Cholesterol Distribution between the Two Halves of the Lipid Bilayer of Human Erythrocyte Ghost Membranes*

(Received for publication, September 6, 1978, and in revised form, October 10, 1978)

Lea Blau and Robert Bittman

From the Department of Chemistry, Queens College of The City University of New York, Flushing, New York 11367

SUMMARY

The binding of filipin with cholesterol in sealed and unsealed ghosts prepared from human erythrocytes and in right-side-out and inside-out vesicles prepared from ghosts follows second order kinetics (first order in each reactant). The second order rate constant of interaction of filipin with cholesterol, determined by stopped flow measurements of the initial rate, is slower in sealed ghosts than in unsealed ghosts by a factor of 2.0, whereas identical rate constants were obtained with right-side-out and inside-out vesicles. These results suggest that the cholesterol accessible to rapid reaction with filipin is distributed symmetrically between the inner and outer halves of the lipid bilayer of erythrocyte ghost membranes.

The molecular organization of the components of the erythrocyte membrane has been studied extensively. The phospholipids, proteins, and carbohydrates have been found to be distributed asymmetrically between the two halves of the membrane bilayer (e.g. Ref. 1). The distribution of cholesterol, a major component of the erythrocyte membrane, in the two halves of the bilayer has been studied using a variety of exchange systems (e.g. Refs. 2 to 5). Two separate membrane pools of free cholesterol have been postulated in erythrocytes, but estimates of the fraction of cholesterol in each pool and of the extent of equilibration between the two pools vary. It therefore seemed worthwhile to apply an independent and novel technique to the important problem of cholesterol localization in the erythrocyte membrane. In the current study, we estimated the distribution of cholesterol between the two halves of the bilayer of human erythrocyte ghost membranes by measuring the initial rates of association of the polyene antibiotic, filipin, with cholesterol. We have recently used this novel technique to the important problem of cholesterol localization in the erythrocyte membrane. In the current study, the binding of filipin with cholesterol in sealed and unsealed ghosts prepared from human erythrocytes and in right-side-out and inside-out vesicles prepared from ghosts follows second order kinetics (first order in each reactant). The second order rate constant of interaction of filipin with cholesterol, determined by stopped flow measurements of the initial rate, is slower in sealed ghosts than in unsealed ghosts by a factor of 2.0, whereas identical rate constants were obtained with right-side-out and inside-out vesicles. These results suggest that the cholesterol accessible to rapid reaction with filipin is distributed symmetrically between the inner and outer halves of the lipid bilayer of erythrocyte ghost membranes.

METHODS

Ghosts were prepared either from blood taken from healthy patients, to which 6 mg of Na2EDTA was added per ml of blood as an anticoagulant, or from recently outdated blood from a blood bank, to which sodium citrate was added as an anticoagulant. The fresh blood was used within 5 days after it was drawn. Blood bank and freshly drawn blood gave very similar results. Ghost preparation was according to the method of Steck and Kant (9) with the exception that in the present study the pH of the buffer was 7.4 instead of 8.0. The serum and buffy coat were removed by aspiration, and the erythrocytes were transferred to 50 ml polycarbonate centrifuge tubes and suspended in approximately 5 volumes of 5 mM phosphate buffer containing 0.15 M sodium chloride. The erythrocytes were centrifuged at 15,000 rpm for 10 min in a Sorvall RC2-B centrifuge equipped with a SS34 fixed angle rotor. The washing procedure was performed three times. All procedures were carried out at 0-4°C unless otherwise stated.

U ghosts were prepared by lysis of the washed erythrocytes. To initiate the hypotonic hemolysis, 1 ml of packed cells was mixed thoroughly with about 40 ml of 5 mM sodium phosphate buffer, pH 7.4. The lysis suspension was stirred for 10 min and then the ghosts were pelleted at 20,000 rpm for 10 min. The supernatant and the small hard button formed under the loosely packed ghosts were removed by aspiration. The ghosts were washed three more times with 5 mM sodium phosphate, pH 7.4 buffer. Creamy white unsealed ghosts were obtained.

The preparation of S ghosts was similar to that of U ghosts, except that 1 mM Mg2+ was present in the buffer (9). Since these ghosts release shortly after lysis, they contain some hemoglobin. To minimize the amount of trapped hemoglobin, a 60-fold dilution was used in the lysis step.

For the preparation of vesicles, 1 ml of concentrated U ghosts was suspended in 40 ml of 0.5 mM sodium phosphate buffer, pH 8.0, and incubated for 16 h at 4°C. RO vesicles were obtained in buffer containing 0.1 mM Mg2+ whereas IO vesicles are formed in the absence of Mg2+ (10). The suspensions were centrifuged at 15,000 rpm in a SS34 rotor for 30 min and the pellets were resuspended in approximately 1 ml of 0.5 mM sodium phosphate, pH 7.4 or 0.5 mM sodium phosphate, pH 7.4, 0.1 mM Mg2+ for RO vesicles and in 0.5 mM sodium phosphate, pH 7.4, 0.1 mM Mg2+ for IO vesicles. To complete the vesiculation, each suspension was passed through a No. 27 gauge needle five times. The homogenates were separated by density gradient centrifugation for 2 h at 40,000 rpm in a Beckman L2-65B ultracentrifuge equipped with a SW 41 rotor. Each cellulose nitrate centrifuge tube was filled with 6.5 ml of dextran 70 (Pharmacia) solution, density 1.09 g/ml and aliquots were layered on 6 ml of IO or RO vesicles. Two bands were obtained in each tube: one with d < 1.03 and the other with d > 1.03. The vesicles (d < 1.03) were collected and diluted to approximately 40 ml with buffer and then centrifuged at 15,000 rpm for 30 min in a SS34 rotor. The pellets were resuspended in buffer and used for further studies within 1 week.

* This work was supported by Grant HL 16660 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The abbreviations used are: S, sealed; U, unsealed; RO, right-side-out; IO, inside-out.
estimated from the activities of enzymes presumed to reside either on the cytoplasmic side of the membrane, such as glyceraldehyde-3-phosphate dehydrogenase, or on the external surface, such as acetylcholinesterase. Triton X-100 (Sigma Chemical Co.) was used at a concentration of 0.2% (w/w) to disrupt the permeability barrier of the membrane and to expose the latent enzyme (9). The activities of these membrane-bound enzymes were assayed as described previously (9). From the accessibility of the enzymes it was found that U ghosts were 100% unsealed, whereas preparations of S ghosts contained 10% or less of U ghosts. About 5 to 10% of the RO vesicles were unsealed, and 15 to 20% of the IO vesicles were unsealed.

Cholesterol and protein were determined as cited previously (6). Total sialic acid was estimated by the method of Warren (11).

Phospholipid vesicles containing 24 mol % of cholesterol and 4 mol % dicetyl phosphoric acid were prepared at 1 mM total phospholipid. The aqueous dispersions in 5 mM sodium phosphate, pH 7.4 buffer were 100% unsealed, whereas preparations of S ghosts contained 10% dicetyl phosphoric acid were prepared at 1 mM total phospholipid. The aqueous dispersions in 5 mM sodium phosphate, pH 7.4 buffer containing 0.05 M KCl were sonicated with a Heat Systems model W-375 sonicator in a nitrogen atmosphere at 0°C at power level 5 for 6 min at 50% pulse rate. Vesicles were formed from phosphatidylcholine and phosphatidylethanolamine, both isolated from egg yolk and purchased from Makor Chemicals Ltd., Jerusalem, Israel, at molar ratios of 9:1, 3:1, and 1:1. The absorbance at 600 nm of the vesicles was 0.08. The ratio of external to total primary amino groups was estimated using picryl sulfonic acid (Sigma Chemical Co.) according to the procedure of Roseman et al. (12); when stored under nitrogen, vesicles were found to have 58% (at 1:1 molar ratio) to 66% (at 9:1 molar ratio) of the phosphatidylethanolamine exposed.

Initial rates of filipin association with cholesterol were measured at 360 nm in a Durrum stopped flow spectrophotometer as described previously (13). The linear part of the transmittance changes (within approximately 20 ms after mixing) was converted into absorbance units per s. The total amplitude in absorbance units was calculated for each reaction. The magnitude of the total amplitude was similar in U and S ghosts at identical cholesterol concentrations. The initial reaction represents about 6% of the total amplitude. Analysis of reaction traces taken at times up to about 1 min indicated that three rate processes were present. During the very short time range used to monitor the initial rate of filipin-cholesterol association, no significant penetration of filipin into the inner bilayer surface of synthetic phospholipid vesicles occurs (13). No transmittance changes were detected at 600 nm (a wavelength where filipin does not absorb, but where light-scattering changes arising from membrane structural alteration could be monitored) during the initial 200 ms after mixing of phosphatidylcholine-cholesterol vesicles with filipin (13). This suggests that filipin does not alter the structure of artificial bilayer membranes in this time range. With phosphatidylcholine vesicles containing 24 mol % cholesterol, the initial rate of filipin-cholesterol association was linearly dependent on the cholesterol concentration in the range of 10 to 60 μM; furthermore, the second order rate constant of filipin-cholesterol association was invariant to changes between 16 and at least 32 mol % cholesterol in dimyristoylphosphatidylcholine vesicles (13). Kinetic measurements were made at 25°C. In absorbance units per s. The ordinate is in absorbance units/s. The absorbance units are 0.08.

RESULTS AND DISCUSSION

The accessibility of cholesterol to filipin was investigated by comparing the second order rate constants of filipin association with U (both surfaces exposed) and S (only the outer surface exposed) ghosts. Similar rate measurements with IO and RO vesicles provided an additional means for analyzing the distribution of cholesterol in the two halves of the bilayer. The dependence of the initial rate of association on the filipin and cholesterol concentrations is shown in Figs. 1 and 2, respectively. The initial rate is first order in filipin (above 2 μM) and in cholesterol in the concentration ranges examined approximately 20 ms after mixing) was converted into absorbance units per s. The total amplitude in absorbance units was calculated for each reaction. The magnitude of the total amplitude was similar in U and S ghosts at identical cholesterol concentrations. The initial reaction represents about 6% of the total amplitude. Analysis of reaction traces taken at times up to about 1 min indicated that three rate processes were present. During the very short time range used to monitor the initial rate of filipin-cholesterol association, no significant penetration of filipin into the inner bilayer surface of synthetic phospholipid vesicles occurs (13). No transmittance changes were detected at 600 nm (a wavelength where filipin does not absorb, but where light-scattering changes arising from membrane structural alteration could be monitored) during the initial 200 ms after mixing of phosphatidylcholine-cholesterol vesicles with filipin (13). This suggests that filipin does not alter the structure of artificial bilayer membranes in this time range. With phosphatidylcholine vesicles containing 24 mol % cholesterol, the initial rate of filipin-cholesterol association was linearly dependent on the cholesterol concentration in the range of 10 to 60 μM; furthermore, the second order rate constant of filipin-cholesterol association was invariant to changes between 16 and at least 32 mol % cholesterol in dimyristoylphosphatidylcholine vesicles (13). Kinetic measurements were made at 25°C. In absorbance units per s. The ordinate is in absorbance units/s. The absorbance units are 0.08.

RESULTS AND DISCUSSION

The accessibility of cholesterol to filipin was investigated by comparing the second order rate constants of filipin association with U (both surfaces exposed) and S (only the outer surface exposed) ghosts. Similar rate measurements with IO and RO vesicles provided an additional means for analyzing the distribution of cholesterol in the two halves of the bilayer. The dependence of the initial rate of association on the filipin and cholesterol concentrations is shown in Figs. 1 and 2, respectively. The initial rate is first order in filipin (above 2 μM) and in cholesterol in the concentration ranges examined approximately 20 ms after mixing) was converted into absorbance units per s. The total amplitude in absorbance units was calculated for each reaction. The magnitude of the total amplitude was similar in U and S ghosts at identical cholesterol concentrations. The initial reaction represents about 6% of the total amplitude. Analysis of reaction traces taken at times up to about 1 min indicated that three rate processes were present. During the very short time range used to monitor the initial rate of filipin-cholesterol association, no significant penetration of filipin into the inner bilayer surface of synthetic phospholipid vesicles occurs (13). No transmittance changes were detected at 600 nm (a wavelength where filipin does not absorb, but where light-scattering changes arising from membrane structural alteration could be monitored) during the initial 200 ms after mixing of phosphatidylcholine-cholesterol vesicles with filipin (13). This suggests that filipin does not alter the structure of artificial bilayer membranes in this time range. With phosphatidylcholine vesicles containing 24 mol % cholesterol, the initial rate of filipin-cholesterol association was linearly dependent on the cholesterol concentration in the range of 10 to 60 μM; furthermore, the second order rate constant of filipin-cholesterol association was invariant to changes between 16 and at least 32 mol % cholesterol in dimyristoylphosphatidylcholine vesicles (13). Kinetic measurements were made at 25°C. In absorbance units per s. The ordinate is in absorbance units/s. The absorbance units are 0.08.

RESULTS AND DISCUSSION

The accessibility of cholesterol to filipin was investigated by comparing the second order rate constants of filipin association with U (both surfaces exposed) and S (only the outer surface exposed) ghosts. Similar rate measurements with IO and RO vesicles provided an additional means for analyzing the distribution of cholesterol in the two halves of the bilayer. The dependence of the initial rate of association on the filipin and cholesterol concentrations is shown in Figs. 1 and 2, respectively. The initial rate is first order in filipin (above 2 μM) and in cholesterol in the concentration ranges examined approximately 20 ms after mixing) was converted into absorbance units per s. The total amplitude in absorbance units was calculated for each reaction. The magnitude of the total amplitude was similar in U and S ghosts at identical cholesterol concentrations. The initial reaction represents about 6% of the total amplitude. Analysis of reaction traces taken at times up to about 1 min indicated that three rate processes were present. During the very short time range used to monitor the initial rate of filipin-cholesterol association, no significant penetration of filipin into the inner bilayer surface of synthetic phospholipid vesicles occurs (13). No transmittance changes were detected at 600 nm (a wavelength where filipin does not absorb, but where light-scattering changes arising from membrane structural alteration could be monitored) during the initial 200 ms after mixing of phosphatidylcholine-cholesterol vesicles with filipin (13). This suggests that filipin does not alter the structure of artificial bilayer membranes in this time range. With phosphatidylcholine vesicles containing 24 mol % cholesterol, the initial rate of filipin-cholesterol association was linearly dependent on the cholesterol concentration in the range of 10 to 60 μM; furthermore, the second order rate constant of filipin-cholesterol association was invariant to changes between 16 and at least 32 mol % cholesterol in dimyristoylphosphatidylcholine vesicles (13). Kinetic measurements were made at 25°C. In absorbance units per s. The ordinate is in absorbance units/s. The absorbance units are 0.08.

RESULTS AND DISCUSSION

The accessibility of cholesterol to filipin was investigated by comparing the second order rate constants of filipin association with U (both surfaces exposed) and S (only the outer surface exposed) ghosts. Similar rate measurements with IO and RO vesicles provided an additional means for analyzing the distribution of cholesterol in the two halves of the bilayer. The dependence of the initial rate of association on the filipin and cholesterol concentrations is shown in Figs. 1 and 2, respectively. The initial rate is first order in filipin (above 2 μM) and in cholesterol in the concentration ranges examined approximately 20 ms after mixing) was converted into absorbance units per s. The total amplitude in absorbance units was calculated for each reaction. The magnitude of the total amplitude was similar in U and S ghosts at identical cholesterol concentrations. The initial reaction represents about 6% of the total amplitude. Analysis of reaction traces taken at times up to about 1 min indicated that three rate processes were present. During the very short time range used to monitor the initial rate of filipin-cholesterol association, no significant penetration of filipin into the inner bilayer surface of synthetic phospholipid vesicles occurs (13). No transmittance changes were detected at 600 nm (a wavelength where filipin does not absorb, but where light-scattering changes arising from membrane structural alteration could be monitored) during the initial 200 ms after mixing of phosphatidylcholine-cholesterol vesicles with filipin (13). This suggests that filipin does not alter the structure of artificial bilayer membranes in this time range. With phosphatidylcholine vesicles containing 24 mol % cholesterol, the initial rate of filipin-cholesterol association was linearly dependent on the cholesterol concentration in the range of 10 to 60 μM; furthermore, the second order rate constant of filipin-cholesterol association was invariant to changes between 16 and at least 32 mol % cholesterol in dimyristoylphosphatidylcholine vesicles (13). Kinetic measurements were made at 25°C. In absorbance units per s. The ordinate is in absorbance units/s. The absorbance units are 0.08.
We attempted to estimate what fraction of the total cholesterol reacts in the rapid reaction with filipin. Minute amounts of Triton X-100 were added to U and S ghosts. The initial rate of association of filipin with ghosts increased, but, at Triton concentrations higher than 0.06%, the initial rates decreased and became undetectable at a concentration of 0.9% Triton (Table I), presumably because of disruption of the filipin-cholesterol complex. Comparison of the kinetic data in the absence and presence of the detergent suggests that about 52% of the total cholesterol of U ghosts and about 28% of the total cholesterol of S ghosts are involved in the fast (0 to 25 ms) filipin-cholesterol association reaction.

Upon removal of some of the surface components from S ghosts, the accessibility of cholesterol toward filipin was increased. For example, we found in preliminary experiments that treatment of U and S ghosts with trypsin or papain, under conditions where about 10 to 15% of the protein and 50 to 70% of the sialic acid residues were removed, caused the k2 rate ratio of S/U to increase from 0.5 to 0.7 to 1.0.

The results we have described with untreated preparations of U and S ghosts indicate that the pool of cholesterol that is accessible to filipin is organized symmetrically in the two halves of the bilayer. This conclusion is corroborated by analysis of the initial rates of filipin-cholesterol association in IO and RO vesicles.

Acknowledgment—We are indebted to Dr. T. L. Steck for helpful discussions and advice throughout the course of this work.

REFERENCES
3. deHollander, F., and Chevallier, F. (1972) J. Lipid Res. 13, 733-744

### TABLE I

<table>
<thead>
<tr>
<th>Triton concentration</th>
<th>k2 (U ghosts)</th>
<th>k2 (S ghosts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>s^-1</td>
<td>s^-1</td>
</tr>
<tr>
<td>None</td>
<td>8.4</td>
<td>4.4</td>
</tr>
<tr>
<td>0.02</td>
<td>8.7</td>
<td>6.9</td>
</tr>
<tr>
<td>0.04</td>
<td>11.7</td>
<td>15.0</td>
</tr>
<tr>
<td>0.06</td>
<td>16.0</td>
<td>15.9</td>
</tr>
<tr>
<td>0.08</td>
<td>14.7</td>
<td>11.3</td>
</tr>
<tr>
<td>0.10</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>0.24</td>
<td>3.1</td>
<td></td>
</tr>
</tbody>
</table>

The second order rate constant for filipin association with cholesterol in phosphatidylcholine vesicles varied with fatty acyl chain length (13). To determine whether initial rates of filipin-cholesterol association are affected by phospholipid composition and asymmetry in the inner and outer halves of the erythrocyte membrane, we measured the initial rates in phosphatidylcholine vesicles containing varying concentrations of phosphatidylcholine. The initial rate did not vary in these vesicles, suggesting that the rate constant of filipin-cholesterol complexation in bilayers formed from mixtures of phospholipids is not sensitive to phosphatidylcholine concentration.

**Cholesterol Distribution in Erythrocyte Ghosts**

Rapid mixing of U and S ghosts (90 μM in cholesterol) and filipin (10 μM final concentration) was performed in the stopped flow apparatus as described under "Methods." Ghosts were treated with the indicated concentration of Triton X-100 for 1 min prior to reaction with filipin. Values of the first order rate constant, k2, were calculated from (dA/dt) - (1/ΔA total), where dA/dt is the initial rate of absorbance change of filipin and ΔA total is the average total amplitude change (0.16).

In ghosts and vesicles. The following values of k2 were calculated from analyses of eight separate concentrations of filipin and cholesterol: U ghosts, 1.3 ± 0.1 x 10^-5 M^-1 s^-1; S ghosts, 0.65 ± 0.07 x 10^-5 M^-1 s^-1; RO vesicles, 0.61 ± 10^-5 M^-1 s^-1; IO vesicles, 0.60 x 10^-5. (In reactions with ghosts, filipin was varied between 6 and 20 μM; cholesterol concentrations varied from 40 to 120 μM. In reactions with vesicles, filipin was varied between 5 and 10 μM; cholesterol concentrations varied from 30 to 150 μM. The S ghost/U ghost ratio of initial rates, calculated at 18 different concentrations of filipin or cholesterol in at least five different preparations of U and S ghosts, was 0.50, with a standard deviation of ±0.05.) These k2 values indicate that the cholesterol concentrations available to filipin are equal in the two leaflets of the membrane bilayer, assuming that no difference in membrane lipid organization exists between U and S ghosts.

The second order rate constant for filipin association with cholesterol in phosphatidylcholine vesicles varied with fatty acyl chain length (13). To determine whether initial rates of filipin-cholesterol association are affected by phospholipid composition and asymmetry in the inner and outer halves of the erythrocyte membrane, we measured the initial rates in phosphatidylcholine vesicles containing varying concentrations of phosphatidylcholine. The initial rate did not vary in these vesicles, suggesting that the rate constant of filipin-cholesterol complexation in bilayers formed from mixtures of phospholipids is not sensitive to phosphatidylcholine concentration.
Cholesterol distribution between the two halves of the lipid bilayer of human erythrocyte ghost membranes.
L Blau and R Bittman


Access the most updated version of this article at http://www.jbc.org/content/253/23/8366

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/253/23/8366.full.html#ref-list-1