The Presence of Dolichol in a Lipid Diphosphate *N*-Acetylglucosamine from *Saccharomyces cerevisiae* (Baker's Yeast)

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The lipid moiety of a lipid diphosphate *N*-acetylglucosamine, an intermediate in glycosylation of proteins, was studied. Ozonolysis of the compound gave evidence for an α -saturated isoprene unit. Alkaline hydrolysis of the glycolipid, followed by high-pressure liquid chromatography, showed the presence of a series of polyprenol homologues identical with those isolated directly from *Saccharomyces cerevisiae* (baker's yeast). No particular homologue was preferred in the enzymic transfer of *N*-acetylglucosamine 1-phosphate to endogenous dolichol monophosphate.

The mechanism of glycosylation of proteins in yeast is not yet fully understood, but it is obvious that lipid intermediates play an important role in this process (Lehle & Tanner, 1975; Reuvers *et al.*, 1977). The evidence that the *N*-acetylglucosaminyl intermediates are really polyprenol phosphate derivatives is not unequivocal, being obtained in an indirect manner; the formation of these glycolipids is inhibited by the antibiotic tunicamycin, and addition of polyprenyl phosphate stimulates the incorporation of radioactive *N*-acetylglucosamine into the lipid fraction. In addition, chromatographic and hydrolytic data are consistent with the assignment without providing unambiguous proof (Reuvers *et al.*, 1977). The present paper provides direct evidence for the presence of polyprenols of different chain length in an *N*-acetylglucosaminylated lipid intermediate of yeast. To prove saturation of the α -isoprene residue of these polyprenols (a characteristic of dolichols), the product after ozonolysis was compared with a standard prepared from the phosphate of citronellol, a diprenol with a saturated α -isoprene residue (Hemming, 1974).

Experimental

GlcNAc-*P-P*-lipid (lipid diphosphate *N*-acetylglucosamine) was extracted with chloroform/methanol (2:1, v/v) from a homogenate of *Saccharomyces cerevisiae* (baker's yeast) that had previously been incubated with UDP-[³H]*N*-acetylglucosamine; it was purified by mild alkaline hydrolysis and by column chromatography on DEAE-cellulose and silica gel G60 (Reuvers *et al.*, 1977), followed by further purification on Sephadex LH-20 (column size 50 cm × 1 cm) in chloroform/methanol (2:1, v/v).

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Yeast polyprenols were isolated from *S. cerevisiae* as described by Burgos *et al.* (1963) for liver dolichols, except that final purification was on Sephadex LH-20 (column size 100 cm × 3 cm) in chloroform/methanol (2:1, v/v) [A. Radomska-Pyrek (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa, Poland), unpublished work]. Lipid was extracted from 4 kg of *S. cerevisiae* and most of the ergosterol was removed by crystallization. The resulting lipid (5.5 g) was applied to the column of Sephadex LH-20 in a small volume of the eluting solvent. Flow rate of the solvent was 1 ml/min. The dolichol (0.2 g) was eluted at an elution volume of 200–250 ml. Citronellol (3,7-dimethyl-6-octen-1-ol) was prepared by reduction of citronellal with NaBH₄ (Burgos *et al.*, 1963).

NaB³H₄ (sp. radioactivity 293 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

To phosphorylate citronellol the diprenol (0.1 g) was dissolved in benzene (2 ml) and treated with trichloroacetonitrile (1 ml). After 15 min, bis(triethylamine) phosphate (0.5 g) dissolved in 30 ml of acetonitrile was slowly added (Popjak *et al.*, 1962). The solution was left overnight at 25°C, then transferred to a separation funnel; 50 ml of 0.1 M-NH₃, followed by 150 ml of diethyl ether, was added. The ether fraction was washed twice with water. The combined water layers were washed with ether, evaporated to dryness and dissolved in chloroform/methanol (2:1, v/v). This fraction was further purified on a column of DEAE-cellulose, essentially as described below for phosphorylated lipids.

For ozonolysis, ozone was produced by passing O₂ gas through a LG-2-L1 Corona generator (Davison Chemical Division, Manchester, U.K.). Phosphorylated citronellol (15 mg) or GlcNAc-*P-P*-lipid (40 000

d.p.m.) was used, dissolved in freshly distilled dichloromethane. This solution was cooled by an acetone/solid CO₂ mixture. Termination of the reaction was indicated by a KI/starch solution. After reaching room temperature (21°C), the solution was reduced with triphenylphosphine (100mg) (Beroza & Bierl, 1967). After 1 h the dichloromethane solution was washed with an equal volume of 0.1 M-NH₃, and this aqueous layer was washed four times with dichloromethane to remove the triphenylphosphine. To label ozonolysed lipids, the water layer after ozonolysis of GlcNAc-*P-P*-lipid was evaporated to dryness and dissolved in 2 ml of 0.1 M-KOH, followed by addition of about 5 mCi of solid NaB³H₄. The reduction was continued overnight. Remaining NaB³H₄ was decomposed by addition of 4 ml of acetic acid/methanol (1:9, v/v) and the solution was evaporated to dryness. Acid hydrolysis of ozonolysed GlcNAc-*P-P*-lipid was performed in 0.1 M-HCl/methanol (1:1, v/v) for 10 min at 100°C under reflux.

To produce free polyprenols, GlcNAc-*P-P*-lipid was treated with alkali; to a dry glycolipid portion 10 ml of ethanol and 5 ml of 60% (w/v) KOH were added. After 1 h refluxing, the mixture was cooled to room temperature and 30 ml of water was added. The solution was extracted with 3 × 50 ml of ether and the combined ether portion was washed three times with water (50 ml) to remove the alkali. The ether fraction was then dried overnight over anhydrous Na₂SO₄ and evaporated.

To purify phosphorylated lipids, columns (10 cm × 1.5 cm) of DEAE-cellulose (acetate form) were used. Elution was performed with chloroform/methanol (2:1, v/v), methanol, 10 mM-ammonium formate in chloroform/methanol (2:1, v/v) and 100 mM-ammonium formate in chloroform/methanol (2:1, v/v) (100 ml of each). These 100 ml fractions were collected and tested for lipids by t.l.c.

To purify the polyprenols after alkaline hydrolysis of GlcNAc-*P-P*-lipid, columns (10 cm × 1 cm) of Brockman grade-III alumina were washed with 100 ml of light petroleum (b.p. 40–60°C), followed by 100 ml each of 2%, 4%, 10% and 20% (v/v) ether in light petroleum.

Separation of the different homologues of the polyprenols was carried out by high-pressure liquid chromatography on Waters Associates (Northwich, Cheshire, U.K.) equipment. Samples were injected in propan-2-ol and separated on a column (30 cm × 0.4 cm) of μ -Bondapak C₁₈, with methanol/propan-2-ol/hexane/water (30:65:5:1, by vol.) as solvent. The flow rate was 0.7 ml/min. U.v. absorbance at 210 nm was recorded by a Pye-Unicam LC3 u.v. detector with the range set at 0.08 (Tavares *et al.*, 1977).

T.l.c. was performed on precoated SG60 plates (Merck, Darmstadt, Germany). Solvent mixtures (by vol.) used were: (A) chloroform/methanol/water

(60:25:4), (B) chloroform/methanol/water (10:10:3), (C) propan-2-ol/NH₃ (sp.gr. 0.88) (6:3:1)/water and (D) di-isobutyl ketone/acetic acid/water (20:15:2). Lipids were detected with I₂ vapour or with an anisaldehyde spray (Dunphy *et al.*, 1967). Phosphorylated lipids were detected by spraying with an ammonium molybdate spray (Burrows *et al.*, 1952).

Results and Discussion

After phosphorylation of citronellol, most of the phosphorylated material was eluted with 100 mM-ammonium formate from the DEAE-cellulose column, and was homogeneous on t.l.c., the *R_F* values in the different solvents being (A) 0.14, (B) 0.44, (C) 0.39 and (D) 0.36. These values are consistent with a diphosphate linked to the diprenol (Warren & Jeanloz, 1975). During ozonolysis the double bond between C-7 and C-8 was attacked, and after mild reduction with triphenylphosphine the resulting product was a C₇ pyrophosphate with an aldehyde group. This new product showed no difference in *R_F* value in solvents C and D, but in solvent B it had a lower *R_F* value (0.34), compared with citronellyl pyrophosphate. The new compound was reduced to 6-hydroxy-3-methylhexyl pyrophosphate with NaBH₄ without any marked change in *R_F* value with the different solvents; only a small quantity was converted into the monophosphate form (*R_F* 0.6 in solvent C).

Ozonolysis of GlcNAc-*P-P*-lipid resulted in a 97% yield of radioactivity in the water layer after phase separation, demonstrating that the lipid is susceptible to ozone. This ozonolysed lipid was treated with NaB³H₄ overnight, followed by mild acid hydrolysis to split off the sugar moiety. After evaporation to dryness, the residue, dissolved in chloroform/methanol (2:1, v/v), was applied to a DEAE-cellulose column and the 100 mM-formate fraction was collected. The radioactivity in this fraction caused by reduction with NaB³H₄ was 1600000 d.p.m. Samples were spotted on t.l.c. plates; in solvent B most of the label coincided with standard 6-hydroxy-3-methylhexyl pyrophosphate (Fig. 1a). A minor quantity running ahead might be due to fragments of the polyisoprenoid chain resulting from incomplete ozonolysis. Solvent C was used to distinguish between mono- and pyro-phosphate; the *R_F* value was characteristic for the pyrophosphate (Fig. 1b).

With this method of ³H labelling it was possible to get an impression about the specific radioactivity of [³H]N-acetylglucosamine in the glycolipid. After reduction of the aldehyde group to an alcohol, the specific radioactivity of this product (6-hydroxy-3-methylhexyl pyrophosphate) is about 25% of the original specific radioactivity of the NaB³H₄. According to this supposition, 1600000 d.p.m. corresponds to about 10 nmol, a value consistent

Fig. 1. Thin-layer chromatograms of products prepared from lipid diphosphate *N*-acetylglucosamine after ozonolysis

After ozonolysis the resulting product was purified as described in the text and subjected to t.l.c. Bands 5 mm wide were scraped off and counted for radioactivity (Reuvers *et al.*, 1977). (a) and (b) show results for runs in solvents B and C respectively. A, B, and C represent positions of standard 6-hydroxy-3-methylhexyl pyrophosphate, citronellyl pyrophosphate and 5-hydroxy-3-methylhexyl monophosphate respectively. Abbreviations: O, origin. F, front.

with the yield of dolichol from the alkaline hydrolysis (an accurate value is not available, since the yield of dolichol was probably less than 100%). Consequently the specific radioactivity of the [^3H]*N*-acetylglucosamine in the original glycolipid was about 2 mCi/mmol. The specific radioactivity of UDP-[^3H]*N*-acetylglucosamine used in the synthesis of labelled glycolipids was originally 6.6 Ci/mmol. This result points to the presence of a large amount of endogenous UDP-*N*-acetylglucosamine, a surmise mentioned previously (Reuvers *et al.*, 1977), or of a pool of unlabelled GlcNAc-*P-P*-Dol (dolichol diphosphate *N*-acetylglucosamine).

The relatively large amount of GlcNAc-*P-P*-Dol allowed detection of the polyprenols directly with high-pressure liquid chromatography by means of u.v. absorbance. For this, 40000 d.p.m. of GlcNAc-*P-P*-Dol was treated with strong alkali and extracted with ether. The ether fraction was dried and purified over an alumina column prepared in light petroleum; the polyprenols were present in the 10% ether fraction. High-pressure liquid chromatography per-

Fig. 2. High-pressure liquid chromatogram of products prepared from lipid diphosphate *N*-acetylglucosamine after alkaline hydrolysis

The high-pressure liquid chromatogram of the 10% ether fraction isolated by chromatography on alumina of the hydrolysis product is shown (see the text). The arrows indicate the positions of the polyprenol homologues isolated from *S. cerevisiae*, and the values above the arrows represent the number of isoprene units in these homologues isolated from yeast. The broad peak between 4 and 7 min represents solvent and polar impurities. Further details are given in the Experimental section.

mitted us to separate the different homologues of the polyprenols (Fig. 2). As a standard mixture we have used polyprenols isolated from *S. cerevisiae*. The pattern of the different polyprenols from GlcNAc-*P-P*-Dol was the same as the composition of the polyprenols isolated from *S. cerevisiae*; the homologue with 15 isoprene units was most abundant, accounting for 42% of the total amount. The conclusion can be drawn that no particular homologue is preferred in glycosylation of polyprenols in yeast.

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