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Posttranslational modifications of the cytochrome P-450 monooxygenase system *,**

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Summary. Two forms of enzymatic posttranslational modifications of the monooxygenase system are described: modification by phosphatase and modification by protein kinase. Phosphatase treatment of microsomes isolated from phenobarbital-pretreated rabbits and rats caused a marked decrease of monooxygenase activity which was paralleled by a comparable decrease of NADPH-cytochrome P-450 reductase activity while the second essential component of the system, cytochrome P-450, remained unaltered. Thus phosphatase attacks monooxygenase via reductase. Protein kinases showed the opposite preference; while cytochrome P-450 was phosphorylated, NADPH-cytochrome P-450 reductase was not. Thus the kinase affects monooxygenase via cytochrome P-450. The phosphorylation of cytochrome P-450 turned out to be a specific reaction observed only with certain cytochrome P-450 isoenzymes and certain protein kinases.

Key words: Monooxygenase – Cytochrome P-450 – NADPH-cytochrome P-450 reductase – Protein kinase – Phosphatase

Introduction

Chemical carcinogens such as arylhydrocarbons require metabolic activation in order to become tumorigenic; bay-region diol epoxides have been proven or strongly implicated to account for the carcinogenic effect (Jerina et al. 1985). The essential epoxidations are catalyzed by cytochrome P-450-dependent monooxygenase, a membrane-anchored enzyme system (Schenkman and Kupfer 1982). It was therefore very tempting to suppose a correlation between the cellular level of monooxygenase activity and the tumorigenicity of a hydrocarbon. Employing the multistep model of chemical carcinogenesis on mouse skin, we demonstrated that tumorigenicity and increased levels of monooxygenase activity due to induction, a well-characterized form of monooxygenase regulation occurring at the genetic level (Pyerin and Hecker 1983; Adesnik and Atchison 1985), are not causally related (Pyerin and Hecker 1979; Pyerin et al. 1980). On the other hand, decreasing the monooxygenase activity using specific monooxygenase inhibitors caused a drastic decrease in tumorigenicity. The extent of this reduction showed a notable dependence on the length of the interval between administration of the carcinogenic hydrocarbon and the monooxygenase inhibitor; to obtain a significant decrease, the interval had not to exceed a few hours (Pyerin and Hecker 1979, 1980). This indicates that it is the "early phase" of monooxygenase-catalyzed metabolism which is decisive for tumor initiation. It follows that mechanisms able to rapidly regulate monooxygenase are of considerable importance. Is there any such mechanism?

The possibility of a short-term control appeared following our recent observation that monooxygenase components can be modified covalently through phosphorylation (Pyerin et al. 1983). Phosphorylation-dephosphorylation of proteins is known as a powerful and rapid control device employed extensively by cells to regulate numerous of their physiological systems (Krebs and Beavo 1979; Flockhart and Corbin 1982; Cohen 1982; Roach 1984). Phosphorylation was observed in vitro when the catalytic subunit of cyclic AMP-dependent protein kinase was incubated in the presence of ATP with rabbit liver cytochrome P-450 isoenzyme 2 (P-450 LM 2) (Pyerin et al. 1983), the component accomodating the active center of the monooxygenase system (Schenkman and Kupfer

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1982). The phosphorylation occurred with physiological "meaningful" characteristics (K_m values etc., Pyerin et al. 1984) and was observed with solubilized P-450 LM 2 as well as with P-450 LM 2 incorporated into model membranes (Pyerin et al. 1984) and also with microsome-bound P-450 LM 2 (Pyerin et al. 1986). Phosphorylation of isolated P-450 LM 2 was accompanied by a decrease in activity of the reconstituted monooxygenase (Pyerin et al. 1984) indicating a deactivation mechanism. This mechanism was found to be a conversion of cytochrome P-450 to enzymatically inactive cytochrome P-420 (Taniguchi et al. 1985) due to phosphorylation of the serine residue at position 128 of the molecule (Müller et al. 1985; Pyerin et al. 1986). This position is in close proximity to one of two highly conserved regions of the protein which are thought to be involved in heme binding (Tarr et al. 1983; Gotoh et al. 1983).

In addition to the phosphorylation, monooxygenase was also found to be affected by dephosphorylating enzymes. Treatment of microsomes obtained from livers of rabbits and rats with alkaline and acid phosphatases caused a marked decrease in monooxygenase activity (Pyerin et al. 1983; Sanghvi et al. 1981; Goodwin et al. 1982). Thus dephosphorylating enzymes have a similar effect on monooxygenase activity as phosphorylating enzymes.

In the present study, we investigated this contradiction. It is shown that the site of action of phosphatase and kinase on the monooxygenase system differ; while the kinase acts on the cytochrome P-450 component, the phosphatase affects the NADPH-cytochrome P-450 reductase. The second part deals with the question whether phosphorylation of cytochrome P-450 is a phenomenon occurring exclusively in isoenzyme 2 of rabbit liver and exclusively with the catalytic subunit of cyclic AMP-dependent protein kinase. It is shown that there is no such restriction; phosphorylation can be obtained both with different cytochrome P-450 isoenzymes and with different protein kinases, although it is not a general but a selective phenomenon observed only with certain P-450 forms and certain kinases.

Materials and methods

Microsomes were prepared as described elsewhere (Pyerin et al. 1986) from livers of New Zealand white rabbits and Sprague-Dawley rats which had been pretreated for 1 week by daily i.p. injection of 50 mg and 80 mg phenobarbital in saline, respectively. Cytochrome P-450 isoenzymes were purified from such microsomes; P-450 LM 2 and LM 3c from rabbit microsomes (Imai et al. 1980; Ingelman-Sundberg and Glaumann 1980), P-450 PB 1a, PB 1b, PB 2a, and PB 3b from rat microsomes (Wolf et al. 1986; several forms generously supplied by Dr. C. R. Wolf, Imperial Cancer Research Fund, Laboratory of Molecular Pharmacology and Drug Metabolism, Edinburgh, UK). NADPH-cytochrome P-450 reductases were isolated from the same microsomes as described by Taniguchi et al. (1984). The catalytic subunit of cyclic AMP-dependent protein kinase was prepared from rat and rabbit skeletal muscle (Pyerin et al. 1979; Kübler et al. 1979). One unit of kinase activity was defined as that amount of enzyme which transfers 1 pmol phosphoryl group in 1 min at 37 °C to "basic" substrate proteins, a histone mixture (type IIA, Sigma). Calcium-phospholipid-dependent protein kinase was isolated from chick oviduct (Horn et al. 1985), and kinase units defined as above. Phosvitin/casein kinase type II was isolated from rat livers (Pyerin et al. 1981; Pyerin et al. submitted), and kinase units defined as above but with phosvitin (Sigma) as the "acidic" substrate protein. NADPH-dependent O-deethylase activity was determined by measuring the formation of hydroxycoumarin from 7-ethoxycoumarin (Ullrich and Weber 1972) and expressed as nanomoles product formed per min and milligram protein at 37 °C. NADPH-dependent cytochrome *c* reductase activity was assayed by the increase in absorbance at 550 nm (Imai 1976), and expressed as reduction of micromoles cytochrome *c* per min and milligram protein. Cytochrome P-450 and P-420 contents were determined from CO difference spectra obtained after dilution of microsomes as given below, bubbling with CO, and reducing with dithionite (Omura and Sato 1964). Protein was determined by the Lowry procedure (Lowry et al. 1951) using bovine serum albumin as standard. The phosphatase treatment of microsomes was carried out in 0.1 *M* Tris-Cl buffer (pH 8.0) with 0.08 units of alkaline phosphatase (Sigma; 1 unit: enzyme amount hydrolyzing 1 μmol p-nitrophenylphosphate/min at pH 8.0 and 37 °C) per milligram microsomal protein at 37 °C. Aliquots were taken after defined periods (see *Results*), immediately diluted with 0.1 *M* potassium phosphate buffer (pH 7.3) containing 0.1 m*M* ethoxycoumarin (for determination of monooxygenase activity), or with 0.3 *M* phosphate buffer (pH 7.3) containing 50 μ*M* cytochrome *c* (for determination of reductase activity), or with 0.1 *M* potassium phosphate buffer (pH 7.3) containing 20% glycerol and 0.2% Emulgen 913 (for determination of cytochrome P-450 and P-420 content). The phosphorylation of cytochromes P-450 and of P-450 reductases was carried out with 1–2 units of the respective protein kinase and 1–5 μg of protein of P-450 or P-450 reductase per assay in presence of 50 m*M* Hepes buffer (pH 7.4), 10 m*M* Mg^{2+}, and 5–150 μ*M* γ-^{32}P ATP (Amersham Buchler, Braunschweig; sp. act. >20 Ci/mmol). If calcium-phospholipid-dependent kinase was employed, assays were additionally supplemented with 1.5 m*M* dithioerythritol and 10 μ*M* $CaCl_2$. Reactions were terminated through addition of sodium dodecyl sulfate-containing sample buffer for electrophoresis which was followed by polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate, protein staining, autoradiography, and scanning (Kübler et al. 1982). Every series of assays included either cytochrome P-450 LM 2 as reference standard (cyclic AMP-dependent protein kinase) or cytochrome P-450 PB 2a (calcium-phospholipid-dependent protein kinase).

Results and discussion

Incubation of hepatic microsomes obtained from both phenobarbital-treated rabbits and rats in the presence of alkaline phosphatase caused a time-dependent decrease in monooxygenase activity (determined as 7-ethoxycoumarin deethylation). After 60 min, monooxygenase activity was diminished to 10% and 30% in rabbit and rat microsomes, respectively (Table 1). No change in activity occurred in microsomes treated in the same way but without phosphatase or in the presence of both phosphatase and phosphatase inhibitor (EDTA, 5 m*M* final concentration). Addition of protease inhibitors (phenylmethylsulfonylfluoride, Nα-p-tosyl-L-lysine-chloromethyl-ketone, pepstatin A, or

Table 1. Effect of alkaline phosphatase on microsomal monooxygenase activity, content of cytochrome P-450 and P-420, and on NADPH-cytochrome P-450 reductase activity
Microsomes prepared from livers of phenobarbital-treated rabbits and rats were incubated for 0 and 60 min in the presence of alkaline phosphatase followed by determination of monooxygenase activity, NADPH-cytochrome P-450 reductase activity, and content of cytochrome P-450 and P-420 as described in *Materials and methods*. The values obtained were referred to the respective zero time values (100%). Deviations from the mean less than 10%

Source of microsomes	Phosphatase treatment [min]	Monooxygenase activity[a] [%]	Cytochrome content[b] P-450	P-420	Reductase activity[c] [%]
Rabbit liver	0	100	86	14	100
	60	10	83	17	8
Rat liver	0	100	89	11	100
	60	30	86	14	23

[a] 7-Ethoxycoumarin 0-deethylation; absolute values (Mean ± SD; $n = 5$) were 2.2 ± 0.1 and 0.9 ± 0.1 nmol/min per mg protein of rabbit and rat microsomes, respectively
[b] Taken from CO difference spectra; quadruplicate samples; sum total of P-450 and P-420 set 100%
[c] Cytochrome *c* reduction; absolute values (mean ± SD; $n = 4$) were 0.22 ± 0.01 and 0.25 ± 0.01 μmol/min per mg protein of rabbit and rat microsomes, respectively

trypsin inhibitor) to the incubates also had no significant influence excluding the possibility that the phosphatase effect might be simulated by a protease impurity of the phosphatase preparation employed.

Because phosphatase removes phosphate groups from proteins, it was obvious to suppose that one or both of the two main components constituting the monooxygenase system were dephosphorylated and that the dephosphorylation changed the component's activity and thereby that of the whole system. Since cytochrome P-450 LM 2 is abundant in microsomes prepared from phenobarbital-treated rabbits, and since LM 2 is phosphorylated (Pyerin et al. 1983, 1984, 1986) and the phosphorylation accompanied by a conversion of P-450 to enzymatically inactive P-420 (Taniguchi et al. 1985), the microsomal content of P-450 and P-420 was followed during phosphatase treatment (determined from CO difference spectra). No significant change was observed either in rabbit or rat microsomes (Table 1). Thus the cytochrome component obviously has little to do with the effect of the employed phosphatase on monooxygenase activity. This is in clear contrast to the effect of the phosphatase treatment noted on the second monooxygenase component, the NADPH-cytochrome P-450 reductase.

The reductase activity (determined as cytochrome *c* reduction) decreased drastically; after 60 min, the remaining activity was 8% and 23% in microsomes from rabbit and rat liver, respectively (Table 1). This decrease is of the same order of magnitude as that of the respective monooxygenase activity and therefore is sufficient to explain the phosphatase effect on the microsomal monooxygenase system.

This finding seems to indicate that NADPH-cytochrome P-450 reductase is also a phosphorylatable protein and that the phosphatase causes a change in activity due to dephosphorylation. However, the reductase isolated from both rabbit and rat liver microsomes was not significantly phosphorylated [below 0.05 mol phosphate (P)/mol reductase] by any of three different types of protein kinases, the catalytic subunit of cyclic AMP-dependent kinase (see also Pyerin et al. 1983, 1984), the calcium-phospholipid-dependent kinase, and the phosvitin/casein kinase II (Table 2). The mechanism of action of the phosphatase on NADPH-cytochrome P-450 reductase therefore is not clear. Preliminary data indicate that it is the prosthetic group of the reductase which is attacked by the phosphatase. This issue is currently under investigation.

So far, rabbit liver cytochrome P-450 LM 2, the main phenobarbital-inducible form, is the only cytochrome P-450 proved to be phosphorylated. It was therefore of interest to determine whether other phenobarbital-inducible P-450 forms from rabbit liver and liver from other animal species are also accepted as kinase substrates. The catalytic subunit of cyclic AMP-dependent protein kinase, the kinase phosphorylating P-450 LM 2 (Pyerin et al. 1983, 1984, 1986), was also active on two rat liver cytochromes, namely P-450 forms PB 3a and PB 3b (Table 2). While the degree of phosphorylation of P-450 PB 3b was comparable to that of P-450 LM 2, i.e., approximately 0.3 mol P/mol P-450 under these conditions (see Pyerin et al. 1984), phosphorylation of P-450 PB 3a was at least twice as high. Phosphorylation of cytochrome P-450 is thus neither a special case restricted

Table 2. Cytochrome P-450 isoenzymes and P-450 reductase as substrates of different protein kinases
Cytochromes P-450 and P-450 reductases were isolated from liver microsomes of phenobarbital-treated rabbits and rats, incubated with γ-^{32}P ATP and the catalytic subunit of cyclic AMP-dependent kinase, calcium-phospholipid-dependent protein kinase, or phosvitin/casein kinase II. The degree of phosphorylation was estimated semiquantitatively by densitometer tracing following polyacrylamide gel electrophoresis (in presence of sodium dodecyl sulfate) and autoradiography. Details are given in *Materials and methods*. Reference: phosphorylation of cytochrome P-450 LM 2 by cyclic AMP-dependent kinase (+; approximately 0.3 mol P/mol P-450), and of P-450 PB 2a by calcium-phospholipid-dependent kinase

Monooxygenase component	Animal species	Relative extent of phosphorylation	
		Catalytic subunit of cAMP-dependent kinase	Calcium-phospholipid-dependent kinase
Cytochrome P-450			
LM 2	Rabbit	+	−
LM 3c	Rabbit	−	(+)
PB1a	Rat	−	+
PB1b	Rat	−	−
PB2a	Rat	−	+
PB3a	Rat	++	++
PB3b	Rat	+	−
NADPH-cytochrome P-450 reductase	Rabbit	−	−
	Rat	−	−

to rabbit liver nor to a single cytochrome P-450 isoenzyme. However, it is obviously not a general phenomenon; P-450 LM 3c, from rabbit liver, and P-450 PB 1a, PB 1b, and PB 2a from rat liver behaved as nonsubstrates (Table 2). When phosphorylation was attempted with calcium-phospholipid-dependent protein kinase, an enzyme accepting "basic" substrate proteins such as histones similar to cyclic AMP-dependent kinase but underlying a different regulation in the cell (Nishizuka 1984), there was again a differential phosphorylation of cytochrome P-450 isoenzymes. This time, however, P-450 LM 2 and P-450 PB 3b were not significantly phosphorylated, while P-450 PB 1a and P-450 PB 2a became substrates. The highest degree of phosphorylation was again noted with P-450 PB 3a, whereas P-450 PB 1b was not accepted as a substrate. The third protein kinase employed is known to prefer "acidic" substrates such as phosvitin or caseins (Pyerin et al. 1981). This kinase accepted none of the cytochromes P-450 investigated.

Conclusion

Protein kinases and phosphatases attack the monooxygenase system at different sites. Phosphatase attack obviously occurs at cytochrome P-450 reductase, kinase attack at the other essential component of the system, cytochrome P-450. While it is clear that the kinase acts by introducing a phosphoryl group into cytochrome P-450 at a critical site causing a conformational change in the protein, the mechanism of action of the phosphatase remains to be established. Cytochromes P-450 and protein kinases show a rather specific pattern of interactions, that is, certain P-450 species are phosphorylated by certain protein kinases while others are not.

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