Chemical modification of microsomal cytochrome P450: role of lysyl residues in hydroxylation activity

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Cytochrome P450 purified from phenobarbital-induced rat liver microsomes was acetylated at 3 lysyl residues. When reconstituted with purified NADPH-cytochrome P450 reductase, the modified cytochrome showed full activity and substrate-induced spectral changes with d-benzphetamine. With 7-ethoxycoumarin, neither enzymic activity nor binding was detected. It is concluded that the positively charged lysine residues of cytochrome P450 are important for metabolism of 7-ethoxycoumarin by cytochrome P450.

Microsomal monoxygenase *Cytochrome P450 activity* *Acetylation* *Reconstitution*

1. INTRODUCTION

Cytochrome P450 and NADPH-cytochrome P450 reductase are key enzymes of the hepatic microsomal monooxygenase system, catalyzing the oxidative metabolism of endogenous substrates and many xenobiotics [1–3]. The reductase ($M_r \sim 78000$) is anchored to the membrane via a small ($M_r \sim 6000$) hydrophobic segment [4,5]. The large hydrophilic part protrudes from the membrane into the cytoplasmic space and accepts electrons from NADPH. Cytochrome P450 ($M_r \sim 50000$), on the other hand, is deeply imbedded in the membrane [6]. The arrangement of the two enzymes in the membrane and their odd stoichiometry (20–30 cytochromes/reductase) raises questions as to the mechanism of electron transfer from the reductase to the cytochrome and the functional interactions of the proteins in the monooxygenase system.

Some progress has been made in our understanding of the structure of cytochrome P450. Amino acid sequences of several species are now available [7,8]. In addition the dimension of the heme pocket has been partially characterized by heme alkylation studies [9,10].

By combining physical, biochemical and immunological techniques we demonstrated the formation of heterodimeric complexes between cytochrome P450 and its reductase co-reconstituted into liposomal vesicles and capable of substrate hydroxylation [11–13]. We are now chemically modifying specific sites of the proteins to improve our understanding of the functional interactions of the proteins. Here, we show that limited acetylation of lysyl residues of cytochrome P450 leads to a complete loss of ethoxycoumarin dealkylation activity whereas the dealkylation of benzphetamine is not affected.

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; EDTA, ethylene diaminetetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid

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2. MATERIALS AND METHODS

Phospholipids [egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), bovine spinal cord phosphatidylserine (PS), all grade I] were purchased from Lipid Products (South Nutfield) and stored at −20°C. Dilauroylphosphatidylcholine was from Sigma (St. Louis, MO). [^{3}H]-Acetic anhydride (500 mCi/mmol) was from the Radiochemical Centre (Amersham). d-Benzphetamine was from Upjohn. 7-Ethoxycoumarin was prepared as in [14]. All other reagents were of the highest grade available.

2.1. *Analytical procedures*

All spectral analyses were performed at 25°C using a Cary 219 spectrophotometer. Cytochrome P450 concentration was determined as in [15]. Protein concentration was measured by the Lowry assay [16] using bovine serum albumin as a standard. SDS–PAGE was performed according to Laemmli [17].

Isolation of cytochrome P450, NADPH-cytochrome P450 reductase and the preparation of proteoliposomes (PC/PE/PS, 10:5:1, by wt) was perfofmed as in [12]. Either protein showed a single band when analyzed on SDS–PAGE. Additional evidence that we have only a single species of cytochrome P450 in our preparation is supported by its similar catalytic activity and substrate specificity to that of cytochrome P450 PB-B described in [18]. For the enzyme assays, acetylated cytochrome P450 containing vesicles were dissociated by cholate. NADPH-cytochrome P450 reductase was added and vesicles were formed again by the cholate-dialysis techniques.

2.2. *Reconstitution in dilauroylphosphatidylcholine*

Cytochrome P450 and NADPH-cytochrome P450 reductase were mixed with freshly sonicated dilauroylphosphatidylcholine (0.2% (w/w) in 0.1 mM EDTA) to give protein/lipid = 1:10 (w/w). The samples were incubated for 10 min at room temperature. In all reconstitution experiments the molar ratio of cytochrome P450 to reductase was 5:1.

2.3. *Acetylation of cytochrome P450*

A method for the acetylation of ϵ-lysyl-amino groups [19] was applied to cytochrome P450. One volume of cytochrome P450 (26 μM), pre-equilibrated with 50 mM HEPES (pH 8.0, 20% glycerol), was mixed with half this volume of a freshly prepared, saturated sodium acetate solution, containing 20% glycerol and [^{3}H]acetic anhydride (10 mM, 30 μCi/ml). The solution was stirred at room temperature. After 10 min the same amount of the above-mentioned sodium acetate acetic anhydride solution was added. Stirring was continued for 20 min. Cytochrome P450 in vesicles was acetylated by the same procedure.

2.4. *Determination of the acetylated amino acids*

^{3}H was measured with an Isocap 300 liquid scintillation counter, after separation of the protein by SDS–PAGE. The gel containing cytochrome P450 was sliced and dissociated in 500 μl hydrogen peroxide (30%) at 60°C overnight, prior to adding the scintillation liquid. The nature of the acetylated residues was affirmed by amino acid analysis after dansylation [20] of the protein.

2.5. *Enzyme assays*

Ethoxycoumarin *O*-de-ethylase activity was measured as in [14] except that cytochrome P450 either in vesicles or in solution in the presence of a NADPH regenerating system (glucose 6-phosphate (5 mM), glucose 6-phosphate dehydrogenase (2.5 units)) was used instead of microsomes. Benzphetamine hydroxylation was determined as in [12]. Substrate-induced difference spectra in the Soret region were recorded in tandem cuvettes with vesicles in 100 mM KP_i (pH 7.4, 20% glycerol).

3. RESULTS

Specific acetylation of lysyl residues can be achieved with acetic anhydride in the presence of sodium acetate [19]. When dimethyl sulfoxide was used as solvent of acetic anhydride to prevent its rapid hydrolysis cytochrome P450 proved unstable. At 5°C and with a 400-fold excess of acetic anhydride over cytochrome P450 in aqueous buffer no significant heme loss (< 5%) or denaturation of the cytochrome as judged by heme·CO spectroscopy was detected. The extent of acetylation was reproducible under these conditions. Fig.1. shows the protein pattern and distribution

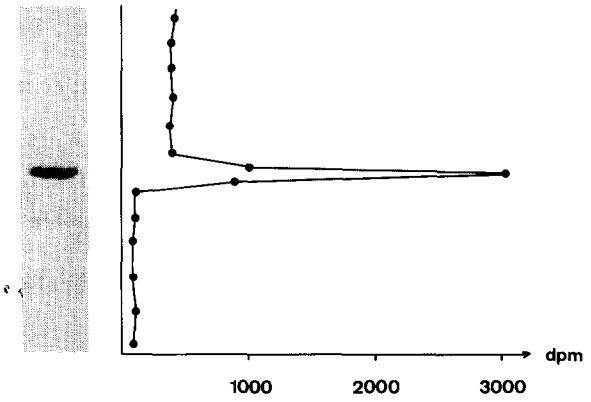

Fig.1. Protein pattern and distribution of radioactivity of acetylated cytochrome P450. 10–15% SDS–PAGE was performed as in [17]; cytochrome P450 was acetylated with [^{3}H]acetic anhydride as in section 2. Each lane was loaded with 0.12 nmol cytochrome P450.

of radioactivity of acetylated cytochrome P450 analyzed on SDS–PAGE. From these experiments, 2.8 ± 0.5 ($n = 23$) acetyl residues were calculated to be bound per one cytochrome molecule. Precipitation of the modified protein by acid and subsequent filtration followed by liquid scintillation indicated attachment of 3.0 ± 0.2 ($n = 3$) acetyl groups to cytochrome P450. The specificity of the modification and the number of lysyl residues modified was confirmed by acid hydrolysis and amino acid analysis as in [21] (not shown). Acetylation of cytochrome P450 in solution (i.e., in the presence of dilauroylphosphatidylcholine) gave similar results. Acetylation of phospholipids was not detected.

Acetylated cytochrome P450 reconstituted into liposomes or in solution showed $110\% \pm 50\%$ ($n = 12$) of the activity of the native protein with benzphetamine as substrate for hydroxylation. Within a single set of experiments reproducibility of these activity measurements was good ($\pm 10\%$). Large variations were observed however between different sets of experiments, with values ranging from 30–194 nmol formaldehyde formed . min^{-1} . nmol cytochrome P450^{-1}. The dealkylation rate of ethoxycoumarin catalyzed by native cytochrome P450 reconstituted into liposomes or in solution was 250 ± 26 pmol ($n = 8$) min^{-1} . nmol cytochrome P450^{-1}. No dealkylation of this substrate by acetylated cytochrome P450 was observed. The presence of poly(lysine) in amounts equimolar to cytochrome P450 completely inhibited the dealkylation of ethoxycoumarin whereas this polycation did not alter the hydroxylation of benzphetamine.

Substrate binding studies of native or acetylated cytochrome P450 reconstituted into liposomes or in solution showed typical type I spectra with benzphetamine (fig.2a,b) while ethoxycoumarin induced spectral changes only in the native (fig.2c) but not in the acetylated cytochrome.

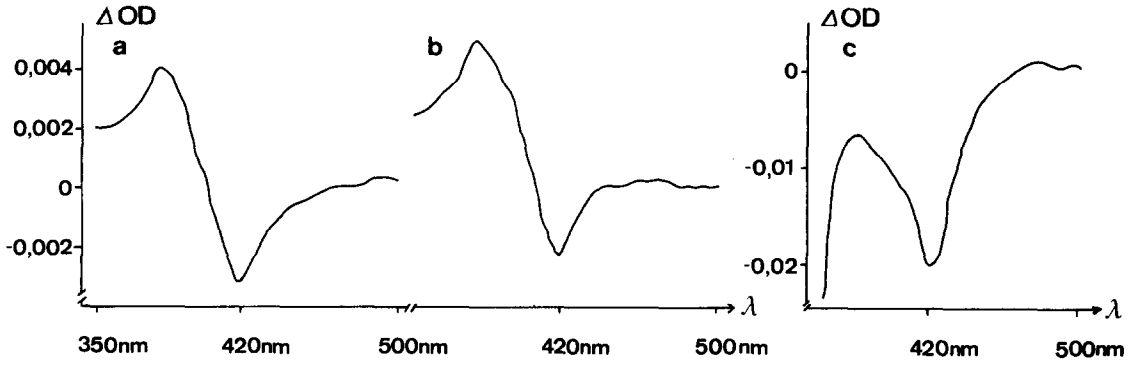

Fig.2. Substrate-induced difference spectra of cytochrome P450 reconstituted in liposomes: (a) native cytochrome P450 (0.26 μM, 1 mM benzphetamine); (b) cytochrome P450 (0.26 μM acetylated at 3 lysyl residues, 1 mM benzphetamine); (c) native cytochrome P450 (2.6 μM, 1.5 mM ethoxycoumarin).

4. DISCUSSION

Assuming the existence of only one species of cytochrome P450 in our preparation (see section 2) the absence in acetylated cytochrome P450 of an ethoxycoumarin binding spectrum and the lack of dealkylation of this compound is most logically explained by an impaired binding to the modified cytochrome. Ethoxycoumarin is a more polar molecule than benzphetamine, the other substrate used in this study. The absence of any metabolism of ethoxycoumarin by cytochrome P450 with a decreased number of positive charges or by native cytochrome P450 in the presence of poly(lysine) suggests the importance of polar residues for channeling of this substrate towards or binding to the active site of the protein.

Cytochrome *c*, an unphysiological electron acceptor of NADPH-cytochrome P450 reductase has a ring of lysyl residues around its active site [22]. Acetylation of lysyl residues of cytochrome *c* inhibits reduction of the cytochrome by NADPH-cytochrome P450 reductase [23]. Whether lysyl residues are also necessary for electron transfer to cytochrome P450, the physiological electron acceptor of the reductase is not known at present. The unchanged hydroxylation activity with benzphetamine as substrate of cytochrome P450 modified at lysyl residues argues against a decreased rate of electron transfer from the reductase to the modified cytochrome P450. Studies of the reduction kinetics of the native and modified cytochrome are currently being undertaken in our laboratory to clarify this point.

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