RECONSTITUTION OF BENZO[a]PYRENE 4,5-OXIDE REDUCTASE ACTIVITY BY PURIFIED CYTOCHROME *P*-450

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1. Introduction

Polycyclic aromatic hydrocarbons are generally known to be oxidatively metabolized to their epoxides, some of which are proximate or ultimate carcinogens [1,2]. These epoxides were reductively metabolized to corresponding hydrocarbons by rat liver microsomes [3].

We demonstrated [4] the possible involvement of cytochrome *P*-450 in the reduction of benzo[a]pyrene 4,5-oxide using liver microsomes. However, purified cytochrome *P*-450 did not catalyze the reduction of benzo[a]pyrene 4,5-oxide [5], in spite of the reduction ability of microsomes, which was inhibited by carbon monoxide.

To dissolve the complexity, we examined the activity of cytochrome *P*-450 for the reduction reaction using purified cytochrome *P*-450 preparations. The results shown in this paper provide direct evidence for the involvement of cytochrome *P*-450 in the epoxide reduction. We also show that among cytochrome *P*-450 species cytochrome *P*-448 isolated from 3-methylcholanthrene-treated rat liver microsomes exhibited the highest activity for the reaction.

2. Materials and methods

2.1. *Purification of cytochrome* P-*450*

Cytochrome *P*-450 (PB *P*-450) was purified from phenobarbital-treated rat liver microsomes by a method essentially as in [6], which was a minor modification of the method in [7,8]. The specific content of the purified PB *P*-450 was 15.4 nmol/mg protein. Two species of cytochrome *P*-450 were purified from 3-methylcholanthrene-treated rat liver microsomes. Rats were given 3 daily intraperitoneal injections of 3-methylcholanthrene at a dose of 40 mg/kg. A species (MC *P*-450) exhibiting a peak in the carbon monoxide difference spectrum at 449–450 nm was purified by the method for purification of PB *P*-450 in [6]. The specific content of this cytochrome was 9.1 nmol/mg protein. The other species of cytochrome *P*-450 (MC *P*-448) exhibiting a peak at 448 nm was purified using two common columns (ω-amino *n*-octyl Seph. 4B and hydroxylapatite columns) for the purification of MC *P*-450, and two columns of CM-Seph. (C-50). The specific content of the purified MC *P*-448 was 17.6 nmol/mg protein. The MC *P*-448 preparation was free of detectable activities of NADPH-cytochrome *P*-450 reductase, NADH-cytochrome b_5 reductase and cytochrome b_5, and was estimated to be a low spin species as judged from the absolute spectrum. Cytochrome *P*-450 was determined as in [9], except that 20% glycerol and 0.2% Emulgen 913, a non-ionic detergent, were added in all determinations. Protein was determined as in [10].

2.2. *Purification of NADPH-cytochrome* P-*450 reductase*

NADPH-cytochrome *P*-450 reductase was purified from phenobarbital-treated rat liver microsomes by a modification of the method in [11]. The purified preparation of NADPH-cytochrome *P*-450 reductase was free of detectable amounts of cytochrome *P*-450, cytochrome b_5 and NADH-cytochrome b_5 reductase. The specific activity of this preparation was 57.2 units/

mg protein. The activity of NADPH-cytochrome *P*-450 reductase was determined using cytochrome *c* as an electron acceptor by the method in [12], and 1 unit reductase activity was defined as 1 μmol cytochrome *c* reduced/min.

2.3. *Measurement of benzo[a]pyrene 4,5-oxide reductase activity*

The incubation mixture for the reconstitution of NADPH-dependent benzo[a] pyrene 4,5-reductase activity contained 0.4 unit NADPH-cytochrome *P*-450 reductase, 15 μg dilauroyl L-3-phosphatidylcholine, 0.1 nmol cytochrome *P*-448, 50 μmol Na,K-phosphate (pH 7.4), 1 μmol NADPH and 0.1 μmol benzo[a]-pyrene 4,5-oxide in final vol. 0.5 ml. The incubation mixture for the determination of microsomal activity of NADPH-dependent benzo[a] pyrene 4,5-oxide reductase activity contained 5 mg protein of microsomes from intact or 3-methylcholanthrene-treated rat livers, 5 μmol NADP, 25 μmol glucose 6-phosphate, 1.5 IU glucose 6-phosphate dehydrogenase, 10 μmol $MgCl_2$, 7.5 μmol 1,2-epoxy 3,3,3-trichloropropane and 500 μmol Na,K-phosphate (pH 7.4) in final vol. 4.0 ml. The incubation tubes were sealed with rubber serum cap and the mixture was bubbled through a needle with oxygen-free nitrogen gas for 2 min on ice. NADPH or NADP was added after bubbling with nitrogen, and the mixture was preincubated at 37°C for 2 min under nitrogen gas stream. The reaction was started by addition of benzo[a] pyrene 4,5-oxide in 20 μl acetone. Incubations were carried out at 37°C for 10 min under nitrogen gas. The incubation mixture for measurement of reduced methylviologen-dependent benzo[a] pyrene 4,5-oxide reductase activity consisted of 0.1 nmol of a species of cytochrome *P*-450, 0.1 mg sodium cholate, 50 μmol Na,K-phosphate (pH 7.4), 0.25 μmol EDTA, 10 μmol methylviologen previously reduced with an equimolar sodium dithionite and 0.1 μmol benzo[a] pyrene 4,5-oxide in final vol. 0.5 ml. The mixture was preincubated at 37°C for 2 min after addition of reduced methylviologen and the reaction was started by addition of the substrate in 20 μl acetone. The reaction was carried out at 37°C for 5 min under the nitrogen gas as above. The reaction rate was estimated by determination of benzo[a] pyrene formed fluorometrically as in [4] using a Hitachi 203 fluorescence spectrophotometer. All determinations were conducted in duplicate, and the mean activities were presented in the tables.

2.4. *Materials*

Benzo[a] pyrene 4,5-oxide was synthesized according to the method in [13]. Nitrogen gas of extra pure grade (99.9995%) was obtained from Yamato Sanki Co. (Japan) and the nitrogen was further freed from oxygen by passing through the alkali-dithionite solution.

3. Results and discussion

The requirements for the reconstitution of NADPH-dependent benzo[a] pyrene 4,5-oxide reductase activity was examined. As can be seen in table 1, cytochrome *P*-450 (MC *P*-448) and NADPH-cytochrome *P*-450 reductase were essential requirements. No detectable activity was observed in the absence of MC *P*-448, indicating that NADPH-cytochrome *P*-450 reductase is not active for the epoxide reduction. In accordance with the reported observation for drug oxidation system [14], lipid was also required for the maximal activity. In the absence of added lipid, the activity for benzo[a] pyrene 4,5-oxide reduction was only 25% of the complete system. Using liver microsomes from intact and 3-methylcholanthrene-treated

Table 1
Requirement for NADPH-dependent benzo[a] pyrene 4,5-oxide reductase activity

Incubation mixture	Activity (nmol/nmol *P*-448 or *P*-450/min)
Reconstituted system	
complete	10.8
reductase omitted	0.0
P-448 omitted	0.0
NADPH omitted	0.0
lipid omitted	2.3
Microsomal system	
intact	0.6
3-Methylcholanthrene-treated	1.7

The experiments with the reconstituted system were carried out as in section 2, except that the indicated component was omitted from the complete system

Table 2
Stimulation of NADPH-dependent benzo[a] pyrene 4,5-oxide reductase activity by riboflavin and its inhibition by carbon monoxide

Addition	Gas phase	Activity (nmol/nmol *P*-448/min)	
–	N_2	10.2	(100%)
–	CO	0.3	(3%)
Riboflavin (100 μM)	N_2	17.3	(170%)
Riboflavin (100 μM)	CO	0.3	(3%)

The experimental conditions were the same as in section 2, except that when necessary oxygen free carbon monoxide was used instead of nitrogen

rats as controls, we found that MC *P*-448 in the reconstituted system exhibited about 18-fold and 6-fold greater activity than intact and 3-methylcholanthrene-treated microsomes, respectively, on the nmole of cytochrome basis. Part of this increased activity may probably be explained by current reduction rate of cytochrome *P*-448 in the reconstituted system, which contained high amounts of NADPH-cytochrome *P*-450 reductase.

In the previous experiments using rat liver microsomes, we observed that benzo[a] pyrene 4,5-oxide reductase activity was enhanced by a flavin nucleotide when added in the incubation mixture [4]. As shown in table 2, the effect of riboflavin on the activity was examined using the reconstituted system. The addition of riboflavin at a concentration of 100 μM resulted in a 1.7-fold enhancement of the benzo[a] pyrene 4,5-oxide reductase activity. The activities in the presence and absence of added riboflavin were almost completely inhibited by carbon monoxide, showing that reduced riboflavin does not chemically reduce the substrate.

Benzo[a] pyrene 4,5-oxide is reduced by cytochrome *P*-450 even in the absence of NADPH and NADPH-cytochrome *P*-450 reductase if a reducing equivalent such as reduced methylviologen, is present. In addition, we have found that lipid is not required for the reaction in this system.

The comparison of the activity of cytochrome *P*-450 species for the epoxide reduction is shown in table 3. MC *P*-448 showed that highest activity, followed by MC *P*-450 and PB *P*-450. This finding was in accordance with the above observation that microsomal benzo[a] pyrene 4,5-oxide reductase activity was induced by pretreatment of rats with

Table 3
Reduced methylviologen-dependent reduction of benzo[a]-pyrene 4,5-oxide by various cytochrome *P*-450 preparations

Cytochrome *P*-450 species	Activity (nmoles/nmoles *P*-448 or *P*-450/min)
MC *P*-448	24.1
MC *P*-450	11.5
PB *P*-450	5.2

The reactions were carried out as in section 2, except that the indicated preparation of cytochrome *P*-450 species was used. The activities corrected by the amount obtained in the absence of the cytochrome are given

3-methylcholanthrene. It is of interest to note that MC *P*-448 is the most active species of cytochrome *P*-450, since MC *P*-448 of rat liver microsomes has been known to be highly active for the oxidation of benzo[a] pyrene [15]. This result suggests that both benzo[a] pyrene and benzo[a] pyrene 4,5-oxide bind to a common site in the cytochrome *P*-450 (MC *P*-448) molecule.

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