Biochimica et Biophysica Acta, 550 (1979) 357—361

BBA Report

BBA 71366

INHIBITION OF THE BINDING OF CYTOCHROME b_5 TO PHOSPHATIDYLCHOLINE VESICLES BY CHOLESTEROL

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(Received August 1st, 1978)

Key words: Cytochrome b_5 binding; Cholesterol; (Phosphatidylcholine vesicle)

Summary

The binding of cytochrome b_5 to single-walled liposomes of egg phosphatidylcholine was inhibited by the presence of cholesterol in the lipid bilayer under conditions where a limited amount of liposomes was incubated with the cytochrome. Since similar conditions seem to apply for the binding of cytochrome b_5 to erythrocyte ghosts, this observation supports the conclusion of Enomoto and Sato (Enomoto, K. and Sato, R. (1977) Biochim. Biophys. Acta 466, 136—147) that the localization of cholesterol on the outer surface of the ghost membrane prevents the binding of cytochrome b_5 to this surface. The finding reported by Roseman et al. (Roseman, M.A., Holloway, P.W. and Calabro, M.A. (1978) Biochim. Biophys. Acta 507, 552—556) that cholesterol did not prevent the cytochrome binding to phosphatidylcholine liposomes in the presence of a large excess of liposomes could be confirmed in the present study, but this does not contradict the abovementioned conclusion.

Cytochrome b_5, an integral membrane protein, has been purified from liver microsomes [1—4] and shown to be capable of binding to a variety of natural and artificial lipid bilayer membranes [5—10]. Strittmatter et al. [5] have, however, reported that it is unable to bind to the membrane of human erythrocytes. Enomoto and Sato [10] have later shown that the cytochrome can actually bind to the inner (cytoplasmic) surface, though not to the outer surface, of the membrane of erythrocyte ghosts, and interpreted this asymmetric binding as due to the preferential localization of cholesterol in the outer leaflet of the bilayer of the ghost membrane [11]. The evidence presented by Enomoto and Sato [10] in support of this conclusion includes, among other things, the observation that the binding of cytochrome b_5 to multilamellar liposomes consisting of egg phosphatidylcholine and cholesterol decreases as the molar ratio of cholesterol to phosphatidylcholine is increased.

However, Roseman et al. [12] have recently reported that cytochrome b_5 can bind almost quantitatively to sonicated single-walled liposomes containing 0—0.8 mol cholesterol per mol egg phosphatidylcholine, and concluded that cholesterol does not prevent the binding of cytochrome b_5 to phosphatidylcholine vesicles and, therefore, to the erythrocyte membrane. They have also argued that the apparently conflicting results obtained with multilamellar liposomes [10] can be explained by taking the following three possibilities into consideration: (1) only a small portion of the lipid bilayer surface of multilamellar liposomes is exposed to the external medium [13] and thus available for cytochrome b_5 binding; (2) multilamellar liposomes contain some small vesicles which can bind cytochrome b_5 effectively but are not sedimentable under the centrifugal conditions used by Enomoto and Sato [10] to recover the bound cytochrome; and (3) inclusion of cholesterol into multilamellar phosphatidylcholine liposomes either decreases the surface available for cytochrome b_5 binding or increases the amount of nonsedimenting vesicles.

By using single-walled liposomes, therefore, we have reexamined the effect of cholesterol on the binding of cytochrome b_5 to phosphatidylcholine vesicles in order to assess the validity of the aforementioned conclusion of Enomoto and Sato [10]. Single-walled liposomes containing 0—0.8 mol cholesterol per mol egg phosphatidylcholine were prepared as described by Barenholtz et al. [14]. Upon Sepharose 4B gel chromatography these liposomes were eluted as a symmetrical band and their elution position exactly corresponded to that of 'fraction II', which has been carefully characterized by Newman and Huang [15] to consist of unilamellar phosphatidylcholine-cholesterol liposomes. Cytochrome b_5 was purified from rabbit liver microsomes by the method of Spatz and Strittmatter [3]. Incubation of cytochrome b_5 with liposomes was performed under nitrogen at 0°C for 70 h, though Roseman et al. [12] incubated the mixture at 37°C for 30 min. This was because we noticed that the binding of cytochrome b_5 to phosphatidylcholine vesicles at 37°C was rather complicated, as will be reported elsewhere. The incubation time of 70 h was chosen based on a report that the cytochrome binding to phosphatidylcholine vesicles at 4°C took about 48 h for completion [8]. The formation of cytochrome b_5-liposome complex was monitored by sucrose density gradient centrifugation as described in the legend to Fig. 1. The floatation, rather than sedimentation, procedure was employed for the gradient centrifugation to obtain better separation of the cytochrome-liposome complexes from unbound cytochrome b_5, which should remain at the bottom of the tube in the floatation procedure.

When a large excess of liposomes were incubated with a small amount of the cytochrome, e.g. at a molar ratio of cytochrome b_5 to phosphatidylcholine of 1 : 1000 (see ref. 12), the cytochrome was almost quantitatively incorporated into the liposomes containing as much as 0.8 mol cholesterol per mol phosphatidylcholine (data not shown), in confirmation of the results of Roseman et al. [12]. However, such a condition does not seem to reflect that under which cytochrome b_5 binding to the ghost membrane was examined. According to the data of Enomoto and Sato [10], the inner surface of the ghost membrane could bind about 2 nmol cytochrome b_5 per mg ghost protein upon incubation at 37°C for 20 min in the presence of an amount of

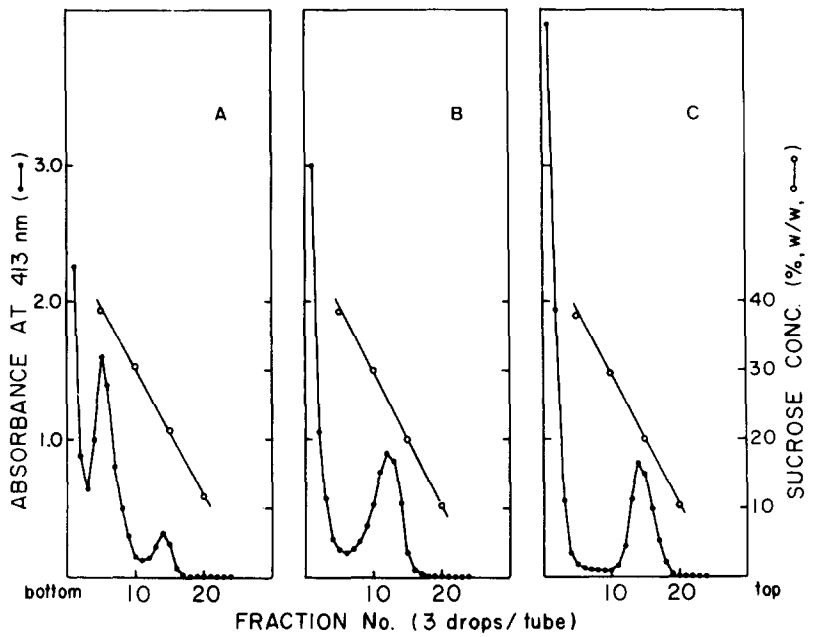

Fig. 1. Binding of cytochrome b_5 to single-walled liposomes containing 0 (A), 0.4 (B), and 0.8 mol (C) cholesterol per mol phosphatidylcholine. Cytochrome b_5 (735 μM) and liposomes (8.07 mM with respect to phosphatidylcholine) were incubated in 0.1 ml of 50 mM Tris-HCl (pH 8.0)/1 mM EDTA for 70 h at 0° C under nitrogen. The incubated sample was mixed with 0.1 g of sucrose and placed at the bottom of a centrifugal tube. 4.9 ml of a linear sucrose concentration gradient from 5 to 45% (w/v) in 50 mM Tris-HCl (pH 8.0)/1 mM EDTA were layered over the sample, and then centrifuged at 50 000 rev./min for 20 h at 2° C in an RPS 50-II rotor of a Hitachi 55P centrifuge. After centrifugation, 3-drop fractions were collected from the bottom of the tube. Each fraction was mixed with 0.5 ml water and assayed for cytochrome b_5 by measuring the absorbance at 413 nm. The sucrose concentration was determined by using an Abbe refractometer.

cytochrome b_5 that was about 50-fold larger than that finally bound. This indicates that in these binding experiments the number of cytochrome b_5-binding sites on the ghost membrane is very limited compared with the amount of cytochrome b_5 added. Therefore, it seemed that the effect of cholesterol on cytochrome b_5 binding to phosphatidylcholine vesicles should be studied in the presence of a limiting amount of liposomes.

From this point of view, we have conducted another series of experiments in which the molar ratio of cytochrome b_5 to phosphatidylcholine in the incubation mixture was kept to about 1 : 11. As shown in Fig. 1, incubation of cytochrome b_5 at this molar ratio with liposomes containing 0, 0.4, and 0.8 mol cholesterol per mol phosphatidylcholine resulted in the formation of the cytochrome-liposome complexes which, upon sucrose density gradient centrifugation, were located at sucrose concentrations of 38.5, 25.5, and 21.0% (w/w), respectively. In the case of liposomes containing no cholesterol, another minor band of the cytochrome-liposome complex was reproducibly formed at about 22% sucrose for unknown reasons. At any rate, the main band of cytochrome b_5-liposome complexes became lighter as the cholesterol content in the liposomes was increased. This was mainly due to the increase in cholesterol content but partly due to the decrease in the amount of cytochrome b_5 bound to the liposomes. Fig. 1 also shows clearly that the

amount of unbound cytochrome b_5 remaining at the bottom of the tube increased as the cholesterol content in the liposomes was increased, indicating that under the conditions employed cholesterol did prevent the binding of cytochrome b_5 to single-walled phosphatidylcholine liposomes.

Each of the main peaks of cytochrome b_5-liposome complexes in Fig. 1 was then isolated, dialyzed thoroughly against 0.1 M NH_4HCO_3, and analyzed for cytochrome b_5, phosphatidylcholine, and cholesterol. In Fig. 2 is plotted the molar ratio of cytochrome b_5 to phosphatidylcholine in each preparation against the molar ratio of cholesterol to phosphatidylcholine. As can be seen, each complex retained practically the same cholesterol to phosphatidylcholine ratio as in the original liposomes. It is also clear that inclusion of cholesterol into the composition of single-walled phosphatidylcholine liposomes actually inhibited the binding of cytochrome b_5. Fig. 2 shows further that the binding of cytochrome b_5 did not decrease linearly as a function of the cholesterol content in liposomes. suggesting that the inhibition of binding was not simply due to the occupation of cytochrome b_5-binding sites by cholesterol. Huang and coworkers [15,16] have shown that in egg phosphatidylcholine liposomes containing high concentrations of cholesterol there occurs asymmetric distribution of cholesterol in favor of the inner layer of bilayers. It is likely that such asymmetric distribution of cholesterol is responsible for the nonlinear decrease of cytochrome b_5 binding as a function of cholesterol concentration, though further work is needed to reach a decisive conclusion.

The results described above indicate clearly that the binding of cytochrome b_5 to single-walled liposomes of egg phosphatidylcholine is in fact inhibited by the presence of cholesterol when the incubation mixture contains a limited amount of liposomes relative to that of cytochrome b_5. Since this condition reflects more precisely what holds for cytochrome b_5 binding to the

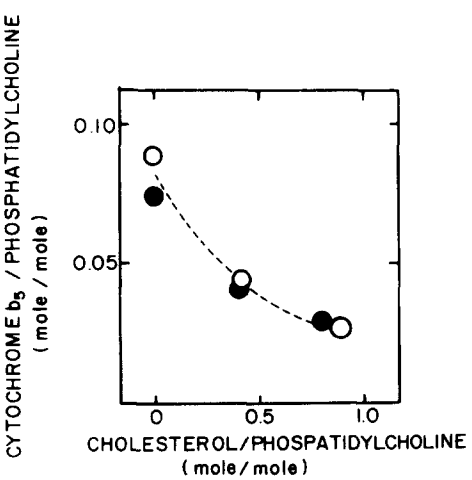

Fig. 2. A plot of the cytochrome b_5/phosphatidylcholine ratio against the cholesterol/phosphatidylcholine ratio of the isolated cytochrome b_5-liposome complexes. The main peak of cytochrome b_5-liposome complexes in Fig. 1 was collected and dialyzed against 0.1 M NH_4HCO_3. Then cytochrome b_5 was determined from the absorbance at 413 nm assuming a molar extinction coefficient of $1.17 \cdot 10^5$ [3]. Phospholipid phosphorus was determined by the method of Bartlett [17] after sulfuric acid digestion. Cholesterol was estimated as described by Zlatkis et al. [18]. The results of two separate experiments are shown (○ and ●).

ghost membrane as discussed above, these results appear to support the conclusion of Enomoto and Sato [10] that the asymmetric binding of the cytochrome to the ghost membrane is due to the preferential localization of cholesterol in the outer leaflet of the bilayer of the membrane, in spite of the argument to the contrary by Roseman et al. [12], who chose unsuitable binding conditions to draw a correct conclusion. Their results, which we could confirm, can be explained by assuming that in the presence of a large excess of liposomes there remain a sufficient number of cytochrome b_5-binding sites even if they contain a high level of cholesterol.

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