METYRAPONE INTERACTION WITH HEPATIC MICROSOMAL CYTOCHROME P-450 FROM RATS TREATED WITH PHENOBARBITAL*

Alfred G. Hildebrandt, Kenneth C. Leibman#, and Ronald W. Estabrook
Department of Biochemistry, University of Texas Southwestern Medical School, Dallas, Texas 75235

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SUMMARY

Metyrapone, an inhibitor of steroid hydroxylation reactions, both stimulates as well as inhibits product formation during many mixed function oxidation reactions catalysed by hepatic microsomes from rats. A similar biphasic effect is found in liver microsomes when studying a) the effects of metyrapone on the enzymatic reduction of cytochrome P-450 and b) the reaction of reduced cytochrome P-450 with either O_2 or CO. These studies permit the spectral and functional resolution of two forms of cytochrome P-450 with apparent differences in their reaction with CO. It is concluded that metyrapone changes the equilibrium between two functionally different forms of cytochrome P-450 which exists in microsomes. The decrease of one form of cytochrome P-450 appears as an "inhibition" of aminopyrine or hexobarbital metabolism; the concomitant increase of the other form of cytochrome P-450 results in a "stimulation" of other types of hydroxylation reactions, for example, the ring hydroxylation of acetanilide.

The question remains unsolved as to how to rationalize substrate interactions with a common cytochrome P-450 yet account for the high degree of specifity for mixed function oxidase reactions. In an attempt to answer this question previous studies have showed (1) the presence of two spectral forms of cytochrome P-450 which are influenced by different types of inducing agents (phenobarbital, benzypyrene, 3-methylcholanthrene). The hypothesis was established (1) that a single cytochrome of liver microsomes is capable of interacting with two different types of substrates causing a modification in the spectral properties of the oxidized and reduced hemoprotein as well as the CO derivative of the reduced hemoprotein. It was further concluded that the Soret band of the CO derivative appears as the mean of the absorbance maxima of two spectrally distinct species (2).

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In a further attempt to answer this question, experiments have been carried out to examine the influence of modifiers of the pattern of drug metabolism by liver microsomes. The present study indicates that metyrapone [(SU - 4885) (2-methyl - 1,2-bis (3 pyridyl) - propanone)] may serve as a useful chemical for this purpose. Metyrapone has been shown (3) to be an inhibitor of the steroid 11β hydroxylase. Recent studies by Leibman (4) and Netter (5) have shown that low concentrations of metyrapone inhibit the oxidative metabolism of hexobarbital and animopyrine, but similar low concentrations of metyrapone stimulate the formation of phenolic metabolites from acetanilide (4). In the latter case inhibition occurs only at very high concentrations of metyrapone.

The present paper reports results demonstrating the nature of the inhibition obtained with metyrapone as well as the modification of the pattern of reduction and oxidation of cytochrome P-450. Associated changes in the binding of CO to reduced cytochrome P-450 result from a change in the equilibrium between the two forms of the reduced pigment.

METHODS

Microsomes were prepared from the livers of male Holtzman rats pretreated with phenobarbital as previously described (1). Prior to homogenization in 0. 25M sucrose solution, the livers were perfused *in situ* with 0. 15M NaCl. The microsomal pellet obtained by differential centrifugation of a 10% homogenate of liver was suspended in 150 mM KCl and resedimented to remove traces of hemoglobin. The washed microsomal fraction was finally suspended in a buffer mixture containing 50mM tris chloride, pH 7.4 in 0. 25M sucrose. Spectral changes were measured with an Aminco-Chance dual wavelength scanning recording spectrophotometer. Metyrapone was obtained from CIBA.

RESULTS AND DISCUSSION

Similar to many other nitrogenous base compounds metyrapone reacts with oxidized

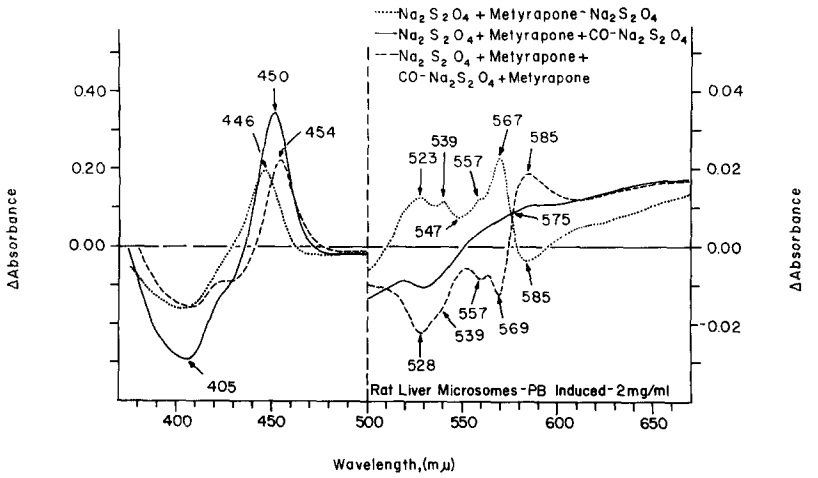

Fig. 1. The binding of metyrapone (MP) as compared to the binding of carbonmonoxide with microsomal cytochrome P-450. Liver microsomes from phenobarbital (PB) treated male Holtzman rats were diluted to a final concentration of 2 mg of protein per ml in 50 mM Tris chloride buffer, pH 7.4, containing 15 mM KCl. $Na_2S_2O_4$ was added prior to the distribution of the suspension into two cuvettes. The difference spectrum obtained upon the addition of 33μM MP is shown by the dotted line curve. The subsequent addition of CO to microsomes containing reduced cytochrome P-450 and MP reveals the spectral changes shown by the solid line curve. Addition of MP to reduced cytochrome P-450 contained in the reference cuvette permits the recording of the difference spectrum resulting from the influence of CO on the reduced cytochrome P-450 MP complex (dashed line curve) (Light path 1 cm. T=25°C).

cytochrome P-450 of liver microsomes resulting in a Type II spectral change, i.e. the appearance in the difference spectrum of a maximum at about 425mμ, with the formation of a trough at about 395 mμ (6,7). In contrast to other compounds metyrapone is also bound to reduced cytochrome P-450 causing a pronounced spectral change (Figure 1) with the appearance in the difference spectrum of maxima at 446, 523, 557 and 568 mμ and minima at 405, 547 and 585 mμ. The binding of metyrapone to reduced cytochrome P-450 varies with microsomes prepared from livers of animals of different species or with animals pretreated with various inducing agents. Of considerable interest is the appearance of an absorption band with a maximum at 446 mμ similar to the absorption band observed when CO interacts with reduced cytochrome P-450 of hepatic microsomes from 3-methylcholanthrene treated animals (1). The fact that this absorption band at 446 mμ , observed when metyrapone is added

to reduced cytochrome P-450, is not due to the presence of adventitious CO has been established by the failure of hemoglobin to modify the magnitude of the spectral change. The subsequent addition of CO to microsomes containing reduced cytochrome P-450 and metyrapone results in a difference spectrum (Figure 1) typical for the CO complex of reduced cytochrome P-450, i.e. a maximum at 450 mμ. Addition of metyrapone to reduced cytochrome P-450 contained in the reference cuvette permits the recording of the difference spectrum (Figure 1) showing the influence of CO on the reduced P-450 metyrapone complex. It appears that the absorption band observed at 450 mμ in the presence of CO is the summation of two distinct absorption bands--one with a maximum at 446 mμ and the other at 454 mμ. This conclusion would support earlier findings (2) of two types of interaction of CO with cytochrome P-450 as determined with hepatic microsomes from animals treated with 3-methylcholanthrene or phenobarbital

The data described above posed the question of how to prove whether the effect observed with CO was the displacement of metyrapone from one common binding

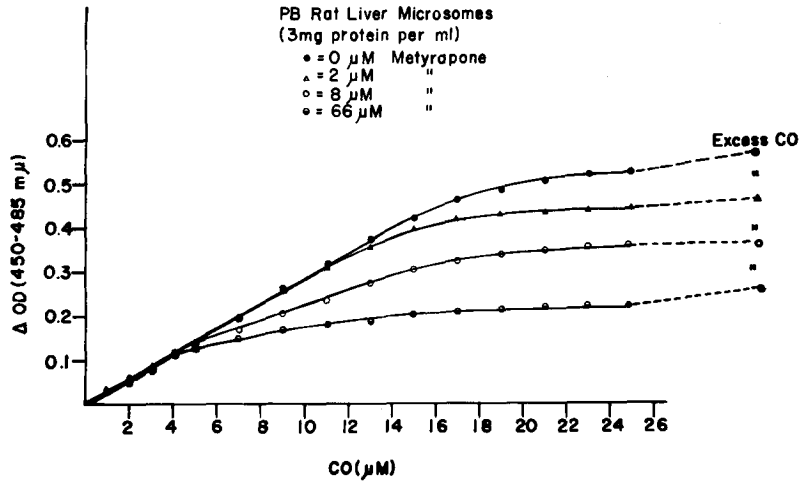

Fig. 2. The effect of MPon the formation of the CO derivative of reduced cytochrome P-450. Liver microsomes from phenobarbital treated rats were suspended in 0.1 M potassium phosphate buffer pH 7.4 to a concentration of 3 mg of protein per ml and reduced with $Na_2S_2O_4$. The changes in absorbance caused by the consecutive addition of CO saturated buffer (0.1 M potassium phosphate + 2mM $Na_2S_2O_4$, pH 7.4) were recorded at 450mμ relative to 485mμ. The presence of different concentrations of MP added prior to the addition of CO is shown by the following signs: ●=0μM MP ▲=2μM MP ○=8μM MP ◉=66μM MP.

site on cytochrome P-450 or whether there exists two distinct binding sites for CO, one of which is occupied in the presence of metyrapone. Figure 2 shows the results of experiments where reduced cytochrome P-450 of microsomes was titrated with CO in the presence and absence of metyrapone. The magnitude of absorbance change obtained in the presence of excess CO was markedly reduced in the presence of metyrapone. Of greatest interest is the failure to see any significant change in the magnitude of absorbance when low concentrations less than 5 μM CO were employed. At concentrations greater than 5 μM CO the presence of metyrapone caused a pronounced suppression of the spectral change observed on addition of CO. Analysis of the CO titration curve in the absence of metyrapone shows that two distinct phases of CO binding are apparent suggestive of two sites of CO interaction with different equilibrium constants. It appears that the presence of metyrapone interferes with one of these binding sites, while stimulating the binding of CO to the other site. If metyrapone had been displaced by CO one would expect a deviation to appear in the titration curve with CO suggestive of a loss of extinction as the reduced cytochrome P-450 metyrapone complex was dissociated and the reduced cytochrome P-450 CO complex formed.

If metyrapone interacts at a site on reduced cytochrome P-450 common with one site of CO interaction, it would suggest that this site was also common with a site of oxygen interaction. Experiments were therefore carried out to measure the influence of metyrapone on the oxidation of reduced cytochrome P-450. Recently a method has been devised[+] for measuring the oxidation or reduction of cytochrome P-450 in the absence of CO. For these experiments a suspension of liver microsomes is made anaerobic by gassing the sample with Argon followed by removal of residual oxygen by lipid peroxidation of microsomes using ascorbate and ADP plus ferric chlorid At the wavelength pair 548 minus 565 mμ (Figure 3) a spectral change attributable to reduction of cytochrome P-450 can be measured upon addition of TPNH. At the wavelength pair 569 mμ minus 575 mμ the oxidation or reduction of cytochrome P-450

[+] Hildebrandt, A. G. and R. W. Estabrook, unpublished result.

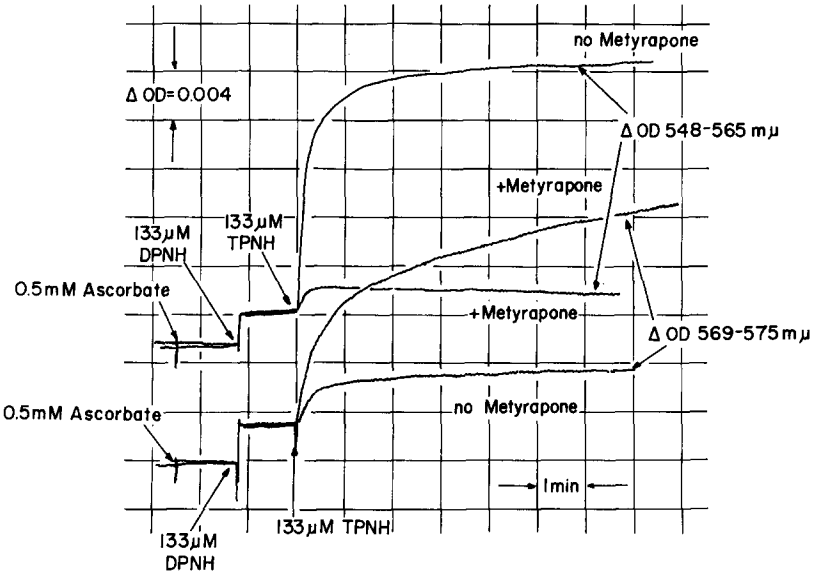

Fig. 3. The kinetics of reduction of the two forms of cytochrome P-450 by TPNH. Liver microsomes from phenobarbital treated rats were suspended in 50mM Tris-chloride buffer (pH 7.4) containing 15mM KCl to a concentration of 4 mg protein per ml and the samples gassed with Argon for 1 minute. The changes of absorbance were recorded at two different wavelength pairs (548-565mμ; 569-575mμ) as indicated. Metyrapone was added prior to the addition of 0.02mM $FeCl_3$ and 2mM ADP. Ascorbate, DPNH and TPNH were added as indicated.

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can be measured in the presence of metyrapone. The subsequent addition of diluted H_2O_2 to the catalase supplemented anaerobic reaction mixture generates sufficient oxygen rapidly to cause a cyclic oxidation of reduced cytochrome P-450. Figure 4 shows the influence of metyrapone on the extent of oxidation (548-565mμ) of one form of reduced cytochrome P-450. A concentration of metyrapone of 2.5μM is sufficient to cause a 50% decrease in the magnitude of this spectral change. The oxidation and reduction reactions of the other form of cytochrome P-450 (569-575mμ) are increased by this concentration of metyrapone. From these experiments it is concluded that metyrapone interacts with reduced cytochrome P-450 by changing the equilibrium between the two forms of the pigment i.e., one form which may be similar to an pyridine hemochrome and another form which is more like a cyanide hemochrome. The presence of metyrapone increases the content of the

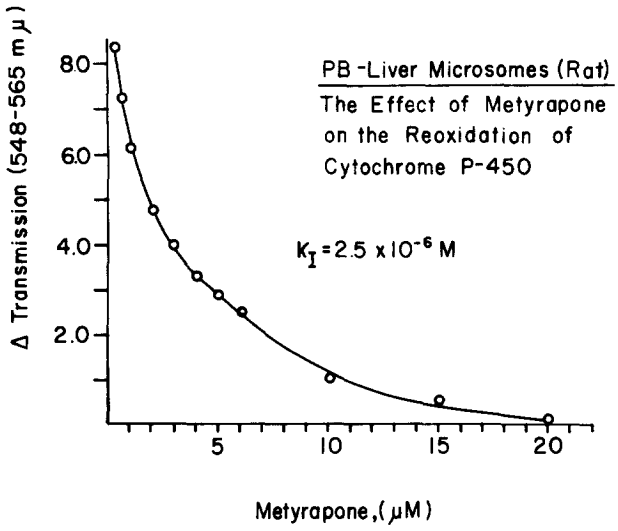

Fig. 4. The effect of MP on the extent of oxidation of enzymatically reduced cytochrome P-450. Microsomes were suspended to a final concentration of 4 mg of protein per ml in 50mM Tris-chloride buffer, (PH 7.4) containing 15mM KCl and incubated with 20 units catalase (Sigma). Anaerobiosis was obtained as indicated in fig. 3. The reduction of cytochrome P-450 was initiated by the addition of 266μM TPNH to a TPNH generating system (2mM $MgCl_2$, 8mM sodium isocitrate, isocitrate dehydrogenase (Sigma Type IV, 0.05 mg protein per ml) and recorded at 548mμ relative to 565mμ. After the maximum of absorbance was obtained MP was added at different concentrations prior to the addition of H_2O_2.

form where a reduction and reoxidation can be monitored at 569 mμ relative to 575 mμ concomitant with a decrease in the amount of the other form as revealed by the decrease of absorbance changes at 548-565mμ upon reduction and reoxidation of this form.

In a similar series of experiments it was shown that metyrapone modifies the enzymatic reduction of one form of cytochrome P-450 (548-565mμ) while increasing the extent of reduction of the other form (569-575mμ). Parallel experiments showed that metyrapone neither altered the rate of TPNH-cytochrome c reduction, the reduction of cytochrome b_5 by DPNH or TPNH, nor the formation of malonaldehyde via TPNH dependent lipid peroxidation.

The exact nature of these two forms of cytochrome P-450 remains to be elucidated, however the following hypothesis (Fig. 5) is proposed to explain their partici-

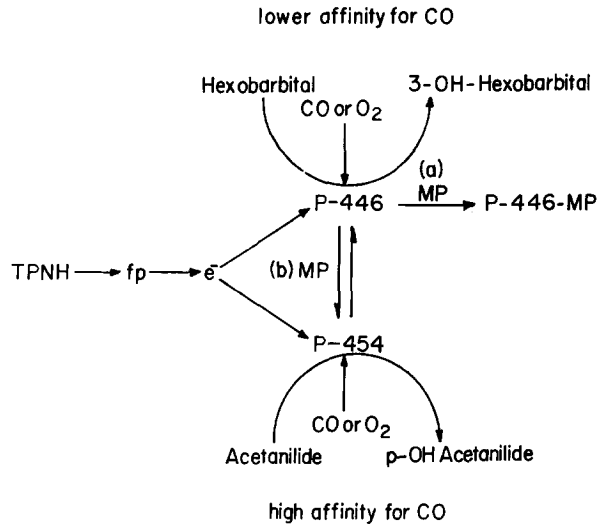

Fig. 5. Scheme showing proposed involvement of cytochrome P-450 in mixed function oxidase reactions with emphasis on the postulated two forms of cytochrome P-450.

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pation in the hydroxylation of the two general classes of substrates metabolized. One form of cytochrome P-450 (absorbance maximum of the CO derivative at 446 mμ, P-446) is involved in the hydroxylation of Type I compounds (hexobarbital, N-ethylmorphine etc.). The other form (absorbance maximum of the CO derivative at 454 mμ, P-454)catalyses the metabolism of type II compounds (aniline, acetanilide etc.). The action of metyrapone is explained either by a) the formation of a non-functional P-446-MP-complex in the ferrous state which would then favour the flow of electrons from TPNH to P-454 or b) by the displacement of the equilibrium of P-454 and P-446 in the presence of metyrapone toward P-454, thereby increasing the amount of functional enzyme for this alternative pathway. The binding of metyrapone with cytochrome P-450 maybe analogous to the reaction of phenylhydroxamine with hemoglobin, suggesting the possible reaction of two hemes interacting in the transfer and reduction of O_2 during hydroxylation reactions.

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