Phenyl-Sepharose-Mediated Detergent-Exchange Chromatography: Its Application to Exchange of Detergents Bound to Membrane Proteins†

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ABSTRACT: Detergent-saturated phenyl-Sepharose was used to exchange detergents for one another in the presence of membrane proteins. The alkyl detergents lauryl maltoside, octyl glucoside, and dodecyl sulfate were each successfully exchanged for Triton X-100, Triton N-101, or Nonidet P-40 present in a solution of either cytochrome *c* oxidase, a mixture of inner mitochondrial membrane proteins, or a mixture of erythrocyte membrane proteins. The method involves (1) saturating a small column of phenyl-Sepharose (1–2 mL) with one of the alkyl detergents at a pH of 8 or 9 and an ionic strength of 0.01, (2) applying a detergent-solubilized membrane protein sample containing as much as 20 mg/mL of Triton X-100, Triton N-101, or Nonidet P-40, and (3) eluting the protein with buffer containing the detergent with which the resin had been saturated. With this approach, 90–99% of the detergent in the initial protein sample was exchanged for the second detergent with an 80–100% recovery of protein. The advantages of this method over previous approaches for exchanging detergents include the rapidity of the technique and the apparent general applicability of the method to a wide variety of detergents and membrane proteins.

A major problem that is faced during the study of intrinsic membrane proteins is the replacement of the detergent that is used to solubilize the protein at one stage of a project with a detergent that is better suited for the next experiments. For example, with cytochrome *c* oxidase, it is often necessary to exchange cholate, deoxycholate, Triton X-100, Nonidet P-40, lauryl maltoside,[1] Tween 20, Tween 80, or Brij 96 for each other. Exchange of these detergents is required since Triton-type detergents, cholate, and/or deoxycholate is often used to purify the complex (Hartzell et al., 1978); Triton X-100, Nonidet P-40, or cholate is excellent for delipidation of the complex (Robinson et al., 1980; Yu et al., 1975); lauryl maltoside, Tween 20, Tween 80, or Brij 96 is the most useful for maximum electron-transport activity [e.g., Thompson & Ferguson-Miller (1983), Robinson & Capaldi (1976), Yu et al., (1975), and Vanneste et al. (1974)]; cholate is the best for reconstitution of the complex into phospholipid vesicles (Carroll & Racker, 1977). The necessity of detergent exchange is not a problem that is unique for cytochrome *c* oxidase. Unfortunately, with most intrinsic membrane proteins, no single detergent is usually well suited for each part of a project.

A number of detergent-exchange methods are available, but most of these are relatively slow and cannot be used with a wide variety of detergents or intrinsic membrane proteins. For example, gel filtration of a Triton X-100 solubilized protein in the presence of excess deoxycholate can be used to exchange deoxycholate for Triton X-100 (Robinson & Capaldi, 1976), but this method can only be used if the size of the protein–detergent complex is sufficiently larger than the size of the detergent mixed micelles to separate by gel filtration chromatography. This is not true with most nonionic detergents and membrane proteins. We, therefore, have developed a method using phenyl-Sepharose-mediated detergent exchange that appears to be an extremely useful approach for exchanging different detergents that are bound to intrinsic membrane proteins. The method is very rapid, quite simple, and generally applicable to a wide variety of intrinsic membrane proteins. In this paper, we describe the experimental basis of the method and illustrate its use with both purified cytochrome *c* oxidase and mixtures of detergent-solubilized membrane proteins.

Experimental Procedures

Materials. Phenyl-Sepharose CL-4B (lot 12514 with ca. 40 μmol of ligand/mL of gel bed) was obtained from Pharmacia Fine Chemicals. Triton X-100, Triton N-101, and cholic acid were obtained from Sigma Chemical Co. (Sodium cholate was prepared from the cholic acid, after its recrystallization from 90% ethanol, by titration of an ethanol solution of cholic acid with NaOH.) Octyl β-D-glucoside and lauryl β-D-maltoside were purchased from Calbiochem-Behring; Nonidet P-40 was purchased from Bethesda Research Laboratories; dodecyl sulfate was purchased from Accurate Chemical and Scientific Corp, a distributor of BDH Chemicals. [^{3}H]Triton X-100 was obtained from New England Nuclear. All other chemicals were reagent grade.

Cytochrome *c* oxidase was prepared from beef heart as previously described (Robinson, 1982). After ammonium sulfate precipitation, the protein pellet was dissolved at 20 mg of protein/mL in either 1% Triton X-100 or 1% Nonidet P-40 in 0.02 M Tris-HCl–0.1 mM EDTA buffer at pH 8.1 and dialyzed against 50 volumes of this buffer for 20 h at 5 °C to reduce the ionic strength. Preparations had 9.5–10.5 nmol of heme *a*/mg of protein and a molecular activity of 375 mol of cytochrome *c* oxidized s^{-1} (mol of cytochrome *c* oxidase)$^{-1}$ when assayed at 25 °C in a 0.025 M sodium phosphate buffer at pH 7.0 containing 1 mg of lauryl maltoside/mL. The preparation was stored at 20 mg of protein/mL in liquid nitrogen until it was used.

Nonidet P-40 solubilized inner mitochondrial membrane proteins were prepared from yeast submitochondrial particles

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[1] Abbreviations: cmc, critical micelle concentration; lauryl maltoside, dodecyl β-D-maltoside; octyl glucoside, octyl β-D-glucoside; Tris-HCl, tris(hydroxymethyl)aminomethane base titrated to the appropriate pH with HCl; EDTA, ethylenediaminetetraacetic acid.

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[prepared as described by Todd et al. (1979)] by extraction of the membranes at 10 mg of protein/mL with 0.5% Nonidet P-40 and separation of detergent-soluble and -insoluble proteins by centrifugation in a Beckman airfuge. The solubilized protein (5 mg of protein/mL) was dialyzed against 0.02 M Tris-HCl–0.1 mM EDTA at either pH 8.0 or pH 9.0 before it was used for detergent exchange.

Triton N-101 solubilized human erythrocyte membrane proteins were obtained as a gift from David Nelson of this department. These proteins had been extracted from purified erythrocyte membranes at 0.1 mg of protein/mL with 0.2% Triton N-101, and the Ca^{2+},Mg^{2+}ATPase had been removed by a calmoldulin–Sepharose affinity column (Nelson & Hanahan, 1985). The erythrocyte membrane proteins that were not retarded by the affinity column (50 mL) were pooled, concentrated to 7 mL (0.2 mg of protein and 6 mg of Triton N-101 per mL) with an Amicon PM-10 membrane and pressure concentrator, and dialyzed vs. 100 volumes of 0.02 M Tris-HCl–0.1 mM EDTA buffer, pH 9.0, before they were used.

Methods. All experiments, unless otherwise noted, were performed at room temperature, ca. 23 °C, in 0.02 M Tris-HCl buffer at pH 9.0 containing 0.1 mM EDTA. Cytochrome *c* oxidase enzymatic assays and polyacrylamide gel electrophoresis in dodecyl sulfate and 2 M urea were performed as previously described [see Robinson (1982) and Robinson et al., (1980), respectively]. Cytochrome *c* oxidase concentrations were determined spectrophotometrically by using a molar extinction coefficient at 422 nm of 1.54×10^5 M^{-1} cm^{-1} (van Gelder, 1978); protein concentrations, for proteins other than cytochrome *c* oxidase, were measured by the method of Lowry et al. (1951). Concentrations of Triton X-100 were determined by using a molar extinction coefficient at 277 nm of 1465 M^{-1} cm^{-1} (Robinson & Tanford, 1975); concentrations of Triton N-101 and Nonidet P-40 were determined by using molar extinction coefficients at 277 nm of 1570 and 1630 M^{-1} cm^{-1}, respectively (these were determined experimentally by using an assumed molecular mass of 640 g/mol). The concentration of Triton X-100 or Nonidet P-40 in solutions also containing cytochrome *c* oxidase was calculated after correcting for the absorbance due to the protein from the measured A_{422} of the solution and a value of $A_{277}/A_{422} = 2.40$ for pure cytochrome *c* oxidase. In the presence of other proteins, these detergents were phase separated into chloroform before the absorbance was measured so that the protein would not interfere with the measurement. With the experiment using inner mitochondrial membrane proteins, the concentration of Nonidet P-40 was determined by adding [^{3}H]Triton X-100 to the solution (Triton X-100 and Nonidet P-40 are chemically identical). This was necessary since some mitochondrial membrane proteins partition into chloroform, thereby making measurement of detergent concentrations on the basis of absorbance at 277 nm an unusable method. When [^{3}H]Triton X-100 was used, the detergent concentrations were determined on a Tracor liquid scintillation counter with a Triton X-100/toluene scintillation fluid that was prepared as we have previously described (Robinson & Tanford, 1975). The concentrations of octyl glucoside and lauryl maltoside were determined by the anthrone assay for glucose (Seifter et al., 1950).

Results

Maximum Binding of Detergents to Phenyl-Sepharose. The hydrophobic sites on phenyl-Sepharose could be saturated with any of the detergents used in this study (Triton X-100, Triton N-101, Nonidet P-40, lauryl maltoside, octyl glucoside, or dodecyl sulfate) by flowing a detergent solution, having a concentration of detergent greater than the cmc, over a small column containing the phenyl-Sepharose resin.[2] With each detergent solution, the concentration of detergent in the column effluent was approximately equal to the cmc until the resin became saturated with detergent; thus, only the detergent that was in excess of the cmc bound to the resin. The maximum binding of several detergents to the phenyl-Sepharose resin was determined by measuring the micromoles of detergent that had been removed from the effluent prior to saturation of the resin, saturation being defined as the point at which the concentration of detergent in the effluent was equal to the concentration in the eluting buffer. Figure 1 illustrates the saturation of phenyl-Sepharose with several detergents. With each detergent, after approximately 40 μmol of detergent had been removed from the effluent, the concentration of detergent increased sharply, indicating that saturation had occurred. For

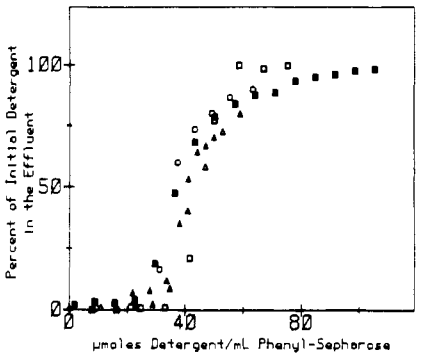

FIGURE 1: Saturation of phenyl-Sepharose by detergents. (Abscissa) Micromoles of detergent that had been passed over the phenyl-Sepharose column minus the micromoles of detergent in the column void volume. (Ordinate) Ratio of the detergent concentration in the effluent of the column divided by the detergent concentration in the eluting buffer. The detergents, buffer, and column size that were used and the corresponding symbol on the graph are as follows: (1) 4 mM lauryl maltoside in 0.02 M Tris-HCl buffer at pH 8.1 and $I = 0.10$ applied to a 0.5 × 2.5 cm bed of resin (open squares); (2) 6 mM Nonidet P-40 in 0.02 M Tris-HCl buffer at pH 9.0 and $I = 0.01$ applied to a 0.5 × 10 cm bed of resin (open triangles); (3) 6 mM Triton N-101 with same buffer and column size as (2) (open circles); (4) 12 mM Triton X-100 in 0.02 M Tris-HCl buffer containing 20% glycerol at pH 8.1 and $I = 0.10$ applied to 0.5 × 5 cm bed of resin (filled squares); (5) 3 mM Triton X-100 with same buffer and column size as (4) (filled triangles).

Table I: Binding of Detergents to Phenyl-Sepharose[a]

	buffer[b]			
detergent	concn of detergent (mM)	pH	I	maximum binding (μmol/mL of resin)
lauryl maltoside	4	8.1	0.1	39
Nonidet P-40	3	9.0	0.01	46
octyl glucoside	17	9.0	0.01	0
	34	9.0	0.01	37
	51	9.0	0.01	45
Triton N-101	3	9.0	0.01	39
Triton X-100	3	9.0	0.01	40
	3	8.1[c]	0.1	44
	6	8.1[c]	0.1	45
	9	8.1[c]	0.1	38
	12	8.1[c]	0.1	36

[a] According to Pharmacia Fine Chemicals, the resin contained 40 μmol of phenyl groups/mL of resin. [b] The pH 8.1 buffer contained 0.02 M Tris-HCl, 0.09 M NaCl, and 0.1 mM EDTA; the pH 9.0 buffer contained 0.02 M Tris-HCl – 0.1 mM EDTA. [c] Also included 20% glycerol (w/v).

[2] If the concentration of detergent in the eluting buffer was less than the cmc, the phenyl-Sepharose bound very little detergent; e.g., with 5 mM octyl glucoside, no significant binding occurred (Table I).

Table II: Summary of Phenyl-Sepharose Detergent-Exchange Results Obtained with Various Intrinsic Membrane Proteins[a]

membrane protein[b]	buffer[c] pH	buffer[c] I	detergent in applied sample[d]	detergent used for exchange[e]	recovery of protein (%)[f]	removal of orginal detergent (%)[g]
cytochrome *c* oxidase	8.0	0.004	5 mg of TX-100	LM	88	
	8.0	0.01	5 mg of TX-100	LM	79	
	8.0	0.10	5 mg of TX-100	LM	75	
	9.0	0.01	12 mg of NP-40	LM	95	99.7
	9.0	0.01	5 mg of NP-40	OG	82	99.1
	9.0	0.01	9 mg of NP-40	$DodSO_4$	76	89.5
inner mitochondrial membrane proteins	8.0	0.01	10 mg of NP-40	OG	79	96
	9.0	0.01	3 mg of NP-40	OG	86	97
	9.0	0.01	10 mg of NP-40	$DodSO_4$	100	
erythrocyte membrane proteins	9.0	0.01	8 mg of TN-101	$DodSO_4$	90	99.6

[a] Detergent exchange was performed on a column (0.5 × 5 or 10 cm) containing 1 or 2 mL of phenyl-Sepharose. [b] The sample applied to the resin contained 1.5–2 mg of protein and the detergent to be removed in 0.5–1.0 mL of the buffer with which the column had been equilibrated. [c] The buffers used were 0.02 M Tris base–0.1 mM EDTA containing the appropriate detergent. The pH was adjusted to either 8.0 or 9.0 with HCl. NaCl was added to adjust the ionic strength (I). [d] The abbreviations used for the detergents are as follows: NP-40, Nonidet P-40; TX-100, Triton X-100; TN-101, Triton N-101. [e] The abbreviations used for the detergents are as follows: $DodSO_4$, dodecyl sulfate; OG, octyl glucoside; LM, lauryl maltoside. The equilibrating and eluting buffer contained either 1 mg of $DodSO_4$/mL, 1 mg of LM/mL, or 15 mg of OG/mL. [f] Percent recovery is reported as the total amount of protein that eluted from the column in the void volume peak. [g] Percent removal of original detergent was based upon the detergent in the two or three fractions that contained the majority of protein.

each detergent or experimental condition that was tested, the maximum binding of detergent to phenyl-Sepharose was very close to 40 μmol of detergent/mL of resin (Table I), a value that is identical with the number of moles of phenyl groups reported to be present on the resin by Pharmacia Fine Chemicals (refer to Materials). Clearly, monomers, not micelles of detergent, are bound to the phenyl groups of the resin.

Detergent Exchange in the Absence of Protein. Once the hydrophobic sites on phenyl-Sepharose were saturated with a particular detergent, the resin could be used for detergent exchange much like the chloride or acetate forms of Dowex 1 or the sodium or potassium forms of Dowex 50 can be used for anion or cation exchange, respectively. Exchange of one detergent for another by detergent-saturated phenyl-Sepharose columns was accomplished with a variety of detergents. In a typical exchange experiment, a 0.5 cm × 10 cm column of resin that had been saturated with a particular detergent, e.g., saturated with lauryl maltoside at pH 8 in 0.02 M Tris-HCl buffer, I = 0.10, could be used to remove at least 99% of the detergent from a 1-mL sample containing 5–15 mg of Triton X-100. In this case, the solution that passed directly through the column of phenyl-Sepharose contained approximately the same molar amount of lauryl maltoside as the molar amount of Triton X-100 in the original sample. Other examples of detergent exchange that were successful were the exchange of lauryl maltoside, octyl glucoside, cholate, or lauryl sulfate for either Triton X-100 or Nonidet P-40. Conversely, Triton X-100 was successfully exchanged for lauryl maltoside. Presumably, the exchange of other detergents would also be possible. The only difficulty that was encountered was a small amount of material in Triton X-100 solutions having A_{277} that did not exchange with other detergents. Whenever Triton X-100 was used, 0.35% of the total absorbance at 277 nm that had been applied to the column was not retarded by the phenyl-Sepharose and contaminated the exchanged sample. This material probably represents a subpopulation of molecules in the Triton X-100 mixture that does not have the same affinity for the phenyl-Sepharose column as do the other detergents.

Exchange of Detergents Bound to Cytochrome c Oxidase. In order for the phenyl-Sepharose exchange method to be applicable to the exchange of detergents that are bound to intrinsic membrane proteins, the association of these proteins with the hydrophobic matrix had to be minimized. If protein binding to the column could be eliminated, the protein complex would elute in the presence of the detergent that had been displaced from the resin, and detergent exchange would be accomplished.

High pH and low ionic strength minimized the association of the cytochrome *c* oxidase complex with detergent-saturated phenyl-Sepharose. At pH 9.0 and an ionic strength of 0.01, only 5–15% of either the Triton X-100 or Nonidet P-40 solubilized complex bound to a column that had been saturated with lauryl maltoside. The remainder of the cytochrome *c* oxidase (85–95%) was not retarded by the column and eluted with the lauryl maltoside that had been displaced from the resin by the Triton X-100 or Nonidet P-40 in the original sample (Table II). Under these conditions, complete exchange of lauryl maltoside for the original Triton X-100 or Nonidet P-40 was accomplished (Figure 2A). Only 0.35% of the original 15 mg/mL Triton X-100 or <0.1% of the original 15 mg/mL Nonidet P-40 remained with the complex after the column. Clearly, both the protein-bound detergent and the free detergent exchanged with the lauryl maltoside that was bound to the phenyl-Sepharose column. Complete exchange could also be accomplished at a lower pH or higher ionic strengths, but a greater percentage of the cytochrome *c* oxidase was bound to the resin, decreasing the recovery of protein in the detergent-exchanged sample (Table II). The exchange of octyl glucoside or dodecyl sulfate for the Triton X-100 or Nonidet P-40 present in cytochrome *c* oxidase solutions was also accomplished by using these optimal conditions of pH 9 and I = 0.01. With each detergent, the cytochrome *c* oxidase was not retarded by the phenyl-Sepharose column and eluted in the presence of the detergent with which the phenyl-Sepharose column had been equilibrated. The association of the Triton X-100 or Nonidet P-40 with the octyl glucoside or dodecyl sulfate saturated phenyl-Sepharose was not as strong as their association with the lauryl maltoside saturated resin, and they eluted from the column shortly after the protein (Figure 2B). Nevertheless, with either octyl glucoside or dodecyl sulfate, the separation of the protein and Nonidet P-40 was sufficient to respectively exchange 99 and 90% of the Nonidet P-40 that was with the cytochrome *c* oxidase (Table II).

The phenyl-Sepharose-mediated detergent-exchange procedure did not significantly alter the cytochrome *c* oxidase

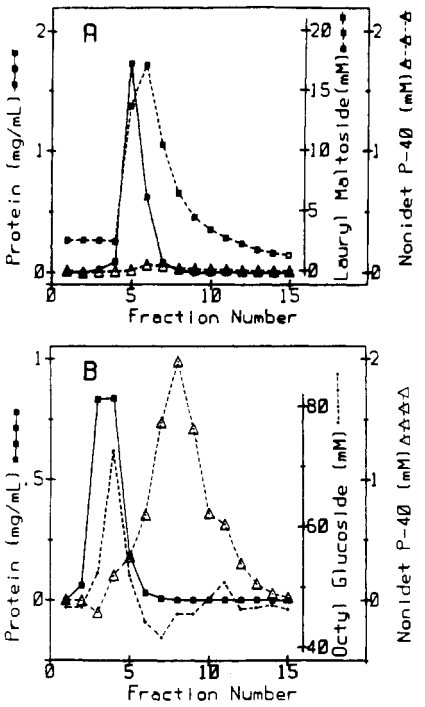

FIGURE 2: Exchange of detergents bound to cytochrome *c* oxidase. (A) Exchange of lauryl maltoside for protein-bound Nonidet P-40 at pH 9: a 0.5 × 10 cm bed of phenyl-Sepharose was presaturated with 13 mL of 10 mM lauryl maltoside in a pH 9.0, $I = 0.01$ buffer, followed by 8 mL of 2 mM lauryl maltoside in the same buffer; a 0.6-mL protein sample was applied that contained 3 mg of protein/mL and 26 mM Nonidet P-40 in the pH 9, $I = 0.01$ buffer to which enough lauryl maltoside was added to make the concentration equal to 2 mM lauryl maltoside; the eluting buffer was 2 mM lauryl maltoside in the pH 9, $I = 0.01$ buffer; 0.68-mL fractions were collected at 4-min intervals. (B) Exchange of octyl glucoside for protein-bound Nonidet P-40 at pH 9: a 0.5 × 5 cm bed of phenyl-Sepharose was presaturated with 3 mL of 51 mM octyl glucoside in a pH 9.0, $I = 0.01$ buffer; a 0.5-mL sample was applied to the column that contained 3.2 mg of protein/mL and 15 mM Nonidet P-40 in the pH 9, $I = 0.01$ buffer to which enough octyl glucoside was added to make the solution 51 mM octyl glucoside; the elution buffer was identical with the presaturation buffer; 0.68-mL fractions were collected at 4-min intervals.

complex in any way other than changing the detergent that was bound to the complex. Neither the amount of bound phospholipid, subunit composition, nor enzymatic activity was significantly altered by the detergent-exchange procedure. For example, after exchange of lauryl maltoside for Nonidet P-40 bound to cytochrome *c* oxidase, the electron-transport activity of the lauryl maltoside solubilized complex was 85–95% its original value, provided both samples were assayed in the presence of 1 mg/mL lauryl maltoside. To achieve this high retention of enzymatic activity, the time that the protein complex remained at pH 9.0 was minimized by collecting the cytochrome *c* oxidase that was eluted from the pH 9 phenyl-Sepharose column in a 0.2 M Tris-HCl buffer at pH 8. The detergent-exchange procedure also did not affect the subunit composition of the complex; i.e., after either lauryl maltoside exchange or dodecyl sulfate exchange, the complex had the seven major subunits and two minor bands typical of bovine heart cytochrome *c* oxidase.

The only detergent exchange that was not successful with cytochrome *c* oxidase was the exchange of cholate for Nonidet P-40 or Triton X-100. Although cholate could be exchanged for these detergents in the absence of protein, the method could not be used if cytochrome *c* oxidase were present since the protein complex tightly bound to the cholate-saturated resin even at pH 9 and $I = 0.01$ and was not eluted even with very high concentrations of cholate.

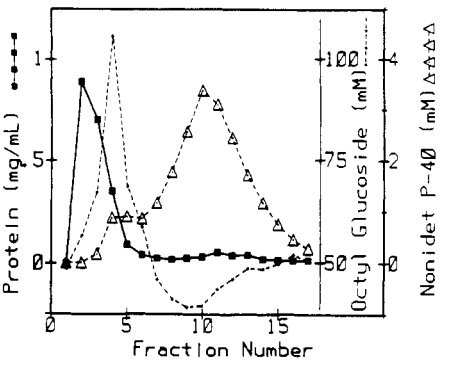

FIGURE 3: Exchange of octyl glucoside for Nonidet P-40 bound to a mixture of inner mitochondrial proteins at pH 8. A 0.5 × 10 cm bed of phenyl-Sepharose was presaturated with 51 mM octyl glucoside in pH 8.0, $I = 0.01$ buffer; a 0.6-ml sample was applied to the column that contained 3.5 mg of protein/mL and 30 mM Nonidet P-40 in the pH 8, $I = 0.01$ buffer to which enough octyl glucoside was added to make solution 51 mM octyl glucoside; the elution buffer was identical with the presaturation buffer; 0.6-mL fractions were collected at 4-min intervals.

Detergent Exchange with Mixtures of Intrinsic Membrane Proteins. To test the general applicability of the phenyl-Sepharose-mediated detergent-exchange method for the exchange of detergents bound to a variety of intrinsic membrane proteins, exchange of octyl glucoside or dodecyl sulfate for the detergent present in Nonidet P-40 solubilized mitochondrial inner membranes was tested. Complete exchange of either of these detergents for protein-bound Nonidet P-40 was possibile at pH 9.0 and $I = 0.01$ with a 85–100% recovery of the inner mitochondrial membrane proteins (Table II). Exchange of octyl glucoside for the protein-bound Nonidet P-40 was also possible at pH 8.0 and $I = 0.01$ with only a slightly lower recovery of protein (Figure 3 and Table II). To determine whether individual proteins were preferentially bound to the hydrophobic resin, the mitochondrial protein composition of the sample after exchange into either octyl glucoside or dodecyl sulfate was compared with the protein composition of the original Nonidet P-40 solubilized membrane by using polyacrylamide slab gel electrophoresis in the presence of dodecyl sulfate and 2 M urea. Within the accuracy of this type of analysis, the protein composition had not been affected by the detergent-exchange procedure, indicating that preferential binding of some proteins to the hydrophobic resin had not occurred.

As a second test of the general applicability of this detergent-exchange method with other detergent-solubilized intrinsic membrane proteins, dodecyl sulfate was exchanged for the Triton N-101 present in a mixture of erythrocyte membrane proteins. Complete exchange of dodecyl sulfate for the protein-bound Triton N-101 was accomplished with a 90% recovery of erythrocyte membrane proteins (Figure 4). Subsequent analysis of the protein composition of the recovered protein by the same polyacrylamide dodecyl sulfate gel electrophoresis system described above indicated that none of the intrinsic membrane proteins were preferentially bound to the phenyl-Sepharose.

Removal of Excess Detergent from Detergent-Solubilized Membrane Proteins. In the previous experiments, phenyl-Sepharose has been shown to preferentially bind detergents rather than detergent-solubilized membrane proteins. This preferential binding of detergents can be utilized to remove excess detergent from solubilized membrane proteins. From the detergent binding data, phenyl-Sepharose was known to bind ~40 μmol of Triton X-100/mL of resin. If one uses this value, 1 mL of resin should be able to remove 25 mg of excess

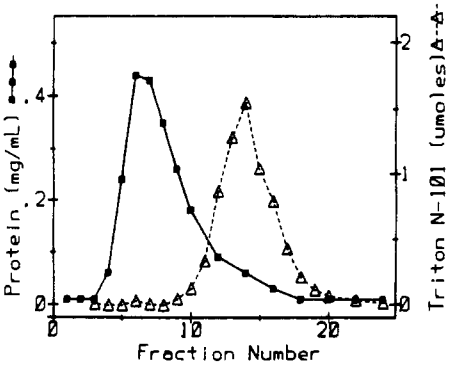

FIGURE 4: Exchange of dodecyl sulfate for Triton N-101 bound to a mixture of erythrocyte proteins at pH 9. A 0.5 × 10 cm bed of phenyl-Sepharose was presaturated with 10 mL of 3.5 mM dodecyl sulfate in the pH 9.0, $I = 0.01$ buffer; a 2.0-mL sample that contained 2.3 mg of protein/mL and 12.6 mM Triton N-101 was applied to the column; the elution buffer was identical with the presaturation buffer; 0.66-mL fractions were collected at 4-min intervals.

Triton X-100 from a protein solution. Removal of Triton X-100 from a protein was demonstrated by applying a 2-mL sample of cytochrome *c* oxidase (1 mg/mL) that was solubilized with 13 mg/mL Triton X-100 to a 1-mL phenyl-Sepharose column at pH 8, $I = 0.10$. The majority of the cytochrome *c* oxidase (68%) was not retarded by the column and eluted in the presence of 1 mg of Triton X-100. Similar removal of excess Triton X-100 or Nonidet P-40 from cytochrome *c* oxidase at pH 9 and $I = 0.01$ resulted in considerably higher recovery of protein.

Discussion

The preceding results illustrate the usefulness of detergent-saturated phenyl-Sepharose as a chromatographic medium for the exchange of detergents bound to intrinsic membrane proteins. The basis of the method appears to be quite similar to the exchange of anions or cations with ion-exchange chromatography; therefore, we have called this procedure "detergent-exchange chromatography". The design of our experimental procedure was as follows: (1) as in the exchange of anions or cations, the resin was first equilibrated with a detergent that would be exchanged for the detergent already bound to the protein; (2) aggregation or precipitation of the membrane proteins was avoided by using detergent concentrations that were greater than the cmc for each detergent; (3) the association of the proteins with the resin was minimized by using a relatively high pH (9.0), by using a low ionic strength (0.01), and by presaturating the resin with detergent. When this approach was followed, the exchange of detergents proceeded as would be expected with any other type of partition chromatography.

Although exchange of several detergents was accomplished, exchange of some detergents was more successful than others. For example, the exchange of either lauryl maltoside or octyl glucoside for protein-bound Nonidet P-40 was routinely accomplished with 99.7 and 97–99% efficiency, respectively. However, exchange of of dodecyl sulfate for the protein-bound Nonidet P-40 was usually only 90% complete. The relative affinities of these detergents for phenyl-Sepharose are clearly not identical. This is also evident from the very slow elution of Nonidet P-40 from a column that had been saturated with lauryl maltoside (refer to Figure 1A) but a relatively rapid elution of the Nonidet P-40 from resin that had been saturated with either octyl glucoside or dodecyl sulfate (refer to Figures 1B, 2, and 3).

An unexpected aspect of the detergent-exchange chromatography method was the elution of the protein from the column prior to the elution of the large detergent peak (refer to Figures 2 and 3). Apparently, the detergent partitioned between the immobile and mobile phases while the protein did not partition because the protein did not bind to the resin. The lack of coincident elution of protein and excess detergent did not cause experimental problems since excess detergent was also present in the elution buffer; therefore, the protein eluted in the presence of excess detergent even if it preceded the detergent peak.

The detergent-exchange chromatography procedure has several advantages over other methods that have been used for the exchange of detergents bound to intrinsic membrane proteins. First, the method is both simple and rapid. Complete detergent exchange can typically be achieved with very small columns, ca. 1–2 mL, in a very short time, e.g., less than 10 min. Second, the membrane proteins are always solubilized by detergent; thus, protein aggregation is avoided while the maintenance of enzymatic activity is maximized.

Other procedures that have used hydrophobic resins for the exchange of detergents bound to proteins have completely removed the detergent while the protein was bound to the resin (Horigome & Sugano, 1983; Steiner & Oesterhelt, 1983). After all of the detergent had been removed, the protein was then displaced from the resin with a second detergent. There are two difficulties with this approach. First, it is quite difficult to remove the last traces of protein-bound detergent in the absence of other amphiphilic molecules. Second, in contrast to our procedure, this approach does not maintain the proper environment at the hydrophobic surface of the protein. It is our experience that complete retention of biological activity is seldom achieved if the aqueous solution rather than detergents or phospholipids contacts these surfaces.

Although experiments using hydrophobic matrices other than phenyl-Sepharose were not described in this paper, similar exchange of detergents with octyl-Sepharose CL-4B were also successful. Conceivably, reverse-phase HPLC column-packing materials would also work well, but the rapidity of the detergent-exchange chromatography method with 1–2-mL columns of phenyl-Sepharose probably means that an HPLC approach is not warranted. From the variety of proteins that were used in this study as well as the variety of detergents that were successfully exchanged, phenyl-Sepharose-mediated detergent-exchange chromatography appears to be a general method that could be used with most detergent-solubilized membrane proteins.

Registry No. Phenyl-Sepharose CL4B, 69106-59-8; Triton X-100, 9002-93-1; Triton N-101, 9016-45-9; cholic acid, 81-25-4; octyl β-D-glucoside, 29836-26-8; lauryl β-D-maltoside, 69227-93-6; Nonidet P-40, 9036-19-5; dodecyl sulfate, 151-41-7.

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Monovalent Cation-Induced Phospholipid Vesicle Aggregation: Effect of Ion Binding†

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ABSTRACT: Aggregation of acidic phospholipid vesicles induced by monovalent cations was studied for vesicles of small and large sizes. It was found that there were two phases in the aggregation of large acidic phospholipid vesicles. In the initial phase, observed in the range of 0.1–0.4 M monovalent salts, aggregation took place spontaneously after a change in salt concentration; in the second phase (>0.4 M salt), aggregation progressed gradually with time. The order of capability for monovalent cations to induce the initial phase of aggregation of large phosphatidylserine vesicles (more than 1000 Å in diameter) was $Li^+ > Na^+ > K^+ > TEA^+$. However, for the second phase of aggregation, the order was $Na^+ > Li^+ > K^+ > TEA^+$, which was the same as that to induce massive aggregation of small phosphatidylserine vesicles (250 Å in diameter). A similar reversal in the order was observed in studies of the surface potential of the phosphatidylserine monolayer. In these studies, the order of the binding strength of monovalent cations was deduced from the change in surface potential produced by successive additions of $MgCl_2$ to the subphase solution, which contained a certain level of monovalent salt initially. These measurements were carried out with monolayers that had a range of areas per molecule. The order was $Na^+ > Li^+ > K^+$ for monolayers of large area (>80 $Å^2$) per molecule and was $Li^+ > Na^+ > K^+$ for those of small area (<80 $Å^2$) per molecule. For phosphatidic acid vesicles, the order of monovalent cations inducing vesicle aggregation was independent of the size of the vesicles and the monovalent salt concentration: $Na^+ > Li^+ > K^+ > Cs^+$. In this case, too, two phases of the aggregation process were observed. From the surface potential experiment of phosphatidic acid monolayers, it was also deduced that the adsorption of Na^+ on the phosphatidic acid membrane surface is slightly stronger than that of Li^+ in the range of areas (60~90 $Å^2$) per molecule. The observed phenomena of monovalent cation-induced vesicle aggregation with respect to variation of the size of vesicle as well as salt concentration were examined in terms of binding constant and accessibility of monovalent cations to the binding sites of lipid polar groups.

In order to elucidate biological membrane adhesion and fusion, a number of studies to mimic such phenomena using model membranes have been done for the last decade (Nir et al., 1983). Among them, it is shown that acidic phospholipid membranes can adhere or fuse in the presence of proper amounts of cations in membrane bathing solutions (Nir et al., 1983a,b; Papahadjopoulos et al., 1974). The degree of such membrane adhesion or fusion seems to depend on the strength of binding of these ions to negatively charged polar groups of lipid molecules (Ohki, 1982). Although monovalent cations can induce aggregation of acidic phospholipid membranes (Ohki et al., 1982), they do not induce fusion of these membranes (Ohki, 1982), probably because of a rather weak binding capability of monovalent cation to phospholipid molecules when compared with those of divalent and polyvalent cations (Ohki, 1982; Düzgünes, et al., 1981). The presence of monovalent cations in the solution, however, does influence the degree of divalent cation binding as well as their fusion capabilities, probably due to a competitive binding to phospholipid polar group binding sites between monovalent and divalent cations (Ohki, 1982; Düzgünes, et al., 1981; Nir et al., 1983a,b).

Recently, we have studied phosphatidylserine vesicle aggregation induced by various monovalent cations (Ohki et al., 1982). It was found that the order of the capability of monovalent cations to induce aggregation of small vesicles made of phosphatidylserine molecules is $H^+ > Na^+ > Li^+ > K^+ > Cs^+ > TEA^+$. From this, we have deduced that the order of binding strength of these ions is the same as the above. However, others (Hauser et al., 1970; Eisenberg et al., 1979) have measured electrophoretic mobility of the large multilamellar vesicles of the same lipid in various monovalent salt suspension and found that the order of the binding of these ions is $H^+ > Li^+ > Na^+ > K^+ > Cs^+ > TEA^+$, which is almost the same as the above except for the relative positions of Na^+ and Li^+ in the series. The same order of ion binding strength was obtained for multilamellar phosphatidylserine membrane systems from the study with X-ray diffraction techniques (Loosley-Millman et al., 1982; Hauser & Shipley, 1983). We have suggested (Ohki et al., 1982) that these differences might be due to the use of different sizes of vesicles in the two different experiments.

In order to elucidate possible reasons for these observed differences, here we have performed experiments on vesicle

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