THE INVOLVEMENT OF CYTOCHROME P-450 IN THE NADH-DEPENDENT O-DEMETHYLATION OF p-NITROANISOLE IN PHENOBARBITAL-TREATED RABBIT LIVER MICROSOMES

Tetsuya Kamataki and Haruo Kitagawa

Department of Biochemical Pharmacology, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Chiba-shi, Chiba, 280 Japan

Received April 14,1977

Summary: Addition of p-nitroanisole to a reaction mixture containing phenobarbital-pretreated rabbit liver microsomes brings about an increase the re-oxidation rate of NADH-reduced cytochrome b_5. Addition of partially purified cytochrome b_5 to a solution containing microsomes results in a marked increase in both NADH- and NADPH-dependent O-demethylation of p-nitroanisole. p-Nitroanisole also increases the rate of NADH mediated cytochrome P-450 reduction. From these and other results described in the Discussion section, we confirm that electrons required for NADH-dependent O-demethylation of p-nitroanisole is transfered from NADH to cytochrome P-450 via cytochrome b_5 and that cytochrome P-450 is the enzyme which catalyzes p-nitroanisole O-demethylation.

INTRODUCTION

It has been widely accepted that liver microsomes contain two electron transport chains. One of these is a NADPH-dependent and the other a NADH-dependent system (1). The current view is that there might be at least two cross-linking sites between the two chains. Thus, the finding by Oshino et al. (2) that stearoyl Co A desaturase activity in liver microsomes is partly supported by NADPH indicates that electron flow from NADPH-cytochrome P-450 reductase to cytochrome b_5 is taking place. The other possible site of cross-linking is from cytochrome b5 to cytochrome P-450. Cohen and Estabrook (3) reported that the activity of NADPH-dependent demethylation of some drugs, including aminopyrine, is significantly increased by the addition of NADH, a poor electron donor for the reaction unless NADPH is present. This NADH-synergism of an NADPH-dependent reaction was interpreted as evidence that the second electron required for the hydroxylation of drugs is transfered via

ISSN 0006-291X

cytochrome b_5 to cytochrome P-450. In support of this transport of electrons from cytochrome b_5 to cytochrome P-450, recent studies have shown that NADH can also act as an electron donor for reduction of cytochrome P-450 (4-6). In addition West et al. (7) have reported that electrons for NADH-dependent hydroxylation of benzo(a)pyrene is transfered from NADH to cytochrome P-450 via cytochrome b_5. However, in spite of these data little is known on the significance of the electron flow from NADH to cytochrome P-450 with respect to drug hydroxylations.

In this communication, we report on data which indicates the NADH-dependent O-demethylation of p-nitroanisole is catalyzed by cytochrome P-450 receiving electrons from cytochrome b_5.

MATERIALS AND METHODS

Male New Zealand white rabbits weighing 1.00 to 1.35 kg and male Wistar rats weighing 203 to 248 g were given 0.05 % and 0.1 % sodium phenobarbital in drinking water for 3 days, respectively. The animals were fasted for 18 hr prior to sacrifice. Hepatic microsomes were prepared as described previously (8). Protein was determined by the method of Lowry et al (9). Unless otherwise stated, values in the tables are means of duplicate determinations. Partial purification of cytochrome b_5 Cytochrome b_5 was partially purified from phenobarbital-treated rat liver microsomes by the method described by Imai (10). The specific content of cytochrome b_5 thus obtained was 19.4 nmoles/mg protein, and was free from cytochrome P-450. The cytochrome b_5 preparation eluted from ω-amino-n-octyl Sepharose 4B was dialyzed against redistilled water for 48 hr and used for the experiment. Cytochrome b_5 was determined by recording NADH- or dithionite-reduced minus oxidized difference spectrum of cytochrome b_5 depending upon the purification step. Assay of NADH-dependent and NADPH-dependent demethylase activities A typical incubation mixture for the assay of NADH-dependent demethylase activities contained a substrate (5 mM), 1 mM NADH, 0.1 M Na,K-phosphate (pH 6.0), 0.1 mM EDTA and rabbit liver microsomes (1 mg protein) in a final volume of 1.0 ml. When NADPH-dependent demethylation activities were measured, an NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 6 mM $MgCl_2$ and 0.045 unit glucose 6-phosphate dehydrogenase) and 0.1 M Na,K-phosphate (pH 7.4) were added in the incubation mixture instead of NADH and 0.1 M Na,K-phosphate (pH 6.0). Incubations were carried out at 37° for 10 min aerobically. Oxidative demethylation activities of N-monomethylaniline and ethylmorphine were estimated by determining formaldehyde by the method of Nash (11). p-Nitroanisole O-demethylation activity was measured by determining p-nitrophenol formed using a minor modification of the method reported by Kato and Gillette (12). Measurement of cytochrome b_5 reoxidation rate A mixture containing 0.2 mM Na_2S, 0.1 M Na,K-phosphate (pH 7.4) and microsomes (1.48 mg protein) was placed in a cuvette, and the cytochrome b_5 reoxidation rate was measured at 25° by recording the decrease of the absorbance difference between 409 nm and 424 nm during several min after addition of 10 μM NADH. Acetone (10 μl) was added as a control since acetone was used as a solvent of p-nitroanisole.

The molar extinction coefficient of 185 $mM^{-1}cm^{-1}$ (4) was used for calculating cytochrome b_5 reoxidation rate.

Measurement of NADH-cytochrome P-450 reduction rate A mixture for measuring the NADH-cytochrome P-450 reduction rate contained a substrate (5 mM), 0.1M Na,K-phosphate (pH 7.4), rabbit liver microsomes (1 mg protein), 10 mM glucose, 15 unit glucose oxidase and 130 unit catalase in a final volume of 3.0 ml. After introduction of oxygen-free carbon monoxide to the reaction mixture, the reaction was started by addition of 1 mM NADH. Cytochrome P-450 reduction was measured by recording the increase of absorbance difference between 450 nm and 490 nm due to formation of the reduced cytochrome P-450-carbon monoxide complex. The reaction was carried out at 25° in an Aminco anaerobic cell (Model A1-65085). The reaction velocity was calculated using a mmolar extinction coefficient of 91 $mM^{-1}cm^{-1}$ between 450 nm and 490 nm (13). All measurements for kinetic experiments were conducted using a Hitachi dual wave length recording spectrophotometer, Model 356. The Aminco recording spectrophotometer, Model DW-2, was used for measuring the difference spectra of cytochrome b_5 and cytochrome P-450.

RESULTS

Initially, the reaction velocities of NADH-dependent and NADPH-dependent demethylation of p-nitroanisole, N-monomethylaniline and ethylmorphine were compared. As can be seen in Table 1, the highest activity was seen for N-monomethylaniline demethylation in both NADPH- and NADH-dependent reactions and only negligible activity was detected for ethylmorphine N-demethylation when NADH was used as the electron donor. The ratios of NADH-dependent activities for p-nitroanisole, N-monomethylaniline and ethylmorphine demethylations to NADPH-dependent activities were 0.48, 0.20 and 0, respectively. In six separate experiments, the NADH/NADPH dependent activity ratios for p-nitroanisole demethylation were seen to vary from 0.48 to 0.91 (0.71 $\pm$ 0.16, mean $\pm$ SD).

Table 1
COMPARISON IN THE NADPH-DEPENDENT AND NADH-DEPENDENT DEMETHYLASE ACTIVITIES OF p-NITROANISOLE, N-MONOMETHYLANILINE AND ETHYLMORPHINE

Substrate	NADPH(T) (nmole/mg protein/10 min)	NADH(D) (nmole/mg protein/10 min)	D/T
p-Nitroanisole	36.5	17.4	0.48
N-Monomethyl-aniline	115.6	22.6	0.20
Ethylmorphine	48.4	0	0

Table 2
EFFECT OF p-NITROANISOLE ON THE REOXIDATION RATE OF CYTOCHROME b_5

Additions	Cyt. b_5 reoxidation rate (nmole cyt. b_5 oxidized/mg protein/min)	-Blank
None	0.63	---
Acetone	0.88	---
p-Nitroanisole	2.07	1.19

Table 3
EFFECT OF EXOGENOUSLY BOUND CYTOCHROME b_5 ON THE NADH-DEPENDENT AND NADPH-DEPENDENT O-DEMETHYLATION ACTIVITIES OF p-NITROANISOLE

Group	Microsomal cyt. b_5 ±fortification (nmole/mg protein)	p-Nitroanisole O-demet. (nmole/mg/10 min) NADPH(T)	NADH(D)	D/T
Control	0.47	40.5	36.9	0.91
+ Cyt. b_5(17.9 nmole)	0.89	50.3	52.0	1.03
+ Cyt. b_5(35.7 nmole)	1.29	48.9	54.1	1.11
+ Cyt. b_5(89.7 nmole)	1.96	44.5	56.5	1.27

Partially purified cytochrome b_5 (amounts shown in the brackets) were added to the flasks containing 0.1 M Na,K-phosphate (pH 7.4) and rabbit liver microsomes (20 nmoles cyt. b_5). The flasks were incubated at 37° for 30 min. The microsomes fortified with cytochrome b_5 were washed once with 0.1 M Na,K-phosphate (pH 7.4) by centrifugation at 105,000 xg for 30 min.

Table 4
EFFECT OF p-NITROANISOLE ON THE CYTOCHROME P-450 REDUCTION RATE

Additions	Cyt. P-450 reduction rate (nmole/mg protein/min)	- Blank
None	1.95 (2)*	---
Ethylmorphine	2.03 (3)	0.08
Acetone	2.00 (2)	---
p-Nitroanisole	4.50 (3)	2.50

* Numbers in brackets are the number of experiments.

The involvement of cytochrome b_5 in the NADH-dependent p-nitroanisole O-demethylation was examined. The results showing the effect of p-nitroanisole on the reoxidation rate of NADH-reduced cytochrome b_5 is shown in Table 2. Acetone slightly increased the cytochrome b_5 reoxidation rate, but p-nitroanisole elevated this rate markedly. Thus the p-nitroanisole-enhanced cytochrome b_5 reoxidation rate (cytochrome b_5 reoxidation rate due to p-nitroanisole) was 1.19 nmole/mg protein/min. To further support the idea that cytochrome b_5 is involved in NADH-dependent O-demethylation of p-nitroanisole, the effect of adding partially purified cytochrome b_5 to rabbit liver microsomes on the activity of p-nitroanisole O-demethylation was examined (Table 3). The addition of cytochrome b_5 to the microsomes resulted in an increase in NADH-dependent O-demethylation activity. NADPH-dependent O-demethylation activity was increased when a small amount of cytochrome b_5 was added. However, the activity gradually decreased with increasing amounts of cytochrome b_5.

The possibility that p-nitroanisole O-demethylation is catalyzed by cytochrome P-450 was examined by an experiment described in Table 4. In this experiment, the effect of ethylmorphine and p-nitroanisole on NADH-cytochrome P-450 reductase activity was determined. Ethylmorphine did not appreciably altered the NADH-cytochrome P-450 reduction rate whereas p-nitroanisole remarkably increased the rate. The NADH-cytochrome P-450 reduction rate due to the addition of p-nitroanisole was 2.5 nmole/mg protein/min.

DISCUSSION

Shigematsu *et al.* (14, 15) demonstrated the possibility that NADH-dependent O-dealkylation of p-nitroanisole and p-nitrophenetole is catalyzed by an enzyme other than cytochrome P-450. This conclusion was based on the evidence that carbon monoxide-oxygen (8:2) inhibited p-nitroanisole O-demethylation by only 10 to 20 %, and enhanced p-nitrophenetole O-deethylation. However, the results shown in this paper supports the idea that the NADH-dependent O-demethylation of p-nitroanisole is catalyzed by cytochrome P-450 (Table 4), and

that electrons for reducing cytochrome P-450 are transfered via cytochrome b_5 (Tables 2 and 3). Recent studies have shown there are multiple species of cytochrome P-450 in liver microsomes (16-19). The substrate difference in the NADH-dependent demethylase activities (Table 1) suggests that one or more cytochrome P-450 species participates in NADH-dependent reaction more readily than others. In accordance with this hypothesis, our recent studies[1] have indicated that antiserum against cytochrome P-450 isolated from the livers of phenobarbital-treated rats inhibited NADPH-dependent O-demethylation of p-nitroanisole to a greater extent than the NADH-dependent reaction. Further, these studies indicated that fortification of rat liver microsomes with purified cytochrome P-448 from 3-methylcholanthrene-treated rats resulted in a greater enhancement of both NADPH-dependent and NADH-dependent activities of p-nitroanisole O-demethylation than that seen using purified cytochrome P-450 from phenobarbital-treated rats. Oshino et al. (2) demonstrated that NADPH-cytochrome P-450 reductase transfers electrons both to cytochrome P-450 and to cytochrome b_5. As shown in Table 3, exogenously bound cytochrome b_5 enhanced NADPH-dependent p-nitroanisole O-demethylation activity as well as the NADH-dependent activity. The enhanced activity is, therefore, assumed to be due to the electron flow from NADPH-cytochrome P-450 reductase to cytochrome P-450 via cytochrome b_5.

ACKNOWLEDGEMENT

We would like to thank Miss Chie Kojima for her skillful technical assistance. We are also indepted to Mr. Mitsukazu Kitada for his useful discussions. Furthermore, We are grateful to Dr. Robert A. Neal, Vanderbilt University, for his kind suggestions in the preparation of this manuscript.

REFERENCES

1. Gillette, J. R., Davis, D. C. and Sasame, H. A. (1972) Ann. Rev. Pharmacol. 12, 57-84
2. Oshino, N., Imai, Y. and Sato, R. (1971) J. Biochem. (Tokyo) 69, 155-167
3. Cohen, B. S. and Estabrook, R. W. (1971) Archs. Biochem. Biophys. 143, 46-53

[1] unpublished results

4. Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370-2378
5. Hildebrandt, A. and Estabrook, R. W. (1971) Archs. Biochem. Biophys. 143, 66-79
6. Hrycay, E. G. and Estabrook, R. W. (1974) Biochem. Biophys. Res. Commun. 60, 771-778
7. West, S. B., Levin, W., Ryan, D., Vore, M. and Lu, A. Y. H. (1974) Biochem. Biophys. Res. Commun. 58, 516-522
8. Kamataki, T. and Kitagawa, H. (1974) Japan. J. Pharmacol. 24, 195-203
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
10. Imai, Y. (1976) J. Biochem. (Tokyo) 80, 267-276
11. Nash, T. (1953) Biochem. J. 55, 416-421
12. Kato, R. and Gillette, J. R. (1965) J. Pharmacol. Exptl. Therap. 150, 279-284
13. Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2379-2385
14. Shigematsu, H. , Yamano, S. and Yoshimura, H. (1976) Archs. Biochem. Biophys. 173, 178-186
15. Shigematsu, H. and Yoshimura, H. (1977) Personal Communication, Chem. Pharm. Bull. (Tokyo) in press
16. Lu, A. Y. H. and Levin, W. (1974) Biochim. Biophys. Acta 344, 205-240
17. Ryan, D., Lu, A. Y. H., Kawalek, J., West, S. B. and Levin, W. (1975) Biochem. Biophys. Res. Commun. 64, 1134-1141
18. Haugen, D. A., van der Hoeven, T. A. and Coon, M. J. (1975) J. Biol. Chem. 250, 3567-3570
19. Hashimoto, C. and Imai, Y. (1976) Biochem. Biophys. Res. Commun. 68, 821-827