The Mannophosphoinositides of *Corynebacterium aquaticum*

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(Received 13 December 1974)

1. Besides the monomannophosphoinositide previously reported in *Corynebacterium aquaticum* small amounts of other, apparently more glycosylated, mannophosphoinositides have been identified in stationary phase cells. Moreover, by labelling cells with [32 P]P_i, phosphatidylinositol was found, comprising about 1.5% of the stationary-phase phospholipids. 2. Pulse-chase experiments performed on cells in the late exponential phase of growth further suggested the sequence phosphatidylinositol → monomannophosphoinositide as the first step in the biosynthesis of the mannophosphoinositides. 3. Di- and tri-mannophosphoinositides are apparently the main mannophosphoinositides present during exponential growth. Monomannophosphoinositide predominates only in late stationary phase; in the earlier stationary phase, phosphatidylinositol comprises 50% of the phosphoinositide lipid, and tetramannophosphoinositide constitutes much of the remainder. 4. The metabolism and functions of the mannophosphoinositides are discussed, particularly in relation to changes in their composition throughout the growth cycle.

Khuller & Brennan (1972) reported that *Corynebacterium aquaticum*, grown in liquid medium for 4–5 days, produced a monomannophosphoinositide and was apparently devoid of other phosphoinositides. Therefore the organism seemed uniquely suitable for the purpose of examining the synthesis of the monomannophosphoinositide. Brennan & Ballou (1967, 1968) had demonstrated mannose incorporation from GDP-[U- 14 C]mannose into the dimannophosphoinositides of *Mycobacterium phlei* and since phosphatidylinositol stimulated the reaction, they postulated sequential mannosylation of phosphatidylinositol as the means of biosynthesis of the dimannophosphoinositides of *M. phlei*. The first mannosylation step was not demonstrable, since monomannophosphoinositide is present in only trace amounts in *M. phlei* (Lee & Ballou, 1965). This circumstance, combined with the apparent absence of phosphatidylinositol in *C. aquaticum*, gave credence to the contention of others (Shaw & Dinglinger, 1969) that monomannophosphoinositide could arise by a route other than by mannosylation of phosphatidylinositol. In the present study, we have posed the question of whether or not phosphatidylinositol is directly involved in monomannophosphoinositide synthesis in *C. aquaticum*. At the outset of the work, we demonstrated that minute quantities of phosphatidylinositol exist in stationary-phase cells of *C. aquaticum*. However, the amounts were such as to preclude experiments *in vitro*. Instead, we have used a combination of pulse-chase experiments to explore the issue.

Experimental

Materials

Phosphatidylinositol was isolated from yeast by the method of Trevelyan (1966). Dimannophosphoinositides were isolated from *M. phlei* as described by Brennan & Ballou (1967). [32 P]P_i (125 Ci/mg of P) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Growth and labelling of cells

C. aquaticum (strain B2252) was kindly provided by Dr. H. Lechevalier, Institute of Microbiology, Rutgers University, New Brunswick, N.J., U.S.A. The organism was maintained on Oxoid Nutrient Agar slopes at 37°C, and grown under shaking conditions, at the same temperature, in the medium of Brennan *et al.* (1970) (phosphate-rich medium), or in the same medium without phosphate salts (phosphate-deficient medium). Cells were harvested by centrifugation and washed with 0.9% (w/v) NaCl. The colonies from two slopes were transferred to 50 ml of phosphate-rich medium and grown for 3 days; this served as inoculum for 3 litres of phosphate-rich medium. Cells grown in phosphate-rich medium for a further 2 days were harvested, washed and resuspended in twice the original volume of phosphate-deficient medium supplemented with 1.0 mCi of [32 P]P_i/l. Growth in this medium continued for various times. In the case of the [32 P]P_i chase experiments, growth was allowed to

continue in the same flasks after the addition of 12 g of Na_2HPO_4 and 6 g of KH_2PO_4 to 1 litre of suspension.

Characterization of lipids

Lipids were extracted with chloroform-methanol (2:1, v/v) and chloroform-methanol (1:2, v/v) and washed by the method of Folch *et al.* (1957). T.l.c. of total lipids were carried out on silica-gel H (Merck) plates (20 cm $\times$ 20 cm and 20 cm $\times$ 40 cm) or on silica-gel H plates impregnated with sodium acetate (Minnikin & Abdolrahimzadeh, 1971). The following solvent systems were used: A, chloroform-methanol-water (65:25:4, by vol.); B, chloroform-methanol-acetic acid-water (30:15:4:2, by vol.); C, chloroform-methanol-7M- NH_3 (12:7:1, by vol.), and a two-dimensional t.l.c. system (Rouser *et al.*, 1967) where the developing solvent of the first dimension was system A above, and the developing solvent of the second dimension was chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1, by vol.) (system D). Radioautograms of t.l.c. plates were obtained by exposing the plates to Kodak 'Blue Brand' BB54 X-ray film. Lipids were deacylated by the method of Ballou *et al.* (1963). The products were chromatographed on Whatman no. 1 paper or on Silica-Gel H plates in the solvent system propan-2-ol-aq. NH_3 (sp.gr. 0.88) (2:1, v/v) (system E) alongside glycerylphosphorylinositol and glycerylphosphorylinositol dimannoside. Standards were located with the periodate-Schiff spray reagents (Shaw, 1968) or with the silver nitrate-NaOH dip reagents (Anet & Reynolds, 1954) and the tracks of the analytical applications were scanned by using a Packard 7201 radiochromatogram scanner. Bands running lower than standard glycerylphosphorylinositol dimannoside were tentatively identified as higher glycerylphosphorylinositol mannosides since they had R_F values similar to the deacylated oligomannophosphoinositides of *M. phlei*. Phosphatidylethanolamine, phosphatidylglycerol and bisphosphatidylglycerol, which were identified in *C. aquaticum* by Khuller & Brennan (1972) were again characterized by the same procedures. To quantify the amounts of the individual lipids, gel areas and portions of paper chromatograms were counted for radioactivity in an Intertechnique SL30 scintillation spectrometer (10 ml of 0.4% 2,5-diphenyloxazole in toluene per vial).

Results and Discussion

Characterization of the phospholipids of *C. aquaticum*

In the examination of the phosphoinositides of *C. aquaticum* by Khuller & Brennan (1972) only the deacylated monomannophosphoinositide was characterized. The intact glycopospholipid was not

Fig. 1. Distribution of the phosphoinositides of *C. aquaticum* on t.l.c.

The deacylated lipids from the four t.l.c. areas delineated in Plate 1 were subjected to radiochromatography (see the text for details). GPI, Glycerylphosphorylinositol; GPIM₂, glycerylphosphorylinositol dimannoside.

Table 1. Amounts of phosphoinositides in stationary-phase *C. aquaticum*

These values were obtained by paper radiochromatography (see the text for details) of the deacylated ^{32}P -labelled lipids. PI, Phosphatidylinositol; PIM₁, PIM₂, PIM₃, PIM₄, PIM₅ and PIM₆, mono-, di-, tri-, tetra-, penta- and hexamannophosphoinositide respectively.

Phosphoinositide	Phospholipid (% of total)
PI	1.5
PIM ₁	20.0
PIM ₂	0.2
PIM ₃	1.0
PIM ₄	2.5
PIM ₅	0.5
PIM ₆	0.5

isolated. Pangborn & McKinney (1966) and Brennan & Ballou (1967, 1968) had demonstrated that, in mycobacteria, a mannophosphoinositide of a fixed degree of glycosylation may contain more than two fatty acyl groups. The dimannophosphoinositides of *Corynebacterium xerosis* and *Corynebacterium diptheriae*, and the phosphatidylinositol of *C. xerosis*, exhibited similar features (Brennan, 1968; Brennan & Lehane, 1971). Since a knowledge of the extent of acylation of the phosphoinositides is important for the understanding of their biosynthesis and function (Brennan & Ballou, 1968; Shaw & Dinglinger, 1969), an effort was

EXPLANATION OF PLATE I

*Two-dimensional t.l.c. (sodium acetate-impregnated plate) in the systems of Rouser et al. (1967) of the ^{32}P -labelled stationary-phase lipids of *C. aquaticum**

Spots on the chromatogram were detected by radioautography (12h exposure). Some 1.2×10^4 c.p.m. were applied to the origin at the bottom left-hand corner and the first dimension was developed vertically.

EXPLANATION OF PLATE 2

T.l.c. in propan-2-ol-aq. NH₃ (sp.gr. 0.88) (2:1, v/v) of the ³²P-labelled deacylated stationary-phase lipids of C. aquaticum

The plate was developed three times. Spots were detected by radioautography (24 h exposure). Some 1.2×10^4 c.p.m. were applied. GPIM₁, Glycerylphosphorylinositol monomannoside; GPI, glycerylphosphorylinositol; GPG, glycerylphosphorylglycerol; diGPG, diglycerylphosphorylglycerol.

made to gauge the degree of acylation of the monomannophosphoinositide of *C. aquaticum*. The small amount of material available precluded a direct estimation of the number of acyl groups. Polydispersity on t.l.c. was sought, since previous work had shown that this reflects varying numbers of fatty acyl groups. Hence the ^{32}P -labelled lipids from stationary-phase cells were subjected to two-dimensional t.l.c. in the systems of Rouser *et al.* (1967) and yielded the highly reproducible pattern of Plate 1. The delineated areas were excised from the t.l.c. plates. The lipids were eluted, deacylated, subjected to paper chromatography in system E and scanned for radioactivity as described above (Fig. 1). The deacylated lipid pattern from area 4 shows that the large spot is not homogeneous. Although it is composed largely of the monomannophosphoinositide, substantial quantities of the higher mannophosphoinositides are also present. In addition, small amounts of phosphatidylinositol occur in this area. The mobility of these lipids in the t.l.c. solvents indicates that they are highly acylated; yeast phosphatidylinositol had R_F values of less than 0.5 in both directions. When the phospholipids from areas 1 and 2 were deacylated, glycerylphosphorylinositol monomannoside, presumably arising from less-acylated monomannophosphoinositides, was one of the deacylated products. The major components of areas 1 and 2 were phosphatidylglycerol and phosphatidylethanolamine respectively; bisphosphatidylglycerol was found in area 4. Chromatography of the deacylated diffuse phospholipid of area 3 showed a single component with an R_F value suggestive of glycerylphosphorylinositol trimannoside. To determine the relative amounts of the various phosphoinositides in stationary-phase lipids of *C. aquaticum*, the entire lipid fraction was deacylated and subjected to t.l.c. (Plate 2) and paper chromatography (Table 1). Plate 2 substantiates the evidence for small amounts of glycerylphosphorylinositol among the deacylated stationary-phase lipids of *C. aquaticum*. Previous attempts, using chemical means of detection (Khuller & Brennan, 1972), failed to demonstrate this product. Phosphatidylinositol comprises 1.5% of the stationary-phase phospholipid; the higher mannophosphoinositides (those with two or more mannose residues), which had not previously been detected in *C. aquaticum*, represent about 5% of the total phospholipids (Table 1). The monomannophosphoinositide, the only member of this group of glycerophospholipids detectable by previously used chemical means, represents about 20% of the total phospholipid.

Growth cycle-dependent change in the phosphoinositides of *C. aquaticum*

The evidence for the presence of phosphatidylinositol in *C. aquaticum* was noteworthy since it again

Fig. 2. Variation of composition of phosphoinositides of *C. aquaticum* with growth

Deacylated ^{32}P -labelled lipids of *C. aquaticum* grown for various times were subjected to paper radiochromatography (see the text for details). PI, Phosphatidylinositol; PIM₁, PIM₂, PIM₃, PIM₄, PIM₅ and PIM₆, mono-, di-, tri-, tetra-, penta- and hexa-mannophosphoinositides respectively.

focused attention on its possible role as a precursor of the monomannophosphoinositide. Attempts to isolate [^{32}P]phosphatidylinositol from stationary-phase lipid by t.l.c. on 20cm×40cm plates in solvent systems B and C were unsuccessful. Thus a demonstration of its direct conversion into monomannophosphoinositide was impractical. Efforts were therefore made to demonstrate a relationship between the two phosphoinositides during earlier phases of the growth cycle and during conditions of pulse and chase labelling. In view of the complexity of the intact phospholipid patterns, the labelling of the deacylated phospholipids only was followed in these experiments.

Observations on the variations in the phosphoinositide composition of *C. aquaticum* with time are summarized in Fig. 2. A number of unexpected conclusions emerge. Di- and tri-mannophosphoinositides together constitute about 70% of very early exponential-phase phosphoinositides; in later exponential phase they are the only mannophosphoinositides present. In the early stationary phase

Scheme 1. *Proposed explanation of the interrelationships of the phosphoinositides of C. aquaticum during the growth cycle*

The basic scheme is that of Brennan & Ballou (1967). For details of abbreviations see Table 1. For details of steps (1)–(4) see the text.

of growth, tetramannophosphoinositide rises to comprise about 50% of the phosphoinositides whereas di- and tri-mannophosphoinositides decrease to low amounts. Phosphatidylinositol, present at low amounts since mid-exponential phase, rises to comprise the other 50% of the phosphoinositide lipid. Finally, the monomannophosphoinositide, which is present at low amounts in mid-exponential phase, builds up to constitute 88% of the phosphoinositides in the late stationary phase of growth.

It is possible to explain the complete sequence of events recorded in Fig. 2 by referring to Scheme 1, which is the main biosynthetic pathway for the mannophosphoinositides of mycobacteria proposed by Brennan & Ballou (1967, 1968). At the early growth stages there is exclusive synthesis of the di- and tri-mannophosphoinositides, presumably connected with the membrane functions peculiar to the rapid growth phase. Subsequently, at early stationary phase, there is a complete conversion of the di- and tri-mannophosphoinositides into the tetramannophosphoinositide, possibly caused by acceleration of step (3) by the induction of the enzyme that catalyses step (4). Essentially this is the end of the sequence, since the penta- and hexa-mannophosphoinositides never rise above 7% of the phosphoinositides after their initial appearance. With the onset of the synthesis of the tetramannophosphoinositide there is renewed production of the trimannophosphoinositide and the shape of both curves suggest that this may occur partly because of the degradation of the tetramannophosphoinositide to the trimannosylated analogue. Concomitant with these events there is a build-up of phosphatidylinositol. This may result from end-product inhibition of the first manno-

Fig. 3. *Pulse-chase experiment demonstrating the precursor-product relationship between phosphatidylinositol and monomannophosphoinositide in C. aquaticum*

Cells were grown for 96 h in ^{32}P -labelled medium. A portion was harvested, and the remainder allowed to grow in excess of unlabelled phosphate for the time intervals indicated. Paper radiochromatography of the ^{32}P -labelled deacylated lipids followed (see the text for details). ●, Phosphatidylinositol; ○, monomannophosphoinositide.

sylation step by the true end-product, the tetramannophosphoinositide.

The appearance of the monomannophosphoinositide in stationary-phase cells is puzzling. The simplest explanation is that the bulk of it arises by

the action of mannosidases on the higher mannophosphoinositides. Part of it could also arise by mannosylation of phosphatidylinositol since the restraints on the primary mannosylation step may be removed by the degradation of the tetramannophosphoinositide. The unique structure of the monomannophosphoinositide may make it likely to be the end-product of a series of degradative steps since the compound from mycobacteria has the mannose attached to the 2 position of inositol (Ballou & Lee, 1964) and all of the higher mannophosphoinositides have the extra mannosyl groups attached to the 6 position of inositol (Lee & Ballou, 1965). Such a structure for the monomannophosphoinositide may ensure that it is not easily susceptible to further degradation.

Relationship of phosphatidylinositol to monomannophosphoinositide

As mentioned above, the most contentious issue of Scheme 1 is the role of phosphatidylinositol. Since in *C. aquaticum*, phosphatidylinositol was present in definite amounts in cells grown for 96h, and since at subsequent times monomannophosphoinositide was very obvious, it was decided to conduct a chase experiment to determine if the label in phosphatidylinositol could end up in monomannophosphoinositide. Cells grown for 96h in ³²P-labelled medium contained phosphatidylinositol, mono-, di- and tri-mannophosphoinositide. Chases with unlabelled phosphate for 1, 4 and 8h followed labelling, and the deacylated lipids were analysed by radiochromatography (Fig. 3). Phosphatidylinositol, present to the extent of about 20% of the phosphoinositides after 96h of growth, dropped to a value of about 14% after 8h of chase. Concomitantly, the monomannophosphoinositide increased from a value of about 12% to 16% after the same chase period. Since the sum of the label in phosphatidylinositol and monomannophosphoinositide is a constant percentage of the total phosphoinositide label during the course of the chase experiments, it appears that degradation of di- to mono-mannophosphoinositide is not occurring under these conditions, unless such label input is being balanced by degradation of phosphatidylinositol to non-lipid material. This seems unlikely, since the cells were growing rapidly under the condition of phosphate depletion.

This evidence supports previous results that phosphatidylinositol is the direct precursor of the monomannophosphoinositide and provides indirect support for the sequence described in Scheme 1 (summarized by Ballou, 1972). Takayama & Goldman (1969) using cell-free preparation of *Mycobacterium tuberculosis*, showed that the [¹⁴C]-mannose incorporated from GDP-[1-¹⁴C]mannose

into the dimannophosphoinositides was exclusively associated with the 6-position of the inositol ring and that the mannose attached to the 2-position was unlabelled. From this evidence the authors suggested that the direct mannosylation of phosphatidylinositol was not operative in this organism. However, it now seems that a likely explanation is that monomannophosphoinositide synthetase activity was inactivated or unexpressed in their preparations and that endogenous monomannophosphoinositide was serving as an acceptor of labelled mannose.

The functions of the mannophosphoinositides are unknown but they probably have important roles in the cell since they are the only glycopospholipids present in the organism. Of the two other major classes of microbial glycopospholipids, the glycoposphingolipids of eukaryotic micro-organisms (Brennan *et al.*, 1974) and the phosphatidylglycosyldiglycerides of Gram-positive bacteria (Shaw & Stead, 1972), only the last have been discussed from a functional viewpoint (Toon *et al.*, 1972). They are thought to be of importance in anchoring the intracellular teichoic acid to the cell membrane, and to have properties favouring the intracellular transport of hydrophilic molecules. It is possible that the mannophosphoinositides may have an analogous function.

Recently, models involving cell-cell interaction mediated by the externally projecting sugar residues of glycoproteins and glycolipids and the enzymes responsible for transferring further sugar residues to these substrates, have come into prominence (Roseman, 1970). It is possible that the synthesis of the di- and tri-mannophosphoinositides in exponential phase growth is confined to the mesosomal-plate area and that lamellar build-up before cell division is closely linked thereto. Cell separation could occur by the inter-conversion of the di- and tri-mannophosphoinositides by specific enzymes, the products would then detach from these enzymes as cell-wall synthesis progresses inward and after a few generations of growth most mannophosphoinositides should occur in non-mesosomal areas. In fact, pulse-chase experiments on cells grown for 4h in ³²P-labelled medium demonstrated the expected lability of phosphatidylinositol, but a remarkable stability of di- and tri-mannophosphoinositide (J. A. Hackett & P. J. Brennan, unpublished work); the label in these compounds after 4h growth on ³²P-labelled medium remained unchanged in quantity and proportion after cells were grown for a further 12h in unlabelled medium. Were a small amount of mannosyltransferases present in non-mesosomal areas, higher mannophosphoinositides should accumulate without affecting cell division. This is in line with the point made by Ballou (1972) that the lower mannophosphoinositides tend to occur preferentially in internal microsomal membranes, whereas the

higher homologues tend to occur preferentially in cell-membrane-cell-wall preparations. Induction of the tetramannophosphoinositide synthetase depletes the di- and tri-mannophosphoinositides by accelerating their through conversion into the tetramannophosphoinositide. This accumulation of the tetramannophosphoinositide may have the effect of inhibiting the first step in mannophosphoinositide biosynthesis (Scheme 1), thereby rendering their resynthesis impossible and, by the above hypothesis, this would tend to stop cell division. After further metabolic changes, degradation of all mannophosphoinositides to the monomannophosphoinositide characteristic of stationary-phase lipids in *C. aquaticum* may ensue.

This work was supported by a grant from the National Science Council (Republic of Ireland).

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