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METYRAPONE - REDUCED CYTOCHROME P-450 COMPLEX: A SPECIFIC METHOD FOR THE DETERMINATION OF THE PHENOBARBITAL INDUCIBLE FORM OF RAT HEPATIC MICROSOMAL CYTOCHROME P-450

V. LUU-THE
J. CUMPS
P. DUMONT

Department of Medicinal Chemistry
Faculty of Medecine
Catholic University of Louvain
Emmanuel Mounier av. 73
1200 Bruxelles, Belgium

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SUMMARY

Using homogeneous cytochrome P-450, we have shown that the well-known metyrapone-dithionite reduced cytochrome P-450 complex is specific for the cytochrome P-450b induced by phenobarbital. A linear relationship was observed between the absorbance of metyrapone-reduced cytochrome P-450 complex and the one of CO-reduced cytochrome P-450 complex, the usual method for the determination of cytochrome P-450. A method has been proposed for the specific determination of the cytochrome P-450b.

INTRODUCTION

Evidence for a variety of forms of cytochrome P-450, an essential component of the liver microsomal oxidase system has been based on visible spectrophotometry (1), electron paramagnetic spectroscopy (2), SDS-polyacrylamide gel electrophoresis (3), immunological (3-4) and in vivo induction studies.

Purification of these forms has been accomplished in many laboratories (4-7). The three most studied forms mentionned by Ryan et al.(6) as cytochrome P-450c, P-450b, and P-450a correspond to the main fractions induced by 3-methylcholanthrene (MC), phenobarbital (PB), and uninduced respectively. By now there are no simple spectrophotometric method which permits to distinguish and quantify specifically these different forms of cytochrome P-450. In using homogeneous forms of

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cytochrome P-450, our studies give evidence that the well known metyrapone-reduced cytochrome P-450 complex (8), absorbing at 446 nm, is specific for the cytochrome P-450b. In addition, a linear relationship was observed between the absorbance of metyrapone-reduced cytochrome P-450b complex and that of the CO-reduced cytochrome P-450 complex, used in the habitual method to determine cytochrome P-450 (9). Based on these observations a method is proposed for the specific determination of cytochrome P-450b.

MATERIALS AND METHODS.

All chemicals were commercially available. Ethylisocyanide was purchased from Sigma chemical Co, metyrapone was a gift from Ciba-Geigy Co.
Cytochrome P-450 from PB and MC treated rats were extensively purified by the method described previously (7). This method is an adaptation of the procedure described by Imai et al.(4), in which the steps of chromatography on exchange ion column are substituted by a second chromatography on an octylamino-Sepharose-4B column.
Cytochrome P-450 was determined by the method described by Omura and Sato(8). Metyrapone and ethylisocyanide difference-dithionite reduced cytochrome P-450 spectra were recorded following the same procedure. Metyrapone dissolved in water and ethylisocyanide dissolved in methanol were added to a concentration of about 1 mM.
Protein was determined by the method of Lowry et al. (10) as modified by Schacterle et al. (11)
Difference spectra were performed on a Perkin-Elmer double wavelength, double beam spectrophotometer model 556, equipped with a baseline corrector accessory.

RESULTS AND DISCUSSION.

Using the isolation procedure reported in materials and methods, cytochrome P-450b[1] induced by PB and cytochrome P-450c[1] induced by MC have been purified to a specific content of 17 nmoles x mg^{-1} protein. Both forms appear homogeneous as judged by SDS-polyacrylamide gel electrophoresis. In agreement with the litterature their CO-reduced complexes absorb at 450 nm and 448 nm respectively (fig. 1A). Only partial purification could be achieved with cytochrome P-450a[1] from untreated rats: polyacrylamide gel electrophoresis showed the presence of one major polypeptide and two minor components corresponding to cytochrome P-450b

[1] terminology used by Ryan et al.(6) for different forms of cytochrome P-450.

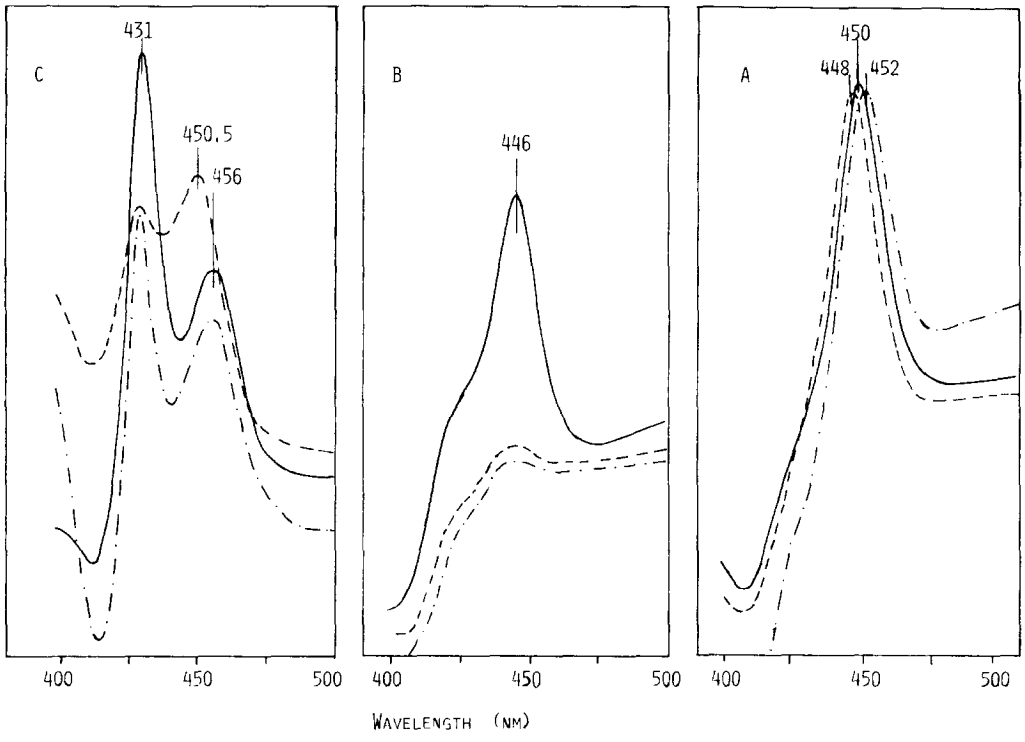

Fig. 1 Difference spectra of reduced cytochrome P-450 induced by PB (——) by MC (– –), and uninduced (— · —) with CO (A), metyrapone (B) and ethylisocyanide (C).

and cytochrome P-450c. The CO-dithionite reduced complex of this cytochrome absorbs at 452 nm (fig. 1A).

These three purified cytochromes P-450 exhibit similar absorption in the 456 nm region, when they are reduced and complexed with ethylisocyanide (fig. 1C). In contrast their metyrapone-dithionite reduced complexes show quite different spectral caracteristics (fig. 1B) : only the PB induced cytochrome P-450 produces a significant absorption, while the others do not. Determination of the apparent dissociation constants (Ks) of the metyrapone-cytochrome P-450 binding strenghens this observation : in the reduced state, the Ks of the metyrapone-cytochrome P-450 complex was 1.8 μm for the PB induced form, but not determinable for the MC induced cytochrome P-450 (table 1). In the oxidized state a Ks value of about 60 μM was obtained with the former and two Ks values of several times greater: 180 μM and 442 μM was obtained with the latter (table 1).

TABLE I.

Apparent dissociation constants, $Ks^{(a)}$, for metyrapone bound to purified cytochrome P_{450}.

	PB induced Cytochrome P_{450}	MC induced Cytochrome P_{450}
oxidized cytochrome	60 ± 5	1) 186 ± 6 (b)
		2) 442 ± 38
Reduced cytochrome	1.8 ± 0.3	--------

(a) Values in μM ± S.E.

(b) Two Ks values were observed.

As shown in fig. 2, a linear relationship between the absorbance given by the metyrapone-dithionite reduced cytochrome P-450 complex and the CO-dithionite reduced cytochrome P-450 complex was observed for the PB induced cytochrome P-450. By contrast, in the case of MC and uninduced cytochrome P-450, the metyrapone complex gives a very small absorbance in comparaison with the CO complex. The observed linear relationship permits to calculate the molar extinction coefficient of the specific metyrapone-dithionite reduced cytochrome P-450b complex (K_{MP}) on the base of the molar extinction coefficient (K_{CO}) of the CO-dithionite reduced cytochrome P-450 complex (0.091 M^{-1} cm^{-1} as reported by Omura and Sato (9) :

$$\frac{A_{446-490}}{A_{450-490}} = m \text{ (slope)}$$

After Lambert-Beer's law :

$$A_{446-490} = K_{MP} . C . d$$

$$A_{450-490} = K_{CO} C . d$$

$$K_{MP} = m . K_{CO} = 0.052 \ M^{-1} \ cm^{-1}$$

The specificity of metyrapone complex for the cytochrome P-450b allows to estimate the proportion of this form in microsomes of rats untreated or pretreated with PB or MC. Results reported in table 2 show that the

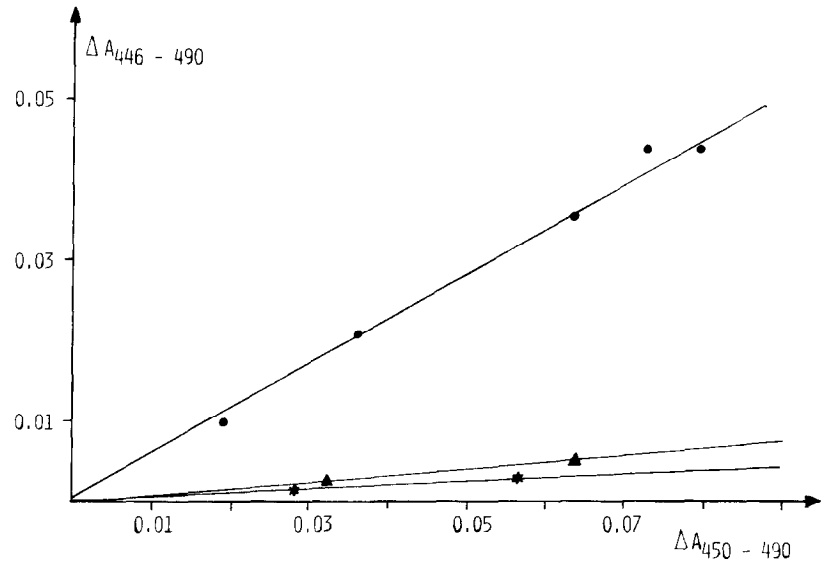

Fig. 2 Relationship between absorbances given by metyrapone-reduced cytochrome P-450 complex ($\Delta A_{446-490}$) and CO-reduced cytochrome P-450 complex ($\Delta A_{450-490}$). (●—●), cytochrome P-450 induced by PB; (▲—▲), cytochrome P-450 induced by MC; (✱—✱), uninduced cytochrome P-450

metyrapone-reduced cytochrome P-450 complex corresponds respectively to 51 %, 72 % and 38.5 % of the total cytochrome P-450 complex. These results are consistent with the report of Jonen et al.(12) : they observed that the binding of metyrapone to reduced cytochrome P-450 was increased by pretreatment with PB but not with MC.

TABLE 2.

Estimation of metyrapone-dithionite reduced cytochrome P_{450} complexes in microsomes of untreated, PB treated and MC treated rats.

	Concentration of metyrapone complex(a)	Concentration of CO complex (b)	(%)
Untreated	0.663	1.3	51
PB treated	0.885	1.23	72
MC treated	0.635	1.65	38.5

(a) Determined from an extinction coefficient value of 52 mM^{-1} cm^{-1}

(b) Determined from an extinction coefficient value of 91mM^{-1} cm^{-1}

Concentration was expressed in nmole/ml: the same cytochrome P-450 was used in (a) and (b).

The origin of the specificity of metyrapone binding toward reduced cytochrome P-450b is still unknown. It is possible to speculate that this property is related to the spin state of cytochrome P-450, indeed the level of low spin cytochrome P-450 was found to be increased in rats pretreated with PB, while the level of high spin cytochrome P-450 was increased in the MC pretreated rats (2).

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