MULTIPLICITY OF CYTOCHROME P_{450} HEMOPROTEINS IN RAT LIVER MICROSOMES

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SUMMARY

Three hemoproteins have been resolved by SDS-polyacrylamide gel electrophoresis of rat liver microsomes. The hemoproteins, identified by staining with benzidine and H_2O_2, have apparent molecular weights of 44,000, 50,000, and 53,000. Pretreating rats with phenobarbital induces the hemoprotein with molecular weight 44,000 while pretreatment with 3-methylcholanthrene induces the hemoprotein of molecular weight 53,000. The hemoprotein of molecular weight 50,000 predominates in microsomes from untreated rats. These observations provide evidence for multiple forms of cytochrome P_{450}.

INTRODUCTION

Rat liver endoplasmic reticulum (microsomes) contains a mixed-function oxidase which utilizes NADPH and O_2 in the hydroxylation of drugs, steroids, fatty acids, and a wide variety of xenobiotics (1,2). The manner by which this system catalyzes the hydroxylation of a large and varied number of substrates is unknown. It is becoming an increasingly popular view that specificity is imposed upon the system by the existence of multiple forms of the substrate-binding, terminal oxidase, cytochrome P_{450}. This hypothesis is based upon the knowledge that different spectral (1,2) and catalytic (3-8) forms of this cytochrome can be induced in rat liver microsomes by pretreatment of animals with phenobarbital (PB) or 3-methylcholanthrene (3-MC). Furthermore, the separation of three spectrally distinguishable forms of this cytochrome on DEAE-cellulose after protease digestion and detergent-solubilization of microsomes has been reported (9).

We have recently presented additional evidence for the multiplicity

of microsomal cytochrome P_{450} based on SDS-polyacrylamide gel electrophoretic analyses of the protein constituents of liver microsomes from control and PB- or 3-MC-pretreated rats (10). While other investigators have suggested that cytochrome P_{450} migrates in SDS-gels with an apparent molecular weight of approximately 50,000 (11,12), we were able to resolve several proteins in this region of SDS-gels and demonstrate specific increases in certain of these microsomal membrane protein constituents upon pretreatment of rats with PB or 3-MC (10). These results have subsequently been confirmed in another laboratory (13); however, it still remained to be shown that the inducible proteins in the 50,000 molecular weight region of SDS-gels were actually different hemoproteins. Since Black and Bresnick (14) suggested that the heme does not appear to completely dissociate from cytochrome P_{450} in SDS-gels, a solution to this problem was found by employing a sensitive staining technique to assay for cytochrome P_{450} in SDS-gels. This method is based upon the observation that in SDS, cytochrome P_{450} is converted to cytochrome P_{420} which has peroxidase activity (15,16). In this paper we report the use of benzidine and H_2O_2 (17) to stain for this peroxidase activity. Using this method, three microsomal hemoproteins, having apparent molecular weights of approximately 50,000, have been resolved in SDS-gels. Furthermore PB- or 3-MC-pretreatment of rats specifically induces different hemoproteins in liver microsomes.

METHODS

Immature male Sprague-Dawley rats weighing between 75-100 g were used in these studies. PB (50 mg/kg) was administered daily by i.p. injection for 5 days prior to sacrifice. 3-MC (20 mg/kg in corn oil) was administered i.p. 36 and 24 hours before sacrifice. Liver microsomes were prepared as previously described (3) and cytochrome P_{450} assays were performed by the method of Imai and Sato (18). Protein was determined by the method of Lowry, et al. (19). The specific activity of cytochrome P_{450} (nmoles/mg protein) in the various types of microsomes was as follows: Control, 1.0; PB, 3.0;

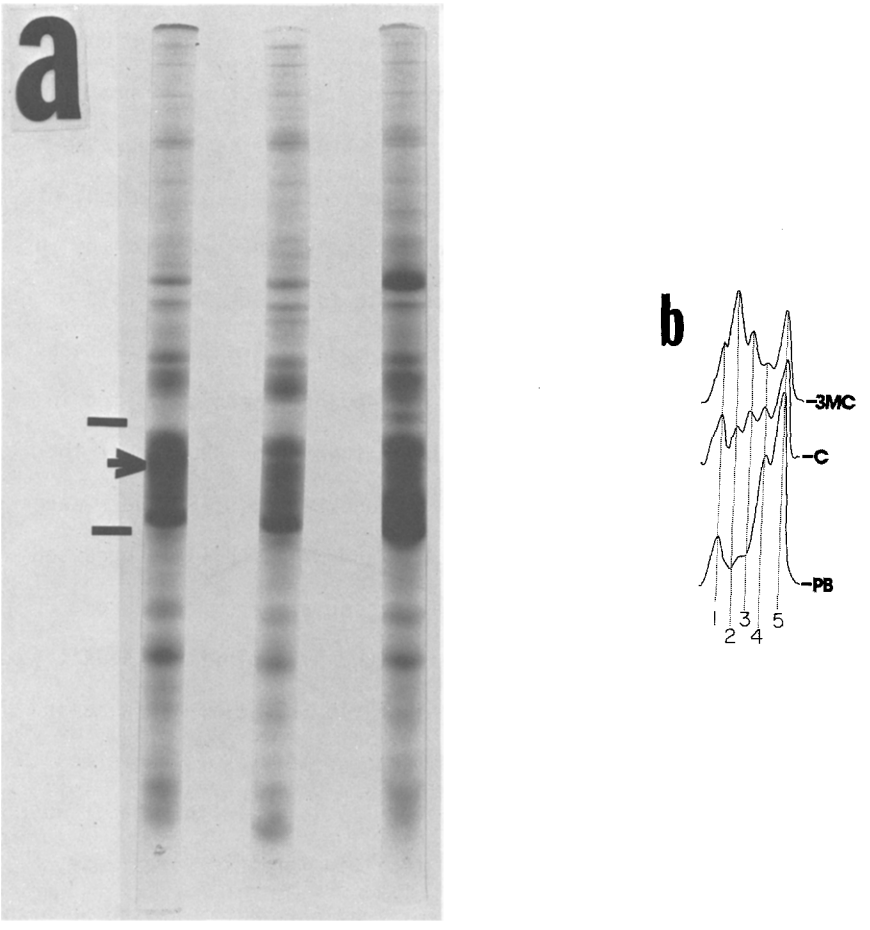

Figure 1. Coomassie blue protein profiles obtained by electrophoresis of liver microsomes from control and PB- or 3-MC-pretreated rats in 0.1% SDS-polyacrylamide gels. Microsomes were electrophoresed as described in the "Methods" after applying 60 ug of protein to each gel.

(a) A photographic representation of the protein profile from each type of microsome. The gel of 3-MC-pretreated rat liver microsomes is on the left, control in the middle, and PB-pretreated on the right. The arrow points to the position to which a protein of molecular weight 50,000 would migrate. The bars denote the region of the gels which was scanned for figure 1b.

(b) Scans of the 50,000 molecular weight region of the gels shown in figure 1a. Absorbance at 550 nm is plotted in the ordinate and migration distance on the abscissa. The upper scan is from the gel run on microsomes from 3-MC-pretreated rats; the middle from control rats; and the lower from PB-pretreated rats. Bands 1, 2, 3, 4 and 5 have the following apparent molecular weights: 57,000 $\pm$ 1,000; 53,000 $\pm$ 1,000; 50,000 $\pm$ 1,000; 47,000 $\pm$ 1,000 and 44,000 $\pm$ 1,000. The molecular weight values presented are the averages and standard deviations from 6 determinations.

and 3-MC, 1.5. Before use the microsomes were washed in 0.3 M Sucrose containing 0.1 M sodium pyrophosphate, pH 7.5 (20).

For 0.1% SDS-polyacrylamide gel electrophoresis, 10 cm gels were prepared according to the method of Fairbanks, et al. (21), except the SDS concentration in the gels and in the electrophoresis buffer was only 0.1%. The microsome sample was suspended to a protein concentration of 6 mg/ml in 1% SDS containing 10% sucrose, 10 mM Tris-HCl (pH 8.0 at 25°C), 1 mM EDTA, and 10 mg/ml pyronin B tracking dye. It was then immediately applied to pre-electrophoresed gels and electrophoresis was performed in the dark at 5°C using an electrophoresis apparatus with a cooling jacket. A voltage gradient of 5V/cm was used (∿ 2ma/tube). Electrophoresis took about 8 hours under these conditions. After electrophoresis the gels were either stained for protein using Coomassie blue (22) or for cytochrome P_{420} peroxidase activity using benzidine and H_2O_2 (17). For benzidine staining, immediately after electrophoresis, the gels were washed for 30 min in 0.02 M Tris-HCl (pH 7.5 at 25°C) containing 50% methanol to lower the SDS concentration within the gels. The gels were then placed in 0.25 M sodium citrate, pH 4.7 containing 0.25% benzidine, 25% methanol and 0.75% H_2O_2. Color development took approximately 15 minutes. Because the gels became opaque during the staining procedure and the stain was not stable for long periods of time, recording the results of these experiments by gel scanning was impossible and photographing the gels was very difficult. Coomassie blue-stained gels could be easily photographed or scanned at 550 nm using a Gilford spectrophotometer.

Molecular weight markers bovine serum albumin, catalase, carbonic anhydrase, and alcohol dehydrogenase were run in parallel with membrane protein samples.

RESULTS AND DISCUSSION

Figure 1a. is a photograph of the 0.1% SDS-polyacrylamide gel electrophoresis protein profiles obtained from liver microsomes prepared from control and PB- or 3-MC-pretreated rats. While there are some minor differences, the major differences are in the distribution of the protein constituents having

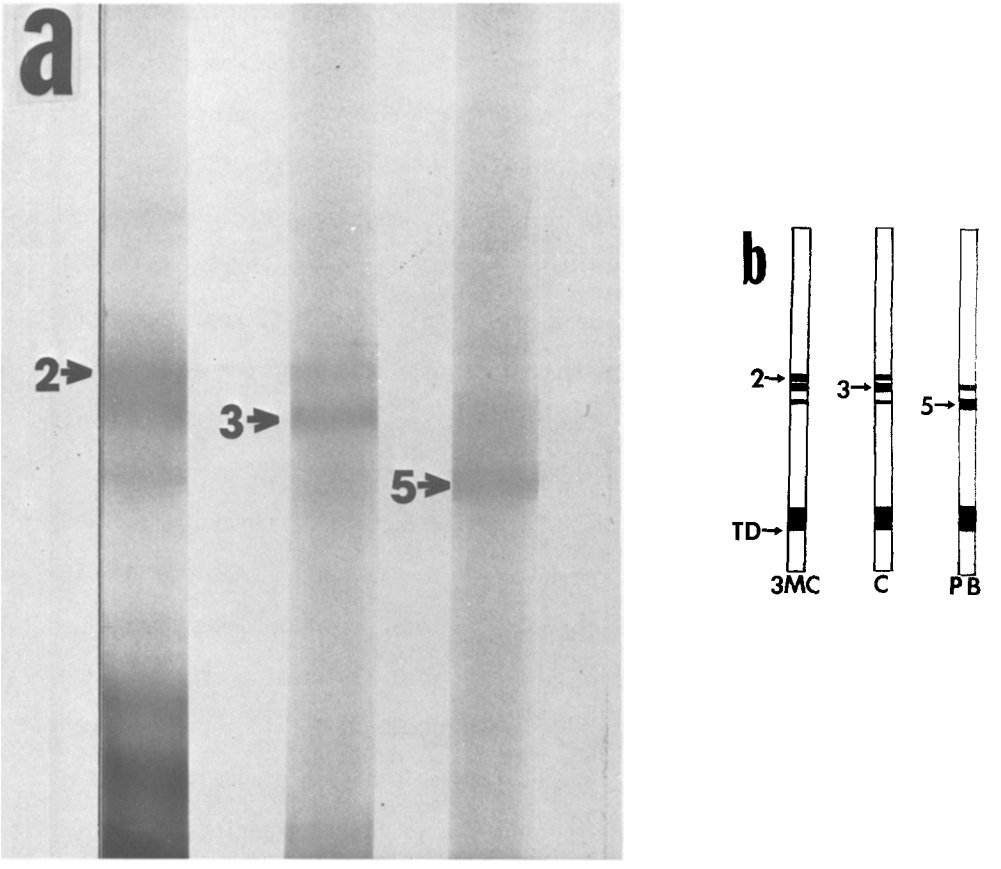

Figure 2. Hemoprotein profiles obtained by staining 0.1% SDS-polyacrylamide gels of liver microsomes from control and PB- or 3-MC-pretreated rats with benzidine and H_2O_2. Microsomes were electrophoresed as described in the "Methods" after applying 100 ug of protein to each gel.
(a) A photographic representation of the hemoprotein profile from each type of microsome. The gel of 3-MC-pretreated rat liver microsomes is on the left; control in the middle; and PB-pretreated on the right.
(b) A graphic representation of the hemoprotein profile from each type of microsome. The dark band migrating immediately above the track-in dye (TD) is heme which dissociates from the hemoproteins during electrophoresis in 0.1% SDS.
In each representation the hemoprotein bands denoted by the numbers 2, 3 and 5 have the following apparent molecular weights: 53,000 ± 1,000; 50,000 ± 1,000; and 44,000 ± 1,000. These molecular weight values are the averages and standard deviations from 6 determinations. Band 3 is the predominant species in the microsomes from control rats, band 5 in those from PB-pretreated rats, and bands 2 and 3 in those from 3-MC-pretreated rats.

apparent molecular weights of approximately 50,000. These differences can best be seen in Figure 1b. which is a scan of the protein banding pattern in

the 50,000 molecular weight region of these gels. Clearly, 3-MC-pretreatment of rats induced a protein of apparent molecular weight 53,000 (Band 2) while PB-pretreatment induces two proteins having apparent molecular weights of 47,000 (Band 4) and 44,000 (Band 5). Since cytochrome P_{450} is known to migrate with microsomal polypeptides having molecular weights of approximately 50,000 in SDS-gels (11,12) and since PB and 3-MC are known to induce different spectral (1,2) and catalytic (3-8) forms of this cytochrome, the results of these electrophoretic comparisons suggested that PB and 3-MC may actually be inducing different cytochrome P_{450} hemoproteins.

To further examine this possibility we sought a method by which the cytochrome P_{450} hemoprotein(s) could be identified in SDS-gels. We found, as suggested by Black and Bresnick (14), that all of the heme is not removed from cytochrome P_{450} upon SDS-polyacrylamide gel electrophoresis of ^{3}H-heme-labeled microsomes. Use of ^{3}H-heme-labeled microsomes required the application of large amounts of microsomal protein to the gels, however, and this resulted in poor resolution of components having molecular weights of approximately 50,000. Since Hrycay and O'Brien (15,16) have shown that SDS-solubilized cytochrome P_{450} (cytochrome P_{420}) has peroxidase activity, we therefore decided to stain the SDS-gels for peroxidase activity using benzidine and H_2O_2 (17). This method was developed using cytochrome P_{450} cam from Pseudomonas putida (a gift of Drs. I. C. Gunsalus and Karl Dus, University of Illinois) which retains a portion of its heme even during pyridine-acetone extraction (Dr. Karl Dus, personal communication). Electrophoresis of this cytochrome in SDS-gels, even in combination with a heme-binding protein such as bovine serum albumin (23), resulted in only one benzidine-stained band. This band had the same migration properties in SDS-gels as cytochrome P_{450} cam. These observations suggested that the method was specific for hemoproteins. Furthermore this method was very sensitive and did not require the application of large amounts of protein to the gels.

Figure 2a is a photograph of the results of staining 0.1% SDS-gels of

the microsomal proteins from control and PB- or 3-MC-pretreated rats with benzidine and H_2O_2. As previously stated, during the staining procedure these gels become opaque, making them difficult to photograph. The results of these experiments are better presented in figure 2b, a graphic representation of these gels. Control microsomes appear to contain three hemoproteins of approximately 50,000 molecular weight. These hemoproteins have molecular weights of 53,000, 50,000, and 44,000 and correspond to bands 2, 3, and 5 observed by Coomassie blue staining. It is possible to compare the relative amounts of these hemoproteins in the benzidine-stained gels if it is assumed that the percentage of heme lost from each hemoprotein is the same. In this case, band 3 (50,000 MW) appears to be the major hemoprotein in control microsomes. The microsomes from 3-MC-pretreated rats also appear to contain all three hemoproteins; however, the level of the hemoprotein corresponding to band 2 (53,000 MW) appears to increase. This agrees well with the 3-MC induction pattern observed in gels stained with Coomassie blue. PB-pretreatment greatly increases the level of the hemoprotein corresponding to band 5 (44,000 MW). This protein band is also one of those observed to be induced by PB in gels stained with Coomassie blue. Actually, induction of hemoprotein 5 by PB appears to occur at the expense of hemoproteins 2 and 3. Band 2 cannot be detected and band 3 is barely detectable by benzidine staining of SDS-gels run on the microsomes from PB-pretreated rats. A similar observation can be made of the PB induction pattern of microsomal proteins after Coomassie blue staining (Figure 1). Thus, the results of SDS-polyacrylamide gel electrophoresis studies of microsomal proteins in which both Coomassie blue and benzidine were used as stains are consistent with the existence and specific inducibility of multiple cytochrome P_{450} hemoproteins.

Prior to the studies reported in this paper, there were several observations which argued for the existence of multiple forms of cytochrome P_{450} in liver microsomes. For example, it has been known for years that spectrally different forms of the cytochrome are inducible in liver microsomes by pre-

treatment of animals with PB or 3-MC (1,2). Kinetic studies on the hydroxylation of drugs which were conducted in this laboratory (3,4) using control and PB- or 3-MC-microsomes have also supported this hypothesis. Similar conclusions have resulted from studies by Lu, et al. (7,8) using a reconstituted drug metabolism system. Furthermore, Comai and Gayler (9) have recently shown that three spectrally distinguishable cytochrome P_{450} species can be separated on DEAE-cellulose after protease digestion and detergent solubilization. The results of all these studies were consistent with two possibilities, however. Either different cytochrome P_{450} hemoproteins existed in microsomes or there was but one cytochrome P_{450} hemoprotein in microsomes whose spectral and catalytic properties were modified by the induction of other membrane constituents by PB or 3-MC. The results presented in this paper clearly indicate that different cytochrome P_{450} hemoproteins do appear to exist in rat liver microsomes and that pretreatment of animals with either PB or 3-MC results in the induction of different levels of these hemoproteins. To gain further insight into the manner by which specificity is imposed upon the mixed-function oxidase system, it will be necessary to isolate and characterize each of the individual cytochrome P_{450} hemoproteins.

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