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PARTICIPATION OF CYTOCHROME P-450 IN NICOTINE OXIDATION

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SUMMARY. Addition of nicotine to phenobarbital-inducible cytochrome P-450 caused a shift of maximum of Soret peak toward the red approximately 3 nm. The difference spectrum produced by nicotine showed a type 2 spectral change with a peak at 427 nm and a trough at 393 nm. A spectral dissociation constant of phenobarbital-inducible cytochrome P-450 was found to be 0.16 mM for nicotine. Nicotine oxidation in the reconstituted system depended on cytochrome P-450, NADPH-cytochrome P-450 reductase and NADPH. These results indicate that phenobarbital-inducible cytochrome P-450 participates in nicotine oxidation.

Hucker et al. reported that a main reaction of nicotine metabolism occurred in hepatic microsomes and required NADPH and O_2 (1). Since then, a number of studies on the metabolism of nicotine has suggested that cytochrome P-450 catalyzed hepatic nicotine oxidation (2-5), but no direct evidence for the participation of purified cytochrome P-450 in the reaction has so far been presented. Cytochrome P-450 plays an important role in bioactivation and detoxication of wide variety of drugs including carcinogens and xenobiotics (6,7). Recent studies on cytochrome P-450 show that the enzyme exists in multiple forms (8) and the biogenesis is postulated to be regulated by cytosolic receptors (9,10). These studies on multiple forms of cytochrome P-450 and its biogenesis seem to be essential for investigation of drug metabolism since the administration of drugs to animal was found to affect specific or several types of cytochrome P-450 (8).

Abbreviation: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PB, phenobarbital; SDS; sodium dodecyl sulfate; dilauroyl-GPC, dilauroylglyceryl-3-phosphorylcholine.

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In the preceding paper, we suggested that two or more types of cytochrome P-450 might participate in nicotine oxidation (11). The present report gives the first evidence of nicotine oxidation with purified cytochrome P-450.

MATERIALS AND METHODS

Seven-week old Wistar rats were obtained from Kiwa Laboratory Animal Company and maintained on lab chow (Oriental MF., Japan) and water *ad libitum* for the experiments. PB was injected each day into the rats intraperitoneally at 60 mg/kg for 5 days. The hepatic microsomal fractions were prepared as previously described except that ultracentrifugation was carried out at 65,000 x g for 1.5 hr (11). PB-inducible cytochrome P-450 was highly purified by 10-16% polyethylene glycol fractionation, DE-52 column chromatography at room temperature and QAE-Sephadex column chromatography according to the procedure of West et al. (12). When the resulting QAE-Sephadex column fractions contained PB-inducible cytochrome P-450 and other minor proteins judging from the result of SDS-polyacrylamide gel electrophoresis, the fractions were further purified by hydroxyapatite column chromatography according to the procedure of Harada and Omura (13). The preparations used in these experiments had specific contents of 16.5 to 17.5 nmoles of cytochrome P-450 per mg protein. When 0.2 to 1.0 μg of the purified cytochrome P-450 were applied to SDS-polyacrylamide gels and then electrophoresed, only a single protein band was observed. Minimum molecular weights of purified cytochrome P-450 were estimated to be 53,000 daltons by comparison to protein standards of known mobility. Emulgen 913 was removed from the preparations using a hydroxyapatite column. After the A_{275} in the elute (due to emulgen 913) decreased to less than 0.005, cytochrome P-450 was eluted from the column with 200 mM potassium phosphate buffer (pH 7.2) containing 20% glycerol. NADPH-cytochrome P-450 reductase was purified by a combination of the method of Dignam and Stroebel (14) and Yasukochi and Masters (15).

All absorption spectra were determined on a Hitachi Model 200-20 Spectrophotometer. The binding of nicotine to the oxidized cytochrome P-450 was determined at cytochrome P-450 concentrations of 1.8 nmole per ml in 0.1 M potassium phosphate buffer (pH 7.25) containing 20% glycerol and 0.1 mM EDTA. Nicotine was dissolved in 0.1 M potassium phosphate buffer (pH 7.25). The spectra were recorded at room temperature after the addition of nicotine to the sample cuvette and buffer to the reference cuvette. Nicotine oxidase was assayed at 37°C as previously described (11). Reconstituted assay mixture contained 0.1 M HEPES-NaOH buffer (pH 7.4), 0.5 mM NADPH, 0.5 mM nicotine, 6 mM $MgCl_2$, 60 μg dilauroyl-GPC, 0.61 nmole cytochrome P-450, 0.28 unit NADPH-cytochrome P-450 reductase in a total volume of 2 ml. The sample of cytochrome P-450, NADPH-cytochrome P-450 reductase and dilauroyl-GPC were first added, mixed and allowed to stand for 2 min at room temperature. The remaining components were added to the solution and then preincubated for 3 min at 37°C. The reaction was started by the addition of nicotine. Cytochrome P-450 content was determined by the method of Omura and Sato (16). Protein was determined by the method of Lowry *et al.* (17).

SDS-polyacrylamide gel electrophoresis was carried out according to the procedure of Laemmli *et al.* (18).

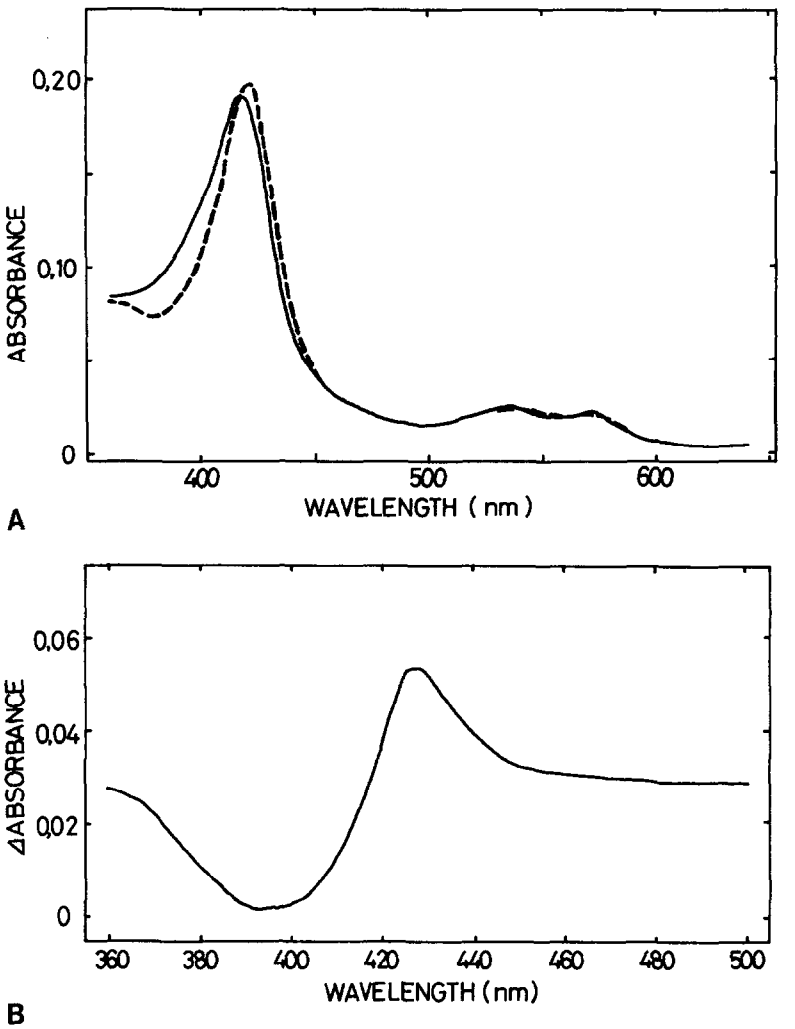

Fig. 1. Effect of nicotine on spectrum of PB-inducible cytochrome P-450 from rat liver microsomes. The experimental procedure is given under MATERIALS AND METHODS.
A: Absolute spectra. The spectra were recorded in the presence (---) or the absence (—) of 0.9 mM nicotine.
B: Difference spectrum. The nicotine difference spectrum was recorded after addition of 0.9 mM nicotine to the sample cuvette.

RESULTS

Absolute spectrum of oxidized cytochrome P-450 in the presence or the absence of nicotine is shown in Fig.1-A. As reported by Ryan et al. (19), the absolute spectrum had a broader peak in the 417 region. Addition of nicotine to cytochrome P-450 caused the shift of maximum of Soret peak toward the red approximately 3 nm and the disappearance of a shoulder between 390 and 400 nm. Fig.1-B shows the difference spectrum produced by addition of

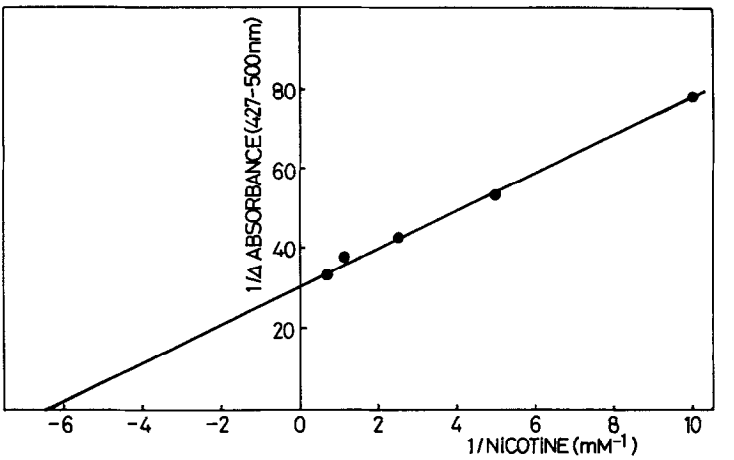

Fig. 2. Double reciprocal plot of the nicotine difference spectral change. ΔAbsorbance values refer to a difference maxima at 427 nm and an isobestic point at 500 nm.

nicotine to the purified cytochrome P-450. The absorption maximum was at 427 nm and the trough was at 393 nm. The spectral dissociation constant was derived from the double reciprocal plot of the change in absorbance at 427 nm relative to 500 nm. Double reciprocal plot of the difference spectral change is shown in Fig. 2. Dissociation constant for nicotine was 0.16 mM at room temperature. Table 1 shows requirements for nicotine oxidation in the reconstituted system. The oxidation proceeded linearly for about 30 min under the reaction conditions. Cytochrome P-450, NADPH-cytochrome P-450 reductase and NADPH were required absolute-

Table 1. Requirements for reconstitution of nicotine oxidation.

System	Nicotine oxidation (nmoles nicotine/min/ml)	% Maximal activity
Complete	0.409	100
− P-450	0	0
− Reductase	0.030	7
− NADPH	0.022	5
− $MgCl_2$	0.273	67

The complete system contained 0.1 M HEPES-NaOH (pH 7.4), 0.5 mM nicotine, 0.5 mM NADPH, 6 mM $MgCl_2$, 60 μg dilauroyl-GPC, 0.61 nmole cytochrome P-450, 0.28 μmoles/min NADPH-cytochrome P-450 reductase in a total volume of 2 ml.

ly in the reaction. Removal of $MgCl_2$ decreased the activity by about 1/3.

DISCUSSION

A major PB-inducible cytochrome P-450 used in the present study is probably identical to forms isolated by other workers (12,13,19-21) on the basis of the criteria such as purification procedure, molecular weights and spectral properties. Schenkman et al. showed a type 2 spectral change resulted from the interaction of nicotine with hepatic microsomal cytochrome P-450 (3). Their observation that nicotine difference spectrum was characterized by a peak at 428 nm and a trough at 395 nm is similar to those found in this study. Similarities in spectral dissociation and Michaelis constants (22) suggest that the spectral change produced by nicotine reflects the formation of a cytochrome P-450-nicotine complex, though type 2 compounds are known to be usually poor substrates (22). Nicotine oxidation by the reconstituted system seems to support the formation of cytochrome P-450-nicotine complex. Michaelis constants of microsomal nicotine oxidase for nicotine in monkeys, dogs and mice were found to be 0.73, 1.4 and 1.9 mM, respectively (2,4). These values are higher by about 4 to 10 folds than the dissociation constant found in this study. After intravenous injection of nicotine, nicotine was found to decrease rapidly in the blood and then be distributed in all tissues (23,24). In this case, the hepatic concentration of nicotine was low for all periods of nicotine metabolism and excretion. The high affinity of PB-inducible cytochrome P-450 for nicotine seems to appropriate to catalyze hepatic nicotine oxidation. There remain some uncertainty as to the mechanism of microsomal nicotine oxidation (5). A simple reconstituted system of nicotine oxidation will be useful for the investigation of nicotine oxidation.

ACKNOWLEDGEMENTS

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REFERENCES

1. Hucker, H. B., Gillette, J. R. and Brodie, B. B. (1960) J. Pharmacol. Exp. Ther. 129, 94-100.
2. Hill, D. L., Laster, W. R. Jr and Struck, R. F. (1972) Cancer Res. 32, 658-665.
3. Schenkman, J. B., Remmer, H. and Estabrook, R. W. (1967) Mol. Pharmacol. 3, 113-123.
4. Dohi, T., Kojima, S. and Tsujimoto, A. (1973) Japan. J. Pharmacol. 13, 748-751.
5. Murphy, P. J. (1973) J. Biol. Chem. 248, 2796-2800.
6. Gillette, J. R., Davis, D. C. and Sasame, H. A. (1972) Annu. Rev. Pharmacol. 12, 57-84.
7. Weisburger, E. K. (1978) Annu. Rev. Pharmacol. & Toxicol. 18, 395-415.
8. Lu, A. Y. H. and West, S. B. (1979) Pharmacol. Rev. 31, 277-295.
9. Poland, A., Glover, E. and Kende, A. S. (1976) J.Biol. Chem. 251, 4936-4946.
10. Guenthner, T. M. and Nebert, D. W. (1977) J. Biol. Chem. 252, 8981-8989.
11. Nakayama, H., Nakashima, T. and Kurogochi, Y. (1982) Biochem. Biophys. Acta 715, 254-257.
12. West, S. B., Huang, M. T., Miwa, G. T. and Lu, A. Y. H. (1979) Arch. Biochem. Biophys. 193, 42-50.
13. Harada, N. and Omura, T. (1981) J. Biochem. 89, 237-248.
14. Dignam, J. D. and Strobel, H. W. (1975) Biochem. Biophys. Res. Commun. 63, 845-852.
15. Yasukochi, Y. and Masters, B. S. S. (1976) J. Biol. Chem. 251, 5337-5344.
16. Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370-2378.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
18. Laemmli, U. K. (1970) Nature 227, 680-685.
19. Ryan, D. E., Thomas, P. E., Korzeniowski, D. and Levin, W. (1979) J. Biol. Chem. 254, 1365-1374.
20. Guengerich, F. P. (1978) J. Biol. Chem. 253, 7931-7939.
21. Guengerich, F. P. and Martin, M. (1980) Arch. Biochem. Biophys. 205, 365-379.
22. Jefcoate, C. R. (1978) Method Enzymol. 52, 258-279.
23. Turner, D. M. (1969) Biochem. J. 115, 889-896.
24. Tsujimoto, A., Nakashima, T., Tanino, S., Dohi, T. and Kurogochi, Y. (1975) Toxicol. Appl. Pharmacol. 32, 21-31.