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Reconstitution of Membranes with Fractions of Triton X-100 Which Are Easily Removed

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Triton X-100 is a neutral detergent which is often employed for reconstitution of lipid–protein membranes. It is, however, difficult to remove entirely from the final reconstituted membrane preparation. We have analyzed the molecular components of the Triton X-100 complex of molecules that remain in reconstituted membranes which are prepared by, first, Triton X-100 solubilization and, second, by detergent removal with polystyrene beads and gel filtration. The analysis was performed with radioactive Triton X-100 and by silica gel chromatography. It showed that the components of Triton X-100 that are difficult to remove from a membrane are the high- and low-molecular-weight ones. Using only an intermediate-molecular-weight, radioactive Triton, lipids and Sindbis virus envelope proteins have been solubilized and used to prepare reconstituted membranes which retain half as many Triton/lipid molecules, or one-fifth as many Triton/protein molecules as do vesicles prepared with complete Triton X-100. The membranes prepared with fractionated Triton are also of more homogeneous size, by gel chromatography, than are membranes prepared with unfractionated Triton.

Triton X-100 is a nonionic detergent which has been used successfully for the reconstitution of a large number of functional membrane proteins (1–5). Despite some inherent drawbacks, such as its low critical micelle concentration (6), which necessitates extensive dialysis (7), and its heterogeneous nature, e.g., containing many different polymers (8) as well as impurities (9), it is often found to be the detergent of choice for the initial solubilization of membrane proteins (2). Since unremoved detergent can influence the measured properties of reconstituted systems (10,11), however, it is imperative that residual detergent be minimized.

In the course of our studies involving the reconstitution of the envelope glycoproteins of Sindbis virus (12), we have examined the removal of Triton X-100 from the reconstituted lipid–protein vesicles in detail. The ease with which the various molecular components of Triton X-100 are removed during the reconstitution procedure is a function of the molecular weight of the species. The component of Triton X-100 which was most easily removed during the reconstitution procedure contained 11 ethyleneoxy units. When this component was isolated by liquid chromatography and used for reconstitution, a reconstituted membrane which retained less detergent was obtained.

MATERIALS

Pure egg phosphatidylcholine was prepared according to the method of Bangham *et al.* (13); 20-μg samples showed a single spot upon thin-layer chromatography (TLC)[1] in a solvent system of chloroform/methanol/water:65/25/4 (vol/vol). Triton X-100 (TX-100) was obtained from the Sigma Chemical Company; radioactive Triton (^{3}H-labeled on benzene ring) was a gift from the Rohm and Haas Company. Polystyrene beads were

[1] Abbreviations used: TLC, thin-layer chromatography; TX-100, Triton X-100; MEK, methyl ethyl ketone; HPLC, high-pressure liquid chromatography.

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from Bio-Rad (Biobeads SM-2) or from Eastman (Amberlite XAD-2) and were washed extensively before use with the procedure of Holloway (14). Sephadex G-25 and Sepharose 2B were products of Pharmacia Fine Chemicals, and silica gel was obtained from Analabs (Anasil H) for preparative thin-layer chromatography and from Mallinkrodt (CC4-silica gel) for column chromatography. All other chemicals and solvents were reagent grade or better. Scintillation cocktail was 3a70B from Research Products International. Methylethyl ketone was purified (to remove yellow impurities) by refluxing the solvent for 1 h with sodium borohydride and the antioxidant butylated hydroxytoluene (1 g each per 1 liter of solvent) and then by distillation at atmospheric pressure under nitrogen. Sindbis virus and radiolabeled Sindbis virus (^{14}C-labeled amino acids) were gifts from B. Sefton of the Salk Institute.

METHODS

Phospholipid analyses were accomplished by an adaptation of the phosphate assay of Rouser *et al.* (15). Protein concentrations were determined by the method of Lowry (16), using bovine serum albumin (Sigma, three times recrystallized) as a standard. All radioactivity determinations were corrected for background and channel spillover. Virus was precipitated before use by dilution with cold water and centrifugation as described by Pfefferkorn and Clifford (17).

Lipid vesicle preparation. Lipid films were prepared by evaporating a chloroform solution of lipid in a test tube on a rotary evaporator. Films were redissolved with diethyl ether and reevaporated twice more. Residual solvent was removed from the films by subjecting them to high vacuum for several hours. Vesicles were prepared by hydrating the lipid film, solubilizing to clarity with the appropriate TX-100 fractions, and then adding polystyrene beads to remove detergent and initiate vesicle formation. The beads were removed (after at least 2 h of incubation at 4°C) and further residual detergent was removed by gel filtration on Sephadex G-25 and Sepharose 2B.

Determination of residual TX-100 in lipid vesicles. Pure lipid films were hydrated with water at a concentration of 5 mg/ml. Unfractionated TX-100 with radioactive label was added to obtain visual clarity. This solution was divided into two equal parts. One part was lyophilized to remove water. To the second part, polystyrene beads (1 g/70 mg TX-100 used) were added and the solution gently agitated for several hours at 4°C. Beads were removed by filtration through a porous polyethylene plug. The vesicles (in the filtrate) were applied to a 1.9 × 10-cm column of Sephadex G-25 and eluted with water; vesicles appeared in the column void volume. After a second passage through this column, water was removed from the vesicles by lyophilization.

Analysis of residual TX-100 in lipid vesicles. To compare the TX-100 remaining in the lipid vesicles prepared by this method with that in the starting material, it was necessary to separate TX-100 and lipid. This was accomplished by preparative TLC, using a solvent system of chloroform/methanol/water (65/25/4, v/v). The TX-100 and lipid bands (R_f values of 0.96 and 0.42, respectively) were visualized with iodine vapor, and the TX-100 isolated and extracted from the silica gel with several washes of chloroform/methanol (2/1, v/v). The TX-100 extract was concentrated on a rotary evaporator and applied to an analytical TLC plate which was developed with water-saturated methyl ethyl ketone (MEK) (8). The resulting, fractionated TX-100 bands were analyzed by dividing the dried plate into 2-mm segments and measuring the radioactivity of each segment. The relative amounts of each TX-100 component remaining after vesicle formation were compared to those with the analogous R_f value in the starting material.

Reconstitution of membranes containing

viral proteins. In a typical reconstitution, a suspension of virus (1.5 to 2.0 mg protein) and ^{14}C-virus as a tracer was divided into two equal aliquots and each aliquot precipitated by dilution. One aliquot was solubilized with a buffer containing 0.2% TX-100 (unfractionated), while the other was solubilized with the fractionated TX-100. Envelope glycoproteins were separated from viral core in each aliquot on sucrose density gradients containing 0.05% of the appropriate TX-100. The fractions containing the envelope proteins of each aliquot were pooled and added to identical dried lipid films. Subsequent steps are as outlined above for lipid vesicle preparation. Thus, for experiments in which the use of fractionated TX-100 was being evaluated, the radiolabeled, fractionated TX-100 component was used exclusively in all steps of this reconstitution protocol.

Preparation of fractionated TX-100. Fractionated TX-100 for use in the reconstitution experiments was prepared by preparative liquid chromatography. Several fractionation procedures for Triton have been published (18–20). In order to minimize exposure to acetic acid, we have employed a chromatography procedure with methyl ethyl ketone as solvent. A 2% solution of 2 g of TX-100 in MEK was applied to a 3.5 × 75-cm column containing 200 g silica gel in MEK and eluted with a linear gradient of 2 liters each of MEK and 8% water in MEK. Fractions were analyzed by TLC using water-saturated MEK as solvent and sulfuric acid charring; appropriate fractions were pooled and the solvent removed under reduced pressure. Radiolabeling of a specific TX-100 fraction was achieved by the coelution of the appropriate TX-100 fraction with unfractionated, radiolabeled TX-100 on preparative TLC plates, followed by isolation of the desired radiolabeled band. When 8 mg was applied, approximately 5 mg of radiolabeled fraction could be recovered from a single 20 × 20-cm plate with a 750-μm layer of silica gel. The purity of the radiolabeled fractions was analyzed further by analytical TLC with water-saturated MEK. The specific activity of the particular fraction used in these studies after dilution with cold detergent was 305.6×10^3 cpm/mg, while that of the unfractionated material was 55×10^3 cpm/mg (cpm/mmol were 2.1×10^8 and 3.5×10^7 for fraction 11 and unfractionated detergent, respectively).

In order to determine whether radioactivity per phenyl group was constant throughout the spectrum of Triton components, two experiments were compared. In one case, the profile of radioactivity per fraction was obtained by thin-layer chromatographic separation of the radioactive material. In the other case, the profile of optical density was obtained using an analytical HPLC separation of cold Triton components. Assuming reasonable base lines for the two profiles, the ratio of radioactivity/optical density appeared constant to within about 20%. For the HPLC separation, 10 μl of a 0.2 M solution of TX-100 in methylene chloride was injected into a 30-cm × 4-mm CN-10 column (Varian MicroPak) and eluted with a 0–60% linear gradient of acetonitrile in methylene chloride.

RESULTS AND DISCUSSION

The identity and relative amounts of various TX-100 fractions remaining after the preparation of lipid vesicles were determined by a quantitative comparison of the TLC profiles of the TX-100 isolated from starting material and from lipid vesicles. Figure 1 displays these results as the ratio of each TX-100 fraction recovered from the lipid vesicles to that present in the starting material. It is quite apparent that both the high- and low-molecular-weight components of TX-100 are preferentially retained in the vesicle preparation, while the middle fractions are efficiently removed by the combination of polystyrene beads and gel filtration. Thus, if these middle fractions could be used exclusively for reconstitution, one

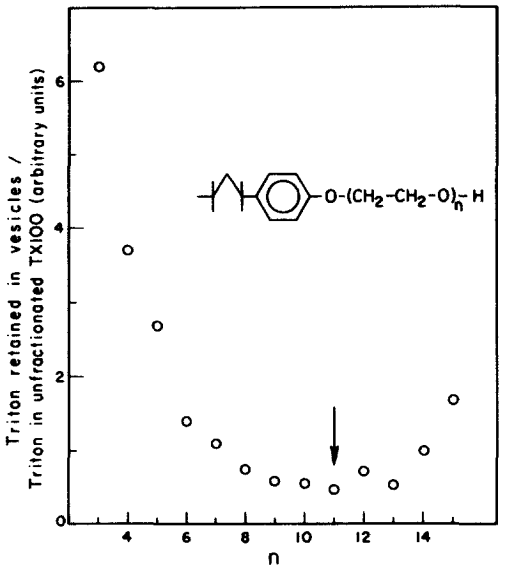

FIG. 1. Comparison of Triton remaining in vesicles with that used for the initial solubilization of the lipid. Analytical TLC plates (solvent front to left) were fractionated and counted; a horizontal line of data points would indicate no preferential retention or loss of individual Triton fractions (indexed by their number of oxyethylene units, *n*) resulting from the vesicle preparation procedure. The arrow indicates the Triton fraction used for the reconstitution of viral envelope proteins.

would expect to be able to minimize residual detergent and its influence on vesicle properties.

To demonstrate the applicability of these middle fractions for reconstitution studies, we have isolated the particular Triton fraction that is least retained in reconstituted membranes (see Fig. 1) and used it exclusively for the reconstitution of Sindbis virus glycoproteins in egg lecithin vesicles. This fraction contains 11 oxyethylene units. Figure 2 compares the Sepharose 2B column profile of the detergent retained by vesicles prepared using unfractionated TX-100 with that of vesicles prepared from fractionated TX-100. In agreement with the findings for pure lipid vesicles, the use of an appropriate TX-100 fraction leads to a significant reduction in the amount of residual detergent. The protein-to-lipid ratio in membranes reconstituted with fractionated Triton is higher than in the membranes prepared from unfractionated Triton. Thus, the extent to which residual Triton is retained in the two cases must be expressed in terms of detergent/protein as well as detergent/lipid ratios. Reconstituted membranes containing the Sindbis virus glycoproteins, when prepared from Triton fraction 11, retain one-fifth the number of detergent/protein molecules and one-half the detergent/lipid molecules found in membranes reconstituted with unfractionated Triton X-100. Additional gel filtration did not reduce the residual TX-100 significantly, with either fractionated or unfractionated detergent. Figure 2 also shows another advantage in the use of the fractionated detergent, namely, it results in a vesicle preparation of more homogeneous size relative to that obtained using the unfractionated detergent. In addition, vesicles prepared with fractionated detergent have an average lipid/protein molar ratio of 70/1, which is nearer the value in

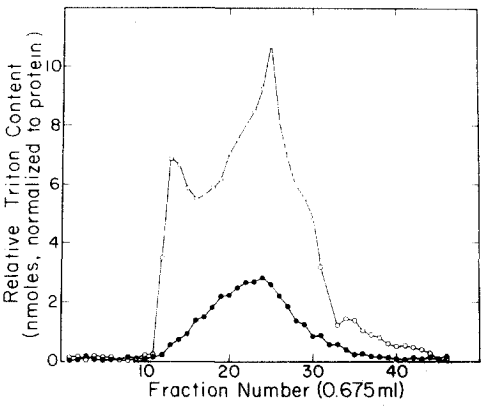

FIG. 2. Gel filtration profiles of Triton remaining in reconstituted vesicles prepared from unfractionated (open circles) and fractionated (closed circles) detergent. Samples from a G25 column containing 7.3 nmol of protein were applied to a 1.2 × 25-cm column of Sepharose 2B and eluted at a flow rate of 0.1 ml/min at 4°C. The void and included volumes of the column occur at fractions 12 and 35, respectively. Triton content was determined as ^3H count per minute and converted (using molar radioactivities) to nanomoles of detergent present in each fraction based on 7.3 nmol of total protein.

the virus (50/1) than the considerably higher average lipid/protein ratio in vesicles prepared with unfractionated detergent.

Reconstitution of small quantities (<1 mg) of membrane proteins in lipid vesicles, with the possibility of trapping substances inside, requires materials which minimize nonspecific adsorption of lipid and protein as well as procedures employing small solution volumes during vesicle formation. The use of polystyrene beads for bulk detergent removal and vesicle formation combined with gel chromatography to remove residual TX-100 satisfies these criteria. Using unfractionated TX-100, this procedure results in vesicles containing approximately 11 molecules of detergent per 100 molecules of phosphatidylcholine. This number is similar to that of 10 found by Allen *et al.* (7) after four passages through a G200 column. Using *fractionated* TX-100, specifically the 11-mer, we find 5 ± 1 molecules of detergent per 100 lipids. Virtually identical results were obtained when pure lipid vesicles were prepared with this procedure. Thus, there appears to be no preferential association of detergent with the Sindbis glycoproteins.

The minimum ratio of TX-100 molecules to lipid molecules obtained when fractionated TX-100 is used is still considerably higher than the molar ratio of detergent to lipid obtained recently in reconstitution procedures employing other detergents. Ratios of 1 deoxycholate/100 lipids (7) and 0.25 octylglucoside/100 lipid molecules (21) have been reported. However, it has been shown (2,22) that residual Triton X-100 in reconstituted membranes may be reduced by a two-step procedure using Triton treatment with a second detergent. Thus, in procedures where Triton is found to be the detergent of choice for retention of the native conformation of a membrane protein or for other reasons (2), fractionated Triton, combined with the two-step procedure is expected to give even more complete Triton removal than in the procedure we report here.

In summary, the use of a particular middle fraction of TX-100 combines the advantages of excellent lipid- and protein-solubilizing ability and ease of removal and, as such, offers a significant improvement in the quality of reconstituted vesicles over those obtained using unfractionated detergent. In addition, vesicle preparations appear to be more homogeneous in size when prepared with the fractionated TX-100. Physical factors likely to influence the removal of a particular TX-100 fraction from a lipid–protein mixture include its critical micelle concentration, its partition coefficients between aqueous, lipid, and protein phases, and its orientation with respect to the bilayer. The presence of residual Triton at the levels found here may be the result of detergent molecules trapped inside the vesicle as well as in equilibrium with external solvent.

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