Acknowledgements—This work was supported by grants from the Australian Research Grants Scheme, the National Health and Medical Research Council and the Anti-Cancer Foundation of the Universities of South Australia.

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Biochemical Pharmacology, Vol. 37, No. 2, pp. 368–370, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Enzymic reduction of *N*-hydroxyamphetamine: the role of electron transfer system containing cytochrome b_5

(*Received* 17 *March* 1987; *accepted* 23 *July* 1987)

Methamphetamine (MP) has been known to be transformed to amphetamine (AP) by *N*-demethylation in mammals [1–4], which proceeds through either *N*-hydroxylation or *C*-hydroxylation pathways. We previously demonstrated that MP was mainly *N*-demethylated by the former pathway in guinea-pigs [4], and suggested further that *N*-hydroxy-MP formed was dehydrogenated to *N*-[(1-methyl-2-phenyl)ethyl]methanimine *N*-oxide (nitrone) which immediately decomposed to formaldehyde and *N*-hydroxy-AP [5]. Furthermore resulting *N*-hydroxy-AP was assumed to be reduced to AP. Kadlubar *et al.* found hydroxylamine reductase system in hog liver microsomes [6] and showed by monitoring the reduction of *N*-methyl-*N*-benzyl-hydroxylamine that the purified system consisted of cytochrome (cyt.) b_5, NADH-cyt. b_5 reductase and unknown SH-protein [7]. Although they have demonstrated also the participation of this hydroxylamine reductase in the reduction of *N*-hydroxy-AP in hog liver microsomes [6], the extent of contribution of this system to the reaction in microsomes has not been evaluated. In the present study, a key component of this system, cyt. b_5 was purified from guinea-pig liver microsomes, and its participation in the reduction of *N*-hydroxy-AP was evaluated by use of anti-cyt. b_5 serum from rabbits.

Materials and methods

Chemicals. The neutral oxalate of *N*-hydroxy-AP was synthesized by the method of Coutts *et al.* [8]. NADH and Freund's complete adjuvant were purchased from Kyowa Hakko Industries, (Tokyo) and Difco Lab. (Detroit) respectively. All other reagents used were from the sources described earlier [4, 5] or of the highest quality commercially available.

Purification of guinea-pig liver cyt. b_5. Cyt. b_5 was purified from the liver microsomes from Hartley guinea pigs according to the method of Spatts and Strittmatter [9]. Purity and molecular weight of this enzyme were determined by use of SDS-polyacryamide gel electrophoresis on 15% acrylamide gels in the presence of 0.1% SDS by the method of Laemmli [10].

Determination of N-hydroxy-AP reductase. Incubation mixture consisted of 5.0 μmol of *N*-hydroxy-AP, 10 μmol of NADH and NADPH, 2–3 mg of liver microsomes and 0.1 M phosphate buffer (pH 6.3) to make a final volume of 6.0 ml. After the incubation for 30 min at 37°, AP formed was determined as a trifluoroacetyl derivative by GLC [3].

Preparation of anti-cyt. b_5 serum. Immunization of the rabbit with purified cyt. b_5, and preparation of anti-serum and nonimmune serum were performed similarly as described elsewhere [11] with some modifications. About 1 mg of cyt. b_5 suspended in Freund's complete adjuvant (1.0 ml) was injected into the foot pads of a rabbit. The rabbit was boosted twice three and four weeks later by s.c. injections of the same amount of the antigen suspension at the back. The anti-serum (about 20 ml) was obtained after a week of the last booster injection. Nonimmune serum was obtained from a nonimmune rabbit. The purified cyt. b_5 formed precipitates only with the serum from the immunized rabbit by Ouchterlony double diffusion method [12].

Results and discussion

N-Hydroxy-AP was effectively reduced to AP with guinea-pig liver microsomes, requiring preferably NADH as a cofactor (Table 1), at optimum pH of 5.0–6.3. These characteristics closely resemble those of hog liver hydroxylamine reductase system containing cyt. b_5 [6]. We therefore attempted to isolate cytochrome b_5 from guinea-pig liver microsomes for preparing anti-cyt. b_5 serum. The purification steps for cyt. b_5 are shown in Table 2. The prep-

Table 1. Cofactor requirement for reduction of *N*-hydroxyamphetamine with guinea-pig liver microsomes

Conditions	Amphetamine formation* (nmol formed/min/mg protein)	% of Control
Microsomes–NADH	5.90 ± 0.39	100
Microsomes–NADPH	1.42 ± 0.18	24
Microsomes only	0.73 ± 0.08	12
Boiled microsomes–NADH	1.29 ± 0.19	22

* Each value represents the mean ± SE of four animals.

Table 2. Purification steps of cytochrome b_5 from guinea-pig liver microsomes

Purification steps	Protein (mg)	Cytochrome b_5 (nmol)	Cytochrome b_5 (nmol/mg protein)	Recovery (%)
Washed microsomes	3462	1430	0.41	100
Solubilized sup.	695	1164	1.67	81.4
DEAE-Cellulose	193	911	4.72	63.7
Sephadex G-100	32.7	626	19.1	43.8
Sephadex G-100	11.3	455	40.3	31.8

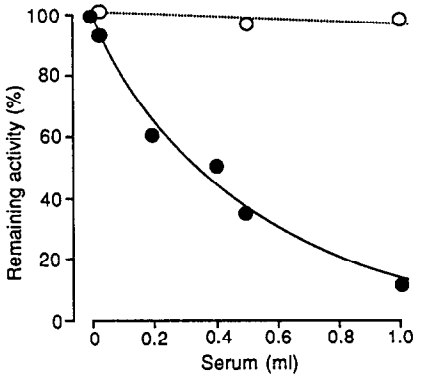

Fig. 1. Effect of anti-cytochrome b_5 (—●—) and non-immune (---○---) serum on *N*-hydroxyamphetamine reductase activity in guinea-pig liver microsomes. The serum contained about 80 mg proteins/ml. The value represents the mean of 2 to 6 determinations.

aration exhibited a single band on SDS-polyacrylamide gel electrophoresis at a molecular weight (M_r) of about 16,500 and absorption maxima of absolute spectrum of the oxidized form at 280 and 413 nm. These properties are very similar to those from rats [13] and rabbits [9]. As shown in Fig. 1, addition of the anti-cyt. b_5 serum strongly inhibited *N*-hydroxy-AP reductase activity in guinea-pig liver microsomes. These results mean that hydroxylamine reductase system containing cyt. b_5 in guinea-pig liver microsomes plays a very important role in the reduction of *N*-hydroxy-AP. We previously observed high activity of *N*-hydroxy-MP formation from MP in purified flavin-containing monooxygenase from guinea-pig liver microsomes [5]. Further, the same enzyme efficiently catalyzed transformation of *N*-hydroxy-MP to *N*-hydroxy-AP [5]. And, in the present study, *N*-hydroxy-AP was transformed to AP by the reductase system. Thus, these steps form another pathway of *N*-demethylation of MP besides C-hydroxylation pathway containing cytochrome P-450.

In summary, the participation of a hydroxylamine reductase system containing cyt. b_5 in the reduction of *N*-hydroxy-AP was proved in guinea pig microsomes by use of anti-cyt. b_5 serum of rabbit. For preparing this antiserum, cyt. b_5 (Mw. 16,500) was purified from guinea-pig liver according to the method for rabbits.

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