THE EFFECT OF EXTRA BOUND CYTOCHROME b_5 ON CYTOCHROME P-450-DEPENDENT ENZYME ACTIVITIES IN LIVER MICROSOMES

Eugene G. Hrycay and Ronald W. Estabrook

Department of Biochemistry
The University of Texas Health Science Center
Dallas, Texas 75235

Received August 7,1974

SUMMARY: Binding of increasing amounts of detergent-purified cytochrome b_5 to rabbit liver microsomes produces a progressive inhibition of NADPH-cytochrome P-450 reductase activity which is accompanied by a similar inhibition of NADPH-supported benzphetamine demethylation. In contrast, NADH-cytochrome P-450 reductase activity in the enriched microsomes is markedly enhanced and this stimulation is accompanied by a similar increase in NADH-peroxidase activity, suggesting that cytochrome b_5 in these two reactions functions as an intermediate electron carrier to cytochrome P-450.

Of the two major hemoproteins present in liver microsomes of mammalian species, cytochrome P-450 has received the most attention because of its unique versatility in being able to catalyze the transformation of a wide variety of lipid-soluble substrates in the presence of NADPH and molecular oxygen (1). The physiological function for the second major hemoprotein, cytochrome b_5, has remained obscure until recently when the hemoprotein was found to function as an intermediate electron carrier in various NADH-supported reactions (2-9). The terminal electron acceptor in these reactions can be one of three enzymes: (a) cytochrome P-450 (e.g. NADH-peroxidase activity, NADH-supported laurate hydroxylation) (4,5); (b) fatty acid desaturase (2,9); and (c) hydroxylamine reductase (3).

Detergent-purified cytochrome b_5 can under optimal conditions be incorporated into the microsomal membrane and this extra bound hemoprotein appears to be functionally indistinguishable from that normally present in microsomes (9,10). Jansson and Schenkman (11) utilizing this incorporation procedure reported a progressive inhibition of NADPH-supported aminopyrine demethylation with increasing concentrations of microsomal bound

cytochrome b_5 and concluded that cytochrome b_5 was not a functional component for cytochrome P-450-catalyzed reactions. However, the response of other cytochrome P-450-mediated activities to cytochrome b_5 enrichment of microsomes has not been examined.

This study reports the effect of extra bound cytochrome b_5 on various cytochrome P-450-dependent enzyme activities in liver microsomes. The results obtained suggest that the inhibition of NADPH-supported drug demethylation in cytochrome b_5-enriched microsomes is the result of the inhibition of NADPH-cytochrome P-450 reductase activity. On the other hand, NADH-cytochrome P-450 reductase and NADH-peroxidase activities in the enriched microsomes are markedly enhanced suggesting that cytochrome b_5 in these two reactions functions as an intermediate electron carrier to cytochrome P-450.

METHODS

Microsomes were prepared from livers of male albino rabbits that had been pretreated with sodium phenobarbital (80 mg/kg) once daily for 4 days as described previously (12). Purified cytochrome b_5 (20 nmoles/mg protein) was prepared from rabbit liver microsomes by detergent solubilization (13). Binding of cytochrome b_5 to microsomes was accomplished essentially as described by Strittmatter et al. (9). Approximately 14 ml of microsomes (6 mg/ml) were incubated at 37° for 30 minutes with varying amounts of purified cytochrome b_5 (ranging from 100-600 nmoles) in 0.1 M Tris-acetate buffer (pH 7.4) containing 1 mM EDTA. A control incubation without added cytochrome b_5 was carried out. The suspensions were centrifuged at 105,000 g for 1 hour, washed once with 20 ml of buffer mixture, and centrifuged again. The pellets were suspended in 5 ml of 0.25 M sucrose-10 mM Tris-HCl (pH 7.4) to a final protein concentration of about 15 mg/ml. Benzphetamine N-demethylation in rabbit liver microsomes (0.8 mg/ml) was measured in the presence of 0.3 mM NADPH or NADH by determining the initial rate of formaldehyde production as described by Estabrook et al. (14). Formaldehyde was determined by the method of Nash (15).

NAD(P)H-cytochrome P-450 reductase activity was measured under anaerobic conditions at 450 nm relative to 490 nm using an Aminco DW-2 spectrophotometer in the dual wavelength mode. Into the anaerobic sample cuvette was placed 3 ml of a CO-saturated solution containing 0.1 M potassium phosphate buffer (pH 7.6), microsomal protein (about 1 mg/ml), 100 mM glucose, 1 mg glucose oxidase (Sigma, type II), and 0.5 mg catalase (Sigma). Into the plunger assembly of the cuvette was placed a solution of NAD(P)H to give a final concentration of 0.15 mM. The air space in the cuvette was flushed for 10 minutes with CO that had been passed through a deoxygenating medium (16) and the reaction was initiated by rapid mixing of NAD(P)H with the cell contents. NAD(P)H-peroxidase activity was assayed at 340 nm by determining the rate of oxidation of NAD(P)H by cumene hydroperoxide in the presence of microsomes (17,18). NAD(P)H-oxidase and NAD(P)H-cytochrome *c* reductase activities were measured as previously described (5,18,19). Cytochromes P-450 and b_5 were determined as reported previously (18). Protein was estimated by the Lowry *et al.* (20) procedure using bovine serum albumin as a standard. All measurements were performed at 24°.

RESULTS

In Table I is presented the effect of extra bound cytochrome b_5 on various enzyme activities in rabbit liver microsomes. Since the specific content of cytochrome P-450 in the preparations varied because of the contribution of extra bound cytochrome b_5 to the total protein, activities dependent on cytochrome P-450 were expressed per nmole of cytochrome P-450. It is noted that the cytochrome b_5 content of the microsomes was increased approximately 4-fold. This increase was accompanied by a similar increase in NADH-cytochrome P-450 reductase and NADH-cytochrome *c* reductase activities. NADH-peroxidase activity (a cytochrome b_5 and cytochrome P-450-dependent reaction) (4) was stimulated 2-fold. In contrast, NADPH-cytochrome P-450 reductase and NADPH-benzphetamine demethylase activities were progressively inhibited with increasing concentrations of bound cytochrome b_5. Addition of an equiva-

TABLE I

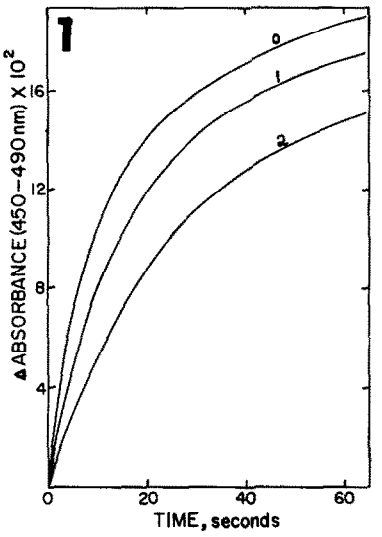
Enzyme Activities in Rabbit Liver Microsomes and Microsomes Containing Extra Bound Cytochrome b_5

Activity	Control Microsomes	Prep. 1	Prep. 2	Prep. 3
Cytochrome P-450[a]	3.66	3.30	3.23	3.04
Cytochrome b_5[a]	1.03	2.22	3.08	3.54
NADPH-cytochrome P-450 reductase[b]	4.56	3.51	1.80	1.53
NADH-cytochrome P-450 reductase[b]	0.62	0.91	1.85	1.90
NADPH-benzphetamine demethylase[c]	5.25	4.00	3.47	3.13
NADH-Benzphetamine demethylase[c]	0.28	0.61	0.64	0.48
NADPH-Peroxidase[d]	33.0	33.0	29.0	29.0
NADH-Peroxidase[d]	66.0	109.0	119.0	131.0
NADH-Cytochrome c reductase[e]	0.54	1.16	1.54	1.58
NADPH-Cytochrome c reductase[e]	0.20	0.19	0.18	0.18
NADH-oxidase[f]	5.1	5.0	5.0	4.9
NADPH-oxidase[f]	10.1	9.5	10.0	10.0

a Hemoprotein content expressed as nmoles/mg protein.
b Activities expressed as nmoles cytochrome P-450 reduced/min/nmole P-450
c Activities expressed as nmoles formaldehyde formed/min/nmole P-450
d Activities expressed as nmoles NAD(P)H oxidized/min/nmole P-450.
e Activities expressed as μmoles cytochrome c reduced/min/mg protein.
f Activities expressed as nmoles NAD(P)H oxidized/min/mg protein.

lent concentration of NADH did not stimulate the initial rate of the two NADPH-supported reactions to any appreciable extent. NADH by itself was a poor electron donor in supporting benzphetamine demethylation. NADPH-peroxidase activity (a cytochrome P-450-dependent reaction) (17) was slightly diminished with increasing concentrations of bound cytochrome b_5 whereas NAD(P)H-oxidase and NADPH-cytochrome c reductase activities remained virtually unchanged (Table I).

In Figure 1 is shown the kinetics of the reduction of microsomal cytochrome P-450 by NADPH in the presence of extra bound cytochrome b_5. The results indicate a progressive decrease in the reaction rate with increasing concentrations of bound cytochrome b_5. In contrast, NADH-cytochrome P-450 reductase activity was progressively enhanced with increasing cytochrome b_5 concentrations (Figure 2). It should be noted that the reaction rate obtained with NADH at the maximal cytochrome b_5 level (3.08 nmoles/mg protein) (Figure 2,



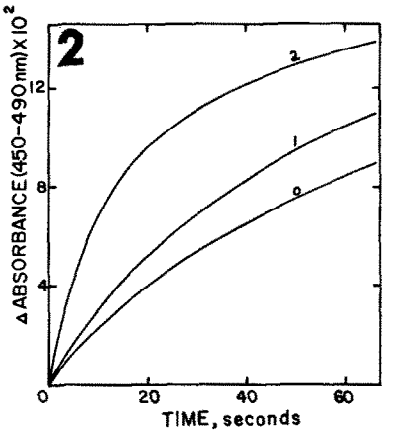


Figure 1: Inhibition of NADPH-cytochrome P-450 reductase activity by extra bound cytochrome b_5. The reaction was measured as described in "Methods" using control liver microsomes (curve 0) prepared from phenobarbital-pretreated rabbits and microsomes containing extra-bound cytochrome b_5 (curves 1 and 2). The NADPH concentration was 0.15 mM and the cytochrome P-450 content was approximately the same in all three assays.
curve 0: cyt. b_5, 0.76 μM; cyt. P-450, 2.69 μM; protein, 0.74 mg/ml.
curve 1: cyt. b_5, 1.79 μM; cyt. P-450, 2.66 μM; protein., 0.80 mg/ml.
curve 2: cyt. b_5, 2.40 μM; cyt. P-450, 2.52 μM; protein, 0.78 mg/ml.

Figure 2: Stimulation of NADH-cytochrome P-450 reductase activity by extra bound cytochrome b_5. Assay conditions were similar to those described in figure 1 except that NADH was substituted for NADPH.

curve 2) was equivalent to the reaction rate observed with NADPH (Figure 1, curve 2) and yet NADPH-supported benzphetamine demethylation proceeded at a much faster rate (6 times) than did NADH-dependent demethylation (Table I). Measurement of NADH-cytochrome P-450 reductase activity in the presence of the type I substrate, benzphetamine (1 mM), revealed a stimulation in the reaction rate.

DISCUSSION

The incorporation of detergent-purified cytochrome b_5 into the microsomal

membrane has recently been used as a means of evaluating the role of the hemoprotein in various NADPH-supported electron transport reactions in liver microsomes. Employing this incorporation procedure, several workers from different laboratories have demonstrated the ability of the extra bound cytochrome $\underline{b}_5$ to function as an electron carrier to the fatty acid desaturase enzyme (9) and to exogenous cytochrome $\underline{c}$ (9-11). We have confirmed the reports of these investigators in this study and have shown that the extra bound cytochrome $\underline{b}_5$ in liver microsomes markedly enhances the NADH-dependent rate of reduction of cytochrome $\underline{c}$. More importantly, the present study has shown that the incorporated cytochrome $\underline{b}_5$ markedly stimulates the NADH-dependent rate of reduction of cytochrome P-450, either in the presence or in the absence of a type I drug substrate, suggesting that the extra bound cytochrome $\underline{b}_5$ can channel electrons to cytochrome P-450 as does the endogenous cytochrome $\underline{b}_5$ of normal liver microsomes (4). This accelerated rate of cytochrome P-450 reduction by NADH appears to be responsible for the increase in NADH-peroxidase activity in the enriched microsomes (4). It is puzzling, however, why the NADPH-benzphetamine demethylase reaction rate in the fortified microsomes was several times greater than the NADH-dependent reaction rate while the rate of cytochrome P-450 reduction by NADPH was similar to that observed in the presence of NADH.

Hildebrandt and Estabrook (21) were the first to raise the possibility that cytochrome $\underline{b}_5$ functions in hepatic microsomal mixed-function oxidase reactions by providing the second electron (which can be donated by either NADPH or NADH) to the oxygenated, substrate-bound cytochrome P-450 complex. Support for this hypothesis and for the involvement of cytochrome $\underline{b}_5$ as an electron carrier to cytochrome P-450 in NADH-dependent reactions has been provided by the reports of a number of investigators (4-7,22-24). On the other hand, Jansson and Schenkman (11) showed that excessive binding of cytochrome $\underline{b}_5$ to liver microsomes stimulates NADH-cytochrome $\underline{c}$ reductase activity but inhibits NADPH-dependent aminopyrine demethylation and concluded that cytochrome $\underline{b}_5$

does not participate in the microsomal mixed-function oxidase reaction. Furthermore, these workers suggested that cytochrome b_5 inhibits the reaction by serving as an alternate electron acceptor diverting electrons away from cytochrome P-450. However, the inability in our study to observe any increase in NADPH-oxidase activity in the enriched microsomes raises a question concerning the validity of the alternate electron acceptor hypothesis. The results obtained in this study support the view that the inhibition of NADPH-dependent drug demethylation by extra bound cytochrome b_5 is the result of the inhibition of NADPH-cytochrome P-450 reductase activity. However, the problem still remains unresolved whether cytochrome b_5 can function as an electron carrier to cytochrome P-450 during NADPH-supported mixed-function oxidation reactions although a recent report by Sasame and coworkers (23) tends to support the view that cytochrome b_5 does participate in certain NADPH-dependent cytochrome P-450-mediated activities.

ACKNOWLEDGMENTS: This study was supported in part by USPHS grant No. GM-16488 and by the Medical Research Council of Canada. The authors are indebted to Dr. J. Werringloer for his stimulating discussions and to Mr. G. Nunn and Miss C. Christian for technical assistance.

REFERENCES

1. Gillette, J.R., Davis, D.C., and Sasame, H.A. (1972) Ann. Rev. Pharmacol. 12, 57-84.
2. Shimakata, T., Mihara, K., and Sato, R. (1972) J. Biochem. 72, 1163-1174.
3. Kadlubar, F.F., and Ziegler, D.M. (1974) Arch. Biochem. Biophys. 162, 83-92.
4. Hrycay, E.G., and Prough, R.A. (1974) Arch. Biochem. Biophys., in press.
5. Sasame, H.A., Thorgeirsson, S.S., Mitchell, J.R., and Gillette, J.R. (1974) Life Sciences 14, 35-46.
6. Correia, M.A., and Mannering, G.J. (1973) Mol. Pharmacol. 9, 455-469.
7. Mannering, G.J., Kuwahara, S., and Omura, T. (1974) Biochem. Biophys. Res. Comm. 57, 476-481.
8. West, S.B., Levin, W., Ryan, D., Vore, M., and Lu, A.Y.H. (1974) Biochem. Biophys. Res. Comm. 58, 516-522.
9. Strittmatter, P., Rogers, M.J., and Spatz, L. (1972) J. Biol. Chem. 247, 7188-7194.
10. Enomoto, K., and Sato, R. (1973) Biochem. Biophys. Res. Comm. 51, 1-7.
11. Jansson, I., and Schenkman, J.B. (1973) Mol. Pharmacol. 9, 840-845.

12. Remmer, H., Greim, H., Schenkman, J.B., and Estabrook, R.W. (1967) Methods Enzymol. 10, 703-708.
13. Spatz, L., and Strittmatter, P. (1971) Proc. Nat. Acad. Sci. U.S.A. 68, 1042-1046.
14. Estabrook, R.W., Matsubara, T., Mason, I., Werringloer, J., and Baron, J. (1973) Drug Metabolism and Disposition 1, 98-110.
15. Nash, T. (1953) Biochem. J. 55, 416-421.
16. Gigon, P.L., Gram, T.E., and Gillette, J.R. (1969) Mol. Pharmacol. 5, 109-122.
17. Hrycay, E.G., and O'Brien, P.J. (1973) Arch. Biochem. Biophys. 157, 7-22.
18. Hrycay, E.G., and O'Brien, P.J. (1974) Arch. Biochem. Biophys. 160, 230-245.
19. Cohen, B.S., and Estabrook, R.W. (1971) Arch. Biochem. Biophys. 143, 46-53.
20. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
21. Hildebrandt, A., and Estabrook, R.W. (1971) Arch. Biochem. Biophys. 143, 66-79.
22. Correia, M.A., and Mannering, G.J. (1973) Mol. Pharmacol. 9, 470-485.
23. Sasame, H.S., Thorgeirsson, S.S., Menard, R.H., Hinson, J.A. Mitchell, J.R., and Gillette, J.R. (1974) Fed. Proc. Fed. Amer. Soc. Exp. Biol. 33, Abstract no. 1208.
24. Sasame, H.A., Mitchell, J.R., Thorgeirsson, S., and Gillette, J.R. (1973) Drug Metabolism and Disposition 1, 150-155.