Functional Reconstitution into Proteoliposomes and Partial Purification of a Rat Liver ER Transport System for a Water-Soluble Analogue of Mannosylphosphoryldolichol†

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ABSTRACT: Mannosylphosphoryldolichol (Man-P-Dol) is synthesized on the cytosolic leaflet of the rough endoplasmic reticulum (ER), and functions as a mannosyl donor in the biosynthesis of $Glc_3Man_9GlcNAc_2$-P-P-Dol after being translocated to the lumenal leaflet. An assay, based on the transport of Man-P-citronellol (Man-P-Dol_{10}), a water-soluble analogue of Man-P-Dol_{95}, into sealed microsomal vesicles, has been devised to identify protein(s) (flippases) that could mediate the thermodynamically unfavorable movement of Man-P-Dol between the two leaflets of the ER. To develop a defined system for the systematic investigation of the properties of the Man-P-Dol_{10} transporter, and as an initial step toward purification of the protein(s) involved in the transport of Man-P-Dol_{10}, the activity has been solubilized from rat liver microsomes with *n*-octyl-β-D-glucoside and reconstituted into proteoliposomes ($\sim$0.1 μm in diameter). The properties of the reconstituted Man-P-Dol_{10} transport system are similar to the Man-P-Dol_{10} uptake activity in microsomal vesicles from rat liver. Man-P-Dol_{10} transport into reconstituted proteoliposomes is time-dependent, reversible, saturable, and stereoselective. The direct role of ER proteins in the functionally reconstituted transport system is supported by the inhibitory effects of trypsin treatment, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), or diethylpyrocarbonate (DEPC). Solubilization and functional reconstitution are shown to provide an experimental approach to the partial purification of the protein(s) mediating the transport process.

The role of polyisoprenoid glycosyl carrier lipids in the biosynthesis of complex glycoconjugates has been extensively documented in prokaryotes (*1*, *2*) and eukaryotes (*3*, *4*). Because the unassisted transbilayer movement of glycerophospholipids (*5*, *6*) and polyisoprenyl phosphate-linked sugars (*7*, *8*) in liposomes is extremely slow, the transverse diffusion of the polar lipids is believed to require a specialized class of membrane proteins termed "flippases" (*9*). In bacteria, flippases are thought to mediate the flip-flopping of a wide variety of intermediates in the synthesis of bacterial O-antigens (*10*, *11*), lipopolysaccharide (*12*), the muramyl-pentapeptide intermediate of the cell wall (*2*), the enterobacterial common antigen (ECA)[1] of *Escherichia coli* (*13*), and many other extracellular polymers (*1*). In eukaryotes, flippases would mediate the transbilayer movement of glycolipid intermediates involved in the synthesis of GPI anchors (*14*), complex glycosphingolipids (*15*, *16*), dolichol-linked saccharide intermediates (*17*−*19*), and the O-mannosyl units of yeast glycoproteins (*20*).

In the synthesis of the dolichol-linked precursor oligosaccharide in the protein N-glycosylation pathway, Man-P-Dol, Glc-P-Dol, and $Man_5GlcNAc_2$-P-P-Dol are formed on the cytoplasmic leaflet of the endoplasmic reticulum (ER) and must diffuse transversely to the lumenal monolayer to participate in the synthesis of $Glc_3Man_9GlcNAc_2$-P-P-Dol (*17*, *18*). Since the unassisted "flip-flopping" of $GlcNAc_2$-P-P-Dol (*7*) and spin-labeled analogues of polyisoprenyl compounds (*8*) in synthetic lipid bilayers occurs at an extremely slow rate, the transbilayer movement of the polar headgroups of these three lipid intermediates is thought to be facilitated by a set of "flippases" in the ER (*9*, *19*, *21*, *22*).

Despite the crucial role of flippases in membrane biology, progress in isolating and characterizing the membrane proteins functioning as flippases in prokaryotic and eukaryotic cells has been extremely slow due to the inherent technical difficulties in assaying these processes in vitro. Several years ago, Bishop and Bell (*23*) followed the transport of a water-soluble analogue of phosphatidylcholine (PC), diC_4PC, into sealed microsomal vesicles as an approach to assaying PC flippase activity in rat liver. This experimental approach was later adapted to follow the transport of Man-P-citronellol (Man-P-Dol_{10}) and Glc-P-citronellol (Glc-P-Dol_{10}), water-soluble analogues of Man-P-Dol and Glc-P-

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[1] Abbreviations: ECA, enterobacterial common antigen; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DEPC, diethylpyrocarbonate; Man-P-Dol_n, mannosylphosphoryldolichol (n = number of carbon atoms in the isoprenyl chain); Glc-P-Dol; glucosylphosphoryldolichol; Man_5-$GlcNAc_2$-P-P-Dol, mannosyl$_5$-chitobiosylpyrophosphoryldolichol; OG, *n*-octyl-β-D-glucoside; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CDG, congenital disorders of glycosylation; PC, phosphatidylcholine; PE, phosphatidylethanolamine; diC_4PC, dibutyroylphosphatidylcholine; ER, endoplasmic reticulum.

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Dol, respectively, into microsomal fractions from rat liver and pig brain, as a means of assaying the corresponding flippase activities (*24*−*26*). In addition, flippase assays using GlcNAc-P-P-nerol, a water-soluble analogue of GlcNAc-P-P-undecaprenol (GlcNAc-P-P-Undec) have provided evidence that the *E. coli* WzxE protein mediates the transbilayer movement of Fuc4NAc-ManNAcA-GlcNAc-P-P-Undec (lipid III), an intermediate in the assembly of the ECA in *E. coli* (*27*).

Reconstitution of membrane proteins into synthetic phospholipid bilayers has proven to be an effective approach for characterizing the properties and function of a variety of membrane transporters and receptors (*28*). To extend the characterization of the transport system, and develop methods to be used in the purification of proteins involved in the transbilayer movement of Man-P-Dol, Man-P-Dol_{10} transport activity was solubilized from rat liver ER-enriched microsomes with *n-octyl-β*-D-glucoside (OG), and reconstituted into synthetic phospholipid bilayers by equilibrium dialysis. The experiments described here demonstrate that solubilized Man-P-Dol_{10} uptake activity with the properties of the Man-P-Dol_{10} transport activity in ER vesicles can be efficiently reconstituted into synthetic proteoliposomes. The procedure described here is the first report of the solubilization and functional reconstitution of a member of this novel family of membrane proteins, and provides a potential approach for the purification and thorough characterization of the putative Man-P-Dol flippase system.

EXPERIMENTAL PROCEDURES

Materials. n-Octyl-*β*-D-glucoside was obtained from Calbiochem (La Jolla, CA). Pig liver phosphatidylcholine (PC) was purchased from Serdary Research Laboratories (London, Ontario, Canada). DEAE-Sephacel and di-oleoyl-phosphatidylethanolamine (PE) were from Sigma Chemical Co (St. Louis, MO). 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was obtained from Molecular Probes, Inc. (Eugene, OR). *S*-Citronellol was obtained from Aldrich Chemical Co. (Milwaukee, WI) and phosphorylated using phosphorus-oxychloride as described previously (*29*). [2-^{3}H]Mannose was purchased from American Radiolabeled Chemicals (St. Louis, MO), and used to synthesize GDP-[^{3}H]mannose and [^{3}H]Man-P-Dol_{10} as described (*29*). Spectrapor 14 000 molecular weight cutoff (08-677 B) dialysis tubing was obtained from Fisher Scientific Co. (Pittsburgh, PA). Millipore HA 0.45 *μ*m filters were purchased from Baxter Scientific Products (Obetz, OH). Econosafe Biodegradeable Counting Cocktail is a product of Research Products International Corp. (Mount Prospect, Il). All other chemicals were obtained from standard commercial sources.

Preparation of Rat Liver Microsomes and Subcellular Fractions. Rat liver ER-enriched microsomes were prepared from 24 h fasted Sprague−Dawley rats by the method of Coleman and Bell (*30*). Subcellular fractions from rat liver were prepared from 24 h fasted Sprague−Dawley rats by a modification of the methods of Leelavathi et al. (*31*) and Fleisher and Kervina (*32*) exactly as described previously (*24*).

Synthesis of Man-P-Dol_{10}. α-Man-P-Dol_{10} and *β*-Man-P-Dol_{10} were synthesized and purified as described previously (*24*, *29*).

Assay for Man-P-Dol_{10} Uptake Activity. Man-P-Dol_{10} uptake was assayed as described previously with a minor modification (*24*). Assay mixtures for Man-P-Dol_{10} uptake contained 100−250 *μ*g of membrane protein, 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM EDTA, and the indicated concentration of [^{3}H]Man-P-Dol_{10} (10−500 cpm/pmol) in a total volume of 0.02 mL. Following incubation of the sample at 21 °C for the indicated period of time, the transport assay mixtures were diluted with 0.5 mL of ice-cold 10 mm Tris-HCl (pH 8.5), 0.25 M sucrose, 5 mM $CaCl_2$ and transferred immediately to a prechilled vacuum filtration manifold equipped with a Millipore HA 0.45 *μ*m filter. The filters were washed with 10 mL of ice-cold 10 mm Tris-HCl (pH 8.5), 0.25 M sucrose, 5 mM $CaCl_2$ and analyzed for radioactivity by liquid scintillation spectrometry using a Packard TR2100 Liquid Scintillation spectrometer after solubilization with 1 mL of 1% SDS and the addition of 10 mL of Econosafe Biodegradeable Counting cocktail. Pilot studies indicated that the recovery of protein by filtration of proteoliposomes on Millipore HA filters is similar to the recovery of liver ER microsomal protein (60−70%) under the conditions used in these transport experiments. A modification of this assay, described in the legend to Figure 7, was employed to normalize for the effect of trypsin inhibitor on the recovery of proteoliposomes.

Assay for the Intactness of Rat Liver ER Microsomes and Reconstituted Proteoliposomes. Man 6-P phosphatase activity was determined as described previously (*33*). The intactness of rat liver ER microsomes was calculated based on the latency of Man 6-P phosphatase (*33*). The stability of reconstituted proteoliposomes to trypsin treatment was assessed by a preloading control with [^{3}H]inositol. Liposomes and proteoliposomes containing preloaded [^{3}H]inositol were formed by detergent depletion of Triton X-100 extracts using polystyrene SM-2 BioBeads (Bio-Rad Laboratories, Hercules, CA) as described by Menon et al. (*34*).

Solubilization and Reconstitution of Rat Liver Man-P-Dol_{10} Uptake Activity into Synthetic Proteoliposomes. Solubilization mixtures contained rat liver microsomes (2−10 mg/mL membrane protein), 20 mM Tris-HCl (pH 7.4), 0.1 M KCl, 10 mM $MgCl_2$, 5 mM EDTA, 0.25 M sucrose, and 2% OG. After 30 min at 4 °C, insoluble material was removed by centrifugation at 140000*g*, 30 min. The supernatant was supplemented with an equal volume of 20 mM Tris-HCl (pH 7.4), 0.1 M KCl, 10 mM $MgCl_2$, 5 mM EDTA, 0.25 M sucrose, and 2% OG containing ultrasonically dispersed pig liver PC (4−20 mg/mL) and di-oleoyl-PE (0.02−1 mg/mL) and transferred to a Spectrapor dialysis bag (Fisher Scientific Co, Pittsburgh, PA) 14 000 molecular weight cutoff (08-677 B). The solubilization mixture was dialyzed at 4 °C against 50−100 vols of 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$ for 4 days, replacing the dialysis buffer every 12 h. Reconstituted proteoliposomes were recovered by centrifugation at 140000*g*, 30 min, resuspended in 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose at a membrane protein concentration of 5−10 mg/mL and frozen quickly in a dry ice/ethanol bath. Reconstituted proteoliposomes were stored at −80 °C until analyzed for Man-P-Dol_{10} uptake activity.

Chromatography of Rat Liver ER Proteins on DEAE-Sephacel. Rat liver ER-enriched membrane proteins were solubilized as described above except that KCl was omitted. Solubilized rat liver ER membrane proteins were fractionated

Table 1: Comparison of Various Detergents for the Solubilization and Reconstitution of Man-P-Dol_{10} Transport Activity[a]

	protein solubilized		protein in proteoliposomes		Man-P-Dol_{10} transport activity		
detergent	(mg)	(%)	(mg)	(%)	(pmol/min/mg)	(pmol/min)	(%)
CHAPS	19.5	63	4	20.6	1.4	0.58	2.5
sodium deoxycholate	25.7	82.5	1.4	5.3	0.1	0.14	0.6
sodium taurocholate	26.2	84.3	0.7	2.6	1.6	0.1	0.5
octyl glucoside	12.6	40.5	1.4	11.1	6.1	0.85	3.6

[a] Assay mixtures for the determination of Man-P-Dol_{10} uptake contained 200 μg of membrane protein, 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM EDTA, and 12 μM [^{3}H]Man-P-Dol_{10} (517 cpm/pmol) in a total volume of 0.02 mL. Following incubation at 21 °C for 5 min, Man-P-Dol_{10} uptake was determined as described in Experimental Procedures.

on a 1 × 8 cm column of DEAE-Sephacel equilibrated in 0.01 M Tris-HCl (pH 7.4), 0.25 M sucrose, 0.5% OG. Following elution with 2 column vols of equilibration buffer, bound proteins were eluted with a gradient of 0 to 1 M NaCl (40 mL) in equilibration buffer. Fractions of 3 mL were collected, supplemented with 1.6 mg of PC and 0.08 mg of PE, as described above, and dialyzed against 10 mM Tris-HCl (pH 7.4), 0.25 sucrose, and 1 mM $MgCl_2$. After 84 h of dialysis, the proteoliposomes were recovered by sedimentation at 140000*g*, 30 min, resuspended in 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose and assayed for protein and for Man-P-Dol_{10} transport activity.

SDS−PAGE. Gel electrophoresis was performed in SDS−12% polyacrylamide minigels according to Laemmli (*35*) at a current of 30 mA in a Hoeffer SE 250 Mighty Small minigel apparatus (San Francisco, CA). Protein bands were detected by staining with the GelCode SilverSNAP Stain kit (Pierce, Rockford, IL) using the manufacturer's instructions.

Electron Microscopy of Reconstituted Rat Liver Proteoliposomes. Reconstituted rat liver proteoliposomes were examined by electron microscopy on a Hitachi 7000 transmission electron microscope at 75 kV following negative staining with 2% uranyl acetate on a copper mesh Formvar carbon-coated grid.

Analytical Methods. Membrane protein concentration was determined by the method of Rodriquez-Vico et al. (*36*) using a commercial protein assay reagent (BCA, Pierce, Rockford, IL). Lipid-phosphorus was determined by the method of Bartlett (*37*). Succinate dehydrogenase assays were performed according to Pennington et al. (*38*). Samples were analyzed for radioactivity by liquid scintillation spectrometry in a Packard Tri-Carb 2100TR liquid scintillation spectrometer following the addition of Econosafe Biodegradeable Counting cocktail (Research Products International Corp., Mt. Prospect, IL).

RESULTS

Comparison of Detergents for the Solubilization and Reconstitution of Man-P-Dol_{10} Transport Activity. To select an effective detergent for the solubilization and reconstitution of Man-P-Dol_{10} uptake activity into proteoliposomes, several detergents were compared for their ability to solubilize rat liver ER membrane proteins. A comparison of several commonly available detergents (Table 1) indicated that all of the detergents tested were capable of solubilizing a substantial amount of membrane protein. However, following centrifugation at 140000*g* and removal of detergent from the soluble fraction by equilibrium dialysis, OG was found to be the most effective for the formation of proteoliposomes capable of transporting Man-P-Dol_{10}. Recovery of reconstituted protein in proteoliposomes following dialysis of the sodium deoxycholate and sodium taurocholate extracts was very poor, consistent with inefficient removal of detergent by dialysis, due to the low CMC of these detergents. Although the total reconstituted transport capacity was similar in the CHAPS and OG preparations, the specific activity of Man-P-Dol_{10} uptake activity in the proteoliposomes reconstituted from OG extracts was approximately 4-fold higher than the activity in CHAPS-reconstituted proteoliposomes. On the basis of this comparison, OG was used for the remainder of these studies on the reconstituted Man-P-Dol_{10} transport system.

Properties of Man-P-Dol_{10} Transport Activity in Synthetic Proteoliposomes. To optimize the formation of Man-P-Dol_{10} transport-competent proteoliposomes, the effects of ionic strength, OG concentration, and protein concentration during solubilization and dialysis time during reconstitution were examined. Preliminary studies indicated that reconstitution of Man-P-Dol_{10} uptake activity was optimal following solubilization in the presence of 150 mM KCl and 2% OG at a membrane protein concentration of approximately 2−10 mg/mL (data not shown). The formation of Man-P-Dol_{10} transport-competent proteoliposomes is highly dependent on dialysis time. Figure 1 shows that, although the bulk of the solubilized membrane protein is incorporated into proteoliposomes during the first 24 h of dialysis (Figure 1, ○), Man-P-Dol_{10} transport activity reconstitutes much more slowly, requiring up to 90 h to reach near maximal transport activity (Figure 1, ●). In Figure 1, the data represent the results of near-equilibrium transport experiments, assessing the formation of transport-competent vesicles, and are expressed as total picomoles of Man-P-Dol_{10} transported into proteoliposomes at equilibrium. All subsequent experiments were performed with proteoliposomes formed after approximately 90 h of dialysis.

Effect of Exogenous Phospholipids on Reconstitution of Man-P-Dol_{10} Transport Activity. To determine if the formation of Man-P-Dol_{10} transport active proteoliposomes could be enhanced by the addition of exogenous phospholipids, the effects of the addition of the indicated lipids to the reconstitution mixtures were evaluated. Figure 2 indicates that the addition of exogenous pig liver PC significantly enhances the formation of the Man-P-Dol_{10} transport system in proteoliposomes. The addition of exogenous pig liver PC to the reconstitution mixtures has only a modest effect on the amount of protein recovered in the proteoliposomes (Figure 2, ○), but dramatically improves the capacity for Man-P-Dol_{10} uptake (Figure 2, ●). As in Figure 1, the data in Figure 2 represent near-equilibrium transport experiments

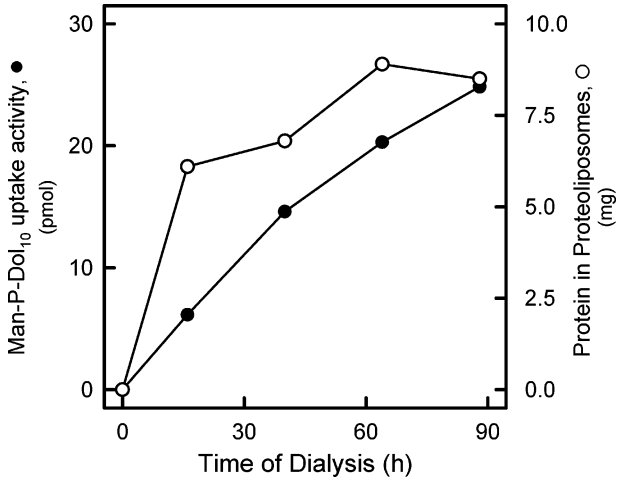

FIGURE 1: Time course of reconstitution of Man-P-Dol_{10} transport activity into synthetic proteoliposomes. Rat liver ER-enriched microsomes (124 mg of membrane protein) were solubilized with 2% OG, centrifuged at 140000*g*, 30 min, supplemented with pig liver PC and PE and dialyzed at 4 °C against 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, and 1 mM $MgCl_2$ as described in Experimental Procedures. Aliquots, containing 23 mg of membrane protein, were withdrawn at the indicated times, centrifuged at 140000*g*, 20 min, resusupended in 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose and assayed for protein (○) and Man-P-Dol_{10} transport activity (●). Assay mixtures for the determination of Man-P-Dol_{10} uptake contained 200 μg of membrane protein, 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM EDTA, and 12 μM [^{3}H]Man-P-Dol_{10} (517 cpm/pmol) in a total volume of 0.02 mL. Following incubation at 21 °C for 5 min, Man-P-Dol_{10} uptake was determined as described in Experimental Procedures.

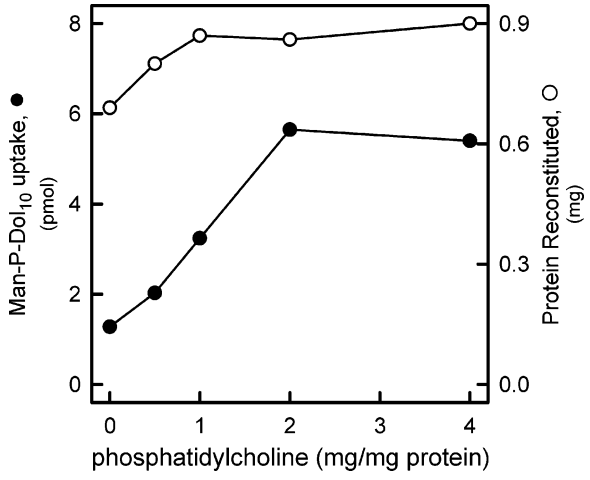

FIGURE 2: Effect of addition of exogenous pig liver PC on the reconstitution of functional Man-P-Dol_{10} transport activity into synthetic proteoliposomes. Rat liver ER microsomes were solubilized with 2% OG as described in Experimental Procedures and centrifuged at 140000*g*, 30 min. The soluble supernatant was assayed for protein, divided into aliquots (2.5 mg of membrane protein), and then supplemented with three vols of solubilization buffer containing ultrasonically dispersed pig liver PC in the indicated ratio to soluble protein and dialyzed at 4 °C as described in Experimental Procedures. After dialysis (84 h), reconstitution reactions were centrifuged at 140000*g*, 20 min, resuspended in 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose and assayed for protein reconstituted into proteoliposomes (○) and Man-P-Dol_{10} transport activity (●). Assay mixtures for the determination of Man-P-Dol_{10} uptake contained 80 μg of membrane protein, 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM EDTA, and 12 μM [^{3}H]Man-P-Dol_{10} (517 cpm/pmol) in a total volume of 0.02 mL. Following incubation at 21 °C for 5 min, Man-P-Dol_{10} uptake was determined as described in Experimental Procedures.

and are, therefore, a reflection of total Man-P-Dol_{10} transport capacity of the proteoliposomes prepared under these conditions. Addition of PC at a phospholipid/protein ratio of approximately 2 mg of PC/mg of solubilized membrane protein was optimal (Figure 2).

Inclusion of exogenous PE had a minimal effect on the reconstitution of "bulk" protein into proteoliposomes and modestly improved the reconstitution of Man-P-Dol_{10} transport activity at a ratio of 0.05 mg of PE/mg of PC (data not shown). Addition of PE beyond 0.1 mg of PE/mg of PC lowered transport activity in the reconstituted vesicles.

The effects of supplementation of the reconstitution mixtures with a variety of other lipids, added at the mol % reported for rat liver ER microsomes (*39*), indicated that the addition of cholesterol at 7.6 mol %, had no effect on the recovery of either protein or phospholipids in the proteoliposomes, but did improve the rate of Man-P-Dol_{10} uptake by approximately 50%, compared to reconstitution in the presence of PC and PE alone. The addition of either phosphatidylserine (2 mol %), phosphatidylinositol (9 mol %), or sphingomyelin (2.1 mol %) had no effect on either the formation of proteoliposomes or on the transport of Man-P-Dol_{10} (data not shown). For the remainder of these studies, reconstitution mixtures were supplemented with only exogenous PC and PE as described in Experimental Procedures. An average of 38% (± 8%) of the initial membrane protein and 14.3% (± 10%) of the exogenously added phospholipid in the OG-soluble extracts were recovered in sedimentable proteoliposomes formed under these conditions.

Electron Microscopic Analysis of Reconstituted Proteoliposomes. To investigate the physical state of the functional reconstituted proteoliposomes described above, the vesicles were examined by electron microscopy following negative staining with 2% uranyl acetate. Figure 3 shows that the reconstituted proteoliposomes are largely unilamellar vesicles, and are approximately 0.05−0.2 μm in diameter, based on three separate analyses. The proteoliposomes formed under these conditions contained an average of 0.72 μmol (± 0.17) of phospholipid/mg of protein (data not shown).

Characterization of Man-P-Dol_{10} Uptake Activity in Reconstituted Proteoliposomes. Previously, Man-P-Dol_{10} transport activity in ER-enriched microsomal vesicles was shown to be (1) time-dependent and stereoselective, (2) reversible, (3) saturable with respect to Man-P-Dol_{10} concentration, (4) protease-sensitive, and (5) enriched in ER vesicles. To verify that the reconstituted activity was similar to the microsomal activity, Man-P-Dol_{10} uptake activity in reconstituted proteoliposomes was characterized as described previously for ER-enriched microsomes (*24*). Man-P-Dol_{10} transport into synthetic proteoliposomes is time dependent (Figure 4A). In addition, β-Man-P-Dol_{10} is transported much more efficiently than α-Man-P-Dol_{10} (Figure 4) into reconstituted proteoliposomes. Furthermore, the time-course and stereospecificity of uptake of β-Man-P-Dol_{10} over α-Man-P-Dol_{10} in reconstituted proteoliposomes is virtually indistinguishable from that in native ER-enriched microsomes (Figure 4B). Native ER vesicles transport Man-P-Dol_{10} at a somewhat higher rate than the reconstituted proteoliposomes.

Consistent with the properties of the rat liver microsomal uptake system, Man-P-Dol_{10} transport in reconstituted proteoliposomes was also reversible. When proteoliposomes,

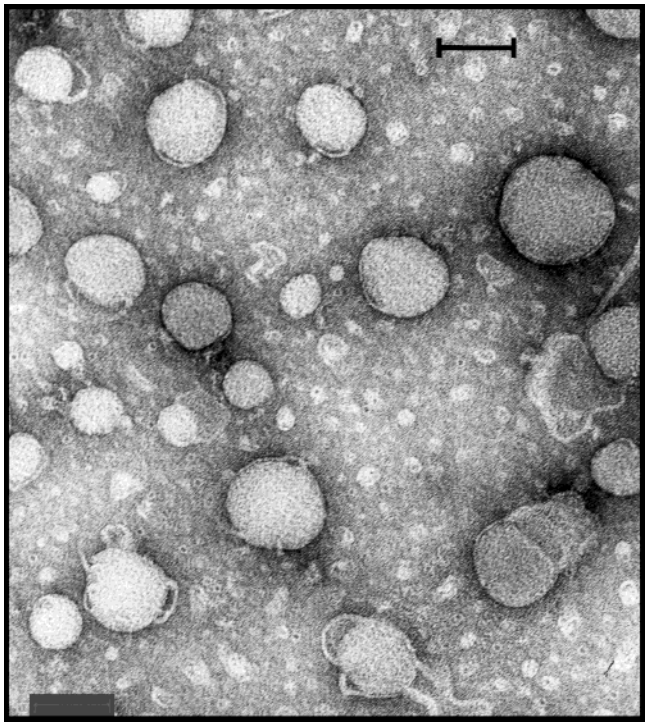

FIGURE 3: Electron microscopy of synthetic proteoliposomes. Reconstituted rat liver proteoliposomes were examined by electron microscopy after negative staining with 2% uranyl acetate as described in Experimental Procedures. A reference bar of 0.1 μm is included in the upper right-hand corner.

preloaded with [^{3}H]Man-P-Dol$_{10}$, are diluted with 25 vols of isotonic buffer, there is a time-dependent efflux of >90% of the internalized [^{3}H]Man-P-Dol$_{10}$ (Figure 5).

Man-P-Dol$_{10}$ transport in reconstituted proteoliposomes was saturable with respect to Man-P-Dol$_{10}$ concentration (Figure 6). The double-reciprocal plots shown in Figure 6, obtained by linear-regression analysis of the transport data (r^2 value = 0.993), indicate that the K_T (Man-P-Dol$_{10}$ concentration at which the transport rate is half-maximal) for Man-P-Dol$_{10}$ uptake in reconstituted proteoliposomes (4 mM, ●) is similar to that of ER-enriched liver microsomes (2 mM, ○). The maximal velocity of Man-P-Dol$_{10}$ transport is higher in native microsomes (6.7 nmol/min/mg) than in the functional reconstituted proteoliposomes (3.3 nmol/min/mg).

To confirm that membrane protein(s) are required for Man-P-Dol$_{10}$ transport in reconstituted proteoliposomes, the effect of trypsin treatment on transport activity was examined. Although Man-P-Dol$_{10}$ transport activity is unaffected by incubation at room temperature for up to 2 h (Figure 7, ○), there is a time-dependent loss of Man-P-Dol$_{10}$ transport following incubation in the presence of trypsin (2.5 mg/mL) (Figure 7, Δ). Trypsin-dependent loss of transport activity was prevented by the addition of trypsin inhibitor (soybean, 25 mg/mL) (Figure 7, ●).

Although the addition of trypsin alone had no effect on the filtration of the proteoliposomes by the HA filters, the combination of trypsin and trypsin inhibitor reduced the recovery of the proteoliposomes on the filters by ~20%. To control for this nonspecific effect, the recovery of phospholipid on the HA filters was determined, as described in the legend to Figure 7, and Man-P-Dol$_{10}$ transport was normalized to phospholipid retained on the filters. Pilot studies

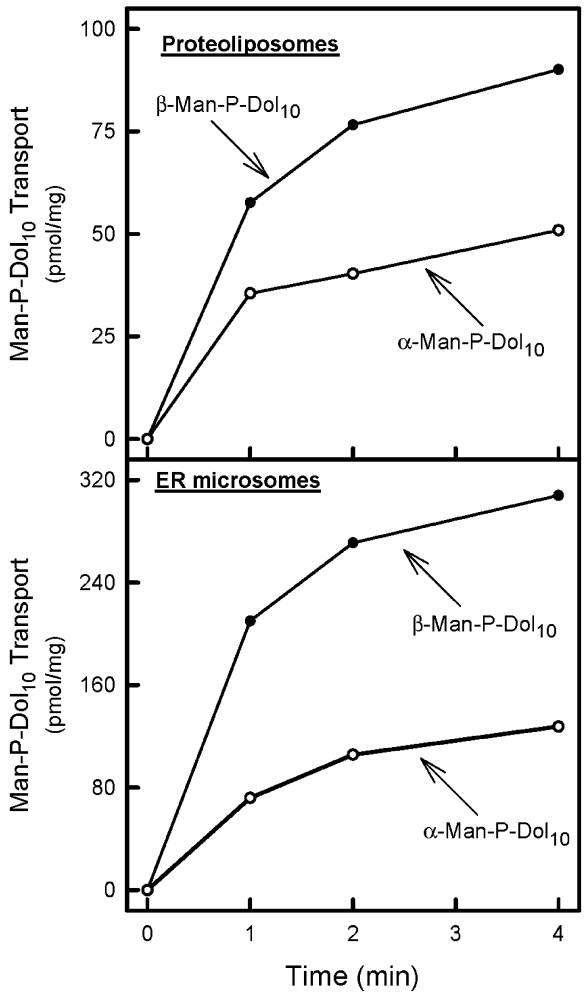

FIGURE 4: Comparison of the time-dependence and stereospecificity of Man-P-Dol$_{10}$ transport in reconstituted proteoliposomes (upper panel) with native rat liver ER-enriched microsomes (lower panel). Reconstituted proteoliposomes (upper panel) and rat liver ER-enriched microsomes (lower panel) were incubated at 21 °C with either α-Man-P-Dol$_{10}$ (○) or β-Man-P-Dol$_{10}$ (●) and assayed for Man-P-Dol$_{10}$ transport. Assay mixtures for the determination of Man-P-Dol$_{10}$ uptake contained either reconstituted proteoliposomes (180 μg of protein) or rat liver ER microsomes (100 μg of membrane protein), 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM EDTA, and either α-Man-P-Dol$_{10}$ (0.15 mM, 9.2 cpm/pmol) or β-Man-P-Dol$_{10}$ (0.15 mM, 23.4 cpm/pmol) in a total volume of 0.02 mL. Following incubation at 21 °C for the indicated periods of time, Man-P-Dol$_{10}$ uptake was determined as described in Experimental Procedures.

employing synthetic liposomes and rat liver ER proteoliposomes, preloaded with [^{3}H]inositol, demonstrated that 97% of the proteoliposomes remained sealed under these incubation conditions (data not included).

To corroborate that Man-P-Dol$_{10}$ uptake in reconstituted proteoliposomes was protein-mediated, the effect of several protein alkylators on Man-P-Dol$_{10}$ transport was investigated. Of the various protein modifying reagents tested, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and diethylpyrocarbonate (DEPC) most effectively blocked Man-P-Dol$_{10}$ transport into rat liver ER vesicles. Stilbene disulfonate inhibitors have been used to study many anion transporters, including the chloride transporter in erythrocytes (*40*) and membrane proteins involved in the transport of lactate (*41*), glucose 6-phosphate (*42*), and ATP (*43*). The transport of Man-P-Dol$_{10}$ into functional proteoliposomes (●) and intact

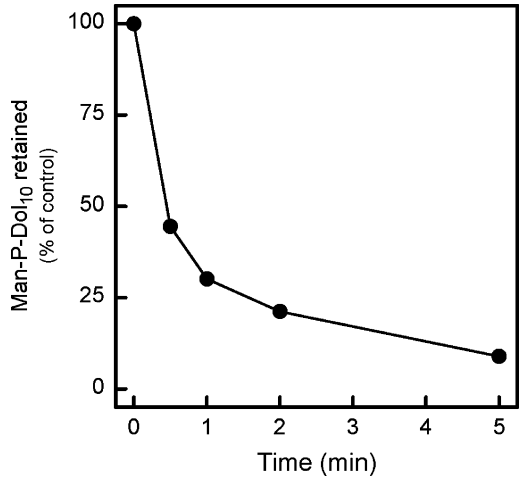

FIGURE 5: Man-P-Dol_{10} uptake into synthetic proteoliposomes is reversible. Assay mixtures for the determination of Man-P-Dol_{10} efflux contained 80 μg of membrane protein, 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM EDTA, and 200 μM [^{3}H]Man-P-Dol_{10} (88 cpm/pmol) in a total volume of 0.02 mL. Following incubation at 23 °C for 5 min, to preload the proteoliposomes, 0.5 mL of 10 mM Tris-HCl, (pH 7.4) and 0.25 M sucrose was added and incubation was continued at 23 °C. After the indicated periods of time, the samples were assayed for [^{3}H]Man-P-Dol_{10} retained within the proteoliposomes using the filtration assay described in Experimental Procedures. The data represent an average of three separate experiments expressed as a percentage of [^{3}H]Man-P-Dol_{10} retained following the 5 min preincubation.

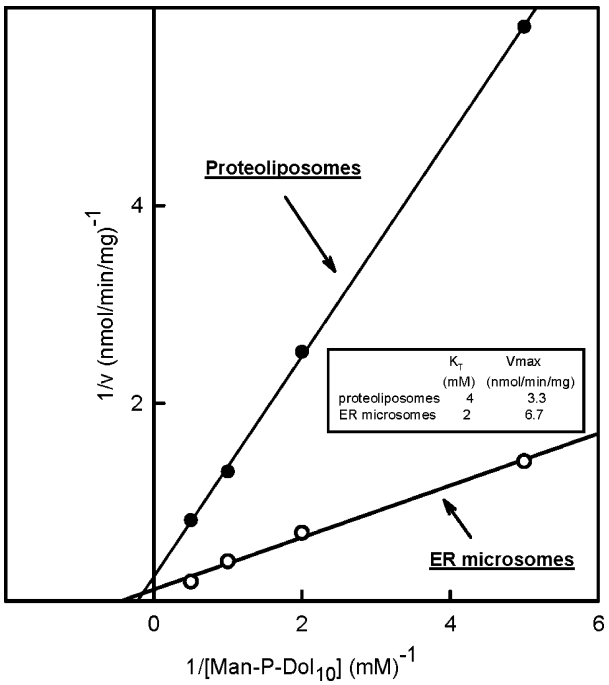

FIGURE 6: Concentration-dependence of Man-P-Dol_{10} transport in reconstituted proteoliposomes (●) and native rat liver ER-enriched microsomes (○). Assay mixtures for the determination of Man-P-Dol_{10} uptake contained either reconstituted proteoliposomes (162 μg of protein) or rat liver ER microsomes (225 μg of membrane protein), 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM EDTA and the indicated concentration of β-Man-P-Dol_{10} (11 cpm/pmol) in a total volume of 0.02 mL. Following incubation at 21 °C for 15 s, Man-P-Dol_{10} uptake was determined as described in Experimental Procedures.

ER-enriched microsomes (○) is blocked in parallel from 0 to 2 mM DIDS (Figure 8). In the concentration range of 2−4 mM DIDS, Man-P-Dol_{10} uptake in proteoliposomes was

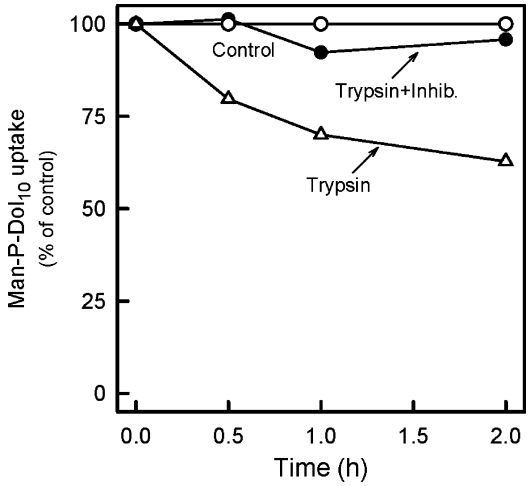

FIGURE 7: Trypsin-sensitivity of Man-P-Dol_{10} transport in reconstituted proteoliposomes. Reconstituted proteoliposomes (0.5 mg of protein) were preincubated at 23 °C in 10 mM HEPES-NaOH (pH 7.4), 0.25 M sucrose, 5 mM $MgCl_2$ in a total volume of 0.1 mL either, with trypsin (△) (2.5 mg/mL), or without trypsin (○), or with trypsin and trypsin inhibitor (soybean, 25 mg/mL, ●). At the indicated times, aliquots (0.017 mL) were withdrawn and assayed for Man-P-Dol_{10} transport. Assay mixtures for the determination of Man-P-Dol_{10} uptake contained reconstituted proteoliposomes (70.8 μg of protein) 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM EDTA, and 0.2 mM β-Man-P-Dol_{10} (206 cpm/pmol) in a total volume of 0.02 mL. Following incubation at 23 °C for 30 s, Man-P-Dol_{10} uptake and phospholipid recovery were determined using a modification of the filtration assay described in Experimental Procedures. Following filtration, the HA filters were extracted with $CHCl_3/CH_3OH$ (2:1). The organic extract was transferred to a centrifuge tube, mixed with 1/5 vol of 0.9% NaCl−10 mM EDTA and centrifuged briefly to separate the phases. The aqueous (upper) phase was removed and the organic phase was washed with $CHCl_3/CH_3OH/H_2O$ (3:48:47) two times. The aqueous phases were combined and dried under a stream of air, and the amount of [^{3}H]Man-P-Dol_{10} recovered was determined by scintillation spectrometry. The organic layer was dried under N_2 and assayed for lipid-phosphorus as described in Experimental Procedures. Man-P-Dol_{10} uptake was normalized for phospholipid recovery and expressed as a percentage of the transport activity in the control reaction (no trypsin or trypsin inhibitor). The data are average values and representative of three separate experiments.

slightly more sensitive to the protein reagent than transport activity in microsomal fractions.

The effect of DEPC treatment on Man-P-Dol_{10} transport in reconstituted proteoliposomes is shown in Table 2. The effect of DEPC treatment on Man-P-Dol_{10} transport in reconstituted proteoliposomes could not be assessed directly, due to abnormally slow filtration of the transport reaction mixtures on the Millipore HA filters following reaction with DEPC. However, pilot experiments indicated that microsomal Man-P-Dol_{10} transport was irreversibly inactivated by incubation with DEPC. To avoid the filtration artifact caused by treating proteoliposomes directly with DEPC, rat liver ER microsomes were first incubated with DEPC, washed with isotonic buffer, and then solubilized with OG. The soluble extracts were then supplemented with PC/PE and dialyzed extensively to form proteoliposomes. Since transport rates in reconstituted proteoliposomes are highly sensitive to vesicle size, the transport data following reconstitution, presented in Table 2, were normalized to proteoliposomal phospholipid content. Man-P-Dol_{10} transport is substantially inhibited by DEPC in native rat liver ER vesicles and in the reconstituted proteoliposomes formed from soluble extracts

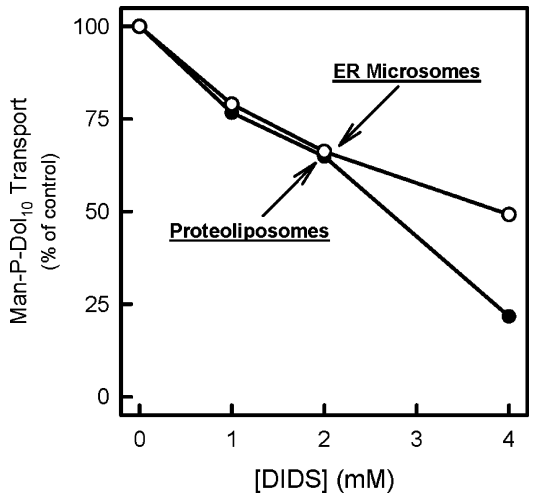

FIGURE 8: Effect of DIDS on Man-P-Dol_{10} transport in reconstituted proteoliposomes (●) and native rat liver ER-enriched microsomes (○). Assay mixtures for the determination of Man-P-Dol_{10} uptake contained either reconstituted proteoliposomes (62 μg of protein) or rat liver ER microsomes (180 μg of membrane protein), 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM EDTA, 1 mM β-Man-P-Dol_{10} (14 cpm/pmol) and the indicated concentrations of DIDS in a total volume of 0.02 mL. Following incubation at 21 °C for 30 s, Man-P-Dol_{10} uptake was determined as described in Experimental Procedures.

Table 2: Effect of Diethylpyrocarbonate on Reconstitution of Man-P-DOL_{10} Transport Activity from Rat Liver Microsomes[a]

	Man-P-Dol_{10} uptake in microsomes		Man-P-Dol_{10} uptake in proteoliposomes	
additions	(pmol/min/mg)	(% inhibition)	(pmol/min/μmol)	(% inhibition)
none	130.4		35.5	
DEPC	13.2	(89.9)	11.5	(68.7)

[a] Rat liver microsomes were incubated either with or without 70 mM DEPC at room temperature. Following incubation for 30 min, reactions were diluted with 6 vol, ice-cold 10 mM HEPES-NaOH, pH 7.4, 0.25 M sucrose, sedimented at 100000*g*, 10 min, resuspended in HEPES-NaOH, pH 7.4, 0.25 M sucrose, sedimented again and resuspended in HEPES-NaOH, pH 7.4, 0.25 M sucrose to a protein concentration of 2 mg/mL. A portion of the resuspended microsomes were then solubilized with OG, supplemented with PC/PE, and dialyzed to form proteoliposomes as described in Experimental Procedures. Assay mixtures for the determination of Man-P-Dol_{10} uptake contained either ER microsomes (100 μg of membrane protein) or reconstituted proteoliposomes (100−130 μmol of phospholipid), 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM EDTA, and 100 μM [^{3}H]Man-P-Dol_{10} (88 cpm/pmol) in a total volume of 0.02 mL. Following incubation at 21 °C for 15 s, Man-P-Dol_{10} uptake was determined as described in Experimental Procedures.

of DEPC-treated microsomes (Table 2). Protein recovery in proteoliposomes was not significantly affected by DEPC treatment.

Consistent with a role in the transbilayer movement of Man-P-Dol, Man-P-Dol_{10} transport activity is enriched in the ER (*24*). To provide additional evidence that the reconstituted transport activity is related to the activity in rat liver ER microsomes, the reconstitution of Man-P-Dol_{10} transport activities from rat liver ER-enriched and mitochondria-enriched subcellular fractions were compared. The results in Table 3 show that Man-P-Dol_{10} transport activity was substantially higher in proteoliposomes reconstituted from ER-enriched microsomes relative to a mitochondrial fraction from rat liver. The very low Man-P-Dol_{10} uptake activity in

Table 3: Reconstitution of Man-P-DOL_{10} Transport Activity from Rat Liver Subcellular Fractions[a]

		Enzyme Markers		
subcellular fraction	Man-P-Dol_{10} uptake (pmol/min)	Man 6-Pase (nmol/min/mg)	SDH (nmol/min/mg)	Man-P-Dol_{10} uptake (pmol/mg)
mitochondria	0.47	6.7	1016	3.7
RER	1.35	18.4	165	8.6

[a] Man 6-P phosphatase and succinate dehydrogenase (SDH) were determined as described in Experimental Procedures. Proteoliposomes from rat liver subcellular fractions (20 mg of membrane protein), enriched in either mitochondria or rough ER, were prepared as described in Experimental Procedures following solubilization with 2% OG. Assay mixtures for the determination of Man-P-Dol_{10} uptake contained 200 μg of membrane protein, 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM EDTA, and 12 μM [^{3}H]Man-P-Dol_{10} (517 cpm/pmol) in a total volume of 0.02 mL. Following incubation at 21 °C for 1 min, Man-P-Dol_{10} uptake was determined as described in Experimental Procedures.

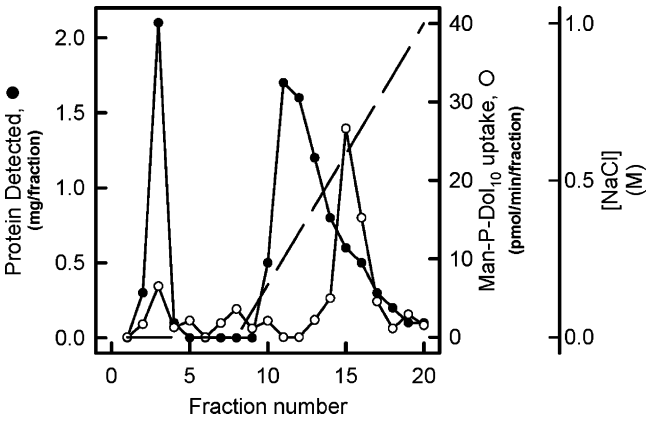

FIGURE 9: Partial purification of Man-P-Dol_{10} transport activity by ion-exchange chromatography on DEAE-Sephacel. Rat liver ER-enriched microsomes were solubilized with 2% OG and the extracts were centrifuged (140000*g*, 30 min). The solubilized supernatant fluid was then fractionated on a DEAE-Sephacel column (1 × 8 cm), and reconstituted into synthetic proteoliposomes as described in Experimental Procedures. Column fractions were assayed for protein (●) and Man-P-Dol_{10} transport activity (○). Assay mixtures for the determination of Man-P-Dol_{10} uptake contained ~2−25 μg of reconstituted protein, 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM EDTA, and 100 μM β-[^{3}H]Man-P-Dol_{10} (85 cpm/pmol) in a total volume of 0.02 mL. Following incubation at 21 °C for 5 min, Man-P-Dol_{10} transport was assayed as described in Experimental Procedures.

proteoliposomes reconstituted from the mitochondrial fraction is probably due to minor contamination with ER since this fraction also contained a small amount of Man 6-P phosphatase activity.

Fractionation of Man-P-Dol_{10} Uptake Activity on DEAE Sephacel. To demonstrate that reconstitution of Man-P-Dol_{10} transport into synthetic proteoliposomes provides a useful means to monitor the purification of the transport activity, OG-solubilized rat liver ER membrane proteins were fractionated by ion-exchange chromatography on DEAE-Sephacel and reconstituted into proteoliposomes. Approximately 75% of the OG-solubilized membrane proteins and about 33% of the membrane phospholipids were retained by DEAE-Sephacel (Figure 9, ●). When proteins in the DEAE-Sephacel fractions were reconstituted into proteoliposomes, the majority of the reconstituted Man-P-Dol_{10} transport activity was recovered in fractions eluting at a salt

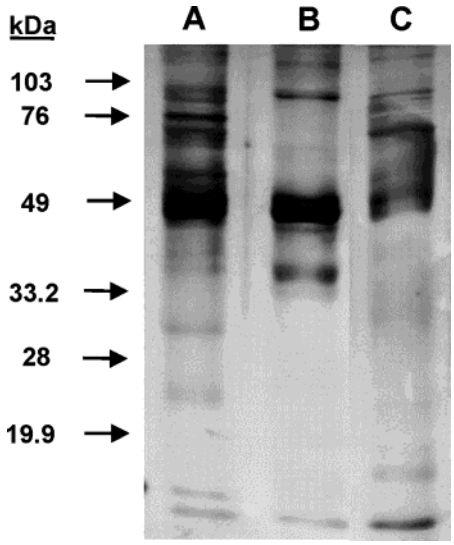

FIGURE 10: SDS−PAGE patterns of detergent extracts and DEAE-Sephacel fractions. Aliquots (1 μg of membrane protein) from either the crude solubilized extract (lane A) or selected DEAE-Sephacel fractions from the experiment described in Figure 9 (lanes B and C) were subjected to SDS−PAGE and the protein bands were visualized by silver staining as described in Experimental Procedures. Lane A, crude detergent extract; lane B, DEAE-Sephacel fraction 3 (Figure 9); lane C, DEAE-Sephacel fraction 15 (Figure 9). Calibration markers are shown on the left.

concentration of approximately 0.5 M (Figure 9, ○). Man-P-Dol_{10} transport activity was enriched approximately 2-fold in fraction 15 (426 pmol/min/mg) when compared to proteoliposomes prepared from the crude OG extracts (272 pmol/min/mg). The phospholipids which were retained by DEAE-Sephacel eluted in a broad peak, overlapping the fractions containing the reconstituted Man-P-Dol_{10} transport activity (data not shown).

To establish that the protein(s) mediating the transport of the water-soluble analogue were effectively resolved from many of the membrane proteins in the OG extracts by DEAE-Sephacel chromatography, selected column fractions were analyzed by SDS−PAGE (12% acrylamide), and the protein patterns were visualized by silver staining. The crude extract contains a large number of proteins with apparent molecular masses ranging from 20 to 103 kDa (Figure 10, lane A. However, a comparison of the unbound fraction (lane B) and fraction 15 (lane C) clearly shows that the fraction containing the highest specific activity of the transport activity was resolved from a large set of other membrane proteins. Thus, assaying Man-P-Dol_{10} uptake in functional proteoliposomal preparations from fractions obtained during chromatography on DEAE-Sephacel and other chromatographic supports has the potential to achieve highly purified preparations of the protein(s) mediating the transport system in rat liver ER.

DISCUSSION

Although the transverse diffusion of dolichyl-linked saccharides is an essential process in protein N-glycosylation, there is very little known about the class of membrane proteins mediating the transbilayer movement of these glycolipid intermediates. Recently, the Rft1 gene has been implicated in the transbilayer movement of $Man_5GlcNAc_2$-P-P-Dol in the ER in *Saccharomyces cerevisiae* (*44*), but the proteins involved in the transbilayer movement of Man-P-Dol and Glc-P-Dol remain to be identified. The work presented here extends earlier studies on a Man-P-Dol flippase activity in rat liver microsomes by describing a procedure for the reconstitution of the transport system into proteoliposomes.

The validity of utilizing citronellol-based analogues to study proteins mediating the flip-flopping of dolichyl-linked intermediates is supported by the observations that several enzymes in the lipid intermediate pathway utilize the corresponding citronellyl derivative as substrates. These enzymes include Man-P-Dol synthase, Glc-P-Dol synthase, Man-P-Dol:$Man_{5-8}GlcNAc_2$-P-P-Dol mannosyltransferase(s), Glc-P-Dol:$Glc_{0-2}Man_9GlcNAc_2$-P-P-Dol glucosyltransferase (*24*−*26*, *29*, *45*), UDP-GlcNAc:Dol-P GlcNAc-phosphotransferase (*27*), and UDP-GlcNAc:GlcNAc-P-P-Dol GlcNAc-transferase (*27*). The bacterial enzymes, Man-P-Undec synthase from *Micrococcus luteus* (*24*, *29*) and UDP-GlcNAc:Undec-P GlcNAc-phosphotransferase (WecA) from *E. coli* (*27*) will glycosylate neryl-P (C10), a water-soluble analogue of Undec-P (C55). Citronellyl-P-P-*N*-acetylmuramyl pentapeptide has also been used as a model substrate for the MurG *N*-acetylglucosaminyl transferase of *E. coli* (*46*−*48*). Moreover, as noted above, in vitro transport assays with GlcNAc-P-P-nerol have implicated the WzxE protein in the transbilayer movement of Fuc4NAc-ManNAcA-GlcNAc-P-P-Undec during the biosynthesis of ECA in *E. coli* (*27*).

Pilot studies with [^{14}C]OG indicate that after 90 h of dialysis the proteoliposomes contain <1 molecule of OG per 360 molecules of phospholipid (data not shown) or ~200 molecules of OG/vesicle, using values reported by Mimms et al. (*49*) for phospholipid vesicles of ~100 nm in diameter. It is not known if the OG is intercalated within the lipid bilayer, or trapped within the lumen of the vesicle.

Reconstitution of Man-P-Dol_{10} transport activity is enhanced by the addition of exogenous PC and PE. Although the total amount of protein reconstituted into proteoliposomes was relatively constant, rates of Man-P-Dol_{10} transport were significantly higher in the presence of exogenously added PC (~2 mg/mg of protein). Because the transport data presented in Figure 2 represent near equilibrium conditions, the increased Man-P-Dol_{10} uptake in the preparations with exogenously added PC probably reflects an increased internal vesicular volume. It is interesting that addition of exogenous PC above 2 mg of PC/mg of protein fails to form additional transport-competent vesicles. The lack of increased transport activity above 2 mg of PC/mg of protein suggests that the proteoliposomes formed above this ratio may lack a functional Man-P-Dol_{10} transporter. Assuming that all exogenously added PC is incorporated into proteoliposomes, the inflection point at 2 mg of PC/mg of protein corresponds to 403 μg of protein/μmol of phospholipid. This represents a somewhat higher protein/phospholipid inflection point than has been reported elsewhere for the transport of a water-soluble analogue of PC in reconstituted proteoliposomes (*34*). This is due in part because the proteoliposomes formed by equilibrium dialysis of OG extracts in this study are somewhat smaller (~100 nm) than the vesicles formed from Triton X-100 extracts following detergent depletion using SM-2 BioBeads (~180 nm). Calculations using the reported phospholipid composition of vesicles of 100 nm in diameter presented by Mimms et al. (*49*) and assumptions concerning

the size of an average ER protein (~50 kDa) proposed by Menon et al. (*34*) suggest that the abundance of the Man-P-Dol_{10} transport system is similar to that of the diC_4PC transport system (~0.15% of ER protein).

PE has been reported to stimulate lactose permease of *E. coli* in reconstituted proteoliposomes (*50*). Addition of pig liver PE, at low levels (PE/PC ratio = 0.05), modestly stimulated the formation of transport-competent proteoliposomes; however, inclusion of higher amounts of PE was inhibitory. It may be that inclusion of small amounts of PE improves the stability of the lipid bilayer, but higher amounts of PE produce nonbilayer H_{II} structures that are not optimal for transport activity (*51*).

As in rat liver ER microsomes, Man-P-Dol_{10} transport in proteoliposomes is stereoselective. It is not known if the lower rate of α-Man-P-Dol_{10} uptake represents inefficient transport by the *β*-Man-P-Dol flippase, or if the α-anomer is bound or transported by a different mechanism. However, previous studies showed conclusively that the water-soluble analogues do not nonspecifically adhere to lipid vesicles by hydrophobic interactions (*24*). *β*-Man-P-Dol_{10} is not an effective competitor for α-Man-P-Dol_{10} transport when added at concentrations up to 10 mM (data not shown), indicating that the α-anomer is bound or transported by a separate process.

Reconstitution by equilibrium dialysis results in the formation of unilamellar vesicles of ~50−200 nm in diameter. Because Man-P-Dol_{10} transport in native ER vesicles is bidirectional, it is difficult to determine from simple transport studies if the transporter has inserted into the proteoliposomes in the native orientation. Man-P-Dol_{10} transport activity was slowly inactivated by incubation with trypsin, similar to the effect of trypsin on Man-P-Dol_{10} transport in native ER vesicles. Furthermore, Man-P-Dol_{10} transport activity is also sensitive to alkylation by the membrane impermeant stilbene disulfonate, DIDS and to DEPC. Sensitivity to proteolysis by trypsin and inhibition by DIDS and DEPC support the conclusion that Man-P-Dol_{10} uptake is, indeed, catalyzed by membrane protein(s) and that the transporter is inserted into the proteoliposomes in its native configuration. The sensitivity to DEPC implies the presence of an essential histidine residue, but this conclusion will require additional experimental support.

Preliminary attempts were also made to test the feasibility of using conventional chromatographic methods, coupled with a reconstitution-based assay employing Man-P-Dol_{10} transport into proteoliposomes, to ultimately purify the protein(s) involved in the transport system. The reproducible increase in maximal transport rates (~426 pmol/min/mg) from partially purified fractions obtained by chromatography on DEAE-Sephacel compared to vesicles reconstituted from detergent extracts (272 pmol/min/mg) is an encouraging result. A substantial fraction of the solubilized protein, lacking transport activity, did not bind to the DEAE-Sephacel column, indicating that Man-P-Dol_{10} transport is catalyzed by a specific subset of liver ER protein(s). Purification by ion exchange chromatography and other supports should yield preparations depleted of endogenous phospholipids, allowing the investigation of the properties of the transporter in proteoliposomes with defined phospholipid mixtures.

The critical role of protein N-glycosylation in the normal function and development of mammalian cells is apparent in the recent identification of a group of inherited metabolic disorders in humans, classified as congenital disorders of glycosylation (CDG) (*52*, *53*). Mutations affecting the transbilayer movement of Man-P-Dol and Glc-P-Dol will certainly affect the synthesis of full-length dolichol-linked oligosaccharides, and it seems inevitable that human patients with Man-P-Dol and Glc-P-Dol flippase disorders will ultimately be identified. The development of a reconstitution-based assay for Man-P-Dol flippase activity could prove useful in the diagnosis of patients with this genetic defect.

In summary, this paper presents evidence that rat liver ER Man-P-Dol_{10} transport activity can be solubilized and reconstituted into synthetic proteoliposomes. The reconstituted activity has all of the properties of the Man-P-Dol_{10} uptake system present in native rat liver ER vesicles and can be partially purified by ion exchange chromatography. The solubilization and reconstitution of the Man-P-Dol_{10} transporter is a crucial step toward the future goals of purifying and characterizing the properties of this novel membrane transporter and identifying the polypeptide components. Successful purification will be essential to understand the mechanism by which the flippase shields the hydrophilic mannosylphosphoryl headgroup as it traverses the hydrophobic core of the ER.

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