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THE OBLIGATORY REQUIREMENT OF CYTOCHROME b_5 IN THE p-NITROANISOLE O-DEMETHYLATION REACTION CATALYZED BY CYTOCHROME P-450 WITH A HIGH AFFINITY FOR CYTOCHROME b_5

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SUMMARY: The role of cytochrome b_5 in the p-nitroanisole O-demethylation was studied with a reconstituted system containing a unique cytochrome P-450, isolated from rabbit liver microsomes as a species with a high affinity for cytochrome b_5. The maximal activity was obtained in the complete system consisting of cytochrome P-450, NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase, and Triton X-100 in addition to cytochrome b_5. The omission of cytochrome b_5 from the complete system entirely abolished the activity. These results clearly show that cytochrome b_5 is obligatory in the reconstituted p-nitroanisole O-demethylation system, and this cytochrome P-450 probably interacts with cytochrome b_5 in such a way that the second electron is transferred from cytochrome b_5 and thus exhibits the demethylase activity.

INTRODUCTION: Attempts to establish the role of cytochrome b_5 in the microsomal cytochrome P-450 containing mixed function oxidase system have been carried out by kinetic studies (1-4), immunochemical studies (5-9), and reconstitution of purified enzyme system (10-15). Hildebrandt and Estabrook (1) and Correia and Mannering (2) have postulated that the synergistic effect of NADH on NADPH-dependent reaction, according to which the second electron of the two electrons required for the drug oxidation is derived either from NADPH or from NADH and supplied to cytochrome P-450 *via* cytochrome b_5. It remains uncertain whether cytochrome b_5 is obligatory , although the NADH synergism is commonly observed in the microsomal mixed function oxidase system. Recently, components in the microsomal drug hydroxylation system have been highly purified and successfully reconstituted in the drug hydroxylation systems, and thus it has become possible to study the role of cytochrome b_5 in the NADPH-supported hydroxylation by using a reconstituted system (10-13). Cytochrome b_5 is stimulatory for chlorobenzene hydroxylation (10), testosterone 16α-hydroxylation (10), and benzphetamine

Abbreviations: cyt b_5, cytochrome b_5 ; P-450, cytochrome P-450 ; Fp_1, NADH-cytochrome b_5 reductase ; Fp_2, NADPH-cytochrome P-450 reductase.

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N-demethylation (11), but when NADPH is the sole electron source in the reconstituted systems, cytochrome b_5 is not required for these and other drug hydroxylations (12-15).

We have recently obtained a characteristic cytochrome P-450, which can be purified by using cytochrome b_5-immobilized Sepharose affinity column chromatography and shows a high affinity for cytochrome b_5. In this communication, we wish to report on the data which indicate that cytochrome b_5 is an obligatory component in the p-nitroanisole O-demethylation reaction catalyzed by this characteristic cytochrome P-450.

MATERIALS AND METHODS: Four components of the microsomal drug oxidation system, NADH-cytochrome b_5 reductase, NADPH-cytochrome P-450 reductase, cytochrome b_5, and cytochrome P-450, were solubilized from rabbit liver microsomes in the presence of 1% Triton X-100. Cytochrome P-450 was purified by a novel technique of cytochrome b_5-immobilized Sepharose affinity column chromatography described in a preliminary report (16). Cytochrome b_5 and NADH-cytochrome b_5 reductase were purified by the methods of Spatz and Strittmatter (17) and Mihara and Sato (18), respectively, with some modifications. NADPH-cytochrome P-450 reductase was purified by using 2',5'-ADP-Sepharose affinity column chromatography as reported by Yasukochi and Masters (19). None of these purified components contaminated with each other.

The p-nitroanisole O-demethylase activity was directly measured by the absorbance increment at 400 nm, which is due to the production of p-nitrophenol according to the method of Netter and Seidel (20), using a millimolar extinction coefficient of 14.5 $mM^{-1}cm^{-1}$ at pH 7.4.

Carbon monoxide inhibition was carried out in a closed vessel (2 ml) equipped with a Galvanic type oxygen electrode and a thermostated water bath. Reaction mixture was saturated with carbon monoxide by bubbling it thoroughly, and oxygen was introduced at an appropriate tension by monitoring with the oxygen electrode in the liquid phase. p-Nitrophenol was extracted by the method of Shigematsu et al.(21), and determined spectrophotometrically using a millimolar extinction coefficient of 18.9 $mM^{-1}cm^{-1}$ at 400 nm above pH 11.

Unless otherwise stated, the incubation mixture contained cytochrome P-450 (0.37 μM), cytochrome b_5 (0.82 μM), NADH-cytochrome b_5 reductase(4 units), NADPH-cytochrome P-450 reductase(0.2 unit), Triton X-100(0.05 %,W/V), NADH(0.1 mM), NADPH(0.1 mM), potassium phosphate(0.1 M, pH 7.4), and p-nitroanisole(1 mM). The reaction was carried out aerobically at 37°C. One unit of the enzyme was defined as the amount of it catalyzing the reduction one μmole of an acceptor per min at 25°C. Ferricyanide and cytochrome c were used as an acceptor for assaying the activity of NADH-cytochrome b_5 reductase and NADPH-cytochrome P-450 reductase, respectively.

RESULTS: Fig. 1 shows what components are required for the O-demethylation of p-nitroanisole in the reconstituted system. The activity was measured by the 400 nm absorbance increment, which is due to the production of p-nitrophenol. In experiment (A), addition of either NADPH or NADH to the reaction mixture, containing cytochrome P-450, NADH-cytochrme b_5 reductase, and NADPH-cytochrome

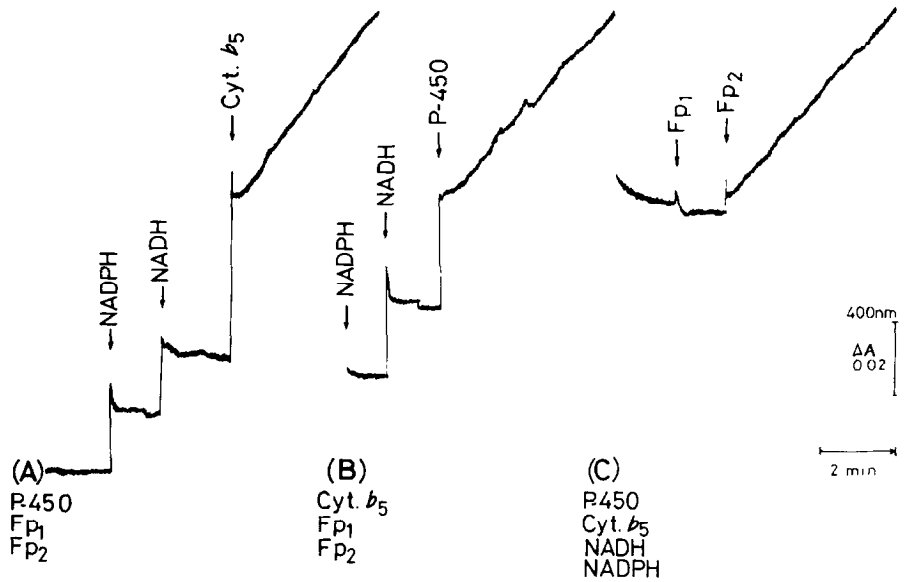

Fig. 1. The demonstration of the obligatory components in the p-nitroanisole O-demethylation by the reconstituted system. The reaction mixture (1.0 ml in 0.1 M potassium phosphate, pH 7.4) contained in the final stage: P-450 (0.37 μM), cyt b_5 (0.82 μM), Fp_1 (4 units), Fp_2 (0.2 unit), Triton X-100 (0.05%,W/V), NADH (0.1 mM), NADPH (0.1 mM), and p-nitroanisole (1 mM). In experiment (A), the reaction mixture was preincubated with P-450, Fp_1, and Fp_2 for 5 min at 37°C and then NADPH, NADH, and cyt b_5 were sequentially added. Experiments (B) and (C) were carried out in the same manner as experiment (A) except the order of addition of the components which are indicated in the figure.

P-450 reductase, did not increase the 400 nm absorbance, but addition of cytochrome b_5 to this reaction mixture caused an immediate increase in the 400 nm absorbance. In experiment (B), the absorbance increment was not observed even in the presence of cytochrome b_5, NADH-cytochrome b_5 reductase, NADPH-cytochrome P-450 reductase, NADPH, and NADH, unless cytochrome P-450 was added. In experiment (C), no absorbance change was observed when NADH-cytochrome b_5 reductase was added to the reaction mixture containing cytochrome P-450, cytochrome b_5, NADH, and NADPH, but further addition of NADPH-cytochrome P-450 reductase was necessary for the demethylase activity.

These results are summarized in Table I. The maximal activity was 2.0 nanomoles of p-nitrophenol produced per nanomole of cytochrome P-450 per min when the reaction mixture contained cytochrome P-450, cytochrome b_5, NADH-cytochrome b_5 reductase, NADPH-cytochrome P-450 reductase, and Triton X-100 as a

Table I
The obligatory components in the reconstituted p-nitroanisole O-demethylation system

System	Activity	
Complete[a]	2.00[b]	(100)[c]
- P-450	0	(0)
- cyt b_5	0	(0)
- Fp_2	0	(0)
- Fp_1	1.73	(86)
- Triton X-100	0.53	(27)

a. The complete system cantained the same as described in Fig. 1.
b. nmol p-nitrophenol produced per nmol P-450 per min.
c. percent

complete system. The activity was abolished or greatly decreased when any one of these components was omitted from the complete system. An omission of NADH-cytochrome b_5 reductase caused a decrease in the activity to 86 % of the maximum, probably because cytochrome b_5 is capable of accepting an electron from NADPH-cytochrome P-450 reductase (22). To obtain the maximal activity, it was also necessary to include 0.05 % of Triton X-100 in the reconstituted system.

The inhibition of p-nitroanisole O-demethylase activity was examined by using this reconstituted system. Under as atmosphere of 90 % of carbon monoxide and 10 % of oxygen, the activity decreased to 10 % of that under an atmosphere of 90 % of nitrogen and 10 % of oxygen. Phenylisocyanide (20 μM) inhibited the activity completely, and metyrapone (0.15 mM) also inhibited it by 70 %. In the presence of 0.25 mM cyanide the activity decreased to 50 % of the maximum. These results lead us to conclude that cytochrome P-450 is the terminal enzyme which catalyzes p-nitroanisole O-demethylation in the reconstituted system.

DISCUSSION: The results suggest that the two electron transport systems, i.e. NADH-linked and NADPH-linked systems, are required for the p-nitroanisole O-demethylation reaction, and exclude the possibility of either NADH-linked or NADPH-linked O-demethylation of p-nitroanisole. This report presents the first

evidence that cytochrome b_5 is an obligatory component in the reconstituted p-nitroanisole O-demethylation system.

As shown in Table I, the activity in the "Complete" system, in which electrons are donated both by NADH and by NADPH, was 1.2 times greater than that in the "Complete-Fp_1" system, in which electrons are donated only by NADPH. The NADH synergism was thus observed in our reconstituted system. The synergism could result from the enhancement of the reduction level of cytochrome b_5 in the steady state by switching the electron flow from NADPH to cytochrome b_5 *via* NADPH-cytochrome P-450 reductase to that from NADH to cytochrome b_5 *via* NADH-cytochrome b_5 reductase. The mechanism of the second electron transfer from NADH or NADPH to cytochrome P-450 *via* cytochrome b_5 remains still obscure.

Our system is considered to be unique in its absolute requirement of electrons transferred both from cytochrome b_5 and from NADPH-cytochrome P-450 reductase, which is in a marked contrast with a number of the reconstituted systems hitherto reported (12-15), where sufficient activities are observed even in the absence of cytochrome b_5 as long as the electrons are transferred from NADPH-cytochrome P-450 reductase. Studies to elucidate the mechanism of the second electron transfer from cytochrome b_5 to cytochrome P-450 using this beneficial reconstituted system are in progress.

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