of P-450, competitively inhibits both the spin-state changes and the fluorescence quenching in equal proportion. The K_d for the interaction of BP with P-450$_c$ is exceptionally low (10 nM) relative to the K_m for monooxygenation (ca. 1 μM). Decreasing the concentration of either dilauroylphosphatidylcholine or dioleoylphosphatidylcholine concomitantly increases the high-spin state (from 30% to 80%) of fully complexed P-450$_c$ and the fluorescence quenching (50–100%) of the complexed BP (half-maximal at 80 μg of lipid/mL). It is concluded that spin state and fluorescence quenching both reflect the same changes in the interaction of the BP with the P-450 heme. These changes also occur in parallel with a 3-fold decrease in BP metabolism as LPC increases from 7.5 to 200 μg/mL and a 20-fold increase in the affinity of P-450$_c$ for membrane-bound BP. The interaction of BP with P-450$_c$ is sensitive to the P-450$_c$:lipid ratio in a manner that directly affects enzyme turnover and may involve different aggregation states of the cytochrome.

Microsomal cytochrome P-450 exists in many forms of broad but nonetheless differing substrate specificity (Ryan et al., 1982; Guengerich et al., 1982). Substrate binding to cytochrome P-450, in most cases, can be measured by changes in its optical spectrum (Remmer et al., 1966; Peterson, 1971; Schenkman et al., 1967). These changes in the optical spectrum correlate with changes in the electron spin resonance spectrum of the cytochrome (Tsai et al., 1970; Stern et al., 1973; Grasdalen et al., 1975) and reflect changes in the spin state of the P-450 heme. Substrate-free cytochrome P-450 generally adopts a low-spin state (absorption maximum at 417 nm), although for a few forms a high-spin state is the preferred configuration in the absence of substrate (Friedrich et al., 1979; Ryan et al., 1980). Liver forms of microsomal P-450 typically adopt a mixed-spin state upon binding substrate. The mixed-spin state of these complexes is substrate dependent, and this has been attributed to a perturbation of an equilibrium between high- and low-spin configurations (Sligar & Gunsalus, 1979; Ristau et al., 1979). Transition of the heme to the high-spin configuration is also frequently associated with an increased rate of reduction of the cytochrome and with increased monooxygenation rates (Backes et al., 1982).

Membrane phospholipids may play a key role in determining both substrate binding and activity of P-450. Since most substrates are lipid soluble, the partitioning of such substrates between the aqueous and lipid phases becomes a major determinant of binding (Al-Gailany et al., 1978; Parry et al., 1976; Canady et al., 1974). In addition, cell membranes may contain regions of differing fluidity that may in turn be perturbed by lipophilic compounds (Sanioto & Schreir, 1975; Shimchick & McConnel, 1973; Ebel et al., 1978; Stier, 1976). Membrane lipids themselves may have direct effects on P-450 itself, as well as on the interaction between P-450 and its reductase (Schenkman et al., 1980; Miwa & Lu, 1981).

The binding of the fluorescent polycyclic hydrocarbon pyrene to cytochrome P-450 results in a quenching of pyrene fluorescence. This approach has been employed to establish the 1:1 stoichiometric binding of pyrene to rabbit hepatic cytochrome P-450 LM$_4$ (Imai, 1982). However, binding of benzo[*a*]pyrene (BP)[1] to LM$_4$ does not induce a spin-state

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[1] Abbreviations: PAH, polycyclic aromatic hydrocarbon(s); BP, benzo[*a*]pyrene; MC, 3-methylcholanthrene; LPC, dilauroylphosphatidylcholine; OPC, dioleoylphosphatidylcholine; cmc, critical micelle concentration; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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change, and this form of P-450 has a low turnover number for BP. It is important to note that optical and electron spin resonance spectroscopies determine the effect of the substrate on the P-450 heme, while fluorescence perturbation examines the effect of the cytochrome on the substrate. Furthermore, polycyclic aromatic hydrocarbons (PAH) fluoresce strongly in organic solvents but display very little if any fluorescence in an aqueous environment (Lackowicz & Hylden, 1978; Vo-Dinh, 1976). Since PAH are extremely hydrophobic, they consequently partition essentially completely from an aqueous solution into lipid membranes, where they fluoresce as if in an organic solvent. Thus, when BP is added to an aqueous suspension of phospholipid micelles, the observed fluorescence can be attributed directly to the concentration of the BP in the membrane.

In this paper we demonstrate that fluorescence quenching due to BP–P-450$_c$ complex formation is closely correlated with the changes in the spin state of cytochrome P-450$_c$ observed by optical absorption spectroscopy. Both optical and fluorescence spectroscopies are used to establish a "true" K_d for the high-affinity binding of BP to cytochrome P-450$_c$ and to examine the effect of two different phospholipids on the binding process. The molecular implications of these observations are then discussed.

Materials and Methods

Materials. Male rats (80–100 g) were obtained from Sprague-Dawley (Madison, WI). Materials for buffers were from Sigma (St. Louis, MO), benzo[*a*]pyrene was from Aldrich (Milwaukee, WI), and [^{3}H]benzo[*a*]pyrene was from Amersham (Arlington Heights, IL). Hydroxylapatite was from Bio-Rad Laboratories (Richmond, CA) and DEAE-trisacryl M from LKB (Rockville, MD). Acetonitrile was spectral grade from Burdick and Jackson, and highly purified synthetic phospholipids were obtained from Serdary Research Laboratory (London, Ontario, Canada) and prepared as a sonicated suspension under nitrogen at a concentration of 1 mg/mL.

Purification of Cytochrome P-450. Cytochrome P-450$_c$ was prepared according to a modification of published methods of Guengerich (1977, 1978). A DEAE-trisacryl M column run at room temperature as described by Ryan et al. (1979) was substituted for the DEAE-Sephacel column of the Guengerich procedure. Detergent removal was carried out on a hydroxylapatite column (1 mL of resin/15 nmol of P-450). The specific content was 15–17 nmol of P-450/mg of protein, and the enzyme was apparently pure by SDS–PAGE. The pooled enzyme was stored at −70 °C for up to 3 months prior to use with no detectable losses.

Purification of Cytochrome P-450 Reductase. Cytochrome P-450 reductase was purified with octylamino-Sepharose 4B and agarose–2′,5′-ADP according to the method of Guengerich (1977). The preparation was assayed spectrally at 456 nm, employing an extinction coefficient of 21.4×10^3 M^{-1} cm^{-1} (French & Coon, 1979). The purified preparation had a specific activity of 32 000 units/mg of protein and a specific content of 12 nmol/mg of protein and was apparently pure by SDS–PAGE.

Phospholipids. Lipid micelles or vesicles were prepared from purified phospholipids by drying a chloroform solution to a thin film on the walls of a conical glass tube under a stream of nitrogen, adding 10 mM potassium phosphate buffer, pH 7.5, and then sonicating with a microtip probe sonicator (Heat Systems Ultrasonics) for 30 min at 4 °C. Suspensions were stored under nitrogen and used within 24 h of preparation.

Optical Spectroscopy. Optical spectra were recorded at room temperature on an Aminco DW-2 spectrophotometer in the split-beam mode. Substrates dissolved in acetonitrile were titrated into both sample and reference cuvettes to final concentrations of 0.4–0.5 μM. Spectra were corrected for dilution due to sample titration prior to data analysis. The high-spin content of the cytochrome was measured from difference spectra by the decrease in the 417/450-nm wavelength pair, employing an extinction coefficient of 55 mM^{-1} cm^{-1} for the low- to high-spin transition (Ristau et al., 1979).

The increase in high-spin heme can also be followed by quantitating the increase in the 390/417-nm wavelength pair with an extinction coefficient of 110 mM^{-1} cm^{-1} or the increase in the 645/700-nm wavelength pair and an extinction coefficient of 4.5 mM^{-1} cm^{-1} (Peterson, 1971). The high-spin heme content measured by both methods was similar.

Fluorescence Titrations. Fluorescence titrations were performed at room temperature with an Aminco-Bowman spectrophotofluorometer using 300/403 nm as the excitation/emission wavelength pair. Sample preparation and titrations were similar to those for optical experiments. Cytochrome P-450 concentration was 0.1 μM unless stated otherwise. Acetonitrile was used for fluorescence titrations since it had no internal filter effects. Titrations were corrected for dilution during data analysis. Fluorescence background due to the P-450 was typically less than 1% of that of the ligands being studied and was subtracted from the data.

Fluorescence titrations in the presence of P-450 were also corrected for the absorbance of the cytochrome at the excitation and emission wavelengths [the inner filter effect described by Parker & Barnes (1957)], by multiplying the observed fluorescence by the antilogs of the absorbances of the P-450 at the excitation and emission wavelengths. The magnitude of the inner filter effects on BP fluorescence was quantitatively confirmed for other materials which absorb at these wavelengths yet do not quench BP, such as hemoglobin and ferricyanide (data not shown).

Fluorescence spectra were recorded by utilizing an SLM 9000 photon-counting spectrophotofluorometer in the ratio mode. Spectra were recorded in 1-nm increments and plotted on a Hewlett Packard digital X-Y plotter. Digitized spectra were traced by hand for smoothing.

Benzo[a]pyrene Metabolism. BP oxidation was measured in 0.5-mL reaction mixtures containing 50 mM potassium phosphate, pH 7.5, 25 nM P-450$_c$, 50 nM cytochrome P-450 reductase, an NADPH generating system, 1 μM BP, and the indicated concentration of LPC. Following a 5-min preincubation, reactions were initiated by the addition of substrate. Extraction and analysis of the oxidation products resulting from 30-s incubations at 37 °C were by the potassium hydroxide/dimethyl sulfoxide assay of Van Cantfort et al. (1977).

Results

Fluorescence Quenching in P-450$_c$ Complexes. In this study, we have investigated the effect of purified cytochrome P-450$_c$, the major isozyme present in MC-induced rat liver microsomes (Ryan et al., 1979), on the fluorescence of BP in both LPC micelles and dioleoylphosphatidylcholine vesicles. Quenching of BP fluorescence was specific for P-450$_c$, since no quenching was observed when BSA or five other purified isozymes of P-450 from rat hepatic microsomes (P-450$_a$, P-450$_b$, P-450$_d$, P-450$_e$, or P-450$_h$) were added to the phospholipid micelles. The decrease in BP fluorescence emission at a single wavelength caused by the presence of P-450$_c$ (Figure 1) is not due to shifts in the excitation or emission spectra. The fluorescence spectrum of BP bound to P-450$_c$ was quite

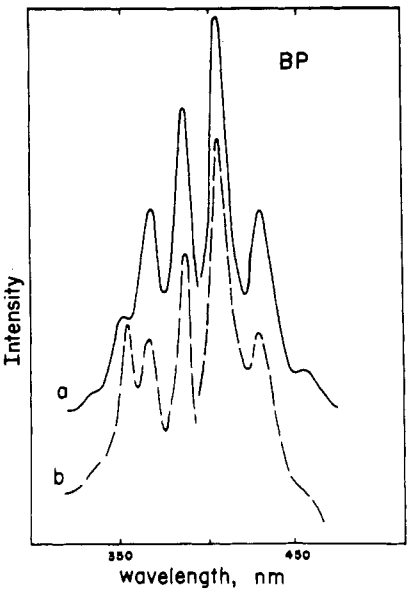

FIGURE 1: Excitation and emission spectra of BP in phospholipid micelles in the absence and presence of purified P-450$_c$. (a) Excitation and emission spectra of BP (10 nM) in dilauroylphosphatidylcholine micelles (60 μg/mL) in 0.1 M potassium phosphate, pH 7.5. (b) Excitation and emission spectra of BP (10 nM) in micelles (60 μg/mL) containing P-450$_c$ (0.2 μM). Under these conditions, 96% of the BP is bound to P-450$_c$.

similar to that of free micellar BP apart from diminished intensity and a slight shift in the relative intensity of two maxima in the excitation spectrum.

Fluorescence quenching of BP in phospholipid micelles was proportional to the concentration of P-450$_c$. The decrease in fluorescence (ΔF) caused by P-450$_c$ was obtained directly from the difference in fluorescence observed between equivalent concentrations of BP in phospholipid micelles, in the absence or presence of P-450$_c$, and is shown in Figure 2A. The affinity of P-450$_c$ for BP is evidently high, since at concentrations of BP substantially less than that of the P-450 (100 nM) essentially all BP is complexed.

The quenching of BP fluorescence by P-450$_c$ was partially blocked by the presence of *n*-octylamine (Figure 2B), a ligand that binds directly to the heme of P-450 cytochromes (Jefcoate et al., 1969). Double-reciprocal plots from separate experiments (data not shown) indicated competitive binding. Competition by *n*-octylamine was employed to shift BP titration curves, allowing more accurate determination of "bound" and "free" BP. Bound BP was subtracted from total BP to determine the true value of free BP utilized in the Scatchard analyses. This correction is described in detail in the Appendix. By use of the standard relationship for competitive binding, the "apparent" K_d for BP binding to P-450$_c$ was calculated from Scatchard plots to be 7.5 ± 0.5 nM.

Since BP partitions essentially completely into the lipid phase, a "membrane" K_d has also been calculated on the basis of the molar ratio of [BP]/[LPC]. On this basis, the K_d is 8.1×10^{-5} mol/mol. This is still very tight binding, especially in comparison to the membrane K_d for the binding of cholesterol to P-450$_{scc}$, which is 3–4 orders of magnitude weaker depending upon the phospholipid employed (Lambeth et al., 1980). The apparent K_d should be inversely proportional to the concentration of lipid present during the titration. Equally, a "true" K_d expressed as [BP]/[lipid] should remain constant when the lipid concentration is changed. In fact, when the LPC concentration was increased from 60 to 200 μg/mL, a range in which the spin state changes, the apparent K_d actually decreased 2-fold instead of increasing 3-fold (Table I). These

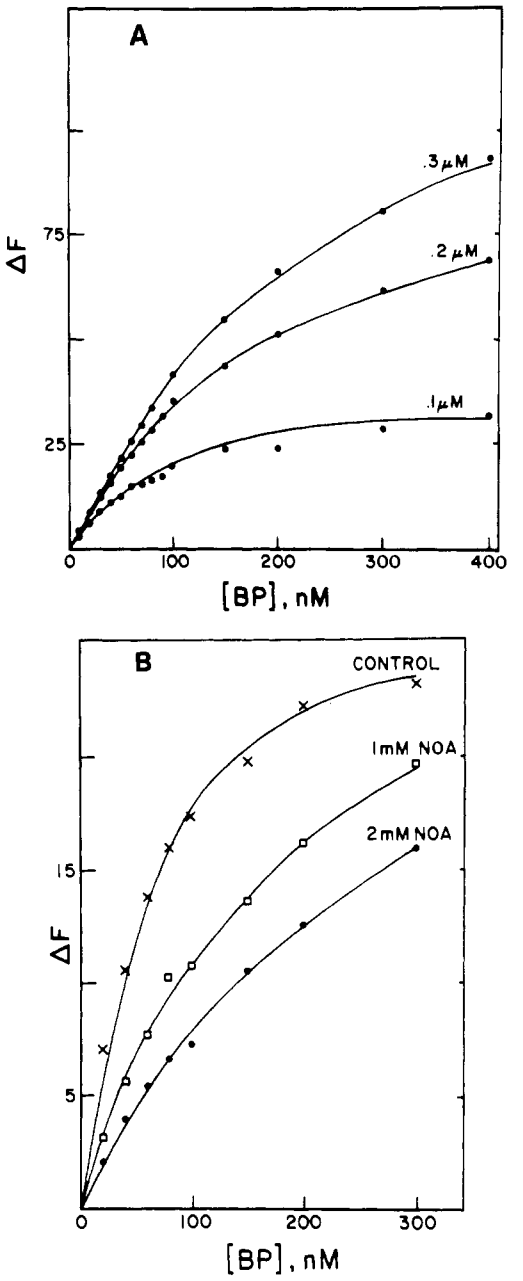

FIGURE 2: (A) Effect of P-450$_c$ concentration on the quenching of BP fluorescence (ΔF). The difference in BP fluorescence between titrations in the absence and presence of P-450$_c$ (ΔF) is plotted against the concentration of BP. Experiments were conducted in the absence or presence of 0.1, 0.2, or 0.3 μM P-450$_c$ in dilauroylphosphatidylcholine micelles (60 μg/mL) in 0.1 M potassium phosphate, pH 7.5. Results shown have been corrected for differential excimer quenching as described in the Appendix. (B) Inhibition of quenching of BP fluorescence by P-450$_c$ by the competitive inhibitor *n*-octylamine (NOA). Titrations were conducted analogous to those shown in (A) containing 0.1 μM P-450$_c$ in dilauroylphosphatidylcholine micelles (60 μg/mL) but in the presence of none (control), 1 mM, or 2 mM NOA.

results varied somewhat with the individual lipid dispersion but nevertheless show that there is an approximately 20-fold decrease in the true K_d in changing from the 80% high-spin complex favored at low lipid concentrations (7.5 μg/mL) to the 30% high-spin complex formed at higher (200 μg/mL) LPC. There is a small additional change in affinity at much higher concentrations of LPC even though there is no further change in fluorescence quenching or spin state.

Correlation between Spin State and Fluorescence Quenching. The absolute spectrum of highly purified deter-

Table I: Effect of Lipid Concentration on K_d of Benzo[a]pyrene with P-450$_c$ in Dilauroylphosphatidylcholine Micelles

[LPC] (μg/mL)	apparent K_d from [BP] (nM)[a,c]	true K_d from [BP]/[LPC] (mol/mol × 10^5)[b,c]
7.5	4.4 ± 2.0 (3)	38.0 ± 17.0
60	9.1 ± 1.3 (3)	9.6 ± 1.4
200	4.5 ± 1.4 (3)	1.5 ± 0.4
500	54.5 ± 17.4 (2)	7.0 ± 2.3

[a] Apparent K_d calculated on the basis of the total concentration of BP in the system. [b] True or membrane K_d calculated on the basis of the concentration of BP in the membrane as a molar ratio (mol/mol). [c] Average ± SEM of the number of experiments shown in parentheses. Titrations were carried out as described under Materials and Methods in the presence of 100 nM P-450$_c$, in 0.1 M potassium phosphate, pH 7.5, containing LPC micelles at the indicated concentration.

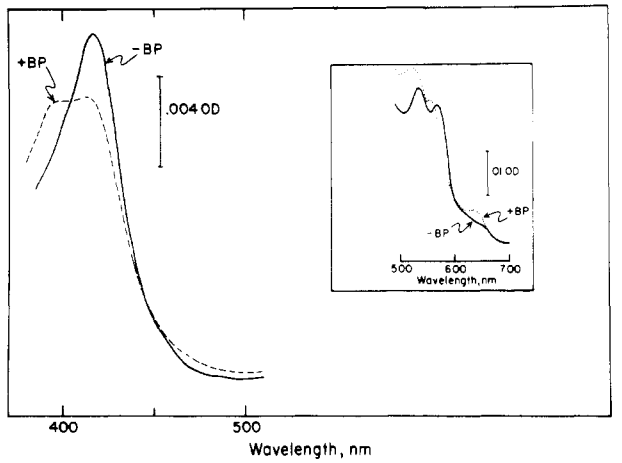

FIGURE 3: Effect of BP on the absorption spectra of purified P-450$_c$. Spectra of P-450$_c$ (0.1 μM) in dilauroylphosphatidylcholine micelles (60 μg/mL) in 0.1 M potassium phosphate, pH 7.5, before (−BP) and after (+BP) the addition of 600 nM BP. (Inset) Absolute spectra of 2.0 μM P-450$_c$ before (−BP) and after (+BP) the addition of 3 μM BP. The conversion of low-spin native enzyme to a typical mixed high- and low-spin state due to ligand (BP) binding is clearly indicated by the decrease in absorbance at 417 nm and the increase in absorbance at 390 and 645 nm.

gent-free P-450$_c$ in the native state (Figure 3) is >95% low spin as has been reported by others investigators (Ryan et al., 1980). Thus it seems unlikely that the purified enzyme contains any residual hydrocarbon inducer still bound to P-450$_c$ (Jefcoate & Gaylor, 1969). Furthermore, optical spectroscopy indicated that LPC itself does not induce a spin-state change in purified P-450$_c$ at the concentrations employed in these studies, since the absolute spectrum of P-450$_c$ in the presence of LPC gave no indication of a detectable high-spin component as measured by either the 417/450- or 645/700-nm wavelength pairs. Difference spectra also indicated no changes in spin state of cytochrome P-450$_c$ due to LPC (data not shown).

Addition of BP to P-450$_c$ produced a change to a partially high-spin state as evidenced by increases in absorbance in the P-450$_c$ spectrum at both 390 and 645 nm (Figure 3). Titration of P-450$_c$ in lipid micelles with BP induces a low to high spin state transition due to complex formation. The maximum percent spin-state change at saturation of binding is inversely proportional to the lipid concentration. Furthermore, this increase in high-spin content was found to be competitively inhibited by *n*-octylamine (1–2 mM) and completely blocked by 1-methylimidazole (2 mM), compounds both known to bind to P-450 heme.

The dependence of the spin-state change of P-450$_c$ on the concentration of BP in the presence of 1 mM *n*-octylamine (analogous to the fluorescence titrations shown in Figure 2)

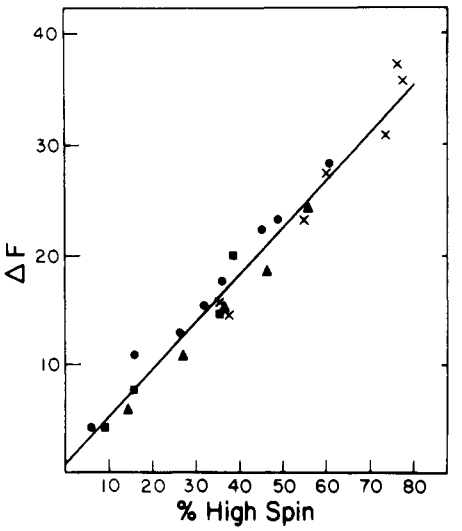

FIGURE 4: Correlation between percent high-spin cytochrome measured by optical spectra and quenching of BP fluorescence (ΔF) due to binding of BP to purified P-450$_c$. Samples contained 0.1 μM P-450$_c$ in LPC micelles (60 μg/mL) and none (●), 1 mM (▲), or 2 mM (■) of the competitive inhibitor *n*-octylamine. Also shown (×) are the maximum spin-state change and fluorescence quenching at saturation of P-450$_c$ (0.1 μM) by BP (300 nM) in LPC micelles prepared at (left to right) 500, 200, 90, 60, 30, 15, or 7.5 μg/mL. Best fit line was $\Delta F = 0.43(\%\text{ high spin}) + 0.8$ ($r^2 = 0.97$).

was also used to calculate the K_d for the binding of BP to P-450$_c$ (data not shown). The apparent K_d determined at 60 μg/mL LPC by this method (as detailed in the Appendix) was found to be 8.2 ± 0.5 nM (8.8 × 10^{-5} mol/mol on the basis of [BP]/[LPC]), in excellent agreement with the K_d determined fluorometrically.

Figure 4 clearly illustrates the high correlation between fluorescence quenching (ΔF) observed in titrations of P-450$_c$ with BP and the increases in high-spin cytochrome observed in equivalent optical titrations ($r^2 = 0.97$). This correlation again demonstrates that fluorescence quenching and spin-state transition are both quantitating the same interaction of BP with P-450$_c$.

Fluorescence Quenching by Microsomal P-450$_c$. Uninduced rat hepatic microsomes contain very little P-450$_c$ (<1%; N. M. Wilson and C. R. Jefcoate, unpublished data), which is the major cytochrome in MC-induced microsomes. Titrations of MC-induced and uninduced microsomes adjusted to equal protein concentration closely resembled the above titrations using micelles with and without P-450$_c$, respectively (data not shown). Titrations of uninduced microsomes showed no evidence of quenching, while titrations of MC-induced microsomes exhibited saturable quenching.

Optical difference spectroscopy utilizing control and MC-induced microsomes (data not shown) gave analogous results. Titration of uninduced microsomes with BP produced little if any detectable spin-state changes, while titrations of MC-induced microsomes indicated considerable spin-state changes, which saturated at concentrations proportional to the P-450 content. Results from fluorescence and optical titrations with phenobarbital-induced microsomes, which also contain little or no P-450$_c$, were similar to those with uninduced microsomes. These findings suggest that the observed differences in BP fluorescence in MC-induced microsomes derived from the induction of P-450$_c$.

BP Fluorescence and Lipid Concentration. The dependence of fluorescence quenching on lipid concentration was examined by performing titrations with BP (0–1000 nM) at several concentrations of LPC both in the absence and in the presence

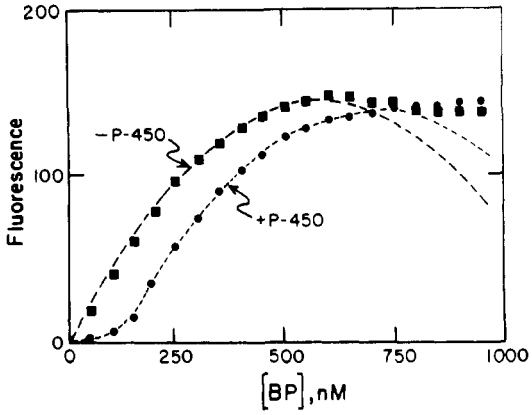

FIGURE 5: Fluorescence of BP in dilauroylphosphatidylcholine (LPC) micelles in the absence (■) or presence (●) of 0.1 μM purified P-450$_c$. Fluorescence is when BP was titrated into micelles containing 7.5 μg/mL LPC in 0.1 M potassium phosphate, pH 7.5. Dashed lines indicate the theoretical fluorescence of BP under these conditions predicted by eq 1. Deviations from the model occur only at concentrations of BP greater than 700 nM in micelles prepared at 7.5 μg/mL (0.06 = [BP]/[LPC], mol/mol), consistent with saturation of the lipid phase with hydrocarbon at this level.

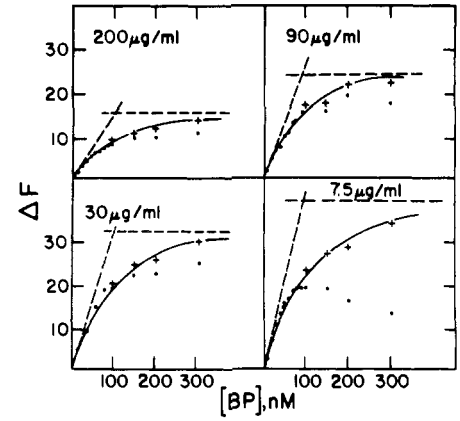

FIGURE 6: Effect of dilauroylphosphatidylcholine concentration on the quenching of BP fluorescence by purified P-450$_c$. Results show the increased quenching (ΔF) of BP fluorescence by purified P-450$_c$ as LPC concentration was decreased (clockwise from top left) from 200 to 7.5 μg/mL. Difference between BP fluorescence in the absence and presence of 0.1 μM P-450$_c$ uncorrected for excimer quenching (●) and corrected for excimer quenching (+) as described in the Appendix.

of P-450$_c$. In the absence of P-450$_c$, the use of lower LPC concentrations resulted in greater deviations from a linear concentration dependence of BP fluorescence. The concentration dependence of these decreases was consistent with fluorescence quenching due to the formation of transient "excimers" resulting from collisions between an excited-state and a ground-state BP molecule (Förster & Kasper, 1955). Thus, fluorescence of BP in the absence of P-450$_c$ for concentrations of BP up to nearly 1 μM, at LPC concentrations ranging from 7.5 to 500 μg/mL, was accurately described by eq 1, where k and k' are constants relating to, respectively,

$$F = k[\mathrm{BP}] - k'[\mathrm{BP}]^2 \quad (1)$$

the fluorescence emission intensity and the quenching due to excimer formation. The value of k and of k' for each lipid concentration was determined experimentally as detailed in the Appendix. On this basis, k' was inversely proportional to the LPC concentration ($r^2 = 0.95$).

Fluorescence Quenching and Lipid Concentration. The fluorescence of BP in LPC micelles containing P-450$_c$ can also be accurately predicted by eq 1, when the K_d of BP for P-450$_c$ is utilized to calculate the amount of "free" hydrocarbon and therefore the amount available for excimer quenching. The close agreement between observed and predicted fluorescence of BP in LPC micelles both in the absence and in the presence of 0.1 μM P-450$_c$ is shown in Figure 5 for the lowest concentration of LPC employed, 7.5 μg/mL. Deviations from the model occur only at [BP]/[LPC] ratios exceeding 0.05 mol/mol (which is 500-fold greater than the K_d of BP for P-450$_c$), consistent with a saturation of the lipid membrane by the hydrocarbon (Vanderkooi & Callis, 1974). At higher concentrations of LPC (i.e., lower [BP]/[LPC] ratios), this saturation does not occur, and eq 1 accurately predicts BP fluorescence in the absence and presence of P-450$_c$ up to 1 μM.

The dependence of fluorescence quenching on lipid concentration in the presence of a fixed concentration of P-450$_c$ (from several of the titrations described above) is represented in Figure 6. By utilizing the direct data (shown by solid circles in Figure 6), the stoichiometry determined was in all cases apparently less than 1:1 (about 0.6 BP:P-450$_c$). The cause of this discrepancy in the stoichiometry is the difference in excimer quenching between titrations in the absence or presence of P-450$_c$. Complex formation by P-450$_c$ decreases excimer quenching by reducing the concentration of free hydrocarbon in the lipid phase. This differential excimer quenching due to the presence of P-450$_c$ is most apparent at the lower concentrations of LPC where membrane concentrations of BP are greatest and results in an apparent premature saturation and subsequent apparent decrease in uncorrected ΔF at high BP concentrations (Figure 6). When a correction for excimer quenching was calculated, the equivalence points for the corrected fluorescence quenching titrations were then consistent with a 1:1 stoichiometry.

Since the critical micelle concentration (cmc) for LPC (28 μg/mL; Coon et al., 1976) is in the range of LPC concentrations studied, we also examined dioleoylphosphatidylcholine (OPC), which has a critical micellar concentration several orders of magnitude lower (Reynolds et al., 1977). At comparable concentrations, OPC produced similar changes in the spin state and fluorescence quenching of the BP–P-450 complex as those seen with LPC (data not shown).

Spin State and Lipid Concentration. In a parallel investigation of the effect of lipid concentration on the optical spectra of the BP–P-450$_c$ complex, the percentage of high-spin cytochrome induced by a saturating concentration of BP varied from 35% to 80% as the concentration of LPC was decreased from 200 to 7.5 μg/mL. The percentage high-spin cytochrome induced by BP binding reached a maximum at about 15 μg of LPC/mL. Analogous to the fluorescence experiments described above, a very similar increase in the high-spin component was also observed in parallel studies with OPC. This close correlation ($r^2 = 0.97$) between the change in BP fluorescence and the change in spin state as a function of lipid concentration is clearly shown in Figure 7 (and Figure 4).

Activity and Lipid Concentration. An increase in the proportion of high-spin cytochrome is frequently associated with an increase in reduction rate of the cytochrome and an increase in monooxygenase activity (Blanck et al., 1983; Sligar, 1976; Sligar et al., 1979). The specific activities for BP oxidation measured at LPC concentrations ranging from 0 to 500 μg/mL are shown in Figure 8. At low concentrations of phospholipid, we observed the typical increase in monooxygenase activity with an increase in phospholipid concentration (Miwa & Lu, 1981; Lu & West, 1978; Strobel et al., 1970; Seybert et al., 1979). This stimulation has been attributed to an increased association of P-450 with its reductase (Coon et al., 1976) and recently confirmed by Muller-Enoch

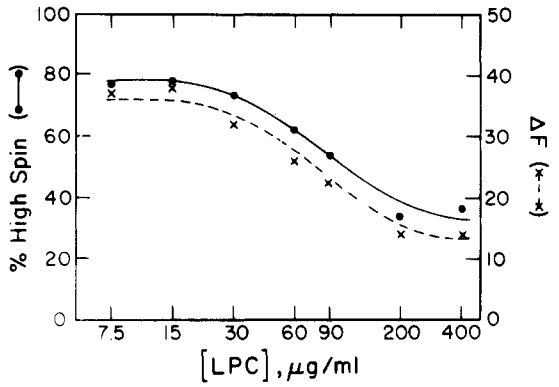

FIGURE 7: Correlation of the effects of dilauroylphosphatidylcholine concentration on the BP-induced change in the spin state of P-450$_c$ and the quenching of BP fluorescence due to complex formation. Results show the correlation (r^2 = 0.97) between the percent high-spin cytochrome (●) measured by optical spectroscopy and the maximum fluorescence quenching ΔF (×). Experiments were similar to those shown in Figure 6. Samples contained 0.1 μM P-450$_c$ in LPC micelles at the indicated concentration, titrated to a saturating concentration of BP (300 nM).

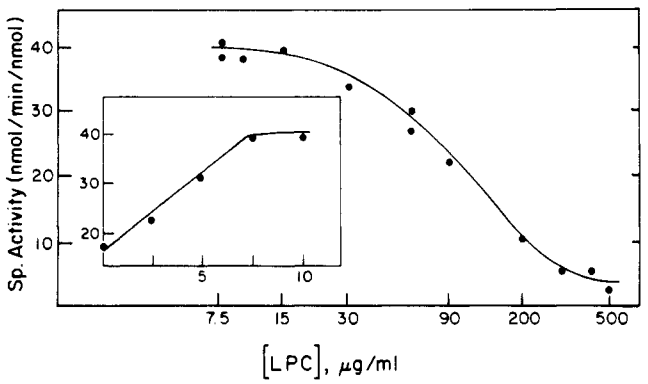

FIGURE 8: Effect of dilauroylphosphatidylcholine concentration on the specific activity of P-450$_c$ for BP metabolism. Results show specific activity [nmol of BP oxidized min^{-1} (nmol of purified P-450$_c$)$^{-1}$] as phospholipid concentration was increased from 7.5 to 500 μg/mL, utilizing a reconstituted system as described under Materials and Methods. Reaction mixtures (0.5 mL) contained 25 nM P-450$_c$, 50 nM P-450 reductase, and 1 μM BP. (Inset) Turnover of BP by purified P-450$_c$ in reconstituted systems containing 0–10 μg/mL LPC.

et al. (1984). However, previous studies have examined the effects of only relatively low concentrations of phospholipids. At higher concentrations of phospholipid (30–200 μg/mL), a sigmoid decrease in activity was observed (Figure 8) that paralleled the decrease in high-spin cytochrome (Figure 7). These changes in BP metabolism and maximal spin state were highly correlated (r^2 = 0.99).

Discussion

The intense fluorescence of PAH has been utilized to examine the effect P-450$_c$ directly on the complexed substrate, as distinct from the effect of the substrate on the spin state of the cytochrome as measured by optical and electron spin resonance spectroscopies. A previous study of the quenching of pyrene fluorescence in the presence of purified rabbit cytochrome P-450 LM$_4$ (Imai, 1982) indicated a 1:1 stoichiometry for the complex and full quenching of the complexed pyrene. However, those experiments were only carried out at a high concentration (1.9 μM) of P-450, and in the absence of lipid. Furthermore, this form of P-450 poorly metabolizes PAH and does not undergo a spin-state change upon complex formation. In this study, we show that rat liver P-450$_c$ also causes quenching of benzo[*a*]pyrene fluorescence as a consequence of very high affinity 1:1 complex formation. There is a nearly exact correlation (Figure 4) between the extent of the spin-state change in the cytochrome as indicated by changes in the Soret region absorption spectrum and the quenching of BP fluorescence due to specific binding by cytochrome P-450$_c$. This indicates that the two changes indeed both derive from the same complex formation. This relationship was also confirmed by a close correlation with the BP-induced increase in the absolute spectrum of P-450$_c$ at 645 nm, which derives exclusively from high-spin cytochrome.

The specific nature of the fluorescence quenching is further demonstrated by the findings that fluorescence quenching (1) is competitively inhibited by other ligands that bind to P-450 heme (*n*-octylamine and 1-methylimidazole), (2) does not occur with other isozymes of rat liver cytochrome P-450 that metabolize BP orders of magnitude more slowly (Ryan et al., 1982; Wilson et al., 1984), and (3) is clearly saturable at nearly 1:1 stoichiometry. Combined with the correlation of substrate-induced spin-state changes, these features of substrate fluorescence quenching clearly indicate that it is associated with binding to the active site of P-450$_c$.

Both optical and fluorescence titrations of BP binding to P-450$_c$ indicate high-affinity binding of BP to P-450$_c$. Ligands other than BP have also been reported to have very high affinities for cytochromes P-450, such as cholesterol with P-450$_{scc}$ (Lambeth et al., 1980) and progesterone with P-450$_{C21}$ (Narasimhulu et al., 1985). The extremely high affinity of P-450$_c$ for BP can only be quantitated by the use of very low concentrations of the cytochrome ($<K_d$) and/or the utilization of competitive inhibitors. One major advantage of the fluorescence quenching technique for measuring complex formation is extreme sensitivity, which allows the use of sufficiently low concentrations of cytochrome to quantitate such high affinities. A second major advantage is that the fluorescence method also provides a direct measure of the concentration of the ligand in the membrane phase. We have therefore established that only the equilibrium of BP between lipid and the P-450$_c$ active site is being measured.

We have shown that the observed fluorescence (F) of BP in phospholipid micelles both in the absence and in the presence of P-450$_c$ can be quantitatively described by a standard relationship that accounts for both fluorescence emission and excimer quenching (Förster & Kasper, 1955). Equation 1 provides a nearly exact fit for the observed fluorescence values in the reconstituted system employed in this study until the hydrocarbon/lipid ratio ([BP]/[LPC]) exceeds 0.05, where saturation of the membrane with hydrocarbon probably occurs (Vanderkooi & Callis, 1974; Galla & Sackmann, 1974; Vanderkooi et al., 1974). Furthermore, since the association of P-450$_c$ with the vesicle affects neither the quantum yield of free BP (k), the diffusion constant (k'), nor the extent of diffusion (concentration) of BP in the membrane, it appears that P-450$_c$ has only a minor effect on the ordering of phospholipid molecules in LPC micelles.

BP fluorescence in uninduced microsomes is also described by eq 1. The constants k and k' derived from the data for uninduced microsomes agree closely with values derived for LPC assuming a microsomal lipid content of ca. 0.33 mg of lipid/mg of protein (DePierre & Dallner, 1975). The quenching of BP fluorescence in MC-induced microsomes can also be quantitatively explained by complex formation with P-450$_c$, the major form of P-450 in these microsomes. A stoichiometry of about 0.5 nmol of BP quenched per milligram of protein is indicated by the dose dependence of quenching. This quenching corresponds to only 40% of the total P-450

(1.2 nmol/mg of protein) and is substantially less than expected from the proportion of P-450$_c$ in the microsomes (75%; Ryan et al., 1982). However, since the BP-induced type I spectral response in MC-induced microsomes is also only about half of what would be expected from this concentration of P-450$_c$ (Keller & Jefcoate, 1984), all of the P-450$_c$ present may not be equally available for substrate binding.

Two effects may both contribute to the close correlation between the spin state of the fully complexed cytochrome and the quenching of the bound BP: (i) The high-spin heme of the cytochrome may be intrinsically more effective than the low-spin heme in quenching BP fluorescence because of the more paramagnetic high-spin heme. It is unlikely that changes in the spectral overlap between heme absorption and BP emission spectra contribute to the spin-state correlation since the overlap is actually greater for the low-spin form, and the correlation is seen with other PAH with very different emission spectra. (ii) The quenching and the proportion of the high-spin state is a common consequence of proximity of the BP molecule to the heme. Recent work suggests that displacement of an endogenous sixth axial oxygen ligand by substrate binding leads to a transition of the heme from a low-spin to a high-spin conformation (White & Coon, 1982; Janig et al., 1984). Since quenching is inversely proportional to the sixth power of the distance separating the fluorescent molecule from the quencher (Förster, 1948; Latt et al., 1965; Badley, 1976), the close approach of BP to the heme is necessary for effective quenching and may at the same time cause displacement of an axial oxygen ligand that directly causes the change to a high-spin state.

Two important correlations between BP binding and monooxygenation emerge from these studies. The K_m for microsomal oxidation of BP at comparable lipid concentrations is approximately 0.35 μM (Fahl, 1982), and preliminary results in this laboratory indicate a value of approximately 1 μM following reconstitution of P-450$_c$ into LPC vesicles. Consequently, these data imply a 100-fold difference between the K_d for binding and the K_m for the oxidation reaction. The very tight binding of BP demonstrated here may account for this shift, if the dissociation rate is indeed much slower than the turnover rate for monooxygenation. Similar discrepancies in K_d and K_m have been reported for a series of benzphetamine analogues, where increased turnover numbers were associated with increased differences between K_d and K_m (Blank et al., 1983).

Cytochrome P-450$_c$–BP complexes apparently exist in two states that are dependent upon the lipid concentration. Low lipid concentrations favor a more high-spin (highly quenched) complex with a weaker affinity for BP while high lipid concentrations favor a more low-spin (weakly quenched) complex in which BP is bound more tightly. This change in quenching is not associated with a change in the stoichiometry of complex formation. Comparable interactions have been observed over this same concentration range when utilizing OPC for which the cmc is at least 4 orders of magnitude less than that of LPC (Huang, 1969). Consequently, these two types of complex must derive from changes in lipid–P-450$_c$ interactions that do not depend on the cmc.

The lipid effect on the spin state and fluorescence quenching of the P-450$_c$–BP complex also correlates closely with changes in turnover. Lower lipid concentrations favor the high-spin state and stimulate BP monooxygenation, while higher lipid concentrations favor the low-spin state and decrease metabolism in direct proportion to the spin-state change. Previous studies [e.g., Miwa & Lu (1981)] have generally focused on lipid activation in the lower range of lipid concentrations and have indicated only an increase in monooxygenation followed by an apparent plateau in activity. The high-spin state enhances reduction of P-450 (Sligar, 1976), and previous studies of structurally related substrates have shown a correlation between the extent of formation of a high-spin complex and the rate of metabolism (Blank et al., 1983). Factors that favor the low-spin state (such as increased lipid concentrations) should conversely slow the rate of reduction and as a consequence decrease monooxygenation activity.

A recent report (Wagner et al., 1984) has shown similar concentration effects on the spin state and activity of rabbit liver P-450 LM_2 by the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Parallel centrifugation studies indicated that at low CHAPS concentration P-450 LM_2 formed pentamers while at high CHAPS concentrations the cytochrome was monomeric. By analogy, this study suggests that P-450$_c$–BP complexes may exist in two states when in contact with phospholipids; a high-activity oligomeric state exhibiting a predominantly high-spin heme and a low-activity monomer (or lower oligomer) exhibiting a predominantly low-spin heme.

In this paper, we have shown that optical and fluorescence spectroscopies together provide the means to accurately describe the equilibrium of one lipophilic fluorescent ligand (BP) between the membrane and the active site of one form of cytochrome P-450. We have utilized these techniques to directly measure for the first time the very high affinity binding of BP to P-450$_c$ (K_d = 8 nM). Furthermore, we have established a major discrepancy between K_d and K_m values for BP with P-450$_c$ and the correlation between spin state and fluorescence quenching of P-450$_c$–BP complexes. These findings indicate a closer approach of substrate to the heme that is controlled in part by lipid–protein or protein–protein interactions.

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APPENDIX

Calculation of K_d of BP for P-450$_c$. The binding constant (K_d) of P-450$_c$ for BP was determined independently by both optical difference and fluorescence spectroscopy.

(A) Optical Spectroscopy Studies. The K_d for BP binding to P-450$_c$ was determined by optical difference spectroscopic techniques in the presence of the competitive inhibitor *n*-octylamine. This ligand is known to bind to the heme of P-450 yet does not induce a spin-state change. Hence, this ligand can function as a competitive inhibitor of BP binding yet does not itself induce spin-state changes that will interfere with spin-state changes arising from BP binding.

P-450$_c$ (0.1 μM) in reconstituted systems containing LPC (60 μg/mL) and *n*-octylamine was titrated with BP (0–500 nM), and the spectral changes were recorded. Equal amounts of solvent were added to the reference cell. The change in absorbance for the 426/450-nm wavelength pair was calculated as a function of the BP concentration. The maximum absorbance change due to BP binding (ΔA_{max}) was calculated by computer-aided least-squares regression analysis of these hyperbolic plots. Assuming a stoichiometry of 1:1 for the interaction between BP and P-450$_c$, the amount of free ligand (BP) at each titration step was calculated from

$$[BP_{free}] = [BP_{tot}] - [BP_{bound}]$$

where

$$[BP_{bound}] = (\Delta A/\Delta A_{max})[P\text{-}450_c]$$

The apparent K_d is derived from -1/slope of the resulting Scatchard plots of bound vs. bound/free ligand. The true K_d is calculated by employing the standard relationship for competitive inhibition:

$$K_{d(apparent)} = K_d([I]/K_i + 1)$$

where [I] is the concentration of inhibitor (*n*-octylamine) and the K_i for *n*-octylamine is 0.12 mM, determined from separate experiments as the K_d of *n*-octylamine for P-450$_c$.

(B) Calculation of K_d from Fluorescence Studies. The K_d for the binding of BP to P-450$_c$ was calculated from fluorescence experiments similar to those conducted with optical spectroscopy, except that fluorescence quenching (ΔF) was utilized instead of absorbance change.

Hyperbolic plots of ΔF (the difference in fluorescence observed in the absence and presence of P-450$_c$) corrected for excimer quenching were analyzed by least-squares regression analysis to obtain $\Delta F_{(max)}$. The amount of BP bound to P-450$_c$ was calculated from

$$BP_{bound} = [\Delta F/\Delta F_{(max)}][P\text{-}450_c]$$

BP_{free} was calculated from

$$BP_{free} = BP_{tot} - BP_{bound}$$

Scatchard analyses of the data as described for the optical spectroscopy studies then yielded K_d.

Calculation of Fluorescence Constants. At high lipid concentrations (ca. 200 μg/mL), the fluorescence of BP is approximated by

$$F = k[BP]$$

where F is the observed fluorescence, [BP] is the total concentration of BP, and k is a constant reflecting the quantum yield of BP in the membrane. The constant k was determined by microcomputer-aided linear-regression analysis of the initial slope from plots of [BP] vs. F at 200 μg of LPC/mL and found to be 0.49 in the LPC and OPC micelles employed in this study.

Since the excited-state lifetime of the BP molecule is relatively long, it is possible for diffusion-mediated collisions to occur between an excited-state BP molecule and a ground-state molecule. Nonradiative energy losses from these transient complexes or "excimers" decrease fluorescence. The extent of fluorescence quenching due to these nonradiative energy losses will be directly proportional to the number of complexes formed. The rate of formation of these transient complexes will be the product of a constant proportional to the lateral diffusion constant of the BP in the membrane (k') and the square of the concentration of BP in the membrane ($[BP]^2$).

Thus, the fluorescence of BP is properly described by

$$F = k[BP] - k'[BP]^2 \quad (2)$$

Computer-aided polynomial regression analysis of plots of [BP] vs. F at the various lipid concentrations employed confirmed the value of k for BP in the lipid and provided k' at each lipid concentration employed (4.2×10^{-4}, 3.2×10^{-4}, 1.0×10^{-4}, 1.4×10^{-4}, and 0.48×10^{-4} at 7.5, 15, 30, 60, and 200 μg of LPC/mL, respectively). k' was found to be inversely proportional to [LPC] with a correlation coefficient of 0.95. The diffusion constant of the hydrocarbon in the lipid should be invariant; therefore, when fluorescence is plotted as a function of [BP]/[LPC], a single value of k (3.55) and k' (.017) is sufficient to describe BP fluorescence in systems containing the various concentrations of LPC. Deviations from the model do appear to occur, but only at extremely high concentrations of BP in the membrane (>6% on a molar ratio). These deviations are almost certainly due to the saturation of the lipid phase with hydrocarbon as has been previously suggested (Vanderkooi et al., 1974).

Corrections of Fluorescence Measurements for Differential Dimer Quenching. Since the formation of transient excimers resulting from the collision of excited and ground-state BP monomers results in fluorescence quenching, BP monomers that become bound to P-450$_c$ are thus immobilized and, hence, unavailable for collision-mediated excimer quenching. Excimer quenching will thus be reduced in the presence of P-450$_c$ due to the reduction in free BP. Therefore, when calculating ΔF, the difference in fluorescence in the absence and presence of P-450$_c$, an excimer quench correction must be calculated to compensate for the difference in free BP between the two systems. In the absence of P-450, the free BP is simply the concentration of BP in the lipid phase. However, in the presence of P-450$_c$, the concentration of free BP is equal to the total concentration minus the amount bound to P-450$_c$. Failure to account for this differential excimer quenching in the absence or presence of P-450$_c$ leads to deviations in the ΔF plots, which are most pronounced at low lipid concentrations when the effective BP concentration in the membrane ([BP]/[LPC]) is greatest (Figure 5).

Plots of ΔF were corrected for this differential excimer quenching by the following method: In the absence of P-450$_c$, BP fluorescence is described by

$$F = k[BP_{tot}] - k'[BP_{tot}]^2$$

where $[BP_{tot}]$ = total concentration of BP. In the presence of P-450$_c$, however, BP fluorescence is described by

$$F = k[BP_{free}] - k'[BP_{free}]^2$$

where $[BP_{free}]$ = total concentration of BP minus amount bound to P-450$_c$ and complete quenching of BP in the complex is assumed.

Thus, the amount of differential excimer quenching becomes

$$k'([BP_{tot}]^2 - [BP_{free}]^2)$$

This quantity must then be added to the observed difference in fluorescence (ΔF) due to the presence of P-450$_c$ as a correction for the differential quenching between the two systems arising from the unequal amounts of free BP available for excimer quenching.

The concentration of free BP (S_{free}) in each system was calculated by assuming a 1:1 stoichiometry for the binding of BP to P-450$_c$, utilizing the known concentration of P-450$_c$ and the appropriate binding constant (K_d) for BP at each lipid concentration as determined in separate experiments. The concentration of free substrate was calculated as described elsewhere (Keller & Jefcoate, 1984) from

$$S_{free} = 0.5[-[K_d + E_t - S_t] + ([k_d + E_t - S_t]^2 + (4K_dS_t))^{1/2}]$$

where E_t = total concentration of enzyme and S_t = total concentration of ligand.

These corrections for differential excimer quenching are illustrated in Figure 6. The observed (uncorrected) values of ΔF are shown by solid circles, while the ΔF values corrected for differential excimer quench are shown by plus signs. These corrections obviously become more significant as the concen-

tration of BP increases, leading to an increase in the rate of excimer formation.

Registry No. BP, 50-32-8; LPC, 18285-71-7; OPC, 10015-85-7; cytochrome P-450, 9035-51-2.

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