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CHARACTERIZATION OF MANNOSYL-TRANSFER REACTIONS CATALYZED BY DOLICHYL-MANNOSYL-PHOSPHATE-SYNTHASE*

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ABSTRACT

Evidence suggesting that a single enzyme catalyzes mannosyl transfer from GDP-mannose to both dolichyl phosphate and to phenyl phosphate was obtained as follows: (a) The two activities were coeluted from columns of DEAE-cellulose and Sepharose CL-6B, (b) both reactions demonstrated similar kinetic constants for the glycosyl donor and for guanosine nucleoside inhibitors, (c) both reactions were sensitive to inhibition by low concentrations of nonionic detergents, and (d) both activities were found to be thermally inactivated at similar rates upon incubation at 55°. The reaction conditions required for optimal mannosyl transfer by the purified enzyme preparation to the hydrophobic and water soluble acceptors, however, were found to be quite different. Whereas mannosyl transfer from GDP-mannose to dolichyl phosphate occurred at maximal rates only in the presence of specific phospholipids, the rate of mannosyl transfer to phenyl phosphate was essentially unaffected by the addition of phospholipid. These results indicate that dolichyl-mannosyl-phosphate-synthase, which has some of the properties of an intrinsic membrane protein, does not have an absolute requirement for phospholipid for catalytic activity, but rather that phospholipid is required for interaction of the enzyme with the long chain polyisoprenol substrate dolichyl phosphate.

INTRODUCTION

The lipophilic glycosyl donor, dolichyl D-mannopyranosyl phosphate (Dol-*P*-Man), is an important intermediate in the biosynthesis of the asparagine-linked carbohydrate chains of glycoproteins[1–3] and the enzyme catalyzing the synthesis of this intermediate has recently been purified 900-fold from rat liver microsomes[4]. The activity of the partially purified mammalian enzyme was found to be optimal in the presence of unsaturated derivatives of phosphatidylethanolamine and the enzyme had no activity in the presence of the nonionic detergents that were tested. The phospholipid specificity of the Dol-*P*-Man-synthase was similar to that

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previously reported[5–7] for mannosyltransferase II, an enzyme that catalyzes the formation of an α-D-mannopyranosyl-(1→3)-D-mannose linkage in dolichyl oligosaccharide diphosphate intermediates. Although specific phospholipids were found to be required for optimal synthase activity under the reaction conditions employed for the enzyme assays, it remained to be determined whether phospholipid was required for catalytic activity of the enzyme or was required to provide a lipophilic matrix for proper interaction of the enzyme with the hydrophobic glycosyl acceptor. One method to resolve this problem would be to utilize alternative mannosyl acceptors that varied in hydrophobicity and to determine whether the same phospholipid requirement was observed.

Kato *et al.*[8] have reported that rat liver microsomes catalyze the formation of phenyl D-mannosyl phosphate (Ph-*P*-Man) from GDP-mannose and phenyl phosphate (Ph-*P*). It was suggested by these investigators that Ph-*P* was a water soluble acceptor for microsomal preparations of Dol-*P*-Man-synthase. We report herein that highly purified preparations of Dol-*P*-Man-synthase catalyze mannosyl transfer to both Dol-*P* and Ph-*P*, providing further evidence that both acceptors are utilized by the same enzyme. Our results show that mannosyl transfer from GDP-mannose to the water soluble acceptor, Ph-*P*, is essentially unaffected by the addition of phospholipids. Thus, the requirement of phospholipid for mannosyl transfer to Dol-*P* appears to be associated with the interaction between the synthase and the long-chain polyprenyl phosphate substrate.

EXPERIMENTAL

Materials. — GDP-D-[^{14}C]mannose (9.9 GBq/mmol) and GDP-D-[^{3}H]mannose (395 GBq/mmol) were purchased from New England Nuclear Corp. C_{95}-Dolichyl phosphate was provided by T. Chojnacki, Warsaw, and amphomycin was a gift from W. F. Minor, Bristol Laboratories. DEAE-cellulose (DE-52) was from Whatman Ltd., phenyl phosphate and *p*-hydroxymercuribenzoate were from Sigma Chemical Co., and Sepharose CL-6B was from Pharmacia Fine Chemicals. Soybean phospholipids were obtained from Avanti Polar Lipids, Inc. All other chemicals, standard compounds, and supplies were purchased from commercial sources.

Analytical methods. — Protein content was determined with Coomassie Blue by following the procedure of Bradford[9] using bovine serum albumin as the protein standard. Phosphate content was determined[10] with an acidic $(NH_4)_2MoO_4$–Malachite green reagent after digestion of the sample by heating with $Mg(NO_3)_2$. Radioactivity was measured in a liquid-scintillation counter using premixed scintillation fluids (Scintiverse and Scintilene, Fisher Scientific Co.).

Chromatographies. — Ascending chromatography was carried out on Whatman No. 1 or 3MM paper in the following solvent systems: (*a*) 7:1:2 propanol–ethyl acetate–water, (*b*) 8:1:2 ethyl acetate–pyridine–water, (*c*) 7:3 ethanol–M amonium acetate, and (*d*) 9:1 ethanol–M ammonium acetate. Descending chromatography

was carried out on Whatman No. 1 paper in solvent (*e*), 5:3 butyric acid–0.5M NH_4OH. Thin-layer chromatography was performed on precoated Silica gel G plates (Analtech, Inc.) in solvent (*f*), 65:25:4 chloroform–methanol–water. Carbohydrates were detected with the *p*-anisidine phthalate reagent[11] and thin-layer chromatograms developed with I_2 vapor or with a Mo spray reagent[12]. Paper chromatograms were assayed for radioactivity with a Packard Model 7201 radiochromatogram strip scanner or by cutting 1-cm segments from the chromatograms and counting the radioactivity in toluene-base scintillation fluid.

Buffers. — All buffers and solutions were prepared with de-ionized water that was further purified by double distillation in a glass still. Buffer (*A*), pH 7.0, and buffer (*B*), pH 7.5, were 0.1M Tris acetate containing 1.3mM EDTA and 1.6mM reduced glutathione. The presence of EDTA was found to be essential for optimal mannosyltransferase activity.

Enzyme assays. — The formation of Dol-*P*-Man by microsomal and crude, solubilized-enzyme fractions was assayed in the presence of nonionic detergent by the following procedure. Dol-*P* (2 μg) in 2:1 chloroform–methanol and 10% (v/v) Nonidet P-40 (4 μL) in toluene were added to 1.5-mL, conical polypropylene centrifuge tubes, and the solvents were removed under a stream of N_2. Water (135 μL), 0.1M $MnCl_2$ (5 μL), buffer *B* (0.05 mL), and GDP-[^{3}H]mannose (5 μL; 3.7 KBq, 26.7 GBq/mmol) were added, and the contents were thoroughly dispersed with a Vortex mixer. The reactions were initiated by the addition of appropriate dilutions of microsomal enzyme (5 μL, 30–40 μg of protein), the incubations terminated after 3 min at 37° by the addition of 2:1 chloroform–methanol (0.25 mL), and Dol-*P*-Man was extracted as previously described[13].

Assays for the formation of Dol-*P*-Man by all other enzyme fractions were carried out in the presence of phospholipids by the following procedure. The assay involved the differential partitioning of tritiated substrate and product in a two-phase, scintillation cocktail that allowed specific determination of the radioactive product. The glycosyl acceptor, Dol-*P* (0.01 mg), and phosphatidylethanolamine (0.4 mg), dissolved in organic solvents, were mixed and the organic solvents were completely removed under a stream of dry N_2. Following the addition of buffer (*B*) (1 mL), the lipid mixture was dispersed by immersion in an ultrasonic, cleaning bath (Branson Model 7) for 4 min. Assays were carried out in reaction mixtures containing the phospholipid–Dol-*P* dispersion (0.05 mL), water (0.135 mL), and 0.1M $MnCl_2$ (5 μL). The enzyme (5 μL, 0.06–0.12 μg of protein) was then added, the contents of the tubes were mixed on a Vortex mixer, and the mixtures were incubated at 37° for 5 min. The transferase reactions were initiated by the addition of GDP-[^{3}H]mannose (5 μL; 3.7 KBq, 26.7 GBq/mmol). After 10 min at 37°, the reactions were terminated by transferring 0.15-mL aliquots of the reaction mixtures into scintillation vials (1.6 × 5.2 cm) containing water-saturated butanol (0.45 mL) and water (0.25 mL). The contents were mixed on a Vortex mixer for 10 sec, a xylene-based scintillation fluid (3.6 mL; Scintilene, Fisher Scientific Co.) was added, and the contents were again mixed on a Vortex mixer for 10 sec. The vials

were centrifuged for 2 min at 3000 r.p.m. in a table top centrifuge and the radioactivity was counted in a Beckman Model LS 8100 scintillation counter. In control reactions that did not contain active enzyme, the blanks ranged between 50 to 75 c.p.m. or less than 0.1% of the introduced radioactivity. Essentially all of the Dol-*P*-Man was partitioned into the upper phase.

The synthesis of Ph-*P*-Man by microsomal and crude, solubilized-enzyme fractions was assayed as follows. Water (5 μL), buffer (*A*) (25 μL), 0.1M $MnCl_2$ (5 μL), 0.1M Ph-*P* (5 μL), and GDP-[^{14}C]mannose (5 μL; 1.85 KBq, 9.9 GBq/mmol) were added to 1.5-mL centrifuge tubes. The reactions were initiated by the addition of enzyme (5 μL, 0.3–0.4 mg of protein) and, after 30 min at 37°, were terminated by heating to 100° for 2 min. The radioactive product was separated by paper chromatography on Whatman 3 MM paper in solvent (*d*). Areas of the chromatogram corresponding to Ph-*P*-Man were excised and the radioactivity was counted. Control reactions containing inactivated enzyme were incubated and assayed under the same conditions. Counts were not corrected for quenching. In some assays, phospholipids were added in the indicated amounts as sonified dispersions in buffer (*A*) following procedures described earlier. Purified enzyme fractions were assayed by the same procedure except that the amount of the DEAE-enzyme fraction (Table I) was varied (0.06–0.3 μg of protein).

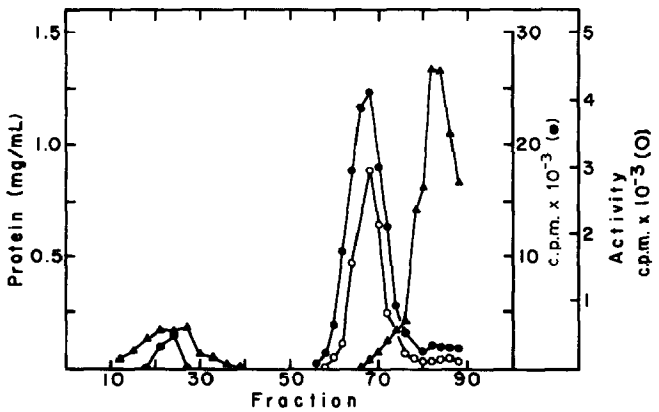

Fig. 1. Chromatography of solubilized Dol-*P*-Man-synthase on DEAE-cellulose. Rat liver microsomes (8 mL, 280 mg of protein) were solubilized by the addition of 2.5% (v/v) Nonidet P-40 (5 mL) to a final concentration of 1.0% of Nonidet P-40. After 10 min, the mixture was diluted to 51 mL by the addition of glycerol (10 mL), water (28 mL), and mercaptoethanol (0.1 mL), and was centrifuged for 1 h at 100 000*g*. The supernatant (46 mL) was applied to a column of DEAE-cellulose (2.5 × 14 cm), previously washed with 150 mL of a 1:10 dilution of buffer (*A*) containing 10% of glycerol, 0.1% of Nonidet P-40, and 0.5mM dithiothreitol. The pH of the eluate was 9.0. After all the enzyme had been applied, the column was washed with the same buffer (50 mL) and, starting at fraction 26, the column was eluted with a linear gradient (250 mL) from 0.01 to 0.1M buffer (*A*) containing 10% of glycerol, 0.1% of Nonidet P-40, and 0.5mM dithiothreitol. Fractions (3.8 mL) were collected at a flow rate of 40 mL/h and aliquots of the fractions were assayed for protein (▲——▲), mannosyl transfer to Dol-*P* (●——●), and mannosyl transfer to Ph-*P* (○——○) as described in the Experimental section. Fractions 57–70 were pooled (53 mL), the pH was immediately adjusted to 7.5 with M acetic acid, and the detergent was removed with a SepPak C_{18} cartridge (Waters Associates, Millipore Corp.) as previously described[5]. This fraction was labeled DEAE-enzyme and stored frozen.

RESULTS AND DISCUSSION

Earlier[4], we reported methods for the preparation of a highly purified fraction of Dol-*P*-Man-synthase from rat liver microsomes that was free of contaminating mannosyl-, glucosyl-, and *N*-acetylglucosaminyl-transferase activities[4]. This purified enzyme fraction catalyzed mannosyl transfer from GDP-mannose to Dol-*P* at maximal rates only in the presence of specific phospholipids. Since the purified enzyme did not appear to associate tightly with phospholipids in the absence of the glycosyl acceptor, Dol-*P*, it was considered possible that a phospholipid was primarily required to provide the proper matrix for interaction of the enzyme with the polyisoprenol acceptor. The results reported by Kato *et al.*[8] using a crude, microsomal-enzyme preparation from rat liver suggested that a water soluble compound, Ph-*P*, could be used as an alternative acceptor for Dol-*P*-Man-synthase. The purified enzyme fraction used in the studies reported herein was also found to catalyze mannosyl transfer to Ph-*P*, and the results suggested that both mannosyl transfer reactions are catalyzed by a single enzyme.

Rat liver microsomes were prepared and solubilized with 1% Nonidet P-40. Following centrifugation at 100 000*g* for 1 h, the supernatant was applied to a column of DEAE-cellulose and column fractions were assayed for mannosyltransferase activities with Dol-*P* and with Ph-*P* as glycosyl acceptors (Fig. 1). The elution profiles from DEAE-cellulose for the two activities were found to be identical, suggesting that both reactions were catalyzed by the same enzyme. When the relative purifications of the two activities were compared (Table I), the Dol-*P*-Man-synthase activity was purified 500-fold and the Ph-*P*-transferase activity 340-fold. Although the purification ratio for the two activities changed somewhat between microsomal and DEAE-enzyme fractions, this difference can be accounted for in part by the different assay requirements for the formation of Dol-*P*-Man by microsomal and DEAE-enzyme fractions. Whereas assays of microsomal

TABLE I

SOLUBILIZATION AND COPURIFICATION OF MANNOSYLTRANSFERASE ACTIVITIES[a]

Fraction	*Volume (mL)*	*Total protein (mg)*	*Acceptor*		
			Dol-P (A) (units)	*Ph-P (B)*	*Ratio A/B (units)*
1. Microsomes	8	280	21	0.56	37
2. Solubilized enzyme	48	247	22	0.73	30
3. DEAE-cellulose	53	0.64	24	0.43	56

[a]The enzyme was solubilized and purified as previously described[4]. Activity units are defined as the amount of enzyme catalyzing the transfer of 1 nmol of mannose/min. The synthesis of Dol-*P*-Man in Fractions 1 and 2 was assayed in the presence of nonionic detergent as described in the Experimental section. Fraction 3 was assayed in the presence of plant phosphatidylethanolamine. The synthesis of Ph-*P*-Man was assayed as described in the Experimental section.

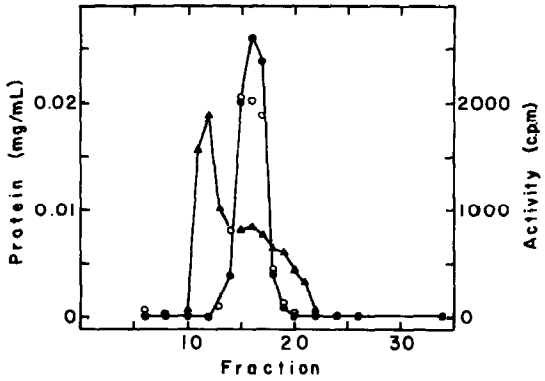

Fig. 2. Chromatography of Dol-*P*-Man-synthase on Sepharose CL-6B. Nonidet P-40 was added to the DEAE-enzyme to a final concentration of 0.03% and the enzyme was concentrated 6-fold by ultrafiltration through a YM-30 membrane (Amicon Corp.). A sample of the concentrated enzyme (1 mL, 0.1 mg of protein) was applied to a column (1.5 × 14 cm) of Sepharose CL-6B equilibrated with 0.01M Tris acetate buffer, pH 7.0, containing 10% of glycerol, 0.03% of Nonidet P-40, and 0.5M NaCl. The column was eluted with the same buffer at a flow rate of 15 mL/h. Fractions (1 mL) were collected and assayed for proteins (▲——▲), mannosyl-transfer activity to Dol-*P* (●——●), and mannosyl-transfer activity to Ph-*P* (○——○). Assays for Dol-*P*-Man formation were carried out with 0.05-mL aliquots of the indicated fractions for 30 min at 37° in the presence of 0.04 mg of phosphatidylethanolamine. Assays for the formation of Ph-*P*-Man were carried out with 0.06-mL aliquots of the indicated fractions for 5 h at 37° in the presence of 0.08 mg of phosphatidylcholine. Excess phospholipid was included in the enzyme assays to reduce the inhibition due to the presence of nonionic detergent. The void volume of the column was at fraction 11, as determined with Blue Dextran, and the total volume was at fraction 25 as determined with mannose. Equine ferritin was eluted at fraction 14.

enzyme required the addition of detergent-solubilized substrate, assays of the DEAE-enzyme fraction required the addition of phospholipid for optimal activity. The quantitative recovery of total enzyme activity for this reaction throughout the purification procedure may indicate that detergent inhibition resulted in the underestimation of Dol-*P*-Man-synthase activity in the microsomal fraction.

The DEAE-enzyme fraction was concentrated by ultrafiltration and applied to a column of Sepharose CL-6B (Fig. 2). The recovery of the total activity applied to the column was low, but coelution of the two activities from the gel-filtration column in an included fraction provided additional evidence that both substrates were utilized by a single enzyme. The enzyme eluted from the gel-filtration column was quite labile and lost 50% of its activity after 2 h when stored at 0–4°. This lability of the enzyme has prevented further purification.

All of the remaining experiments were carried out with the DEAE-enzyme fraction. This enzyme fraction was stable for 3 months when stored frozen after removal of the nonionic detergent with a reverse-phase affinity matrix[4]. Mannosyl transfer from GDP-mannose to Ph-*P* was assayed as described in the Experimental section and the ^{14}C-labeled reaction product was identified as Ph-*P*-Man by the methods of Kato *et al.*[8]. Mannosyl transfer to Ph-*P* was linear for at least 30 min and was approximately linear for 1 h. The reaction was directly proportional to enzyme concentration up to 0.3 μg of protein per assay. The K_m value for Ph-*P* was

TABLE II

COMPARISON OF MANNOSYL-TRANSFER REACTIONS TO DOLICHYL PHOSPHATE AND PHENYL PHOSPHATE

Property	*Acceptor*	
	Dol-P[a]	*Ph*-P
K_m for GDP-mannose (μM)	0.69	1.6
K_m for acceptor (μM)	0.3	2300
Effect of divalent cations (relative activity)[b]		
5mM Mn^{2+}	1.00	1.00
5mM Mg^{2+}	0.70	0.06
5mM Co^{2+}	0.90	0.43
Inhibitors (concentration required for 50% inhibition)		
GMP (μM)	16.0	10.0
GDP (μM)	1.3	0.8
GTP (μM)	3.0	5.0
Nonidet P-40 (%)	0.01	0.006
Amphomycin (μg/mL)	30	560
p-Hydroxymercuribenzoate (μM)	3.0	[c]
Phospholipid required	yes	no

[a]Data for mannosyl transfer to Dol-*P* taken from ref. 4. [b]Activity was maximal in the presence of 5mM $MnCl_2$ for both reactions. The chloride salts of Zn, Ca, and Ni did not stimulate transfer to Ph-*P*. [c]Stimulation.

found to be 2.3mM. The Lineweaver–Burk plots were nonlinear at high Ph-*P* concentrations and substrate inhibition was noted at Ph-*P* concentrations >8mM. Other properties of the reaction are shown in Table II with comparable information for the mannosyl-transfer reaction catalyzed by the purified DEAE-enzyme fraction with Dol-*P* as the acceptor.

Similarities in a number of reaction properties were found, within experimental error, that suggested that the two substrates were utilized by the same enzyme (Table II). The affinity of the enzyme for GDP-mannose, as assessed by the K_m value for the nucleotide sugar, was similar for both mannosyl-transfer reactions. Both reactions were inhibited to about the same extent by guanosine nucleotides and by low concentrations of nonionic detergent, as judged by the concentrations of inhibitors required for a 50% inhibition. Both reactions were found to have an absolute requirement for divalent cations with Mn^{2+} providing optimal activity, but the relative activities differed with some other divalent cations. When the activity was assayed as a function of pH, the two reactions had similar pH optima, although the curve of mannosyl transfer to Ph-*P* was relatively flat over a wide pH range. In cross-inhibition experiments, mannosyl transfer from GDP-mannose to Dol-*P*, assayed in the presence of phosphatidylethanolamine, was inhibited to 50% by Ph-*P* at a concentration of 3mM, which is equal to the K_m value of 2.3mM for Ph-*P*. The reaction was inhibited to 95% by 16mM Ph-*P*. When the reverse experiment was carried out, mannosyl transfer to Ph-*P* was inhibited to

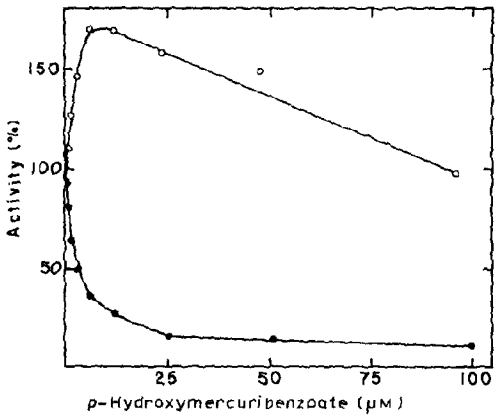

Fig. 3. Mannosyl transfer as a function of *p*-hydroxymercuribenzoate concentration. Mannosyl transfer to Dol-*P* (●——●) and to Ph-*P* (○——○) was assayed as described in the Experimental section, except that *p*-hydroxymercuribenzoate was present in the reaction mixtures at the indicated concentrations. Reaction mixtures containing *p*-hydroxymercuribenzoate, incubated at 0–4° for 10 min in the presence of substrates prior to raising the temperature to 37° for assay of mannosyl-transferase activities, gave identical results.

45% by 23μM Dol-*P* in the presence of 20 μg of either phosphatidylethanolamine or phosphatidylcholine added as a matrix for the polyisoprenylphosphate. Although the apparent K_m value for Dol-*P* was found to be 0.3μM, the amount of a hydrophobic substrate in a lipid matrix has no relevence to the Michaelis–Menten kinetic constants. Therefore, it might be expected that the apparent K_m value for Dol-*P* and the amount of Dol-*P* required to inhibit mannosyl transfer to Ph-*P* by 50% would differ by a significant amount. Additional evidence was also obtained from heat-inactivation studies. When the DEAE-enzyme fraction was incubated at 55° in the absence of reducing agents, both mannosyl-transfer activities were inactivated at approximately the same rate with a half-life of 4 min. The overall results are strongly indicative that a single enzyme catalyzes mannosyl transfer to both Dol-*P* and to Ph-*P*.

The results of the current investigation also show several significant differences for reactions utilizing the two substrates. Whereas mannosyl transfer to Dol-*P* was inhibited to 50% by amphomycin at a concentration of 30 μg/mL, 560 μg/mL of the antibiotic was required to inhibit mannosyl transfer to Ph-*P* to the same extent. This result was not totally unexpected as amphomycin is believed to block Dol-*P*-Man synthesis by specifically interacting with the polyisoprenol substrate[14]. The two mannosyl transfer activities were also differentially affected by the sulfhydryl-forming reagent *p*-hydroxymercuribenzoate when the reagent was incubated with the enzyme in complete reaction mixtures containing substrates (Fig. 3). Mannosyl transfer to Dol-*P* was inhibited to 50% at a *p*-hydroxymercuribenzoate concentration of 3μM. Interestingly, mannosyl-transfer to Ph-*P* was

stimulated by the addition of *p*-hydroxymercuribenzoate and the activation curve was almost the inverse of the inactivation curve at the lower reagent concentrations. The sulfhydryl reagent stimulated mannosyl transfer to Ph-*P* to the same extent, whether phospholipid was present or not in the incubation mixture, indicating that phospholipid had no effect on the reactivity of sulfhydryl group(s) on the enzyme. Both the inhibitory and stimulatory effects could be partially reversed (50–70%) by the addition of either mM dithiothreitol or 20mM mercaptoethanol prior to raising the incubation temperature to 37°. Essentially no effect on enzyme activity was noted when the enzyme was incubated with the sulfhydryl-forming reagent in the absence of substrates. The enzyme was incubated in the presence of 16μM *p*-hydroxymercuribenzoate for 20 min at 3° and in the absence of substrates. This concentration of reagent produced almost maximal effects on the enzyme in complete reaction mixtures as shown in Fig. 3. When the enzyme was diluted 20-fold into reaction mixtures for assays of mannosyltransferase activities, the concentration of the thiol reagent was diluted to 0.8μM, and the effect noted on enzyme activities was that expected with 0.8μM *p*-hydroxymercuribenzoate in the presence of substrates. Therefore, these results suggested that *p*-hydroxymercuribenzoate reacts with a unique sulfhydryl group(s) that was exposed only in the presence of substrates and that the reagent might be an uncompetitive effector of the mannosyltransferase reaction.

The differential effect of *p*-hydroxymercuribenzoate on the rate of mannosyl transfer to the two acceptors could be interpreted as evidence that separate enzymes catalyzed the reactions. It is likely, however, that the effects noted were caused by dissimilar interactions of the enzyme with two substrates that differ considerably in molecular weight and hydrophobic properties, especially since the sulfhydryl-forming reagent was only active in the presence of the glycosyl acceptors. Modification of a single amino acid (arginyl) residue on carboxypeptidase A has been shown to markedly decrease peptidase activity while simultaneously increasing esterase activity 3-fold[15]. More recently, the irreversible modification of lysine residues on phospholipase A_2 has been shown to inhibit activity towards phosphatidylcholine while stimulating activity towards the relatively poor substrate phosphatidylethanolamine[16]. Thus, evidence that was presented earlier and was based on a variety of different criteria still supports the postulate that a single mannosyltransferase catalyzes transfer to both Dol-*P* and to Ph-*P*. It is also unlikely that other mannosyltransferases, which utilize carbohydrate acceptors, would catalyze transfer to the phosphate residue of this nonphysiological acceptor. Final resolution of this question must await eventual isolation of a pure enzyme, although all previous attempts to further purify the synthase from mammalian sources have proven unsuccessful because of the lability of the enzyme.

We have previously shown that the purified enzyme fraction did not catalyze mannosyl transfer from GDP-mannose to Dol-*P* in the presence of nonionic detergents and demonstrated that the reaction rate was optimal when the hydrophobic substrate was reconstituted in the presence of phosphatidylethanolamine or

phosphatidylethanolamine–phosphatidylcholine mixtures. In the present investigation, mannosyl transfer from GDP-mannose to the water-soluble acceptor Ph-*P* was found to be essentially unaffected by the addition of phosphatidylethanolamine at concentrations ranging from 5 to 100 μg per assay. Phosphatidylcholine, which does not support mannosyl transfer from GDP-mannose to Dol-*P*, was found to slightly stimulate the mannosyl transfer to Ph-*P* (0.5-fold) at a concentration of 20 μg per assay. This slight activation can be compared to the greater than 5-fold stimulation observed with phosphatidylethanolamine for transfer to Dol-*P*.

In summary, this investigation showed that purified Dol-*P*-Man-synthase demonstrates a requirement for specific phospholipids when catalyzing mannosyl transfer from GDP-mannose to the long-chain polyprenol substrate Dol-*P*, but not for mannosyl transfer to the water-soluble acceptor Ph-*P*. Therefore. these results indicate that this membrane enzyme does not have a specific requirement for phospholipid for catalytic activity, but rather that a phospholipid is required for proper interaction of the enzyme with the hydrophobic glycosyl acceptor. Consequently, the purified rat liver enzyme differs from the Dol-*P*-Man-synthase that has been purified from yeast membranes[17]. The yeast enzyme was shown to catalyze the transfer from GDP-mannose to Dol-*P*, both in the presence of detergent and when the substrate and enzyme were reconstituted with a commercial lecithin preparation, although the exact composition of the phospholipid matrix was not determined. A comparable lecithin preparation from the same source did not support the synthesis of Dol-*P*-Man by the rat liver enzyme.

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