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Posttranslational Modification of Hepatic Cytochrome P-450. Phosphorylation of Phenobarbital-Inducible P-450 Forms PB-4 (IIB1) and PB-5 (IIB2) in Isolated Rat Hepatocytes and in Vivo†

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ABSTRACT: Phosphorylation of hepatic cytochrome P-450 was studied in isolated hepatocytes incubated in the presence of agents known to stimulate protein kinase activity. Incubation of hepatocytes isolated from phenobarbital-induced adult male rats with [^{32}P]orthophosphate in the presence of N^6,$O^{2'}$-dibutyryl-cAMP (diBtcAMP) or glucagon resulted in the phosphorylation of microsomal proteins that are immunoprecipitable by polyclonal antibodies raised to the phenobarbital-inducible P-450 form PB-4 (P-450 gene IIB1). Little or no phosphorylation of these proteins was observed in the absence of diBtcAMP or glucagon or in the presence of activators of Ca^{2+}-dependent protein kinases. Two-dimensional gel electrophoresis revealed that these ^{32}P-labeled microsomal proteins consist of a mixture of P-450 PB-4 and the closely related P-450 PB-5 (gene IIB2), both of which exhibited heterogeneity in the isoelectric focusing dimension. Phosphorylation of both P-450 forms was markedly enhanced by diBtcAMP at concentrations as low as 5 μM. In contrast, little or no phosphorylation of P-450 forms reactive with antibodies to P-450 PB-1 (gene IIC6), P-450 2c (gene IIC11), or P-450 PB-2a (gene IIIA1) was detected in the isolated hepatocytes under these incubation conditions. Phosphoamino acid analysis of the ^{32}P-labeled P-450 PB-4 + PB-5 immunoprecipitate revealed that these P-450s are phosphorylated on serine in the isolated hepatocytes. Peptide mapping indicated that the site of phosphorylation in hepatocytes is indistinguishable from the site utilized by cAMP-dependent protein kinase in vitro, which was previously identified as serine-128 for the related rabbit protein P-450 LM2. Phosphorylation of P-450s PB-4 and PB-5 (as well as immunoreactive P-450 forms PB-1 and PB-2a) was also shown to occur in the liver in vivo in a process that is facilitated by, but not obligatorily dependent on, administration of diBtcAMP + theophylline to stimulate cAMP-dependent protein kinase. In vitro analyses revealed that phosphorylation of P-450 PB-4 leads to a loss of monooxygenase activity, suggesting that the posttranslational modification of this P-450 enzyme by cAMP-dependent protein kinase may play a role in the modulation of P-450-dependent monooxygenase activity in vivo.

Cytochrome P-450 (P-450)[1] is comprised of a superfamily of heme protein monooxygenase enzymes that catalyze oxidative metabolism of a broad range of endogenous and exogenous compounds including many steroids, drugs, and chemical carcinogens. P-450 expression in liver and other tissues is modulated by exposure to foreign compounds, many of which can differentially induce and suppress the levels of individual P-450 enzymes (Guengerich, 1987). Many P-450-dependent activities are also regulated by gonadal and pituitary hormones, which play an important role in the sex-dependent developmental regulation of P-450 gene expression in rodent liver and kidney (Skett, 1987; Waxman, 1988). Hormonal regulation of P-450 enzyme activity is also suggested by studies on the modulation of P-450-dependent mono-

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[1] Abbreviations: P-450, cytochrome P-450; SDS, sodium dodecyl sulfate; cAMP, cyclic AMP; diBtcAMP, N^6,$O^{2'}$-dibutyryl-cAMP; the terms P-450 form and P-450 enzyme are used interchangeably in this report.

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oxygenase activity in liver by the cAMP analogue diBtcAMP (Weiner et al., 1972; Ross et al., 1973; Banhegyi et al., 1988; Berry & Skett, 1988) as well as by hormones known to elevate cAMP levels within cells (Botham et al., 1984). The rapid response of hepatic P-450 activities to elevated cAMP levels suggests that the effects of these hormones may be mediated by posttranslational events, such as protein phosphorylation, rather than through alterations in P-450 gene expression.

Protein phosphorylation has been proposed to play a key role in the short-term regulation of several P-450 enzymes, in particular cholesterol 7α-hydroxylase and other monooxygenase enzymes of bile acid biosynthesis (Sangvhi et al., 1981; Botham & Boyd, 1984; Holsztynska & Waxman, 1987). This proposal is largely based on indirect evidence, for instance, the observation that bile acid biosynthesis is generally stimulated by diBtcAMP in isolated hepatocytes and the finding that microsomal cholesterol 7α-hydroxylase activity can be modulated by conditions likely to influence protein phosphorylation/dephosphorylation states. That P-450 enzymes can, in principle, be regulated by phosphorylation is supported by the observation that several of these cytochromes serve as substrates for one or more purified protein kinases in vitro (Pyerin et al., 1987). Best studied is the phosphorylation of purified rabbit liver P-450 form LM2 (P-450 gene IIB4) by cAMP-dependent protein kinase, which results in the incorporation of 0.2–0.6 mol of phosphate/mol of P-450 and a 30–60% decrease in enyzme activity (Pyerin et al., 1984; Jansson et al., 1987). This activity loss has been associated with conversion of the cytochrome to the inactive P-420 form (Taniguchi et al., 1985). Phosphorylation of this cytochrome occurs on serine-128, which is located within the cAMP-dependent protein kinase recognition sequence Arg-Arg-Phe-Ser, and has been proposed as a possible step in the pathway of P-450 degradation (Muller et al., 1985). Direct evidence for the occurrence of P-450 phosphorylation in whole cells is lacking, however, raising questions about the relevance of these in vitro phosphorylation reactions. The current study was therefore undertaken to directly evaluate whether phosphorylation of one or more hepatic P-450 enzymes does occur in hepatocytes and whether it might provide a mechanism for modulation of monooxygenase activity in vivo.

Materials and Methods

Animal Treatment. Male Fischer 344 rats (200–300 g) were purchased from Harlan Sprague-Dawley, Inc., and were fed commercial rat chow ad libitum. Phenobarbital dissolved in saline and adjusted to pH 8.5–9 (20 mg/mL) was administered by four daily intraperitoneal injections (80 mg/kg). Twenty-four hours following the final injection, rats were used directly for in vivo phosphorylation experiments or were anesthetized with ether and prepared for hepatocyte isolation.

In Vivo Administration of [^{32}P]Orthophosphate and Isolation of Phosphorylated Rat Liver Microsomes. Uninduced and phenobarbital-induced adult male rats (240–250 g) were administered a total of three intraperitoneal injections of diBtcAMP + theophylline (2 mg of each compound/100 g body weight per injection and suspended in 0.5 mL of 0.9% NaCl) at 90-min intervals. A single intraperitoneal injection of [^{32}P]orthophosphate (6 mCi/100 g body weight; carrier-free, ICN) was administered to the rats 30 min after the second diBtcAMP + theophylline injection, the rats were sacrificed by ether anesthesia and cervical dislocation 2 h later. Livers were homogenized in buffer A (120 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM EDTA, 50 mM NaF, 5 μM sodium orthovanadate, 500 μM molybdic acid, and 6 mM Na_2ATP), and microsomes were prepared by differential centrifugation. Final microsomal pellets (105000g × 60 min) were resuspended in microsome storage buffer (100 mM KP_i, pH 7.4, 20% glycerol, 1 mM EDTA, 5 mM NaF, 50 μM sodium orthovanadate, and 500 μM molybdic acid). Protein was determined by using the Bio-Rad protein assay reagent and bovine serum albumin as standard. The isolated microsomes had specific activities ranging from 1300 to 2500 cpm of ^{32}P/μg of protein.

Isolation of Rat Hepatocytes. Rat hepatocytes were prepared from collagenase-perfused phenobarbital-induced adult male Fischer 344 rats according to Seglen (1973), with all buffers and media containing 0.6 mM phenobarbital to stabilize cytochrome P-450 (Canepa et al., 1985). Hepatocytes were washed twice and then diluted to 2 × 10^6 cells/mL in either Leibovitz L-15 media (media A), Krebs–Ringer bicarbonate media (119 mM NaCl, 4.7 mM KCl, 24 mM $NaHCO_3$, 2.4 mM $MgSO_4$, and 2.3 mM $CaCl_2$) containing 15 mM glucose and 10 mg/mL bovine serum albumin (Sigma A-7030) (media B), or Krebs–Ringer bicarbonate media containing 16 mM lactate, 4 mM pyruvate, 0.1 mM KP_i (pH 7.4), and 10 mg/mL bovine serum albumin (media C). Cells were then incubated at 37 °C in air (media A) or under 95% O_2/5% CO_2 (media B and C) in the presence of [^{32}P]orthophosphate (0.4–0.7 mCi/mL; carrier-free, ICN). Hormones and protein kinase activators (obtained from Sigma and dissolved in a small volume of 0.9% NaCl, pH 10, unless indicated otherwise) were added to the hepatocytes either 30 min (diBtcAMP, cAMP), 45 min (glucagon, phenylephrine), or 50 min (Ca^{2+} ionophore A23187, dissolved in dimethyl sulfoxide) after the addition of [^{32}P]orthophosphate. Hepatocytes incubated in the presence of glucagon also contained theophylline (1 mM) to inhibit cAMP phosphodiesterase activity. In control experiments, theophylline alone was shown to have no effect on hepatocyte P-450 phosphorylation. After a total incubation time of 60 min at 37 °C, hepatocytes were pelleted by centrifugaion at 70g for 2 min and then resuspended in an equal volume of buffer A. The cell suspension was quick-frozen, thawed to 4 °C, and homogenized. Preparation of microsomes was as described above, with a yield of 0.8–1.2 mg of microsomal protein/8 × 10^6 cells.

Immunoprecipitation of Microsomal P-450s. Phosphorylated microsomes (200 μg) were adjusted to a protein concentration of 2 mg/mL containing 1% SDS, 50 mM Tris-HCl, pH 7.8, 1 mM $MgCl_2$, and 10 mM dithiothreitol and then boiled for 3 min. Samples (0.1 mL) were cooled to 20–22 °C and diluted with buffer B (50 mM Tris-HCl, pH 7.5, 190 mM NaCl, 6 mM EDTA, and 2.5% Triton X-100; 0.29 mL), and rabbit anti-P-450 antisera (10 μL) were then added. Samples were incubated overnight at 4 °C, then 40 μL of a 10% suspension of formalin-fixed *Staphylococcus aureus* cells (Sigma P-7155) was added, and incubation was continued for 60 min at 20–22 °C with continuous mixing. *S. aureus* cells were pelleted in an Eppendorph centrifuge (0.5 min) and then washed 5 times with 1 mL of buffer B containing 0.25% SDS. The final cell pellets were resuspended in SDS gel sample buffer (62 mM Tris-HCl, pH 6.8, 1% SDS, 0.001% bromophenol blue, 10% glycerol, and 5% 2-mercaptoethanol), heated for 10 min at 100 °C, and centrifuged for 2 min, and the supernatants were analyzed on 12.4% Laemmli SDS gels prepared on Gel-Bond PAG film using Acrylaide (FMC Corp.). Gels were dried at 70 °C and autoradiographed with Kodak X-AR5 film at −80 °C with one Du Pont lightning-plus intensifying screen.

Two-Dimensional Gel Electrophoresis. Liver microsomes (100–150 μg) and isolated P-450s (30–60 pmol) were analyzed

by two-dimensional gel electrophoresis using the methods of Vlasuk and Walz (1980).

Phosphoamino Acid Analysis and Tryptic Peptide Mapping of Hepatocyte ^{32}P-Labeled P-450 PB-4 + PB-5.[2] ^{32}P-Labeled microsomes (0.8 mg) prepared from glucagon-stimulated rat hepatocytes were solubilized, immunoprecipitated with anti-P-450 PB-4 antibodies as described above, and then electrophoresed through an SDS–polyacrylamide gel (14 × 16 cm, 0.75 mm thick). Samples of purified P-450 PB-4 (20 pmol) that had been phosporylated by [γ-^{32}P]ATP + cAMP-dependent protein kinase catalytic subunit (see below) were electrophoresed in a parallel lane on the gel. The gel was fixed for 60 min in 10% acetic acid/45% ethanol, then incubated at 20–22 °C for 24 h with two changes of 10% acetic acid/15% ethanol (200 mL of each), washed 3 times in 200 mL of double-distilled H_2O (ddH_2O) (30 min/wash), dried onto Whatman 3MM filter paper, and autoradiographed with Kodak X-AR5 film. Gel slices containing the ^{32}P-labeled P-450 bands were excised (without the filter paper) and then incubated with TPCK-treated trypsin (50 μg/mL; Worthington Biochemical Corp.) in 0.4 mL of 50 mM ammonium bicarbonate buffer, pH 8, for 5 h at 37 °C. An additional 0.2-mL aliquot of TPCK-trypsin (50 μg/mL) was then added, and the samples were incubated a further 19 h. Samples were spun; the supernatants were lyophilized repeated to remove residual ammonium bicarbonate and then resuspended in 6 μL of 0.033% 2-mercaptoethanol. An aliquot (2 μL) was removed for tryptic peptide analysis while the remainder was lyophilized and then subjected to partial acid hydrolysis and phosphoamino acid analysis.

Samples prepared for phosphoamino acid analysis were resuspended in 0.1 mL of ddH_2O, lyophilized, and then subjected to partial acid hydrolysis (Martensen, 1984) by heating at 100 °C in 5.7 N HCl (0.1 mL/tube; Pierce Chemical Co.) under N_2 for 90 min. HCl was removed by repeated lyophilization, and the samples were then dissolved in ddH_2O (10 μL/tube) containing 8 μg each of phosphoserine, phosphothreonine, and phosphotyrosine standards (Sigma) and a 1:250 dilution of Dye Mix A (0.3% each of Xylene cyanole FF, Orange G, and Acid fuschin; Eastman) to standardize migration distances. Aliquots (5 μL) were spotted onto a 20 cm × 20 cm thin-layer cellulose chromatography plate (Eastman Kodak 13255) which was carefully moistened with pH 3.5 electrophoresis buffer (pyridine/acetic acid/H_2O; 5:50:945) (Hunter & Sefton 1980). Plates were electrophoresed for 75 min at 800 V in a Shandon, Inc. (Sewickley, PA), thin-layer electrophoresis apparatus. ^{32}P-Labeled phosphoamino acids and partial hydrolysis products were detected by autoradiography (3-day exposure) and compared to the phosphoamino acid standards as detailed in Table I. Phosphoamino acid standards were visualized with 0.25% ninhydrin (Sigma) dissolved in ethanol followed by heating at 100 °C for 3 min.

Table I: Phosphoamino Acid Analysis of ^{32}P-Labeled P-450[a]

	migration distance (cm)			
	hepatocyte-labeled anti-P-450 PB-4 immunoprecipitate		in vitro labeled P-450 PB-4	
	^{32}P label	ninhydrin react.	^{32}P label	ninhydrin react.
orthophosphate	15.8	[b]	16.3	
serine	12.7	12.7	12.9	12.9
threonine	[c]	11.5	[c]	11.8
tyrosine	[c]	10.0	[c]	9.9
phosphopeptide I[d]	7.1		7.1	
phosphopeptide II[d]	6.1		6.0	

[a] ^{32}P-Labeled P-450 PB-4 + PB-5 was immunoprecipitated from glucagon-stimulated hepatocytes, purified by SDS gel electrophoresis, and then digested with trypsin. Parallel analyses were carried out with P-450 PB-4 that was ^{32}P labeled by cAMP-dependent protein kinase for 90 min at 37 °C (see Materials and Methods) and then purified by SDS gel electrophoresis. Tryptic digests were subjected to partial acid hydrolysis under conditions where about one-third of the ^{32}P label was released as free phosphoamino acid, one-third hydrolyzed to [^{32}P]-orthophosphate, and one-third converted to a mixture of two ^{32}P-labeled phosphopeptides (designated I and II), as is typically observed (Martensen, 1984). Tabulated are the mobilities of the ^{32}P-labeled phosphoamino acids, phosphopeptides, and orthophosphate (detected by autoradiography; columns 2 and 4) in comparison to the mobilities of the three phosphoamino acid standards (visualized with ninhydrin; columns 3 and 5). [b] Not applicable. [c] No radiolabel detected. [d] Only one tryptic peptide was observed in Figure 5, indicating that the ^{32}P-labeled phosphopeptides I and II arise from partial acid hydrolysis of the same ^{32}P-labeled tryptic peptide. Phosphopeptides I and II derived from hepatocyte-labeled [^{32}P]P-450 immunoprecipitates exhibited the same migration distances as those derived from in vitro ^{32}P-labeled P-450 PB-4 (7.1- and 6.1-cm mobility, column 2, versus 7.1- and 6.0-cm mobility, column 3). This further supports the conclusion (Figure 5) that the same site is phosphorylated in hepatocytes as in vitro.

Samples for tryptic peptide analysis were co-spotted with ~0.5–1 μL of Dye Mix A on 10 cm × 10 cm cellulose-coated thin-layer plates (EM Science 5577). Plates were electrophoresed with pH 1.9 buffer (formic acid/acetic acid/H_2O; 1:3:16) for 20 min at 1000 V, air-dried for 60 min, and then chromatographed in a second dimension at 20–22 °C with 1-butanol/pyridine/acetic acid/H_2O (6.5:5:1:4). Plates were air-dried and then autoradiographed (3-day exposure).

Phosphorylation of P-450 PB-4 by cAMP-Dependent Protein Kinase. Bovine heart cAMP-dependent protein kinase catalytic subunit (10 units; Sigma P-2645) was dissolved in 14 μL of dithiothreitol (50 mg/mL) and incubated on ice for 10 min. P-450 PB-4 (75 pmol), magnesium acetate (pH 6.5; 15 mM final concentration), and [γ-^{32}P]ATP (1–3 μCi; 100 μM ATP final concentration) were added to give a final volume of 25 μL. Samples were incubated for 0–90 min at 37 °C and aliquots (12 pmol of P-450) then reconstituted with purified NADPH P-450 reductase and dilauroylphosphatidylcholine and assayed for 7-ethoxycoumarin *O*-deethylase activity as previously described (Waxman & Walsh, 1982).

P-450 Forms and Antibodies. Rat hepatic P-450 forms PB-4 and PB-5 were purified as described previously (Waxman & Walsh, 1982). Polyclonal antibodies to P-450 forms PB-4, PB-2a, PB-1, and 2c were raised in rabbits and characterized with respect to their P-450 form specificities by using methods described (Waxman, 1984). In general these antibodies were at least partially cross-reactive within a P-450 subfamily (e.g., anti-PB-4 with PB-5) but not between P-450 subfamilies.

Results

P-450 Phosphorylation in Isolated Hepatocytes. Phosphorylation of cytochrome P-450 was studied by incubation

[2] The designations given to the rat hepatic P-450 forms included in this study can be related to the standardized gene designations (Nebert et al., 1989) and alternative protein nomenclatures used by other investigators, as follows: *P-450 forms PB-4, PB-5, PB-1, and 2c* (Waxman & Walsh, 1982, 1983; Waxman, 1984) (P-450 genes IIB1, IIB2, IIC6, and IIC11, respectively) correspond to preparations respectively designated b, e, k, and h (Thomas et al., 1987); preparations PB-B, PB-D, PB-C, and UT-A (Guengerich et al., 1982); preparations PBRLM5, PBRLM6, RLM5a, and RLM5 (Schenkman et al., 1987); and preparations PB3a, PB3b, PB1b, and PB2a (Wolf, 1986). *P-450 PB-2a* purified in this laboratory (Waxman, 1984; Waxman et al., 1985) appears to be part of the same gene subfamily (IIIA) as the phenobarbital/dexamethasone/troleandomycin/pregnenolone 16α-carbonitrile-inducible form(s) designated p (Wrighton et al., 1985), PCNa, PCNb (Graves et al., 1987), and PCN-E (Guengerich et al., 1982).

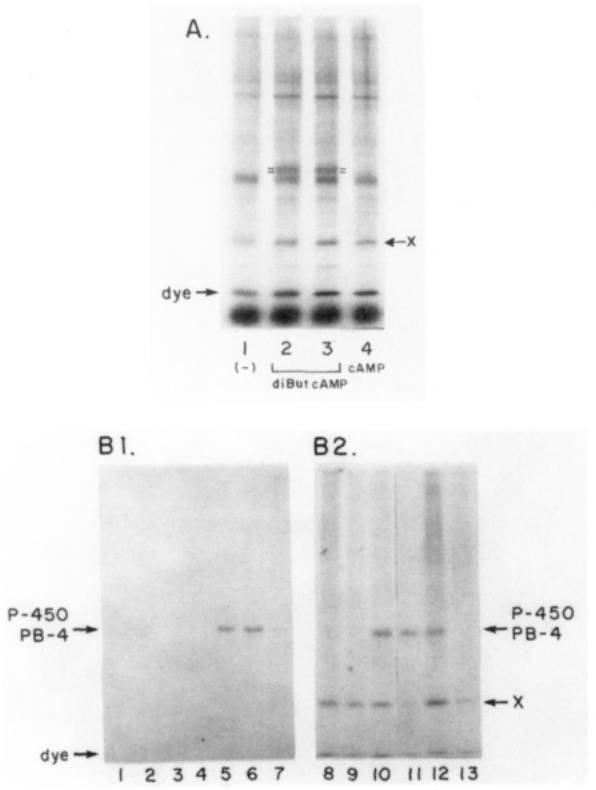

FIGURE 1: (A) Phosphorylation of microsomal proteins in phenobarbital-induced hepatocytes. Hepatocytes were incubated for 60 min with [^{32}P]orthophosphate alone (lane 1) or in combination with 100 μM diBtcAMP (lane 2), 1 mM diBtcAMP (lane 3), or 100 μM cAMP (lane 4). cAMP and diBtcAMP were added to the hepatocyte culture media after 30 min of incubation as described under Materials and Methods. Microsomes were isolated and then analyzed (40 μg of protein/lane) for ^{32}P-labeled proteins by SDS gel electrophoresis and autoradiography (2-day exposure to X-ray film). *Dashes* alongside lanes 2 and 3 indicate a pair of microsomal proteins whose phosphorylation is markedly stimulated by diBtcAMP. The more rapidly migrating of the two proteins corresponds to P-450 PB-4 and P-450 PB-5 in its electrophoretic mobility.[3] Band X indicates a ^{32}P-labeled microsomal polypeptide seen to contaminate some immunoprecipitates (e.g., Figure 1B2). (B) Immunoprecipitation of P-450 PB-4 from ^{32}P-labeled hepatocytes. Phenobarbital-induced hepatocytes were incubated with [^{32}P]orthophosphate alone (lane 4) or in combination with 100 μM diBtcAMP (lanes 1, 2, 5, 9, and 10), 1 mM diBtcAMP (lane 6), 100 μM cAMP (lanes 7 and 8), glucagon (1 μM, lane 11; 10 μM, lane 12), or phenylephrine (10 μM, lane 13) as described in Figure 1A and under Materials and Methods. Purified P-450 PB-4 standard (unlabeled) (lane 3) migrated at the position marked by arrows. Microsomes were isolated then solubilized and immunoprecipitated with anti-P-450 PB-4 (lanes 4–8 and 10–13), anti-P-450 PB-2a (lane 2), or nonimmune sera (lanes 1 and 9), and then analyzed as described under Materials and Methods. Shown are autoradiographs of gels from separate experiments carried out with hepatocytes incubated in media B (lanes 1–7) or media C (lanes 8–13) (3-day exposure to X-ray film) (see Materials and Methods). No major influence of media choice on the diBtcAMP-stimulated phosphorylation of P-450 PB-4 was observed. Immunoprecipitates shown in lanes 8–13 were contaminated to a variable extent by a nonspecifically associated phosphoprotein (band X) which corresponds to a major ^{32}P-labeled hepatocyte microsomal protein labeled in isolated hepatocytes (Figure 1A) and in vivo (Figure 4A, below).

of hepatocytes isolated from phenobarbital-induced rats with [^{32}P]orthophosphate in the presence of diBtcAMP, a membrane-permeable cAMP analogue that stimulates cAMP-dependent protein kinase activity. Hepatocyte microsomes were isolated by differential centrifugation and then analyzed by SDS gel electrophoresis and autoradiography for the presence of phosphorylated microsomal proteins (Figure 1). Phosphorylation of two microsomal proteins of M_r ~50 000 was found to be markedly stimulated by diBtcAMP (Figure 1A; bands marked by *dashes* in lanes 2 and 3) but was not enhanced by phenylephrine (10 μM) or the Ca^{2+} ionophore A23187 (10 μM) (not shown), which both serve to activate Ca^{2+}-dependent protein kinases (Hems & Winton, 1980). The electrophoretic mobility of the lower phosphoprotein band is identical with that of P-450 form PB-4,[2] as determined by electrophoresis of the purified cytochrome in an adjacent lane. Immunoprecipitation analysis revealed that this phosphorylated microsomal polypeptide is precipitated by anti-P-450 PB-4 antibody (Figure 1B, lanes 5, 6, and 10) but not by nonimmune sera (lanes 1 and 9). In contrast, little or no phosphorylation of P-450 forms reactive with antibodies to P-450 PB-2a (lane 2), P-450 PB-1, or P-450 2c was detected (data not shown). Significant phosphorylation was observed in the anti-P-450 PB-4 immunoprecipitates derived from hepatocytes incubated in the presence of diBtcAMP at concentrations ranging from 100 μM to 1 mM (lanes 5, 6, and 10), with ~25% as much phosphorylation observed at 5 μM diBtcAMP as compared to 1 mM diBtcAMP (data not shown). No significant radioactivity was associated with anti-PB-4 immunoprecipitates prepared from hepatocytes incubated in the absence of diBtcAMP (lane 4) or in the presence of the Ca^{2+}-dependent protein kinase activators phenylephrine (lane 13) and A23187 (data not shown). Furthermore, addition of cAMP (which does not permeate intact hepatocyte membranes) did not lead to significant phosphorylation of immunoreactive P-450 PB-4 (lanes 7 and 8), indicating that hepatocyte integrity was effectively maintained throughout the incubation period. Finally, glucagon, which activates adenylate cyclase to increase cAMP levels within the cell, stimulated P-450 PB-4 phosphorylation to a level comparable to that obtained with diBtcAMP (lanes 11 and 12 versus lane 10).

Polyclonal antibodies raised to P-450 PB-4 are known to react with both P-450 PB-4 and P-450 PB-5 (Waxman & Walsh, 1982; Ryan et al., 1982), which share 97% amino acid sequence identity, as well as with several allozymic variants of these two P-450 forms that are poorly resolved from each other on SDS gels[3] (Rampersaud & Walz, 1983). ^{32}P-Labeled hepatocyte microsomes prepared from diBtcAMP-stimulated cells were therefore analyzed by two-dimensional gel electrophoresis to ascertain which form or forms of P-450 are phosphorylated in the intact hepatocytes. These analyses revealed that diBtcAMP stimulates phosphorylation of at least four discrete microsomal polypeptides of M_r ~ 50 000 at concentrations as low as 5 μM (Figure 2B,C versus Figure 2A). Two of these phosphorylated polypeptides, designated spots 1 and 2, comigrate with P-450 PB-4 in the SDS gel dimension but are more acidic as judged from their mobility in the isoelectric focusing dimension. These spots correspond to acidic (i.e., phosphorylated) derivatives of the two major isoelectric species present in highly purified preparations of P-450 PB-4, designated spots a and b in Figure 2D. This identification was confirmed by the near-complete conversion of spots a + b to spots 1 + 2 upon incubation of purified P-450 PB-4 with cAMP-dependent protein kinase (data not shown). Similarly, the other two phosphorylated microsomal polypeptides (spots 3 and 4, Figure 2B,C) correspond to more acidic forms of the two major P-450 PB-5 polypeptides (spots c and d, Figure 2D). diBtcAMP enhanced the phosphorylation of all four PB-4/PB-5 polypeptides to a similar extent [com-

[3] Although P-450 PB-4 can be resolved from P-450 PB-5 on SDS gels under suitable conditions of electrophoresis [e.g., see Waxman et al. (1983)], no resolution of these cytochromes is achieved on the Acrylaide-containing gels used to analyze the ^{32}P-labeled hepatocyte microsomes and immunoprecipitates (Figure 1A,B).

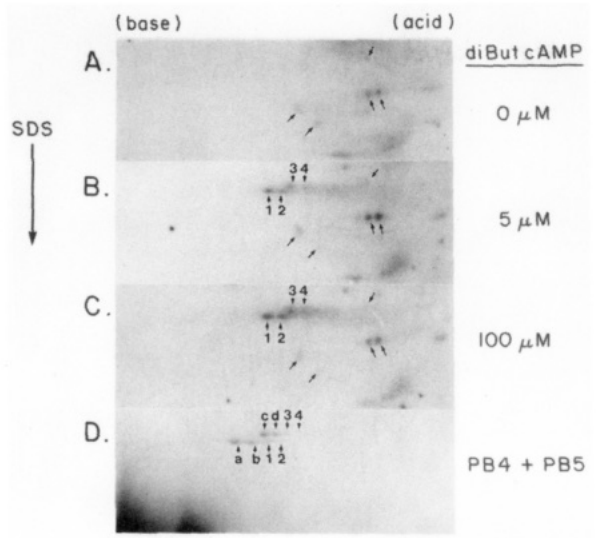

FIGURE 2: Two-dimensional gel electrophoretic analysis of ^{32}P-labeled hepatocyte microsomal proteins. Phenobarbital-induced hepatocytes were incubated with [^{32}P]orthophosphate alone (panel A) or in combination with 5 μM diBtcAMP (panel B) or 100 μM diBtcAMP (panel C) and then analyzed on two-dimensional gels as detailed under Materials and Methods. Shown are autoradiographs (5-day exposure) of portions of each gel. Phosphorylated forms of P-450 PB-4 (arrows 1 and 2) and P-450 PB-5 (arrows 3 and 4) are undetectable in the absence of diBtcAMP (panel A). The five unmarked diagonal arrows in panels A–C identify polypeptides whose phosphorylation was not significantly affected by diBtcAMP; these serve as reference points for comparison of the gels. Shown in panel D is a Coomassie-stained gel of a mixture of purified P-450 PB-4 + PB-5, demonstrating the microheterogeneity exhibited by these proteins in the isoelectric focusing dimension. Both P-450s are composed of two major polypeptides [respectively marked a and b (PB-4) and c and d (PB-5)] and one minor polypeptide which comigrated with the ^{32}P-labeled polypeptides marked 1 (P-450 PB-4) and 3 (P-450 PB-5). These minor Coomassie-stained spots (~15% of total PB-4/PB-5 staining) appear to correspond to endogenously phosphorylated forms of the respective cytochromes (see Discussion). Patterns similar to those shown in panel D were obtained upon two-dimensional gel analysis of phenobarbital-induced liver microsomes immunoblotted with anti-P-450 PB-4 antibodies (not shown).

pare relative intensities of ^{32}P-labeled spots 1–4 (Figure 2B,C) to relative intensities of Coomassie-stained PB-4/PB-5 polypeptides a–d (Figure 2D)], indicating that the cAMP-dependent hepatocyte protein kinase does not exhibit high selectivity within the P-450 gene subfamily representing forms PB-4 and PB-5 (gene subfamily IIB).

Phosphorylation of Hepatic P-450s in Vivo. To determine whether the phosphorylation of P-450 PB-4 and P-450 PB-5 observed in isolated hepatocytes reflects processes that occur in vivo, phenobarbital-induced adult male rats were injected with [^{32}P]orthophosphate in combination with diBtcAMP + theophylline, a treatment regimen designed to stimulate cAMP-dependent processes in whole animals (Wicks et al., 1972). Liver microsomes were isolated and then analyzed for ^{32}P-labeled P-450 forms by immunoprecipitation, SDS gel electrophoresis, and autoradiography. Immunoprecipitation with anti-P-450 PB-4 antibodies indicated that P-450 forms PB-4 and/or PB-5 are phosporylated in vivo (Figure 3, lane 4). Antibodies to P-450 forms PB-2a (lane 5) and to a lesser degree PB-1 (lane 6) immunoprecipitated corresponding radiolabeled polypeptides, indicating that these (or related)[4]

[4] The precise identity of these phosphorylated P-450s is not known, since polyclonal antibodies to P-450 PB-2a and to P-450 PB-1 are each cross-reactive with at least one other P-450 member of their respective gene subfamilies (designated IIIA and IIC, respectively; see footnote 2).

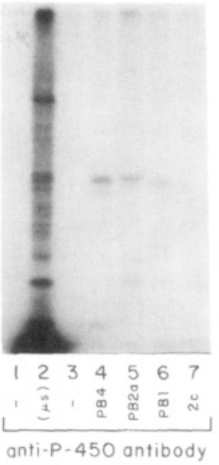

FIGURE 3: Immunoprecipitation analysis of hepatic P-450s labeled with [^{32}P]orthophosphate in vivo. Cytochrome P-450 immunoprecipitates (lanes 4–7) and phosphorylated microsomal proteins (lane 2) from a phenobarbital-induced rat injected with [^{32}P]orthophosphate, diBtcAMP, and theophylline (see Materials and Methods) were analyzed by SDS gel electrophoresis and autoradiography (6-day exposure). Immunoprecipitations were conducted using nonimmune sera (lane 1) and antibodies reactive with P-450 forms PB-4 + PB-5 (lane 4), PB-2a (lane 5), PB-1 (lane 6), and 2c (lane 7). For a control solubilized microsomes were incubated in the presence of *Staphylococcus aureus* cells alone (lane 3).

P-450 forms are phosphorylated in vivo. However, no significant radiolabeled immunoprecipitates were observed using antibodies reactive with the constitutively expressed P-450 form 2c (IIC11) (lane 7), even when the experiments were carried out in an uninduced adult male rat, which expresses high levels of P-450 2c (Waxman et al., 1985). Further experiments with uninduced rats, which do not express significant levels of P-450 PB-4 or PB-5, and which have much lower levels of immunoreactive P-450 PB-2a, revealed that levels of the corresponding phosphoproteins are strikingly reduced in the absence of phenobarbital induction (Figure 4A lane 3 versus lane 4 and lane 6 versus lane 7), consistent with the identification of the in vivo phosphorylated forms as P-450 PB-4/PB-5 and PB-2a, respectively. Interestingly, although P-450 PB-4/PB-5 phosphorylation was stimulated by diBtcAMP + theophylline, significant levels of phospho-P-450 PB-4/PB-5 (Figure 4A, lane 5) as well as phospho-P-450 PB-2a (lane 8) and phospho-PB-1 (data not shown) were also detected in the absence of these activators of cAMP-dependent protein kinase. Thus, the in vivo situation contrasts with that in isolated hepatocytes, where phosphorylation of P-450 forms PB-4 and PB-5 was more strikingly dependent upon diBtcAMP, and where significant phosphorylation of immunoreactive P-450 PB-2a was not observed (Figure 1B).

Two-dimensional gel analysis of in vivo ^{32}P-labeled P-450 PB-4 + PB-5 partially purified from the phenobarbital-induced liver samples revealed a pattern of phosphorylated P-450 polypeptides 1–4 (Figure 4B) indistinguishable from that present in the ^{32}P-labeled isolated hepatocytes (Figure 2B,C). Corresponding P-450 fractions prepared from ^{32}P-labeled uninduced rats did not exhibit these phosphorylated microsomal polypeptides (data not shown), consistent with the near-absence of P-450 PB-4 and P-450 PB-5 in the uninduced animals. Thus, both P-450 PB-4 and PB-5 are phosphorylated in vivo.

Identification of Phosphorylation Site. P-450s PB-4 + PB-5 were immunoprecipitated from glucagon-stimulated ^{32}P-labeled hepatocytes and then purified by SDS gel electrophoresis. The ^{32}P-labeled cytochromes were digested with trypsin and then subjected to partial acid hydrolysis to liberate free

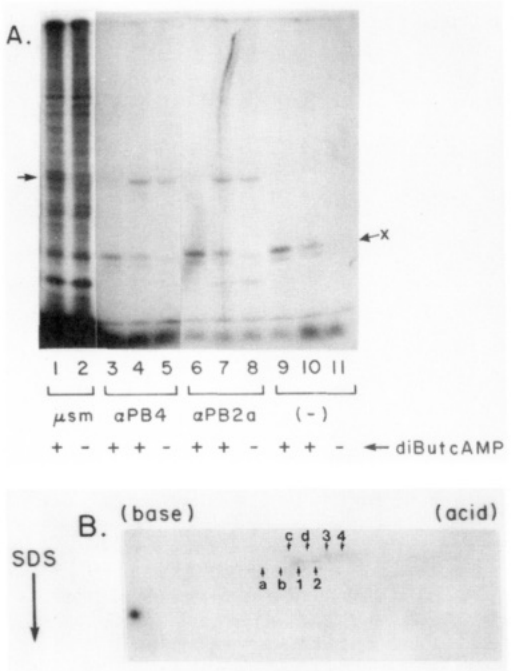

FIGURE 4: Phosphorylation of P-450s in vivo: dependence on diBt-cAMP and phenobarbital induction. (A) Shown is an autoradiograph of an SDS gel analyzing microsomal proteins phosphorylated in a phenobarbital-induced rat in vivo (lanes 1 and 2) and immunoprecipitates of microsomal P-450s isolated from an uninduced rat injected with [^{32}P]orthophosphate and diBtcAMP + theophylline (lanes 3, 6, and 9), a phenobarbital-induced rat injected with [^{32}P]orthophosphate and diBtcAMP + theophylline (lanes 4, 7, and 10), and a phenobarbital-induced rat injected with [^{32}P]orthophosphate alone (lanes 5, 8, and 11) (10-day exposure, lanes 3–11; 4-day exposure, lanes 1 and 2). Immunoprecipitations were carried out using nonimmune sera (lanes 9–11) and antisera reactive with P-450 forms PB-4 (lanes 3–5) and PB-2a (lanes 6–8). The unmarked arrow at the left indicates a microsomal band with the electrophoretic mobility of P-450 PB-4 and P-450 PB-2a whose phosphorylation in vivo was enhanced by diBtcAMP + theophylline treatment (lane 1 versus 2). Band X (also see Figure 1A,B2) contaminated the immunoprecipitates to a variable extent, particularly in diBtcAMP-stimulated samples. [Note the similar patterns of band X in the control precipitates (lanes 9–11) as compared to the immunoprecipitates (lanes 3–5 and 6–8).] (B) Autoradiograph of a portion of two-dimensional gel electrophoretic separation of partially purified microsomal protein [8–16% poly(ethylene glycol) fraction of cholate-solubilized liver microsomes] isolated from a phenobarbital-induced rat injected with [^{32}P]orthophosphate in the absence of diBtcAMP and theophylline (7-day exposure). The partial purification employed was found to remove the majority of ^{32}P-labeled microsomal proteins resolved on the two-dimensional gel (cf Figure 4A, lane 2). Isoelectric points of the major unlabeled forms of microsomal P-450 PB-4 (arrows a and b) and P-450 PB-5 (arrows c and d), visualized by Coomassie blue staining of the same gel, were as indicated.

phosphoamino acids. Thin-layer electrophoretic analysis of the acid hydrolysate revealed the presence of ^{32}P-labeled phosphoserine, with no phosphothreonine or phosphotyrosine detectable (Table I, columns 1 and 2). Parallel analysis of purified P-450 PB-4 phosphorylated by incubation with [γ-^{32}P]ATP + cAMP-dependent protein kinase (see Materials and Methods) indicated that phosphorylation also occurs exclusively on serine residues under these cell-free phosphorylation conditions (columns 3 and 4). In order to ascertain whether the same site is phosphorylated in isolated hepatocytes as in vitro, comparative tryptic peptide mapping analyses were carried out. The results obtained (Figure 5) indicated that P-450 PB-4 is phosphorylated on a single peptide that is the same in hepatocytes as in vitro.

Effects of Phosphorylation on Monooxygenase Activity. Phosphorylation of purified P-450 PB-4 by cAMP-dependent

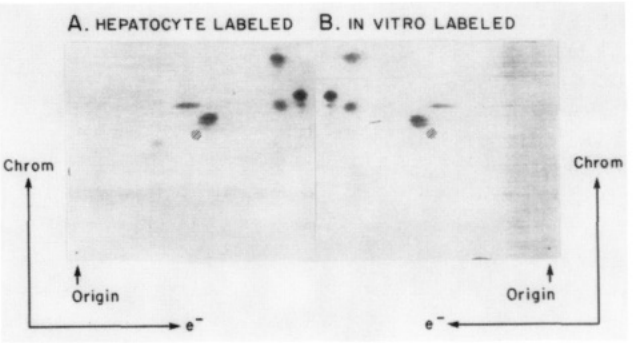

FIGURE 5: Tryptic peptide mapping of [^{32}P]P-450 PB-4/PB-5 immunoprecipitate derived from glucagon-stimulated hepatocytes (panel A) and [^{32}P]P-450 PB-4 labeled by purified cAMP-dependent protein kinase in vitro (panel B). ^{32}P-Labeled P-450 samples were isolated from SDS gels, digested with trypsin, and then subjected to two-dimensional analysis as described under Materials and Methods. The mobility in two dimensions of the ^{32}P-labeled peptide detected in each sample by autoradiography is indicated by a hatched circle. Chromatographic (*Chrom*) and electrophoretic (e^-) mobilities of the hepatocyte-labeled ^{32}P peptide in panel A were indistinguishable from those of the in vitro-labeled ^{32}P peptide in panel B, as indicated by a comparison of their migration relative to the mixture of colored marker dyes that were co-spotted with the tryptic digest (see Materials and Methods) and that appear as dark spots in this figure.

Table II: Influence of Phosphorylation on Monooxygenase Activity of P-450 PB-4[a]

time (min)	*O*-deethylase act. [nmol of 7-hydroxycoumarin min^{-1} (nmol of PB-4)$^{-1}$] −ATP	+ATP
0	8.1 (100)[b]	7.6 (94)
10	7.6 (94)	5.0 (62)
20	7.4 (91)	3.9 (48)
40	7.6 (94)	3.6 (44)
60	7.7 (95)	3.4 (42)
90	7.6 (94)	2.8 (35)

[a] Purified P-450 PB-4 was incubated with cAMP-dependent protein kinase for 0–90 min at 37 °C as described under Materials and Methods. Aliquots (4 μL) containing 12 pmol of P-450 were withdrawn from the kinase reaction mixture at the indicated times and diluted into buffer (80 μL) containing NADPH–P-450 reductase and dilauroylphosphatidylcholine. Samples were reconstituted (10 min at 20–22 °C) and then diluted to a final volume of 0.4 mL in buffer containing 1 mM 7-ethoxycoumarin substrate and 0.3 mM NADPH and *O*-deethylase; activity was then determined as previously described (Waxman & Walsh, 1982). P-450 PB-4 activity was unaffected unless the kinase incubation was carried out in the presence of ATP. [b] Percent.

protein kinase resulted in a time-dependent loss of monooxygenase activity that reached 65% under the condition employed (Table II). In control incubations carried out in the absence of ATP, no loss of activity was observed, indicating that the inactivation of the cytochrome is directly dependent on protein phosphorylation.

Discussion

Phosphorylation of rat hepatic P-450 forms PB-4 and PB-5 was shown to occur in isolated hepatocytes and in vivo in a process stimulated by the membrane-permeable cAMP analogue diBtcAMP. Glucagon, which elevates intracellular cAMP levels, also stimulated phosphorylation of these cytochromes, while activators of Ca^{2+}-dependent protein kinases did not. These data strongly suggest that the observed P-450 phosphorylation is mediated by a hepatic cAMP-dependent protein kinase. This conclusion is supported by the demonstration that the hepatocyte kinase phosphorylates these P-450s at the same site that is utilized by purified cAMP-dependent protein kinase when incubated with purified P-450 PB-4 in

vitro. Although significant P-450 PB-4 and PB-5 phosphorylation was observed in vivo in the absence of added kinase activators, phosphorylation of these P-450s in isolated hepatocytes was obligatorily dependent on the kinase activators. This result suggests that circulating hormones stimulate phosphorylation of these P-450s in vivo and that these hormones are absent (or present at reduced levels) in the isolated cells.

Two-dimensional gel analysis of ^{32}P-labeled P-450s PB-4 and PB-5 revealed the presence of two distinct phosphorylated derivatives of each P-450 form, respectively designated as spots 1 and 2 (PB-4-derived) and spots 3 and 4 (PB-5-derived) (Figure 2). Spots 1 and 2 differed from each other only in their isoelectric points and appear to correspond to phosphorylated derivatives of the two major isoelectric components of highly purified P-450 PB-4 (spots a and b, Figure 2D). Similarly, ^{32}P-labeled spots 3 and 4 appear to correspond to phosphorylated derivatives of the two major components of purified P-450 PB-5, respectively designated spots c and d. Two-dimensional gel analysis of purified P-450 PB-4 also revealed the presence of a third immuno-cross-reactive isoelectric species, comprising ~15% of the total Coomassie-staining intensity, which was indistinguishable in its electrophoretic migration and its isoelectric point from the phosphorylated spot 1. Similarly, purified P-450 PB-5 preparations included an isoelectric species corresponding to phosphorylated spot 3. This suggests that P-450s PB-4 and PB-5 are both phosphorylated to a significant extent in vivo, where their phosphorylation status and catalytic activity may be influenced by hormonal or environmental factors. The precise relationship between PB-4 spot a and spot b and between PB-5 spot c and spot d is not known but may involve posttranslational modification via processes other than phosphorylation (e.g., acetylation, glycosylation, deamidation), or perhaps may reflect charge heterogeneity due to the presence of allozymic variants of these cytochromes (Rampersaud & Walz, 1983). This "microheterogeneity" of P-450s and PB-4 and PB-5 was also observed upon Western blot analysis of microsomes prepared from phenobarbital-induced rat liver or from rat hepatocytes induced with phenobarbital in culture (data not shown), indicating that it is not an artifact of enzyme isolation.

The site of P-450 PB-4 and PB-5 phosphorylation by the hepatocyte kinase was identified as a serine residue localized to a single tryptic peptide that is also phosphorylated by purified cAMP-dependent protein kinase in vitro. In the case of rabbit P-450 LM2, which is structurally related to rat P-450 PB-4, a corresponding tryptic phosphopeptide has been isolated (Arg-126–Arg-133) and the site of phosphorylation identified as serine-128 (Muller et al., 1985). Since this peptide contains only one serine residue and is 100% conserved in the corresponding regions of rat P-450s PB-4 and PB-5 (Fujii-Kuriyama et al., 1982), it seems highly likely that P-450s PB-4 and PB-5 are also phosphorylated on serine-128. Serine-128 is contained within a recognition sequence (Arg-Arg-X-Ser) utilized by many cAMP-dependent protein kinases (Krebs & Beavo, 1979), further supporting the involvement of a cAMP-dependent protein kinase in the hepatocyte phosphorylation reaction. This recognition sequence is conserved in several other rat liver P-450 gene family II members (i.e., genes IIA1, IIC11, IID1, IID2, and IIE1) as well as many rabbit and human P-450 family II genes (data not shown). Thus, one or more of these other P-450 forms may also be phosphorylated by cAMP-dependent protein kinase in liver tissue. The presence of the Arg-Arg-X-Ser recognition sequence does not, however, ensure phosphorylation by the kinase, as indicated by the absence of significant P-450 2c (gene IIC11) phosphorylation in isolated hepatocytes (data not shown). Little or no phosphorylation of P-450 forms PB-1 (gene IIC6) or PB-2a (gene IIIA1) was observed in isolated hepatocytes in the present study, consistent with the absence of the cAMP-dependent kinase recognition sequence in these P-450 forms. This sequence is also absent in P-450 forms IIA2, IIB3, IIC7, and IIC12 and all members of gene families I, III, and IV sequenced to date [for references to sequence data, see Nebert et al. (1989)]. Interestingly, although P-450 forms PB-1 and PB-2a lack the cAMP-dependent kinase recognition sequence and were not detectably phosphorylated in isolated hepatocytes, they (or immunochemically related forms[4]) were phosphorylated in vivo. This suggests that the phosphorylation of these latter P-450 forms is mediated by protein kinase(s) distinct from the cAMP-dependent kinase active in P-450 PB-4 and PB-5 phosphorylation and that their phosphorylation is probably subject to independent regulatory control.

Phosphorylation of P-450 PB-4 was found to lead to a loss of monooxygenase activity toward 7-ethoxycoumarin (Table II) and testosterone (data not shown) as substrates. In the case of rabbit P-450 LM2, phosphorylation has been associated with denaturation of the cytochrome to the inactive P-420 form (Taniguchi et al., 1985). Whether dephosphorylation and enzyme reactivation can occur in vivo or whether phosphorylation necessarily leads to P-420 formation followed by heme loss and apoprotein degradation is not known. Attempts to examine the reversibility of the activity loss by incubation of phosphorylation P-450 PB-4 with alkaline phosphatase in vitro were unsuccessful owing to the low activity of the phosphorylated cytochrome toward this phosphatase under the conditions employed (unpublished experiments). Deactivation of hepatic P-450-dependent monooxygenase activity has been observed upon administration of cAMP-dependent kinase activators in vivo [e.g., see Ross et al. (1973)], suggesting that the loss of P-450 activity upon phosphorylation in vitro reflects processes that occur in vivo and that these processes may contribute to the short-term regulation of hepatic monooxygenase activity. Longer term exposure of hepatocytes to elevated cAMP levels leads, however, to an increase in P-450 activity in a process dependent on protein synthesis (Canepa et al., 1985; Berry & Skett, 1988). Thus, the responses of P-450 activity to hormones that alter intracellular cAMP levels are likely to be complex.

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Registry No. P-450, 9035-51-2; monooxygenase, 9038-14-6.

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Mutation-Induced Perturbation of the Cytochrome *c* Alkaline Transition†

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ABSTRACT: The possible influence of residue Phe-82 in the cytochrome *c* alkaline isomerization has been evaluated by spectrophotometric pH titrations of a family of mutant yeast iso-1-cytochromes *c* in which the identity of the residue at this position has been varied. The pK_a for the exchange of the Met-80 heme iron ligand was determined from pH titrations in which the S → Fe charge-transfer band (695 nm) was monitored and was found to be 8.5 for the wild type, 7.7 for Ser-82, 7.7 for Gly-82, 7.2 for Leu-82, and 7.2 for Ile-82. pH-jump experiments [Davis et al. (1974) *J. Biol. Chem. 249*, 2624] established that substitutions at position 82 affect the alkaline isomerization by lowering the pK_a of the titrating group by as much as 1.4 pK units; for the Ser-82 and Gly-82 variants, there is also a small effect on the K_{eq} for the ligand exchange equilibrium. On the basis of these findings, we conclude that one critical role for Phe-82 in the wild-type protein is stabilization of the native heme binding environment.

Residue Phe-82 of eukaryotic cytochromes *c* is phylogenetically conserved and has been implicated in the mechanism of electron transfer between cytochrome *c* and cytochrome *c* peroxidase (Poulos & Kraut, 1980). By use of the technique of site-directed mutagenesis, this residue has been replaced in yeast iso-1-cytochrome *c* by Ser, Tyr, Gly, Ile, or Leu (Pielak et al., 1985; Liang et al., 1988), and the resulting proteins have been found to demonstrate significant differences in their rates of electron transfer in photoexcited electron-transfer reactions with Zn-CCP (Liang et al., 1987, 1988). During subsequent studies we have discovered that mutations produced at position 82 unexpectedly destabilize the oxidized form of the cytochrome by lowering the pK_a for the conversion

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