HETEROGENEITY OF CYTOCHROME *P*-450 IN RAT LIVER MICROSOMES: SELECTIVE INTERACTION OF METYRAPONE AND SKF 525-A WITH DIFFERENT FRACTIONS OF MICROSOMAL CYTOCHROME *P*-450

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1. Introduction

It is well established that different substrates affect the light absorption of microsomal cytochrome *P*-450 differently. The substrates have been classified into two types [1]: type I substrates which cause a shift of the Soret band absorption to shorter wavelength, indicating an increase in high spin character, and type II substrates which cause a shift of the Soret band to longer wavelength, indicating an increase in low spin character. The different spectral changes resulting from the interaction of various substrates with microsomal cytochrome *P*-450 poses the question whether the substrates interact with different cytochrome *P*-450 species or with different sites on a single species of cytochrome *P*-450. Many studies concerned with purification of liver microsomal cytochrome *P*-450 show evidence for different forms of the hemoprotein [2–7].

We will report here observations by light absorption and EPR suggesting that SKF 525-A (diethylaminoethyl-2,2-diphenylvalerate) and metyrapone (MP), inhibitors of cytochrome *P*-450-linked reactions [8], react selectively with different fractions of microsomal cytochrome *P*-450. The reactions are independent of each other, strongly indicating the presence of at least two forms of the hemoprotein. The ratio between the two fractions of cytochrome *P*-450 is different in microsomes from normal rats and from rats pretreated with phenobarbital (PB); essentially only the form reacting with MP is induced by PB treatment.

In addition the interaction of other inhibitors and substrates with cytochrome *P*-450 are being investigated using the same technique. These seem to bind with less selectivity and show different degrees of competition.

In general binding to cytochrome *P*-450 occurs in two phases: a strong binding phase and a gradual binding of low affinity.

2. Materials and methods

The animals used were male Sprague-Dawley rats weighing 150–250 g. Liver microsomes were isolated [9] and washed in 0.15 M KCl. Phenobarbital, sodium salt, (80 mg/kg body weight) was injected intraperitoneally once daily for three consecutive days. Cytochrome *P*-450 and protein were determined using the methods of Omura and Sato [10] and Lowry et al. [11], respectively.

Metyrapone was obtained from Ciba-Geigy and camphor from the British Drug Houses Ltd. SKF

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525-A (Smith, Klein & French laboratories) and lidocaine (Astra Pharmaceutical Co.) were used as the hydrochlorides.

The spectral changes produced by addition of various reactants to suspensions of microsomes at a temperature of about 25°C were recorded with an Aminco DW 2 UV-VIS spectrophotometer. The microsomal suspension, containing 50 mM Tris–Cl, pH 7.5, 15 mM KCl and 3–6 mg protein/ml in a total vol of 6 ml, was equally distributed between reference and sample cuvettes having a 1 cm light path. The substrates were dissolved in water and added successively to the sample cuvette or to both cuvettes, and in some cases finally to the reference cuvette in the order specified in the figure legends. An equivalent volume of water was added to the other cuvette. Care was taken to measure the initial part of the titration curve whenever a new substrate was used.

The EPR spectra were recorded and g-values were measured as described elsewhere [12]. Samples were measured at 77 K using a conventional liquid N_2 cold finger and at 3.6 K using a He-flow system (Oxford Instruments, SCL 5002 ESR 9). The concentrations of high and low spin components were determined according to Aasa and Vänngård [13] using 1 mM Cu^{2+} in 10 mM EDTA solution as reference. For the high spin signals a correction was applied assuming a zero field splitting of 8.63 cm^{-1} between the lowest and middle Kramers doublets [14] and Boltzmann distribution between the levels.

3. Results and discussion

As can be seen from fig.1, the presence of MP was practically without effect on the light absorption change resulting from titration of oxidized microsomal cytochrome *P*-450 with SKF 525-A. This behaviour is further indicated by the data shown in fig.2, where addition of equal amounts of MP to the sample and reference cuvettes had no or only a very minor effect on the SKF 525-A titration curves. Both the spectral amplitude and the shift in the wavelength of maximum absorbance induced by SKF 525-A were unaffected by MP. Similar results were obtained in analogous experiments where titration with MP was carried out in the absence or presence of SKF 525-A. The general

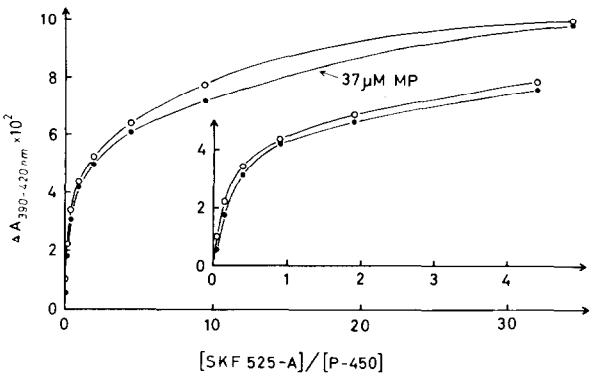

Fig.1. Titration with SKF 525-A of oxidized cytochrome *P*-450 in microsomes from untreated (control) rats in absence (○——○) and in presence (●——●) of 37 μM metyrapone (MP) in both cuvettes. The absorbance difference between 390 nm and 420 nm was measured after consecutive additions of SKF 525-A to the sample cuvette. The concentration of cytochrome *P*-450 was 3.2 μM. The inset shows the low concentration region on an expanded scale.

nature of the titration curves was the same, irrespective of whether microsomes from control or from PB treated rats were used. The spectral changes obtained by adding both MP and SKF 525-A were always the algebraic sum of the type I (SKF 525-A) and type II (MP) difference spectra. This finding demonstrates

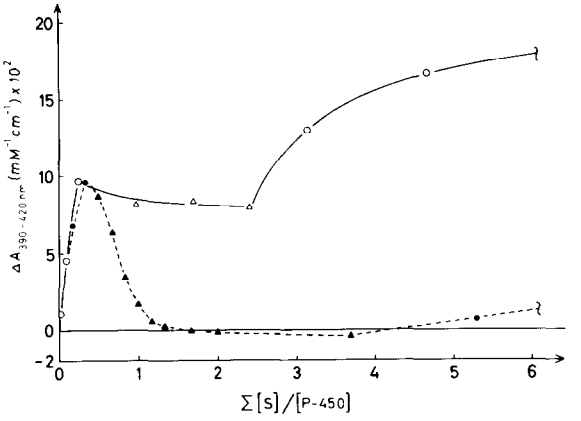

Fig.2. Optical difference titrations of microsomes from phenobarbital treated rats with SKF 525-A (○——○) and lidocaine (●---●) added to the sample cuvette. Intermediately metyrapone (△, ▲) was added to the sample as well as to the reference cuvette. The concentration of cytochrome *P*-450 was 10 μM.

that there is essentially no interaction between the binding of MP and SKF 525-A and suggests that these two reactants bind independently of each other to different fractions of microsomal cytochrome *P*-450.

As shown in fig.2, a quite different pattern is observed for the interaction between lidocaine, which like SKF 525-A gives a type I difference spectrum, and MP. It can be seen that the spectral change induced by lidocaine is completely abolished by addition of MP. The subsequent addition of lidocaine gives only a very minor spectral change. This strong interaction is only possible if the two reactants compete for the same binding site or at least bind to the same molecule.

An outstanding feature of the curves shown in figs.1 and 2 are the relatively large spectral changes that occur with reactant/*P*-450 molar ratios $<<1$. The initial parts of the curves clearly demonstrate the presence of a strong substrate-binding portion of cytochrome *P*-450 responsible for about one half of the maximal spectral change. At higher reactant concentrations, binding to sites of lower affinity causes a considerable further increase in the spectral change. However, even at very high substrate/*P*-450 molar ratios, no single substrate studied (SKF 525-A, lidocaine and several other type I substrates) was able to convert the total amount of cytochrome *P*-450 to a high spin state.

If we apply to our system the difference extinction coefficient ($\Delta\epsilon_{390-420\ nm} \simeq 130\ mM^{-1}\ cm^{-1}$) reported for the conversion of purified *Pseudomonas putida* cytochrome *P*-450 from a low spin to a high spin state [15], we find that in control microsomes 20–35% of the total cytochrome *P*-450 may be converted to a high spin state by reaction with SKF 525-A; the corresponding figure for microsomes from PB treated rats in which the level of cytochrome *P*-450 was 2 to 3 times higher than in control microsomes was only 5–15%. These values are in reasonable agreement with our preliminary analysis of optical absolute spectra of microsomal cytochrome *P*-450 and indicate that about the same absolute amount of cytochrome *P*-450 reacted with SKF 525-A in the two types of microsomes. This is particularly well demonstrated in fig.3, where the initial titrations with SKF 525-A show that, on the basis of total protein, very nearly the same optical change is obtained with microsomes from control and from PB treated rats. Treatment of rats

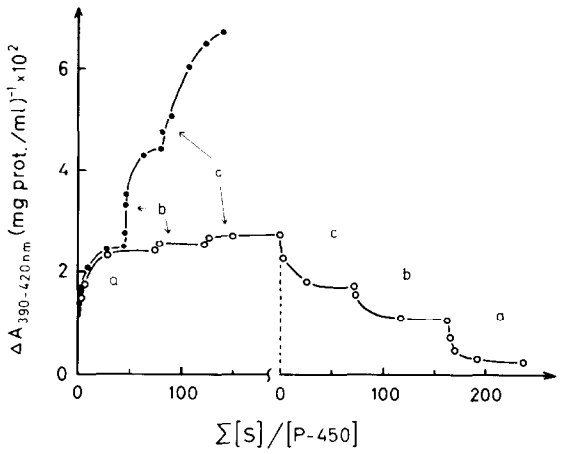

Fig.3. Optical titrations with three type I substrates of cytochrome *P*-450 in microsomes from control rats (○——○) and from phenobarbital treated rats (●——●). In the first part the substrates were added to the sample cuvette in the order: (a) SKF 525-A, (b) lidocaine and (c) camphor. In the second part, in the case of microsomes from control rats, they were added in the reverse order to the reference cuvette. The concentrations of cytochrome *P*-450 were: 3.4 and 9.2 μM and of protein 4.1 and 4.6 mg/ml in microsomal suspensions from control and phenobarbital treated rats, respectively.

with PB is known to induce more cytochrome *P*-450 with only relatively small change of the total protein content of the microsomes (approx. 30% after 72 h PB treatment) [16]. On the other hand, the spectral changes induced by MP in microsomes from control rats and in microsomes from PB treated rats are closely proportional to the concentrations of cytochrome *P*-450. These findings demonstrate that the cytochrome *P*-450 induced by PB treatment is almost entirely in a form which binds MP and not SKF 525-A.

Fig.3 also provides evidence that liver microsomal cytochrome *P*-450 can be further divided into subfractions which differ in their binding specificity for type I substrates such as SKF 525-A, lidocaine, and camphor. The relative amounts of these subfractions are altered by pretreatment of animals with PB. The remarkably abrupt increase in the spectral change associated with the initial addition of each new substrate clearly demonstrates the presence of different binding sites or fractions. Furthermore, the limited spectral change obtained for each substrate presumably reflects the amount of free cytochrome *P*-450

possessing a high affinity binding site for the substrate in question. Apart from this heterogeneity of liver microsomal cytochrome *P*-450, the curves in fig.3 also reveal that there is partial competition between the substrates, an overlap in the specificities of different subfractions. For example, when SKF 525-A is added alone to control microsomes, it gives a spectral change that is about three times as large as when it is added in the presence of an excess of camphor and lidocaine. Conversely, if camphor is added alone to the same microsomes, it causes a spectral change that is 6 to 7 times larger than that obtained in the presence of an excess of SKF 525-A and lidocaine.

Some EPR results that complement the optical observations are summarized in table 1. The low field absorption-like wings of the low spin EPR spectra of cytochrome *P*-450 measured at 77 K are shown in fig.4. Microsomes from control rats without added reactant show one low spin component (g = 2.42, 2.24, 1.92) which accounts for 64% of the total cytochrome *P*-450. Upon addition of MP two low spin components are found, the earlier component now accounting for 41% and one or possibly two other components with shifted g-values (g = 2.47, 2.26, 1.90) accounting for 49%. Microsomes from PB treated rats without any addition give two closely situated

Table 1
Fractions of cytochrome *P*-450 appearing in the EPR signals observed

Sample source (nmoles *P*-450/mg protein)	Added reactant	Amount of *P*-450 on which fractional figures are based	Original low spin component(s) centered at g = 2.42, 2.24, 1.92	Low spin component(s) of MP complex centered at g = 2.47, 2.26, 1.90	High spin component centered at g = 8.1, 3.6, 1.7
			measured (calculated)	measured (calculated)	measured (calculated)
Control	none	total	0.64	0	0.11
rats	MP	total	0.41	0.49	0.06
(0.50)	SKF 525-A	total	0.69	0	0.30
PB treated	none	total	0.86	0	0.07
rats	MP	total	0.20	0.69	0.01
(1.70)	SKF 525-A	total	0.80	0	0.20
(0.50)	MP	control fraction	(0.41)	(0.49)	
(1.20)	MP	PB induced fraction	(0.11)	(0.77)	
(0.50)	SKF 525-A	control fraction			(0.19)
(1.20)	SKF 525-A	PB induced fraction			(0.10)

The abbreviations used are: MP, metyrapone; SKF 525-A, diethylaminoethyl-2,2-diphenylvalerate; PB, phenobarbital.

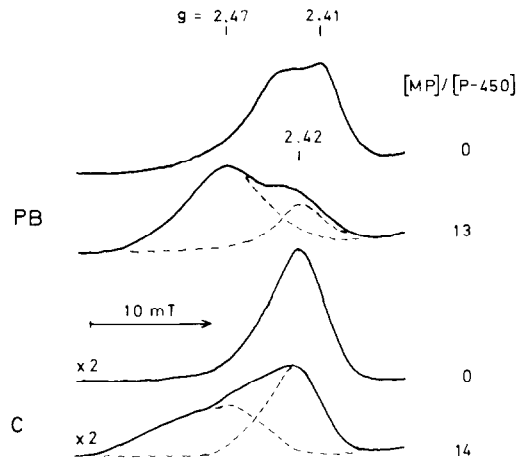

Fig.4. EPR spectra of low spin microsomal cytochrome *P*-450 without and with added metyrapone (MP). The low field absorption-like wings were recorded at 77 K. Microsome suspensions from control rats at bottom (C) and from phenobarbital treated rats at top (PB). The column at the right indicates the concentration of MP added. The concentration of cytochrome *P*-450 was in microsomes from control rats, 42 μM and from PB treated rats, 105 μM. The EPR conditions were: microwave power, 30 mW; microwave frequency, 9.12 GHz; modulation amplitude, 1.2 mT; scanning rate, 20 mT/min. The receiver gain was increased by a factor of 2 for the control samples.

components centered at the same g-values as for microsomes from control rats. The total signal intensity corresponds to 86% of all cytochrome *P*-450. Addition of MP gives in this case a major fraction of 69% at the shifted g-values and a minor fraction of 20% at the original g-values. The spectral components (g = 2.47, 2.26, 1.90) appearing after addition of MP is believed to represent the complex of cytochrome *P*-450 with MP. In agreement with the optical data much more of this complex is formed in microsomes from PB treated rats than from control rats, i.e. 69 and 49%, respectively, of total cytochrome *P*-450. The microsomes from PB treated rats contained 1.70 nmol cytochrome *P*-450 per mg of protein, whereas those from control rats contained 0.50 nmol cytochrome *P*-450 per mg of protein. If we assume that in the microsomes from PB treated rats a 'control' fraction of 0.50 nmol cytochrome *P*-450 per mg of protein behaves identically to the cytochrome *P*-450 in control microsomes, we find that for the PB *induced* fraction (1.20 nmol per mg of protein) upon addition of MP 77% is found in the signal of the MP complex and 11% in the original signal. These figures should be compared with 49 and 41%, respectively, for the control fraction.

We also measured at 3.6 K the high spin signals at g = 8.1 (not shown) assuming the complete set of g-values to be g = 8.1, 3.6, 1.7. In microsomes from control rats addition of MP decreased this component from 11 to 6%, whereas addition of SKF 525-A increased it to 30%. In microsomes from PB treated rats addition of MP decreased the high spin component from 7 to 1% and addition of SKF 525-A increased it to 20%. Again, we may calculate that addition of SKF 525-A to the PB induced fraction increases the high spin component with 10% as compared with 19% in the control fraction.

While these results obtained by low temperature EPR are not completely consistent with the light absorption titrations at room temperature, they nevertheless show that the PB induced fraction of cytochrome *P*-450 has less ability to react with SKF 525-A than the control fraction. The difference between the two sets of experiments might be due to differences in temperature dependent spin equilibria of cytochrome *P*-450 in the different microsomes.

Other series of EPR experiments have been evaluated in the same way and gave similar results. It is worth noting that the sum of the low spin component measured at 77 K and the high spin component measured at 3.6 K usually was close to 100% of the total cytochrome *P*-450.

It was observed that addition of SKF 525-A slightly changed the shape of the low spin EPR signal without noticeable changing the corresponding concentration. It was also observed that addition of both MP and SKF 525-A resulted in low spin and high spin contributions intermediate to those obtained with either reactant alone. These results suggest a competition between the two reactants, in contrast to the optical results. However, we hesitate to draw any extensive conclusions from these EPR observations without making more detailed studies of factors such as concentration and temperature dependencies. In general it is dangerous to generalize from low temperature EPR results to conditions in an aqueous solution at room temperature, especially if corroborating light absorption data are not available.

The main conclusions remain as follows: There are at least two species or modifications of cytochrome *P*-450 in liver microsomes. PB treatment of rats induces the formation of a form of cytochrome *P*-450 which binds MP tightly and only to a very much lesser extent combines with SKF 525-A. The optical experiments at room temperature suggest complete independence of the two binding processes, whereas the low temperature EPR results indicate some competition and interaction between them.

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