Studies on the substrate-binding sites of liver microsomal cytochrome *P*-448

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The interaction of substrates of the microsomal mixed-function oxidases with cytochromes *P*-450 and *P*-448 was investigated by using liver microsomes from rats pre-treated with phenobarbital or 3-methylcholanthrene, and with purified forms of the cytochromes isolated from rabbit liver. The two forms of the cytochrome have different substrate specificities; cytochrome *P*-450 has one type 1 substrate-binding site that can accommodate a large variety of substrates, but in contrast cytochrome *P*-448 may possess two type 1 substrate-binding sites, one of which is different to that of cytochrome *P*-450 in that it shows a specificity for substrates such as safole and 9-hydroxy-ellipticine. These findings explain why the two forms of the cytochrome have different substrate specificities and play contrasting roles in the activation and deactivation of xenobiotics.

The microsomal mixed-function oxidase system, which is involved in the detoxification and activation of exogenous chemicals, and in the turnover of many endogenous materials, is comprised essentially of three components, namely, cytochrome *P*-450, the flavoprotein cytochrome *P*-450 reductase and the membrane lipid electron coupler phosphatidylcholine. Purified cytochrome *P*-450 and its reductase exist in several forms, and the isoenzymes of cytochrome *P*-450 may be induced selectively by various substrates (Guengerich, 1979). One such form, namely cytochrome *P*-448, is preferentially induced by chemical carcinogens, and differs from the native cytochrome *P*-450 in its spectral, electrophoretic and immunological properties, amino acid composition and substrate and reaction specificity (Haugen *et al.*, 1975; Thomas *et al.*, 1976a). Perhaps most important, cytochrome *P*-448 is able to effect oxygenation of molecules in sterically-hindered positions, a reaction not catalysed by other isoenzymes of cytochrome *P*-450 (Jerina & Daly, 1974; Levin *et al.*, 1977). For example, cytochrome *P*-448, but not cytochrome *P*-450, catalyses the 2-hydroxylation of biphenyl, *O*-de-ethylation of ethoxyresorufin and the 9,10-epoxidation of benzo[*a*]pyrene (Creaven & Parke, 1966; Burke & Mayer, 1975; Levin *et al.*, 1977). Different forms of the cytochrome therefore

tend to play contrasting roles in the metabolism of toxic chemicals, cytochrome *P*-450 directing metabolism towards detoxification, whereas cytochrome *P*-448 promotes activation to electrophiles and mutagens (Ioannides & Parke, 1980). The activation of benzo[*a*]pyrene (Ioannides *et al.*, 1981) and tryptophan pyrolysis products (Nebert *et al.*, 1979) to mutagens, and the activation of paracetamol to covalently-bound intermediates (C. Ioannides, C. Steele & D. V. Parke, unpublished work) are all catalysed most readily by cytochrome *P*-448.

Recognition that cytochrome *P*-448, unlike cytochrome *P*-450, may oxygenate substrates at sterically hindered positions, and that induced cytochrome *P*-448 may contain traces of the inducing agent or its metabolite (Schenkman *et al.*, 1969) has led to speculations concerning the substrate-binding site of cytochrome *P*-448 and the mechanism of oxygenation. Three major alternatives are possible, namely; (i) that the type 1 substrate-binding site of cytochrome *P*-448 is much larger than that of cytochrome *P*-450; (ii) possibly because of bound inducing agent, cytochrome *P*-448 is not able to accept another carbon substrate but activates O₂ to superoxide or peroxide, which then hydroxylates the carbon substrate non-enzymically; or (iii) the cytochrome and reductase become decoupled and the reductase catalyses the oxygenation of the substrate. The first of these possibilities is now investigated, by means of spectral interaction studies of the cytochromes with different classes of substrates, and by the effects of inhibitors.

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Materials and methods

Phenobarbital, hexobarbital, aniline hydrochloride (BDH Chemicals, Poole, Dorset, U.K.), 3-methylcholanthrene (Sigma, Poole, Dorset, U.K.), perhydrofluorene, 1-phenylhexane (Aldrich Chemical Co., Milwaukee, WI, U.S.A.), safrole (Hopkin and Williams, Chadwell Heath, Essex, U.K.) and pentobarbital (May and Baker, Dagenham, Essex, U.K.) were all purchased from the respective suppliers. 9-Hydroxyellipticine and benzphetamine were gifts from P. Lesca Laboratoire de Pharmacologie et de Toxicologie Fondamental du CNRS, Toulouse, France, and Upjohn Co., Kalamazoo, MI, U.S.A., respectively.

Male Wistar albino rats (170–200g) from the Animal Breeding Unit, University of Surrey, were used in all experiments. Induction of the mixed-function oxidases was achieved by intraperitoneal administration of either phenobarbital (80 mg/kg body wt.) or 3-methylcholanthrene (20 mg/kg body wt.) for 3 days. 9-Hydroxyellipticine was administered intraperitoneally (20 mg/kg body wt.) 1 h before killing. Animals were killed 24 h after the last administration of the inducer, and the livers were immediately excised and washed. Microsomes were prepared as previously described (Ioannides & Parke, 1975) and stored until use (Delaforge *et al.*, 1980a). Rabbit LM₂ (cytochrome P-450) and LM₄ (cytochrome P-448) preparations from phenobarbital- and β -naphthoflavone-induced animals respectively were prepared as previously described (Coon *et al.*, 1978).

Substrate binding to cytochromes P-450 and P-448 with type I and type II substrates was studied by using a Varian Cary double-beam spectrophotometer coupled with automatic base-line correction (Schenkman *et al.*, 1967).

Results

The type II substrate aniline (0.5–10 mM) gave a typical type II spectrum with liver microsomal preparations from both phenobarbital-induced and 3-methylcholanthrene-induced rats, and with LM₂ and LM₄ purified cytochromes. Similarly, the type I substrates pentobarbital (1–10 mM), benzphetamine (0.05–0.10 mM), hexobarbital (0.5–2.0 mM), perhydrofluorene (1 μ l) and 1-phenylhexane (1 μ l) interacted with the microsomal preparation from phenobarbital-induced rats and with the LM₂ (cytochrome P-450) purified cytochrome, eliciting type I spectra, but none of the type I substrates interacted with the microsomes from 3-methylcholanthrene-treated rats, or with the LM₄ (cytochrome P-448) cytochrome to give a type I spectral change.

In contrast, safrole interacted with both cytochromes P-450 and P-448, as well as with both puri-

Fig. 1. Interactions of safrole with phenobarbital- and 3-methylcholanthrene-induced microsomes and with LM₂ and LM₄ rabbit preparations before and after saturation of the type I substrate-binding site with perhydrofluorene

(a) ———, Both cuvettes contained microsomes from 3-methylcholanthrene-pretreated rats, but safrole (0.2 mM) was only added to the test cuvette. Perhydrofluorene (1 μ l) and microsomes from 3-methylcholanthrene-pretreated animals were added to both reference and test cuvettes and safrole (0.2 mM) in the test cuvette. —, Microsomes from phenobarbital-pretreated animals were added in both cuvettes and safrole (0.2 mM) in the test cuvette only. ———, Microsomes from phenobarbital-pretreated rats and perhydrofluorene (1 μ l) were added in both cuvettes. Safrole (0.2 mM) was added only in the test cuvette. (b) ———, LM₄ was added in both cuvettes and safrole (0.2 mM) in the test cuvette only. LM₄ and perhydrofluorene (1 μ l) were added in both cuvettes and safrole (0.2 mM) in the test cuvette only. —, LM₂ was added in both cuvettes and safrole (0.2 mM) in the test cuvette only. ———, LM₂ and perhydrofluorene (1 μ l) was added in both cuvettes and safrole (0.2 mM) in the test cuvette only. Phenobarbital-induced microsomes (1.2 nmol of cytochrome P-450/ml), 3-methylcholanthrene-induced microsomes (0.7 nmol of cytochrome P-450/ml), LM₂ and LM₄ (1 nmol of cytochrome P-450/ml) were all suspended in 0.1 M-phosphate buffer, pH 7.4.

fied rabbit cytochromes, to give typical type I spectral changes (Fig. 1). When perhydrofluorene was added to both reference and test cuvettes at

Fig. 2. Interaction of 9-hydroxyellipticine with microsomes from phenobarbital- and 3-methylcholanthrene-induced rats and with LM₂ and LM₄ rabbit preparations (a) —, Interaction of 9-hydroxyellipticine (1 μM) with microsomes from 3-methylcholanthrene-pretreated rats. ----, Interaction of 9-hydroxyellipticine (1 μM) with microsomes from phenobarbitone-pretreated rats. (b) —, Interaction of 9-hydroxyellipticine (5 μM) with LM₄ preparation. ----, Interaction of 9-hydroxyellipticine (5 μM) with LM₂ preparation. Microsomes (0.7 nmol of cytochrome *P*-450/ml) and solubilized preparations (1.0 nmol of cytochrome *P*-450/ml) were suspended in 0.1 M-phosphate buffer, pH 7.4.

concentrations that saturate the type I binding site, microsomal preparations from phenobarbital-induced animals, or the LM₂ purified cytochrome, showed greatly decreased type I binding spectra with safole; in contrast the microsomal preparation from 3-methylcholanthrene-induced animals, and the LM₄ preparation, showed little change (Fig. 1).

9-Hydroxyellipticine interacts with liver microsomes from both 3-methylcholanthrene- and phenobarbital-induced rats to give a type II spectral change having an asymmetric trough (Fig. 2). Similar interactions were observed with purified cytochromes

Fig. 3. Interactions of 9-hydroxyellipticine with microsomes from phenobarbital-induced rats and rabbit LM₂ preparations before and after saturation of type I substrate-binding site with benzphetamine and perhydrofluorene respectively

(a) —, Both cuvettes contained microsomes from phenobarbital-pretreated rats but 9-hydroxyellipticine (5 μM) was added only to the test cuvette. ----, Both cuvettes contained microsomes from phenobarbital-pretreated rats and benzphetamine (0.1 mM), and 9-hydroxyellipticine (5 μM) was added only to the test cuvette. (b) —, Both cuvettes contained the LM₂ preparation and 9-hydroxyellipticine (5 μM) was added only to the test cuvette. ----, Both cuvettes contained the LM₂ preparation and perhydrofluorene (1 μl), but 9-hydroxyellipticine (5 μM) was only added to the test cuvette. Microsomes and solubilized preparations were suspended in buffer as described in the legend to Fig. 1.

P-450 (LM₂) and *P*-448 (LM₄) isolated from rabbits pretreated with phenobarbital and β-naphthoflavone respectively (Fig. 2). Addition of the type I substrate

Fig. 4. Interactions of 9-hydroxyellipticine with microsomes from 3-methylcholanthrene-induced rats and rabbit LM_4 preparations before and after saturation of the type I substrate-binding site with safrole

(a) —, Both cuvettes contained microsomes from 3-methylcholanthrene-pretreated rats, but 9-hydroxyellipticine ($5\mu M$) was added only to the test cuvette. ----, Both cuvettes contained microsomes from 3-methylcholanthrene-pretreated rats and safrole ($0.2mM$), but 9-hydroxyellipticine ($5\mu M$) was added only to the test cuvette. (b) —, Both cuvettes contained the LM_4 preparation but 9-hydroxyellipticine ($5\mu M$) was only added to the test cuvette. ----, Both cuvettes contained the LM_4 preparation and safrole ($0.2mM$) but 9-hydroxyellipticine ($5\mu M$) was only added to the test cuvette. Microsomes and solubilized preparations were suspended in buffer as described in the legend to Fig. 1.

benzphetamine ($0.1mM$) to both reference and test cuvettes to saturate the type I binding site resulted in an increase in the spectral interaction of 9-hydroxyellipticine with the liver microsomes from phenobarbital-induced rats, which was accompanied by disappearance of the asymmetry and a shift of the spectrum to lower wavelengths (Fig. 3); however,

Fig. 5. Effect of 9-hydroxyellipticine administration on the spectral interaction of safrole with hepatic microsomes from rats pretreated with phenobarbital or 3-methylcholanthrene

(a) ○, Phenobarbital; □, phenobarbital + 9-hydroxyellipticine. (b) ●, 3-Methylcholanthrene; ■, 3-methylcholanthrene + 9-hydroxyellipticine. Microsomes were suspended ($1mg$ of protein/ml) in $0.1M$ -phosphate buffer, pH 7.4.

no similar changes in the spectra were seen when microsomes from the 3-methylcholanthrene-pretreated rats were used. When the solubilized cytochromes from LM_2 and LM_4 preparations, isolated from rabbit liver, were used, similar results were obtained; perhydrofluorene augmented the 9-hydroxyellipticine spectrum, eliminated the asymmetry and shifted the spectrum with LM_2 but not with LM_4 (Fig. 3).

In contrast, when safrole ($0.2mM$) instead of perhydrofluorene was added to both test and reference cuvettes, there was an increase in the spectral interaction of 9-hydroxyellipticine, a shift to lower wavelengths and loss of spectral asymmetry, with the liver microsomes from 3-methylcholanthrene-induced rats (Fig. 4), but this time no similar effect was seen with microsomes from phenobarbital-pretreated animals. With the purified rabbit hepatic LM_2 and LM_4 preparations, similar results were obtained.

Administration of 9-hydroxyellipticine 1 h before killing the animals resulted in competitive inhibition of the binding of safrole to the microsomes from 3-methylcholanthrene-induced rats but had very

little effect on the binding to the microsomes from phenobarbital-induced animals (Fig. 5).

Discussion

In the present study we examined the binding of various substrates to rat and rabbit liver cytochromes *P*-450 and *P*-448. Type I substrates hexobarbital, benzphetamine, pentobarbital, perhydrofluorene and 1-phenylhexane gave the expected type I spectral interactions with rat and rabbit cytochrome *P*-450, but none elicited any spectral interaction with cytochrome *P*-448. The decrease or absence of type I spectral changes with cytochrome *P*-448 have been reported by a number of workers (Schenkman *et al.*, 1969; Remmer *et al.*, 1969; Shoeman *et al.*, 1969). These observations indicate that either cytochrome *P*-448 has no typical type I binding site or the binding site is occupied by the inducing chemical and/or a metabolite. The binding to cytochrome *P*-448 of radioactivity associated with the inducing chemical (3-methylcholanthrene) lends support to the latter possibility (Schenkman *et al.*, 1969; Jefcoate & Gaylor, 1969). However, the binding of some product of the inducer at an allosteric site, preventing the interaction of type I substrates, cannot be excluded.

In contrast, safole interacts with both cytochrome *P*-450 and *P*-448, yielding typical type I spectral changes (Fig. 1), indicating that safole binds to a type I site of cytochrome *P*-448 to which other type I substrates do not bind, or that safole can displace the inducer or its metabolite bound on the normal type I site of this cytochrome. The latter possibility is unlikely, as safole does not have a high affinity for cytochrome *P*-448 ($k_s = 0.13$ mM; Delaforge *et al.*, 1980b). Furthermore, when the type I site is blocked with perhydrofluorene, safole elicits an unchanged type I spectrum with cytochrome *P*-448, whereas the spectral change with cytochrome *P*-450 is greatly decreased by perhydrofluorene (Fig. 1). This indicates that the type I site of cytochrome *P*-450 accommodates safole and other type I substrates, whereas cytochrome *P*-448 probably has two distinct and independent 'type I' binding sites, one of which interacts preferentially with safole.

9-Hydroxyellipticine, a potent inhibitor of cytochrome *P*-448 (Delaforge *et al.*, 1980a), interacts with both cytochrome *P*-450 and *P*-448 to give type II spectral changes (Fig. 2) (Lesca *et al.*, 1978; Sautereau & Lesca, 1979). The asymmetry of these spectra, as in the case of aniline (Schenkman, 1970), disappeared and the spectrum shifted to lower wavelengths when the type I binding site was blocked (Figs. 2 and 3), indicating that the spectrum of 9-hydroxyellipticine has a type I component. 9-Hydroxyellipticine is displaced by benzphetamine or

perhydrofluorene from the type I site of cytochrome *P*-450 (Fig. 3), but no displacement occurs with cytochrome *P*-448 (result not shown). In contrast, safole displaces 9-hydroxyellipticine from cytochrome *P*-448 with loss of spectral asymmetry (Fig. 4), but not from cytochrome *P*-450 (result not shown), thus providing further evidence that cytochrome *P*-448 has a type I binding site, but different from that of cytochrome *P*-450, the former being shared by safole and 9-hydroxyellipticine. Administration of 9-hydroxyellipticine to rats inhibited competitively the binding of safole to cytochrome *P*-448, confirming that these two compounds interact with the same type I binding site of cytochrome *P*-448 (Fig. 5). However, 9-hydroxyellipticine administered to phenobarbital-pretreated animals had little effect on the binding of safole to cytochrome *P*-450 (Fig. 5), which does not accord fully with 9-hydroxyellipticine and safole, together with other substrates, binding at the same type I site of cytochrome *P*-450. This may be due to the poor affinity of 9-hydroxyellipticine for cytochrome *P*-450 (Delaforge *et al.*, 1980a) as 9-hydroxyellipticine (10 μ M) inhibited competitively the type I interaction of safole with cytochrome *P*-450 *in vitro* (M. Delaforge, C. Ioannides & D. V. Parke, unpublished work).

Previous studies demonstrated that cytochromes *P*-450 from rat and rabbit have different properties (Thomas *et al.*, 1976b). In the present study the rabbit solubilized preparations and the crude microsomal preparations from induced rats exhibited the same specificity towards the various compounds investigated, indicating that the rabbit and rat forms of the cytochrome may have similar substrate-binding sites. The observations presented here indicate that the different substrate specificities of cytochromes *P*-450 and *P*-448 may be due, at least partly, to the presence of different type I binding sites. It appears that cytochrome *P*-450 has only one type I substrate-binding site, which can accommodate various substrates of different chemical structure and molecular dimensions. However, with cytochrome *P*-448 this substrate binding site is either absent or occupied by the inducing agent or its metabolite, but a second type I substrate-binding site, distinct from that of cytochrome *P*-450, is also present. Possibly the binding of the inducing agent, or its metabolite, to cytochrome *P*-450 produces a conformational change in the normal type I site so that its substrate specificity and site of substrate oxygenation are changed to that characteristic of cytochrome *P*-448.

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