Effects of Cytochrome b5 on Aniline Hydroxylation Catalyzed by a Reconstituted System Containing Acetone or 2,2'-Bipyridine

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Accepted November 22, 1985

Abstract—Cytochrome b5 did not exert any effect on NADPH-dependent aniline hydroxylation in the absence of acetone or 2,2'-bipyridine, whereas cytochrome b5 exhibited a stimulatory effect on the reaction in the presence of acetone or 2,2'-bipyridine. In addition, cytochrome b5 did not have any significant effect on the cumene hydroperoxide-dependent reaction in the presence of acetone or 2,2'-bipyridine.

It has been reported that cytochrome b5 is an obligatory component in p-nitroanisole O-demethylation (1). Most drug oxidations, however, have been shown to be catalyzed by the reconstituted system containing NADPH-cytochrome P-450 reductase, cytochrome P-450 and phospholipids (2-6).

On the other hand, based on the synergistic effect of NADH on NADPH-dependent reactions, it has been proposed that cytochrome b5 is also involved in the NADPH-dependent oxidations (7). Imai et al. (8, 9) have shown that the second electron is supplied preferentially via cytochrome b5 in the reconstituted system even when only NADPH is used as the electron donor. We have previously reported (10) that the synergistic effect of NADH on microsomal aniline hydroxylation was observed only when acetone or 2,2'-bipyridine was added to the reaction mixture. However, from the results that NADPH-dependent microsomal aniline hydroxylation is not affected by antibody against cytochrome b5, Noshiro et al. (11) have concluded that cytochrome b5 does not participate in aniline hydroxylation.

We report herein that cytochrome b5 stimulates aniline hydroxylation catalyzed by a reconstituted system containing acetone or 2,2'-bipyridine.

Cytochromes P-450 and b5 and NADPH-cytochrome P-450 reductase were purified from phenobarbital-treated rat liver microsomes (0.1% sodium phenobarbital in drinking water for 3 days) by the method described by Imai et al. (2-4), Imai (12), and Yasukochi and Masters (13), respectively. The formation of p-aminophenol from aniline and protein were determined according to the methods of Imai et al. (14) and Lowry et al. (15), respectively. The redox behavior of cytochrome b5 was followed as reported by the method of Oshino and Sato (16).

As can be seen in Table 1, about 60% enhancement of NADPH-dependent aniline hydroxylation due to acetone was observed in the absence of cytochrome b5. The addition of cytochrome b5 to a reconstituted system containing acetone resulted in a further increase in the activity. On the other hand, heat treated-cytochrome b5 did not affect the activity of aniline hydroxylation in the presence or absence of acetone (none, 0.42 nmole/nmole P-450/min; heat treated-cytochrome b5 alone, 0.40 nmole/nmole P-450/min; acetone alone, 0.64 nmole/nmole P-450/min; acetone and heat treated-cytochrome b5, 0.61 nmole/nmole P-450/min; acetone and untreated-cytochrome b5, 1.07 nmole/nmole P-450/min). The effect of cytochrome b5 on aniline hydroxylation was also observed when 2,2'-bipyridine was added to the system in place of acetone.
The decrease in the activity observed by the addition of higher concentration of cytochrome \( b_5 \) in the presence of 2,2'-bipyridine might be due to the competition between cytochromes P-450 and \( b_5 \) for the reductase, although the exact mechanism is not known. On the other hand, acetone did not stimulate the cumene hydroperoxide (CHP)-dependent reaction in the absence of cytochrome \( b_5 \) (1.93 versus 1.93 nmole/nmole P-450/min) as reported elsewhere (17). 2,2'-Bipyridine was also found to be slightly inhibitory, rather than stimulatory under the same condition (1.50 versus 1.93 nmole/nmole P-450/min). In addition, there was a slight increase in the CHP-dependent reaction due to cytochrome \( b_5 \) (2.26 versus 1.93 nmole/nmole P-450/min), but the extent of the increase was essentially unchanged by the addition of acetone (2.27 versus 2.26 nmole/nmole P-450/min).

As expected from the results reported by Noshiro et al. (11), the NADPH-dependent reaction in the absence of acetone was unaffected by cytochrome \( b_5 \). It has been, however, demonstrated (10, 18) that the synergistic effect of NADH on NADPH-dependent microsomal aniline hydroxylation was observed in the presence of acetone or 2,2'-bipyridine which is known to be enhancing agents of aniline hydroxylation (19). The present study shows that cytochrome \( b_5 \) stimulates NADPH-dependent aniline hydroxylation in the presence of acetone or 2,2'-bipyridine. Imai and Sato (8) have demonstrated that cytochrome \( b_5 \) plays two roles in the reconstituted system: 1) improvement of coupling of NADPH oxidation and 2) it supplies the second electron to cytochrome P-450. Furthermore, the contribution of cytochrome \( b_5 \) to cytochrome P-450 catalyzing oxidations has been shown to be dependent on the relative rates of the two electron flows (9). It was, however, impossible to estimate not only the relative rates of the two electron flows but the extent of coupling of NADPH-oxidation during aniline hydroxylation since aniline inhibits the reduction rate to cytochrome P-450 by NADPH and the NADPH-oxidation.

No effect of cytochrome \( b_5 \) on CHP-dependent aniline hydroxylation was observed in the presence of acetone, suggesting that cytochrome \( b_5 \) may affect NADPH-dependent aniline hydroxylation at the step before the introduction of the second electron to the oxyferrous cytochrome P-450-aniline complex. Furthermore, preliminary experiments have shown that the reduced level of cytochrome \( b_5 \) by NADPH in the presence or absence of acetone or 2,2'-bipyridine was 75%, 76% or 74% of the dithionite-reduced level, and the rate of reoxidation of cytochrome \( b_5 \) reduced by NADPH was virtually unchanged by acetone or 2,2'-bipyridine (aniline alone, 0.132 nmole/min; aniline and acetone, 0.14 nmole/min).

Table 1. Effect of cytochrome \( b_5 \) on aniline hydroxylation catalyzed by a reconstituted system in the presence or absence of acetone or 2,2'-bipyridine

<table>
<thead>
<tr>
<th>Cytochrome ( b_5 ) added (nmole)</th>
<th>Aniline hydroxylation</th>
<th>Aniline hydroxylation</th>
<th>2,2'-Bipyridine (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>Acetone (0.8 M)</td>
<td>2,2'-Bipyridine (1 mM)</td>
</tr>
<tr>
<td>(nmole ( p )-aminophenol/nmole cytochrome P-450/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.41±0.03</td>
<td>0.66±0.04</td>
<td>0.71±0.02</td>
</tr>
<tr>
<td>0.05</td>
<td>0.41±0.02</td>
<td>1.21±0.09 (+83)</td>
<td>1.10±0.02 (+55)</td>
</tr>
<tr>
<td>0.1</td>
<td>0.38±0.04</td>
<td>1.09±0.21 (+65)</td>
<td>0.74±0.02 (+4)</td>
</tr>
</tbody>
</table>

The reconstituted system consisted of 0.1 nmole of cytochrome P-450 (12.1 nmole/mg), 0.2 unit of reductase (53.3 units/mg), 15 µg of dilauroyl L-3-phosphatidylcholine, aniline (2 mM), the NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 0.1 unit of glucose 6-phosphate dehydrogenase and 6 mM MgCl\(_2\)) and 50 mM Tris-HCl (pH 7.4), in a final volume of 0.5 ml. Each value represents the mean±S.D. of duplicate determinations. Numbers in parentheses represent percent of change by cytochrome \( b_5 \).
min; aniline and 2,2'-bipyridine, 0.146 nmole/min). Therefore, it is unlikely that acetone facilitates the transfer of a second electron to cytochrome P-450. Further studies will be required to establish the mechanism(s) of the interaction of cytochrome b₅ with cytochrome P-450 in the presence of acetone or 2,2'-bipyridine.

References

12 Imai, Y.: The use of 8-aminooctyl Sepharose for the separation of some components of the hepatic microsomal electron transfer system. J. Biochem. 80, 267–276 (1976)