THE BINDING OF CYTOCHROME b_5 TO PHOSPHATIDYLCHOLINE VESICLES

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SUMMARY

Cytochrome b_5 was isolated from rabbit liver by a detergent procedure and by a proteolytic procedure. Only cytochrome b_5 isolated by the detergent procedure would bind to phosphatidylcholine vesicles and the cytochrome b_5 was not removed by 1 M KCl. The E_o' and visible absorption spectrum of the cytochrome b_5 and its rate of reduction by NADH plus NADH-cytochrome b_5 reductase did not change appreciably upon binding. These data indicate that cytochrome b_5 is bound to phospholipid by a hydrophobic interaction which leaves the heme portion in the aqueous environment.

Previous reports from this and other laboratories have demonstrated the involvement of cytochrome b_5 in fatty acid desaturation (1, 2, 3). It has been shown that lipid is required for the desaturase reaction (1, 4) and also in the component reactions of the microsomal electron transport chain (5, 6). The available data suggest the lipid is interacting with the cytochrome b_5 in these systems.

The present study was undertaken to examine the interaction, if any, of cytochrome b_5 with purified phosphatidylcholine bilayer vesicles. While this work was in progress, two groups reported the binding of cytochrome b_5 to microsomal membranes (3, 7).

METHODS

Detergent-cytochrome b_5 (d-b_5)* was prepared from rabbit liver microsomes by the method of Spatz and Strittmatter (8). Trypsin-cytochrome b_5 (t-b_5)* was isolated from rabbit liver microsomes according to the procedure

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*Cytochrome b_5, isolated by a detergent procedure (detergent-cytochrome b_5), is referred to as d-b_5.

*Cytochrome b_5, isolated by a trypsin procedure (trypsin-cytochrome b_5), is referred to as t-b_5.

of Omura and Takesue (9). Both preparations were found to be essentially homogeneous upon polyacrylamide disc gel electrophoresis. The d-b_5 contained less than 0.26 molecule of phospholipid per molecule of cytochrome and less than one molecule of deoxycholate per molecule of cytochrome. Lipid phosphorus was determined by the method of Chen *et al.* (10). Cytochrome b_5 was assayed spectrophotometrically by measuring the reduced minus oxidized difference spectrum, assuming the extinction difference of the cytochrome between 424 nm and 409 nm to be 185 cm^{-1} mM^{-1} (11).

The oxidation-reduction potential of cytochrome b_5 was determined by a modification of the method of Okada and Okunuki (12). The reaction mixture in an anaerobic gas-tight cuvette contained 250 mM potassium oxalate, 66 mM phosphate buffer pH 7.2, 1 mM ferric ammonium sulfate and approximately 0.18 μM cytochrome b_5. Additions of 0.1 M ferrous ammonium sulfate were made to the sample cuvette through a septum. After each addition and a five minute equilibration at 4.5°, the reduced minus oxidized difference spectrum was recorded between 400 nm and 600 nm. The E_o' was calculated from a plot of log (b_5^{+2}/b_5^{+3}) versus log (Fe^{+2}/Fe^{+3}) using a Hewlett Packard 9100A.

A solution containing 0.18 μM cytochrome b_5, 20 mM Tris acetate- 1 mM EDTA (pH 8.0) was reduced anaerobically by 40 μM NADH and 3.4 nM NADH-cytochrome b_5 reductase. The rate of reduction of the cytochrome was followed at 424 nm.

Phosphatidyl-[methyl-^{3}H] choline was prepared from purified egg phosphatidylcholine via phosphatidic acid (13). The phosphatidic acid was reesterified with [methyl-^{3}H] choline acetate by the procedure of Aneja and Chadha (14). The purified product was homogeneous by thin layer chromatography and had the same fatty acid composition as the original egg phosphatidylcholine. The specific radioactivity of the phosphatidyl-[methyl-^{3}H]choline was 2 μCi per μ mole and was diluted with unlabeled phosphatidylcholine before use to 0.16 μCi per μ mole. Radioactivity was measured in a Packard Tricarb Model 3375 in a Triton-toluen scintillator (15).

RESULTS AND DISCUSSION

As shown in Fig. 1a, d and e, cytochrome b_5 and phosphatidylcholine vesicles equilibrated at different densities during the sucrose density gradient centrifugation. The lipid vesicles floated to near the top of the gradient while both d-b_5 and t-b_5 remained near the bottom of the gradient, where the sample was applied. If the two forms of cytochrome b_5 were incubated individually with the lipid vesicles at 22° for 60 minutes prior to

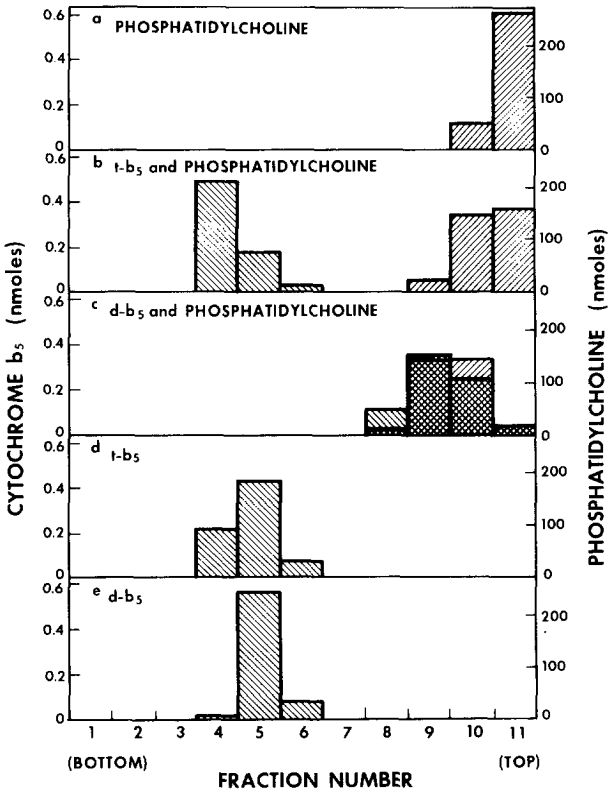

Fig. 1. Interaction of cytochrome b_5 with phosphatidylcholine vesicles. In a final volume of 0.4 ml of 1 M sucrose- 20 mM Tris acetate- 1 mM EDTA (pH8.0) d-b_5 or t-b_5 (1 nmole) were incubated either alone or with phosphatidylcholine vesicles (1 μ mole) as indicated in the Figure . After one hour at 22° the sample was layered on top of the 1.5 M sucrose layer in a stepwise sucrose density gradient consisting of 1 ml aliquots of: 2 M, 1.5 M, 0.75 M, 0.5 M and 0.25 M sucrose- 20 mM Tris acetate- 1 mM EDTA (pH 8.0). The gradients were centrifuged at 134,000 x g (average) for 21.5 hrs. at 4.5° and 0.5 ml fractions were pumped off from the bottoms of the tubes. Cytochrome b_5 (\\\\\) and lipid phosphorus associated with the phosphatidylcholine (/////) were assayed as described under Methods.

centrifugation, the distribution of lipid and cytochrome b_5 shown in Fig. 1b and c was obtained. Whereas a mixture of t-b_5 and phosphatidylcholine vesicles readily separated into their component parts, d-b_5 equilibrated in the same position as the lipid. This indicates that d-b_5, but not t-b_5, will bind to vesicles of phosphatidylcholine. These results parallel those of Strittmatter *et al.* (3) and Enomoto and Sato (7). Both these groups reported that only d-b_5 would bind to microsomal membranes. In order to better quantitate the binding of d-b_5 to phosphatidylcholine vesicles, it was necessary to

improve the sensitivity of the assay for lipid. This was achieved by the synthesis of phosphatidyl-[methyl-^{3}H]choline. We chose to synthesize phosphatidylcholine labeled in the choline moiety to avoid any ambiguities which can arise if the label is in a single fatty acyl chain. When d-b_5 (0.5 nmoles) was incubated at 37° for 30 minutes with increasing concentrations of vesicles made from phosphatidyl-[methyl-^{3}H]choline (50- 800 nmoles phosphatidylcholine), the results shown in Fig. 2a-g were obtained. At low ratios of lipid to d-b_5 all the lipid and d-b_5 remained where the sample was applied. As the ratio increased, the d-b_5 and associated lipid equilibrated at decreasing sucrose densities. In none of the experiments was there a constant ratio of d-b_5 to lipid across the density gradient. This could indicate there exists some degree of cooperativity in the binding reaction or, alternatively, the existence of vesicle populations with different affinities. In the latter respect it should be emphasized that although the lipid vesicles were prepared by a procedure which produces lipid vesicles of homogeneous size (16), this present lipid vesicle preparation was not rigorously characterized.

From Fig. 2 it is obvious that no preferred binding stoichiometry exists. The results in Fig. 2b demonstrate that the number of d-b_5 molecules per vesicle of 2678 phosphatidylcholine molecules (16) may vary from four to less than one. In contrast, in Fig. 2b the ratio may be as high as 30. It should be emphasized that the numbers of d-b_5 molecules per vesicle just referred to are quoted for comparison only. We have no evidence that the lipid is still in a vesicular form, containing 2678 molecules of phosphatidylcholine, in any of the binding experiments. The numbers of molecules of d-b_5 per vesicle in Fig. 2b are much lower than the number of molecules thought to comprise the polymer of d-b_5 as it exists in aqueous solution (8). This probably indicates that depolymerization of the d-b_5 has occurred before it binds to the vesicle.

The strong binding of d-b_5 to phosphatidylcholine vesicles suggests that a different form of association exists between d-b_5 and lipid as compared to that between cytochrome c and lipid. In the latter instance cytochrome c is unable to bind to the zwitterionic phosphatidylcholine but will bind to negatively charged lipid (17). In unpublished experiments we have found similar binding of d-b_5 to both the zwitterionic phosphatidylcholine vesicles and to negatively charged vesicles. It was also found that 1 M KCl, when added to d-b_5 which had already been bound to vesicles, was not able to release the bound d-b_5. Upon sucrose density gradient centrifugation both d-b_5 and lipid were found in fractions 5, 6 and 7 (cf Fig. 2) in a pattern identical to Fig. 2b. These results suggest the binding of the d-b_5 is hydrophobic rather than electrostatic in nature.

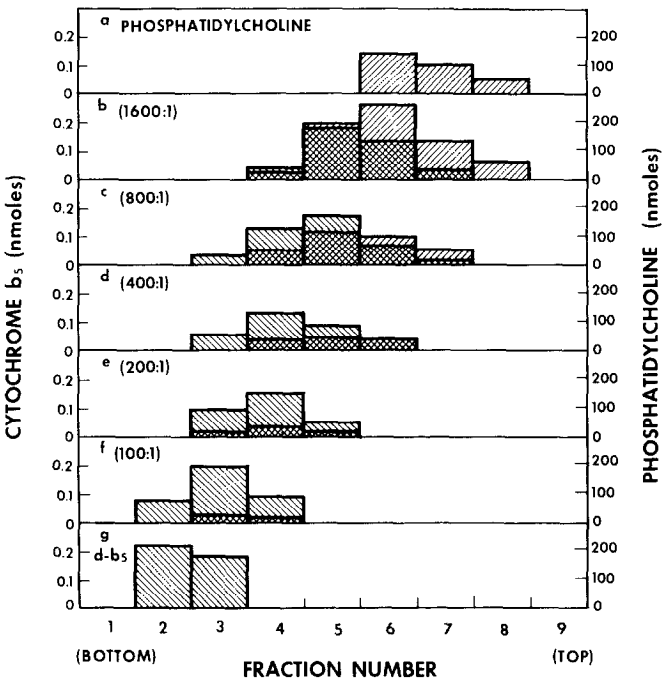

Fig. 2. The interaction of d-b_5 with increasing amounts of phosphatidylcholine vesicles. Aliquots of d-b_5 (0.5 nmoles) were incubated in 0.2 ml of 20 mM Tris acetate- 1 mM EDTA (pH 8.0) at 37° for 30 minutes: (g) alone or with (f) 50 (e) 100 (d) 200 (c) 400 or (b) 800 nmoles phosphatidyl-[methyl-^{3}H]choline. Each sample was diluted with 0.2 ml 2 M sucrose- 20 mM Tris acetate- 1 mM EDTA (pH 8.0) and layered on top of 1 ml of 1.5 M sucrose- 20 mM Tris acetate- 1 mM EDTA (pH 8.0). Successive 1 ml aliquots of 0.75 M, 0.5 M, 0.35 M and 0.25 M sucrose in 20 mM Tris acetate- 1 mM EDTA (pH 8.0) were layered on top of the samples. The sucrose density gradients were centrifuged at 134,000 x g (average) for 18 hours at 0° and 0.6 ml fractions were pumped off from the bottom of the tubes. Cytochrome b_5 (\\\\) and radioactivity associated with the phosphatidylcholine (////) were determined as described under Methods. The ratios in parenthesis refer to the original ratio of phosphatidylcholine molecules to cytochrome b_5 molecules in each incubation mixture.

We attempted to examine the orientation of the d-b_5 in the phospholipid by monitoring some properties of the d-b_5 which are associated with the heme region. As shown in Table 1 the E_o' of t-b_5 and d-b_5 are quite similar, as reported by others (18), and little change occurs upon the addition of phosphatidylcholine vesicles. We confirmed that the d-b_5 was bound to the lipid under the conditions of the E_o' determination by sucrose density gradient centrifugation. The lipid and d-b_5 equilibrated in fractions 5, 6 and 7 in a pattern very similar to Fig. 2b.

Although these results support the suggestion that the hydrophobic

Table I. Effect of phosphatidylcholine vesicles on the E'_o of cytochrome b_5.

Components	E'_o	Correlation Coefficient
d-b_5	0.031	0.999
t-b_5	0.025	0.999
d-b_5 plus phosphatidylcholine	0.025	0.999
t-b_5 plus phosphatidylcholine	0.029	0.999
microsomes	0.027*	0.999
microsomes	0.022+	0.999

*Using NADH to completely reduce the cytochrome b_5.
+Using dithionite to completely reduce the cytochrome b_5.

tail of the d-b_5, rather than the heme portion, is interacting with the lipid vesicles, they do not explain the large difference between the literature values of isolated t-b_5 and cytochrome b_5 in intact microsomes 0.02V (19) versus -0.12V, respectively (20). Surprisingly, when we measured the E'_o of cytochrome b_5 in intact microsomes by the ferric oxalate technique, it too had a similar value to t-b_5 and d-b_5 (Table 1). Some uncertainty is introduced by the ferric oxalate technique as a procedure must be obtained for fully reducing the cytochrome b_5 at the end of the titration experiment. NADH would tend to under reduce and dithionite would also reduce cytochrome P_{450}. In spite of these uncertainties , our values of 0.027V and 0.022V for the two reducing agents are very different from the literature value of -0.12V. Our value for the E'_o of cytochrome b_5 in intact microsomes is, however, precisely what one would predict from the values for d-b_5 and d-b_5 plus phosphatidylcholine shown in Table 1.

In reporting this discrepancy between our values for the E'_o of cytochrome b_5 and those previously published, it should be noted that we measured the E'_o by the ferric oxalate technique, whereas others working with intact microsomes have used the dye method. Although the dye and ferric oxalate techniques may give identical results with soluble systems, it is possible that a membrane system could yield different results by the two procedures. This could result from a partitioning of the dye into the lipid phase or from the alteration of the E'_o of the dye by the hydrophobic environment of the lipid. The same reasoning could be applied to the results of Kawai *et al.* (21), who reported that the E'_o of cytochrome b_5 in microsomes was raised from -0.13V to 0.02V only by a procedure which destroyed the lipid. Kawai *et al.* also re-

ported that the E_o' of t-b_5 was lowered to -0.12V by total microsomal lipid. This latter observation is surprising in view of the inability of t-b_5 to bind to microsomes (3, 7) or to zwitterionic or negatively charged lipid vesicles (Fig.1 and unpublished observations).

The visible spectrum of d-b_5 in both reduced and oxidized states was identical in the presence or absence of phosphatidylcholine vesicles but there was some indication that the lipid vesicles increased the intensity of the absorbance in the ultraviolet region. Measurements in the ultraviolet are hampered by the presence of the lipid vesicles.

The rates of reduction of d-b_5 by NADH plus NADH-cytochrome b_5 reductase were measured at 4.5° and found to be 0.16 nmoles per minute. This was decreased slightly to 0.12 nmoles per minute when the d-b_5 was bound to lipid. Again these results suggest the heme portion of the d-b_5 is quite accessible when the d-b_5 is bound to lipid. The NADH-cytochrome b_5 reductase used was isolated by lysosomal digestion (22) and it does not bind to the lipid vesicles, as determined by sucrose density gradient centrifugation.

This communication supports the suggestions of Strittmatter *et al.* (3) and Enomoto and Sato (7) that the incorporation of d-b_5 into microsomal membranes can be accounted for by an association of the hydrophobic "tail" of the d-b_5 with the phospholipid of the microsomal membrane.

The mechanism of the interaction of d-b_5 with lipid vesicles, the extent of the penetration of the d-b_5 into the lipid membrane and the physiological significance of the process must await the detailed studies with vesicles of different composition which are in progress.

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REFERENCES

1. Holloway, P.W., and Katz, J.T. (1972) Biochemistry 11, 3689 - 3696.
2. Shimakata, T., Mihara, K., and Sato, R. (1972) J. Biochem. (Tokyo) 72, 1163 - 1174.
3. Strittmatter, P., Rogers, M.J., and Spatz, L. (1972) J. Biol. Chem. 247, 7188 - 7194.
4. Jones, P.D., Holloway, P.W., Peluffo, R.O. and Wakil, S.J. (1969) J. Biol. Chem. 244, 744 - 754.

5. Jones, P.D. and Wakil, S.J. (1967) 242, 5267 - 5273.
6. Rogers, M.J. and Strittmatter, P. (1973) J. Biol. Chem. 248, 800 - 806.
7. Enomoto, K. and Sato, R. (1973) Biochem. Biophys. Res. Commun. 51, 1 - 7.
8. Spatz, L. and Strittmatter, P. (1971) 68, 1042 - 1046.
9. Omura, T. and Takesue, S. (1970) J. Biochem. (Tokyo) 67, 249 - 257.
10. Chen, P.S., Jr., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756 - 1758.
11. Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370 - 2378.
12. Okada, Y. and Okunuki, K. (1969) J. Biochem. (Tokyo) 65, 581 - 596.
13. Dawson, R.M.C. and Hemington, N. (1967) Biochem. J. 102, 76 - 86.
14. Aneja, R. and Chadha, J.S. (1971) Biochim. Biophys. Acta 248, 455 - 457.
15. Patterson, M.S. and Greene, R.C. (1965) Anal. Chem. 37, 854 - 857.
16. Huang, C. (1969) Biochemistry 8 344 - 352.
17. Kimelberg, H.K., Lee, C.P., Claude, A. and Mrena, E. (1970) J. Membrane Biol. 2 235 - 251.
18. Ito, A. and Sato, R. (1968) J. Biol. Chem. 243, 4922 - 4923.
19. Velick, S.F. and Strittmatter, P. (1956) J. Biol. Chem. 221, 265 - 275.
20. Strittmatter, C.F. and Ball, E.G. (1952) Proc. Nat. Acad. Sci. U.S. 38 19-25.
21. Kawai, Y., Yoneyama, Y. and Yoshikawa, H. (1963) Biochim. Biophys. Acta 67, 522 - 524.
22. Holloway, P.W. (1971) Biochemistry 10, 1556 - 1560.