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# THE ROLE OF CYTOCHROME P-450 FORMS IN 2-AMINOANTHRACENE AND BENZ[α]PYRENE MUTAGENESIS

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SUMMARY: The role of four forms of cytochrome P-450 from rabbit liver in the metabolic activation of two suspected carcinogens, 2-aminoanthracene and benz-[α]pyrene, was investigated with a *S. typhimurium* tester strain, TA 98. Each of the forms, 2,3,4 and 6 was reconstituted with NADPH-cytochrome P-450 reductase and lipid, and assay conditions were established such that the cytochrome P-450 concentration was rate-limiting. Under these conditions, cytochrome P-450 form 4, but not the other forms, converted 2-aminoanthracene into a potent mutagen. In contrast, form 6 was the only form which metabolized benz[α]pyrene to a mutagen. These results indicate that specific cytochrome P-450 forms preferentially activate particular mutagens.

The microsomal mixed function monooxygenase system activates a wide variety of chemicals to carcinogenic products (1). Multiple forms of the terminal oxidase, cytochrome P-450, have been isolated in several laboratories (2-15). Four forms have been purified from adult rabbit liver in our own laboratory; form 2, the principal phenobarbital-induced species (6), form 3, prepared from untreated animals[1], form 4, the major 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD)[2]-induced species in adults (4,6), and form 6 which is also induced by TCDD in adults and is the only TCDD-induced form in newborn rabbit liver (3,16). Different enzyme activities are associated with each of these forms and these functional differences suggest that individual cytochrome P-450 forms may preferentially activate specific mutagens.

[1] Johnson, E.F., manuscript in preparation.

[2] The abbreviations used are: 2AA, 2-aminoanthracene; 2AAF, 2-acetylamino-fluorene; BαP, benz[α]pyrene; BαP 4,5-diol, *trans*-4,5-dihydroxy-4,5-dihydro-benz[α]pyrene; BαP 4,5-oxide, benz[α]pyrene 4,5-oxide; BαP 7,8-diol, *trans*-7,8-dihydroxy-7,8-dihydrobenz[α]pyrene; BαP 7,8-oxide, benz[α]pyrene 7,8-oxide; BαP 7,8-diol-9,10-epoxide, both stereoisomers of 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenz[α]pyrene; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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Activation of compounds to mutagens can be measured with *S. typhimurium* auxotrophs developed by B. Ames and his colleagues (17). The production of mutagenic metabolites is detected by an increase in the number of histidine-independent revertants following the incubation of these strains with a pro-mutagen and an activating system. Mutagenicity in this system correlates with the known carcinogenicity of many compounds which suggests a close relationship between the mutagenic and carcinogenic processes (18,19).

In the present study, the *S. typhimurium* tester strain TA 98 was employed as an *in vitro* assay system to measure the activation of two suspected carcinogens, 2-aminoanthracene (2AA) and benz[α]pyrene (BαP), by purified forms of rabbit liver cytochrome P-450. A comparison of the four forms, 2,3,4 and 6, showed that 2AA mutagenesis was mediated most effectively by form 4 while BαP was activated most rapidly by form 6.

MATERIALS AND METHODS:

NADPH-cytochrome P-450 reductase (2) and cytochrome P-450 forms 2 (6), $3^1$, 4 (2,4) and 6 (3) were isolated from adult rabbit liver microsomes. All assay components were sterilized before use by filtration through 0.45 μ Millipore filters, except NADPH and dilauroyl-L-α-lecithin which were filtered through 0.22 μ Millipore filters. All Millipore filters were Triton-free. Turnover numbers for the O-deethylation of 7-ethoxyresorufin (forms 4 & 6) (20) or the N-demethylation of aminopyrine (forms 2 & 3) (21) were comparable for both filtered and unfiltered preparations.

*S. typhimurium* tester strain TA 98, a gift from Dr. B. Ames (University of California, Berkeley, Ca.) was harvested during the exponential growth phase. The culture was centrifuged at 1000 x g for 30 minutes, and the pellet of bacteria resuspended in 0.15 M NaCl, 10 mM sodium phosphate buffer, pH 7.0, to give a final concentration of approximately 2 x $10^9$ bacteria/ml.

A sonicated dispersion of the lipid, dilauroyl-L-α-lecithin (3 mg/ml), was prepared on the day of the assay. To compensate for losses of material during filtration the volume of filtered lipid necessary to produce optimal levels of 7-ethoxyresorufin O-deethylase activity was determined.

Varying amounts of cytochrome P-450, 0.5 units of reductase and the optimal volume of lipid, determined as outlined above, were combined in that order. After a 3 minute incubation of the components at room temperature, buffer (0.15 M NaCl, 10 mM Tris-HCl, pH 7.7), 100 μl of strain TA 98 suspension and either 20 μl of 0.625 mM 2AA (Aldrich) in dimethylsulfoxide or 20 μl of 2.0 mM BαP (Aldrich, gold label) in methanol were added to give a total volume of 0.49 ml. The mixture was preincubated for 2 minutes at 37°C and the reaction was initiated with 10 μl of 20 mM NADPH. The reaction was terminated by the addition of 0.1 ml of 5 mM cytochrome c. Addition of top agar and plating were performed as described by Ames *et al* (17). After 48 hours at 37°C, the number of revertant colonies per plate was counted. In the experiments illustrated, each value represents the mean ± S.D. of 3 replicate incubations. Each experiment was performed 2 or more times and qualitatively similar results were obtained. The spontaneous mutation rate for strain TA 98 in these experiments was 22 ± 3 revertants per plate.

RESULTS:

In preliminary experiments, form 4 incubated with 2AA and form 6 incubated with BαP produced significant increases in the number of strain TA 98 revertants. Therefore, optimal reaction conditions were determined for 2AA and BαP mutagenesis with forms 4 and 6 respectively. Initially, we selected substrate concentrations of 25 μM 2AA, similar to that used by McCann *et al* (18), and 80 μM BαP, the concentration used in the aryl hydrocarbon hydroxylase fluorescence assay (22) These concentrations appear to be saturating since no significant change in the number of TA 98 revertants was observed when the final concentration of 2AA was varied from 5 to 25 μM or when BαP was varied from 20 to 120 μM (experiments not shown).

As depicted in Figure 1, the number of revertants increased in a linear fashion for both 2AA and BαP mutagenesis with increasing incubation time at 37°C. Incubation times of 15 minutes for 2AA mutagenesis and 20 minutes for BαP mutagenesis were chosen for subsequent experiments. Next, the relationship between the number of revertants and cytochrome P-450 concentration was examined. The results are illustrated in Figure 2. Metabolic activation of both 2AA and BαP to mutagens increased as a linear function of the concentrations of form 4 or form 6, respectively, up to 100 pmoles of cytochrome P-450 per incubation. These results demonstrate that under the conditions of this assay the amount of cytochrome P-450 is rate-limiting.

The rates of activation of 2AA and BαP by the individual cytochrome P-450 forms, 2,3,4 and 6, were then compared (Table I). Form 4 produced 10 to 30 times more revertants with 2AA than a control in which the cytochrome P-450 was omitted. BαP mutagenesis appeared to be catalyzed almost exclusively by form 6 which produced 6 to 15 times the number of control revertants. In both cases, the other forms produced at most 3 times the control values.

DISCUSSION:

The results in Table I demonstrate that specific cytochrome P-450 forms selectively metabolize particular carcinogens to mutagenic products. These

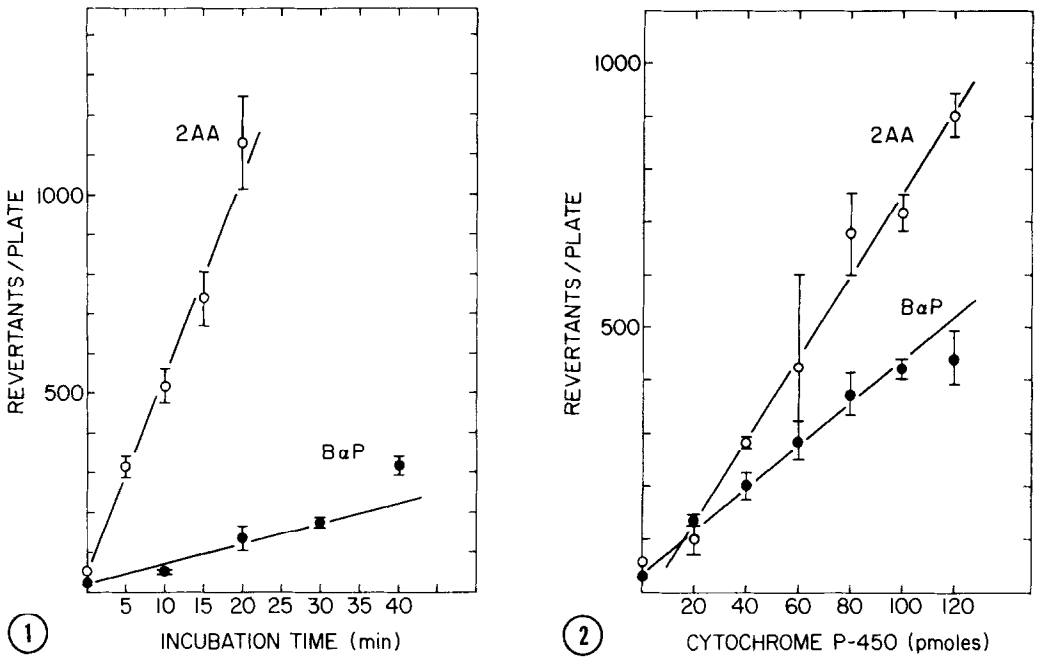


Figure 1: The dependence of the number of strain TA 98 revertants on incubation time at 37°C. 2AA mutagenesis (○) was catalyzed by form 4, BαP mutagenesis (●) by form 6. Both forms were present at 100 pmoles of cytochrome P-450 per incubation. Assay conditions are described in the Materials and Methods section.

Figure 2: The dependence of the number of strain TA 98 revertants on cytochrome P-450 concentration. Varying amounts of cytochrome P-450 form 4 were incubated with 2AA (○) for 15 minutes and varying amounts of form 6 with BαP (●) for 20 minutes. Other assay conditions are as described in Materials and Methods.

products have not been identified at present. It has recently been observed that form 4 is more effective than the other 3 forms in the N-hydroxylation of another arylamine, 2-acetylaminofluorene (2AAF)[3]. The N-hydroxylated metabolite of 2AAF is a potent mutagen in the *S. typhimurium* test system (18). If 2AA metabolism is similar to that of 2AAF, the primary mutagenic metabolite of 2AA produced by form 4 is probably the N-hydroxylated species.

In the case of BαP more information on the nature of the primary metabolites is available. Under the conditions employed in this assay, the primary products

[3]Johnson, E.F., Levitt, D., Muller-Eberhard, U., and Thorgeirsson, S.S., manuscript in preparation.

TABLE I

DEPENDENCE OF MUTAGENESIS ON CYTOCHROME P-450 FORM

| | 2-Aminoanthracene | | | Benz[α]pyrene | | |
|---|---|---|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 1 | Expt. 2 | Expt. 3 |
| Form 2 | 165 | 49 | 105 | 49 | 31 | 43 |
| Form 3 | -- | 48 | 108 | -- | 35 | 40 |
| Form 4 | 1095 | 1094 | 480 | 37 | 44 | 37 |
| Form 6 | 213 | 36 | 48 | 159 | 421 | 393 |
| Minus P-450 | 65 | 33 | 51 | 19 | 31 | 28 |
| Carcinogen only | -- | 28 | 39 | 18 | 22 | 26 |

One hundred pmoles of each cytochrome form were incubated with 2AA for 15 minutes or with BαP for 20 minutes. Other reaction conditions are outlined in Materials and Methods. The results are expressed as the number of strain TA 98 revertants/100 pmoles of cytochrome P-450. In control incubations, either the cytochrome P-450 (Minus P-450) or the entire reconstituted system was omitted (Carcinogen only).

of BαP metabolism are arene oxides, phenols and quinones (23,24). Most of these have been tested with the Ames *S. typhimurium* tester strains and the majority are not mutagenic *per se* (25). In the absence of an activating system, the most potent mutagen is BαP 4,5-oxide which produces about 200 revertants in strain TA 98 at a concentration of 100 μM (25). Since the reconstituted system contains no added epoxide hydrase which would convert BαP 4,5-oxide into the non-mutagenic BαP 4,5-diol, BαP 4,5-oxide is the most likely mutagenic BαP metabolite produced by form 6 in these experiments.

In the presence of epoxide hydrase further metabolism of the initial products can occur leading to, among others, BαP 7,8-diol-9,10-epoxide. In the pathway leading to the production of this metabolite, the initial product of cytochrome P-450 metabolism, BαP 7,8-oxide, is converted to BαP 7,8-diol by epoxide hydrase. The diol is then recycled through the cytochrome P-450 mono-oxygenase system to produce the highly mutagenic BαP 7,8-diol-9,10-epoxide

(26,27,28). In the absence of added epoxide hydrase only the initial metabolite in this pathway, BαP 7,8-oxide, will be produced in the reconstituted cytochrome P-450 system. However, BαP 7,8-oxide is a very weak mutagen (25). Consequently, activation of BαP via this pathway is probably not detected in our assay and it is not clear whether form 6 or another cytochrome P-450 form catalyzes the first step.

It is possible that two forms of cytochrome P-450 are involved in the activation of BαP to the BαP 7,8-diol - 9,10-epoxide, one producing the BαP 7,8-oxide, a second converting the diol into the BαP 7,8-diol - 9,10-epoxide. Recently, Deutsch *et al* have reported that form 4 was more effective than form 2 in converting BαP 7,8-diol to BαP 7,8-diol - 9,10-epoxide which suggests that form 4 may catalyze the last step in the pathway (29). However, the role of form 6 in this reaction is not known since the metabolism of BαP 7,8-diol has not been evaluated with this cytochrome.

Our results demonstrate that specific forms of cytochrome P-450 activate specific mutagens. This suggests that tissue response to a carcinogen may be strongly influenced by its cytochrome P-450 composition. This composition is a function of many variables including species, age, sex and history of exposure to inducers (30). Consequently, activation of chemical carcinogens will depend both on the factors which regulate the tissue occurrence of individual cytochrome P-450 forms as well as on the substrate preferences of each form. Both of these factors, tissue occurrence and substrate preference, must therefore be considered in the rational design of systems for carcinogen detection.

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