Electron Flow and Complex Formation During Cytochrome P-450-Catalyzed Hydroxylation Reactions in Reconstituted Membrane Vesicles

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Purified preparations of rabbit liver microsomal NADPH-cytochrome P-450 reductase, NADH-cytochrome b₅ reductase, and the cytochromes b₅, P-450 LM₃, P-450 LM₄ and P-450 LM₅, were incorporated in various combinations by the cholate gel filtration technique into membrane vesicles. Cytochrome P-450-catalyzed hydroxylation reactions were detected after addition of NADH and/or NADPH to the vesicles. It was found that the substrate and the type of cytochrome P-450 used, to a great extent determined the route of electron transport to cytochrome P-450. Kinetic evidence is given for the formation of a functionally active ternary 1:1:1 complex in the membrane between NADPH-cytochrome P-450 reductase, cytochrome b₅ and cytochrome P-450 during catalysis.

During the last decade, evidence has accumulated in many laboratories indicating that cytochrome b₅ participates in the liver microsomal hydroxylase system (cf. Refs. 1–11). Recently, the most unequivocal evidence has come from the fact that cytochrome b₅ is necessary for reconstitution of p-nitroanisole demethylation catalyzed by a specific form of cytochrome P-450.¹⁰ Other contributing facts are that the two hemoproteins may exchange electrons in microsomal⁷,¹² as well as in reconstituted systems¹³ and that cytochrome b₅, present in a NADPH-supported reconstituted system, enhances product formation, and in a concomitant manner decreases the amount of superoxide anions formed and thus the hydrogen peroxide production.⁹ In addition to other reports,⁷,¹⁴ the latter studies are consistent with the fact that one role for cytochrome b₅ in the liver microsomal hydroxylase system is to donate the second electron to cytochrome P-450. This electron transfer may occur via NADPH-cytochrome P-450 reductase, but at a slower rate.

In the present paper we present evidence that, in reconstituted membrane vesicles using an appropriate substrate, cytochrome b₅ may efficiently donate both electrons to cytochrome P-450. The route of the electron flow to cytochrome P-450 is apparently determined by the type of substrate undergoing hydroxylation, but also by the form of cytochrome P-450 participating in the reaction. Kinetic evidence is presented, indicating that during NADPH-supported reactions, NADPH-cytochrome P-450 reductase, cytochrome b₅ and cytochrome P-450 form a functional 1:1:1 stoichiometric complex in the membrane. Preliminary accounts of some of these results have been given.¹⁵,¹⁶

EXPERIMENTAL

Abbreviations. P-450 LM₂, P-450 LM₃ and P-450 LM₅ are forms of rabbit liver microsomal cytochrome P-450, designated according to their electrophoretic properties. The form P-450 LM₃ is essentially identical to cytochrome P-450 LM₃₈ isolated in Prof. M. J. Coons laboratory (cf. Ref. 32).

Lipids. Microsomal phospholipids were extracted from liver microsomes isolated from phenobarbital-treated rabbits according to Bligh and Dyer.¹⁷ Dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine were pur-

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chased from Serday Research Laboratories (Ontario, Canada). All phospholipids were stored in sealed tubes under nitrogen at −20 °C.

\textbf{Enzymes.} Cytochromes P-450 LM2, P-450 LM3, P-450 LM4, NADPH-cytochrome P-450 reductase and cytochrome b5 were purified from liver microsomes of phenobarbital-treated rabbits as previously described.9,16,19 Rabbit liver NADH-cytochrome b5 reductase was purified essentially as described by Schafer and Hultqvist.20 The specific contents were: Cytochrome P-450 LM2, 12.5–16 nmol/mg; cytochrome P-450 LM3, 13 nmol/mg; cytochrome P-450 LM4, 15.2 nmol/mg; NADPH-cytochrome P-450 reductase, 22 nmol of flavin per mg; cytochrome b5, 32 nmol per mg; NADH-cytochrome b5 reductase, 8 nmol of flavin per mg.

\textbf{Preparation of vesicles.} Unilamellar phospholipid vesicles containing the various protein components, were prepared by the cholate gel filtration technique as previously described.19,21 In certain experiments vesicles were prepared devoid of cytochrome b5. The subsequent addition of this protein to the vesicles resulted in a rapid and complete incorporation of cytochrome b5, as judged from the results obtained by Sepharose 4 B chromatography of the vesicles (cf. Ref. 19). Usually, the molar ratio of cytochrome P-450 LM2 to phospholipid was kept at 1:1300 and the amount of the other protein components varied in relation to cytochrome P-450, keeping the number of phospholipid molecules constant.

\textbf{Assay methods.} Cytochrome P-450 was quantitatively determined from the absorbance at 450 nm of the CO-reduced protein complex, using 91 mM$^{-1}$ cm$^{-1}$ as coefficient for the absorption difference between 450 and 490 nm. Cytochrome b5 was measured by the absorption difference between the reduced and the oxidized form of the protein at 424 nm, using 100 mM$^{-1}$ cm$^{-1}$ as absorption coefficient. Flavin was quantified at 456 nm according to Iyanagi and Mason.22 O-demethylation of p-nitroanisole was determined spectrophotometrically at 417 nm23 and O-dealkylation of 7-ethoxycoumarin or 7-ethoxyresorufrin spectrofluorimetrically, as described by Prough et al.24 Androstenedione metabolism was analyzed according to methods described by Hryncar et al.25, whereas aminopyrine or ethylmorphine N-demethylation was determined by monitoring formaldehyde production.26 NADPH- and NADH-oxidation was determined by following the decrease of absorption at 340 or 360 nm, using absorption coefficients of 6.2 or 4.7 mM$^{-1}$ cm$^{-1}$, respectively, and corrected for contribution to the absorption at the wavelengths by product or substrate. Benz[a]pyrene metabolism was determined by HPLC as previously described27 and protein was quantified according to Lowry et al.28

\section*{RESULTS}

\textbf{Effect of cytochrome b5 on the rates of NADPH-supported hydroxylations reactions.}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Substrate & Catalytic activity, nmol formed per nmol P-450 and min \\ & LM2 & & & & & \\
& b5 & +b5 & b3 & +b5 & b4 & +b5 \\
\hline
Androstenedione, 350 & 0.05 & 0.17 & 1.6 & 8.7 & 0.001 & 0.001 \\
\mu M\textsuperscript{b} & & & & & & \\
Benzo[a]pyrene, 25 & 0.072 & 0.59 & <0.001 & 0.001 & 0.017 & 0.057 \\
\mu M\textsuperscript{c} & & & & & & \\
Ethoxycomarain, 50 & 0.59 & 3.50 & 0.33 & 0.24 & 0.24 & 0.37 \\
\mu M & & & & & & \\
p-Nitroanisole, 1 & 0.9 & 5.1 & <0.1 & <0.1 & <0.1 & <0.1 \\
mM & & & & & & \\
Ethylmorphine, 1.3 & 8.5 & 5.3 & nd & nd & nd & nd \\
mM & & & & & & \\
Ethoxyresorufrin, 5 & 0.035 & 0.05 & nd & nd & 0.094 & 0.094 \\
\mu M & & & & & & \\
Aminopyrine, 2.3 & 0.95 & 1.9 & nd & nd & 0.99 & 0.62 \\
mM & & & & & & \\
\hline
\end{tabular}
\caption{Effect of Cytochrome b5 on the Rate of NADPH-supported Cytochrome P-450-catalyzed Hydroxylation Reactions in Reconstituted Membrane Vesicles.\textsuperscript{a}}
\end{table}

\textsuperscript{a} Incubations were carried out with vesicles prepared by the cholate gel filtration technique corresponding to 0.1–0.3 nmol of cytochrome P-450. The vesicles contained about equimolar amounts of the protein components and a 400 molar excess of microsomal phospholipids. The vesicles, as isolated from the Sephadex G-50 column, were diluted with 50 mM potassium phosphate buffer, pH 7.4, to 1 ml. Substrate was added during a preincubation period of 3 min at 37 °C and the incubations were started by adding NADPH to 0.3 mM. Product formation was monitored as described under "Experimental Procedures". 9 The products detected were 16-hydroxyandrostenedione (LM2) and 6β-hydroxyandrostenedione (LM3). 9 Represents the sum of all benzo[a]pyrene metabolites as isolated by HPLC;\textsuperscript{27} nd, not determined.

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Cytochrome \( b_5 \) was incorporated into vesicles prepared from microsomal phospholipids, NADPH-cytochrome P-450 reductase and cytochrome P-450 LM2, P-450 LM3 or P-450 LM4. The molar ratio phospholipid–P-450 reductase–\( b_5 \)–P-450 was kept at approximately 1200:1:1:1 and various cytochrome P-450-catalyzed hydroxylation reactions were reconstituted, using NADPH as electron donor. As is evident from Table 1. The incorporation of cytochrome \( b_5 \) in most cases resulted in enhanced hydroxylase activities. However, the extent of stimulation was dependent upon (i) the substrate used and (ii) the type of cytochrome P-450 used.

**Complex formation during cytochrome P-450-catalyzed, NADPH-supported hydroxylation reactions.** Vesicles were prepared by the cholate gel filtration technique from microsomal phospholipids, NADPH-cytochrome P-450 reductase, cytochrome \( b_5 \) and cytochrome P-450 LM2, at a molar ratio of protein to lipid of about 1:1300. The relation between the different protein components was altered in various vesicle preparations and the rate of vesicle-catalyzed \( O \)-demethylation of \( p \)-nitroanisole was examined. The results obtained are shown in Fig. 1, when the amount of NADPH-cytochrome P-450 reductase was kept constant and the relation between the two hemoproteins varied. In vesicles having small amounts of cytochrome \( b_5 \), the \( O \)-demethylation activity reached a maximum at low levels of cytochrome P-450 LM2. When more cytochrome \( b_5 \) was introduced into the vesicles, the saturation of the oxygenase activity required more and more P-450 present in the membrane. Maximal activity always was obtained at a 1:1 molar ratio between \( b_5 \) and P-450, despite the presence of excess NADPH-cytochrome P-450 reductase in the vesicles.

Similar results were obtained when vesicles, prepared from microsomal phospholipids, fixed amounts of cytochrome P-450 but variable amounts of NADPH-cytochrome P-450 reductase, were titrated with cytochrome \( b_5 \) (Fig. 2). More cytochrome \( b_5 \) had to be added to vesicles containing higher levels of NADPH-cytochrome P-450 reductase before a constant rate of the \( O \)-dealkylation reaction was reached. Saturation was obtained at a 1:1 molar ratio between NADPH-cytochrome P-450 reductase and cytochrome \( b_5 \), despite the presence of excess cytochrome P-450 LM2 in the vesicles. The results thus indicate that during NADPH-supported \( O \)-demethylation of \( p \)-nitroanisole, the participating proteins, i.e. NADPH-cytochrome P-450 reductase, cytochrome \( b_5 \) and cytochrome P-450 LM2, form a functional 1:1:1 complex in the membrane vesicles. Excess amounts of the third component in the membrane will not affect the rate of the hydroxylase activity.

**NADH-supported hydroxylation reactions in phospholipid vesicles containing NADH-cytochrome \( b_5 \) reductase, cytochrome \( b_5 \) and cytochrome P-450 LM2.** Vesicles were prepared

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*Fig. 1.* The rate of cytochrome P-450 LM2-catalyzed \( O \)-demethylation of para-nitroanisole as a function of the amount of cytochromes P-450 and \( b_5 \) in vesicles having a constant amount of NADPH-cytochrome P-450 reductase. Phospholipid vesicles were prepared by the cholate gel filtration technique from microsomal phospholipids, NADPH-cytochrome P-450 reductase and cytochrome \( b_5 \) in a mol ratios from 2000:1:0 to 2000:1:1. To aliquots of the vesicular preparation corresponding to 0.0226 nmol of NADPH-cytochrome P-450 reductase in a volume of 211 μl of 10 mM Tris-HCl, pH 7.4, containing 50 mM NaCl and 0.2 mM EDTA, various amounts of a 5 μM solution of cytochrome P-450 LM2 was added. The vesicles were subsequently incubated at 37 °C for 3 min and the volume adjusted with 50 mM potassium phosphate buffer, pH 7.4, to 1.95 ml. 20 μl of a 50 mM solution of \( p \)-nitroanisole was added, the sample distributed into two 1 ml cuvettes and the reaction was initiated after 3 min preincubation at 37 °C by the addition of 10 μl 10 mM NADPH to the sample cuvette. The rate of product formation was monitored at 417 nm.

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from microsomal phospholipids, various amounts of NADH-cytochrome \( b_5 \) reductase and equimolar amounts of cytochrome \( b_5 \) and cytochrome P-450 LM2. Increasing levels of \( b_5 \) reductase in the vesicles, resulted in enhanced rate of vesicle-catalyzed \( O \)-demethylation of \( p \)-nitroanisole from 0 to 3.5 nmol of product formed per nmol P-450 and min. Maximal activity was reached at a 1:1 molar ratio between the flavoprotein and the hemoproteins (Fig. 3). NADH-supported hydroxylations were also accomplished using other substrates (Table 2). The efficiency of electron utilization was determined and compared with vesicular systems composed of NADPH, NADPH-cytochrome P-450 reductase and cytochrome P-450 LM2. When both electrons were donated to cytochrome P-450 LM2 from NADH, via \( b_5 \) reductase and cytochrome \( b_5 \), and \( p \)-nitroanisole was used as substrate, the relative efficiency of \( O \)-demethylation of \( p \)-nitroanisole was 3.8-fold higher than when NADPH-cytochrome P-450 reductase served as electron donor. When other substrates were used, rather similar ratios between product formation and cofactor utilization were obtained (Table 2), but the absolute number of electrons transported was about two-fold higher in the NADPH-driven system.

**Reconstitution of \( p \)-Nitroanisole \( O \)-demethylation Using Various Electron Transport Systems.** All appropriate combinations of the microsomal electron transfer components were introduced.

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**Fig. 2.** The rate of cytochrome P-450 LM2-catalyzed \( O \)-demethylation of \( p \)-nitroanisole as a function of the amount of NADPH-cytochrome P-450 reductase and cytochrome \( b_5 \) in vesicles having a constant amount of cytochrome P-450. Vesicles were prepared from microsomal phospholipids, cytochrome P-450 LM2 and NADPH-cytochrome P-450 reductase in molar ratios from 2000:1:0 to 2000:1:1. Vesicles corresponding to 0.2 nmol of cytochrome P-450 LM2 in 205 \( \mu \)l of 10 mM Tris-HCl, pH 7.4, containing 50 mM NaCl and 0.2 mM EDTA, were incubated with indicated amounts of cytochrome \( b_5 \) at 37 °C for 3 min. Subsequent dilution and incubations were performed as described in legend to Fig. 1.

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**Fig. 3.** The rate of cytochrome P-450 LM2-catalyzed \( O \)-demethylation of \( p \)-nitroanisole as a function of the amount of NADH-cytochrome \( b_5 \) reductase in the vesicles. Vesicles were prepared from microsomal phospholipids, cytochrome \( b_5 \), cytochrome P-450 LM2 and NADPH-cytochrome \( b_5 \) reductase in molar ratios from 2000:1:1:0 to 2000:1:1:2 by the gel filtration technique. Incubations were performed with vesicles corresponding to 0.25 nmol of cytochrome P-450 LM2 as outlined in legend to Fig. 1 using NADH instead of NADPH.
Table 2. Relation between the Rate of Cytochrome P-450 LM₂-catalyzed Hydroxylation Reactions and NADH/NADPH Utilization in Reconstituted Membrane Vesicles.*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>NADH→b₅-reductase→b₅→LM₂→NAD⁺ prod/NAD⁺ nmol formed per nmol P-450 and min</th>
<th>Electron transport system used</th>
<th>NADPH→P-450 reductase→LM₂→NAD⁺ prod/NAD⁺ nmol formed per nmol P-450 and min</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Nitroanisole, 1 mM</td>
<td>3.2</td>
<td>0.9</td>
<td>0.53</td>
</tr>
<tr>
<td>Aminopyrine, 2.3 mM</td>
<td>1.5</td>
<td>3.6</td>
<td>0.43</td>
</tr>
<tr>
<td>Androstenedione, 350 μM</td>
<td>0.2</td>
<td>0.6</td>
<td>0.05</td>
</tr>
<tr>
<td>Ethoxycoumarin, 50 μM</td>
<td>0.23</td>
<td>0.45</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* Vesicles were prepared by the cholate gel filtration technique from the protein components in equimolar amounts with about a 400-fold molar excess of microsomal phospholipids. The incubations were performed as outlined in Table 1.

Table 3. Reconstitution of cytochrome P-450 LM₂-catalyzed O-demethylation of paranitroanisole in phospholipid vesicles using various electron transport systems.*

<table>
<thead>
<tr>
<th>Electron transport system</th>
<th>p-Nitrophenol nmol formed per nmol P-450 and min</th>
<th>NAD⁺/NAD⁺ Ratio to product</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH→b₅ reductase→b₅→P-450</td>
<td>3.2</td>
<td>6.0</td>
</tr>
<tr>
<td>NADH→b₅ reductase→b₅→P-450</td>
<td>2.9</td>
<td>5.8</td>
</tr>
<tr>
<td>[P-450 reductase→b₅→P-450] P-450 reductase→</td>
<td>6.5</td>
<td>9.8</td>
</tr>
<tr>
<td>NADPH→P-450 reductase→P-450</td>
<td>0.9</td>
<td>10.1</td>
</tr>
<tr>
<td>NADPH→b₅ reductase→b₅→P-450</td>
<td>11.4</td>
<td>15.1</td>
</tr>
<tr>
<td>NADPM→P-450 reductase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Incubations were carried out with vesicles corresponding to 0.2 nmol cytochrome P-450 as outlined in Table 1. The membranes contained microsomal phospholipids and cytochrome P-450 at a molar ratio of 1300:1 and otherwise equimolar amounts of the proteins.

into phospholipid vesicles and the rate of cytochrome P-450 LM₂-catalyzed O-dealkylation of p-nitroanisole was examined. As seen from Table 3, the ordinary NADPH-dependent electron transport route via NADPH-cytochrome P-450 reductase was the least effective concerning product formation in relation to NADPH utilization and in the absolute amount of product formed. As soon as cytochrome b₅ was introduced into the system, the extent of electron utilization increased to about 50%, despite NADH-cytochrome b₅ reductase or NADPH-cytochrome P-450 reductase being the electron donor to b₅.

DISCUSSION

The results presented indicate that during catalysis, NADPH-cytochrome P-450 reductase cytochrome b₅ and cytochrome P-450 form a ternary complex in the membrane. Previously,

Table 4. NADH and NADPH-supported, cytochrome P-450 LM2-catalyzed O-demethylation of p-nitroanisole in reconstituted membrane vesicles. Vesicles were prepared from equimolar amounts of NADPH-cytochrome P-450 reductase, NADH-cytochrome b5 reductase, cytochrome b5 and cytochrome P-450 LM2 and micromolar phospholipids. The mol ratio of protein to lipid was about 1:100. Vesicles corresponding to 0.3 nmol of cytochrome P-450 in 10 mM Tris-HCl, pH 7.4, containing 50 mM NaCl and 0.2 mM EDTA in a volume of 200 μl were diluted with 50 mM potassium phosphate buffer, pH 7.4, to 2 ml. p-Nitroanisole was added to 50 mM concentration and the solution was distributed between two cuvettes. Subsequently, NADH and/or NADPH was added to the sample cuvette to a final concentration of 10 μM after a preincubation period of 3 min at 30 °C.

<table>
<thead>
<tr>
<th>Cofactor used</th>
<th>Product formed nmol per nmol P-450 LM2 and min</th>
<th>NADH/NADPH oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH and NADPH</td>
<td>10.4</td>
<td>15.1</td>
</tr>
<tr>
<td>NADPH</td>
<td>7.6</td>
<td>11.0</td>
</tr>
<tr>
<td>NADH</td>
<td>2.4</td>
<td>4.0</td>
</tr>
</tbody>
</table>

French et al. and Miwa et al. have given unequivocal evidence of the formation of a functional binary 1:1 complex between cytochrome P-450 and the flavoprotein, whereas Chiang and Bonfils et al. have described the formation of a binary complex between the two hemoproteins. In both cases, the binding of the second component to cytochrome P-450 apparently induces a structural change in the P-450 molecule, as revealed by the enhanced binding affinity for substrate after binding. In view of these findings, it seems natural that the effect of cytochrome b5 in the intact electron transport system on the rate of cytochrome P-450-catalysed hydroxylation reactions, was found to be dependent upon both the type of cytochrome P-450 and the type of substrate used (Table 1). This is in line with the results of others. It is noteworthy that with some substrates, e.g. p-nitroanisole the NADH-dependent electron transport chain can serve cytochrome P-450 much more efficiently than the usual NADPH-driven chain. A nearly four-fold higher rate of hydroxylase activity was observed when both electrons were donated by cytochrome b5, than when the electrons were transferred via NADPH-cytochrome P-450 reductase. To the main extent this fact seems to be inherent in the high degree of "uncoupling" in the system when P-450 reductase serves as electron donor, the extent of cofactor utilization being similar in both cases. It is obvious that the presence of cytochrome b5 in the membrane is important for efficient coupling between NADPH and product (Table 3). Electron supply via P-450 reductase results in only 9% electron utilization for product formation, whereas donation of at least the second electron to P-450 from cytochrome b5, results in more than 50% efficiency due to a lower steady state level of the oxyctytochrome P-450-complex (cf. Refs. 7 and 9). The highest extent of electron utilization (75%) was observed in the complete system containing both the NADH- and the NADPH-donor electron transport chains. When comparing rates of hydroxylase activity in membranes containing NADH-cytochrome b5 reductase, it is evident that NADH exerts a pronounced synergistic effect in the P-450-catalysed hydroxylation reaction. Obviously, the rate of electron transfer between NADPH-cytochrome P-450 reductase and cytochrome b5 in NADPH-supported hydroxylation reactions may be rate-determining. The rate constant for the reductase-mediated reductions of cytochrome P-450 is about 3-fold higher than for cytochrome b5 reduction. Accordingly, a more efficient electron supply to cytochrome b5 from NADH via NADH-cytochrome b5 reductase will increase the overall hydroxylase activity two-fold (Table 3).

The current knowledge unequivocally indicates that in the case of the common cytochrome P-450 substrates, NADPH-cytochrome P-450 reductase serves as the most efficient donor of the first electron to cytochrome P-450, whereas cytochrome b5 delivers the second electron at a higher rate. Thus, the formation of a ternary complex between these electron transport components in the membrane, as indicated in the

present paper, would be of substantial importance to a more efficient electron utilization and for a faster hydroxylation process.

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