THE ROLES OF CYTOCHROME b_5 IN A RECONSTITUTED N-DEMETHYLASE SYSTEM CONTAINING CYTOCHROME P-450

Yoshio Imai and Ryo Sato

Institute for Protein Research, Osaka University,
Suita, Osaka 565, Japan

Received January 24, 1977

SUMMARY: The possible role of cytochrome b_5 (cyt b_5) in NADPH-dependent microsomal N-demethylations has been studied in a system reconstituted from cytochrome P-450 (cyt P-450), NADPH-cytochrome c reductase, and sodium cholate. Although the amount of formaldehyde formed from benzphetamine or N,N-dimethylaniline in this system was considerably less than that of NADPH oxidized, this stoichiometry became close to 1:1 on addition of cyt b_5. When both cyt b_5 and NADH-cyt b_5 reductase were included in the system, the second of two electrons required for the overall activity could be supplied from NADH, though NADPH and NADPH-cytochrome c reductase were obligatorily required for the supply of the first electron to cyt P-450. It is suggested that, at least in the reconstituted system, cyt b_5 can play two roles, i.e. a) improvement of coupling of NADPH oxidation to demethylation, and b) supply of the second electron to cyt P-450.

Liver microsomes catalyze various drug oxidizing reactions including N-demethylations in the presence of NADPH and oxygen, and an electron transfer chain consisting of cytochrome P-450 (cyt P-450) and NADPH-cytochrome c reductase (fp_2) is responsible for these activities. In fact, a number of drug oxidizing activities can be reconstituted from purified cyt P-450, fp_2, and phosphatidylcholine or a suitable detergent (1,2). Although NADPH is the preferred electron donor, NADH can also support these reactions at slower rates in microsomes (3). Moreover, NADH exerts a synergistic effect on the NADPH-dependent drug oxidations by microsomes (4). Based on these and other findings, it has been proposed that cyt b_5 is involved in the NADH-supported microsomal oxidations (4) and that the second of two electrons required for the overall oxidation process is supplied to cyt P-450 via cyt b_5 even in the NADPH-dependent reactions (4). It has also been reported that cyt b_5 is required for maximal activity of chlorobenzene oxidation by a reconstituted system containing cyt P-450 and fp_2 (5). We report here that in a reconstituted N-demethylase system the coupling of NADPH oxidation to demethylation can be improved by the presence of cyt b_5 and in the presence of both

Abbreviations: cyt b_5, cytochrome b_5; cyt P-450, cytochrome P-450; fp_1, NADH-cytochrome b_5 reductase; fp_2, NADPH-cytochrome c reductase.

Copyright © 1977 by Academic Press, Inc.
All rights of reproduction in any form reserved.

ISSN 0006-291X

TABLE I. Effects of cyt b_5 and fp_1 on benzphetamine N-demethylation by the reconstituted system. The composition of the standard system is described in MATERIALS AND METHODS. When NADH (0.1 mM) was added, the NADPH concentration was reduced to 0.1 mM. The NADPH-generating system consisted of 0.1 mM $NADP^+$, 10 mM glucose-6-phosphate, and 1.5 unit/ml of glucose-6-phosphate dehydrogenase. The concentrations of cyt b_5 and fp_1 added were 0.1 μM and 0.7 unit/ml, respectively. Partially purified fp_2 was used in Expts. 1, 2 and 4, and highly purified fp_2 was used in Expt. 3 where sodium cholate was replaced by 50 μM dilauroyl phosphatidylcholine. The reaction was run at 25° for 10 min, and the spectrophotometrically detectable oxidation of NAD(P)H (ΔNAD(P)H), formation of formaldehyde (ΔHCHO), and oxygen consumption (ΔO_2) were determined.

Expt.	System	Electron donor	ΔNAD(P)H (nmole/min/nmole cyt P-450)	ΔHCHO	ΔO_2	ΔHCHO / ΔNAD(P)H
1	Standard	NADPH	36.9	22.1	29.7	0.60
	+ cyt b_5	NADPH	28.8	26.2	29.4	0.91
2	Standard	NADPH	51.6	28.6	-	0.55
	+ cyt b_5	NADPH	31.9	24.7	-	0.77
	+ cyt b_5 + fp_1	NADPH + NADH	41.0	34.1	-	0.83
3	Standard	NADPH	22.6	12.0	-	0.53
	+ cyt b_5	NADPH	20.0	17.5	-	0.88
	+ cyt b_5 + fp_1	NADPH + NADH	29.2	26.2	-	0.90
4	+ cyt b_5 + fp_1	NADPH-generating system	0	36.4	-	-
	+ cyt b_5 + fp_1	NADPH-generating system + NADH	23.5	49.4		2.10

cyt b_5 and NADH-cyt b_5 reductase (fp_1) the second of two electrons to be utilized for demethylation can be supplied from NADH.

MATERIALS AND METHODS Purified cyt P-450 (6) and partially purified fp_2 (7) were prepared from liver microsomes of phenobarbital-pretreated rabbits and rats, respectively, as described. The intact form of cyt b_5 (8) and fp_1 (9), both purified from rabbit liver microsomes, were kindly supplied by Dr. K. Mihara. The apo-protein of intact cyt b_5 was prepared as described by Strittmatter (10). Highly purified fp_2 (11) and the proteolytically solubilized form (hydrophilic fragment) of cyt b_5 (12), both purified also from rabbit liver microsomes, were generous gifts from Dr. H. Satake. 3-Acetylpyridine NADH and dilauroyl phosphatidylcholine were purchased from Pabst Laboratories and Sigma Chemical Co., respectively. Benzphetamine was kindly supplied by Dr. T. Kamataki. The standard reconstituted system for N-demethylation assay contained 0.1 μM cyt P-450, 0.1-0.2 unit/ml of fp_2, 0.04 % sodium cholate, 0.15 mM NADPH, 1 mM benzphetamine or 8 mM N,N-dimethylaniline, and 0.1 M potassium phosphate buffer, pH 7.25. The demethylase activity was assayed by determining the formation of formaldehyde by the method of Nash (13). Oxidation of NADPH and NADH was measured by following the absorbance decrease at 340 nm. Oxygen consumption was determined in a Beckman-Toshiba Oxygen Analyzer. Spectrophotometric measurements were carried out at room temperature in a Cary 14 spectrophotometer.

TABLE II. Effects of cyt $\underline{b}_5$ and its derivatives on NADPH-dependent N-demethylation of N,N-dimethylaniline. The conditions were the same as in Expt. 1 of Table I, except that dimethylaniline was used as substrate and the apo-protein of intact cyt $\underline{b}_5$ (apo-cyt $\underline{b}_5$) and the hydrophilic (protease-solubilized) fragment of cyt $\underline{b}_5$ (cyt $\underline{b}_5$ fragment) were also tested.

System		ΔNADPH (nmole/min/nmole cyt P-450)	ΔHCHO (nmole/min/nmole cyt P-450)	ΔHCHO / ΔNADPH
Standard		57.7	13.7	0.24
+ cyt $\underline{b}_5$	(0.11 μM)	51.3	43.2	0.84
+ apo-cyt $\underline{b}_5$	(0.12 μM)	61.1	17.0	0.28
+ cyt $\underline{b}_5$ fragment	(0.12 μM)	56.3	14.0	0.25

RESULTS AND DISCUSSION In the presence of benzphetamine the reconstituted system consisting of cyt P-450, fp_2, and cholate oxidized NADPH rapidly with concomitant oxygen uptake, but the amount of formaldehyde formed was appreciably less than those of NADPH oxidized and oxygen consumed (Table I). The resultant stoichiometry was roughly 0.6 mole of formaldehyde formed per 1 mole of NADPH oxidized and 0.8 mole of oxygen utilized, indicating that about 40 % of NADPH oxidation was not coupled to demethylation process. When the intact form of cyt $\underline{b}_5$ was added to the system, the benzphetamine-dependent NADPH oxidation was significantly inhibited, though the extent of inhibition varied depending on the ratio of cyt $\underline{b}_5$ to cyt P-450 and the fp_2 preparation used. The formaldehyde formation, on the other hand, was less extensively inhibited than NADPH oxidation or even slightly stimulated. Consequently, the ratio of NADPH oxidized to formaldehyde formed approached to 1:1 on addition of cyt $\underline{b}_5$ at an equimolar concentration to cyt P-450 (Table I). Similar results were obtained when dilauroyl phosphatidylcholine, instead of cholate, was used for the reconstitution. With N,N-dimethylaniline as substrate, only about 0.2 mole of formaldehyde was produced per mole of NADPH oxidized, but the stoichiometry again became close to 1:1 upon addition of cyt $\underline{b}_5$ (Table II). It is thus clear that in the reconstituted system cyt $\underline{b}_5$ somehow interacted with the catalytic machinery to improve the coupling of NADPH oxidation to the demethylation reaction. Since both the

TABLE III. Effect of cyt $\underline{b}_5$ on H_2O_2-dependent benzphetamine demethylation by cyt P-450. The control system contained 0.18 μM cyt P-450, 50 μM dilauroyl phosphatidylcholine, 50 mM H_2O_2, 1 mM benzphetamine, and 0.1 M potassium phosphate buffer, pH 7.25. The reaction was run at 25° for 5 min.

System	HCHO formed (nmole/min/nmole cyt P-450)
Control	22
+ cyt $\underline{b}_5$ (0.19 μM)	38
- cyt P-450 + cyt $\underline{b}_5$	0

apo form of intact cyt $\underline{b}_5$ and the hydrophilic, heme-containing fragment of cyt $\underline{b}_5$ were inactive (Table II), it appears that the whole molecule of the intact hemoprotein is required for the coupling effect.

In an attempt to locate the site of cyt $\underline{b}_5$ interaction, we examined the effect of the hemoprotein on the hydrogen peroxide-dependent demethylation of benzphetamine by cyt P-450 in the absence of fp_2 and NADPH (14). It was thus found that this reaction was stimulated about 2-fold by the addition of cyt $\underline{b}_5$ (Table III). Both apo-cyt $\underline{b}_5$ and the hydrophilic fragment were again ineffective (data not shown). Since it has been postulated that hydrogen peroxide reacts with the substrate complex of ferric cyt P-450 to form directly a highly reactive, oxygenated intermediate which is also produced in the NADPH- and oxygen-linked reaction (14), it is tempting to conclude that the observed effects of cyt $\underline{b}_5$ are due to its interaction with this intermediate. Guengerich et al. (15) have reported that cyt $\underline{b}_5$ acts as an effector on an oxygenated intermediate of the substrate-cyt P-450 complex (Complex II) and stimulates its decomposition to oxidized cyt P-450 and the products. However, the possibility cannot be excluded that the effect observed in the hydrogen peroxide-dependent reaction is unrelated to the coupling effect in the reconstituted system. Further studies are needed to clarify the mechanism of coupling effect of cyt $\underline{b}_5$. It is also to be clarified whether or not cyt $\underline{b}_5$ exerts the same coupling effect on drug oxidations in intact microsomes.

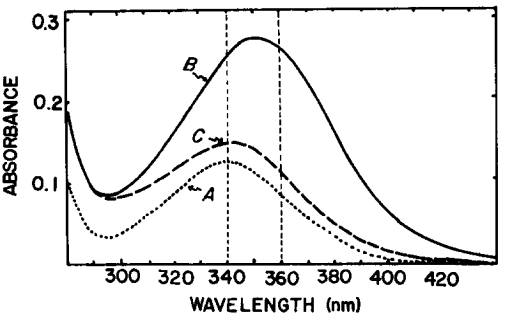

Fig. 1. Utilization of acetylpyridine NADH for benzphetamine N-demethylation. The reaction was run in a cuvette of 2-mm optical path. The reaction mixture was the same as in Expt. 4 of Table I, except that 0.1 mM acetylpyridine NADH was added instead of NADH. Curve A, in the absence of acetylpyridine NADH and benzphetamine. Curve B, just after the addition of benzphetamine. Curve C, 30 min after the addition of benzphetamine.

Lu et al. (16) have reported that cyt b_5 added to a reconstituted N-demethylase system inhibited the formaldehyde formation, but this inhibition was reversed completely by further addition of fp_1 and NADH. In the present study, the addition of fp_1, cyt b_5, and NADH to the standard reconstituted system catalyzing the NADPH-dependent benzphetamine demethylation caused a significant increase in NAD(P)H oxidation and the rate of formaldehyde formation was further enhanced to a level that was considerably higher than that observed in the standard system. As a result, an almost 1:1 stoichiometry for NAD(P)H to formaldehyde was obtained (Table I). To see if the NADH added was actually utilized as electron donor, the NADPH in the system was replaced by an NADPH-generating system to keep the level of NADPH constant. Under these conditions, the addition of benzphetamine decreased the absorbance at 340 nm in the presence of NADH but not in its absence (Table I, Expt.4). If either fp_1 or cyt b_5 was omitted from the system, no decrease in the 340 nm absorbance took place even in the presence of NADH. Similar results were obtained with N,N-dimethylaniline as substrate. That the NADH added was actually oxidized could be further confirmed by an experiment in which acetylpyridine NADH was used in place of NADH. As shown in Fig. 1, the absorption spectrum of the whole system just after the addition of benzphetamine exhibited a broad peak around 350 nm due to the presence of equimolar amounts of both NADPH

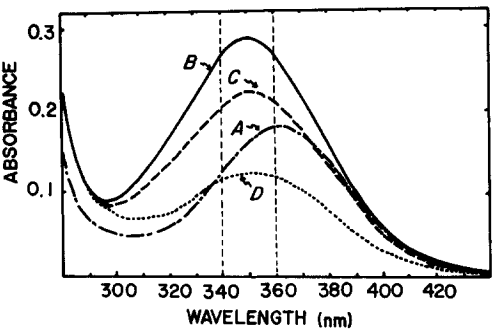

Fig. 2. Utilization of both NADPH and acetylpyridine NADH for benzphetamine N-demethylation. The conditions were the same as in Fig. 1, except that the NADPH-generating system was replaced by 0.1 mM NADPH. Curve A, in the absence of NADPH and benzphetamine. Curve B, in the absence of benzphetamine. Curve C, 10 min after the addition of benzphetamine. Curve D, 50 min after the addition of benzphetamine.

(absorbing at 340 nm) and acetylpyridine NADH (absorbing at 363 nm). As the demethylation reaction proceeded, however, the absorbance in the 350 nm region was decreased with a concomitant shift of the peak position to 340 nm, and finally the spectrum became almost identical with that of NADPH alone.

In the system containing both the NADPH-generating system and either NADH or acetylpyridine NADH, about 2 moles of formaldehyde were produced per mole of NADH or acetylpyridine NADH oxidized (see Table I, Expt. 4). This stoichiometry suggests that one of the two electrons required for the overall demethylase reaction was almost exclusively provided by NADH or acetylpyridine NADH via fp_1 and cyt $\underline{b}_5$ under these conditions.

When NADPH, instead of the NADPH-generating system, and acetylpyridine NADH were added to the reconstituted system supplemented with both fp_1 and cyt $\underline{b}_5$, an absorption spectrum having a peak around 350 nm was again observed. On addition of benzphetamine, a time-dependent decrease in the absorbance took place, but this time no shift of the peak was detectable (Fig. 2). This indicates that both NADPH and acetylpyridine NADH were utilized at an equimolar ratio to drive the demethylation reaction under the conditions employed. When NADPH or fp_2 was omitted from the system, however, neither NADH (or acetylpyridine NADH) oxidation

nor formaldehyde formation occurred on addition of benzphetamine even though the system contained fp_1 and cyt b_5. Moreover, cyt P-450 remained oxidized in a system containing NADH, fp_1 and cyt b_5 in CO, whereas immediate reduction of cyt P-450 was observed on addition of NADPH if fp_2 was present in the system.

These results indicate clearly that the supply of at least one electron to cyt P-450 from NADPH via fp_2 is obligatorily required for the demethylase activity of the reconstituted system and that this electron is used to reduce the substrate complex of ferric cyt P-450. It can also be concluded that the second electron is introduced almost exclusively from NADH (or acetylpyridine NADH) via fp_1 and cyt b_5 under the conditions employed. However, since NADPH-dependent demethylation can take place in the system in which no electron flow from NADH exists, it is certain that the second electron can also be supplied from NADPH via fp_2. The almost exclusive supply of the second electron via cyt b_5 demonstrated above is probably due to the fact cyt b_5 is reducible by NADH and fp_1 much faster than NADPH and fp_2.

REFERENCES

1. Lu, A.Y.H., Strobel, H.W., & Coon, M.J. (1970) Mol. Pharmacol. 6, 213-220.
2. Lu, A.Y.H., Levine, W., & Kuntzman, R. (1974) Biochem. Biophys. Res. Commun. 60, 266-276.
3. Gillette, J.R., Davis, D.C., & Sasame, H.A. (1972) Ann. Rev. Pharmacol. 12, 57-84.
4. Hildebrandt, A., & Estabrook, R.W. (1971) Arch. Biochem. Biophys. 143, 66-79.
5. Lu, A.Y.H., Levin., Selander, H., & Jerina, D.M. (1974) Biochem. Biophys. Res. Commun. 61, 1348-1355.
6. Imai, Y., & Sato, R. (1974) Biochem. Biophys. Res. Commun. 60, 8-14.
7. Imai, Y. (1976) J. Biochem. 80, 267-276.
8. Spatz, L., & Strittmatter, P. (1971) Proc. Nat. Acad. Sci. USA 68, 1042-1046.
9. Mihara, K., & Sato, R. (1975) J. Biochem. 78, 1057-1073.
10. Strittmatter, P. (1960) J. Biol. Chem. 235, 2492-2497.
11. Satake, H., Imai, Y., & Sato, R. (1972) Seikagaku 44, 765.
12. Omura, T., & Takesue, S. (1970) J. Biochem. 67, 249-257.
13. Nash, T. (1953) Biochem. J. 55, 416-421.
14. Nordblom, G.D., White, R.E., & Coon, M.J. (1976) Arch. Biochem. Biophys. 175, 524-533.
15. Guengerich, F.P., Ballou, D.P., & Coon, M.J. (1976) Biochem. Biophys. Res. Commun. 70, 951-956.
16. Lu, A.Y.H., West, S.B., Vore, M., Ryan, D., & Levin, W. (1974) J. Biol. Chem. 249, 6701-6709.