THE EFFECT OF CHARCOAL TREATMENT ON MICROSOMAL CYTOCHROME *P*-450

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1. Introduction

The interaction of a drug with microsomal cytochrome *P*-450 may cause Type I, Type II and modified Type II difference-spectra or no spectral change at all [1]. Which of these effects is actually observed depends as well on properties of the drug – like hydrophobicity and availability of lone-pair electrons – as on the state of the cytochrome [2].

The heme portion of cytochrome *P*-450 exists preferentially in a low-spin state which – under change of its absorbance characteristics – is partially converted to high-spin state, when Type I binders are added [3]. Many Type I substrates interact so strongly with cytochrome *P*-450 that they remain bound to a certain degree during preparation of microsomes and beyond that also during purification of cytochrome *P*-450. Cytochrome *P*-450 from animals treated with phenobarbital [4] and methylcholanthrene [5] was isolated in form of complexes with those substrates or their metabolites.

'Endogeneous' substrates of that kind will complete with in vitro added Type I compounds in such a way that only diminished Type I binding spectra are observed and the apparent affinities and presumably the metabolism of the drugs are influenced. Reliable experiments on the nature of drug–cytochrome *P*-450 interactions therefore can only be performed with a cytochrome as far as possible free of endogeneous substrates.

The purpose of this paper is to show a simple method for removing substrates from cytochrome *P*-450: Incubation with charcoal leads to an extensive release of firmly bound ligands without any detectable destruction of the cytochrome and its catalytic properties.

2. Materials and methods

Chemicals and biochemicals of the highest purity available were obtained from E. Merck (Darmstadt), Boehringer (Mannheim), Sigma and Atlas chemical company. ^{3}H-labelled Tiamutin^{R*}, a derivative of pleuromutilin, was prepared by F. Battig (Sandoz Forschungsinstitut) with a specific activity of 22 μCi/mg.

If not otherwise indicated, experiments were performed in isotonic KCl/Tris buffer (50 mM Tris, 0.12 M KCl, pH 7.4).

Microsomes were prepared from livers of male rats (Sprague-Dawley, 200 g) and rabbits (New Zealand, 1.5 kg) as previously described [6]. Cytochrome *P*-450 from rabbit liver was partially purified about 3 times by the procedure of Van der Hoeven and Coon [7]. Its content was determined using the method of Omura and Sato [8] protein was measured according to Lowry [9]. The metabolizing activities of microsomes were checked by their ability to hydroxylate aniline and demethylate aminopyrine [10,11].

Charcoal was activated by heating it gently in 0.25 M NaEDTA at 90°C for 1 h. Afterwards it was washed with excess volume of distilled water and finally suspended in KCl/Tris buffer to a storage suspension of 100 mg/ml. For the removal of cytochrome *P*-450-bound substrates aliquots of freshly prepared charcoal suspension were put either to microsomes or to partially purified cytochrome *P*-450 (final concentration of cytochrome *P*-450 in both cases about

* 14-deoxy-14((2'-diethyl-aminoethyl)-mercaptoacetoxy)-mutilin hydrogen fumarate.

10 μM). The resulting suspensions were stirred at 0°C usually for 20 min and afterwards charcoal was removed by centrifugation at 11 000 X *g* for 10 min.

In order to determine the extent of released substrate, a Type I binder, ^{3}H-labelled Tiamutin^R, was added to microsomes (initial Tiamutin^R-concentration 0.1 mM). The charcoal concentration was varied between 0.12 and 2.4 mg/mg microsomal protein.

After 10, 20, 40 min incubation at 0°C samples were centrifuged and the remaining radioactivity in the supernatant determined. Simultaneously the content of protein, cytochrome *P*-450 and its metabolizing activities were measured.

The spectral changes produced by addition of Tiamutin^R to microsomes and partially purified cytochrome *P*-450 at 25°C were recorded on a Perkin Elmer EPS-3T spectrophotometer, as described in [6].

^{3}H-radioactivity measurements were performed in Instagel (Packard Instruments GmbH) on a Packard Tri-Carb 3375 liquid scintillation spectrometer.

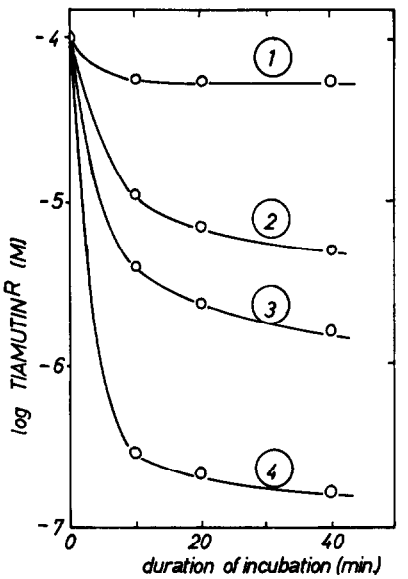

Fig.1. Removal of microsomal bound [^{3}H]Tiamutin^R by charcoal at 0°C in KCl/Tris buffer (pH 7.4). Microsomes from rat liver (cytochrome *P*-450 10 μM). Charcoal concentration ① 0.12 mg/mg microsomal protein, ② 0.59 mg/mg microsomal protein, ③ 1.15 mg/mg microsomal protein, ④ 2.4 mg/mg microsomal protein. Tiamutin^R, total concentration of Tiamutin^R in the supernatant after sedimentation of charcoal.

3. Results and discussion

In order to demonstrate the charcoal mediated release of cytochrome *P*-450 bound ligands we used the antibiotic Tiamutin^R as a probe which interacts exclusively in Type I manner at low drug concentrations ($K_a \geqslant 1\ \mu M^{-1}$, KCl/Tris buffer, 25°C). At elevated Tiamutin^R concentrations ($C > 20\ \mu M$) the formation of Type I complex is followed by the emergence of modified Type II complex. ([12,13] and [6] describe the very similar properties of hydrated Tiamutin^R.) As we could show by stopped-flow kinetics this latter spectral change is obviously caused by a conformational change of the cytochrome. It is

Table 1
Some properties of charcoal treated microsomes from rat liver

Contents/activities	Control	After charcoal treatment[a]
Protein (mg/g liver)	11.6	8.8
Cytochrome *P*-450 (nmol/mg protein)	0.76	0.80
Hydroxylation of aniline (nmol/min/mg protein)	0.70	0.87
Demethylation of aminopyrine (nmoles/min/mg protein)	6.3	9.9

[a] 2.4 mg charcoal/mg protein, 20 min

Some properties of charcoal treated microsomes from rat liver. Microsomes from rat liver (cytochrome *P*-450, 10 μM) treated with 2.4 mg charcoal/mg protein, 20 min.

probably due to high enrichment of the compound in the phospholipid bulk of microsomal membranes respectively around the enzyme itself [13].

Dependent on charcoal concentration, [^{3}H]Tiamutin^R is removed from its unspecific phospholipid binding sites and from cytochrome *P*-450 (fig.1). At the highest charcoal concentration less than 0.016 m/mol cytochrome *P*-450 remain bound, whereas – calculated on the basis of difference spectra between 420 and 490 nm [14] – at least 0.1–0.15 m/mol cytochrome *P*-450 had been complexed in Type I manner before. The small amount of ligand still bound was largely released by repeated treatment with charcoal.

Charcoal treatment does neither denature cytochrome *P*-450 significantly nor diminish its catalytic activities (table 1). In most cases a slight increase of relative cytochrome *P*-450 content was observed – presumably due to removal of non-microsomal protein by charcoal. The high increase of demethylating activity is obviously caused by a release of some endogeneous inhibitor.

Tiamutin^R was also used as a probe for the spectral properties of cytochrome *P*-450: Charcoal treatment leads to highly increased Type I difference spectra. This was proved with microsomes from rat and rabbit, of control and phenobarbital or methylcholanthrene induced animals. Cytochrome *P*-450 – partially purified from rabbits after [7] has lost some Type I sites during purification – presumably due to detergent binding. Charcoal treatment enhances the magnitude of Type I spectra very markedly (fig.2A). Evaluation of the binding isothermes shows that the number of Type I sites has doubled and the apparent affinity is increased about 4-times from 0.06–0.25 μM^{-1} at 25°C (in KCl–Tris/Tris acetate glycerol). In contrast the modified Type II spectrum observable at high Tiamutin^R concentrations is not influenced by charcoal treatment (fig.2B).

This result is in accordance with the assumption that the conformational change of the cytochrome [13] which leads to modified Type II emergence is mediated by Tiamutin^R–phospholipid interactions. (A certain amount of phospholipid adhered to the protein also in the partially purified preparation [12].) This type of interaction should not depend so much on the nature of Type I ligand.

'Endogeneous' substrates will inhibit the binding

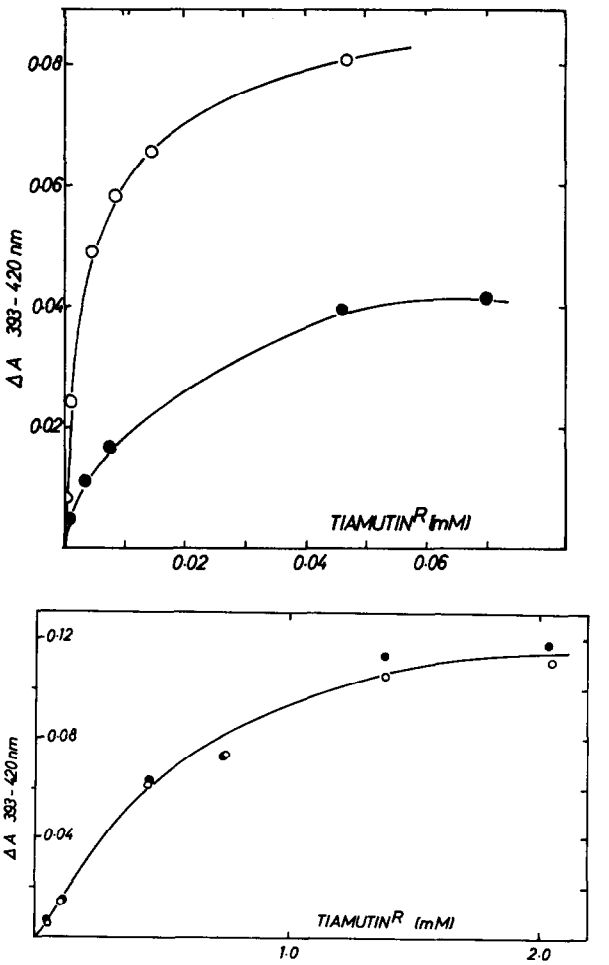

Fig.2. Titration of partially purified cytochrome *P*-450 from rabbit liver with Tiamutin^R before and after charcoal treatment (pH 7.4, buffer = 40% KCl/Tris, 60% Tris–acetate–glycerol, Tris acetate 10 mM, glycerol 20%, EDTA 0.1 mM). (●—●) Before charcoal treatment, (○—○) after 2.4 mg charcoal/mg protein, 20 min $C_{P\text{-}450}$ = 9.0 μM (3.0 nm/mg protein). Tiamutin^R, total concentration of Tiamutin^R. (A) Type I spectrum, (B) modified Type II spectrum.

as well as the metabolism of a certain drug to an unknown degree. Their removal seems to us a crucial prerequisite before starting an experiment. The use of charcoal for this purpose has some advantage over the use of albumin as proposed by Holtzman et al. [4]. Activated charcoal can easily be prepared in a standard quality whereas albumin may have bound varying amounts of minor components like fatty acids

which can dissociate drugs from cytochrome *P*-450. Endogeneous substrates are removed together with the charcoal from the sample under investigation. No coupled equilibria, as in the case of ligand binding to the cytochrome and albumin, have to be considered.

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