The Binding of Cytochrome $b_5$ to Liver Microsomes*

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SUMMARY

The 40-44 hydrophobic peptide segment, present in cytochrome $b_5$, obtained by nonhydrolytic means, but absent from lipase or trypsin-extracted cytochrome $b_5$, has been found to be required for the binding of a 10- to 20-fold molar excess of cytochrome $b_5$ to liver microsomes. At saturation, the bound cytochrome $b_5$ constitutes nearly 20% of the total protein of the vesicle preparations. This extra bound hemoprotein is indistinguishable from endogenous cytochrome $b_5$ as an electron acceptor from microsomal NADH cytochrome $b_5$ reductase and as an electron donor for external cytochrome $c$ or the rat liver microsomal stearyl coenzyme A desaturase. On the basis of earlier work of others and present data, it appears that these enzymes are localized on the outside of microsomal vesicles. A model, consistent with these data, proposes that the extra, as well as endogenous cytochrome $b_5$, is bound to microsomes by interactions between the nonpolar peptide segment and the phospholipid of the membrane. This permits interactions of the polar catalytic heme binding segment at the surface of the outer membrane with either reductase, cytochrome $c$, or the desaturase system. The latter catalytic events require only the translational movement of the nonpolar portion of the protein in the lipid membrane of several molecular diameters.

We have described the utilization of detergent extraction procedures to isolate a form of rabbit liver cytochrome $b_5$ (cytochrome $b_5$ (detergent)) which contains an additional hydrophobic sequence of 40 to 44 amino acid residues attached to the functional hemoprotein structure isolated by pancreatic lipase extraction (cytochrome $b_5$ (lipase)) (1). Hemebinding to lipase-extracted preparations from both rabbit and calf liver has been characterized (2-4), and it has been found that this binding stabilizes the heme peptide core as a globular hydrophilic structure (5-7). The primary structure of the lipase preparations from these two (8-10), and other (11, 12), species are now known. Moreover, Mathews et al. (13) have described x-ray diffraction studies of rabbit cytochrome $b_5$ (lipase) at 2-A resolution which clearly show that the heme is inserted into a tight nonpolar crevice in a compact globular molecule which contains most of the polar residue on the surface. The additional nonpolar peptide segment of rabbit cytochrome $b_5$ (detergent), attached to the globular heme peptide, is readily cleaved by trypsin. Crystallographic studies (13) indicate that both the NH$_2$- and COOH-terminal residues of cytochrome $b_5$ (lipase) are highly flexible and would therefore presumably provide access to trypsin and other proteolytic enzymes. In the case of the rabbit detergent preparation, indirect evidence initially suggested that the hydrophobic peptide is attached to the NH$_2$ terminus of the heme binding segment (10), but subsequent sequence studies by 0zols (12) on human cytochrome $b_5$ (detergent) indicate that the hydrophobic peptide is probably at the COOH terminus. The essential structural feature, in either case, is that intact cytochrome $b_5$ (detergent) is an amphipathic molecule in which the nonpolar region promotes polymerization in aqueous solution and apparently provides the firm attachment to the phospholipid matrix of the endoplasmic reticulum, thus orienting the hydrophilic heme binding segment at the membrane surface.

It appears that the flavoprotein which catalyzes the reduction of cytochrome $b_5$, cytochrome $b_5$ reductase, is also an amphipathic protein (14). In this instance, the polar catalytic portion, isolated by lysosomal digestion (15), is a single peptide chain of 292 residues. Detergent extraction and fractionation procedures (14) yielded a flavoprotein with an additional 98 residues which are more than 60% nonpolar and again promote the formation of a large polymer in aqueous solutions.

In the experiments reported below we have utilized the sensitive spectral characteristics of cytochrome $b_5$ to measure the incorporation of additional hemoprotein directly into microsomal preparations. These studies show that a large molar excess of the detergent preparation, compared to the constitutive cytochrome $b_5$, will bind selectively to microsomes, and that this bound cytochrome is functionally indistinguishable from the hemoprotein originally present in these vesicles. The dependence of this binding upon the nonpolar segment of the cytochrome and the large excess of hemoprotein that will bind imply direct interactions between microsomal membrane phospholipids and the hydrophobic segment of cytochrome $b_5$.

EXPERIMENTAL PROCEDURE

Absorption and difference spectra were recorded with 0.4-ml samples in a Bausch and Lomb Spectronic 505 recording spectrophotometer which has an expanded scale and a 5 A band width from 200 to 700 nm. The indicated temperatures were maintained with a thermostated cell holder. A Gilford model 240 recording spectrophotometer was employed for experiments in which the extent of cytochrome $b_5$ reduction was monitored at a constant wave length (121 nm) during kinetic experiments.

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Cytochrome b₅ concentrations were calculated either from the absorbance of the oxidized form at 413 nm and an extinction coefficient of 117,000 (5), or from the difference between the reduced and the oxidized spectra at 424 nm (extinction difference = 100,000). The reduction of cytochrome c was followed at 550 nm on the basis of a difference of 18,500 in the extinctions of the reduced and oxidized hemoprotein (16). Stopped flow experiments were performed with the micro stopped flow apparatus described previously (17). Protein concentrations were determined by the method of Lowry et al. (18).

Stearyl-CoA desaturase activities of microsomal preparations were assayed both by the method of Jones et al. (19), using the thin layer chromatography system to determine the amount of [14C]oleyl-CoA formed from [14C]stearyl-CoA, and by following the reoxidation of cytochrome b₅ after NADH oxidation in the presence and absence of stearyl-CoA, as suggested by the studies of Oshino et al. (20). The latter assay involves the addition of 1 nmole of NADH to a dilute suspension of microsomes (0.4 to 0.5 mg of protein per ml) in 0.2 ml of 0.1 M Tris-acetate buffer, pH 8.1, containing either 4 nmoles of stearyl-CoA or no substrate. This incubation mixture is then monitored at 424 nm and 25°C. Microsomal cytochrome b₅ is reduced completely by the NADH and microsomal cytochrome b₅ reductase, remains reduced until all of the NADH becomes oxidized, and then becomes reoxidized in turn. The difference between the time for NADH oxidation in the presence and absence of stearyl-CoA, indicated by the onset of cytochrome b₅ reoxidation, was used to calculate the desaturase activity. It was assumed that 1 mole of NADH is required for the formation of 1 mole of oleyl-CoA.

Table I shows that this simple and rapid spectrophotometric procedure agrees well with results obtained by the usual radioactivity assay. With microsomal preparations containing additional bound cytochrome b₅ (detergent) all of the NADH was initially exhausted in the rapid reduction of cytochrome b₅. Then the rate of cytochrome b₅ reoxidation was used to measure desaturase activity by a similar calculation.

NADH and cytochrome c (type VI) were obtained from Sigma Chemical Company and [14C]stearyl-CoA was obtained from New England Nuclear. Rabbit or calf liver cytochrome b₅ (detergent) and cytochrome b₅ (lipase) were prepared as described previously (1, 2, 5).

**Table I**

<table>
<thead>
<tr>
<th>Micromolar concentration</th>
<th>Time for NADH oxidation (min)</th>
<th>[%] Oleate formed</th>
<th>Calculated [%] Oleate formed</th>
<th>[%] Oleate assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>− Stearyl-CoA</td>
<td>+ Stearyl-CoA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.2</td>
<td>2.7</td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>2</td>
<td>3.3</td>
<td>2.3</td>
<td>0.30</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Livers for microsomal preparations were obtained from rabbits and rats promptly after the animals were killed. In the case of rabbit livers, perfusion with 0.25 M sucrose containing 1 mM EDTA and 10 mM Tris-acetate buffer, pH 8.1 and 2°C, was used to chill the liver rapidly and to remove most of the blood. Sprague-Dawley rats, weighing 120 to 180 g, were fasted 48 hours and then refed a high carbohydrate diet for 20 hours, as described by Oshino et al. (20), to obtain livers with high stearyl-CoA desaturase activity. Our modified high carbohydrate diet consisted of 92.9% cornstarch, 15% sucrose, 15% casein (vitamin-free, Nutritional Biochemical Corporation), 4% salts (salt mix w, Nutritional Biochemical Corporation), 2% cellulose, 1% vitamins (vitamin diet fortification mix, Nutritional Biochemical Corporation), and 0.1% choline chloride (Nutritional Biochemical Corporation).

Plasma membranes were obtained from sheep and rabbit erythrocytes by collecting the cells from whole blood by centrifugation, lysing these cells with 10 volumes of H₂O at 2°C, collecting the plasma membrane fraction as a precipitate at 10,000 x g for 10 min, and washing the membrane preparation twice at 2°C with the same volume of 0.1 M Tris-acetate buffer, pH 8.1. Inner and outer mitochondrial membranes of rat liver mitochondria were prepared as described by Sottocasa et al. (21).

**Preparation of Microsomes**—All procedures were performed in the cold room at 2-4°C. Livers from either rabbits or rats was homogenized with 9 ml per g of liver with 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-acetate buffer, pH 8.1, in a glass homogenizer with a loose fitting Teflon pestle. Mitochondria, nuclei, and cell debris were removed by centrifuging twice at 18,000 x g for 15 min, the pellet being discarded in each case. The supernatant fluid was centrifuged at 120,000 x g for 30 min to obtain the microsomal fraction as a tightly packed pellet. The pellets were then resuspended in 0.5 M NaCl and 0.1 M Tris-acetate buffer, pH 8.1, and centrifuged again at 120,000 x g for 30 min to assure removal of adsorbed proteins. This microosomal fraction was washed two additional times with either 0.1 or 0.02 M Tris-acetate buffer, pH 8.1, and suspended in the same Tris-acetate buffer used in the washing procedure to give a final protein concentration of approximately 10 mg per ml. These suspensions of microsomes were sonicated for 40 s at 38 watts to facilitate subsequent spectral observations. By electron microscopy the sonicated preparations were composed largely of microsomal vesicles with minimal contamination by mitochondria or mitochondrial fragments.

**Binding of Cytochrome b₅ to Microsomes**—Usually 0.5 ml of a microsomal suspension in either 0.1 or 0.02 M Tris-acetate buffer, pH 8.1, (10 mg of protein per ml) was mixed with a 1- to 5-fold molar excess of cytochrome b₅ compared to the endogenous microsomal cytochrome b₅ and incubated at 2°C for 16 to 18 hours, or at 37°C for 20 min. The suspension was then diluted with 8.0 ml of the same Tris-acetate buffer, and centrifuged at 120,000 x g for 30 min to recover the packed microsomes, which were then washed twice with 9.0 ml of the buffer. The final pellet of packed microsomes was suspended in 1.0 ml with the same buffer concentration as the initial microsomal suspension.

**RESULTS**

**Binding of Cytochrome b₅ to Microsomes**—Fig. 1 shows that the incubation of rabbit liver microsomes with cytochrome b₅ (detergent) resulted in the binding of additional cytochrome to these vesicles (●), with apparent saturation at a 10- to 11-fold molar excess of cytochrome b₅ (detergent). In contrast, no
binding of cytochrome \( b_5 \) (lipase) occurred under similar incubation conditions \((\circlearrowright)\), suggesting that there is specific interaction of the additional nonpolar segment of the detergent preparation with the microsomal membrane. The fact that high salt \((x)\), additional washes of the microsomes with water, various concentrations of Tris-acetate buffer, pH 8.1, and mechanical disruption by sonication or homogenization failed to remove the bound cytochrome \( b_5 \) (detergent) also implies that nonspecific protein binding is not the basis of the cytochrome interaction with microsomes.

Variation of the incubation conditions (Table II) showed that, whereas the ionic strength is not critical, binding of the hemoprotein is relatively slow and temperature-dependent. At 2° and 25° saturation required several hours, whereas binding was complete in 20 min at 37°.

The first three lines of Table III emphasize two further characteristics of cytochrome \( b_5 \) (detergent) binding to microsomes. First, with microsomal preparations from three different species, saturation by the hemoprotein occurs at approximately the same level, although there is considerable variation in the content of endogenous microsomal cytochrome \( b_5 \) in the isolated vesicles. Rat liver microsomes, which contain less than 50% as much cytochrome \( b_5 \) as rabbit microsomes, bind nearly as much extra hemoprotein on the basis of total protein. This results in a 21-fold molar excess of bound to endogenous cytochrome \( b_5 \) in the rat liver preparation. Second, it is clear that the bound cytochrome is a very significant part of the total protein. At 12 nmoles per mg of protein the hemoprotein represents nearly 20% of the total protein of the preparation. The large amount of phospholipid in endoplasmic reticulum provides ample opportunity for the extensive lipid-protein interactions that would be required by this high level of binding.

The last four lines of Table III suggest that there is membrane specificity for binding of cytochrome \( b_5 \) (detergent). Erythrocyte plasma membranes failed to bind detectable quantities of cytochrome by the sensitive difference spectrum method. There is some binding to preparations of both outer and inner mitochondrial membranes, but the extent of binding is considerably greater to the outer membrane preparation.

Binding of cytochrome \( b_5 \) (detergent) to outer mitochondrial membrane preparations was not unexpected since this membrane does contain cytochrome \( b_5 \) \((21)\). Although the inner membrane fraction is devoid of cytochrome \( b_5 \) \((21)\), this binding does not appear to be an artifact of contamination by outer mitochondrial membranes. There was no detectable endogenous cytochrome \( b_5 \) in the inner mitochondrial fraction, and addition of NADH alone to the inner membrane preparation containing bound cytochrome \( b_5 \) (detergent) did not result in the reduction of the cytochrome in the presence or absence of 0.5 \( \text{mM} \) KCN. Outer mitochondrial membranes contain an NADH cytochrome \( b_5 \) reductase \((21)\) which will catalyze the reduction of bound cytochrome \( b_5 \) (detergent), and, if present as a contaminant, would have caused the reduction of cytochrome \( b_5 \) by NADH in

**FIG. 1.** The binding of cytochrome \( b_5 \) to microsomes. Binding was carried out as described under "Experimental Procedure," at 2°, with an 18-hour incubation period. \( \bullet \), cytochrome \( b_5 \) (detergent) binding; \( \circlearrowright \), cytochrome \( b_5 \) (lipase) binding; \( \times \), cytochrome \( b_5 \) (detergent) binding followed by 0.5 \( \text{mM} \) NaCl, 0.1 \( \text{mM} \) Tris-acetate buffer, pH 8.1, washing to remove any adsorbed hemoprotein.

**Table II**

*Effect of incubation conditions on cytochrome \( b_5 \) binding to microsomes*

The incubations of rabbit liver microsomes were carried out as described under "Experimental Procedure" with a concentration of 2 \( \times 10^{-4} \) \( \text{m} \) cytochrome \( b_5 \) (detergent), in Tris-acetate buffer, pH 8.1.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Temperature</th>
<th>Time</th>
<th>Cytochrome ( b_5 ) bound/ endogenous cytochrome ( b_5 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>2°</td>
<td>18</td>
<td>10.7</td>
</tr>
<tr>
<td>0.02</td>
<td>2</td>
<td>18</td>
<td>10.5</td>
</tr>
<tr>
<td>0.1</td>
<td>2</td>
<td>72</td>
<td>10.6</td>
</tr>
<tr>
<td>0.1</td>
<td>2</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>0.1</td>
<td>25</td>
<td>1</td>
<td>5.6</td>
</tr>
<tr>
<td>0.1</td>
<td>37</td>
<td>3.5</td>
<td>9.7</td>
</tr>
<tr>
<td>0.1</td>
<td>37</td>
<td>0.3</td>
<td>10.2</td>
</tr>
<tr>
<td>0.1</td>
<td>37</td>
<td>1.0</td>
<td>10.3</td>
</tr>
</tbody>
</table>

**Table III**

*Specificity of cytochrome \( b_5 \) (detergent) binding to membrane preparations*

See "Experimental Procedure" for details of preparations and methods. Cytochrome \( b_5 \) was determined both from the difference spectra of the samples in the presence and absence of NADH and purified cytochrome \( b_5 \) reductase, and from the oxidized spectrum of cytochrome \( b_5 \).
the inner membrane preparation. In any case, the amount of cytochrome b₅ (detergent) bound to inner mitochondrial membranes is quantitatively only 15 to 16% of the binding to microsomes.

**Enzymatic Properties of Bound Cytochrome b₅—**By several criteria, bound cytochrome b₅ (detergent) appears to be indistinguishable from the endogenous microsomal cytochrome b₅. First, the addition of NADH to microsomes containing bound cytochrome b₅ (detergent) results in complete reduction of all of the hemoprotein. The endogenous microsomal cytochrome b₅ reduce can therefore effect the reduction of the bound detergent hemoprotein. Stopped flow measurements (Table IV, Lines 1 and 2) indicate that the rate of cytochrome reduction with microsomes containing additional hemoprotein is actually more rapid. The complete reduction of cytochrome b₅ in either preparation does not follow simple first order kinetics, but the half-time for reduction of the preparation containing additional cytochrome b₅ (detergent) is 3.6 times lower.

The rapid reduction of bound cytochrome b₅ (detergent) contrasts with the extremely slow NADH-dependent reduction of unbound cytochrome b₅ (detergent) by microsomes. Cytochrome b₅ (detergent), prior to binding to the vesicle preparations or the excess which remains in solution after incubation, is not reduced at an appreciable rate by microsomes and NADH. The observed rate is at least 4 orders of magnitude slower than the rapid reduction of bound cytochrome b₅ (detergent), and the rapid reduction of cytochrome b₅ as it binds to microsomes therefore provides a simple, rapid method for following the kinetics of binding.

The stopped flow experiments also show that, within microsomes, the rate-limiting step in the reduction of cytochrome b₅ is electron transfer from cytochrome b₅ reductase to hemoprotein, rather than reduction of the flavoprotein by NADH, as is the case in solution with the purified reductase (23). The last two lines of Table IV indicate that ferricyanide reduction by microsomes shows a marked deuterium rate effect when NADH is labeled with this isotope in position A of C-4 of the dihydrogen bond. The rate-limiting step is therefore cleavage of this C—H bond. This is not the case for cytochrome b₅ reduction in microsomes, since the deuterated substrate has no effect upon the rate of cytochrome reduction. Thus, subsequent electron transfer must be rate-limiting and involve either reductase interaction with cytochrome b₅ or, perhaps, electron transfer between molecules of the hemoprotein.

The NADH cytochrome c reductase activity of microsomes provided a second test for the reactivity of bound cytochrome b₅ (detergent). Fig. 2 shows that the rate of cytochrome c reduction by NADH and microsomes increases in proportion to the bound cytochrome b₅ (detergent) to a rate nearly S-fold greater in microsomal preparations containing 10.7 eq of the hemoprotein.

A third, more significant functional criterion that bound cytochrome b₅ is an effective electron donor was the observation that the hemoprotein reacts directly in a physiologically significant reaction. Data from the laboratories of both Wakil (19) and Sato (20) suggest that cytochrome b₅ is the electron donor for microsomal stearyl-CoA desaturase. We were able to confirm this proposed participation of the hemoprotein in the oxygen and NADH-dependent formation of oleyl-CoA by a simple and rapid spectrophotometric assay (Fig. 3A). Microsomal suspensions from livers of rats induced for the desaturase were monitored at 424 nm. Upon the addition of NADH (Curve 2), the cytochrome was reduced rapidly and completely, remained in that state until the 1 n mole of NADH was oxidized, and was then oxidized at a measurable rate. This sequence of cytochrome b₅ reduction and oxidation was accelerated markedly by stearyl-CoA (Curve 1). This difference in the interval required for NADH exhaustion was used to calculate the desaturase activity as described under “Experimental Procedure” and Table I. Moreover, it is clear that the rate of cytochrome b₅ reoxidation is also stimulated by stearyl-CoA, in agreement with the suggestion of Oshino et al. (20) that reduced cytochrome b₅ is the electron donor for the desaturase.

A similar experiment was carried out with rat liver microsomes containing 10.7 n moles of cytochrome b₅ (detergent) per mg of protein (Fig. 3B). The added NADH was rapidly exhausted during the reduction of the large excess of cytochrome b₅. The rate of oxidation of reduced cytochrome b₅ was then followed to completion at 424 nm. In the absence of substrate this occurred slowly (Curve 2), but in the presence of stearoyl-CoA the rate was increased approximately 5-fold (Curve 1). Extrapolation of the linear rate for each reaction yields a maximum velocity for cytochrome b₅ oxidation, and from the difference in these values, a maximum velocity for oleyl-CoA formation. This rate actually exceeds the level of desaturase activity with only endogenous cytochrome b₅, and together with the gradual decrease in the rate of cytochrome oxidation, suggests that electron transfer from cytochrome b₅ to the terminal segment of the desaturase system may become rate-limiting in stearyl-CoA desaturase. As expected from earlier characterizations of the desaturase (19, 20), the acceleration of the oxidation by substrate was cyanide-sensitive (Fig. 3B, circles). This sequence of experiments demonstrates unequivocally that cytochrome b₅ is in fact the electron donor for stearoyl-CoA desaturase since, with excess cytochrome b₅, the reduced hemoprotein is the only source of electrons. Furthermore, it is clear that the extra bound cytochrome is functional in the desaturase reaction.

**Localization of Cytochrome b₅ in Microsomal Vesicles—**On the basis of nearly quantitative proteolytic release of cytochrome b₅ and other enzymes from microsomes, Ito and Sato (24) concluded that cytochrome b₅ is located on the outer surface of microsomal vesicles, and suggested that this may also be true of NADH cytochrome b₅ reductase. The observations that endogenous as well as bound cytochrome b₅ (detergent) are reduced rapidly by NADH, and that stearyl-CoA desaturase accepts electrons

### Table IV

**Reduction of endogenous and bound cytochrome b₅ by reduced pyridine nucleotides**

Solutions of microsomes containing only endogenous cytochrome b₅ (1.2 n moles per mg of protein) or bound cytochrome b₅ (detergent) (13 n moles per mg of protein) were mixed with the indicated nucleotide in the micro stopped flow apparatus. The half-times for reduction of all of the hemoprotein are presented and compared to the NADH ferricyanide reductase activities of the preparations, measured as described previously (22).

<table>
<thead>
<tr>
<th>Cytochrome b₅ content</th>
<th>Nucleotide</th>
<th>Half-time for cytochrome b₅ reduction (s)</th>
<th>Relative ferricyanide reductase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>n moles/mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>NADH</td>
<td>0.47</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>NADH</td>
<td>0.13</td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>NADH</td>
<td>0.47</td>
<td>100</td>
</tr>
<tr>
<td>1.2</td>
<td>NADH(D₅₃)</td>
<td>0.49</td>
<td>27</td>
</tr>
</tbody>
</table>
containing 0.09 nmole of endogenous cytochrome bs (endogenous + bound) in 0.1 M Tris-acetate buffer, pH 8.1, was followed at 25°C by the absorbance change at 550 nm as described under "Experimental Procedure." From the difference in (1) and (2) and the method described under "Experimental Procedure" the desaturase activity was 0.47 nmole of oleyl-CoA formed per min. B, at zero time 1 nmole of NADH was added to a suspension of microsomes containing 0.09 nmole of endogenous cytochrome bs and, either (1) 4 nmole of stearyl-CoA or (2) no substrate in 0.20 ml of 0.1 M Tris-acetate buffer, pH 8.1 and 25°C. The extrapolated linear portions of the two curves were used to calculate a maximum velocity for desaturase activity of 0.78 nmole of oleyl-CoA formed per min. The circles represent a repeat of the experiment of (1) that included 0.5 mM KCN.

**FIG. 3 (right).** Stearyl-CoA desaturase activity of microsomes containing endogenous and bound cytochrome bs. A, at zero time, 1 nmole of NADH was added to a suspension of microsomes containing 0.09 nmole of endogenous cytochrome bs and, either (1) 4 nmole of stearyl-CoA, or (2) no substrate in 0.20 ml of 0.1 M Tris-acetate buffer, pH 8.1 and 25°C. The extrapolated linear portions of the two curves were used to calculate a maximum velocity for desaturase activity of 0.78 nmole of oleyl-CoA formed per min. The circles represent a repeat of the experiment of (1) that included 0.5 mM KCN.

**Table V**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Addition</th>
<th>Reduction by NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicated without cytochrome bs</td>
<td>None</td>
<td>0.0</td>
</tr>
<tr>
<td>Sonicated with cytochrome bs</td>
<td>None</td>
<td>0.0</td>
</tr>
<tr>
<td>Sonicated with cytochrome bs</td>
<td>20 μl of 10% deoxycholate</td>
<td>14.4</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Structural information on cytochrome bs (1) and, more recently, cytochrome bs reductase (14) indicates that these microsomal enzymes are amphipathic molecules composed of a globular catalytic polypeptide segment and a smaller, highly apolar segment. The present experiments provide direct evidence for the previous suggestions (1, 2) that interactions between membrane phospholipids and the nonpolar peptide segment of both the cytochrome and reductase result in the firm binding of these proteins to endoplasmic reticulum. In this arrangement, the hydrophilic portions containing the catalytic sites would be oriented toward the cytoplasm at the surface of the membrane. It is clear that cytochrome bs (detergent) binding to microsomes involves the nonpolar peptide sequence. There is no binding of cytochrome bs (lipase) which lacks only these amino acid residues. Moreover, it appears most probable to us that nonpolar residues interact directly with the phospholipid, because, even at a concentration of 12 nmole of cytochrome bs per mg of microsomal protein, there is still a large molar excess of this dominant lipid species. Since the endogenous and extra bound cytochrome bs are indistinguishable as both electron acceptors and donors in several catalytic sequences...
characteristic of microsomes, the cytochrome \( b_5 \) (detergent) interactions presumably also represent an accurate model for the endogenous hemoprotein binding.

The consequences of the present data are emphasized by Fig. 4, which is a hypothetical and schematic representation of a microsomal surface depicting the spatial relations between the phospholipid molecules, cytochrome \( b_5 \), and cytochrome \( b_5 \) reductase. It is based upon the approximate surface area of a phospholipid molecule in a lipid bilayer (25), the diameter of cytochrome \( b_5 \) (13), an estimated diameter for the reductase based upon its globular structure and molecular weight (26), and the molar ratios of these molecules in microsomes (1). As one of the simplest models, a random distribution of the proteins was assumed. The proteolytic release of the catalytic portions of both enzymes and their reactivities with polar substrates is also consistent with the orientation of the larger catalytic portion of each protein at the surface of the membrane. The area shown, which includes the limitation that cytochrome \( b_5 \) and the reductase appear only on the outer microsomal surface, contains 1 reductase, 10 cytochrome \( b_5 \), and approximately 4000 phospholipid molecules. The left hand portion of the figure shows this density of the endogenous proteins, and the right side, the molecular density with microsomes saturated with cytochrome \( b_5 \) (detergent). A model of this type requires lateral movement of the proteins along the membrane to achieve reduction of cytochrome \( b_5 \) by the reductase. The stopped flow data on microsomal cytochrome \( b_5 \) reduction are consistent with this requirement. The rate-limiting step is electron transfer to the hemoprotein. Furthermore, the half-time for cytochrome reduction of 0.47 s is several orders of magnitude slower than the translational movement of phospholipids in membranes observed by Kornberg and McConnell (26) using spin-labeled phosphatidylcholine. Complete reduction of all cytochrome \( b_5 \) by direct electron transfer from the reduced form of cytochrome \( b_5 \) reductase would involve movement of the proteins by only several molecular diameters, and, in the event of significant hemoprotein-hemoprotein electron transfer, this distance would be even less. Saturation of the membrane with 10 additional molecules of cytochrome \( b_5 \) also produces a decrease in the half-time for reduction to 0.13 s, as would be expected from the closer proximity of interacting molecules. At the present time, the relative extent of electron transfer involving direct contact of hemoprotein with reduced cytochrome \( b_5 \) reductase, in contrast to transfer between molecules of cytochrome \( b_5 \), cannot be estimated. Stopped flow measurements on the rate of hemoprotein reduction in microsomal preparations containing varying molar ratios of cytochrome \( b_5 \) (detergent), and a derivative in which the heme is replaced by deuteroheme, may provide more restrictive information on these alternate routes for cytochrome reduction.

The most direct interpretation of the present data is that the desaturase, as an electron acceptor for reduced cytochrome \( b_5 \), is also on the outer surface of microsomes. Information concerning the concentration and size of this cyanide-sensitive enzyme, as well as the question of whether cytochrome \( b_5 \) and the desaturase interact without intervening electron transfer components, is still not available. However, this model does place an entire NADH- and oxygen-dependent electron transport sequence, involving interactions of at least three firmly bound enzymes on the cytoplasmic side of endoplasmic reticulum.

The fact that microsomes are not isolated with saturating levels of cytochrome \( b_5 \) also suggests that the regulation of the intracellular concentration of this hemoprotein in endoplasmic reticulum is not limited by the number of membrane receptor sites. It is therefore necessary to examine alternatives, e.g. the rates of apoprotein and heme synthesis and the turnover of the hemoprotein (27), to understand the mechanisms which control the concentration of microsomal cytochrome \( b_5 \).

The mechanism of electron transport involving these enzymes, their precise molecular arrangement and lateral motility in the membrane, remain as major questions. Preliminary data, which indicate that liposomal preparations will serve as simpler model vesicles for both cytochrome and reductase interactions with lipid bilayers, and antibodies to these proteins, offer at least two possible approaches to their solution.

Acknowledgments—We are indebted to Dr. Peter Goldblatt, in whose laboratory the electron microscopy was done, and to Ronald E. Barry for the preparation of cytochrome \( b_5 \) (detergent).

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The Binding of Cytochrome \( b_2 \) to Liver Microsomes
Philipp Strittmatter, Michael J. Rogers and Lawrence Spatz


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