INCORPORATION IN VITRO OF PURIFIED CYTOCHROME b_5 INTO LIVER MICROSOMAL MEMBRANES

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Received January 9, 1973

Summary: Incubation of liver microsomes with cytochrome b_5, purified after solubilization with detergents, caused an effective incorporation of the cytochrome into the microsomal membranes. The incorporated cytochrome was reducible by NADH and could not be removed by repeated washing with 0.3 M KCl or 10 mM EDTA. The incorporation was much more efficient at 37°C than at 0°C. Trypsin-solubilized cytochrome b_5, which lacks the hydrophobic tail of the native protein, could not be inserted into the membranes. These findings confirm the view that the hydrophobic tail of the cytochrome molecule is responsible for its tight binding to the microsomal membranes.

Previous work in this laboratory (1,2) showed that cytochrome b_5 of liver microsomes, purified after solubilization with detergents (called "detergent-solubilized cytochrome b_5" or simply "d-b_5"), is an amphipathic protein consisting of a hydrophilic head containing the heme and a hydrophobic tail, and that the protein exists as an oligomer in aqueous solution. It was also shown that cytochrome b_5 preparations, purified after solubilization with proteases (3,4), are heme-containing fragments of the native molecule (d-b_5) produced by proteolytic scission of the hydrophobic moiety (1,2). Furthermore, it was suggested that cytochrome b_5 is anchored to the microsomal membranes by a hydrophobic interaction between the tail of the protein and the membrane matrix (1,2). Recently, Spatz and Strittmatter (5) also purified d-b_5 by an improved method and advanced a similar hypothesis.

In the present study, an attempt was made to verify the above hypothesis by examining the incorporation in vitro of the two types of cytochrome b_5 preparations into liver microsomes. It was thus found that d-b_5, when mixed with microsomes, could be effectively incorporated into the membranes in a form which was capable of interacting with microsomal NADH-cytochrome b_5 reductase and resistant to repeated washing with KCl or EDTA. However, trypsin-solubilized cytochrome b_5 (termed "t-b_5"), which lacks the hydrophobic tail,

could not be incorporated to a significant extent.

METHODS

Rabbit liver microsomes, used for most experiments, were prepared in 0.15 M KCl and washed with 0.15 M KCl-10 mM EDTA, pH 7.0, and 0.1 M phosphate buffer, pH 7.5, as described previously (6). The microsomes thus obtained contained 0.9 - 1.0 nmole of cytochrome $\underline{b}_5$ per mg of protein. Rat liver microsomes, used for sucrose density gradient centrifugation experiments, were prepared and washed as above, except that 0.25 M sucrose was used as the homogenizing medium. The method of Spatz and Strittmatter (5) was employed to purify d-$\underline{b}_5$ from rabbit livers. The preparation thus obtained (43 - 46 nmoles of cytochrome $\underline{b}_5$ per mg of protein) was essentially homogeneous upon polyacrylamide gel electrophoresis. It was also free from phospholipids and detergents (5). A t-$\underline{b}_5$ preparation (70 nmoles of cytochrome $\underline{b}_5$ per mg of protein) was purified also from rabbit livers by a modification of the method of Omura and Takesue (6).

Cytochrome $\underline{b}_5$ in solution was estimated spectrophotometrically from the intensity of its oxidized Soret peak at 413 nm (117 $mM^{-1} \cdot cm^{-1}$) (7). Microsomal bound cytochrome $\underline{b}_5$ was determined from the NADH-reduced minus oxidized difference spectrum as described previously (8). Microsomal NADH-cytochrome $\underline{b}_5$ reductase was assayed by measuring its NADH-ferricyanide reductase activity by the method of Mihara and Sato (9). The procedure of Dallner (10) was employed to determine the NADH-cytochrome $\underline{c}$ reductase activity, which is a manifestation of the cooperative action of NADH-cytochrome $\underline{b}_5$ reductase and cytochrome $\underline{b}_5$. Protein was determined by the Lowry method (11), using bovine serum albumin as the standard.

The standard procedure used for incorporation studies was as follows. A mixture (usually 0.3 ml) containing microsomes (11 mg of protein per ml), 50 mM potassium phosphate buffer, pH 7.5, and a desired amount of d-$\underline{b}_5$ or t-$\underline{b}_5$ was incubated at 0°C or 37°C for 15 min, and the incorporation was "stopped" by diluting the mixture at least 10-fold with cold 0.1 M potassium phosphate buffer, pH 7.5. The diluted mixture was centrifuged at 105,000 × g for 60 min (at 0°C), and the microsomal pellet resuspended in 0.1 M phosphate buffer, pH 7.5, was assayed for cytochrome $\underline{b}_5$ and other activities.

RESULTS AND DISCUSSION

As shown in Fig. 1, rabbit liver microsomes could bind large

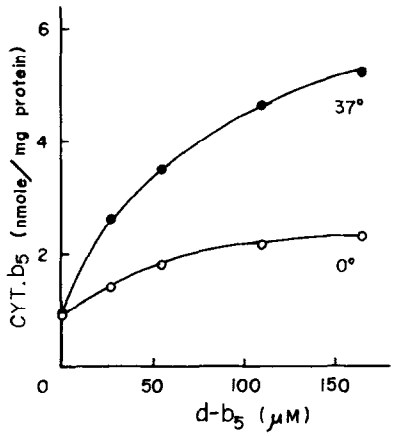

Fig. 1. Incorporation of d-b_5 into rabbit liver microsomes at 0°C and 37°C. The incubation was conducted under the standard conditions using the indicated concentration of d-b_5. The reaction was "stopped" by diluting the mixture 21-fold.

amounts of externally added d-b_5 during incubation at 37°C for 15 min. The extent of binding was dependent on the d-b_5 concentration in the incubation mixture; at the highest concentration tested (165 μM), the microsomes recovered from the mixture contained 5-fold as much cytochrome b_5 as found in the original microsomes. Since the d-b_5 thus bound was detected by its reducibility by NADH, it was certain that at least this portion of d-b_5 was in a form capable of interacting with NADH-cytochrome b_5 reductase of the microsomes. In contrast to the results obtained at 37°C, incorporation was much less efficient when the incubation was conducted at 0°C.

To confirm that the d-b_5 thus bound was actually associated with microsomes, a sucrose density centrifugation study was carried out essentially under the conditions described by Ito and Sato (12). Fig. 2A and 2B show the sedimentation profiles obtained when d-b_5 and rat liver microsomes, respectively, were separately subjected to the centrifugation. Under the centrifugal conditions employed, the d-b_5 still remained afloat near the top of the tube. Both the NADH-ferricyanide and NADH-cytochrome c reductase activities of the microsomes, on the other hand, formed a symmetrical band at a sucrose concentration of about 30 %. When a mixture of d-b_5 and the microsomes, that had been incubated at 37°C for 15 min, was centrifuged, most of the d-b_5 activity added (measured by the NADH-cytochrome c reductase activity reconstituted by adding a sufficient amount of partially purified NADH-cytochrome b_5 reductase (9) essentially as described

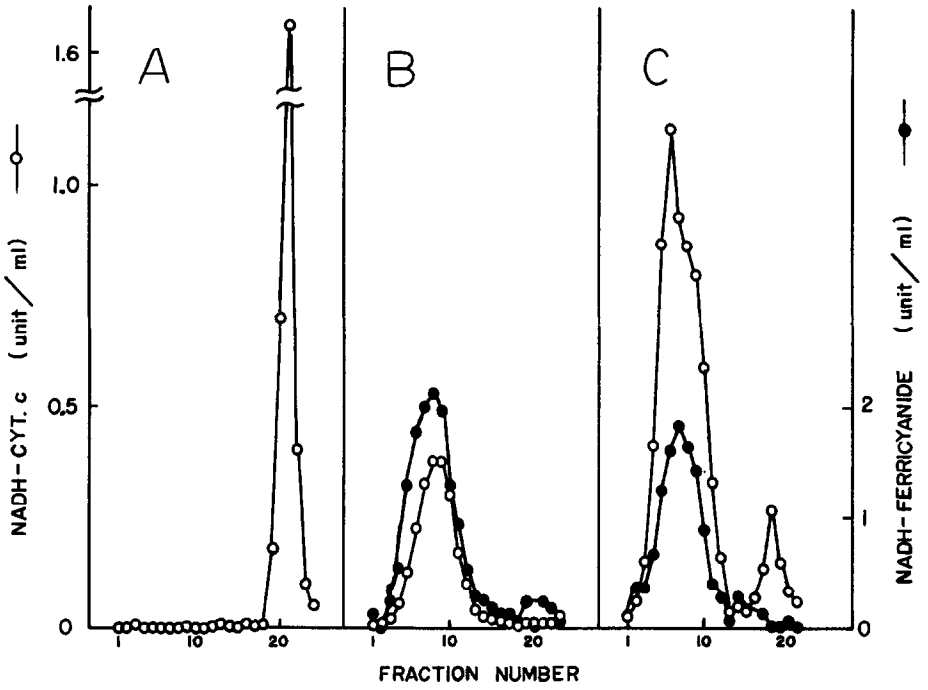

Fig. 2. Demonstration of microsomal localization of incorporated d-b_5. The sample indicated below was diluted 5-fold with 50 mM phosphate buffer, pH 7.5, and 0.3 ml of the diluted sample was layered over 4.4 ml of a linear sucrose concentration gradient (11 - 39 % (w/w) in 50 mM phosphate buffer, pH 7.5). The tube was centrifuged at 155,000 × g for 4 hr (at 2°C), and three drop fractions were collected from the bottom of the tube. Cytochrome b_5 in each fraction was estimated from the NADH-cytochrome c reductase activity reconstituted by adding a sufficient amount of partially purified NADH-cytochrome b_5 reductase (9). NADH-ferricyanide reductase was measured as a microsomal marker. The samples used were: (A) 72.8 μM d-b_5 in 50 mM phosphate buffer, pH 7.5; (B) rat liver microsomes (10.4 mg protein/ml) in the buffer; (C) a mixture of 72.8 μM d-b_5 and microsomes (10.4 mg protein/ml) incubated at 37°C for 15 min.

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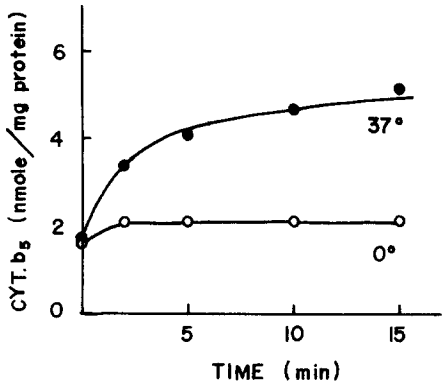
by Shimakata et al. (13)) migrated with the NADH-ferricyanide reductase activity of the microsomes (Fig. 2C). This finding indicated that the d-b_5 had actually been incorporated into the microsomes. It is also seen that a small amount of unbound d-b_5 was recovered near the top of the tube.

Fig. 3 shows the time courses of d-b_5 incorporation at 0°C and 37°C. The considerable amounts of bound d-b_5 observed at time zero were probably due to the binding during the centrifugal separation of the microsomes (note that microsomes become concentrated during



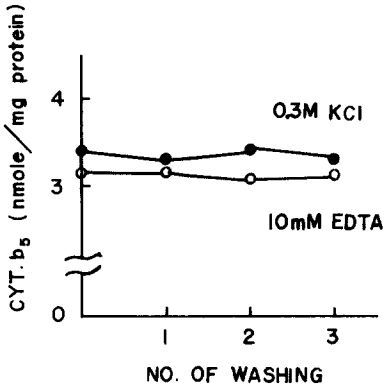


Fig. 3 (left). Time courses of d-b_5 binding to rabbit liver microsomes at 0°C and 37°C. 0.15 ml of 160 μM d-b_5 and 0.15 ml of microsomes (22 mg protein/ml), both in 50 mM phosphate buffer and preincubated at 0°C or 37°C for 1 min, were mixed and the mixture was incubated at the indicated temperature for 2, 5, 10 or 15 min. The reaction was "stopped" by adding 8 ml of cold 0.1 M phosphate buffer, pH 7.5. For the time zero control, the preincubated d-b_5 and microsomes (both 0.15 ml) were separately diluted with 4 ml of the buffer and then mixed.

Fig. 4 (right). Effect of washing on microsomal bound d-b_5. Incorporation of d-b_5 into rabbit liver microsomes was conducted at 37°C under the standard conditions using 50 μM d-b_5. After the incubation, the mixture was diluted 10-fold with 0.1 M phosphate buffer, pH 7.5, and centrifuged at 105,000 × g for 60 min. The cytochrome b_5 content in the resultant pellet was taken as the standard. The pellet was resuspended in 0.1 M phosphate buffer, pH 7.5, containing either 0.3 M KCl or 10 mM EDTA and centrifuged to effect the first washing. The second and third washings were performed in the same way.

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centrifugation). At any rate, it can be seen that the binding was a relatively slow process even at 37°C, approaching completion only after 15 min. This fact suggested that the binding was not due to simple, nonspecific adsorption. Moreover, as shown in Fig. 4, the d-b_5 once bound to microsomes could not be removed by repeated washing with 0.3 M KCl or 10 mM EDTA in 0.1 M phosphate buffer, pH 7.5. The possibility could, therefore, be excluded that the binding was mediated by electrostatic interactions or divalent cations such as Mg^{2+} and Ca^{2+}. Intervention of membrane-bound ribosomes could also be ruled out, because the microsomes used had been stripped of ribosomes by washing with 10 mM EDTA (14). Furthermore, it was not conceivable that d-b_5, being a macromolecule, could penetrate the membranes of microsomal vesicles (cf. ref. 12). It was, therefore, concluded that the bound d-b_5 had been captured by the

TABLE I. LACK OF t-$\underline{b}_5$ BINDING TO RABBIT LIVER MICROSOMES

Incubation Conditions	Specific Activity in Incubated Microsomes		
	Cytochrome $\underline{b}_5$ (nmole/mg)	NADH-Cyt. $\underline{c}$ Reductase (unit/mg)	NADH-Ferricyanide Reductase (unit/mg)
0°C, no addition	0.90	0.58	2.83
0°C, plus 55 μM t-$\underline{b}_5$	0.96	0.60	2.89
0°C, plus 55 μM d-$\underline{b}_5$	1.81	1.18	2.86
37°C, no addition	0.95	0.63	3.00
37°C, plus 55 μM t-$\underline{b}_5$	1.02	0.64	3.02
37°C, plus 55 μM d-$\underline{b}_5$	3.50	1.49	2.60

The incubation was conducted under the standard conditions. The reaction was "stopped" by diluting the mixture 21-fold.

membrane matrix through a hydrophobic interaction.

If a hydrophobic interaction is actually involved in the binding, then it is expected that t-$\underline{b}_5$, which has lost the hydrophobic tail of the native molecule by partial proteolysis (1,2), will not be capable of being incorporated into microsomes. The results shown in Table I indicate that this was in fact the case. Practically no incorporation of t-$\underline{b}_5$ was detected both at 0°C and 37°C under conditions permitting substantial binding of d-$\underline{b}_5$. Table I also shows that the incorporation of d-$\underline{b}_5$ was accompanied by roughly proportional increases in the microsomal NADH-cytochrome $\underline{c}$ reductase activity, again indicating that the bound d-$\underline{b}_5$ could interact with preexisting NADH-cytochrome $\underline{b}_5$ reductase. It is also shown that the NADH-ferricyanide reductase activity of the microsomes was unaffected by d-$\underline{b}_5$ incorporation.

The inability of t-$\underline{b}_5$ to be incorporated into microsomes is a clear indication of the importance of the hydrophobic moiety of the native cytochrome (d-$\underline{b}_5$) in the tight binding of the protein to the microsomal membranes. Although the precise mechanism of this binding is still to be explored, it is likely that the oligomeric form of d-$\underline{b}_5$ undergoes a depolymerization process and the monomer thus formed inserts its hydrophobic tail into the hydrophobic region of the membrane matrix, exposing its hydrophilic head to the surrounding aqueous phase. This picture fits well the previous finding that cytochrome $\underline{b}_5$ is located at the outer surface of microsomal

vesicles (12). Thermodynamically, the state of d-b_5 inserted into the membrane matrix appears to be more stable than the oligomeric state in which a certain number of d-b_5 molecules are held together by an intermolecular hydrophobic interaction. However, it is likely that the depolymerization of the oligomer requires a certain amount of activation energy, as reflected by the more efficient binding at higher temperatures and the relatively slow rate of incorporation. The higher efficiency of binding at 37°C may also be due to a structural alteration in the phospholipid bilayer of the membranes.

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