of configuration at the phosphorus center. These results are consistent with a single in-line displacement by an activated water molecule directly at the phosphorus center.

ACKNOWLEDGMENTS

We thank David P. Dumas for the phosphotriesterase used in this study. We also thank Dr. Joe Ribenspeis for the X-ray structural analysis.

SUPPLEMENTARY MATERIAL AVAILABLE

Atomic coordinates, bond lengths, bond angles, anisotropic displacement parameters, hydrogen atom coordinates, and a table of experimental results for the X-ray structure determination of *O*-ethyl phenylphosphonothioic acid (9 pages). Ordering information is given on any current masthead page.

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Evidence That the Catalytic Differences of Two Structurally Homologous Forms of Cytochrome P-450 Relate to Their Heme Environment

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ABSTRACT: Cytochromes P-450 PB_{3a} and PB_{3b}, which appear to be equivalent to forms *b* and *e* described by Ryan et al. [Ryan, D. E., Thomas, P. E., & Levin, W. (1982) *Arch. Biochem. Biophys. 216*, 272–288], have been shown to share 97% sequence homology [Suwa, Y., Mizukami, Y., Sogawa, K., & Fujii-Kuriyama, Y. (1985) *J. Biol. Chem. 260*, 7980–7984] yet exhibit an intriguing difference in enzymatic activity. Studies to establish the basis for this difference, including a development of the technique of surface-enhanced resonance Raman spectroscopy (SERRS), are reported. Studies on substrate binding, metabolism, and redox properties, as well as SERRS, indicate a significant difference in the heme environment of these two proteins. No significant difference in the interaction of the two proteins with P-450 reductase could be established. However, this interaction appeared sensitive to changes in ionic strength, suggesting ionic interactions are important in the functional coupling of these electron-transport components. A marked variation in the ratio of PB_{3a} to PB_{3b} activity in the metabolism of different substrates, which included a series of structurally similar resorufin analogues, provided further evidence that reductase coupling was not a critical factor. Therefore, the few amino acid differences observed between these proteins indicate sites that may be important in influencing the heme environment of these cytochrome P-450's.

Cytochrome P-450 dependent monooxygenases are a supergene family of proteins that catalyze the oxidation of lipophilic chemicals through the insertion of one atom of molecular oxygen into the substrate (Adesnik & Atchison, 1986; Wolf, 1986). Reactions catalyzed by cytochrome P-450 can be split into various major categories. These are hydroxylation reactions resulting from insertion of oxygen into C–H bonds, epoxidation reactions, and oxidations at nitrogen and sulfur atoms (Wislocki et al., 1980; Wolf, 1982). The products of the latter categories are often electrophilic and bind to DNA, causing mutations, toxicity, and cancer.

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A vast variety of compounds are metabolized by cytochrome P-450, and for many years the reason for the diverse substrate specificity was obscure. However, the multiplicity of the cytochrome P-450 system is now known to partially explain these early observations.

The relationship between cytochrome P-450 structure, substrate specificity, and function as a monooxygenase is still unclear. Two proteins of particular interest for studies on this theme are the phenobarbital-inducible enzymes [termed *b* and *e* by Ryan et al. (1982)], which are approximately 97% homologous at both the protein and DNA levels (Suwa et al., 1985). However, they exhibit significant differences in the metabolism of monooxygenase substrates (Ryan et al., 1982; Guengerich et al., 1982; Wood et al., 1983). The major difference in primary structure between the proteins lies in a hypervariable region in exon seven spanning approximately 50 amino acids, where there are six differences. There are also four amino acid differences in exon eight (Suwa et al., 1985). The catalytic differences between the two proteins could be explained by either an altered affinity or coupling with cytochrome P-450 reductase or an alteration in the heme environment of the cytochrome, which could alter both the substrate binding and the redox properties of the heme iron. In this paper we have investigated these possibilities in order to establish what the differences between these proteins are.

Materials and Methods

Male Wistar rats (200 g) treated with sodium phenobarbital (80 mg/kg ip on three consecutive days) were used. Washed liver microsomal samples were prepared as described previously (Wolf & Oesch, 1983). Cytochromes P-450 PB_{3a} and PB_{3b}[1] were prepared to high purity using the following procedure. Microsomes at a concentration of 10 mg/mL in 10 mM phosphate buffer, pH 7.7, were solubilized with sodium cholate (stock solution 10% w/v, 1 mg/mg protein) for 30 min at 4 °C. Glycerol was then added to give a final concentration of 20% w/v. The ionic strength of this mixture was then increased by the addition of KCl (final concentration 400 mM). The sample was then applied to a column of octyl-Sepharose 4B (approximately 500 mg of protein/100 mL packed column volume) previously equilibrated with 200 mM phosphate buffer, pH 7.7, containing 20% glycerol, 0.1 mM dithiothreitol, and 0.1 mM EDTA[2] (buffer A). The column was then washed with 200 mM buffer A, and 200 mM buffer A containing 0.4% cholate and a protein fraction containing P-450 PB_{3a} and PB_{3b} eluted when 100 mM buffer A containing 0.4% cholate and 0.1% Emulgen 911 was used. The fractions were checked on SDS gels and the purest samples pooled. The sample was then concentrated (10–20-fold), diluted (10-fold) with 20% glycerol, and applied to a DEAE column equilibrated with 5 mM buffer A (50 mL of DEAE/1000 nmol of P-450). This column was then washed with 5 mM buffer A and 5 mM buffer A containing 0.2% cholate, and the cytochrome eluted with this buffer plus 0.1% Emulgen 911. By use of this procedure, two clear bands separated on the column, the lower being P-450 PB_{3b}. This band eluted under these conditions. The second band was eluted by increasing the phosphate ion concentration to 15 mM. Both proteins were highly purified by this stage, the only contaminant being P-450 reductase. This was removed by passing the cytochromes through a 2′,5′-ADP–Sepharose column, the affinity column used for P-450 reductase purification. In addition to the reductase some of the cytochrome P-450 bound to the column. This was eluted by increasing the ionic strength of buffer A to 200 mM and by the addition of Emulgen 911 (0.1%). In order to remove all detectable Emulgen 911, the samples were diluted with 20% glycerol to a phosphate ion concentration of less than 20 mM and an Emulgen 911 concentration of less than 0.5% and applied to a hydroxylapatite and column equilibrated with 10 mM buffer A. Following extensive washing of the column, the cytochromes were eluted with 100 mM buffer A containing 0.2% cholate. Samples were then concentrated by ultrafiltration, using an Amicon PM30 membrane, to 10–20 nmol of P-450/mL, dialyzed against 10 mM buffer A overnight, and stored at −40 °C. The specific contents of P-450 PB_{3a} and PB_{3b} were 15.2 and 16.6 nmol/mg of protein, respectively. The 418:280-nm absorbance ratio was greater than 1.8:1, and there was no spectroscopically detectable Emulgen 911 present. Cytochrome P-450 reductase was eluted from the octyl-Sepharose 4B column with 100 mM buffer A containing 0.4% cholate and 0.5% Emulgen 911. This protein was purified to apparent homogeneity by using 2′,5′ADP–Sepharose 4B and the method of Yasukochi and Masters (1976). Final preparations were dialyzed against 10 mM buffer A and stored at approximately 10^5 units/mL.

Cytochrome P-450 concentration was determined from the ferric absorption peak at 418 nm by using an extinction coefficient of 106 mM^{-1} cm^{-1} (Wolf et al., 1980). Cytochrome P-450 reductase was assayed by measuring the NADPH-dependent reduction of cytochrome *c*. One unit of activity is defined as the amount of reductase required to reduce 1 nmol of cytochrome *c*/min at 30 °C. Protein determinations were by the method of Lowry et al. (1951).

The reconstitution of monooxygenase activity was carried out by two methods, one of which involved mixing concentrated P-450 and reductase solutions (10^4 units/nmol of P-450) and dilauroylphosphatidylcholine (500 μg/nmol of P-450) as described previously (Wolf et al., 1981). In the other method, cytochrome P-450 and reductase were mixed in the absence of added lipid (up to 10^4 units/nmol of P-450) directly in the incubation mixture (1–3 mL). The metabolism of the following substrates was measured. Benzphetamine was measured by formaldehyde production (Nash, 1953), resorufin analogue O-deethylation by the method of Burke and Mayer (1974) and Wolf et al. (1986), and 7-ethoxycoumarin deethylation by the method of Ullrich and Weber (1972).

Peptide maps of the purified proteins using either papain, chymotrypsin, or *Staphylococcus* V8 protease were determined by using the method of Cleveland et al. (1977) as modified by Slaughter et al. (1981).

Surface-Enhanced Resonance Raman Spectroscopy (SERRS). The heme environment, particularly the spin state, of PB_{3a} and PB_{3b} was probed by using the vibrational technique of surface-enhanced resonance Raman spectroscopy (SERRS). This technique combines the inherent selectivity of resonance Raman spectroscopy with the large enhancement obtained from a species, for example, protein, adsorbed at a metal surface. SERR spectra of other proteins on silver colloids and a silver electrode have been reported (Smulevich & Spiro, 1985; Hildebrandt & Stockburger, 1986; de Groot & Hester, 1987; Cotton et al., 1980).

The colloid used in this study was prepared from the reduction of silver nitrate by sodium citrate in aqueous solution following the method of Lee and Meisel (1982) to obtain a

[1] According to recent nomenclature recommendations (Nebert et al., 1987), PB_{3a} and PB_{3b} are part of the rat P-450 family termed family IIB.

[2] Abbreviations: EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl; ADP, adenosine 5′-diphosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

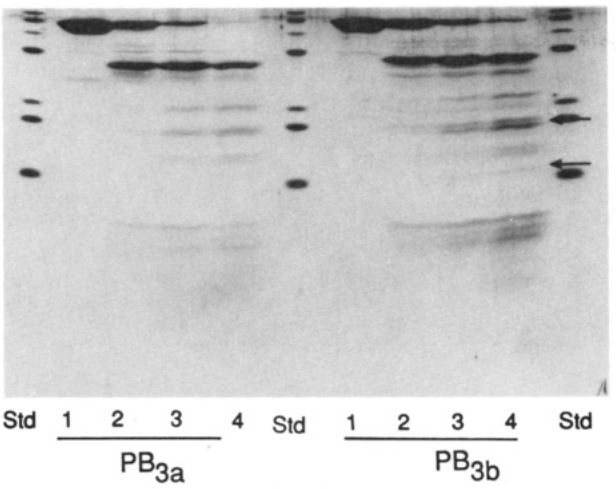

FIGURE 1: Peptide map of cytochrome P-450's PB_{3a} and PB_{3b} using *Staphylococcus* V8 protease. Cytochrome samples (0.5 mg) in 250 μL of 10 mM phosphate buffer, pH 7.4, were mixed with V8 protease (250 μg/mL). Samples were incubated at 37 °C and aliquots removed at 0, 8, 25, and 60 min (lanes 1, 2, 3, and 4, respectively). The reaction was quenced by boiling with SDS and mercaptoethanol. Ten micrograms of protein was run per track. Arrows represent position of differences between PB_{3a} and PB_{3b}.

very finely dispersed sol whose particle size is less than the wavelength of visible light (100–300-Å particle diameter). The cytochrome P-450 sample was added to the sol to give a final concentration of 10^{-7}M. Denaturation was a problem unless careful control was exercised over sol pH. A modification of the technique of Kelly et al. (1987) was used. Following the acidification of the sol with 20 μL of fresh ascorbic acid (1% w/v), neutralization was achieved by the addition of 100 μL of buffer A (without glycerol)/3.0 mL of solution. The final pH of the sol plus adsorbed protein was 7.5–8.0. SERR spectra were obtained from the adsorbed protein by using laser excitation wavelengths of 457.9 and 514.5 nm.

RESULTS

In this study it was first important to establish PB_{3a} and PB_{3b} were closely related proteins equivalent to forms *b* and *e*. Cytochromes PB_{3a} and PB_{3b} were purified with high yield to high purity by using the method described. They had different mobilities on SDS gels and had apparent molecular weights of 53 300 and 54 700 for PB_{3a} and PB_{3b}, respectively. The proteins' NH_2-terminal sequences were identical over the 30 amino acids tested, and the sequence obtained was the same as that reported for P-450b and P-450e (Suwa et al., 1985). The peptide maps of the two proteins were very similar. No differences were observed with either papain or chymotrypsin; however, some minor differences were observed when V8 protease was used (Figure 1). This is also in agreement with the high degree of structural homology. The absolute ferric cytochrome P-450 spectra were, however, different, PB_{3b} having a shoulder in the 395-nm region and a peak at 648 nm indicating that a proportion of the cytochrome was in the high-spin configuration. All the above data are consistent with PB_{3a} and PB_{3b} being equivalent to forms *b* and *e* described by Ryan et al. (1982) and indicate that the differences observed represent intrinsic differences between the proteins and are not due to contamination with external perturbants.

The increased proportion of PB_{3b} in the high-spin state provides evidence that the heme environment is different between these proteins. This was exemplified both by the rate of reduction of the enzymes by sodium dithionite and in the SERR spectra. Interestingly, PB_{3b} could be reduced at a 3.8-fold faster rate by dithionite than PB_{3a}, indicating a significant difference in the redox properties of the two proteins. At a cytochrome concentration of 2 nmol/mL the initial reduction rates were 0.06 and 0.25 nmol/min for PB_{3b} and PB_{3a}, respectively. The SERR spectra are shown in Figure 2. In the high-frequency region (Figure 2a), the well-known spin-

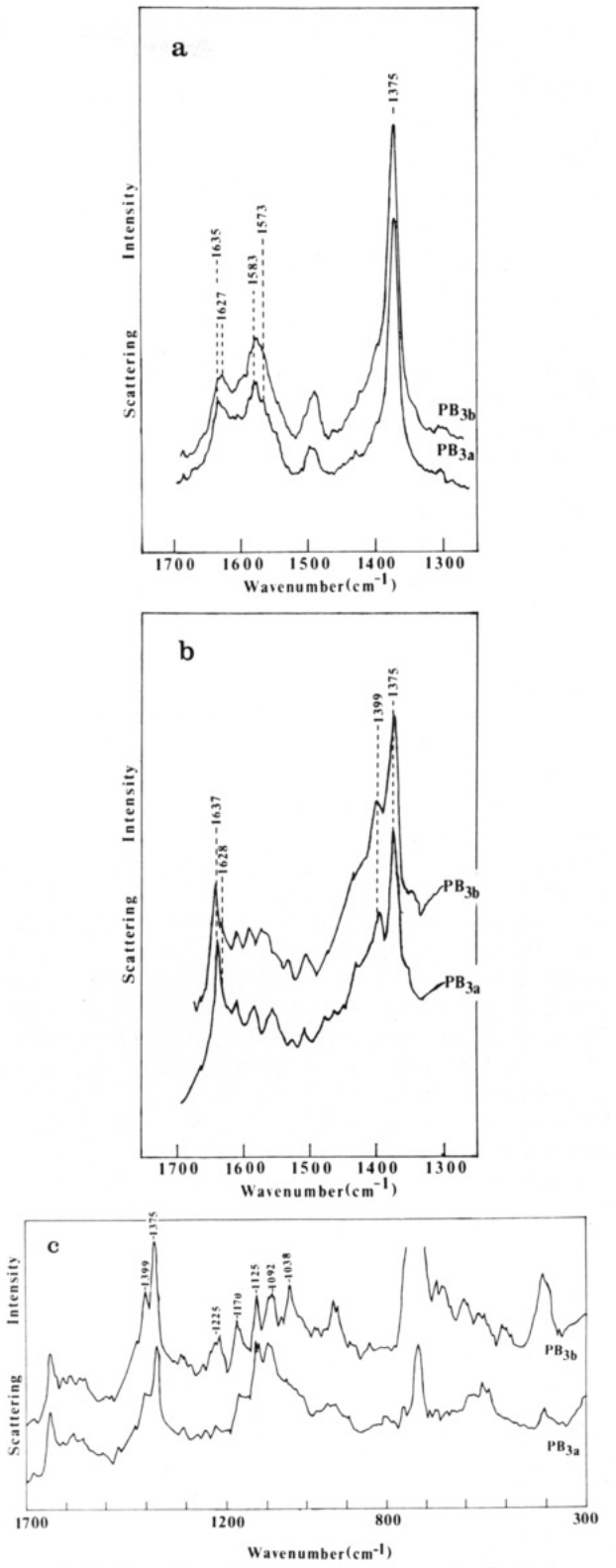

FIGURE 2: Surface-enhanced resonance Raman spectra of cytochromes P-450 PB_{3a} and PB_{3b}. Spectra obtained by using an excitation wavelength of (a) 457.9, (b) 514.5, and (c) 514.5 nm. All spectra were recorded by using 100-mW power and 5-cm^{-1} slit width. A 1-cm fluorometer cell cooled to approximately 5 °C by flushing the cell wall with cold nitrogen was used. In each case the final concentration of P-450 used was approximately 10^{-7} M.

state marker bands ν_{10} (B_{1g}) and ν_{19} (A_{2g}), which are influenced by the C_aC_m stretching modes[3] of the porphyrin ring, were observed (Kitagawa et al., 1978; Abe et al., 1978). ν_{10} is located between 1615 and 1640 cm^{-1} and appeared as a single band at 1635 cm^{-1} for PB_{3a} and at the same frequency with a shoulder at 1627 cm^{-1} for PB_{3b}. This reflects the greater high-spin content of PB_{3b}, as the lower frequency band at 1627 cm^{-1} is associated with high-spin heme, whereas the 1635-cm^{-1} band is attributed to low-spin heme. Consistent behavior was observed for ν_{19}, for which the high-spin band was at 1573 cm^{-1} and the low-spin band at 1583 cm^{-1}.

ν_4 (A_{1g}), the oxidation state marker (Kitagawa et al., 1978; Abe et al., 1978) was at 1375 cm^{-1}, confirming that each protein is as expected in the ferric state.

With use of 514.5-nm excitation (Figure 2b) the ν_{10} spin-state marker band at 1637 cm^{-1}, characteristic of low-spin heme, was greatly enhanced over all the other bands in the 1500–1700-cm^{-1} region. The high-spin component was not enhanced to the same degree but was evident for PB_{3b} and absent from the PB_{3a} spectrum (shoulder at 1628 cm^{-1}). An intense band at 1399 cm^{-1} was observed adjacent to the 1375-cm^{-1} band. There is a large difference in the intensity of this band between PB_{3b} and PB_{3a}. The 1399-cm^{-1} band has been previously assigned (Kitagawa et al., 1978; Abe et al., 1978) to two related and almost degenerate modes, ν_{20} (A_{2g}) and ν_{29} (B_{2g}), neither of which have been explicitly mentioned as spin-state-sensitive bands in previous studies. This band therefore may be sensitive to different factors in the heme environment.

The high-energy region (1300–1700 cm^{-1}) tends to be influenced by the nature of the coordinating axial ligands and coordination number with concomitant changes (expansions/contractions) of the heme core, rather than other protein-induced differences on the heme environment (Spiro et al., 1979). Previous work on structural correlations (Choi et al., 1982b) between different proteins has revealed that the 600–1300-cm^{-1} region yields bands susceptible to heme–protein interactions. Inspection of the PB_{3a} and PB_{3b} spectra in this region revealed significant differences at 514.5-nm excitation (Figure 2c). Porphyrin modes involving C_b–S (Choi et al., 1982a) stretching vibrations and those coupling with peripheral vinyl C–H bending vibrations are affected by the protein influence on the heme. Bands attributable to such modes at 1225, 1170, 1125, 1092, and 1038 cm^{-1} appeared more intense for PB_{3b} than for PB_{3a}. Unfortunately, the bands at 1225, 1092, and 1038 cm^{-1} coincided with those observed from SER scattering of phosphate alone adsorbed on the sol. This also includes the band in the 730-cm^{-1} region. In spite of this, it appears that the difference in protein structure plays a role in the intensity difference observed.

Interestingly, ν_{29} and ν_{20} are known to couple strongly with the vinyl inplane-scissors C–H bending vibration, and thus the stronger intensity of this mode in PB_{3b} may be attributable to the same effect observed with these lower energy vibrations. These modes involve movement on the periphery of the porphyrin ring and are sensitive to changes in the protein environment. Differences in intensity but not frequency suggest quite subtle alterations in the heme pocket, between PB_{3a} and PB_{3b}, reflecting the small differences in the amino acid sequence around the heme site that could induce subtle electronic changes in the heme.

[3] The nomenclature for assigning vibrations to particular parts of the heme ring is not consistent in the literature. We choose to use that of Choi et al. (1982), which is that carbon atoms of the pyrrole ring are labeled C_a (inner) and C_b (outer), the carbon in the macrocycle bridge is C_m, and S refers to the side chain. Kitagawa et al. (1978) and Abe et al. (1978) describe C_aC_m as $C_\alpha C_m$, whereas we use C_α as the carbon of the vinyl side chain attached to the porphyrin ring.

Table I: Cyclohexane- and Benzphetamine-Induced Spin-State Change for PB_{3a} and PB_{3b}[a]

	PB_{3a}		PB_{3b}	
	K_s	ΔA_{max}	K_s	ΔA_{max}
cyclohexane, 25 mM	0.17	8.0	0.50	44.4
cyclohexane, 200 mM	0.18	10.0	ND	ND
benzphetamine, 25 mM	0.33	9.1	0.03	38.4
benzphetamine, 200 mM	0.15	18.0	ND	ND

[a] Spin change was determined by difference spectroscopy at 395 and 420 nm. Assays were carried out in either 25 or 200 mM phosphate buffer, pH 7.4. K_s (mM) and ΔA_{max} ($A_{394-420nm}$) values were obtained from double-reciprocal plots. ND = not determined.

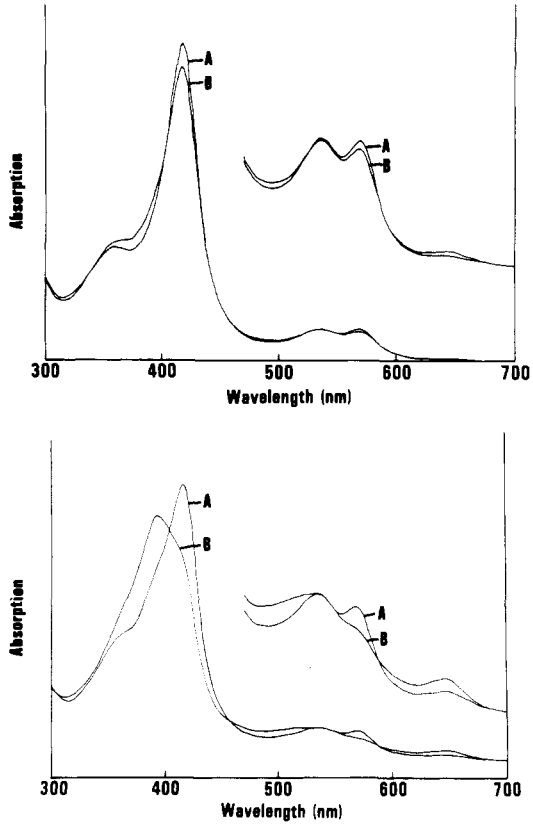

FIGURE 3: Effect of benzphetamine on the spin state of cytochromes PB_{3a} and PB_{3b}. (A) Ferric cytochrome spectrum. (B) Spectrum obtained in the presence of 1 mM benzphetamine. The upper spectrum is PB_{3a} and the lower PB_{3b}.

In addition to the above differences in the heme environment of PB_{3a} and PB_{3b}, a significant difference in the spin change associated with substrate binding was also observed (Table I). On addition of either cyclohexane or benzphetamine to the cytochromes, much smaller spin-state changes were observed with PB_{3a} relative to those with PB_{3b}. Interestingly, on addition of benzphetamine or cyclohexane (1 mM) to PB_{3b}, almost all of the protein was converted to the high-spin configuration (Figure 3). This difference was also reflected in the binding parameters obtained. The ΔA_{max} for PB_{3b} was 4–5-fold higher than that for PB_{3a} (Table I). The binding constants (K_s values) for the proteins were very similar for cyclohexane, but PB_{3b} had a 10-fold higher affinity in benzphetamine binding. The effect of high ionic strength (i.e., 200 mM phosphate buffer) was investigated because of the effects on reconstituted monooxygenase activity (see below). Increasing the ionic strength significantly increased the ΔA_{max} (2-fold) obtained with PB_{3a} when benzphetamine was used as substrate. The change ob-

Table II: Activity of PB_{3a} and PB_{3b} toward Various Monooxygenase Substrates[a]

	activity [nmol min^{-1} (nmol of P-450)$^{-1}$]												
			resorufin analogues										
	7-ethoxy-coumarin	benzphetamine	O	C_1	C_2	C_3	C_4	C_5	C_6	benz	cyclo	isoprop	isobut
PB_{3a}	2.06	14.84	1.06	0.03	0.05	0.13	0.49	4.91	1.04	9.1	0.12	0.08	0.09
PB_{3b}	0.44	4.00	0.04	0	0.03	0.02	0.0	0.04	0.06	0.16	0.01	0.02	0.02

[a] Reconstituted monooxygenase assays were carried out by preincubation of the purified components, including dilauroylphosphatidylcholine, before the addition of 25 mM phosphate buffer, pH 7.4. Other details are given under Materials and Methods and in the legend to Figure 5. O–C_6 represents the length of the alkoxy side chain on the resorufin molecule. Other analogues were the benzyl, cyclohexyl, isopropyl, and isobutyl ethers.

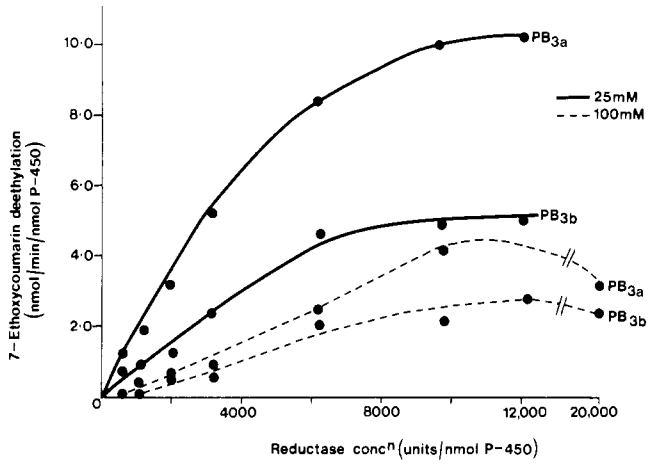

FIGURE 4: Dependence of PB_{3a} and PB_{3b} mediated 7-ethoxycoumarin metabolism on cytochrome P-450 reductase concentration. Cytochrome P-450 (0.1 nmol) was mixed with dilauroylphosphatidylcholine (50 μg) and various concentrations of P-450 reductase in a final volume of 30 μL. Following incubation for 15 min at 37 °C, phosphate buffer (pH 7.4) (25 or 100 mM) containing the substrate (0.1 mM) and NADPH (1 mM) was added. Reactions were then followed at 37 °C according to the method of Ullrich and Weber (1972).

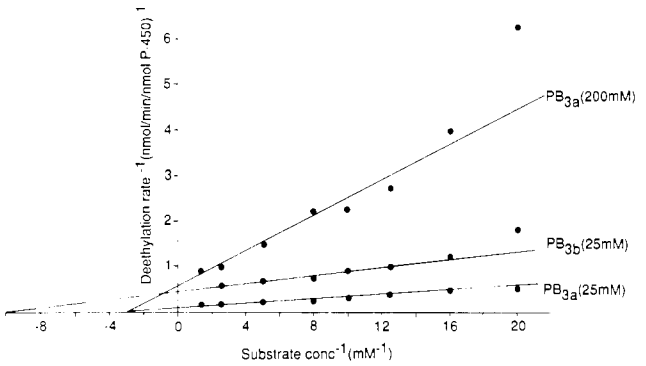

FIGURE 5: Effect of ionic strength on PB_{3a}-mediated metabolism of 7-ethoxycoumarin. Reactions were carried out at either 25 or 200 mM as described in the legend to Figure 5.

tained with cyclohexane, however, was marginal, and the binding constants remained essentially unchanged.

Both P-450's were active in the metabolism of a variety of cytochrome P-450 substrates including 7-ethoxycoumarin, benzphetamine, and a series of resorufin analogues (Table II). In agreement with the general observations of others (Ryan et al., 1982; Guengerich et al., 1982; Wood et al., 1983), PB_{3a} had higher activities than PB_{3b}. The rate difference, however, was not constant and ranged from approximately 3-fold in the case of benzphetamine to over 100-fold in the case of some of the resorufin analogues. This was mainly due to the variation in the PB_{3a} activity, the PB_{3b} activity being uniformly low. It is worthy of note that marked differences in the activity of PB_{3a} toward the resorufin substrates were measured, the pentoxy and benzyloxy derivatives being the two best substrates. This is in agreement with the marked increase in this activity on treatment of animals with phenobarbital.

The experiments summarized in Table II were carried out at saturating cytochrome P-450 reductase. In order to establish whether PB_{3a} and PB_{3b} have different affinities for the reductase, the amount of reductase required to saturate the enzymes was determined (Figure 4). Maximum rates of 7-ethoxycoumarin deethylation were obtained with both enzymes at approximately 10 000 units/nmol of P-450. Fifty percent saturation was obtained at approximately 3000 units/nmol of P-450. The same curves were generated whether the reaction components were combined as concentrated solutions in the presence of phospholipid and preincubated before the addition of 2 mL of assay buffer or whether the cytochrome and reductase were added directly to the assay buffer immediately before the addition of NADPH (not shown). This indicates that under these conditions both enzymes equilibrated with the reductase at equivalent rates and that this was a rapid interaction even in free solution.

At high ionic strength the activity of both enzymes was significantly reduced relative to assays in 25 mM phosphate buffer. Although both enzymes exhibited sensitivity to ionic strength changes, a difference between PB_{3a} and PB_{3b} in this effect was observed. PB_{3a} was initially activated by increasing the phosphate ion concentration, but as the ionic strength increased, the activity reduced sharply. At 25 and 60 mM phosphate the activity was 4-folder higher than the activity measured at 20 mM. In fact, at the higher ionic strength there was no longer a significant difference in activity between PB_{3a} and PB_{3b}. PB_{3b} activity was also affected by phosphate ion concentration but to a lesser degree. A kinetic plot for 7-ethoxycoumarin metabolism at 25 and 200 mM for PB_{3a} compared to that for PB_{3b} at 25 mM is shown in Figure 5. No change in the K_m for PB_{3a} was measured. However, an 8-fold change in V_{max} was observed. It appeared that increasing the ionic strength was altering the coupling of P-450 with PB_{3a} and PB_{3b} with the reductase. This possibility was investigated by mixing PB_{3a} and PB_{3b} with the reductase at low ionic strength (5 mM) and applying the mixture to a column of 2′,5′ ADP–Sepharose, the affinity column that binds P-450 reductase. The reductase together with the cytochromes remained bound to the column. The sequential elution of the components was then attempted by a stepwise increase in the ionic strength (Figure 6). In the experiment shown, both cytochromes could be dissociated from the reductase by high phosphate ion concentration and were eluted together. In a further experiment using 25 mM step increases in phosphate concentration, both cytochromes started eluting at 75 mM. In all cases both PB_{3a} and PB_{3b} eluted concomitantly.

Discussion

On the basis of molecular weight, peptide maps, spectral properties, properties during purification, NH_2-terminal sequence analysis, and inducibility (not shown), PB_{3a} and PB_{3b} are distinguishable proteins that are part of the same P-450 subfamily. The properties of these enzymes are consistent with

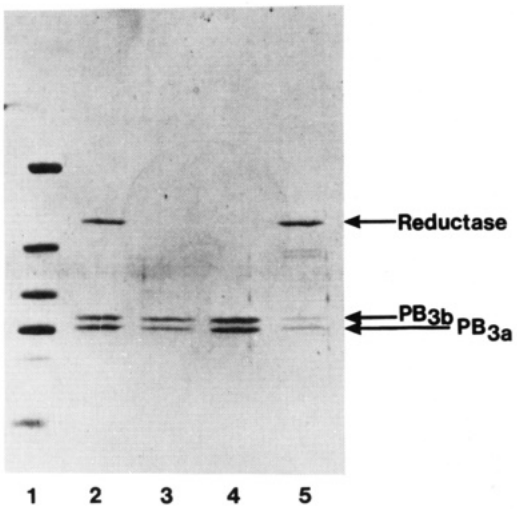

FIGURE 6: Effect of ionic strength on the binding of PB_{3a} and PB_{3b} to cytochrome P-450 reductase. PB_{3a} and PB_{3b} (5 nmol) were mixed with 15000 units of P-450 reductase (10 mM phosphate, pH 7.4) and applied to a column of 2′,5′-ADP–Sepharose 4B. Samples were sequentially eluted from the column, concentrated, and run on SDS–PAGE. (Lane 1) Standards: phosphorylase *a*, serum albumin, catalase, glutamate dehydrogenase, fumarase, and aldolase, 92, 67, 60, 53, 49, and 40 kDa, respectively. (Lane 2) Material loaded onto column. (Lane 3) Sample eluted with 200 mM buffer A. (Lane 4) Sample eluted with 200 mM buffer A + 0.2% cholate. (Lane 5) Sample eluted with the previous buffer containing $NADP^+$ (2 mM).

PB_{3a} being equivalent to form *b* and PB_{3b} being equivalent to form *e* as described by Ryan et al. (1982). The large difference in activity of these proteins toward a variety of substrates would appear to be at least partially due to differences in the heme environment. This conclusion is based on the significant differences in the Raman spectra, the degree of spin conversion on the addition of the monooxygenase substrates, and also on the redox properties of the two enzymes. In addition to the data presented here, Waxman and Walsh (1982) have reported differences between PB_{3a} and PB_{3b} in their sensitivity to inhibition by the heme–ligand metyrapone.

The use of surface-enhanced resonance Raman spectroscopy represents a novel and powerful technique for probing the heme environment of cytochrome P-450. The positions of the resonance Raman bands obtained by using the SERRS technique were consistent with the spectra published for $P\text{-}450_{cam}$ obtained by using conventional resonance Raman spectroscopy (Champion et al., 1978), although changes in band intensity were observed as expected. This, together with the finding that the spectra were very different from those of cytochrome P-420, a denatured form of the enzyme (Kelly et al., 1987), and the fact that up to 70% conversion of PB_{3b} to the high-spin state could be measured in the SERR spectrum on addition of the substrate benzphetamine (not shown) indicate that the P-450 is in its active form when absorbed onto the silver sol.

It was intriguing that PB_{3b} could be almost completely converted to the high-spin configuration by substrate addition. Substrate-induced spin-state changes have been directly equated with enzyme activity. This was clearly not the case here, where a much lower proportion of the enzymatically more active PB_{3a} was converted to the high-spin state on substrate addition. The difference in spin equilibrium between PB_{3a} and PB_{3b} may account for the difference in the rate of reduction of the proteins by sodium dithionite (Backes et al., 1985). However, neither spin state nor the redox potential for the addition of the first electron would appear to be important in determining substrate turnover number. The possibility that the differences between PB_{3a} and PB_{3b} are related to the interaction of the cytochrome with P-450 reductase could not be substantiated. In all the parameters studied the interaction of the two P-450's with the reductase appeared similar. On mixing the enzymes in free solution, both PB_{3a} and PB_{3b} interact well with P-450 reductase to give an enzymatically active complex. This was exemplified by the finding that when this mixture was applied to the P-450 reductase affinity column, both PB_{3a} and PB_{3b} remained bound to the column. The strength of this binding was similar for both proteins (Figure 6). This finding was substantiated by the observation that the same amount of reductase was required to saturate both P-450's. The cytochrome–reductase complex could be dissociated both physically and enzymatically by increasing the ionic strength of the phosphate buffer used. Although it is possible that other bonding interactions are also involved in the coupling of these two components, these data indicate that ionic interactions may be important in the functional interaction of these proteins. The reason why PB_{3a} was significantly activated at lower ionic strengths is unclear and is not necessarily related to reductase coupling. High ionic strength did appear to have some effects on the heme environment of PB_{3a}, as seen in the increase in A_{max} for benzphetamine binding.

The higher enzymic activity of PB_{3a} vs PB_{3b} appears related to differences in the heme environment. Cytochromes P-450b and P-450e (PB_{3a} and PB_{3b}) are highly homologous proteins with 97% sequence homology in the amino acid sequence (Suwa et al., 1985). It is therefore intriguing that these amino acid differences could give rise to such large differences in substrate metabolism. The finding that difference in turnover number varied between substrates (from 1.6- to 122-fold) further supports the evidence that the differences between the two proteins are not related to a reductase coupling, as a change in reductase coupling would be expected to change the turnover rate by an equal proportion for all substrates if this was a rate-determining step in the reaction.

Which of the amino acid differences between PB_{3a} and PB_{3b} are responsible for the different properties of the proteins remains unclear. In a recent paper by Poulos et al. (1987) the X-ray structure of cytochrome $P\text{-}450_{cam}$ from *Pseudomonas putida* has been used to provide an indication of the spatial position of the amino acid differences of P-450b and P-450e. In relation to this work certain conclusions of these workers are worthy of note. The difference at amino acid 303 (PB_{3a} = Ser, PB_{3b} = Gly) may be significant, as it is adjacent to a threonine 302, which is highly conserved between P-450's and involved in oxygen binding. Also, of the 14 amino acid differences, 8 are in the helix-poor domain, which has been proposed to be important in determining substrate specificity. The helix-rich domain has been associated with the formation of the heme pocket (Poulos et al., 1987). Precise conclusions as to the implications of the substitutions cannot be made at this time.

We hope to develop the SERRS technique and use site-directed mutagenesis in order to clarify this question.

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