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REACTION OF OPTICALLY ACTIVE S- AND R-FORMS OF DOLICHYL PHOSPHATES WITH ACTIVATED SUGARS

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Summary: Chemical synthesis was used to produce optically active isomeres of dolichol (S- and R-forms) with 18 and 19 isoprene residues. The phosphorylated polyprene was studied in rat liver microsomal GDP-mannosyl and UDP-N-acetyl-glucosaminyl transferase systems. The two dolichol-P forms in both transferase systems gave V_{max} values which for the S-form exceeded 4-6 times what was obtained with the R-form. The K_m values were also higher for the S-form. The hepatocyte appears to contain a large excess of dolichyl-P, by 100 times exceeding that of the K_m values. For this reason the S-form of dolichyl-P seems to be one of the requirements for the normal establishment of the N-glycosidically linked oligosaccharide chain.

The established role of dolichyl-P is participation in the biosynthesis of N-glycosidically linked oligosaccharide chain (1). The great variety of protein bound oligosaccharides, and the specificity of both sugar sequence and terminal monosaccharid, demonstrate that strict regulation of biosynthesis take place mediated by the numerous glycosyl transferase systems and by the informations built into the protein acceptors.

Contrary to other carriers of lipid nature, such as coenzyme Q, dolichyl-P has a number of forms (2). In animal tissues the chain length varies between 17 and 22 isoprene units, but the functional importance of this variation is not yet established. Fully unsaturated polyprenyl phosphates are poor glycosyl acceptors in comparison with the α-saturated counterparts (3, 4). Dolichyl-P in

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animal tissues and in yeast contains two trans isoprene units, the rest are in cis form. The importance of this arrangement has not been studied in detail, but it is probably not decisive for the sugar transfer capacity (5).

Natural dolichyl phosphate has a center of asymmetry at C-3, giving an S configuration (6-8). Recent advances in the chemical synthesis of mammalian dolichols made it possible to produce pure forms of both R and S dolichols which can be chemically phosphorylated (9). In this study we have analyzed the two most commonly occurring R- and S-forms of mammalian dolichyl-P acting as sugar acceptors in the liver microsomal glycosyl transferase systems.

MATERIALS AND METHODS

Total microsomes from livers of starved rats were prepared as previously (10). For measurement of the glycosyl transferase activity, the appropriate dolichyl phosphate in chloroform-methanol (2:1) was evaporated in the presence of $MgCl_2$ and EDTA. The dried mixtures were dissolved in 0.5% Triton X-100. Samples were added to the incubation tube which in a volume of 100 µl contained 30 mM Tris-HCl, pH 7.8, 5 mM EDTA, 10 mM $MgCl_2$ and 2 mM ATP in the final concentrations. The final Triton concentration was adjusted to 0.04% in the case of GDP-mannosyl and UDP-glycosyl transferase and 0.18% for the UDP-GlcNAc transferase. The microsomes were solubilized in Triton and preincubated for 15 min at 0°C before addition to the incubation mixture. Each tube contained 0.03 mg (mannosyl transferase) or 0.12 mg (glucosaminyl and glucosyl transferases) protein. The substrates added were: 0.25 µCi GDP-[^{14}C]mannose (290 mCi/mmol) or 0.25 µCi UDP-[^{14}C]GlcNAc (260 mCi/mmol) or 0.25 µCi UDP-[^{14}C]glucose (296 mCi/mmol), all from Amersham. When radioactive dolichyl-P was used, the labeling of the lipid was done according to Keenan and Kruczek using NaB^3H_4 (11). The incubation was started by the addition of nucleotide sugars, or by microsomes when labeled dolichyl-P was used. After incubation at 30°C (3 min for mannosyl and 10 min for glucosaminyl and glucosyl transferases) the reaction was stopped by addition of 1.0 ml chloroform-methanol (2:1) and 0.1 ml water.The mixture was extracted at 40°C for 30 min. After centrifugation the upper layer was discarded and the lower phase was washed with theoretical upper phase by rinsing twice and mixing and centrifuging once. The washing procedure was repeated three times. The chloroform phase was used for chromatography and for measurement of radioactivity.

For the synthesis of dolichols with 18 and 19 isoprene residues, polyprenols were isolated from leaves of Ginkgo biloba (12); the acetate form of these were used when adding optically active saturated isoprene units, using a Gringard coupling reaction (9). Phopshorylation of dolichol was performed according to Danilov and Chojnacki (13).

Dolichyl phosphates were identified by thin layer chromatography (TLC) on RP-18 HPTLC plates (Merck) using acetone as a solvent, or on silica plates (Merck) with ethylacetate-benzene (5:95) as a solvent. Silica Gel 60 HPTLC plates with concentrating zone (Merck) were used for separation of dolichyl-P-bound sugars; the solvent in the case of bound mannose and glucose was chloroform-methanol-ammonia (65:35:5) and for glucosamine n-butanol-acetic acid-water (60:20:20).

RESULTS AND DISCUSSION

The chemically synthesized dolichols were entirely identical with those obtained from pig liver as indicated by ^{1}H NMR, ^{13}C NMR and IR analyses. The optical purities of both S- and R-dolichol were determined to be more than 97% e.e. by the method described previously (9). The $[\alpha]_D^{25}$ values were -0.51° (neat) and +0.53° (neat), respectively. The chromatographic behavior of the two stereoisomers was the same (Fig. 1).

The V_{max} and the K_m values for the various dolichols were determined by the use of enzymic systems known to utilize dolichyl-P as acceptor: transferases specific for the substrate GDP-mannose, UDP-GlcNAc and UDP-glucose. In studying these reactions several factors will have to be taken into consideration, one of which is the detergent concentration during enzyme activity measurements. In the case of mannosyl transferase - under the given conditions - 0.04% Triton X-100 gives maximal activation; this concentration is four-fold lower than what is required for the UDP-GlcNAc transferase (Fig. 2). On the other hand, the UDP-glucosyl transferase is greatly influenced by the detergent and is inhibited already at low concentrations.

The Lineweaver and Burk plot was used for the determination of V_{max} and K_m for the individual dolichyl-P forms in the three glycosyl transferase systems (Table I). As known from earlier experience the mannosyl transferase system has

1 2 3 4 5 6 7 8 9 10

Fig. 1. Thin layer chromatography of dolichols on reversed phase. Polyprenols were applied to RP-18 HPTLC plates (Merck) and developed in pure acetone. The lipids were visualized by iodine staining. 1, Natural dolichol-18; 2, dolichol-18-S; 3, dolichol-18-R; 4, natural dolichol-19; 5, dolichol-19-S; 6, dolichol-19-R; 7, natural dolichol-17; 8, natural dolichol-18; 9, natural dolichol-19; 10, natural dolichol-20. The natural dolichols, in S-form, were isolated from human liver.

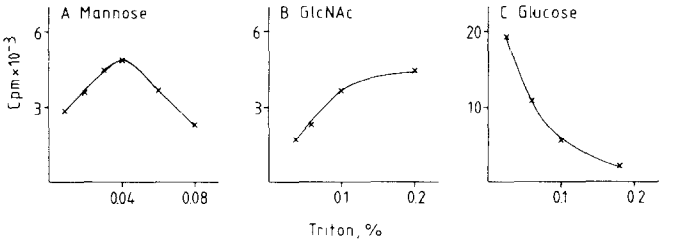

Fig. 2. Effect of Triton X-100 on the various glycosyl transferase activities. Dolichyl-19-P, S-form was used as acceptor. The substrates used were GDP-mannose (A), UDP-GlcNAc (B) and UDP-glucose (C).

a much higher velocity than the glucosaminyl counterpart. In spite of the great V_{max} difference of the two transferase systems, both dolichyl-P forms that were used displayed a pronounced difference between S- and R-forms in both reactions, the former giving a 4-5-fold higher value. The V_{max} for both dolichyl-P forms is also higher in the glucosyl transferase reacton; however, in this case the R-form is operating with 50% of the maximal velocity or higher in comparison with the S-form. Dolichyl-P with 18 and 19 isoprene residues in the S-form gives a 2-3 fold higher K_m value than the comparable R-form when GDP-mannose or UDP-GlcNAc are employed as substrate. Again, the picture is different in the case of the UDP-glucosyl transferase reaction where the values for both isomers are the same.

In order to control the validity of the principle that the S-form of dolichyl-P is the effective carrier of sugars in the glycosyl transferase reactions, experiments were also performed using excess of labeled dolichyl-P and

TABLE I. V_{max} and K_m values for dolichyl-P in mannosyl, N-acetylglucosaminyl and glucosyl transferase reactions

	GDP-mannos		UDP-GlcNAc		UDP-glucose	
	D18-P	D19-P	D18-P	D19-P	D18-P	D19-P
V_{max}[1]						
S-form	1.43	1.15	0.014	0.010	0.033	0.025
R-form	0.36	0.25	0.002	0.004	0.019	0.018
K_m[2]						
S-form	66	61	17	10	80	49
R-form	32	22	9	12	78	81

All values are the mean of 7-9 separate experiments.
[1]nmol/min/mg protein; [2]nM.

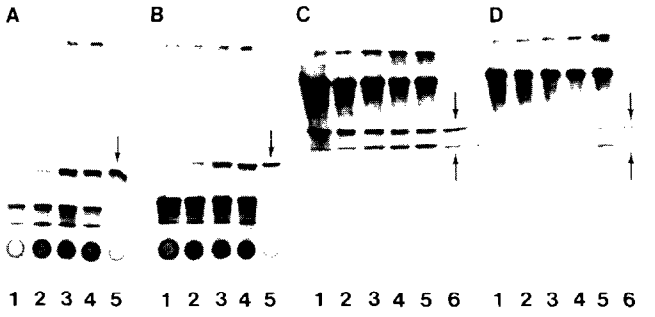

Fig. 3. Thin layer chromatography of dolichyl-P- and dolichyl-PP-sugars on Silica Gel 60 HPTLC plates. The lipids were extracted after incubation of microsomes with dolichyl-P and nucleotide sugars. A. Incubation of dolichyl[^{3}H]-18-P-S with microsomes and GDP-mannose. Incubation times were: 0 min (1), 1 min (2), 5 min (3), 30 min (4), and 30 min in the presence of unlabeled dolichyl-18-P (natural) and GDP[^{14}C]-mannose (5). The dolichyl-P mannose that was formed is indicated with an arrow. B. Incubation of dolichyl[^{3}H]-18-P-R with microsomes and GDP-mannose. 1-5, Conditions as in A. C. Incubation of dolichyl[^{3}H]-18-P-S with microsomes and UDP-GlcNAc. Incubation times were: 5 min (1), 15 min (2), 30 min (3), 60 min (4), 90 min (5), and 30 min in the presence of unlabeled dolichyl-18-P (natural) and UDP[^{14}C]-GlcNAc (6). The upper arrow indicates dolichyl-PP-N-acetyl-glucosamine while the lower arrow points to dolichyl-PP-diacetylchitobiose. D. Incubation of dolichyl[^{3}H]-18-P-R with microsomes and UDP-GlcNAc. 1-6, Conditions as in C.

increasing concentrations of activiated sugar substrate. The reaction product was separated by reversed phase TLC. Autoradiography detects higher amounts of dolichyl-P-mannose in the initial phase of the incubation when the S-form is employed (Fig. 3A and B). In the case of the UDP-GlcNAc transferase reaction, two products are formed; dolichyl-PP-GlcNAc and dolichyl-PP diacetylchitobiose (Fig. 3C and D). The monosaccharide form is the dominating product, and initially only small amounts of dolichyl-PP disaccharide are produced. For this reason it appears acceptable to calculate V_{max} and K_m values for dolichyl-P as presented in Table I. The difference between the two optical isomeres is evident and, as expected, the S-form is much more effective.

The V_{max} and K_m values for GDP-mannose and UDP-glucose in the GDP-mannosyl and UDP-glucosyl transferase reactions were determined using both isomeres (Table II). Significantly higher V_{max} values where obtained for GDP-mannose and also for UDP-glucose using the S-form rather than the R-form. The K_m for the two sugars did not differ when the S- and the R-form of dolichyl[^{3}H]-18-P were used as acceptors.

TABLE II. V_{max} and K_m values for GDP-mannose and UDP-glucose in glycosyl transferase reactions using dolichyl[^{3}H]-18-P as acceptor

	V_{max}[1]		K_m[2]	
	S-form	R-form	S-form	R-form
GDP-mannose	71	37	42	98
UDP-glucose	38	22	50	53

All values are the mean of 4 experiments.
[1]pmol/min/mg protein; [2]nM.

It is uncertain at present which type of molecular organization of dolichyl-P is important for its appropriate function and which structural features serve as organizers for putting the lipid in the right position in the membrane or in the transferase complex. The chain length, the α-saturation and the number of cis-trans residues have been investigated so far, and were in some cases found to have a modifying role in sugar transfer reactions (3-5). It is clear that configuration at C-3 is an important factor for sugar acceptor capacity, since the R type of stereochemical orientation does not eliminate but greatly reduce the function as sugar acceptor. This is not apparent when UDP-glucose is used as substrate; however, it may well be the result of the incubation conditions. We minimized the amount of detergent for solubilization of the polyprene. Nevertheless a strongly inhibitory effect on the UDP-glucosyl transferase reaction was recorded.

The analysis reveald high K_m values for the S-form. This raises the question as to what extent the affinity influences the reaction. One gram of liver contains 10-15 nmol dolichyl-P (14), which should be compared to the K_m values that were obtained (around 0.03 nmol per ml). It therefore appears that the V_{max} value is a true measure of the rate of the glycosylation of lipid intermediates.

The proper optical isomeric form of dolichyl-P seems to be necessary to accomodate this lipid in the right position in the membrane and fit it into the transferase complex. The distribution of various polyprenols in model membranes is specific and very much dependent on the type, nature and configuration of both polyprenols and surrounding phopsholipids (15). The appropriate arrangement and location of polyprenes in membranes influences to a large extent the func-

tion of the lipid itself and interferes with membrane structural properties, such as stability and fluidity (15-17). It is possible that the R-form of dolichyl-P represents a configuration which does not fit in with the neighbouring phospholipids; consequently, the lipid acquires a position which is unsuitable for its function as an acceptor or donor of sugar on the outer and inner surfaces of the microsomal membranes.

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