The Identification of the Heat-stable Microsomal Protein Required for Methoxyflurane Metabolism as Cytochrome \( b_5 \)*

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Methoxyflurane is an anesthetic whose metabolism by cytochrome \( P-450_{LM} \) has been shown to be dependent upon a heat-stable microsomal protein (Canova-Davis, E., and Waskell, L. A. (1982) Biochem. Biophys. Res. Commun. 108, 1264–1270). Treatment of this protein with diethylpyrocarbonate, which modifies selected amino acids, caused a dose-dependent loss in its ability to effect the metabolism of methoxyflurane by purified cytochrome \( P-450_{LM} \). This protein factor has been identified as cytochrome \( b_5 \) by demonstrating that cytochrome \( b_5 \) and the heat-stable factor coelute during cytochrome \( b_5 \) purification. Neither ferriheme nor apocytochrome \( b_5 \) was able to substitute for the activating factor, while cytochrome \( b_5 \) reconstituted from apocytochrome \( b_5 \) and heme exhibited an activity similar to that of native \( b_5 \). Examination of the cytochrome \( b_5 \) molecule by computer graphics suggested that diethylpyrocarbonate did not inactivate \( b_5 \) by reacting with the anionic surface of the cytochrome \( b_5 \) molecule. Maximal rates of methoxyflurane metabolism were obtained at a ratio of 1:1:1 of the three proteins, cytochrome \( P-450_{LM} \):reductase:cytochrome \( b_5 \). In summary, it has been demonstrated that the heat-stable protein, cytochrome \( b_5 \), is obligatory for the metabolism of methoxyflurane by cytochrome \( P-450_{LM} \). These data also suggest that cytochrome \( b_5 \) may be acting as an electron donor to \( P-450_{LM} \) in the \( O \)-demethylation of methoxyflurane.

Methoxyflurane (2,2-dichloro-1,1-difluoroethyl methyl ether) is a volatile anesthetic whose biotransformation is effected in hepatic microsomes. It is readily \( O \)-demethylated to formaldehyde, dichloroacetic acid, and fluoride ion which is nephrotoxic. The participation of cytochrome \( P-450 \) in this reaction is suggested by the following observations: 1) methoxyflurane produces a Type I difference spectrum when incubated with microsomes indicating that the anesthetic binds to the monoxygenase (Takahashi et al., 1974); 2) its metabolism is inhibited by carbon monoxide (Ivanitech et al., 1979) and by antibodies to NADPH cytochrome \( P-450 \) reductase (Waskell and Gonzales, 1982); and 3) its biotransformation is induced 10-fold by phenobarbital (Greenstein et al., 1975).

In spite of the fact that methoxyflurane metabolism was induced by phenobarbital, a purified preparation of the phenobarbital-induced \( P-450 \), known as \( LM_{b} \), was unable to metabolize methoxyflurane.† Hence, an effort was made to isolate the \( P-450 \) isozyme responsible for methoxyflurane metabolism by monitoring activity in addition to the customary tabulation of \( P-450 \) content. It was discovered that although the methoxyflurane-metabolizing ability of \( P-450 \)-containing fractions was lost after ion exchange chromatography, the activity could be restored by the addition of heat-treated polyethylene glycol-precipitated fractions of the solubilized microsomes (Canova-Davis and Waskell, 1982). The results presented herein demonstrate that cytochrome \( b_5 \) is the essential component in these fractions and probably functions by transferring the second electron to \( P-450_{LM} \).

**EXPERIMENTAL PROCEDURES**

**Purification of Microsomal Enzymes**—Liver microsomes were prepared from phenobarbital-treated white New Zealand male rabbits as described by Haugen and Coon (1976). The procedure for obtaining the heat-stable factor from microsomes has been previously published (Canova-Davis and Waskell, 1982). The method of Dignam and Strobel (1975) as modified by Vermilion and Coon (1978) was followed for the isolation of NADPH-cytochrome \( P-450 \) reductase from pyrophosphate-extracted phenobarbital-induced rabbit liver microsomes. The preparation was electrophoretically homogeneous and had a specific activity of 51 μmol of cytochrome \( c \) reduced/min/mg of protein in 0.3 M potassium phosphate buffer, pH 7.7 at 30°C.

The purification to electrophoretic homogeneity of \( P-450_{LM} \), from phenobarbital-treated rabbits was performed essentially according to Johnson et al. (1979). Pyrophosphate-washed microsomes were solubilized with sodium cholate and fractionated with polyethylene glycol. A 6–10% polyethylene glycol fraction was obtained and applied to a DE52 cellulose column equilibrated with 10 mM Tris acetate, pH 7.4 at 25°C, 20% glycerol, 0.3% Nonidet P-40, and 1 mM EDTA (Buffer A). After the \( P-450_{LM} \)-containing fraction was eluted, a \( P-450_{LM} \)-enriched fraction was obtained by eluting with 0.06 M KCl in Buffer A. Increasing the KCl content to 0.3 M released the remainder of the proteins from the column. The \( P-450_{LM} \)-containing peak was treated with calcium phosphate gel and chromatographed on a CM52 cellulose column as described by Johnson et al. (1979) except that the Nonidet P-40 concentration was increased to 0.3%. The \( P-450_{LM} \)-containing peak from this CM52 cellulose column was treated with calcium phosphate gel and applied to an hydroxyapatite-gelose gel column equilibrated with 10 mM KPO4, pH 7.4, 20% glycerol, 0.1 mM EDTA, and 0.3% Nonidet P-40. The column was then washed with 3 column volumes of starting buffer and purified \( P-450_{LM} \) was eluted with 55 mM buffer. The other cytochromes were removed from the column with 200 mM buffer. The concentration of cytochrome \( P-450 \) was determined by the method of Omura and Sato (1964). The specific activity of the cytochrome \( P-450 \) was expressed as micromoles of cytochrome \( c \) reduced/min/mg of protein.

† L. A. Waskell, unpublished observation.

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content of the purified sample was 17.2 nmol of P-450/mg of protein. The partially purified P-450 used in some studies was obtained after the DE52 cellulose chromatography; specific activity: 7.2–7.9 nmol of P-450/mg of protein.

The method of Chiang (1981) was used for the purification of b₅ from detergent-solubilized microsomes of phenobarbital-treated rabbits. Modifications to this method are described elsewhere. Detergent-solubilized microsomes were applied to a DE52 cellulose column equilibrated with 10 mM Tris-chloride, pH 7.7 at 4 °C, 10% glycerol, 0.1 mM EDTA, and 0.4% Nonidet P-40. The hemoprotein fraction which eluted with 0.1 M KCl in the equilibration buffer was reserved for the isolation of b₅ and partially purified P-450LM₂. The reductase was adsorbed to the column and was purified as previously published (Vermilion and Coon, 1978). The hemoprotein fraction was applied to a DE52 cellulose column as described for the isolation of P-450LM₂. The protein fraction which was eluted with 0.3 M KCl was enriched in cytochrome b₅. This fraction was further purified on DE52 cellulose and Sephadex G-100 columns as described by Chiang (1981). The concentration of purified b₅ was ascertained from the absolute spectrum of the ferric protein using an absorption coefficient of 117 mM⁻¹ cm⁻¹ at 413 nm (Strittmatter and Velick, 1956). In cruder preparations the amount of b₅ was estimated from the reduced versus oxidized spectra (Esta-brook and Warrington, 1978; Oszlányi, 1979). Reconstitution of Apocytochrome b₅ with Ferriheme—Acytochrome b₅ was prepared by an acid-acetone treatment as described by Cinti and Oszlányi (1975) as modified by Vatsis et al. (1982). The reconstitution of b₅ was affected according to Vatsis et al. (1982) except that excess hemin was removed by dialysis through Spectra/Por 6 membrane tubing (molecular weight cutoff, 3,500; Spectrum Medical Industries).

Benzphetamine N-Demethylation in the Reconstituted Enzyme System—Reaction mixtures were prepared in a total volume of 300 μl of 0.05 M Tris acetate buffer, pH 7.4 at 37 °C, containing an NADPH-generating system consisting of 10 mM glucose 6-phosphate, 1 mM NADP, and 0.3 unit of glucose-6-phosphate dehydrogenase. The P-450LM₂ preparation was first combined with the reductase followed by 20 μg of dilauroylglyeryl-3-phosphorylcholine before addition of the aqueous components. The reaction was begun by the addition of benzphetamine to a final concentration of 1 mM. Incubation was conducted at 37 °C in a shaking water bath for 20 min. The reaction was terminated by the addition of 33 μl of 70% trichloroacetic acid. After centrifugation for 3 min in an Eppendorf microfuge the form-dehydroxy content of the supernatant solution was determined.

Methoxyflurane O-Demethylation in the Reconstituted Enzyme System—Reaction mixtures were prepared in a total volume of 300 μl of 0.1 M Tris acetate buffer, pH 7.4 at 37 °C, containing an NADPH-generating system consisting of 10 mM glucose 6-phosphate, 1 mM NADP, and 0.3 unit of glucose-6-phosphate dehydrogenase. The P-450LM₂ preparation was first combined with the NADPH-P-450 reductase, followed by 20 μg of dilauroylglyeryl-3-phosphorylcholine and either the heat-stable factor was indeed b₅. As the specific activity of b₅ in the various steps increased, so did the activation properties of the heat-stable polyethylene glycol fraction.

RESULTS

Proteinaceous Character of the Heat-stable Microsomal Factor—As reported previously (Canova-Davis and Waskell, 1982) incubation with trypsin destroyed the activity of the heat-stable factor. Further evidence that the factor was a protein was provided by the heat-stable fractions containing the factor with increasing concentrations of diethylpyrocarbonate, which modifies selected amino acids in proteins (Osterman-Golkar et al., 1974). A dose-dependent inactivation of the heat-stable factor was observed (Fig. 1). Restoration of the activating properties of the heat-treated polyethylene glycol fraction occurred upon decarboxylation with hydroxylamine, suggesting the involvement of histidine, serine, threonine, or tyrosine in the unknown protein.

Specifically, calmodulin and bovine serum albumin were inactive. Micromoles isolated in the presence of fluorode ion metabolized methoxyflurane to the same extent as microsomes isolated without fluorode ion present, suggesting that the necessary protein was probably not a kinase (Goodwin et al., 1982).

Copurification of the Protein-activating Factor with Cytochrome b₅—Studies of P-450-mediated microsomal reactions have led to proposals that b₅ can play a role in the transfer of the second electron in substrate oxidations (Hildebrandt and Estabrook, 1971). Hence, purification of b₅ was undertaken to determine if the heat-stable factor was indeed b₅. As the specific activity of b₅ in the various steps increased, so did the activation properties of the heat-stable factors, and b₅ might be identical (Table I).

Role of the Heme Prosthetic Group of Cytochrome b₅ in P-450LM₂-catalyzed Methoxyflurane O-Demethylation—To rule out the possibility that the copurification of the activating protein and holocytochrome b₅ was not merely coincidental,

![FIG. 1. Inhibition of the heat-treated polyethylene glycol fraction with diethylpyrocarbonate and subsequent reactivation with hydroxylamine hydrochloride. The heat-treated polyethylene glycol fraction (250 μg) was precipitated with the indicated concentrations of diethylpyrocarbonate (DEP) at room temperature for 30 min. The assay was conducted as described under “Experimental Procedures”: partially purified P-450LM₂, 1.8 nmol/ml; reductase, 0.32 nmol/ml; incubation time: 60 min. Controls at each point consisted of identical components except the heat-treated polyethylene glycol fraction was added after the diethylpyrocarbonate and assayed immediately. Reactivation was accomplished by adding hydroxylamine hydrochloride at the concentrations indicated to the diethylpyrocarbonate incubation mixture and further incubated at room temperature for 30 min before assay.](http://www.jbc.org)
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TABLE I
Copurification of b₅ and activation factor

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein</th>
<th>Cytochrome b₅</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrophosphate-treated microsomes</td>
<td>4465</td>
<td>1.4</td>
<td>0.9</td>
</tr>
<tr>
<td>First DE52 cellulose column eluate</td>
<td>1214</td>
<td>3.6</td>
<td>19.4</td>
</tr>
<tr>
<td>Second DE52 cellulose column eluate</td>
<td>221</td>
<td>6.6</td>
<td>55.6</td>
</tr>
<tr>
<td>Third DE52 cellulose column eluate</td>
<td>74</td>
<td>12.9</td>
<td>92.0</td>
</tr>
<tr>
<td>First Sephadex G-100 column eluate</td>
<td>19</td>
<td>33.0</td>
<td>579.3</td>
</tr>
</tbody>
</table>

* Assayed as described under "Experimental Procedures": partially purified P-450LM₂, 2.7 nmol/ml; reductase, 0.32 nmol/ml. Incubation was conducted for 30 min. Activation is normalized to 1.0 nmol of P-450/ml.

FIG. 2. Role of cytochrome b₅ versus apocytochrome b₅ in the metabolism of methoxyflurane. The assay was conducted as described under "Experimental Procedures": partially purified P-450LM₂, 2.7 nmol/ml; reductase, 0.25 nmol/ml; ---, apocytochrome b₅; --O--O, reconstituted b₅; ×--×, cytochrome b₅; incubation time, 30 min.

![FIG. 2](http://www.jbc.org/)

FIG. 3. Methoxyflurane metabolism by P-450LM₂ as a function of reductase concentration. The assay was performed as described under "Experimental Procedures": partially purified P-450LM₂, 0.9 nmol/ml; b₅, 0.9 nmol/ml; reductase as indicated; incubation time, 30 min.

![FIG. 3](http://www.jbc.org/)

FIG. 4. Methoxyflurane metabolism of P-450LM₂ as a function of cytochrome b₅ concentration. The assay was performed as described under "Experimental Procedures": partially purified P-450LM₂, 0.9 nmol/ml; b₅, 0.9 nmol/ml; reductase as indicated; incubation time, 30 min.

![FIG. 4](http://www.jbc.org/)
Fig. 5. Effect of dilauroylglyceryl-3-phosphorylcholine (dilauroyl-GPC) concentration on methoxyflurane metabolism by P-450M. The assay was performed as described under “Experimental Procedures”: purified P-450M, 0.64 nmol/ml; b, 0.64 nmol/ml; reductase, 0.64 nmol/ml; dilauroylglyceryl-3-phosphorylcholine, as indicated; incubation time, 30 min.

TABLE II

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Inhibition of P-450M by allylisopropylacetamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoxyflurane</td>
<td>Benzyphenetamine</td>
</tr>
<tr>
<td>Microsomes*</td>
<td>25</td>
</tr>
<tr>
<td>P-450M</td>
<td>25</td>
</tr>
</tbody>
</table>

* Microsome concentration, 0.6 nmol of P-450/mL.
* Assayed as described under “Experimental Procedures”: partially purified P-450M, 2.7 nmol/ml; reductase, 0.64 nmol/ml; incubation time, 30 min. The P-450M concentration was reduced to 0.27 nmol/ml for the metabolism of benzphetamine.

DISCUSSION

Besides demonstrating that the heat-stable factor was a protein, the experiments with diethylpyrocarbonate suggest that either a histidine, tyrosine, serine, or threonine residue on the b5 molecule, which was subsequently identified as the heat-stable factor, is necessary for stimulating methoxyflurane degradation. This was deduced from the observation that hydroxylamine could restore activity to the factor. Modifications on lysine or arginine residues or on α-amino groups are not reversed by treatment with hydroxylamine (Peterson et al., 1974). If diethylpyrocarbonate reacted with an amino acid residue on the anionic surface of b5 and thus sterically inhibited its interaction with cationic groups on either P-450M and/or P-450 reductase, electron transfer would be inhibited (Heinemann and Ozols, 1983; Dailey and Strittmatter, 1980). This possibility was eliminated by the examination of the three-dimensional structure of the b5 molecule on the computer graphics system at the University of California at San Francisco (Fig. 6). Inspection of this anionic surface revealed that susceptible amino acids were not found on this face of the molecule. Three possible sites for the carbethylation by diethylpyrocarbonate exist. 1) The two ligands to the heme of b5 are histidine residues (Argos and Mathews, 1975). 2) The hydrophilic tail of b5, which is essential for electron transfer to the P-450 system (Chiang, 1981) contains susceptible residues (Dailey and Strittmatter, 1981). 3) Serine 64 (Fig. 6), which is immediately adjacent to this surface, may function in stabilizing the heme in its pocket (Argos and Mathews, 1975).

Since several laboratories (Vatsis et al., 1980; Kusunose et al., 1981; Okita et al., 1984) had reported that boiled b5 was unable to stimulate P-450-mediated oxidation, the heat-stable factor was not initially considered to be b5. In addition Kita- gawa and co-workers (1982) reported that above 55 °C irreversible denaturation of b5 occurred. However, after publication of the initial findings of the heat-stable factor (Canova and Waskell, 1982) it was learned that b5 had to be boiled for several hours to significantly decrease its activity. Consequently, the purification of b5 was effected to determine if the heat-stable factor was indeed b5. As the specific activity of b5 in the various steps increased, so did the activation properties (Table I), establishing their identity.

The role of b5 in the P-450-catalyzed oxidation of methoxyflurane was investigated in a number of ways. Removal of the heme prosthetic group, and the subsequent inactivity of the apocytochrome b5 suggested that electron transfer had been disrupted. Recovery of activity was effected upon reconstitution of b5 by insertion of ferriheme into the apocyto- chrome b5 molecule. The involvement of b5 in a 1:1 molar ratio with P-450M and the reductase (Figs. 3 and 4) supports its postulated role as the second electron donor in the metabolism of methoxyflurane. The inhibition studies with allylisopropylacetamide (Table II) indicated that different enzyme systems participate in the metabolism of benzphetamine as opposed to methoxyflurane. This latter observation probably reflects the requirement for the additional protein-protein interaction of b5 with P-450 in the metabolism of methoxyflurane.

The observed obligatory role for b5 in the NADPH-dependent monoxygenation of methoxyflurane with purified P-450M extends the list of substrates with such a requirement. Cytochrome b5 has been found to be necessary in a number of systems (Vatsis et al., 1980; 1982; Kuwahara and Omura, 1980; Sugiyama et al., 1980). It appears that the involvement of b5 in reconstituted drug oxidation systems varies according to the substrate being metabolized, even when the molecular species of P-450 is the same; e.g. note the difference in the requirement of b5 in the metabolism of...
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I. Role of Cytochrome b₅ in Hydroxylations by Cytochrome P-450

II. FIG. 6. A stereoscopic view of the anionic surface (in red) of the cytochrome b₅ molecule

methoxyflurane as compared to that of benzphetamine (Canova-Davis and Waskell, 1983). Furthermore, the metabolism of the same substrate by different forms of P-450 also exhibits different requirements for b₅, as demonstrated by the work of Vatsis et al. (1982) with the prostaglandins. In addition to its obligatory role, b₅ has been implicated as a participant in the reconstituted P-450 system in a facilitory role (Imai and Sato, 1977; Ingelman-Sundberg and Johansson, 1980; Okita et al., 1981; Bösterling et al., 1982; Vatsis et al., 1982).

The results discussed above demonstrate that b₅ plays an important role in the biotransformation of the anesthetic methoxyflurane. The function of b₅ in this reaction is presumably to transfer the second of the two electrons that are required for substrate oxidation (Bonfils et al., 1981). At the present time there is no satisfactory explanation for the biological function or mechanism of this phenomenon. The question of how the oxyferro-substrate complex of a presumably unique species of P-450 is able to accept the second electron from the reductase versus b₆ is intriguing. One may envision that substrate-induced allosteric effects on a given P-450 can result in an altered affinity of the oxyferro-substrate complex for the reductase versus b₅.

The in vivo role of b₅ is to provide electrons for a host of important biosynthetic reactions (Passon et al., 1972; Oshino et al., 1971; Okayasu et al., 1977; Reddy et al., 1977; Grinstead and Gaylor, 1982; Paltauf et al., 1974; Keyes et al., 1979; Strittmatter et al., 1982). The oxidation by P-450 of selected drugs and/or endogenous substrates which requires b₅ competes for these electrons. Such oxidation reactions may shift the balance between the various pathways, with possible significant consequences on the biochemical homeostasis of an organism.

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