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# Characterization of cytochromes *P*-450 purified from untreated and $^{14}$C-2,3,7,8-tetrachlorodibenzo-*p*-dioxin – treated marmoset monkeys: Identification of the major form as a possible orthologue of *P*-450 1A2

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**Abstract**

Three purified cytochrome *P*-450 (*P*-450) forms obtained from liver microsomes of marmoset monkeys induced with $^{14}$C-2,3,7,8-tetrachlorodibenzo-*p*-dioxin ($^{14}$C-TCDD) were characterized. Comparison of electrophoretic and spectral properties and reconstituted methoxy-and ethoxyresorufin *O*-dealkylase (MROD and EROD) activities with those of forms isolated from untreated marmosets indicated that one of these (form F) is likely constitutive. Another form (D) had MROD and EROD activities which were 100 and 15 times those observed for form F. A form having biophysical properties similar to those of form D was also found in untreated animals. A third form (C) had an appreciable capacity to bind its inducing agent and showed a TCDD-to-*P*-450 molar ratio for detergent-free solutions of $0.66 \pm 0.13$ to 1. In immunoblot analyses of these forms with antibodies raised against specific peptide sequences derived from rat *P*-450 1A1 and 1A2, the only positive reactions seen were those for untreated and inducible forms D with anti-rat 1A2. This provides evidence that the main or sole *P*-450 1A form in marmoset liver microsomes is 1A2, as in humans, and that this is inducible by TCDD.

*Key words:* Cytochrome *P*-450; *P*-450 1A2; Purification; Monkey; Dioxin

Interest in studying TCDD has arisen from the fact that this substance, which is capable of inducing *P*-450 enzymes of the *P*-450 gene subfamily 1A, represents one of the most long-lasting environmental hazards [1,2]. Evidence has been accumulating that ubiquitous, polycyclic aromatic hydrocarbons like TCDD [3], pentachlorodibenzofuran [4], and coplanar polyhalogenated biphenyls [5] not only can induce *P*-450 but also bind to it, thus raising the question as to whether any such *P*-450 form(s) may serve as a source of stabilization [5] or even possibly a 'storage site' [4] for these chemicals in vivo. These investigations have been carried out with the rat as a source of *P*-450 isozymes, and while both 1A1 and 1A2 are present in rat livers, only 1A2 has been found in human liver [6]. We have therefore used the marmoset for our studies. This New World monkey is a typical and convenient non-human primate which, quite in contrast to humans, can be induced in a controlled manner. We have previously described that in comparison to rats, both induced and untreated marmosets display differing features regarding metabolic activities, notably towards otherwise specific substrates such as ethoxy- and pentoxyresorufin [7,8]. The aim of this study was to characterize three purified hepatic microsomal *P*-450 forms derived from TCDD-induced marmosets in order to compare their properties with *P*-450 forms of rats and humans.

Untreated (UT) male marmosets (*Callithrix jacchus*) and TCDD-induced marmosets of both sexes were used. The animals were 9 to 10 months old, i.e. close to maturity. One single injection of 300–1000 ng $^{14}$C-TCDD per kg body weight was given s.c. four days prior to sacrifice. Microsomes were prepared as described [7] and solubilized in sodium cholate. The present communication concerns three of seven *P*-450 fractions isolated from TCDD-induced marmosets as

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Abbreviations: *P*-450, cytochrome *P*-450; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; UT, untreated; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; EROD, ethoxyresorufin *O*-deethylase; MROD, methoxyresorufin *O*-deethylase.


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described in detail elsewhere [9]. This involved 8-aminooctyl-Sepharose, Mono Q and Mono S columns in an FPLC system (Pharmacia, Uppsala), and residual non-ionic detergents were removed by using 'Hydroxylapatite HPLC Grade' (Calbiochem, La Jolla). At this stage of purification, the specific contents of forms C, D and F were 13.7, 13.5 and 15.1 nmol *P*-450 per mg protein, and the SDS-PAGE apparent molecular weights as re-examined in the present work were 53 500, 52 500 and 47 500 dalton. Forms UT-D and UT-F were obtained from untreated animals and eluted at the same positions as did TCDD-D and TCDD-F. For comparison with these, *P*-450 forms from TCDD-treated Wistar rats were purified using the same procedures [9]. NADPH-*P*-450-reductase was isolated from phenobarbital-treated rats [7] and used in reconstituted sytems involving *P*-450 forms from both species. TCDD-concentrations in fractions were ascertained by measuring the radioactivity with liquid scintillation counting. It had been shown previously that under these experimental conditions, radioactivity represents only the non-metabolized TCDD [10]. The specific activity of the $^{14}$C-TCDD used in this study was 3.96 Bq/pmol. Immunoblot analyses [11] and assays of catalytic, spectral and electrophoretic properties [7] were also carried out as described previously.

Many studies of environmental chemical-induced 1A1 and 1A2 monooxygenases derived from human and rat sources have concerned cigarette smoking, and TCDD (and related agents), respectively. It was therefore considered to be of great importance to establish whether TCDD-treated marmosets would more closely reflect those inductions in smokers, or in TCDD-treated rats. The *P*-450 contents (mean ± SD) found for TCDD-treated marmoset microsomes of 535 ± 66 pmol per mg protein represent one-third more than those found for untreated animals [8] and approximately the same values found elsewhere for liver microsomes obtained from wedge biopsy samples from human non-smokers (490 ± 50) and smokers (550 ± 30) [12]. Individual NADPH-*P*-450-reductase activities found here for untreated marmoset microsomes of 141 ± 87 pmol (cytochrome *c* reduced) (mg protein)$^{-1}$min$^{-1}$ also reflect basically the same values reported for non-smokers (149 ± 11) or rats (110 ± 3) [12]. Reductase activities were increased by only 16% in TCDD-induced marmosets, coinciding with 10% in smokers [12]. EROD activities in non-smoker liver microsomes are lower than in the rat [13] but can nonetheless be significantly increased in the livers of smokers [14,15]. EROD activities in TCDD-induced marmoset microsomes of 1065 ± 148 pmol resorufin/mg protein/min were about 10 times those found for untreated microsomes. Again, these values are practically the same as those found for smokers (1045 ± 980) and non-smokers (318 ± 174) [14].

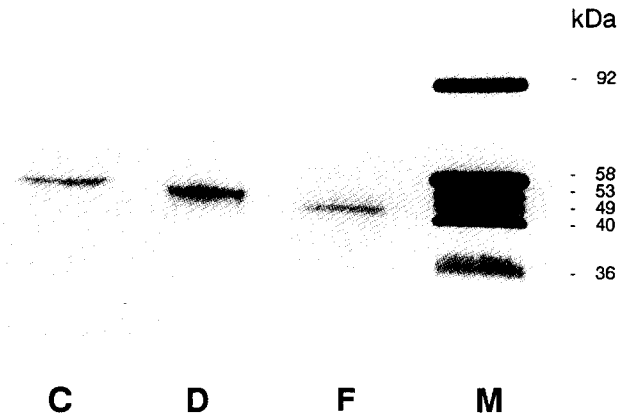


Fig. 1. SDS-PAGE gel of *P*-450 forms C (30 ng of protein), D (50 ng) and F (20 ng) purified from TCDD-treated marmoset monkeys. M: marker proteins with molecular weights in kilodalton (kDa) as described in reference [9]. The 10–15% gradient gel was stained with silver.

All in all, concerning this data, marmoset liver microsomes appear to reveal monooxygenase contents and activities very similar to those obtained from human sources. The same TCDD dose (300–1000 ng/kg body weight) in Wistar rats causes an EROD-induction of more than 200-fold [8]. Since we found that, in contrast to rats, at these doses an apparent plateau in inductive capacity of EROD activity in marmosets was reached, higher doses of TCDD were not used in our studies. No striking sex-based differences in marmoset total *P*-450 content or EROD activity were evident. TCDD induction was, however, found to suppress in male but not in female liver microsomes the aminopyrine and ethylmorphine *N*-demethylation rates by 25 and 50%, respectively as had been shown previously for TCDD-treated rats [16]. Sex differences concerning purified marmoset *P*-450 forms were not investigated here.

An SDS-PAGE gel of the TCDD-induced fractions discussed here is illustrated in Fig. 1. The absolute spectra of oxidized solutions indicate that all three forms are present exclusively in the low-spin state. By contrast, in rats treated with polycyclic aromatic hydrocarbons, the two inducible *P*-450 forms are 1A1 which is low-spin, and 1A2 which is high-spin [4,17,18]. Use of both EROD and MROD measurements was chosen here on the basis that these reactions have been shown to display some specificity towards rat 1A1 and 1A2, respectively [19]. In TCDD-induced marmoset livers, forms C, D and F were seen to exhibit middle, high, and low catalytic activities, respectively, and also different EROD-to-MROD ratios [9]. Form F was also isolated from untreated animals, suggesting that this en-

zyme is constitutive. A form having the same electrophoretic and spectral properties and chromatographic behaviour as form D was also found in untreated animals but had a somewhat lower EROD activity. In one study [17], 1A2 in 45 samples of human liver was found to exhibit varying phenacetin *O*-deethylase specific activities, thus suggesting that differential levels of immunoreactive 1A2 can exist in livers. Whether forms TCDD-D and UT-D described here also reflect a difference in their expression, or whether they are two completely different enzymes is at present unknown.

Assay of $^{14}$C-TCDD radioactivity indicated that after use as an inducer, TCDD is also bound to *P*-450, and is associated preferentially with *P*-450 form C. Detergent-free fraction C was found to bind $0.66 \pm 0.13$ nmol TCDD per nmol of isolated cytochrome (mean values $\pm$ SD for three purifications), whereas for detergent-free fractions D and F, these values were less than $0.07 \pm 0.01$ and $0.06 \pm 0.01$, respectively. Those ratios for fraction C are similar to the values ranging between 0.5 and 0.9 nmol substrate per nmol *P*-450 reported for detergent-free purified rat 1A2 after treatment with TCDD [3], pentachlorodibenzofuran [4], hexabromobiphenyl [5] and isosafrole [20], and for the corresponding rabbit form isolated after induction with 3-methylcholanthrene [21].

Interestingly, the TCDD-binding marmoset *P*-450 form (C) in the presence of non-ionic detergent always bound to, and was eluted from, Mono Q (anion-exchange) columns, whereas similar purifications of rat *P*-450 [4,18,20] consistently yielded the inducer-binding form (1A2) in gradient elutions from cation-exchangers. Prior to hydroxyapatite column chromatography, detergent-containing fractions of marmoset form C bind as much as $5.1 \pm 1.6$ nmol TCDD/nmol *P*-450. This was not the case with the other two forms discussed here: in detergent-containing fractions of D and F, the TCDD binding was as little as in detergent-free solutions (see above).

In such *P*-450 fractions isolated from detergent-containing solutions, this *P*-450 has been assumed elsewhere to be located in a lipid environment which would also enhance the binding of TCDD to the cytochrome [3]. Such a situation could thereby also be hypothetically envisageable in vivo, where phospholipids in place of detergent are present, indicating that inducer-binding may possibly serve as a source of storage for such agents in the liver as hypothesized by Kuroki et al. [4]. On the other hand, Edwards [22] has provided evidence that most of the *P*-450 protein is in the cytosol and that only the N-terminus is in the membrane. Indeed, reference [9] showed that the highly lipophilic TCDD can be separated off, along with the detergent in which it is dissolved. Thus, most of the TCDD appears to be associated more with the non-ionic detergent than with *P*-450, so that the significance of *P*-450 as a 'storage site' must remain questionable.

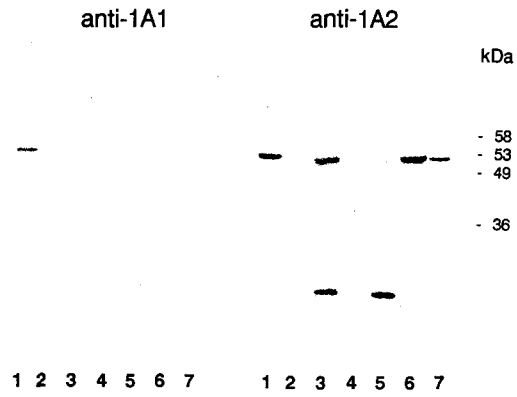


Fig. 2. Immunoblots of microsomes and purified TCDD-marmoset *P*-450 fractions with polyclonal antibodies specific for peptide sequences [11] derived from rat *P*-450 1A1 and 1A2. Left: with anti-1A1; right: with anti-1A2. Lanes 1: 25 $\mu$g of TCDD-induced rat liver microsomal protein. Lanes 2, 3 and 4: 10 pmol each of TCDD-marmoset *P*-450 fractions C, D and F. Lane 5: 10 pmol of untreated marmoset *P*-450 fraction D. Lanes 6 and 7: 25 $\mu$g each of TCDD-induced and untreated, marmoset liver microsomal protein. The appropriate molecular weight regions are given in kilodalton (kDa).

Immunoblot analyses of these TCDD forms (C, D and F) and UT forms (D and F) were performed with antibodies raised against specific peptide sequences derived from rat *P*-450 1A1 and 1A2 according to the technique of Edwards et al. [11]. Four further TCDD and three UT fractions as isolated in reference [9] were also screened. With anti-1A1, which bound to rat, mouse and rabbit antigens [23], no reactions were seen for any TCDD- or UT-*P*-450 forms (Fig. 2). An antibody which recognized 1A2 in rat, mouse, rabbit, hamster, and human liver microsomes (Dr. R. Edwards, personal communication) gave a very strong reaction with TCDD-D and reacted to a similar degree with UT-D (Fig. 2). With anti-1A2, TCDD-D and UT-D also gave a reaction with an additional antigen (Fig. 2, lanes 3 and 5). Since this protein is not a *P*-450 form but a protein of much lower molecular weight (28 000 dalton), the cross-reactivity with anti-1A2 seen here may have resulted from a proteolytic fragment present in the purified fractions. Evidence for this is indeed lent upon closer examination of the lane for the TCDD-induced microsomal sample (Fig. 2, lane 6), whereby a very faint band does appear to exist.

It is of interest that this *P*-450 form (TCDD-D) is not the same form which is capable of binding TCDD or other such agents as is however the case with rat or rabbit *P*-450 (1A2) [3,4,20,21]. With the same anti-1A2, *P*-450 TCDD-C and F did not indicate any binding. Thus, the main or sole *P*-450 1A form in untreated or TCDD-induced marmoset liver appears to be 1A2. The lack of binding of anti-1A1 to these purified fractions,

however, does not mean that 1A1 is absent in marmoset liver; it may well be that this antibody does not bind to marmoset 1A1 simply because of sequence differences. Immunoblot procedures have been used previously to demonstrate inter-relatedness of various rat and human *P*-450 isozymes [13,17,24]. Particularily Sesardic et al. [6] have shown that, concerning the gene subfamily 1A in human liver microsomal fractions, practically only 1A2 is present, and this appears to hold true as well for human fetal liver purified *P*-450 forms [25]. Although our data suggest the absence of 1A1 in marmoset liver as well, final proof of this would have to await further studies with specific antibodies against marmoset 1A1. Such a protein, however, has yet to be isolated or detected, and this may likely necessitate investigation of extrahepatic marmoset tissue.

Maurel and colleagues [26] have meanwhile shown that in primary human hepatocytes, gene products CYP1A1 are indeed highly inducible by TCDD, β-naphthoflavone, and other substances. The strong heterogeneity in the expression of 1A1 and its capacity for induction, as well as pharmacokinetics, however, may be the reason why others have not detected this gene product in a limited number of smoker and non-smoker autopsy samples [27].

Recently, a monoclonal antibody recognizing rat 1A1 – but not 1A2 – has been produced by Burke and co-workers, which also recognized a similar protein band in immunoblots of human liver microsomes [28]. In this case, a heterogeneity of the distribution of such enzymes among various liver zones was observed.

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