Activation of CYP3A4: Evidence for the Simultaneous Binding of Two Substrates in a Cytochrome P450 Active Site

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ABSTRACT: A unique characteristic of the CYP3A subfamily of cytochrome P450 enzymes is their ability to be activated by certain compounds. It is reported that CYP3A4-catalyzed phenanthrene metabolism is activated by 7,8-benzoflavone and that 7,8-benzoflavone serves as a substrate for CYP3A4. Kinetic analyses of these two substrates show that 7,8-benzoflavone increases the V_{max} of phenanthrene metabolism without changing the K_m and that phenanthrene decreases the V_{max} of 7,8-benzoflavone metabolism without increasing the K_m. These results suggest that both substrates (or substrate and activator) are simultaneously present in the active site. Both compounds must have access to the active oxygen, since neither phenanthrene nor 7,8-benzoflavone can competitively inhibit the other substrate. These data provide the first evidence that two different molecules can be simultaneously bound to the same P450 active site. Additionally, structure–activity relationship studies were performed with derivatives of 7,8-benzoflavone structure. The effects of 13 different compounds on the regioselectivity of phenanthrene, chrysene, and benzo[*a*]pyrene metabolism were determined. Of the 13 compounds studied, 6 were activators, 2 were partial activators, and 5 were inhibitors. Analyses of the data suggest that (1) naphthalene substituted with a ketone in the 2-position can activate 3A4 and (2) the presence of an activator results in a narrower effective substrate binding site. Since the CYP3A enzymes are very important in drug metabolism, the possibility of activation, and autoactivation, must be considered when *in vitro–in vivo* correlations are made and when possible drug interactions are considered.

Cytochrome P450 enzymes in the CYP3A subfamily have an important role in the metabolism of drugs and other xenobiotics. These enzymes are capable of oxidizing a wide variety of compounds ranging in size from monosubstituted benzenes to cyclosporin (Wrighton et al., 1985; Aoyama et al., 1989; Brian et al., 1990). A unique characteristic of the CYP3A enzymes is their ability to be activated by certain compounds. It was first shown in this laboratory that benzo[*a*]pyrene metabolism is stimulated by the presence of 7,8-benzoflavone with control microsomes but not with methylcholanthrene-induced microsomes (Wiebel et al., 1971, 1974). Later studies by Schwab et al. (1988) established that purified CYP3A enzymes are directly activated by 7,8-benzoflavone.

Studies by Johnson and co-workers (Johnson et al., 1988; Schwab et al., 1988) on the rabbit CYP3A6 isoform showed that CYP3A substrates could act as activators of their own metabolism. For example, the metabolism of progesterone by CYP3A6 gives nonlinear Lineweaver–Burk kinetics, which become linear by the addition of 5 μM 7,8-benzoflavone (Schwab et al., 1988). Further, 7,8–benzoflavone increased the spectral binding affinity of the type II inhibitor 22-amino-23,24-bis(nor-5-cholen-3β-ol) to CYP3A6 (Johnson et al., 1988). This led the authors to suggest that a separate activator binding site exists for the 3A P450s and that binding of the activator affects the conformation of the active site.

In this study, we report kinetic data on the activation and inhibition of the metabolism of the polycyclic aromatic hydrocarbons (PAHs)[1] shown in Figure 1. The data suggest that the substrate and activator bind simultaneously to the same "active site", with both having access to the active oxygen.

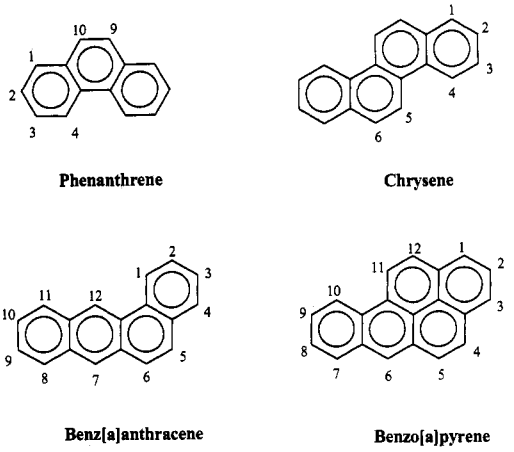

FIGURE 1: Polycyclic aromatic hydrocarbons used in this study.

In addition, we report structure–activity data which suggest that specific interactions between the activator and the P450 change the effective dimensions of the active site.

MATERIALS AND METHODS

Materials. [G-^{3}H]Phenanthrene (specific activity = 440 mCi/mmol), [G-^{3}H]benz[*a*]anthracene (508 mCi/mmol) and metabolites *trans*-1,2-, 3,4-, 5,6-, 8,9-, and 10,11-dihydro-

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[1] Abbreviations: P450, cytochrome P450; PAH, polycyclic aromatic hydrocarbon; diol, dihydrodiol; B[*a*]P, benzo[*a*]pyrene; 3MC, 3-methylcholanthrene; 4-chr, 4-chromanone; 1MeN, 1-methylnaphthalene; 2MeN, 2-methylnaphthalene; 1NMeOH, 1-naphthalenemethanol; 2NMeOH, 2-naphthalenemethanol; 2MeNOH,2-methyl-1-naphthol; 1OH2AcN, 1-hydroxy-2-acetonaphthone; 2AcN, 2-acetonaphthone; 1MO2AcN, 1-methoxy-2-acetonaphthalene; CYP3A4, cDNA-expressed human cytochrome P450 3A4.

diols, [G-^{3}H]chrysene (629 mCi/mmol) and metabolites *trans*-1,2-, 3,4-, and 5,6-dihydrodiols and 1- and 3-phenols, [G-^{3}H]-benzo[*a*]pyrene (466 mCi/mmol) and metabolites *trans*-4,5-, 7,8-, and 9,10-dihydrodiols, 1-, 3-, 6-, 7-, and 9-phenols, and 1,6-, 3,6-, and 6,12-quinones were purchased from NCI Chemical Carcinogen Repository. Since phenanthrene metabolites were not commercially available, *trans*-1,2-, 3,4-, and 9,10-dihydrodiols were obtained by the metabolism of phenanthrene by liver microsomes of rats pretreated with 3-methylcholanthrene (3MC) and were separated on a Nova-Pak C_{18} column. These diols were dehydrated by HCl/acetone (1:1 v/v) to form corresponding phenols which were readily isolated by reversed-phase HPLC. The identity of three diols and five isomeric phenols was established by comparing UV absorption spectra identical to authentic standards previously reported (Jerina et al., 1976; Bao & Yang, 1991). NADPH was purchased from Boehringer Mannheim. Flavone derivatives 7,8-benzoflavone, 5,6-benzoflavone, and flavone and naphthalene derivatives 1-methylnaphthalene, 2-methylnaphthalene, 1-naphthalenemethanol, 2-naphthalenemethanol, 2-methyl-1-naphthol, 1-hydroxy-2-acetonaphthone, 2-acetonaphthone, 4-chromanone, and α-tetralone were purchased from Aldrich Chemical Co. 1-Methoxy-2-acetonaphthalene was synthesized by the reaction of 1-hydroxy-2-acetonaphthalene with excess CH_3I and K_2CO_3 at reflux overnight. The solvent was removed and the product analyzed by GC–MS (m/z 200).

Expression of Human P450 3A4 in Hep G2 Cells. Human TK$^-$ 143 (thymidine kinase-deficient human embryoblast) was used to produce stocks of vaccinia virus coding cytochrome P450 3A4 as previously described (Gonzalez et al., 1991a). Hepatoma Hep G2 cells were grown to >90% confluence on 175 cm^2 plastic flasks and infected with the recombinant vaccinia virus stock containing human 3A4 cDNA. Infected Hep G2 cells were harvested 24 h after infection. The P450 3A4 content was determined by Fe^{2+}–CO vs Fe^{2+} difference spectroscopy (Omura & Sato, 1964). For metabolism studies, the cells were lysed by sonication and centrifuged for 10 min at 500000*g* (Beckman TL100 ultracentrifuge), and the pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.4.

Metabolism of PAHs by CYP3A4. A typical 1-mL incubation contained 50 pmol of P450 3A4 and 50–100 nmol of respective [G-^{3}H]PAH substrates, in 50 mM potassium phosphate buffer (pH 7.4). The incubation mixture was preincubated for 2 min at 37 °C in a shaking water bath, and the reaction was initiated by addition of 1 μmol of NADPH. The metabolism was terminated after 30 min by the addition of 3 mL of dichloromethane. The organic solvent extracts were evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved in methanol, and cold metabolite standards were added as markers for HPLC analysis. Turnovers (picomoles per minute per nanomole of P450 3A4) for metabolite formation were determined from the areas under the radiochromatogram peaks. For the CYP3A4 activation assays, 10, 50, 100, 150, and 200 μM of 13 naphthalenes and flavones were added to the incubation mixture along with the [G-^{3}H]PAH.

Enzymatic Kinetics. Kinetic parameters for phenanthrene metabolism were determined by quantitating the metabolism of radiolabeled substrate. Although the metabolites for 7,8-benzoflavone were identified (see below), authentic metabolite standards were not available. In addition, the quantities generated by our expression system are too low to allow for the isolation and purification of the quantities necessary to determine extinction coefficients. Therefore, the turnover number for 7,8-benzoflavone metabolism was estimated by the disappearance of the substrate. The kinetic analysis of the 7,8-benzoflavone metabolism used the UV absorption of the metabolites to obtain relative quantitative data, which was scaled to the 7,8-benzoflavone turnover number. Substrate concentrations ranged from 5 to 100 μM for both phenanthrene and 7,8-benzoflavone. Michaelis parameters and statistics were determined for CYP3A4 by fitting the experimental data to a hyperbola using the least-squares program of Cleland (1979).

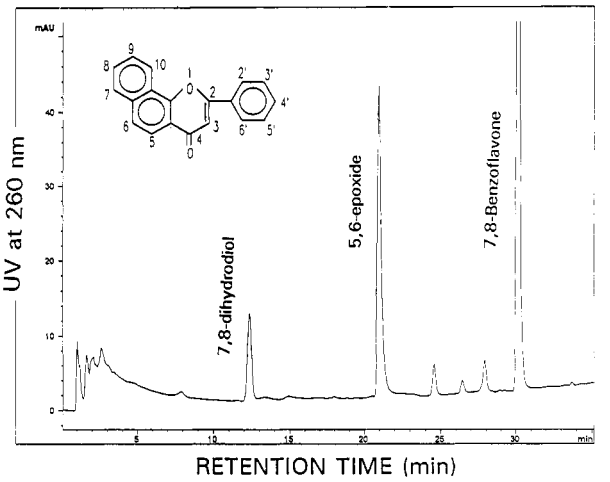

FIGURE 2: HPLC chromatogram for 7,8-benzoflavone metabolism by CYP3A4. HPLC conditions are given under Materials and Methods.

Isolation and Properties of 7,8-Benzoflavone Metabolites. Incubation mixtures contained 50 mM potassium phosphate buffer (pH 7.5), 1 mM NADPH, 500 pmol of CYP3A4 protein or liver microsomes of rats treated with 3-methylcholanthrene (3MC), and 200 μM 7,8-benzoflavone in a final volume of 10 mL. The reaction was carried out at 37 °C for 30 min, terminated, and extracted by the addition of 30 mL of dichloromethane. The organic solvent extracts were evaporated to dryness at 40 °C under a stream of nitrogen. Metabolites were loaded on a DuPont Golden Series ODS column (4.6 mm i.d. × 25 cm) and eluted with a linear gradient from 50% methanol/water to 100% methanol over 50 min with a flow rate of 1 mL/min. Preparations of CYP3A4 and 3MC-induced microsomes formed two major metabolites with retention times of 13 and 21 min (Figure 2). Mass spectra analysis (CI-NH_3) of the first metabolite gave a molecular ion at m/z 307 $(M + H)^+$, suggesting a dihydrodiol. The second peak gave a molecular ion at m/z 289 $(M + H)^+$ with a large peak at m/z 273, indicative of an epoxide. The results are consistent with a previous study on the metabolism of 7,8-benzoflavone by rat liver microsomes pretreated with 3MC (Andries et al., 1990) in which these two metabolites were identified as *trans*-7,8-dihydrodiol (retention time = 13 min) and 5,6-epoxide (retention time = 21 min).

High-Performance Liquid Chromatography. HPLC was performed on a Hewlett-Packard Model HP1050 liquid chromatograph equipped with an HP Model 1050 autosampler, a ternary solvent delivery system, and a multiple-wavelength detector. For HPLC/radiochemical analysis, a Radiomatic FLO-ONE\β Model A-500 detector system with digitally controlled liquid scintillator pump and eluate mixer was used. Gradient quench curves and DPM were used for quantitation.

B[*a*]P and its metabolites were separated on a DuPont Golden Series Zorbax ODS (C_{18}) column (4.6 mm i.d. × 25

Table 1: Influence of 7,8-Benzoflavone on the Kinetic Parameters for Phenanthrene Metabolism with Expressed CYP3A4[a]

[7,8-benzoflavone] (μM)	K_m (μM)	SE	V_{max} (min^{-1})	SE
0	67.9	0.8	1.35	0.01
10	36.0	1.4	1.55	0.03
25	42.2	9.2	2.7	0.3
50	25.0	2.3	4.1	0.2
100	30.4	2.5	6.3	0.2
150	42.0	4.2	7.3	0.3
200	35.5	2.3	7.6	0.2

[a] See Materials and Methods for incubation conditions. Kinetic parameters and standard errors were determined according to the method of Cleland (1979).

Table 2: Influence of Phenanthrene on the Kinetic Parameters for 7,8-Benzoflavone Metabolism with Expressed CYP3A4[a]

[phenanthrene] (μM)	K_m (μM)	SE	V_{max} (min^{-1})	SE
0	48.8	9.9	21.7	1.5
10	41.6	4.9	13.8	0.5
50	34.3	7.1	8.7	0.5
100	56.0	11.7	9.3	0.7
200	86.5	16.6	5.8	0.5
500	42.2	9.8	3.4	0.3

[a] See Materials and Methods for incubation conditions. Kinetic parameters and standard errors were determined according to the method of Cleland (1979).

cm) eluted with an 80-min linear gradient from 60% methanol in water (v/v) to 100% methanol at 1 mL/min. Phenanthrene and its metabolites were separated with a Waters Associates RCM 8 × 10 radial compression module fitted with a Nova-Pak C_{18} cartridge (8 mm i.d. × 10 cm; 4-μm particle size). Peaks were eluted using methanol/water gradients at 1 mL/min. After 5 min at 50% methanol/water, a linear gradient from 50% to 60% was used over 35 min, followed by a linear gradient from 60% to 70% over 10 min and finally a linear gradient from 70% to 100% over 10 min. Chrysene and its metabolites were separated with a Nova-Pak C_{18} cartridge eluted with a 50-min linear gradient from 50% to 100% methanol in water. Benzanthracene and its metabolites were separated on Brownlee Spheri-5 ODS column (4.6 mm i.d. × 25 cm) eluted with 50% methanol/water for 5 min followed by a 50-min linear gradient from 50% to 80% and finally a linear gradient from 80% to 100% methanol over 20 min. Absolute quantitation was accomplished using G-^{3}H-labeled substrates and a Radiomatic Flow-ONE\β Model A500 radioactivity detector. An HP1050 multiwavelength UV detector was used to observe UV standards. Metabolites formed were identified by comparing retention times with those of cold synthetic standards added, and quantitation was achieved by electronically integrating the area under the radiochromatogram peaks.

Theoretical Calculations. Coordinates for each position of metabolism of B[*a*]P, chrysene, and phenanthrene were determined using the AM1 formalism (Dewar et al., 1985) provided within the InsightII molecular modeling package (Biosym Technologies, San Diego, CA). Geometries were optimized using the EF routine in MOPAC 6.0 (QCPE 455). The molecules were aligned and coordinates were determined using Insight II.

RESULTS AND DISCUSSION

Kinetic Analyses. Full kinetic analyses were performed on the effect of 7,8-benzoflavone on phenanthrene metabolism by vaccinia virus expressed CYP3A4. The kinetic parameters and statistics are given in Table 1. These results show that 7,8-benzoflavone dramatically increases V_{max} with no major effect on the K_m. These data alone would suggest that there are independent binding sites for substrate and activator, as has been suggested previously (Johnson et al., 1988; Schwab et al., 1988). However, we found that 7,8-benzoflavone is also a substrate for CYP3A4. The HPLC–UV chromatogram (Figure 2) shows two major metabolites, tentatively identified as *trans*-7,8-dihydrodiol (retention time = 13 min) and 5,6-epoxide (retention time = 21 min) by mass spectra and previously reported data (Andries et al., 1990). The turnover number for CYP3A4 metabolism of 7,8-benzoflavone, as determined by the rate of substrate consumption, is 22 min^{-1}. The kinetic analysis of 7,8-benzoflavone metabolism and the effect of phenanthrene on the kinetic parameters are given in Table 2. These data show that phenanthrene causes a decrease in V_{max} with little or no effect on K_m.

These results provide compelling evidence that both substrates (or substrate and activator) are simultaneously present in the active site. We are defining the active site as the area within the cytochrome P450 protein that can bind a substrate, giving it access to the active oxygen. Both compounds must have simultaneous access to the active oxygen, since neither the phenanthrene nor 7,8-benzoflavone can competitively inhibit the other. Increasing concentrations of a competitive inhibitor would cause the apparent K_m of the substrate to increase. Although large changes in K_m were not observed for phenanthrene–7,8-benzoflavone interactions, this is not true for all other substrates. Weibel et al. showed a dramatic change on K_m for the stimulation of benzo[*a*]pyrene metabolism by a very large benzoflavone derivative (Wiebel et al., 1974). Schwab eta l. also showed large changes in K_m for the metabolism of both estradiol and progesterone by rabbit and human microsomes (Schwab et al., 1988). In fact, it might be expected that the K_m of most substrates will be different in the presence of another. This would result in a saturable change in K_m of one substrate in the presence of increasing concentrations of the other substrate. The lack of appreciable changes in the K_m values for phenanthrene and 7,8-benzoflavone suggests that the presence of one substrate does not greatly change the binding affinity of the other. Although phenanthrene may be unusual in this respect, the relatively constant K_m values provide clear evidence that these two substrates do not displace each other from the active site. Thus, whereas previous papers suggested that multiple binding sites are involved (Johnson et al., 1988; Schwab et al., 1988), we have shown that the compounds occupying both binding sites have simultaneous access to the active oxygenating species.

Structure–Activity Relationships. To determine the structural features necessary for the activation of CYP3A4, a series of partial or modified structures of 7,8-benzoflavone (Figure 3) were tested for their ability to activate or inhibit the metabolism of phenanthrene, chrysene, and B[*a*]P. The turnover numbers and regioselectivity for the PAH substrates in the absence of activators/inhibitor are given in Table 3. Table 4 gives the relative amount of activation or inhibition for each position of the PAH molecules. Of 13 compounds tested, 6 activated the total metabolism of all three PAHs. Two of the compounds were partial activators, activating total chrysene metabolism but inhibiting phenanthrene and/or B[*a*]P metabolism. Five of the compounds inhibited the total metabolism of all three PAHs. As can be seen in Table 4, the presence of many of the activators greatly alters the regioselectivity of the enzyme. Although the data in Table 4 only represent an activator/inhibitor concentration of 200 μM, at

Table 3: Turnover Numbers and Regioselectivity for PAH Metabolism

phenanthrene metabolism (pmol min^{-1} nmol^{-1})				chrysene metabolism (pmol min^{-1} nmol^{-1})				benzo[*a*]pyrene metabolism (pmol min^{-1} nmol^{-1})			
metabolite	amt	region	amt	metabolite	amt	region	amt	metabolite	amt	region	amt
1,2-diol	23.1	1,2	27.8	1,2-diol	151.4	1,2	152.3	9,10-diol	4.5	9,10	10.1
3,4-diol	4.4	3,4	19.5	3,4-diol	170.6	3,4	171.8	4,5-diol	14.4	4,5	14.4
9,10-diol	170.6	9,10	195.5	5,6-diol	1.8	5,6	1.8	7,8-diol	14.5	7,8	14.5
1-OH	3.9			1-OH	0.9			3-OH	11.4	2,3	11.4
2-OH	0.6			3-OH	1.2			9-OH	5.6		
3-OH	4.7			total	326.6			1,6-dione	8.5		
4-OH	10.4							3,6-dione	14.2		
9-OH	24.9							6,12-dione	18.0		
total	242.7							total	91.2		

Table 4: Effect of Activators and Inhibitors on CYP3A4 Regioselectivity

	phenanthrene metabolism				chrysene metabolism[a]				benzo[*a*]pyrene metabolism[a]				
act/inhib[b]	3,4-	9,10-	1,2-	total	5,6-	3,4-	1,2-	total	9,10-	4,5-	7,8-	2,3-	total
none	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
7,8-BF	21.10	10.50	4.60	10.68	0.91	1.03	2.61	1.90	1.85	1.35	2.13	2.80	2.03
flavone	2.51	4.52	3.46	4.30	1.32	1.08	1.64	1.48	1.42	1.26	1.62	2.09	1.68
1MO2AcN	0.92	1.46	1.27	1.41	0.97	1.49	1.39	1.43	1.28	1.08	1.40	1.71	1.40
4-chr	1.39	1.52	1.35	1.49	1.20	1.55	1.70	1.50	1.41	1.11	1.37	1.74	1.42
2-AcN	1.48	1.60	1.41	1.59	1.04	1.16	1.27	1.21	1.34	1.22	1.56	1.72	1.44
α-tetralone	1.26	1.22	1.23	1.23	0.96	1.60	1.31	1.47	1.40	1.24	1.73	2.06	1.67
1-NMeOH	0.41	0.38	0.79	0.40	0.77	1.30	1.29	1.29	0.79	0.71	1.04	1.13	1.13
2-NMeOH	0.43	0.46	0.43	0.45	0.77	1.25	1.28	1.26	0.74	0.53	0.73	0.91	0.69
2-MeN	0.46	0.28	0.25	0.29	0.60	0.83	0.68	0.76	0.81	0.76	0.98	0.95	0.96
5,6-BF	0.42	0.63	0.80	0.63	0.60	0.11	0.19	0.15	0.81	0.77	0.81	1.40	0.97
1OH2AcN	0.44	0.28	0.27	0.29	0.76	0.79	0.80	0.79	0.91	0.64	0.83	0.39	0.75
1-MeN	0.44	0.27	0.20	0.27	0.67	0.56	0.57	0.56	0.79	0.71	1.04	1.13	1.13
2-MeNOH	0.37	0.42	0.34	0.42	0.50	0.64	0.66	0.65	0.46	0.49	0.51	0.40	0.45
substrate dimension													
x coord	2.15	0.00	3.17	3.52	0.84	3.41	4.21	4.62	3.42	1.44	4.20	4.34	4.62
y coord	1.61	2.10	0.28	2.09	1.96	1.40	0.55	2.04	1.41	2.88	0.54	0.65	2.52

[a] Amounts shown are relative to the absence of activator/inhibitor. [b] Abbreviations are given in Figure 3.

least four concentrations between 10 and 200 μM were used. All effects on regioselectivity were shown to be concentration dependent. The changes in regioselectivities are particularly striking for chrysene metabolism, for which metabolism in the 5,6-position is either inhibited or activated to a lesser degree relative to metabolsim in the 1,2-position.

The data in Table 4 show that, of the compounds tested, those with an appropriately placed ketone on the naphthalene ring (e.g., 2-acetonaphthone) activate all three substrates. The necessity of the proper orientation of the ketone is suggested by the inhibitory effect of 2-aceto-1-naphthol. This compound can form an intermolecular hydrogen bond when the keto group is rotated 180° from its position in the flavones (see Figure 3). Methylation of this compound to give 1-methoxy-2-acetonaphthone results in an activator. This apparent requirement for a particular orientation of a functional group suggests that specific interactions with the protein may be involved. Additionally, 5,6-benzoflavone is an inhibitor of CYP3A4, possibly due to a steric interaction with the different orientation of the naphthalene ring. This also implies that activation is due to specific activator–protein interactions.

Further analysis of the activator/inhibitor data indicates that the effective dimensions of the active site are altered during activation. For the three PAH substrates, the coordinates of each position of oxidation were calculated using the AM1 optimized geometries, with the origin at the center of mass, the *x*-axis along the longest dimension, and the *y*-axis in the plane of the PAH. Plots of $x_{max} - x$ (i.e., the distance from the edge of the molecule) vs the relative amount of activation for each position of metabolism are shown in Figure 4. As can be seen, both B[*a*]P and chrysene show decreased activation for positions in the middle of the molecule relative to positions on the narrow portion of the molecule. This was also seen with benzanthracene metabolism for which 7,8-benzoflavone activates 5,6-metabolism significantly less than the other positions (Figure 5). These data suggest that new steric constraints are introduced into the active site by the activator. These constraints allow the narrower portions of the substrate to access the active oxygen more easily than the wider parts of the substrate. This is most easily seen with chrysene (Table 4), for which most compounds inhibit metabolism of the 5,6-position while activating the 1,2- and 3,4-positions. Although there are changes in the regioselectivity for phenanthrene activation, the changes do not show a predictable pattern based on the geometry of the substrate. This suggests that the rotation of the substrate molecule in the active site is not further restricted by the presence of the activators, possibly because phenanthrene is a smaller substrate than chrysene and B[*a*]P. This is consistent with the small or insignificant changes in the K_m values for both phenanthrene and 7,8-benoflavone metabolism in the presence of the other molecule (see Tables 1 and 2).

This change in regioselectivity is also in accordance with data reported by Imaoka et al. (1992), who observed an increase in 2β-hydroxylation of testosterone by CYP3A4 in the presence of 7,8-benzoflavone, without an increase in 6β-hydroxylation. We find that 7,8-benzoflavone activates metabolism at the 2β-positions of testosterone while slightly decreasing 6β- and 15β-hydroxylation (data not shown).

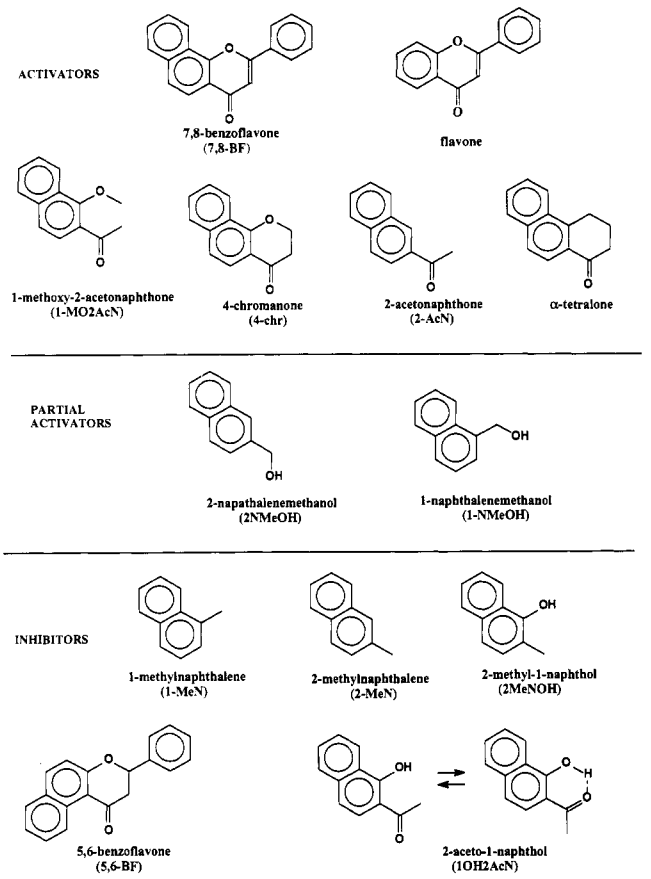

FIGURE 3: Compounds used in the structure–activity study on the activation and inhibition of the metabolism of polycyclic aromatic hydrocarbons.

Together, the above data suggest that (1) both substrates are simultaneously bound to the enzyme in positions that have access to the activated oxygen and (2) specific interactions between the activator and the protein result in a decreased effective size of the PAH binding site. The decrease in the V_{max} for 7,8-benzoflavone metabolism in the presence of phenanthrene (Table 2) suggests that there is competition for the active oxygen between the two substrates. However, this competition does not result in competitive displacement from the active site. The apparent requirement for an appropriately placed ketone functionality and the inability of 5,6-benzoflavone to activate the enzyme together suggest that there is a specific binding site within the active site for the activators. Since the effective dimensions of the PAH binding site decrease in the presence of an activator, the activator binding site may be adjacent to and partially overlapping with the binding site of the other substrate. This suggests that the active site of CYP3A4 is very large, which is consistent with its broad substrate selectivity and its ability to metabolize very large substrates such as cyclosporin and erythromycin (Smith & Jones, 1992).

Although the above data do not address the mechanism of activation, they do suggest that the activators influence the active site. An important determinant for the V_{max} of a P450 oxidation is likely to be the significance of uncoupling reactions, i.e., hydrogen peroxide release (Gillette et al., 1957; Nordblom & Coon, 1982) and excess water formation (Gorsky et al., 1982), during oxygen activation. Raag and Poulos (1991) have suggested that uncoupling to hydrogen peroxide may be associated with the presence of water molecules in the active site. We have suggested recently that large changes in V_{max} for some CYP2A1 mutants may be a result of an increase in

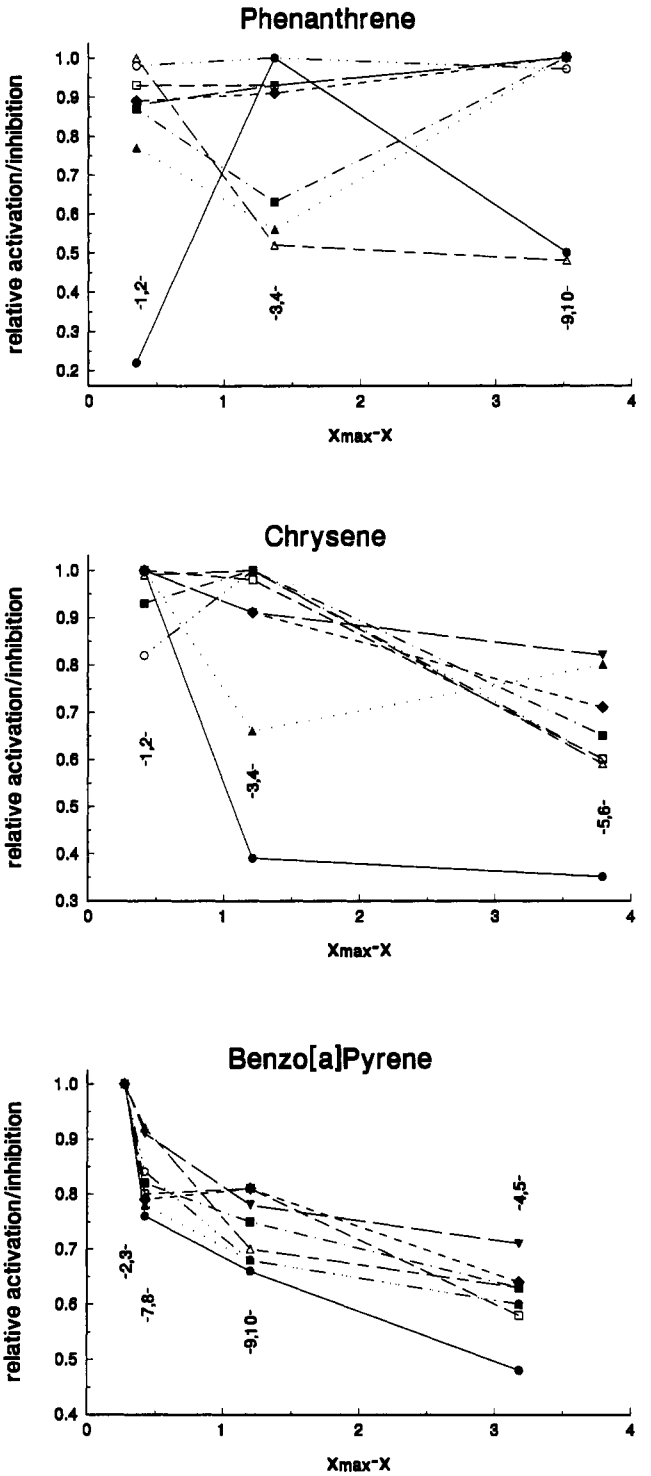

FIGURE 4: Plots of relative activation for each position vs the distance from the edge of the molecule. The coordinates for each position were calculated for the AM1 optimized geometries, with the origin at the center of mass, the x-axis along the longest dimension, and the y-axis in the plane of the PAH. The position activated the most is assigned a value of one and activation in other positions is scaled accordingly. (●) 7,8-Benzoflavone; (▲) flavone; (■) 1-methoxy-2-acetonaphthone; (◆) chromanone; (▼) 2-acetonaphthone; (○) α-tetralone; (△) 1-naphthalenemethanol; (□) 2-naphthalenemethanol.

hydrogen peroxide release, possibly due to increased hydration of the active site (Hanioka et al., 1992). As one possible mechanism for CYP3A4 activation, the activators may be excluding water molecules from the active site, preventing hydrogen peroxide release. A second possibility is that the activator, in some other way, provides an environment for

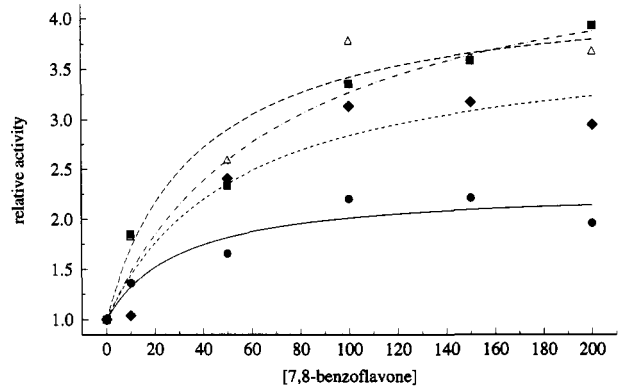

FIGURE 5: Concentration dependence and effect on regioselectivity for the activation of benzanthracene metabolism by 7,8-benzoflavone. (●) 5,6-Position, turnover number = 130 pmol min^{-1} $nmol^{-1}$; (△) 8,9-position, turnover number = 330 pmol min^{-1} $nmol^{-1}$; (■) 10,11-position, turnover number = 105 pmol min^{-1} $nmol^{-1}$; (◆) 3,4-position, turnover number = 18 pmol min^{-1} $nmol^{-1}$.

more efficient or rapid oxygen activation. This could also include interactions that result in more rapid reduction of the P450 by cytochrome P450 reductase. This would allow the bound substrate complexes to see a greater "concentration" of active oxygen species. Finally, these data do not preclude that the mechanism of activation involves the binding of the activator to a separate site on the cytochrome P450 molecule. However, since activation occurs in the same concentration range as metabolism of the activator, then either (1) the substrate and two activator molecules are simultaneously bound to the P450 or (2) activation occurs before the substrate and activator are simultaneously present in the active site.

Many human cytochrome P450s have now been cloned and expressed (Nebert et al., 1991; Gonzalez et al., 1991a,b; Clark & Waterman, 1991; Porter & Larson, 1991; Doehmer & Oesch, 1991; Crespi, 1991) and are likely to be used for future *in vitro–in vivo* correlations. These correlations will involve the determination of kinetic parameters for each human P450 involved in the metabolism of a particular compound. These parameters can be used to determine probable routes of metabolism and possible drug interactions at an early stage in the development of new drugs. The importance of CYP3A4 in the metabolism of many drugs increases our need to understand this activation phenomenon. It will be necessary to look for nonlinearities due to autoactivation at physiologically relevant concentrations. In addition, drug interactions due to CYP3A4 activation as well as inhibition may also need to be considered.

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