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BINDING OF CYTOCHROME b_5 TO CHOLESTEROL-CONTAINING PHOSPHATIDYLCHOLINE VESICLES

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Summary

Cytochrome b_5 was found to bind readily to sonicated vesicles containing as much as 0.8 mol cholesterol per mol egg phosphatidylcholine. This observation conflicts with the suggestion of Enomoto and Sato ((1977) Biochim. Biophys. Acta 466, 136–147) that cholesterol prevents binding of this protein to erythrocyte membranes.

Cytochrome b_5 is an intrinsic membrane protein that is isolated from microsomes by detergent extraction [1–3]. The purified protein, which is soluble in water, binds spontaneously to phospholipid vesicles and to a variety of natural membranes. There are, however, two possible exceptions to this nonspecificity of binding; Dufourcq et al. [4] have presented evidence which suggests that cytochrome b_5 does not bind to negatively charged phospholipid vesicles, and Strittmatter et al. [5] have found that the protein does not bind to erythrocyte membranes. We are particularly interested in the specificity of binding since, as we have recently shown, cytochrome b_5 exchanges rapidly between sonicated vesicles of egg phosphatidylcholine [6]. If this exchange occurs in vivo, the distribution of cytochrome b_5 among the various cellular membranes might well be determined by the different compositions of the membranes.

Enomoto and Sato [7] have recently suggested that the lack of binding to erythrocytes is due to the high levels of cholesterol in the red blood cell membrane. Their evidence to support this conclusion is based on two observations: (1) the binding of cytochrome b_5 to multilamellar liposomes composed of egg phosphatidylcholine and cholesterol decreased as the

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mole ratio of cholesterol was increased, and (2) the binding of cytochrome b_5 to erythrocyte ghosts was increased by partially depleting the ghosts of cholesterol.

This report presents evidence which suggests that this conclusion may be tenuous. These authors used multilamellar liposomes as a measure of the binding of cytochrome b_5 to cholesterol-containing membranes. These liposomes are heterogeneous with respect to size and surface area, and these properties may well vary with the method of preparation and composition of lipid (for a general review, see ref. 8). Bangham et al. [9] have shown that liposomes formed from egg phosphatidylcholine have about 10% of the lipid bilayer exposed to the external medium. Recently, Schullery and Miller [10] have shown that the available surface for binding UO_2^{2+} decreases as the liposome concentration increases; from these results, they suggest that multilamellar vesicles may undergo a reversible aggregation.

Because of these uncertainties, we have repeated the cytochrome b_5-binding experiments with sonicated unilamellar vesicles containing 0—0.8 mol cholesterol per mol egg phosphatidylcholine*. The mole ratio of protein to phospholipid in the incubation mixture was varied from 0.4:1000 to 1:1000. Binding of cytochrome b_5 to the vesicles was determined by sucrose density gradient centrifugation [12]. In all cases, less than 4% of the protein remained unbound; a representative result is shown in Table I for vesicles

TABLE I

BINDING OF CYTOCHROME b_5 TO SONICATED VESICLES CONTAINING 0.8 mol CHOLESTEROL PER mol EGG PHOSPHATIDYLCHOLINE

Cytochrome b_5 (1.82 nmol) and vesicles (4.64 μmol with respect to egg phosphatidylcholine) were incubated in 0.5 ml 50 mM Tris·Cl/1 mM EDTA, pH 8.0 for 30 min at 37°C under argon. 0.5 ml of 2.0 M sucrose solution was then added to bring the final sucrose concentration to 1.0 M, and the sample was layered over 1.0 ml of 2 M sucrose in a 9.0 ml centrifuge tube. 5 ml 0.6 M sucrose, and 1.0 ml buffer were layered over this in sequence. Sucrose solutions contained the same buffer as the sample. Following centrifugation for 15 h at 96 592 × g at 4°C, fractions were collected from the bottom of the tube. Sucrose was determined from the refractive index; cytochrome b_5 was determined from a reduced minus oxidized difference spectrum [18], and lipid phosphorus was determined by a modification of the Bartlett procedure [19]. Cytochrome b_5 (detergent extracted) was isolated from rabbit livers by the method of Ozols [3]; egg phosphatidylcholine was isolated from hen egg-yolks by alumina- and silic acid-column chromatography [19] and cholesterol was purified by the method of Schwenk and Werthessen [20].

Homogeneous populations of vesicles were prepared by the methods of Huang [21] or Barenholz et al. [22].

Fraction	Cytochrome b_5 (μM)	Lipid phosphorous (mM)	% Sucrose
1	0.030	<0.03	44.6
2	0.035	<0.03	34.8
3	0.012	<0.03	28.6
4	0.036	0.186	24.2
5	0.106	0.429	21.0
6	0.167	0.555	18.6
7	0.243	0.626	16.0
8	0.352	0.825	13.4
9	0.492	1.17	11.2
(Top) 10	0.447	1.61	10.4

*The structures and properties of these vesicles have been carefully characterized by Newman and Huang [11].

containing 0.8 mol cholesterol per mol egg phosphatidylcholine. The ratio of protein to lipid is not the same in each fraction because cytochrome b_5 exchanges between vesicles [6]. This experiment shows that cholesterol does not prevent the binding of cytochrome b_5 to sonicated vesicles under conditions where the ratio of protein to lipid is small.

Although this conclusion apparently conflicts with those of Enomoto and Sato [7], we believe that there really is no discrepancy between their data and ours. These investigators found that when cytochrome b_5 was incubated with egg phosphatidylcholine multilamellar vesicles (without cholesterol) in a ratio of 3 mol protein per 1000 mol phospholipid, only 50% of the protein bound. Their assay for binding consisted of sedimenting the multilamellar vesicles, then analyzing the supernatant and pellet for cytochrome b_5. This incomplete binding is important since it has been shown that binding to sonicated vesicles does not reach saturation until the ratio of cytochrome b_5 to lipid is in the range of 1:11 [13] to 1:35 [14]. There are three possible explanations for the fact that only half the protein bound.

First, it is possible that the small amount of external surface in the multilamellar vesicle preparation had already become saturated with protein when half the protein had bound. Second, it is possible that the fraction of protein which did not sediment was actually bound to small vesicles. Even if these small vesicles comprise a very small fraction of the total lipid, their contribution to the total surface area available for binding could be significant. Third, it is conceivable that the intrinsic binding constant and/or rate constant for binding of cytochrome b_5 to multilamellar vesicles is very much smaller than it is for small vesicles; perhaps the nearly planar surface of the multilamellar vesicles is more difficult for the protein to penetrate than is the 'strained' surface of vesicles with a small radius of curvature [15].

We have performed some experiments with multilamellar vesicles to be described shortly, in order to decide which of these possibilities apply. By combining our results with those of Enomoto and Sato, and performing some simple calculations, we draw the following conclusions: (1) multilamellar vesicle preparations may well contain some small (nonsedimenting) vesicles; (2) cytochrome b_5 does bind effectively to the available surface of multilamellar liposomes; (3) cytochrome b_5 probably does bind more readily to sonicated vesicles than to an equal external surface of multilamellar liposomes. Our experiments were performed as follows. Multilamellar vesicles were prepared by the method of Bangham [16], in 50 mM Tris·Cl, 1 mM EDTA, pH 8.0 then centrifuged in a 5 ml volume for 30 min at 105 000 × g at 4°C. The buffer and conditions of centrifugation are the same as those used by Enomoto and Sato [7]. Analysis of the supernatant showed that 1–2% of the phospholipid did not sediment, indicating that some small vesicles are indeed present. It should be pointed out, however, that 'small' vesicles obtained in a multilamellar vesicle preparation may not be identical to those prepared by sonication. When cytochrome b_5 (2.6 μM) was incubated with multilamellar vesicles (5 mM in lipid phosphorus) 68% of the protein sedimented with the bulk of the lipid. The ratio of protein to lipid in the supernatant was 1 mol protein per 120 mol lipid, which suggests that

the vesicles were not saturated with protein. If we assume that approximately 10% of the surface of sedimented lipid is available for binding*, the mol ratio of cytochrome b_5 to lipid on the external surface was about 1:283. Finally, we incubated cytochrome b_5 with a mixture containing multilamellar vesicles and sonicated vesicles in a ratio of 10:1 by total lipid phosphorus. We estimate, therefore, that the external multilamellar vesicle surface area was approximately equal to the vesicle surface area. Under these conditions, we found that more than 99% of the cytochrome b_5 remained with the vesicles in the supernatant; if our estimates of the surface areas is correct this result indicates that the protein binds more readily to the sonicated unilamellar vesicle than to multilamellar vesicles. We do not know if this represents a difference in the affinity constants or in the rate constants of binding. We are investigating this problem further.

By applying the same treatment to the results of Enomoto and Sato [7], with egg phosphatidylcholine, we may calculate that the mole ratio of protein to lipid on the external surface of the sedimented lipid is about 1:60. This is very efficient binding, since as indicated earlier, this number is not far from one of the values reported for saturation of sonicated vesicles. The remaining protein may be unable to bind because the multilamellar vesicle surface is saturated, or alternatively it may be bound to non-sedimenting lipid. Unfortunately, these authors did not indicate that they assayed the supernatant for phospholipid.

As the mol ratio of cholesterol to phosphatidylcholine was increased to 0.8, the amount of sedimented cytochrome b_5 decreased from 50 to 20%. From the previous discussion, it should be clear that this modest drop in binding could result from a smaller external surface of multilamellar vesicles containing cholesterol or from a greater fraction of non-sedimenting lipid. Even if these two parameters are the same for all the multilamellar vesicle preparations, the fact that 20% of the protein does bind indicates to us that cytochrome b_5 does, in fact, bind readily to egg phosphatidylcholine-cholesterol bilayers, as we have shown to be the case with small vesicles. These results certainly cannot explain the observation of Strittmatter et al. [5] that no cytochrome b_5 binds to the membrane of the red blood cell.

Finally, we would like to comment on Enomoto and Sato's observation [7] that more cytochrome b_5 binds to cholesterol-depleted ghosts than to untreated ghosts. These authors expressed their own reservations about interpreting this result because the depleted ghosts become leaky during the high temperature incubation with cytochrome b_5. Although, following the incubation, they were able to isolate 'non-leaky' ghosts which contained cytochrome b_5, we suggest that the non-leaky ghosts may have

*As indicated earlier, Schullery and Miller [10] have shown that the external surface area varies with the concentration of liposome. However, they determined the surface area at only two concentrations of phospholipid, 0.052 and 1.34 mM. Since we cannot assume that the external surface area varies linearly with lipid concentration, we cannot extrapolate their values to the much higher concentrations of lipid that we and Enomoto and Sato [7] have employed. For multilamellar vesicles at 1.34 mM in lipid phosphorus, Schullery and Miller estimate that the external monolayer surface is 10% of the total surface. Unfortunately, Bangham et al. did not report the concentration of lipid they used. We have therefore used the 10% figure as the best approximation for our preparations.

resulted from leaky ghosts which resealed during the incubation period.

There is perhaps another explanation, albeit highly speculative. It has been shown that depleting red blood cells of cholesterol causes the cells to change shape (Ginsburg, H., unpublished results and Cooper et al. [17]). It is possible that this alteration in morphology is accompanied by local alterations in the composition of the membrane, and it is to one or more of these unusual domains that cytochrome b_5 binds.

In conclusion, we feel that there is as yet no simple explanation for the fact that cytochrome b_5 does not bind to erythrocyte membranes.

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