IMMUNOCHEMICAL EVIDENCE FOR THE PARTICIPATION OF CYTOCHROME b_5 IN THE NADH SYNERGISM OF THE NADPH-DEPENDENT MONO-OXIDASE SYSTEM OF HEPATIC MICROSOMES

Gilbert J. Mannering[1], Shin-ichi Kuwahara and Tsuneo Omura
Department of Biology, Faculty of Science, Kyushu University
Fukuoka 812, Japan

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SUMMARY. Anti-cytochrome b_5 immunoglobulin (AIg) from a rabbit was used to establish the role of cytochrome b_5 in the transfer of electrons from NADH or NADPH to the hepatic microsomal mono-oxidase system of the rat. AIg inhibited ethylmorphine (EM) N-demethylase when both NADH and NADPH were present, but had little effect when NADPH was the only source of electrons. Inhibition was reversed when AIg was preincubated with pure cytochrome b_5. Specificity of AIg was shown by its inhibitory effect on NADH cytochrome c reductase activity; it was without effect on NADPH-cytochrome P-450 reductase or aniline hydroxylase activities. It is concluded that the second electron required for EM N-demethylation can be donated by NADH via cytochrome b_5.

The first of the two electrons required for the N-demethylation of ethylmorphine (EM) and the oxidation of many other xenobiotics by the hepatic mono-oxidase system involving cytochrome P-450 is donated by NADPH; the second is thought to be deriviable from NADH as well as from NADPH (1-3). The well-known synergistic effect of NADH on the NADPH-dependent reaction has been postulated by Hildebrandt and Estabrook (1) and by Correia and Mannering (2,3) to be due to the more efficient utilization of second electrons from NADH via cytochrome b_5. Evidence for the involvement of cytochrome b_5 in the overall reaction is based largely on the observation that substrate (EM) lowered the steady state of reduction of cytochrome b_5 in a medium containing both NADPH and NADH (1), and by studies where steroyl CoA lowered the rate of metabolism of EM by shunting electrons away from cytochrome b_5 to the microsomal fatty acyl-CoA system, or where cyanide increased the rate by preventing a loss of electrons to this system (2,3). While supporting a role of cytochrome b_5 in the hepatic mono-oxidase system, this evidence is not conclusive and is subject to alternative interpretations. The current study employed immunochemical methods to establish the participation of cytochrome b_5 in the transfer of electrons from NADH to the cytochrome P-450 mono-oxidase system.

MATERIALS AND METHODS. Preparation of microsomes. Male Sprague-Dawley strain rats (180-260 g) were fed a commercial diet until killed. All were

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administered Na phenobarbital intraperitoneally (100 mg/kg) on each of 3 or 4 mornings preceding the morning they were killed. By greatly increasing activity of the mono-oxidase system without increasing the cytochrome b_5 content of the microsomes appreciably (4), phenobarbital administration enabled the harvest of microsomes which were most suitable for the immunochemical studies in mind. Livers were excised and perfused with isotonic KCl solution. Microsomes were prepared as described previously (2) except that a motor driven Potter-Elvehjem homogenizer was used (7 passes) instead of a Dounce homogenizer; they were used the same day.

Preparation of rabbit antiserum against rat cytochrome b_5. Anti-cytochrome b_5 was prepared as described previously (5). Purified cytochrome b_5 was prepared from rat liver microsomes by the method of Omura and Takesue (6). It gave a single protein band when examined by acrylamide gel electrophoresis (6). A total of 2.4 mg of this cytochrome b_5 in Freund's complete adjuvent (Difco Co.) was injected subcutaneously into 5 different places on the back of an adult white rabbit[2]. This was followed 21, 32 and 35 days later by intravenous (ear vein) injections of 3.6 mg of cytochrome b_5 given in 1.2 mg doses. Five days after the last injection immunoglobulin was prepared from blood collected from the ear vein. Control immunoglobulin (CIg) was prepared from blood of rabbits which had not been immunized.

Assays. EM N-demethylase activity of microsomes was determined as described previously (2) using the Nash method (7) for the measurement of HCHO. Aniline hydroxylase activity of microsomes was determined by measuring p-aminophenol formation as described by Imai et al. (8). NADH-cytochrome c reductase activity of microsomes was determined as described previously (6). NADPH-cytochrome P-450 reductase activity of microsomes was determined by the method of Gigon et al. (9) except that NADPH (400 μM) was used instead of $NADP^+$ plus NADPH generating system and the temperature was 10°C. The cytochrome P-450 and b_5 contents of microsomes were measured by the methods of Omura and Sato (10). Protein was determined by the method of Lowry et al. (11).

RESULTS. Effect of AIg on NADH-cytochrome c reductase activity. The participation of cytochrome b_5 in the microsome catalyzed reduction of added cytochrome c is well established (12). Using 0.02 mg of microsomal protein/ml of medium, 50, 90 and 93% inhibition of NADH-cytochrome reductase activity was achieved with ratios of AIg : microsomal protein of 20, 100 and 165, respectively. This assay was repeated several times during the course of these studies with very similar results.

[2] Donated by Takeda Chemical Industry Co., Osaka, Japan.

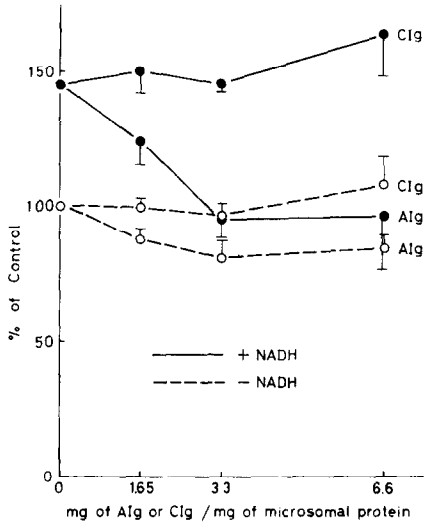

Figure 1.

Effects of anti-cytochrome b_5 immunoglobulin (AIg) and control immunoglobulin (CIg) on ethylmorphine (EM) N-demethylation by hepatic microsomes. A mixture (final vol: 1.0 ml) containing 0.5 mg of microsomal protein from phenobarbital-treated rats, specified amounts of AIg or CIg, isocitrate (4 mM), isocitrate dehydrogenase (0.4 units), $NADP^+$ (0.4 mM), Mg^{++} (2 mM), semicarbazide (7.5 mM), and Na-K PO_4 buffer (40 mM, pH 7.4) in 1.15% KCl solution was preincubated in an open test tube for 5 min (37°C) with shaking (180 oscillations/min); the N-demethylation reaction was initiated by the addition of EM (2.0 mM) or EM (2.0 mM) + NADH (1.0 mM), and terminated 10 min later by the addition of $ZnSO_4$ (7). Rates of EM N-demethylation were obtained by duplicate analyses of HCHO formed (7). Values are means (± S.E.) of 3 experiments; combined microsomes from 2 livers were used in each experiment. Control = velocity of HCHO formation in the absence of AIg, CIg and NADH (64.5±2.4 nmoles/mg of microsomal protein/min). Microsomes contained 1.14 ± 0.27 and 0.49 ± 0.07 nmoles of cytochromes P-450 and b_5/mg of protein, respectively.

Effect of AIg on NADPH-cytochrome P-450 reductase activity. Rates of NADPH reduction of cytochrome P-450 in microsomes (1 mg protein/ml) in the presence of 3.3 mg of AIg, with or without the addition of EM (0.2 mM), were 23 and 30 nmoles/min, respectively. When CIg was substituted for AIg, corresponding rates were 22 and 28 nmoles/min. Values are means of 2 experiments.

Effect of AIg on ethylmorphine N-demethylase activity. Fig. 1 illustrates the inhibitory effect of AIg on EM N-demethylation. The effect is seen to be almost entirely on NADH synergism with little inhibition observed when NADPH was the only source of electrons. For example, at the 3.3 mg level of AIg addition, where the maximum inhibitory effect had been reached, EM metabolism in the absence of NADH was reduced by only 15%, whereas in the presence of NADH, inhibition was about 50%. If it can be assumed that AIg exerts the same degree of inhibition of the transfer of electrons from NADPH when NADH is present as when it is absent, it can be calculated that about 80% of NADH

Table 1. Inhibition of ethylmorphine (EM) N-demethylation with anti-cytochrome b_5 immunoglobulin (AIg) and its reversal with cytochrome b_5

mg AIg/ml	Addition	HCHO Formed -NADH v*	-NADH % of control	+NADH v*	+NADH % of control
1.65 (n = 2)	None (control)	66.3	-	86.6	-
	Cytochrome b_5	65.8	99	78.5	91
	AIg	56.6	85	61.9	71
	Cytochrome b_5 + AIg	64.7	98	82.1	95
3.3 (n = 4)	None (control)	48.6 ± 5.2	-	74.5 ± 9.5	-
	Cytochrome b_5	47.5 ± 6.1	98	76.2 ± 8.9	102
	AIg	41.4 ± 3.6‡	85	50.5 ± 3.2†	68
	Cytochrome b_5 + AIg	50.3 ± 4.4	103	73.0 ± 4.1	98

*nmoles of HCHO/mg of microsomal protein/min S.E.

†statistically different ($P < 0.02$) than corresponding control value using t test of log of $(\text{experimental/control})_1$; other values where S.E. is given are not statistically different than control values at the $P < 0.05$ level.

‡$P < 0.1$

The minimum amount of pure cytochrome b_5 previously determined to completely neutralize the inhibitory effect of AIg on the cytochrome c reducing activity of highly purified NADH-cytochrome b_5 reductase (14), namely, 0.6 nmoles of cytochrome b_5/mg of AIg, was incubated with 1.65 or 3.3 mg of AIg for 2 min at 37°C in 0.1 ml of 1.15 KCl solution. Components of the reaction mixture given in Fig. 1 were added and the mixture was incubated for 5 min in the absence of substrate; EM was then added and the incubation was continued for 10 min as described in Fig. 1. Each experiment used combined microsomes from 2 rats which had been treated with phenobarbital. The final reaction mixture (1.0 ml) contained 3.3 - 0.5 mg of microsomal protein with concentrations of cytochrome P-450 and cytochrome b_5 of 1.53 ± 0.02 and 0.56 ± 0.04/mg of protein, respectively.

synergism is inhibited by AIg. Moreover, it is seen that increasing the concentration of AIg to the 6.6 mg level did not further increase the inhibition of EM metabolism either in the presence or absence of NADH, again showing that the effect involves electrons from NADH almost exclusively. In other words, since there is little effect of AIg on the transport of electrons from NADPH, and because NADH synergism is already almost abolished at the 3.3 mg level of AIg, increasing the level of AIg cannot further increase the degree of inhibition of the overall reaction.

If the inhibitory effect of AIg on EM metabolism is due to specific cytochrome b_5 antibodies, the incubation of AIg with pure cytochrome b_5 should neutralize the inhibitory effect of AIg on EM metabolism. As can be seen in Table 1, the specific role of cytochrome b_5 antibody in the inhibition of EM metabolism was determined in this manner.

Table 2. Effects of anti-cytochrome b_5 immunoglobulin (AIg) and control immunoglobulin (CIg) on aniline hydroxylation by microsomes.

Addition	p-Aminophenol Formed (nmoles/mg microsomal protein/min) -NADH	+NADH
None	0.39 ± 0.035	0.37 ± 0.035
CIg	0.35 ± 0.024	0.33 ± 0.021
AIg	0.38 ± 0.033	0.32 ± 0.025

A mixture (final vol: 1.0 ml) containing 1.0 mg of microsomal protein from phenobarbital-treated rats, 3.3 mg of AIg or CIg, glucose-6-PO_4 (16 mM), glucose-6-PO_4 dehydrogenase (0.4 units), $NADP^+$ (1.6 mM), Mg^{++} (8 mM), K-PO_4 buffer (40 mM, pH 7.4) in 1.15% KCl solution was preincubated in an open test tube for 5 min (37°C) with shaking (180 oscillations/min); the hydroxylation reaction was initiated by the addition of aniline (0.2 mM) or aniline (0.2 mM) + NADH (1.0 mM) and terminated 20 min later by the addition of trichloroacetic acid (8). Rates of aniline hydroxylation were obtained by duplicate analyses of p-aminophenol formed (8). Values are means (±S.E.) of 4 experiments; combined microsomes from 2 livers were used in each experiment. Microsomes contained 1.57 ± 0.27 and 0.53 ± 0.06 nmoles of cytochromes P-450 and b_5/mg of protein, respectively.

Effect of AIg on aniline hydroxylase activity. AIg did not inhibit aniline hydroxylase either in the presence or absence of NADH (Table 2) This was predictable from studies of Correia and Mannering (3) which showed that the hydroxylation of aniline is not synergized by NADH. Imai and Omura (13) showed previously that AIg does not inhibit aniline hydroxylation.

DISCUSSION. The specificity of AIg as an inhibitor of cytochrome b_5 activity was demonstrated by its effect on microsomal and purified NADH-cytochrome c reductase. That it was not a general inhibitor of microsomal enzymes was shown by its failure to inhibit NADPH-cytochrome c reductase and aniline hydroxylase activities or to inhibit effectively the NADPH-supported N-demethylation of EM.

While the failure of AIg to inhibit aniline hydroxylation, or to inhibit appreciably EM N-demethylation when NADPH was the only source of electrons, suggests that cytochrome b_5 is not involved in the transfer of the second electron from NADPH to the reduced cytochrome P-450 substrate complex, or involved to only a very limited degree, these observations do not rule out this possibility. The amount of microsomal cytochrome b_5 which may not have reacted with AIg in these experiments may have been considerable, and while unreacted cytochrome b_5 might then have been insufficient to support the synergized rate of EM metabolism in the presence of both NADPH and NADH, it may have been sufficient to support the maximal rate of EM metabolism that is possible in the absence of NADH. The turnover rate of aniline hydroxylation

is so slow compared to that of EM N-demethylation that NADPH can readily satisfy the need for second electrons without benefit from NADH (3). Thus, while these studies favor the concept that cytochrome b_5 is involved only in the transfer of electrons from NADH, the question of its participation in the transfer of electrons from NADPH in microsomes remains unresolved.

Correia and Mannering (2,3) explain NADH synergism as follows: a) The electron required for the reduction of oxidized cytochrome P-450 substrate complex (first electron) must come from NADPH; b) The electron required for the reduction of reduced cytochrome P-450 substrate complex (second electron) may come from either NADPH or NADH; c) When second electrons are derived from NADH, they must be transfered via cytochrome b_5; whether or not second electrons from NADPH can be transfered via cytochrome b_5 remains a question; d) second electrons from NADH are used by the mono-oxidase system more efficiently than second electrons from NADPH; this may be because NADH maintains a higher steady state of reduced cytochrome b_5 than can be maintained by NADPH; e) The addition of NADH to the NADPH-dependent reaction shifts the rate limitation from the site of entry of the second electron to the site of entry of the first electron, which is now relieved of the restriction formerly imposed at the former site and is thus free to give full expression to its ability to accept electrons; this results in an increased turnover rate of the system. The current study supports this concept.

REFERENCES.

1. Hildebrandt, A. and Estabrook, R. W., Arch. Biochem. Biophys. 143, 66-79 (1971).
2. Correia, M. A. and Mannering, G. J., Mol. Pharmacol. 9, 455-469 (1973).
3. Correia, M. A. and Mannering, G. J., Mol. Pharmacol. 9, 470-485 (1973).
4. Ernster, L. and Orrenius, S., Federation Proc., 24, 1190-1199 (1965).
5. Oshino, N. and Omura, T., Arch. Biochem. Biophys. 157, 395-404 (1973).
6. Omura, T. and Takesue, S., J. Biochem. 67, 249-256 (1970).
7. Nash, T., Biochem. J. 55, 416-421 (1953).
8. Imai, Y., Ito, A. and Sato, R., J. Biochem. 60, 417-428 (1966).
9. Gigon, P. L., Gram, T. E. and Gillette, J. R., Mol. Pharmacol. 5, 109-122 (1969).
10. Omura, T. and Sato, R., J. Biol. Chem. 239, 2370-2378 (1964).
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem. 193, 265-275 (1951).
12. Velick, S. F. and Strittmatter, P., J. Biol. Chem. 221, 265-275 (1956).
13. Imai, Y. and Omura, T., unpublished results.
14. Okada, Y. and Omura, T., unpublished results.