Selectivity in the Binding of Hydroxylated Benzo[a]pyrene Derivatives to Purified Cytochrome P-450$_c$†

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Received June 18, 1984; Revised Manuscript Received April 10, 1985

ABSTRACT: The interaction of rat liver microsomal cytochrome P-450$_c$ with potential benzo[a]pyrene (BP) metabolites has been compared with the binding of BP by optical and fluorescence spectroscopy. Fluorescence quenching of the phenolic derivatives of BP derives from 1:1 complex formation with P-450$_c$, is a function of the position of the hydroxyl substituent, and correlates with the concomitant increase in high-spin cytochrome observed in parallel optical titrations. The proportion of high-spin cytochrome seen when P-450$_c$ was reconstituted in dilauroylphosphatidylcholine vesicles (60 μg/mL) ranged from about 7% for the 3- and 7-phenols to 75% for 11- and 12-phenols. BP and all 12 methyl-BP derivatives have comparable high affinities for P-450$_c$ (50–70% high spin). K_d determinations with purified P-450$_c$ indicated very strong binding of BP phenols that induce high-spin complexes (4-, 5-, 9-, 10-, 11-, and 12-phenols; K_d = 3–25 nM). Inhibition of *n*-octylamine binding by the 3- and 7-phenols indicated weak interactions (K_d = 80–90 nM), even though low-spin complexes were formed. Inhibition of BP metabolism catalyzed by P-450$_c$ with BP phenols correlated with their respective dissociation constants. These results suggest that phenolic substitution at certain positions on BP (1, 2, 3, 7, or 8) interferes with binding to the active site while substitutions at the other positions either enhance or have no effect on binding. BP dihydrodiols [including the (+)- and (−)-BP 7,8-dihydrodiols] were relatively ineffective in forming high-spin complexes (~20%), and fluorescence quenching of dihydrodiols by P-450$_c$ also saturated at low levels. Both titrations indicated high-affinity binding (K_d = 50 nM), although weaker than that of BP (K_d = 8 nM). In contrast, *n*-octylamine binding to the heme of P-450$_c$ was poorly inhibited by dihydrodiols. This apparent discrepancy is accounted for by an uncompetitive binding of dihydrodiols and *n*-octylamine to P-450$_c$ that has been described by two models. The weaker binding of BP dihydrodiols to the P-450$_c$ active site explains the greater susceptibility of 7,8-dihydrodiol metabolism (relative to BP) to competitive inhibition.

Microsomal cytochromes P-450 catalyze the monooxygenation of benzo[a]pyrene (BP)[1] to form arene oxides that either are hydrated by epoxide hydrase to form dihydrodiols or rearrange nonenzymatically to form phenols (Sims & Grover, 1981; Yang et al., 1977a,b). Four of the possible BP dihydrodiols have been identified as microsomal products: the 4,5-, 7,8-, 9,10-, and 11,12-dihydrodiols (Weston et al., 1982; Gozukara et al., 1982). Three BP quinones (the 1,6-, 3,6- and 6,12-quinones) and five BP phenols (1-, 3-, 6-, 7-, and 9-phenols) have also been identified as metabolites of microsomal oxidation (Selkirk et al., 1976a,b; Gelboin, 1980). The polycyclic hydrocarbon dihydrodiols and phenols are key intermediates in the production of ultimate carcinogens and mutagens through secondary monooxidation (Cooper et al., 1983; Conney, 1982; Sims & Grover, 1981; Prough et al., 1981, Gelboin, 1980; Vigny et al., 1980; Yang et al., 1976). Conversion of BP to the carcinogenic (+)-*anti*-benzo[a]pyrene 7,8-dihydrodiol 9,10-oxide is critically controlled by competition between these BP metabolites and BP (Keller & Jefcoate, 1984; Shen et al., 1979). Nonethless, little is known of the interaction of PAH metabolites with cytochrome P-450$_c$.

We have recently demonstrated the utility of fluorescence quenching as a method by which to study BP binding to cytochrome P-450$_c$ (Marcus et al., 1985). Fluorescence quenching of the hydrocarbon was shown to be directly related to the spin state of the hydrocarbon–P-450$_c$ complex (determined by optical spectroscopy), and both methods were subsequently utilized to quantitate the extremely tight binding of BP to P-450$_c$ (K_d = 8 nM). This paper describes experiments in which optical spectroscopy and fluorescence spectroscopy have been used to investigate the binding of several BP metabolites and derivatives to P-450$_c$. Structure–activity relationships governing the effect of hydroxyl substitution on spin state and fluorescence characteristics of hydrocarbon–P-450$_c$ complexes are described. Dissociation constants of BP phenol–P-450$_c$ complexes have been determined and correlated with their inhibitory effect on monooxygenation. A comparison between the dissociation constants of BP and BP 7,8-dihydrodiol with P-450$_c$ suggests an explanation for the greater sensitivity of 7,8-dihydrodiol oxidation to competitive inhibition.

Materials and Methods

Chemicals. Benzo[a]pyrene (BP), BP phenols, BP dihydrodiols, and 6-(hydroxymethyl)-BP were obtained from the chemical repository of the National Cancer Institute. Each compound was checked for purity prior to use by high-pressure liquid chromatography, employing an Altex Ultrasphere-ODS column. Enzyme purifications and all other materials and methods were identical with those reported previously (Marcus et al., 1985).

† This work was supported by National Institutes of Health Grant CA 16265.
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[1] Abbreviations: BP, benzo[a]pyrene; BP phenol, a monohydroxylated derivative of benzo[a]pyrene; BP dihydrodiol, a dihydroxydihydrobenzo[a]pyrene; 6-OHMe-BP, 6-(hydroxymethyl)benzo[a]pyrene; NOA, *n*-octylamine; MC, 3-methylcholanthrene; LPC, dilauroylphosphatidylcholine.

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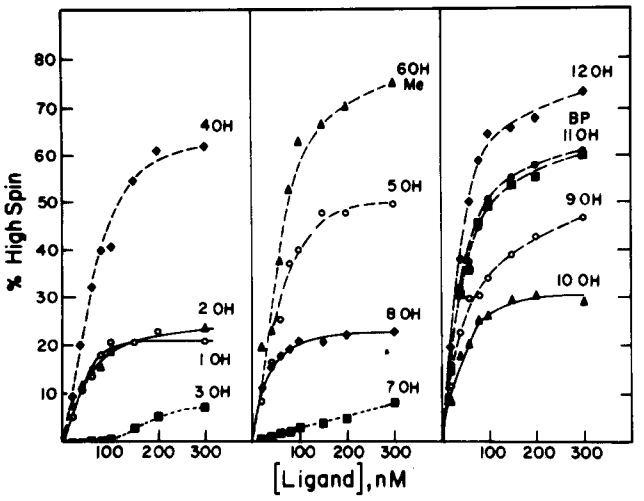

FIGURE 1: Spin-state change of P-450$_c$ due to binding of BP phenols. The percent high-spin cytochrome calculated from the absorption spectrum of P-450$_c$ is shown as a function of the total concentration of hydrocarbon in phospholipid micelles. Samples contained purified P-450$_c$ (0.1 μM) in dilauroylphosphatidylcholine micelles (60 μg/mL) and the indicated concentration of hydrocarbon. High-spin cytochrome was quantitated from the 417/450nm wavelength pair and a millimolar extinction coefficient of 55 for the low- to high-spin transition (Ristau et al., 1979). High (--), intermediate (—), and low (···) high-spin content groups.

Spectral and Fluorescence Techniques. Optical and fluorescence titrations were performed as described in the accompanying paper (Marcus et al., 1985). The high-spin content of cytochrome P-450 was measured from the difference spectra by the decrease in the 417/450-nm wavelength pair by employing an extinction coefficient of 55 mM^{-1} cm^{-1} for the low- to high-spin transition (Ristau et al., 1979). Excitation and emission wavelengths used for each ligand were chosen to minimize the internal filter effect of P-450 (spectral overlap) and maximize fluorescence yield. Spectral and fluorescence characteristics for the 11 BP phenols were obtained from previously published reports (Jerina et al., 1976; Holder et al., 1975; McCaustland et al., 1976). Fluorescence data were normalized to preclude differences in fluorescence quenching (ΔF) caused by differences in fluorescence yield and were corrected for the inner filter effect of P-450$_c$, as described in the accompanying paper. Complex formation was measured as fluorescence quenching (ΔF), and this correlates almost exactly with spin-state changes measured by optical spectroscopy. This quantity is determined by direct substraction of hydrocarbon fluorescence in lipid micelles containing P-450$_c$ from that in identical micelles without P-450$_c$. Dissociation constants for the binding of BP derivatives to P-450$_c$ were determined by optical spectroscopy as previously described in greater detail for BP (accompanying manuscript), by increasing the "apparent" K_d by the addition of the competitive inhibitor *n*-octylamine.

Benzo[a]pyrene Metabolism. BP oxidation was measured as described previously (accompanying paper) 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, 30 μg of dilauroylphosphatidylcholine (LPC), 1 μM BP, BP phenols (up to 1 μM), and an NADPH-generating system. All reaction components except the hydrocarbons were incubated at 37 °C for 5 min. Hydrocarbons were added in 10 μL of acetonitrile to initiate the reaction. Extraction and analysis of the oxidation products resulting from 30-s incubations were by the potassium hydroxide/dimethyl sulfoxide assay of Van Cantfort et al. (1977).

Table I: Spin-State Changes and Fluorescence Quenching due to Binding of Benzo[*a*]pyrene and Hydroxylated Benzo[*a*]pyrene Derivatives to P-450$_c$

ligand	% high spin[a]	K_d (nM)	% quench[b]
BP	61	8[c]	56
BP phenols			
1	22		17
2	24		13
3	7		1
4	62	21[c]	41
5	50		47
7	8	84[d]	11
8	23		16
9	47	23,[c] 25[d]	39
10	30	24[c]	20
11	60	6[c]	56
12	73		56
6-OHMe-BP	75		66
Me-BP's[e]	65		31
BP dihydrodiols			
(+) 7,8	25		21
(-) 7,8	21	250[d]	18
(±) 4,5	24		12
(±) 9,10	24		14

[a] Determined by optical difference spectrocopy as described under Materials and Methods. [b] Maximum fluorescence quenching (ΔF_{max}) due to complex formation with P-450 divided by fluorescence of free hydrocarbon at a concentrtaion equal to the concentration of P-450$_c$ (ΔF_{equiv}). [c] Determined in the presence of 1 mM *n*-octylamine. [d] Determined as the K_i for *n*-octylamine binding. [e] Average of all 12 methyl-BP derivatives.

RESULTS

Hydrocarbon Binding and Spin-State Changes. The Soret region of the absolute spectrum at 390, 417, and 645 nm of purified P-450 indicated this cytochrome to be completely low spin when compared to P-450$_{scc}$ in 0.3% Tween-20, which has previously been shown to be completely low spin under these conditions (Hanukoglu et al., 1981). Figure 1 shows the increase in the proportion of high-spin cytochrome calculated from changes in absorption spectra (7–75%, Table I) when 0.1 μM P-450$_c$ was titrated (0–300 nM) with each of the twelve BP phenols, with the exception of the unstable BP 6-phenol. Differences in the interaction of the enzyme with the various phenols are readily apparent. The percentage of high spin state cytochrome induced by saturation with hydrocarbon ranged from about 7% for the 3- and 7-phenols to 75% for the 12-phenol and 6-(hydroxymethyl)-BP. The phenolic BP derivatives can be divided into three groups on the basis of their effect on spin state: (a) derivatives that increase the proportion of high spin state cytochrome to greater than 50% [BP-4, 5-, 11-, and 12-phenols and 6-(hydroxymethyl)-BP], (b) derivatives that induce only a minimal spin-state change (BP 3- and 7-phenols), and (c) derivatives inducing spin-state changes intermediate to those induced by the first two groups (BP 1-, 2-, 8-, 9-, and 10-phenols).

The spin-state change in P-450$_c$ that is derived from the spectral change following binding of two BP dihydrodiols is shown in Figure 2. Both dihydrodiols appear to bind tightly to P-450$_c$ (K_d ca. 50 nM) yet convert a maximum of only 20% of the cytochrome to the high-spin state at saturation of binding. Furthermore, these spectrophotometric titrations indicated that P-450$_c$ showed no significant selectivity for binding between the optical isomers [(+) and (-)] of BP 7,8-dihydrodiol. Similar results were obtained in experiments utilizing (±)-BP 4,5- and 9,10-dihydrodiols (data not shown).

The apparent binding constants of several of the BP derivatives were determined by optical difference spectroscopy (Table I). In order to determine binding constants (K_d) of these ligands with very high affinity for P-450$_c$, the apparent

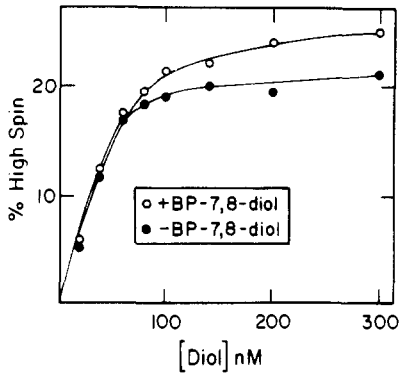

FIGURE 2: Spin-state change of P-450$_c$ due to binding of BP dihydrodiols. The percent high-spin cytochrome calculated from the absorption spectrum of P-450$_c$ is shown as a function of the total concentration of hydrocarbon in phospholipid micelles. Samples contained purified P-450$_c$ (0.1 μM) in dilauroylphosphatidylcholine micelles (60 μg/mL) and the indicated concentration of (+)- or (−)-BP 7,8-dihydrodiol. High-spin cytochrome was quantitated from the 417/450-nm wavelength pair and a millimolar extinction coefficient of 55 for the low- to high-spin transition (Ristau et al., 1979).

K_d was increased by addition of *n*-octylamine, a heme binding ligand that competitively inhibits BP binding. The dissociation constants for the BP derivatives determined in this way (Table I) indicate that BP 11-phenol bound to P-450$_c$ with an affinity very similar to that of BP and that several other BP phenols bind only slightly more weakly.

Although many of the BP metabolites studied appear to possess affinities for P-450$_c$ similar to that of BP itself, the BP 7-phenol and (−)-BP 7,8-dihydrodiol have substantially lower affinities for the cytochrome when determined from their competitive effect upon *n*-octylamine binding. Assuming direct competition with *n*-octylamine, the K_d for BP 7-phenol (84 nM) was substantially weaker than those for phenols exhibiting strong quenching upon binding. Significantly, BP 9-phenol exhibited the same K_d when measured both directly and by the competitive method. However, (−)-BP 7,8-dihydrodiol was very ineffective in displacing *n*-octylamine from P-450$_c$. The apparent affinity of (−)-BP 7,8-dihydrodiol determined by inhibition of *n*-octylamine binding when assuming full competition (250 nM) is approximately 5-fold lower than that determined directly by optical spin-state titrations (50 nM, Figure 2). This discrepancy strongly suggests that *n*-octylamine and BP 7,8-dihydrodiol may not be fully competitive for cytochrome P-450$_c$, as distinct from what has been demonstrated for BP.

Hydrocarbon Binding and Fluorescence Quenching. Binding of BP to P-450$_c$ results in a quenching of the fluorescence of BP concomitant with the change in spin state of the cytochrome. This quenching due to binding to P-450$_c$ was shown to be due primarily to a decrease in total fluorescence emission of the hydrocarbon rather than to the small shifts in the excitation and emission spectra of the hydrocarbon that were also observed (accompanying manuscript). The decreased fluorescence of the major fluorescence emission maxima only slightly overestimated the decrease in quantum yield and has for convenience been used in these studies. Similar quenching effects were also observed when hydroxylated BP derivatives bound to P-450$_c$. However, substantial spectral shifts in both excitation and emmision spectra were also observed when some of these hydrocarbons were bound to P-450$_c$, as shown in Figure 3 for BP 9-phenol. The effect of binding on the fluorescence maxima of both the excitation and emission spectra of BP 9-phenol indicates that the environment of the binding site in the protein is substantially different from that provided by the lipid.

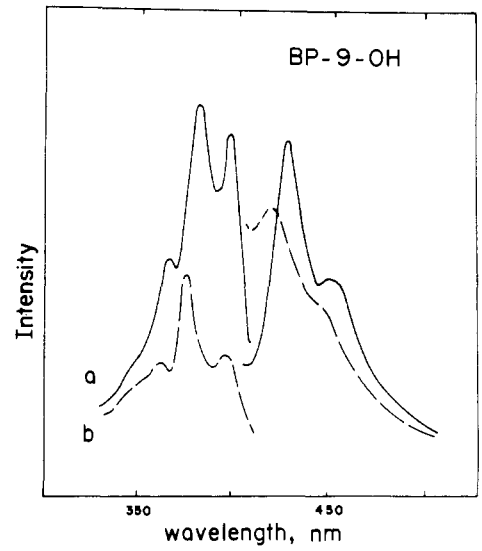

FIGURE 3: Excitation and emission spectra of BP 9-phenol in phospholipid micelles in the absence and presence of purified P-450$_c$. (a) Excitation and emission spectra of BP 9-phenol (2.5 nM) in LPC micelles (60 μg/mL); (b) excitation and emission spectra of BP 9-phenol (2.5 nM) in LPC micelles (60 μg/mL) containing P-450$_c$ (0.2 μM). Under these conditions, 90% of the BP 9-phenol is bound to P-450$_c$, and BP 9-phenol is 35% quenched.

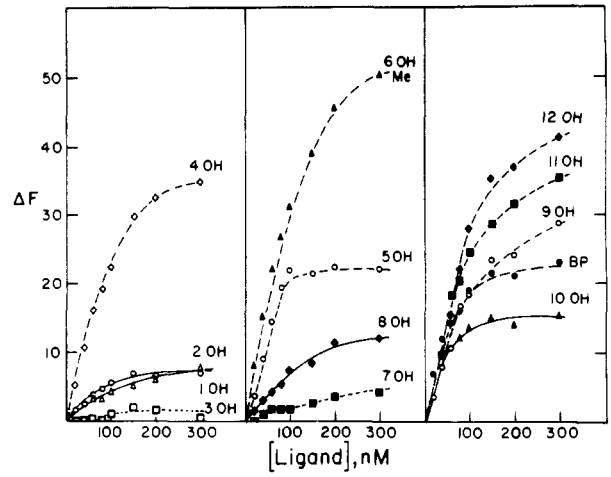

FIGURE 4: Fluorescence quenching of BP phenols due to binding to P-450$_c$. Fluorescence quenching (ΔF) is plotted against the total concentration of hydrocarbon in phospholipid micelles. ΔF represents the fluorescence of the hydrocarbon in the absence of P-450 minus the fluorescence of the hydrocarbon in the presence of P-450$_c$. Samples contained purified P-450$_c$ (0.1 μM) in dilauroylphosphatidylcholine micelles (60 μg/mL) and the indicated concentration of hydrocarbon. High- (−−), intermediate- (—), and low- (···) efficiency quench groups.

The maximum fluorescence quenching (ΔF) at saturating concentrations of these ligands (300 nM) provides the most accurate measure of complex formation. The percent fluorescence quenching (% quench) is equal to the ratio of the maximum fluorescence quenching of a ligand due to binding to P-450$_c$ (ΔF_{max}) to the fluorescence of a concentration of unbound ligand equivalent to the concentration of P-450$_c$ (ΔF_{equiv}):

$$\% \text{ quench} = \Delta F_{max}/\Delta F_{equiv}$$

The dependence of ΔF upon the total ligand concentration is shown for the various BP phenols in Figure 4 and indicates that P-450$_c$ exhibits considerable selectivity in its interactions with these phenolic derivatives of BP. Analogous to their effects on spin state as described above, BP phenols can be

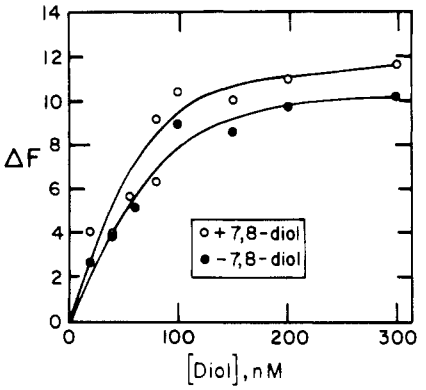

FIGURE 5: Fluorescence quenching of BP dihydrodiols due to binding to P-450$_c$. Fluorescence quenching (ΔF) is plotted against the total concentration of hydrocarbon in phospholipid micelles. ΔF represents the fluorescence of the hydrocarbon in the absence of P-450 minus the fluorescence of the hydrocarbon in the presence of P-450$_c$. Samples contained purified P-450$_c$ (0.1 μM) in dilauroylphosphatidylcholine micelles (60 μg/mL) and the indicated concentration of (+)- or (−)-BP 7,8-dihydrodiol.

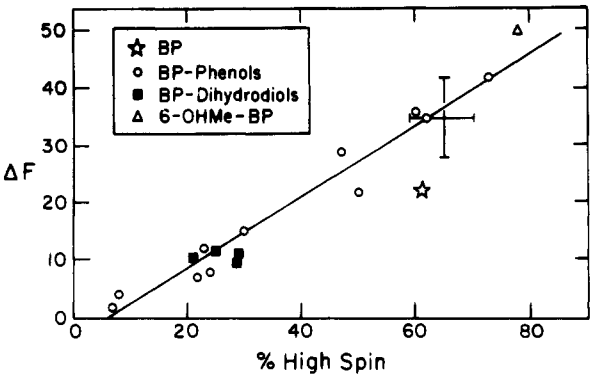

FIGURE 6: Correlation between fluorescence quenching (ΔF) and percent high-spin P-450$_c$ due to complex formation with BP and BP derivatives. Fluorescence quenching (ΔF) of BP and BP derivatives is plotted against the proportion of high-spin cytochrome induced by the hydrocarbon. Values shown are for saturating concentrations (300 nM) of each hydrocarbon with P-450$_c$ (0.1 μM) in dilauroylphosphatidylcholine micelles (60 μg/mL). The cross at the upper right depicts the average and standard deviation for the 12 methyl-BP derivatives. Best fit line was $\Delta F = (0.62 \times \%$ high spin$) - 4.13$ ($r^2 = 0.96$).

divided into three groups on the basis of the fluorescence quenching by cytochrome P-450$_c$. The 6-(hydroxymethyl)-BP derivative and the BP 4-, 5-, 9-, 11-, and 12-phenols comprise a group of compounds that are quenched at least to a similar extent as BP itself. A second group consisting of the BP 3- and 7-phenols exhibit little or no quenching. The BP 1-, 2-, 8-, and 10-phenols form a third group for which quenching is intermediate between these two extremes.

The effect of complex formation with P-450$_c$ on dihydrodiol fluorescence is shown in Figure 5. In contrast to the BP phenols, binding of both BP dihydrodiols to P-450$_c$ resulted in only low fluorescence quenching, even though the fluorescence data indicated tight binding (K_d ca. 50 nM). The low dihydrodiol fluorescence quenching was not due to limited solubility of the dihydrodiols in the membrane. There was again no significant selectivity in the interaction of P-450$_c$ with the two optical isomers of BP 7,8-dihydrodiol, and similar results were obtained with (±)-BP 4,5- and 9,10-dihydrodiols.

These results are almost identical with those obtained from optical spectroscopy spin-state titrations with dihydrodiols (Figure 2), which also indicate a 5-fold higher affinity of the (+)-BP 7,8-dihydrodiol (50 nM) for P-450$_c$ than do spectral titrations with *n*-octylamine (K_i = 250 nM; Table I).

Correlation of Spin-State Change and Fluorescence Quenching. The maximum fluorescence quenching (ΔF) of BP and of the various derivatives by P-450$_c$ and the corresponding proportion of high-spin state induced as a result of binding to the cytochrome were highly correlated ($r^2 = 0.93$) as shown in Figure 6. The relatively weakly binding BP dihydrodiols appear in the group at the lower left, while BP itself, the 12 methyl-BP's, and the high-affinity BP phenols are in the group in the upper right. These results suggest that methyl substitution does not substantially hinder approach of the BP nucleus to the heme and that steric factors alone are not responsible for the selectivity of the BP phenols for the P-450$_c$ binding site.

Competitive Inhibition of BP Metabolism. When BP metabolism by purified P-450$_c$ in reconstituted systems was measured in the presence of the various BP phenols (1 μM), inhibition ranged from 15% to 93% as shown in Table II. Inhibition constants (K_i) were estimated from these data by assuming both competitive inhibition and a K_m for BP with P-450$_c$ of 0.35 μM (Fahl, 1982).

The rank order of the K_i's determined for five of the BP phenols (Table II) was the same as for their K_d's (Table I).

Table II: Inhibition of Benzo[*a*]pyrene Metabolism by P-450$_c$ in an in Vitro Reconstituted System by Hydroxylated Benzo[*a*]pyrene Derivatives

ligand	% inhibition[a]	K_i[b] (μM)
BP phenols		
1	49	0.27
2	36	0.46
3	15	1.47
4	62	0.16
5	61	0.17
7	37	0.44
8	18	1.18
9	44	0.33
10	49	0.27
11	93	0.02
12	76	0.08
6-OHMe-BP	72	0.10

[a] Percent inhibition of BP metabolism [45 nmol min^{-1} (nmol of P-450$_c$)$^{-1}$] compared were less by 1 μM derivative with 1 μM BP. Results shown are averages of three to six tests, and standard deviations were less than 5%. [b] Estimated K_i assuming competitive inhibition and K_m = 0.35 μM, at an inhibitor concentration of 1 μM.

The latter were each moderately correlated ($r^2 = 0.83$) with both the K_d and the ability of the phenol to induce the high-spin state (Table I). It should be noted that the K_i's for the phenols and the K_m for BP are all substantially greater than the respective K_d's for the binding of these same ligands to P-450$_c$.

Discussion

The optical and fluorescence spectroscopy techniques developed previously for the study of BP binding to P-450$_c$ (Marcus et al., 1985) have been extended in this study to include hydroxylated derivatives of BP that are potential secondary metabolites or inhibitors. This study presents evidence that the specific location of a hydroxyl substituent on the BP nucleus substantially affects the formation of the P-450$_c$–hydrocarbon complex.

Addition of a hydroxyl group to BP may either increase or decrease the proportion of high spin state cytochrome induced by binding of the hydrocarbon. This response is not simply a bulk-steric effect, since substitution by methyl groups at the same positions as the hydroxyl groups on the BP nucleus does not alter binding of the hydrocarbon to P-450$_c$ (Figure 6). Although complex formation by BP 1-, 2-, and 7-phenols with

P-450$_c$ produces only small changes of spin state and low fluorescence quenching (Figures 1 and 4), effective inhibition of BP metabolism by P-450$_c$ by these compounds (Table II) suggests effective complex formation with P-450$_c$. Since the weak binding observed for BP 7-phenol (Table I) was sufficient for substantial binding, the minimal spin-state change induced by BP 7-phenol reflects a preferential stabilization of a low-spin state, rather than a failure to bind to the cytochrome. BP 3-phenol and 8-phenol on the other hand are substantially weaker inhibitors of BP metabolism, suggesting that the small spectroscopic changes may in part derive from an inability of these compounds to bind to P-450$_c$ in the active site. The effect of hydroxyl substitution on the binding of the BP nucleus to the active site of P-450$_c$ is illustrated by Figures 1 and 4. Hydroxyl substitutents located along the "sides" (position 4, 5, 6, 11, or 12) or at position 9 of the molecule either enhance or have a minimal effect on the approach of the BP nucleus to the heme. Hydroxyl substitution on the "ends" (position 1, 2, 3, 7, or 8) of the molecule (except for position 9) apparently hinders access of the hydrocarbon to the active site. Limited access to the active site, however, does not preclude monooxygenation, since the BP 1- and 3-phenols are known to be substrates for further oxidation (Prough et al., 1981; Caspary et al., 1981; Wood et al., 1975, 1976) and the BP 2-phenol is activated to mutagenic or carcinogenic metabolites as effectively as BP (Chang et al., 1979; Slaga et al., 1978; Wislocki et al., 1977).

The high correlation between the spin-state change and fluorescence quenching for the various BP phenols ($r^2 = 0.96$) confirms that fluorescence quenching and spin-state change both reflect binding of the hydrocarbon to the same P-450$_c$ site. The fluorescence quenching presumably derives from binding close to the heme, consistent with occupancy of the active site. Further evidence that specific binding close to the heme of P-450$_c$ is the cause of these correlated changes is provided by the close relationship between both K_d and spin-state changes for hydrocarbon binding to P-450$_c$ (Table I) and the K_i for competitive inhibition of monooxygenation (Table II). A shift in the overlap of heme and excited PAH spectra does not account for the effect of spin state on quenching since this spectral overlap is actually decreased for the high-spin form. The hydroxyl substituent apparently orients the BP molecule within the active site of P-450$_c$ so as to affect the relative stabilizations of the high- and low-spin states. Orientations that permit close association of the hydrocarbon with the heme favor the high-spin state and also produce proportionately greater quenching of hydrocarbon fluorescence. A close approach of the hydrocarbon to the heme facilitates displacement of the endogenous sixth axial ligand (White & Coon, 1982; Janig et al., 1984) and at the same time provides greater exposure of the hydrocarbon to the quenching effect of the paramagnetic heme.

The small spin-state changes (20%) induced by both (+)- and (−)-BP 7,8-dihydrodiols are unexpected since both are comparable to BP as substrates for monooxygenation by P-450$_c$ (K_m and V_{max}). Direct spectral measurements of these interactions indicate a high-affinity 1:1 binding (Figures 2 and 4). However, a weaker interaction of the dihydrodiols (compared to BP) is suggested by the much greater susceptibility of BP 7,8-dihydrodiol monooxygenation to competitive inhibition (Keller & Jefcoate, 1984; Shen et al., 1979). Low-affinity binding of dihydrodiols to the active site of P-450$_c$ is further indicated by the very inefficient displacement of *n*-octylamine (a heme binding ligand) by dihydrodiols (Table I). Thus, there is a discrepancy between the high affinity of

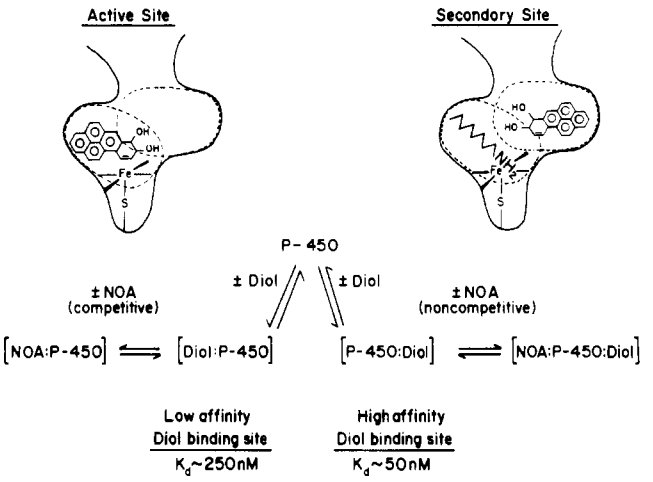

FIGURE 7: A model representing the binding of (±)-BP 7,8-dihydrodiol to purified P-450$_c$. The model illustrates the existence of two sites capable of interacting with dihydrodiol. *n*-Octylamine competes with dihydrodiol for binding only for the low-affinity (heme) site. The space required for optimum binding of 7,8-dihydrodiol to each site is denoted by the area enclosed by the two dotted lines. Thus, *n*-octylamine can still fit in the active site when 7,8-dihydrodiol occupies the secondary site. Overlap of the two areas outlined by the dotted lines denotes that only one site can be occupied by 7,8-dihydrodiol at any one time.

the stoichiometric complex (K_d = 50 nM) and the far weaker interactions indicated by methods directly probing the active site (K_d = 250 nM; Table I).

Two models are consistent with these binding characteristics for dihydrodiols. The first is a *single-site* model, in which BP 7,8-dihydrodiol preferentially binds with high affinity in the active site sufficiently removed from the heme for fluorescence quenching to be relatively low. The binding of the sixth ligand of the low-spin cytochrome is only weakly perturbed, causing only a small high-spin contribution (Figure 7, secondary site only). In this model BP 7,8-dihydrodiol binding allows sufficient space at the heme for coordination by *n*-octylamine, albeit with 4-fold weakening of the interaction.

A second, *two-site* model is analogous to the spin-state equilibria proposed by Sligar (1976). In this model BP 7,8-dihydrodiol can equilibrate between a binding site close to the heme (fully quenched, full high-spin state, and competitive with *n*-octylamine; Figure 7 active site) and a secondary site more removed from the heme than that in the first model (no quenching, full low-spin complex, and no effect on *n*-octylamine binding). Heme and secondary sites cannot be occupied simultaneously by BP 7,8-dihydrodiol. According to this model, the effect of BP 7,8-dihydrodiol on *n*-octylamine binding reflects a 4:1 distribution of dihydrodiol between secondary and active sites with K_d's of 50 and 250 nM, respectively. Clearly, this also represents the distribution between low- and high-spin states and between fluorescent and fully quenched ligand. The 20% spin-state change and the limited fluorescence quenching of BP 7,8-dihydrodiol also fit this distribution. The stereoselectivity of polycyclic hydrocarbon monooxygenation (Van Bladeren et al., 1984) also provides evidence for a large active site for cytochrome P-450$_c$ that can accomodate BP and its derivatives either close to the heme or well removed from the heme. Evidence for double occupancy of a large ligand binding site by polycyclic hydrocarbons and small inhibitors has been described by Imai (1982).

The scheme shown in Figure 7 is a simple model incorporating only the minimum elements required to describe the interaction of the dihydrodiol with P-450$_c$. Other factors,

including conformational changes in protein tertiary structure that most likely occur concomitant with substrate binding and spin-state transition, are not excluded by this model. However, rather than serving as an alternative, this model (based on information obtained by different experimental techniques) provides another viewpoint on these interactions and is complementary and fully consistent with other similar models of P-450–ligand interactions (Sligar, 1976; Van Bladeren, 1984).

The difference in the affinities of BP and BP 7,8-dihydrodiol for P-450$_c$ explains the greater potency of BP quinones and BP phenols as inhibitors of dihydrodiol oxidation compared to BP oxidation (Shen et al., 1979; Keller & Jefcoate, 1984;. It is also clear that microsomal BP oxidation is not limited by substrate binding, since the K_m for this reaction (0.35 μM; Fahl, 1982) is approximately 40-fold greater than the K_d of BP (8 nM; accompanying manuscript) with P-450$_c$, the form of P-450 primarily responsible for hepatic metabolism of BP (Ryan et al., 1982). The oxidation of BP 7,8-dihydrodiol, however, may be limited by substrate binding, since the K_d for the active site (0.2 μM) is close to the microsomal K_m at comparable lipid concentrations (0.4 μM; Shen et al., 1979).

These results demonstrate for the first time a direct measurement of the affinity of cytochrome P-450$_c$ for polycyclic hydrocarbon metabolites. The extremely high affinities of BP and many of its hydroxylated metabolites for P-450$_c$ have major implications for the mechanism of metabolism. The generation of high-affinity products may result in reactions that become limited by product dissociation, substantial product inhibition, and even multiple monooxygenation reactions, as proposed for cholesterol side-chain cleavage in which the second monooxygenation reaction occurs without primary product dissociation (Hume et al., 1984; Stevens et al., 1984). These kinetic considerations have important implications in determining the dominant activation pathway for formation of a bay-region dihydrodiol epoxide from polycyclic hydrocarbons such as BP, dimethylbenzanthracene, methylcholanthrene, and chrysene where multiple hydroxylation steps in activation have been implicated.

ACKNOWLEDGMENTS

We are extremely grateful to Dr. H. A. Lardy, Enzyme Institute, University of Wisconsin, for the use of his photon-counting spectrofluorometer.

Registry No. 1-BP-phenol, 13345-23-8; 2-BP-phenol, 56892-30-9; 6-BP-phenol, 33953-73-0; 8-BP-phenol, 13345-26-1; 4,5-BP-dihydrodiol, 28622-84-6; 9,10-BP-dihydrodiol, 24909-09-9; 6-OHMeBP, 21247-98-3; MeBP, 25167-89-9; 3-BP-phenol, 13345-21-6; 7-BP-phenol, 37994-82-4; 11-BP-phenol, 56892-32-1; 12-BP-phenol, 56892-33-2; 4-BP-phenol, 37574-48-4; 5-BP-phenol, 24027-84-7; 9-BP-phenol, 17573-21-6; 10-BP-phenol, 56892-31-0; 7,8-BP-dihydrodiol, 13345-25-0; cytochrome P-450, 9035-51-2.

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Kinetics of Cytochrome P-450 Reduction: Evidence for Faster Reduction of the High-Spin Ferric State†

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Received July 5, 1984; Revised Manuscript Received January 3, 1985

ABSTRACT: Results are presented that support our hypothesis [Backes, W. L., Sligar, S. G., & Schenkman, J. B. (1980) *Biochem. Biophys. Res. Commun. 97*, 860–867] that the multiphasic reduction kinetics of cytochrome P-450 are, in part, due to the spin equilibrium of the ferric hemoprotein. The disappearance of the high-spin charge-transfer band at 650 nm during reduction of the hemoprotein by NADPH was fast, exhibiting a rate constant greater than that of the fast phase of reduction measured by formation of the carbon monoxide adduct. In contrast, the disappearance of the ferric low-spin form of the cytochrome was at a considerably slower rate. A mathematical expression of the fractional content of high-spin cytochrome P-450 was obtained by comparing the ratio of the initial rate of change in the fraction of total oxidized cytochrome remaining to the initial rate of change in the fraction of high-spin ferric P-450 remaining. Results supporting the model were obtained by using both microsomes and purified cytochrome P-450 RLM5. The calculation from experimental data yielded results that were similar to those obtained by different extrapolation methods used for estimation of the amount of high-spin cytochrome P-450, supporting further the proposed relationship between the spin equilibrium and the reduction kinetics of this hemoprotein.

The metabolism of substrates by hepatic microsomal cytochrome P-450 requires, in addition to substrate and dioxygen binding, two single electron transfers from NADPH–cytochrome P-450 reductase (White & Coon, 1980). The first electron input to cytochrome P-450 from NADPH has been studied by a number of groups and seen to be at least a biphasic process (Gigon et al., 1967; Peterson et al., 1976; Taniguchi et al., 1979; Oprian et al., 1979; Backes et al., 1980, 1982; Ruf, 1980; Blanck et al., 1983) when formation of carbon monoxy–ferrous cytochrome P-450 is monitored. Some of these investigators (Peterson et al., 1976; Taniguchi et al., 1979; Oprian et al., 1979) have attributed the multiphasic reduction kinetics to a functional interaction between the reductase and cytochrome P-450 or to a characteristic of the reductase, while other laboratories (Backes et al., 1980, 1982; Tamburini et al., 1984) have attributed the kinetics to be a consequence of the spin equilibrium between low-spin and high-spin ferric cytochrome P-450.

In previous reports (Backes et al., 1980, 1982) the apparent biphasic kinetics of reduction of hepatic microsomal cytochrome P-450 was shown to be consistent with a sequential model where high-spin cytochrome is initially reduced followed by a slower reduction of low-spin cytochrome subsequent to its conversion to the high-spin state. The kinetics of reduction would therefore be regulated, at least in part, by the preequilibrium between high-spin and low-spin states of the cytochrome. According to the hypothesis, a rapid depletion of the high-spin hemoprotein would be observed on addition of NADPH. This concept requires that a step(s) slower than the direct reduction of high-spin cytochrome exist in order to obtain the observed multiphasic plots. At least one of these steps (though, not necessarily the slow step itself) would be the interconversion between low-spin and high-spin states. Evidence supporting this hypothesis was shown in studies where the extent of reduction in the fast phase was compared to the prereduction levels of high-spin cytochrome P-450 with liver microsomes from untreated rats. A positive correlation was obtained, when the spin state was manipulated either by the addition of substrates or by varying the temperature of the reaction (Backes et al., 1980, 1982). These results demonstrated the relationship between a thermodynamic parameter (the prereduction level of high-spin ferric cytochrome) and the kinetics of reduction.

A general model describing the states of the cytochrome during reduction (Sligar, 1976) has been simplified by the assumption that low-spin cytochrome P-450 is not directly reduced (Sligar et al., 1980; Rein et al., 1979). Results supporting this assumption have been reported by Pierre et al.

† This work was supported in part by U.S. Public Health Service Research Grants GM26114 and GM31756 from the National Institutes of Health. S.G.S. is a Research Career Development Awardee of the U.S. Public Health Service (KO4, AMO778). This work was also supported in part by grants from the U.K. Medical Research Council. Completion of this collaborative study was made possible by a travel grant for J.B.S. to the University of Surrey by the Burroughs Wellcome Fund.

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0006-2960/85/0424-5130$01.50/0