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Phosphorylation of hepatic phenobarbital-inducible cytochrome P-450

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The major phenobarbital-inducible cytochrome P-450 purified from rat liver, a member of family II of the cytochrome P-450 gene superfamily, is rapidly phosphorylated by cAMP-dependent protein kinase. The phosphorylation reaches >0.5 mol phosphate/mol P-450 after 5 min and is accompanied by a decrease in enzyme activity. The serine residue in position 128 was shown to be the sole phosphorylation site and a conformational change of the protein was indicated by a shift of the carbon monoxide difference spectrum of the reduced cytochrome from 450 to 420 nm. Comparison of amino acid sequences of various cytochrome P-450 families revealed a highly conserved arginine residue in the immediate vicinity of the phosphorylated serine residue which constitutes the kinase recognition sequence. It also revealed that only the members of the cytochrome P-450 family II carry this kinase recognition sequence. To find out whether this phosphorylation also occurs *in vivo*, the exchangeable phosphate pool of intact hepatocytes derived from phenobarbital-pretreated rats was labeled with $^{32}P_i$ followed by an incubation of the cells with the membrane-permeating dibutyryl-cAMP or with the adenylate cyclase stimulator glucagon to activate endogenous kinase. As a result, a microsomal polypeptide with the same electrophoretic mobility as cytochrome P-450 became strongly labeled. Peptide mapping and immunoprecipitation with monospecific antibodies identified this protein as the major phenobarbital-inducible cytochrome P-450. It becomes phosphorylated at the same serine residues as in the cell-free phosphorylation. Since the degree of phosphorylation attained in hepatocytes is high enough to be of physiological significance, it is suggested that in liver cells and in liver tissue, phosphorylation may serve as an isoenzyme-specific post-translational regulatory mechanism of cytochrome P-450.

Key words: drug metabolism/enzyme control/hepatocytes/membrane proteins/protein phosphorylation

Introduction

Cytochrome P-450 (P-450) is an endomembrane-anchored heme-protein that occurs in a variety of isoenzymic forms. It catalyzes the mono-oxygenation of a broad spectrum of compounds of exogenous and endogenous origin. Thus, P-450 plays key roles in such diverse fields as steroid metabolism, fatty acid and lipid metabolism, bile acid production and detoxification of drugs and chemical carcinogens as well as the activation of the latter to harmful metabolites (Jakoby, 1980; Schenkman and Kupfer, 1982; Ortiz de Montellano, 1986; Nebert and Gonzalez, 1987).

Some of the P-450 enzymes are inducible by exogenous compounds. This is a well-known and extensively studied process that requires *de novo* RNA and protein synthesis and leads to an inducer-specific increase of distinct sets of P-450 isoenzymes. For example, liver cells of rats respond to phenobarbital, a narcotic drug and liver tumor promoter (Kaufmann *et al.*, 1988), by a marked increase of two closely related isoenzymes, P-450b and P-450e (forms P-450IIB1 and P-450IIB2 according to the nomenclature by Nebert *et al.*, 1989), with little or no change of the other dozen hepatic P-450 isoenzymes known today (Nebert and Gonzalez, 1987). Because of the necessary *de novo* synthesis, induction of P-450 is a relatively slow process. The question then arises whether faster regulatory mechanisms such as post-translational modification by protein phosphorylation may also exist. This is significant both for tuning of the activity of a P-450 once synthesized and the half-life of a P-450 because degradation of proteins is known frequently to depend on their phosphorylation state. In fact, there are several indications that phosphorylation of P-450 may occur.

Various treatments that cause an increase in the cellular concentration of cyclic 3',5'-adenosine monophosphate (cAMP), such as the administration of catecholamines, have been known to depress hepatic drug metabolism (Fouts, 1962). The direct involvement of cAMP in the process was later demonstrated. A membrane-permeating derivative of cAMP, N^6,$O^{2'}$-dibutyryl cAMP (dbcAMP), was shown to decrease microsomal mono-oxygenase activities and the concentration of P-450 in rat liver (Weiner *et al.*, 1972a,b; Ross *et al.*, 1973). The dbcAMP treatment was also shown to inhibit the induction of cytochrome P-450 by phenobarbital (Hutterer *et al.*, 1975). The mechanism by which cAMP affects the mono-oxygenase system, however, remained unknown.

We have shown for the first time that purified P-450 LM_2 (P-450IIB4 according to the nomenclature by Nebert *et al.*, 1989), the major phenobarbital-inducible isoenzyme in rabbit liver, is phosphorylated by the catalytic subunit of cAMP-dependent protein kinase, and suggested that the main cellular target of cAMP action may be P-450 itself (Pyerin *et al.*, 1983, 1984, 1986). The phosphorylation was observed in soluble and in reconstituted liposome-bound states as well as in microsome-bound form, it showed physiologically meaningful K_m values and was accompanied by a decrease in enzyme activity (Pyerin *et al.*, 1984), which is caused by a conversion of P-450 to its enzymatically inactive form, P-420 (Taniguchi *et al.*, 1985). The phosphorylation was found to be isoenzyme specific, i.e. observed only with certain combinations of P-450 isoenzymes and protein kinases (Pyerin *et al.*, 1987). However, the effect of phosphorylation on P-450 isoenzymes other than the rabbit isoenzyme remained unknown. Moreover, all of the

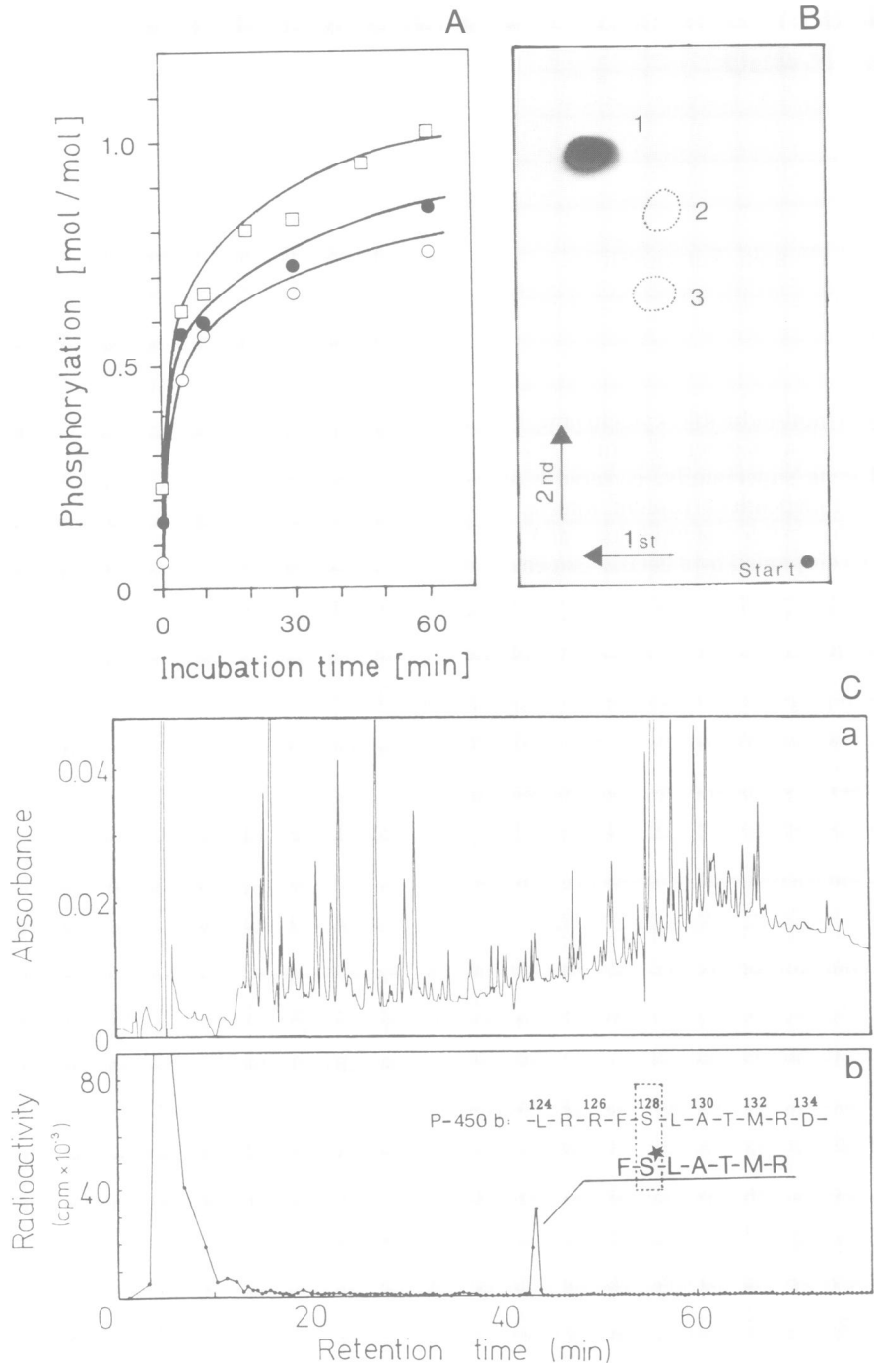

Fig. 1. Phosphorylation of rat hepatic phenobarbital-inducible cytochrome P-450b by catalytic subunit of cAMP-dependent protein kinase. (**A**) Time course of phosphorylation. Purified P-450b was incubated for the indicated times in the presence of [γ-^{32}P]ATP and varying amounts of catalytic subunit of cAMP-dependent protein kinase (○, 0.1 μM; ●, 0.5 μM; □, 1.0 μM) and the acid-precipitable radioactivity was determined. (**B**) Phosphorylated amino acid side chain. Radioactively phosphorylated P-450b was precipitated by trichloroacetic acid and subjected to partial hydrolysis by 6 N HCl followed by two-dimensional high voltage thin layer electrophoresis (1st dimension at pH 1.9, 2nd dimension at pH 3.5), ninhydrin staining of added standard phosphoamino acids and autoradiography. Shown is an autoradiogram with positions of standard phosphoamino acids indicated (1, phosphoserine; 2, phosphothreonine; 3, phosphotyrosine). (**C**) Phosphorylation site within P-450b. Radioactively phosphorylated P-450b was digested with trypsin and the resulting tryptic peptides were separated by HPLC with a Vydac C18 reverse-phase column using a linear gradient of acetonitrile as described in Materials and methods. The column eluate was monitored at 210 nm (**a**), the elucated fractions were collected and tested for radioactivity (**b**). The only peak which contained radioactivity (except for the large unbound radioactive peak due to ATP) is shaded.

phosphorylation experiments so far were carried out in cell-free systems with purified P-450 isoenzymes and purified protein kinases, thus bringing the *in vivo* relevance into question. This led us to investigate the phosphorylation of the major phenobarbital-inducible P-450 of rat liver (i) in its isolated state by the catalytic subunit of cAMP-dependent protein kinase and (ii) in primary cultured rat hepatocytes by activating the cellular kinase with the membrane-permeating analog of cAMP, dbcAMP, as well as with glucagon, a proteohormone known to activate adenylate cyclase.

The results obtained clearly indicate that the major

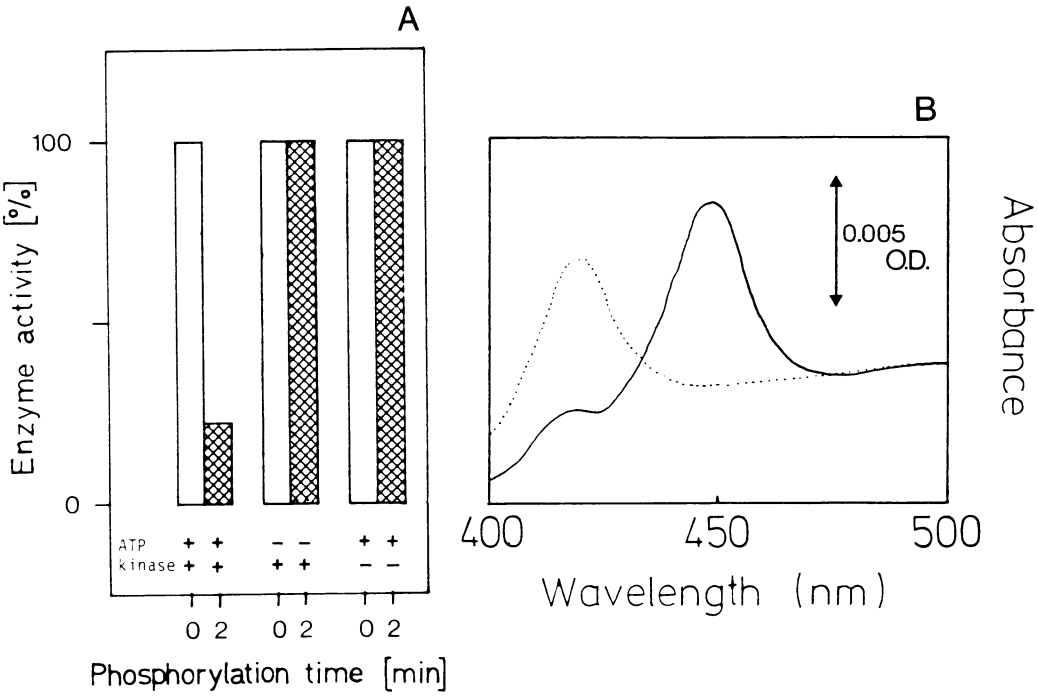

Fig. 2. Effect of phosphorylation of P-450b on enzyme activity and conformation. (**A**) Phosphorylation of P-450b and reconstituted mono-oxygenase activity. P-450b, reductase and lipids were supplied with ATP, catalytic subunit of cAMP-dependent kinase and Mg^{2+} and incubated at 37°C for 0 and 2 min followed by determination of 7-ethoxycoumarin *O*-deethylase activity as described in Materials and methods. (**B**) Phosphorylation and CO difference spectrum of P-450b. Purified P-450b was incubated with kinase and ATP as described in Materials and methods. After incubation at 37°C for 30 min, the incubation mixture was diluted with 100 mM potassium phosphate buffer (pH 7.3) containing 20% glycerol and 0.2% Tergitol NP-10 and CO difference spectrum was measured. ——, before incubation; - - -, after 30 min incubation.

phenobarbital-inducible rat P-450 is phosphorylated at the equivalent site and with the same effect as the rabbit liver counterpart and, most importantly, that the same phosphorylation does occur in intact hepatocytes as well, i.e. within P-450's native environment and through endogenous protein kinase activity. This suggests that in addition to the well-known induction phenomenon there exists the possibility of an isoenzyme-specific regulation of P-450 by post-translational modification through protein phosphorylation.

Results

Cell-free phosphorylation of rat liver P-450b

Purified rat liver P-450b was phosphorylated to a significant degree within minutes by the catalytic subunit of cAMP-dependent protein kinase. After 5 min, incorporation of 0.5−0.6 mol phosphate/mol P-450 was obtained (Figure 1A). Varying the kinase concentration within 0.1−1.0 μM, the cellular concentration range of the kinase (Beavo *et al.*, 1974) had little influence on the kinetics. Both the rate and the degree are comparable to protein phosphorylation reactions with established physiological significance (Krebs and Beavo, 1979). The phosphorylation plateau was reached in 30−60 min at a level of 0.7−1 mol phosphate/mol P-450 depending on the kinase concentration employed. A similar time course was observed with rabbit P-450 LM_2, although in this case the degree of phosphorylation did not exceed 0.6−0.7 mol phosphate/mol P-450 (Pyerin and Taniguchi, 1989).

The phosphoamino acid analysis of phosphorylated P-450b after acid hydrolysis showed exclusively serine phosphate (Figure 1B). Tryptic digestion of radioactively phosphorylated P-450b followed by separation of the phospho-peptides by reverse-phase HPLC resulted in only one radioactive peak (Figure 1C). Amino acid analysis of this peptide revealed the amino acid composition SRTAMLF. According to the known primary structure of P-450b (Fujii-Kuriyama *et al.*, 1982) and the known recognition sequence of the kinase (Krebs and Beavo, 1979), the corresponding sequence of the peptide was unambiguously assigned as F-S*-L-A-T-M-R with the phosphorylated serine residue in position 128 of the P-450 molecule (see Figure 1C). This is, as expected, the same phosphorylation site as that identified in rabbit liver LM_2 (Müller *et al.*, 1985; Pyerin *et al.*, 1986).

Effect of phosphorylation on P-450b

The phosphorylation of P-450b caused a dramatic decrease of its enzymatic activity (Figure 2A). The capability of metabolizing 7-ethoxycoumarin (*O*-deethylase activity) dropped within minutes to <20%. On the other hand, incubation of P-450b with either ATP or kinase alone had no effect at all. Thus the phosphorylation of Ser128 obviously exerts a severe effect on the activity of P-450b, most probably by altering the conformation.

A sensitive indicator of the conformational status of P-450 is its light absorption spectrum, especially the carbon monoxide difference spectrum of reduced cytochrome P-450. Upon phosphorylation, the typical P-450 absorption peak at 450 nm decreased while the shoulder at 420 nm developed into a peak (Figure 2B). Phosphorylation of a single serine residue therefore converts P-450b into its P-420 form which is known to be enzymatically inactive (Schenkman and Kupfer, 1982; Taniguchi *et al.*, 1985). The addition of a heat- and acid-stable inhibitory protein specific for the kinase (Walsh *et al.*, 1971) prevented both the phosphorylation and the conversion (data not shown).

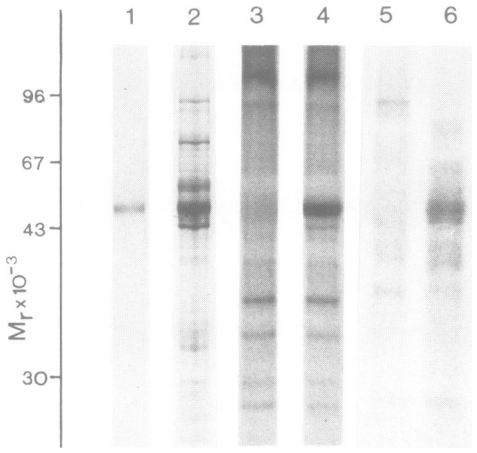

Fig. 3. Phosphorylation of microsomal proteins in intact hepatocytes. Hepatocytes isolated from phenobarbital-pretreated rats were incubated in the presence of $^{32}P_i$ with and without dbcAMP or glucagon. Microsomes were obtained by differential centrifugation from homogenates and analyzed by SDS−PAGE followed by autoradiography as described in Materials and methods. Shown are Coomassie Blue stains (**lanes 1** and **2**) and autoradiograms (**lanes 3−6**). **Lane 1**, isolated cytochrome P-450b (3 μg protein);. **lane 2**, microsomal protein pattern of hepatocytes (40 μg protein); **lanes 3** and **4**, microsomal phosphoprotein pattern of hepatocytes incubated in the absence and presence of dbcAMP (0.1 mM) respectively; **lanes 5** and **6**, microsomal phosphoprotein pattern of hepatocytes incubated in the absence and presence of glucagon (10 μM) respectively, with theophylline (1.1 mM) present in both. Postions of mol. wt standard proteins are indicated on the left.

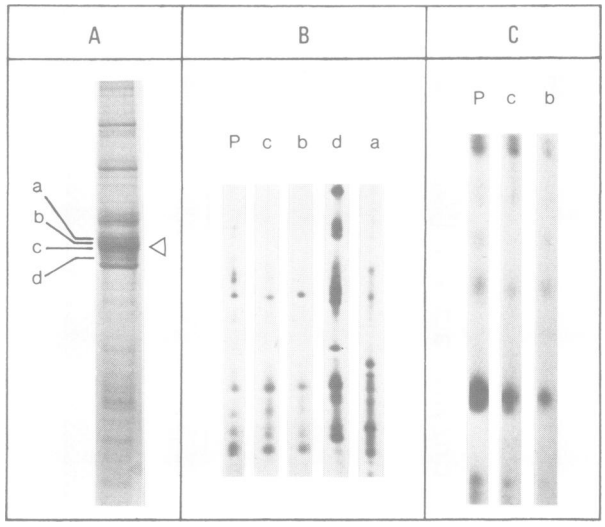

Fig. 4. Comparative peptide mapping of cytochrome P-450b and identically migrating microsomal protein. (**A**) Microsomal protein pattern of hepatocytes from phenobarbital-pretreated rats obtained by SDS−PAGE as in Figure 3. Indicated bands (**a−d**) were cut out from the gel for the mapping. ◁ marks position of P-450b. (**B**) Peptide patterns of *S.aureus* V_8 protease digests (silver stains). Microsomal proteins (**a−d**) and authentic P-450b (**P**) were digested under identical conditions, and peptides separated by SDS−PAGE.
(**C**) Phosphopeptide pattern of *S.aureus* V_8 protease digests (autoradiograms). Radioactively phosphorylated P-450b (**P**) and microsomal protein bands **c** and **b** were treated as in (**B**) and analyzed by autoradiography.

Phosphorylation of microsomal proteins in intact rat hepatocytes

In order to examine whether phosphorylation also occurs in intact hepatocytes, the exchangeable phosphate pools of the cells were labeled with $^{32}P_i$. It was presumed that $^{32}P_i$ becomes incorporated into ATP at the γ-position and finally donated to proteins. In primary cultured hepatocytes obtained from phenobarbital-treated rats, a number of microsomal proteins became radioactively labeled. Addition of dbcAMP to the cells, an agent known to permeate the plasma membrane and to cause the release of the catalytic subunit from regulatory subunits of protein kinase holoenzymes (Kübler *et al.*, 1979), or addition of the adenylate cyclase activator glucagon (together with the phosphodiesterase inhibitor theophylline) caused a dramatic increase in the degree of phosphorylation of microsomal protein(s) with an electrophoretic mobility corresponding to that of purified P-450b, while the rest of the phosphoprotein pattern remained nearly unaltered (Figure 3). Neither the addition of cAMP to hepatocyte cultures nor to buffers used in the preparation of microsomes had any effect on the phosphorylation of this protein (data not shown). This excludes both the possibility that the observed increase of phosphorylation in the presence of dbcAMP or glucagon is due to damaged cells and the possibility that phosphorylation occurred not within cells but rather during homogenization of cells and microsome preparation. Moreover, supplementation of preparation buffers with cold ATP at a concentration sufficiently high enough to stop any radioactive phosphorylation during microsome preparation did not influence the result. When hepatocytes prepared from untreated rats were examined in a similar way, the addition of dbcAMP had little effect on the phosphorylation pattern (data not shown). Thus, the strongly phosphorylated microsomal protein is inducible by phenobarbital and is a substrate of cAMP-dependent protein kinase.

Identification of phosphorylated microsomal protein as cytochrome P-450

The phosphorylated microsomal protein was identified as P-450 in two different ways, by comparative limited proteolysis and by immunoprecipitation.

For comparative limited proteolysis (i) purified P-450b was phosphorylated with the catalytic subunit of cAMP-dependent protein kinase in the presence of [γ-^{32}P]ATP and (ii) microsomes were prepared from hepatocytes isolated from livers of phenobarbital-pretreated rats and incubated in the presence of $^{32}P_i$ and dbcAMP. Both preparations were subjected to SDS−PAGE, and P-450b and the corresponding microsomal protein band as well as adjacent protein bands were cut out from the gel and digested under identical conditions by V_8 protease from *Staphylococcus aureus*. The peptide patterns as well as the phosphopeptide patterns of P-450 were highly similar to those of the corresponding microsomal protein (Figure 4). In contrast, microsomal proteins migrating slightly above and below the protein in question produced clearly different patterns. Thus the method is sensitive enough to indicate that the phosphorylated microsomal protein possesses extremely high sequence similarities to cytochrome P-450b (and its close relative P-450e).

For immunoprecipitation, antibodies were raised in rabbits against purified P-450b, and made monospecific by affinity chromatography on antigen-bound Sepharose. The microsomes were radioactively labeled in cells as above, solubilized with detergents and incubated with the antibody preparation. The antibody precipitated only one radioactive protein which migrated in SDS−PAGE identically to P-450b (Figure 5).

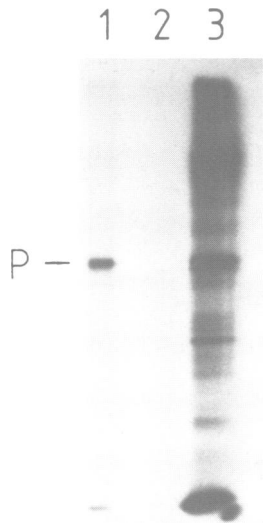

Fig. 5. Immunoprecipitation of microsomal protein co-migrating with P-450b by anti cytochrome P-450 antibody. Hepatocytes obtained from phenobarbital-pretreated rats were incubated in the presence of $^{32}P_i$ and dbcAMP as in Figure 3. Microsomes prepared by differential centrifugation were solubilized with detergents, incubated with monospecific antibody against P-450b or with IgG obtained from preimmune serum, and precipitates subjected to SDS−PAGE (**lane 1**, monospecific antibody; **lane 2**, IgG from preimmune serum; **lane 3**, solubilized microsomes). The position of purified P-450b is marked (P).

As a control, the solubilized microsomes were treated with immunoglobulin obtained from preimmune rabbit serum. In this case, very little, if any, radioactivity was precipitated from the labeled microsomes.

These results clearly established the identity of the phosphorylated microsomal protein as cytochrome P-450.

Phosphorylation of P-450 in hepatocytes and its effects on drug metabolism

The phosphorylation of P-450 caused by the addition of dbcAMP to hepatocytes shown in Figure 3 was observed within minutes and reached a plateau after 60 and 30 min in the presence of 0.1 and 1 mM dbcAMP, respectively. The degree of P-450 phosphorylation did not remain at a maximum level. Incubation of hepatocytes for prolonged periods in the presence of dbcAMP resulted in a lower phosphorylation at both dbcAMP concentrations employed (by roughly a quarter after 90 min and two-thirds after 180 min), while, on the other hand, the microsomal protein pattern remained unchanged (data not shown). The only amino acid phosphate ester detectable upon acidic hydrolysis was serine phosphate as in the cell-free phosphorylation of P-450b (see Figure 1). Together with the close similarity of the patterns of peptide maps of P-450 phosphorylated cell-free and within hepatocytes (see Figure 4), this clearly indicated that the same serine residue is phosphorylated in both cases.

The degree of phosphorylation was roughly assessed by the England−Walsh procedure (1976) by determining the specific radioactivity of ATP of cell extracts via ^{32}P-incorporation into phosphorylase b by phosphorylase kinase. Under the conditions employed, the degree of P-450 phosphorylation reached was of the order of 10%. This is sufficiently high enough to support its regulatory significance. Correspondingly, the P-450-dependent metabolic activity of hepatocytes measured with pentoxyresorufin as a substrate decreased faster in the presence of dbcAMP than in controls (data not shown). However, primary hepatocyte cultures are not suitable for this purpose because the P-450 content is not stable; it decreases rather rapidly even in the control cells.

Discussion

The phosphorylation of the purified phenobarbital-inducible rat liver P-450 isoenzyme by the catalytic subunit of cAMP-dependent protein kinase reaches ~1 mol phosphate/mol P-450b at the plateau phase and, of note with respect to its possible physiological function, a transfer of 0.5−0.6 mol phosphoryl groups/mol P-450b occurs within the first several minutes. This means that >50% of the P-450b molecules present in the assay mixture become phosphorylated quite rapidly if one phosphorylation site per P-450 molecule is assumed. Such an assumption appears valid, since (i) the primary structure of P-450b (Fujii-Kuriyama *et al.*, 1982) contains, as was determined for P-450 LM_2 (Heinemann and Ozols, 1982; Müller *et al.*, 1985; Pyerin *et al.*, 1986), the typical recognition sequence for the kinase -Arg-Arg-X-Ser- (Krebs and Beavo, 1979) only once, with the serine residue in position 128 as the amino acid side chain to the phosphorylated, (ii) phosphoserine was the only phosphoamino acid found in hydrolysates, (iii) only one radioactive tryptic peptide was obtained from radioactively phosphorylated P-450b and (iv) P-450 isoenzymes lacking the kinase recognition sequence such as rat P-450d (P-450IA2) and P-450c (P-450IA1), rabbit P-450 4 (P-450IA2) or bovine P-450 scc (P-450XIA1) (Black and Coon, 1986) are not phosphorylated (Pyerin *et al.*, 1987; see also below, Figure 6).

As demonstrated here, the same phosphorylation of P-450 can also occur in intact cells. Hepatocytes prepared from phenobarbital-treated rats showed a significant phosphorylation of microsomal protein(s) co-migrating in SDS−PAGE with purified P-450b. The identity of this protein as phenobarbital-inducible P-450 was established by several criteria. This protein shows highly similar peptide maps to those obtained with purified P-450b both in protein staining and in autoradiography, is selectively precipitated by monospecific antibodies against P-450b from solubilized microsomes and is found in measurable amounts only in hepatocytes isolated from rats pretreated with phenobarbital. However, the criteria employed cannot distinguish between P-450b and P-450e. These two forms show a 97% identity of their primary structure (Nebert *et al.*, 1987, 1989), are phosphorylated by the same kinase *in vitro* (Pyerin *et al.*, 1987), have no difference in their amino acid sequences near the phosphorylation site (see below), are immunologically cross-reactive, poorly resolved by SDS−PAGE and are highly similar in their peptide maps (Black and Coon, 1986). Since P-450e constitutes <10% the amount of P-450b in rat liver microsomes, its participation in the measured phosphorylation reaction cannot, therefore, be estimated.

The phosphorylation of P-450 in intact hepatocytes was triggered by increasing the cellular cAMP level either indirectly by activating adenylate cyclase through glucagon or directly by dbcAMP, a membrane-permeating analog of cAMP (Weiner *et al.*, 1972a,b; Ross *et al.*, 1973; Hutterer *et al.*, 1975). Binding of cAMP and of its analog to

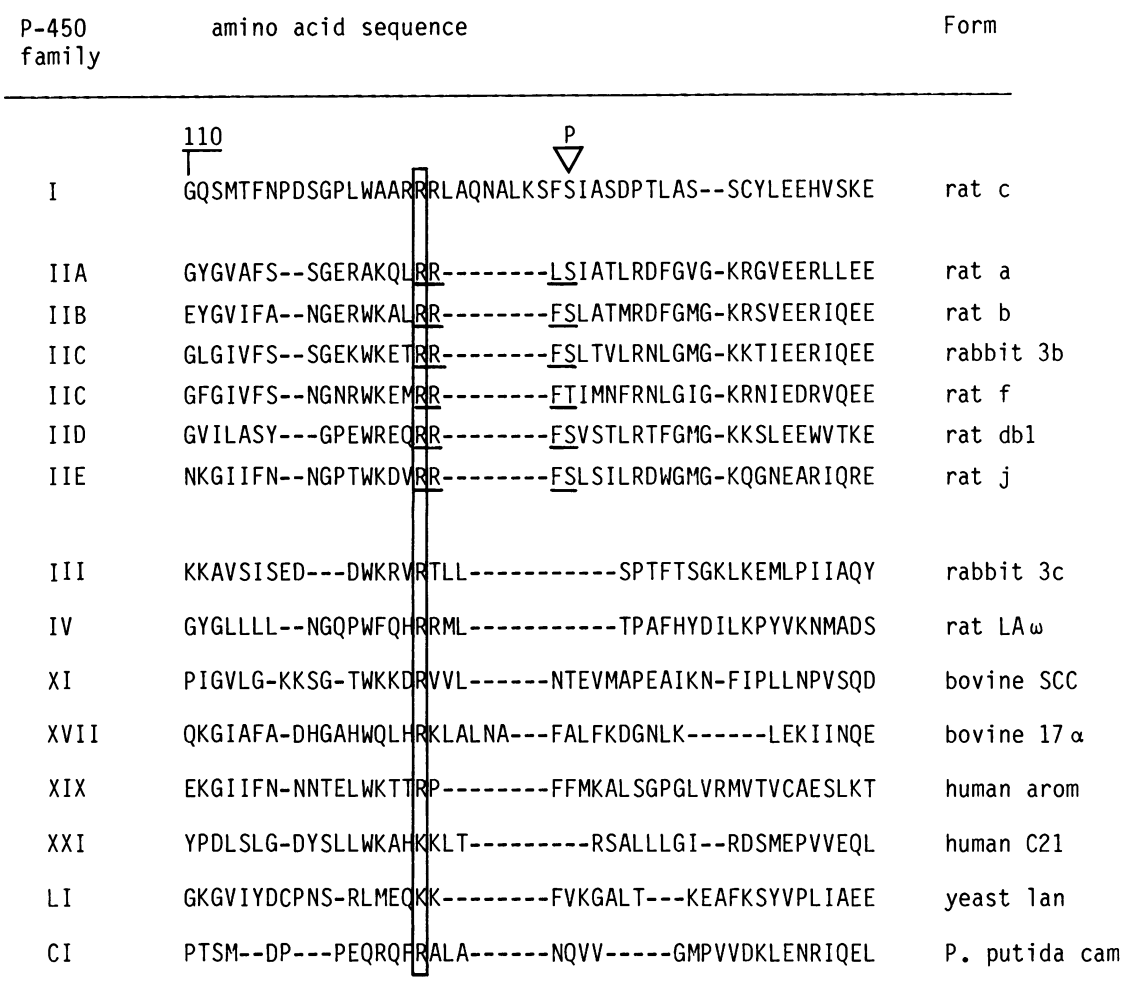

Fig. 6. Comparison of amino acid sequences near the phosphorylation site of representatives of each family of the P-450 gene superfamily. Classification and designations are according to Nebert and Gonzalez (1987). Alignment of the sequences was carried out as described in Materials and methods. The kinase recognition sequence is underlined. The phosphorylated serine residue is marked with P. The highly conserved arginine is boxed. Amino acid sequences were obtained from the following references: rat c (IA1) (Sogawa *et al.*, 1984); rat a (IIA1) (Nagata *et al.*, 1987); rat b (IIB1) (Fujii-Kuriyama *et al.*, 1982); rabbit 3b (IIC3) (Ozols *et al.*, 1985); rat f (IIC7) (Gonzalez *et al.*, 1986); rat db1 (IID1) (Gonzalez *et al.*, 1987); rat j (IIE1) (Song *et al.*, 1986); rabbit 3c (IIIA6) (Dalet *et al.*, 1988); rat LAω (IVA1) (Hardwick *et al.*, 1987); bovine scc (XIA1) (Morohashi *et al.*, 1984); bovine 17α (XVIIA1) (Zuber *et al.*, 1986); human arom (XIXA1) (Simpson *et al.*, 1987); human c21 (XXIA2) (Higashi *et al.*, 1986); yeast lan (LI) (Kalb *et al.*, 1987); *P.putida* cam (CI) (Haniu *et al.*, 1982).

regulatory subunits of cAMP-dependent protein kinase releases catalytic subunits from the holoenzyme (Kübler *et al.*, 1979; Pyerin *et al.*, 1979). Within cells, freed catalytic subunits obviously have access to the kinase recognition sequence of the membrane-bound P-450 molecules. This is in line with our previous observations where phosphorylation of rabbit P-450 LM_2 was observed in microsome- and liposome-bound states (Pyerin *et al.*, 1984, 1986). It has been reported that various *in vivo* treatments causing an increase of the cellular cAMP concentration decreased the P-450-dependent drug oxidase activities (Fouts, 1962; Kato and Gillette, 1965; Weiner *et al.*, 1972a,b) and that the administration of dbcAMP competes with the induction of P-450 by phenobarbital suggesting the involvement of protein phosphorylation in the protein biosynthesis (Hutterer *et al.*, 1975). The action of dbcAMP, however, occurs rather rapidly, i.e. the effects on drug metabolism were observed within minutes, while the protein turnover rate of P-450 is comparatively slow with a half-life of ~20 h (Sadano and Omura, 1983). A decrease in protein biosynthesis, therefore, cannot account for the rapid decrease in activity.

Such a rapid decrease can, however, easily be explained at the molecular level by the direct phosphorylation of P-450, since it is accompanied by a decrease in enzyme activity. The reason for this decrease is a conformational change indicated by an alteration of the carbon monoxide absorption spectrum of reduced P-450. The absorption maximum is shifted from 450 to 420 nm. The resulting 'P-420' is known to be enzymatically inactive (Schenkman and Kupfer, 1982; Taniguchi *et al.*, 1985). Since decrease in activity and conversion has also been found with other P-450 isoenzymes carrying the kinase recognition sequence (Pyerin *et al.*, 1984; Taniguchi *et al.*, 1985; Pyerin and Taniguchi, 1989), it is tempting to extend the regulation potential at the cellular level of P-450 activity. In addition to induction, a post-translational modification by phosphorylation has to be considered as a regulatory device. It is not known at the moment whether this is reversible or irreversible. The observed decrease in the degree of phosphorylation of P-450 in cells upon prolonged phosphorylation times may indicate reversibility. However, it could also reflect accelerated degradation mediated by phosphorylation.

The phosphorylation of P-450 and thus the post-translational regulation shows a strict isoenzyme specificity.

The kinase recognition sequence -Arg-Arg-X-Ser- is not generally found in P-450 isoenzymes. It is found rather exclusively in family II of the P-450 gene superfamily which is involved in the metabolism of foreign compounds such as drugs (Figure 6). Except for a few isoenzymes belonging to subfamily IIC, where Ser is substituted by Thr (which can also serve as phosphoryl group acceptor) all sequences of family II isoenzymes contain this sequence (Khani *et al.*, 1987; Umbenhauer *et al.*, 1987). Arginine residue 125 of the region, which is also part of the kinase recognition sequence and resides in the close vicinity of the phosphorylated serine residue, is highly conserved in P-450 isoenzymes. It is found in all P-450 isoenzymes including even P-450 of *Pseudomonas putida*, except a few isoenzymes where arginine is substituted by a lysine residue, i.e. by another basic amino acid residue. Arg125 can therefore be assumed to be part of an essential structural element of P-450 isoenzymes. It is not surprising that the introduction of two negative charges in its immediate vicinity by the phosphorylation of Ser128 can cause a conformational change; a change that obviously suffices to convert P-450 to P-420 and thus to 'turn off' the P-450 isoenzyme by phosphorylation.

After submission of the present manuscript, a report by J.A.Koch and D.J.Waxman (*Biochemistry*, **28**, 3145–3152, 1989) appeared confirming our results on the phosphorylation of phenobarbital-inducible P-450 in isolated hepatocytes and showing in addition that the corresponding phosphorylation occurs also in the liver of whole animals. The physiological relevance of the phosphorylation of certain P-450 isozymes, therefore, seems to be established.

Materials and methods

Animal treatment

Male Sprague–Dawley rats (180–230 g body weight; Zentralinstitut für Versuchstierzucht, Hannover, FRG), fed *ad libitum* on laboratory chow, were treated with phenobarbital dissolved in phosphate-buffered saline by i.p. injections of 80 mg/kg body weight once a day for 3–4 days. One day after the last injection and after 12–18 h of fasting, the animals were used for hepatocyte preparation or isolation of liver microsomes.

Hepatocytes

Hepatocytes were prepared according to the published method (Rumruen and Pool, 1984) in which liver perfusion of anesthetized rats with collagenase-containing solution is followed by excision of livers, liberation of hepatocytes and filtration through cheesecloth. The cells were washed twice by and suspended in Dulbecco's modified minimal essential medium (Flow Laboratories, Bonn, FRG) containing 10% fetal calf serum (Gibco, Paisley, UK). The freshly isolated hepatocytes were tested for viability by Trypan blue exclusion (0.2% in phosphate-buffer saline; Serva, Heidelberg, FRG) and adjusted with medium to 5×10^6 cells/ml. Suspensions used showed viabilities of >90%.

Phosphorylation in hepatocytes

Phosphorylation was carried out in plastic Petri dishes (Becton-Dickinson, Oxnard, USA) kept at 37°C in humidified air/CO_2 (95%/5%). In order to label the exchangeable phosphate pools, the cells were incubated for 30–45 min with occasional shaking in the presence of $^{32}P_i$ (Amersham Buchler, Braunschweig, FRG; 0.2–1 mCi/10^6 cells). Then, phenobarbital (0.8 mM final concentration) solubilized in prewarmed cell medium was added, together with dbcAMP (0.1 or 1.0 mM final concentration) or with glucagon (0.2 or 10 μM final concentration) and theophylline (1.1 mM final concentration), or with cAMP (0.1 or 1.0 mM final concentration) and incubation was continued. After the indicated times, the cells were sedimented at 8000 *g* for 2 min and washed twice with 0.15 M KCl/10 mM EDTA (pH 7.4). Cells were further processed either immediately or after storage at −75°C. The phenobarbital was added to cell suspensions in order to maintain P-450 levels as high as possible; in the absence of the drug, a considerable decrease has been observed within the time frame (up to 4 h) employed (Cánepa *et al.*, 1985).

The specific radioactivity of ATP in cell extracts was determined in cell homogenates prepared in 0.5 M $HClO_4$ at 0°C followed by centrifugation and neutralization with saturated $KHCO_3$ by the method developed by England and Walsh (1976). Standard [γ-^{32}P]ATP solutions were adjusted with neutralized $HClO_4$ solution and ATP concentration in cell extracts assayed by a luciferin-luciferase assay kit following the manufacturer's instructions (Boehringer Mannheim, FRG).

Microsomes

Microsomes were obtained by differential centrifugation from homogenates of hepatocytes (Pyerin *et al.*, 1986). Hepatocytes were homogenized in 0.15 M KCl/10 mM EDTA (pH 7.4) using a Potter–Elvehjem homogenizer (5×10^6 cells/ml). Microsomes were resuspended in 0.15 M KCl/10 mM EDTA (pH 7.4) or directly solubilized in SDS–PAGE buffer (see below) for 30 min at 60°C.

Cytochrome P-450b was purified from liver microsomes of phenobarbital-pretreated rats as described elsewhere (Funae and Imaoka, 1985; Taniguchi and Pyerin, 1989) to a specific concentration of 16–19 nmol/mg protein.

NADPH-cytochrome P-450 reductase was purified from the same liver microsomes used for cytochrome P-450 isolation according to previously published procedures (Taniguchi *et al.*, 1984; Taniguchi and Pyerin, 1989).

Catalytic subunit of cAMP-dependent protein kinase and the specific heat- and acid-stable inhibitory protein (Walsh *et al.*, 1971) were isolated from bovine heart and rat skeletal muscle respectively, and characterized as described elsewhere (Kübler *et al.*, 1979; Pyerin *et al.*, 1979).

Phosphorylation of cytochrome P-450

Purified P-450b was phosphorylated as described previously (Pyerin *et al.*, 1983, 1984, 1987; Taniguchi *et al.*, 1985). The incubation mixture contained 50 mM Hepes (pH 7.4), 10 mM Mg-acetate, 0.3 mM ATP, indicated concentrations (0.1–1.0 μM) of the catalytic subunit of cAMP-dependent protein kinase, and 6 μM P-450b. Incubation was carried out at 37°C. Aliquots were taken at indicated times and acid-precipitable radioactivity determined. When examining the influence of phosphorylation of P-450b on the reconstituted mono-oxygenase system, P-450b was mixed with purified NADPH-cytochrome P-450 reductase (ratio 1:1) and dilauroyl phosphatidylcholine, incubated at 37°C for 5 min, supplied with purified catalytic subunit of cAMP-dependent protein kinase, ATP, Mg-acetate and Hepes as above, further incubated at 37°C for the times indicated and finally investigated for 7-ethoxycoumarin *O*-deethylase activity in the presence of NADPH as described previously (Pyerin *et al.*, 1984).

For the determination of the phosphorylation site of P-450, P-450 (1 nmol) was incubated with 6 μg of the kinase in the presence of 100 μM [γ-^{32}P]ATP (100 μCi) in 0.35 ml of 60 mM Hepes (pH 7.4) containing 6 mM $MgCl_2$. After 30 min of incubation at 37°C, the reaction was stopped by adding 3.5 μl of 10% trifluoroacetic acid (TFA). The reaction mixture was directly absorbed to a SepPak C18 cartridge (Waters), and the cartridge was first washed with 5 ml of 0.1% TFA to remove most of the ATP, and P-450 was eluted with 70% acetonitrile in 0.1% TFA. After lyophilization, the sample was redissolved in 100 μl of 4 M urea in 0.1 M Tris–HCl (pH 8.0), and the mixture diluted with 100 μl of 0.1 M Tris–HCl buffer (pH 8.0). Five micrograms of TPCK-treated trypsin (Interchem, München, FRG) dissolved in 5 μl of 0.1 mM HCl was added and the mixture incubated for 18 h at 37°C. The reaction was stopped by adding 2 μl of 10% TFA, and the mixture was directly injected into an LKB HPLC apparatus. The resulting trypsin peptides were separated in a Vydac C18 reverse-phase column (4.6 × 250 mm, The Separation Group, CA, USA) using a linear gradient of 0–70% acetonitrile in 0.1% TFA for 120 min. The column eluate was monitored at 210 nm, and the collected fractions were measured for radioactivity in a LKB liquid scintillation counter. The amino acid composition of the peptides was determined after the hydrolysis in 6 N HCl by coupling with PITC as described by Tarr (1986).

Antibodies against cytochrome P-450b

The antibodies were raised in rabbits and purified by affinity chromatography on P-450-Sepharose (H.Taniguchi and W.Pyerin, in preparation). Only one protein band corresponding to P-450b was stained with the antibody in Western blots of liver microsomes from phenobarbital-induced rat liver.

Immunoprecipitation of P-450

Microsomes prepared from hepatocytes (~1 mg protein) were solubilized in 0.2 ml of 0.1 M potassium phosphate buffer (pH 7.3) containing 10% glycerol, 0.75% cholate and 0.25% Emulgen 913 (solubilization buffer) and the mixture centrifuged at 11 000 *g* for 15 min. Thirty microliters of the supernatant was diluted with 200 μl of solubilization buffer, and then incubated with 30 μl of the appropriate immunoglobulin preparation (corresponding to ~10 μg protein) at 4°C overnight. One hundred microliters of Protein A–Sepharose 4B equilibrated in solubilization buffer

was then added to the mixture and incubated at 4°C for 2 h. Protein A–Sepharose was collected by centrifugation and washed three times with the solubilization buffer and once with water. The immunoprecipitates were eluted from the Sepharose by incubating the Sepharose with 60 μl of 10 mM Tris–HCl buffer (pH 6.8) containing 1% SDS, 10% glycerol and 1% mercaptoethanol at 95°C for 20 min. After centrifugation, the supernatant was subjected to SDS–PAGE.

Analytical methods

SDS–PAGE, staining and autoradiography were carried out as described (Kübler *et al.*, 1982). Peptide mapping of excised protein or phosphoprotein bands from SDS–polyacrylamide gels was obtained by digestion with V_8 protease from *S.aureus*, silver staining and autoradiography as described (Pyerin *et al.*, 1986). Phosphoamino acids formed in P-450 were determined as described (Kübler *et al.*, 1982; Pyerin *et al.*, 1986). Protein determination in SDS–polyacrylamide gels as well as quantitation of autoradiograms was carried out densitometrically (Elscript 400, Fisher Scientific), protein in solutions was determined according to the procedure of Lowry *et al.* (1951). CO difference spectra of P-450 were measured according to Omura and Sato (1964) as described elsewhere (Taniguchi *et al.*, 1985). Mono-oxygenase activities were measured in cell-free systems by ethoxycoumarin deethylase activity as described (Pyerin *et al.*, 1984). In hepatocytes, the pentoxyresorufin dealkylase activity was determined in cell homogenates according to the published method (Lubet *et al.*, 1985). The alignment of P-450 sequences was performed according to the method of Needleman and Wunsch (1970) modified as described (Gotoh, 1982).

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