AFLATOXIN B1 OXIDATION BY HUMAN CYTOCHROME P450s

Yune-Fang UENG*, Tsutomu SHIMADA#, Hiroshi YAMAZAKI#, and F. PETERGUENGERICH§

*National Research Institute of Chinese Medicine, Taipei, Taiwan, R. O. C., #Osaka Prefectural
Institute of Public Health, Osaka, Japan, and §Department of Biochemistry and Center in
Molecular Toxicology, School of Medicine, Vanderbilt University, TN, USA

INTRODUCTION

Cytochrome P450 (P450)-dependent monooxygenases play an important role in the detoxication and bioactivation of xenobiotics including drugs, pollutants, and chemical carcinogens. There is considerable evidence that variations in the levels of P450s in experimental animals can influence tumor formation (Conney, 1982). It is necessary to identify the roles of individual P450 enzymes in various reactions. P450 3A4 is the major P450 enzyme present in human liver and the variations of P450 3A4 in human livers is 40-fold. P450 3A5 is expressed in most fetal liver samples and presents in about 25% of individuals. When P450 3A5 is expressed in humans, the levels is usually $\leq 30\%$ that of P450 3A4 (Guengerich 1995). The reconstitution of P450 3A enzymes has usually been problematic. We have optimized a reconstituted system containing *Escherichia coli*-expressed recombinant human P450 3A4 and 3A5.

Aflatoxin (AF) B₁, the secondary metabolite of *Asparagillus flavus* and *A. parasiticus*, has been implicated as a causative compound in human hepatic carcinogenesis. The bioactivation of AFB₁ to its bioactive intermediate is mainly attributed to P450. Some metabolic reactions of AFB₁ and its oxidation products catalyzed by human P450s and other enzymes have been identified (Raney *et al.*, 1992, Ueng *et al.*, 1995). However, there has been some controversy in the literature regarding the roles of P450 1A2 in AFB₁ oxidation (Eaton and Gallagher, 1994), and careful evaluations of products had not been done with human enzymes. We have analyzed the oxidation products of AFB₁ by P450 3A enzymes and comparisons are made with recombinant human P450 1A2. Buening *et al.* (1978) first reported that α -naphthoflavone (α -NF) and certain other flavones could stimulate the activation of AFB₁ to genotoxic oxidation products in human

liver microsomes. The effects of α -NF on the oxidation of AFB₁ by P450 3A4 have been studied.

MATERIALS AND METHODS

Enzyme Assays and Data Analysis: Recombinant human P450s were purified from *E. coli* membranes. Reconstituted systems involving purified recombinant P450 1A2 were generally set up with 0.2 μ M P450 1A2, 0.3 μ M rabbit NADPH-P450 reductase, and 30 μ M L- α -dilauroyl-sn-glycero-3-phosphocholine (DLPC) in 50 mM potassium phosphate buffer, pH 7.4 with substrate and an NADPH-generating system. P450 3A4 and 3A5 reconstituted monooxygenase systems were set up under various conditions as described in the "RESULTS and DISCUSSIONS" section. The optimal reconstituted system used for P450 3A4 contained 20 μ g of phospholipid mixture (DLPC/L- α -dioleoyl-sn-glycero-3-phosphocholine/bovine brain phosphatidylserine, 1:1:1, w/w/w), 30 mM MgCl₂, 40 nM P450 3A4 (or 0.2 μ M 3A5), 80 nM NADPH-P450 reductase (or 0.4 μ M in the case of P450 3A5), 80 nM (or 0.2 μ M in the case of P450 3A5) human or rat cytochrome b₅ (b₅), 0.5 mM sodium cholate, 50 mM potassium HEPES buffer (pH 7.4), and an NADPH-generating system (Ueng *et al.*, 1995). AFB₁ metabolite formation and α -NF oxidation were determined by using HPLC as described by Ueng *et al.* (1995 and 1997). Values of v at various substrate concentrations were fit using nonlinear least-squares regression without weight.

Genotoxicity Test

The umu response was measured with *Salmonella typhimurium* TA 1535/pSK1002 (Oda *et al.*, 1985). Bacteria were incubated with AFB₁ for a 2 hr period prior to measurement of β -galactosidase activity. β -galactosidase activity was determined by using 2-nitrophenyl- β -D-galactopyranoside as a substrate and measuring the absorbance at 420 nm. The bioactivation

Correspondence : Yune-Fang Ueng

activity was expressed in β -galactosidase units per min per nmol P450.

RESULTS AND DISCUSSION

Optimization of Reconstitution Conditions for P450 enzymes

In the course of optimization of conditions, we found a surprisingly strong increase of nifedipine oxidation, testosterone 6 β -hydroxylation, and AFB₁ oxidation activities when 30 mM MgCl₂ was added (Ueng *et al.*, 1995). The complete system consisted of a phos-

Fig. 1. Dependence of AFB₁ oxidation on components of the optimized reconstitution system for recombinant P450 3A4. ■, AFB₁; ▨, the GSH conjugate of AFB₁ exo-8,9-oxide.

Fig. 2. Effects of reconstitution conditions on AFB₁ oxidation activities of P450 3A5. ▨, the GSH conjugate of AFB₁ exo-8,9-oxide; ■, AFB₁.

pholipid mixture, sodium cholate, b₅, MgCl₂, and GSH (Fig. 1). In the absence of b₅ and MgCl₂, the catalytic activities were dramatically decreased. Similar to P450 3A4, AFB₁ oxidation activity of P450 3A5 was considerably stimulated by the addition of MgCl₂ (Fig. 2). We have reported that MgCl₂ enhanced rates of reduction of b₅ by NADPH-P450 reductase in reconstituted systems (Yamazaki *et al.*, 1995). This phenomenon may be contributed to the greater catalytic activities in the presence of divalent cations. The role of GSH in the stimulation of catalytic activities in P450 3A4-reconstituted system was not clear. In the particular study of AFB₁ oxidation, GSH and GSH S-transferase (GST) were added to trap the epoxidation products.

Oxidation of AFB₁ by recombinant human P450s

Direct measurements of AFB₁ oxidation products are necessary to define the roles of individual enzymes in activation and detoxification. Under the particular conditions used here a small amount of dihydrodiol was recovered, apparently not trapped by the GST used in this assay (Fig. 3). The major metabolites by P450 1A2 and 3A4 were AFM₁, and AFQ₁, respectively. In all cases P450 3A4 formed only the exo-oxide and P450 1A2 formed a roughly equimolar mixture of both epoxides (Fig. 3). P450 3A4 was more effective in forming total 8,9-oxides than P450 1A2 at all AFB₁ concentrations (Fig. 4). The exo-oxide is the most strong biological active product and the endo-oxide is essentially not genotoxic (Ueng *et al.*, 1995). These results support the conclusion that P450 1A2 is considerably less effective than P450 3A4 in producing genotoxic product AFB₁-exo-8,9-oxide while it predominantly detoxicates AFB₁ via the AFM₁ pathway. Of particular interest, P450 3A5 (with 88% identity to P450 3A4) formed mainly the AFB₁ exo-epoxide. The rates measured with P450 3A4 were approximately two-fold greater than with P450 3A5 (Fig. 1 and 2). However, the rate of 3 α -hydroxylation by P450 3A5 was only ~1% that of P450 3A4 under these conditions. The ratio of AFQ₁ and epoxide formation is approximately opposite that of P450 3A4 (Fig. 2). Aoyama *et al.* (1990) reported that P450 3A4 was approximately five-fold more active than P450 3A5 in the activation of AFB₁ to a mutagen, although results are expressed on a protein and not P450 basis. This result may be compared to the two-fold difference in the epoxidation rate seen between P450s 3A4 and 3A5.

Genotoxicity Activation by P450 enzymes

The umu test is highly sensitive and conveniently

utilized with microsomes and purified P450s. Direct comparisons of umu responses produced by recombinant human P450s 1A2 and 3A4 were made.

The same pattern was seen in the umu test as in the analysis of production formation of AFB₁ oxidation. *E. coli* membranes containing P4503A4 were more effective in activating AFB₁ than those containing P450 1A2. This effect was seen over a wide concentration range of AFB₁ (10 μ M and 1.0 μ M)(Ueng *et al.*, 1995). The above experiments were repeated with the enzymes after purification and reconstitution, with the same results. Some adjustments to the optimized reconstitution system were made. Even though the conditions may not be completely optimal for AFB₁ activation by P450 3A4, P450 3A4 is clearly more active than P450 1A2(Fig. 5). This result provides further support for the usefulness of this system (umu) in detecting the formation of the genotoxic *exo* isomer of AFB₁ 8,9-oxide.

Modulatory effects of α -NF on AFB₁ oxidation

α -NF stimulated the umu response by P450 3A4-catalyzed AFB₁ activation and rates of AFB₁ 8,9-oxide formation in human liver microsomes, while decreasing the rate of 3 α -hydroxylation (Shimada and Guengerich, 1989, Raney *et al* 1992). In line with the general strong inhibition of P450 1A2 in all animal species by α -NF, we found that the addition of 1-2 μ M

α -NF resulted in potent inhibition of all AFB₁ oxidation reactions catalyzed by recombinant human P450 1A2 (Ueng *et al.*, 1995). The pattern of α -NF response in the optimized reconstituted bacterial recombinant P450 3A4 system is very similar to that observed in human liver microsomes. Particular care was taken to be sure that reactions at the lower substrate concentrations still reflect the initial velocities. Plots of rates of AFB₁ 3 α -hydroxylation and 8,9-epoxidation in P450 3A4-

ACKNOWLEDGEMENT

We thank C. G. Turvy for preparing rabbit NADPH-P450 reductase.

REFERENCES

- Aoyama, T., Yamano, S., Guzelian, P. S., Gelboin, H. V., and Gonzalez, F. J. (1990): Five of 12 forms of vaccinia virus-expressed human hepatic cytochrome P450 metabolically active aflatoxin B₁. *Proc. Natl. Acad. Sci. USA.*, **87**, 4790-4793.
- Buening, M. K., Fortner, J. G., Kappas, A., and Conney, A. H. (1978): 7,8-Benzoflavone stimulate the metabolic activation of aflatoxin B₁ to mutagens by human liver. *Biochem. Biophys. Res. Commun.*, **82**, 348-355.
- Conney, A. H. (1982): Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons. *Cancer Res.*, **42**, 4875-4917.
- Eaton, D. L. and Gallagher, E. P. (1994) Mechanisms of aflatoxin carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.*, **34**, 135-172.
- Guengerich, F. P. (1995): Human cytochrome P450 enzymes. In *Cytochrome P450* (Ortiz de Montellano, P. R., Ed) pp 473-535, Plenum Press, New York.
- Oda, Y., Nakamura, S., Oki, I., Kato, T., and Shinagawa, H. (1985): Evaluation of the new system (umu-test) for the detection of environmental mutagens and carcinogens. *Mutat. Res.*, **147**, 219-229.
- Raney, K. D., Shimada, T., Kim, D.-H., Groopman, J. D., Harris, T. M., and Guengerich, F. P. (1992): Oxidation of aflatoxins and sterigmatocystin by human liver microsomes: significance of aflatoxin Q₁ as a detoxication product of aflatoxin B₁. *Chem. Res. Toxicol.*, **5**, 202-210.
- Ueng, Y.-F., Shimada, T., Yamazaki, H., and

Fig. 3. Oxidation products of AFB₁ formed by recombinant human P450s 1A2 (B) and 3A4 (A). The absorbance scales for the two traces are not identical.

- Guengerich, F. P. (1995): Oxidation of aflatoxin B1 by bacterial recombinant human cytochrome P450 enzymes. *Chem. Res. Toxicol.*, **8**, 218-225.
- Ueng, Y.-F., Kuwabara, T., Chun, Y.-J., and Guengerich, F. P. (1997): Cooperativity in oxidations catalyzed by cytochrome P450 3A4. *Biochemistry.*, **36**, 370-381.
- Shimada, T. and Guengerich, F. P. (1989) Evidence for cytochrome P450NF, the nifedipine oxidase, being the principal enzyme involved in the bioactivation of aflatoxins in human liver. *Proc. Natl. Acad. Sci.*, **86**, 462-465.
- Yamazaki, H., Ueng, Y.-F., Shimada, T., and Guengerich, F. P. (1995) Roles of divalent ions in oxidations catalyzed by recombinant cytochrome P450 3A4 and replacement of NADPH-cytochrome P450 reductase with other flavoproteins, ferredoxin, and oxygen surrogates. *Biochemistry.*, **34**, 8380-8389.