THE ISOLATION AND BIOSYNTHESIS OF THE CERAMIDE-PHOSPHOINOSITOL OF *ASPERGILLUS NIGER*

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1. Introduction

The phosphosphingolipids of plants contain a glycosylated *myo*-inositol-1-phosphate moiety linked via a phosphodiester bridge to the primary hydroxyl of a ceramide [1,2]. Such compounds have also been isolated from the fungi *Saccharomyces cerevisiae, Aspergillus niger* [3–5] and *Agaricus bisporus* (Brennan et al., unpublished results). The biosynthesis of the phosphospingolipids is presumably completed by sugar residue donation from the appropriate sugar nucleotide to the protype lipid, ceramide-phosphoinositol, the biosynthesis of which is thus fundamental to phosphosphingolipid biosynthesis in general. Ceramide-phosphoinositol has not, to date, been isolated from a plant source, but Smith and Lester [4] have chacterised this material in lipid extracts of *S. cerevisiae,* and in vivo pulse-chase experiments with this organism led Angus and Lester [6] to propose that the biosynthesis of ceramide-phosphoinositol proceeded via donation of intact *myo*-inositol-1-phosphate units from phosphatidyl inositol to endogenous ceramide. In the present work, the isolation of ceramide-phosphoinositol from *A. niger* is reported, and the biosynthesis of this compound in vitro is shown to proceed by transfer of inositol-phosphate units from [^{32}P]phosphatidyl [^{3}H]inositol to ceramide containing 2-hydroxy fatty acids. The reaction is catalysed by a soluble enzyme system.

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2. Materials and methods

2.1. *Materials*

Silicic acid for column chromatography was purchased from Mallinckrodt Ltd., Missouri, USA, and was activated at 100°C overnight before use. Silica gel H for t.l.c. was the product of Merck AG, Darmstadt, Germany. The filter aid Hyflo Super-Cel was purchased from Johns Manville, New York, USA. Dithiothreitol (Grade A) was obtained from Calbiochem, California, USA. The silylating reagents hexamethyldisilazane and trimethylchlorosilane were the products of Sigma Ltd., UK. [^{32}P]P_i (71 Ci/mg of P) and [^{3}H]*myo*-inositol (4.5 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks., UK. All other chemicals were analytical reagent grade.

2.2. *Fungus*

A. niger (strain A.T.C.C. 26522) was grown in the laboratory in the glucose–casein hydrolysate medium described elsewhere [7]. When incorporation of [^{32}P] P_i into phospholipid was required, mycelium was grown prior to isotope addition, in a glucose–casein hydrolysate medium that contained only 0.1 g K_2HPO_4/l (phosphate-deficient medium). Cells were harvested by filtration and washed with 0.9% (w/v) NaCl.

2.3. *Analytical methods*

Methanolysis of lipid was effected with 1.25 M HCl in methanol, at 75°C for 15 h. When more severe cleavage conditions were required, hydrolysis of samples proceeded in 3 M HCl at 100°C. Prior to g.l.c.

analysis, the reaction mixtures were dried and the released sugars or polyols derivatised with pyridine–hexamethyldisilazane–trimethylchlorosilane (10 : 4 : 2, v/v/v) for 15 min, and the trimethylsilylated derivatives analysed on a Pye Unicam Series 104 instrument fitted with a flame ionization detector. The column was 3% (w/w) OV-1 on 80/100 Supelcoport (Supelco Ltd., Cheshire, UK), and was purged at 200°C overnight before use. The carrier gas was nitrogen (45 ml/min).

Autoradiography of t.l.c. plates (for the detection of [^{32}P]phospholipid) was effected with Kodak 'Blue Brand' BB54 X-ray film. Labeled liquid samples, or portions of t.l.c. or paper chromatogram tracks, were counted in an Intertechnique SL-30 scintillation spectrometer after addition of scintillation fluid (0.4% (w/v) 2-5-diphenyloxazole in toluene, 10 ml per vial).

2.4. *Preparation of labeled phosphoinositides from A. niger*

Spores of *A. niger* were transferred to 2 l of P_i-deficient medium and grown at 25°C, under shaking conditions for 1 day. Then [^{32}P]P_i (10 mCi) and [^{3}H]*myo*-inositol (200 μCi) were added and growth continued at 25°C for a further 2 days. The harvested cells were washed with 4 l of 0.5% (w/v) K_2HPO_4 and the lipids were extracted with chloroform–methanol (2 : 1, v/v) and chloroform–methanol (1 : 2, v/v), and washed twice by the method of Folch et al. [8]. The organic phase was evaporated to dryness. To the residue was added cold (−25°C) acetone (50 ml) and the suspension stirred on an ice-salt bath for 10 min. The precipitate was pelleted by centrifugation at 11 000 *g* for 10 min (−10°C). The supernatant was evaporated to dryness and the residue resuspended in 10 ml of chilled acetone. The resulting precipitate was isolated. The combined acetone supernatants contained 1.1×10^8 c.p.m. of [^{32}P] and 6.6×10^6 c.p.m. of [^{3}H] while the combined pellets (0.2 g) had 1.2×10^9 c.p.m. of [^{32}P] and 7.2×10^7 c.p.m. of [^{3}H]. The combined pellets were suspended in ethanol (20 ml) and the suspension stirred at 20°C for 45 min, followed by centrifugation. Reextraction of the pellet with ethanol (20 ml) followed. The ethanol supernatants contained 1.1×10^9 c.p.m. of [^{32}P] and did not contain any [^{3}H] counts, while the pellet (51 mg) had 1.2×10^8 c.p.m. of [^{32}P] and 7.2×10^7 c.p.m. of [^{3}H]. The pellet was suspended in chloroform–methanol (7 : 1, v/v) (1 ml).

The ethanol-insoluble phospholipid was further purified by the method of Hanahan et al. [9]. Silica gel (6.7 g) and Hyflo Super-Cel (3.3 g) were mixed and washed with chloroform–methanol (7 : 1, v/v) (150 ml). A glass column (23 cm × 1.5 cm) was packed with this material. The gel was washed with chloroform–methanol (7 : 1, v/v) (150 ml). The suspended lipid (1 ml) was applied and the column eluted successively with chloroform–methanol (7 : 1, v/v) (25 ml), chloroform–methanol (4 : 1, v/v) (50 ml), chloroform–methanol (3 : 2, v/v) (60 fractions of 2 ml), and chloroform–methanol–water (10 : 10 : 3, v/v) (54 fractions of 2 ml). The flow rate was maintained at approximately 1 ml/min by applying slight nitrogen pressure. The first eluate contained 2.5×10^7 c.p.m. of [^{32}P] and the second had 2.0×10^7 c.p.m. of [^{32}P].

The distribution of label in the last two eluates was ascertained by counting of 20 μl portions of each fraction. In both cases, a single peak eluted with the solvent front; the peak fractions were amalgamated. The peak eluting with the third solvent had 4.8×10^7 c.p.m. of [^{32}P] and 1.6×10^7 c.p.m. of [^{3}H] and was designated phosphoinositide 1. The peak of the final eluate had 1.6×10^7 c.p.m. of [^{32}P] and 0.55×10^7 c.p.m. of [^{3}H] and was designated phosphoinositide 2. Total [^{32}P] recovery from the column was 90%.

2.5. *Preparation of ceramides from A. niger*

Ceramides were prepared from lipid extracts of *A. niger* by the method of Weiss and Stiller [10]. Final purification was achieved by preparative t.l.c. in the system chloroform–methanol (38 : 3, v/v) [11]. Detection of ceramides containing 2-hydroxy fatty acids and 2,3-dihydroxy fatty acids was effected with the periodate-Schiff spray reagents [12,13].

2.6. *Breakage of A. niger mycelium and preparations of subcellular fractions*

Fresh-grown mycelium was suspended in a minimal volume of 0.1 M Tris-HCl buffer, pH 7.1, 0.5 mM in $MgCl_2$, and the suspension disrupted with an LKB X-press (30 ml capacity) at −25°C. All further operations were carried out at 0°C. After thawing, cell debris was removed by centrifugation at 11 000 *g*

for 10 min, and the resulting supernatant was subjected to high-speed centrifugation (2×10^5 *g*, 30 min) in a Sorvall OTD-2 ultracentrifuge. The supernatant thus obtained was the source of biosynthetic activity. When microsomal activity was sought, the pellet was suspended by homogenisation in a small volume of the buffer used for cell disruption, here also 0.5 mM in dithiothreitol.

2.7. *Assay conditions for ceramide-phosphoinositol biosynthesis*

The typical incubation mixture contained 1–2 mg protein (estimated by the Biuret reaction using bovine serum albumin as standard), 70 μmol Tris-HCl, 0.35 μmol $MgCl_2$, 0.35 μmol dithiotheritol, [^{32}P]phosphatidyl [^{3}H]inositol (about 10^4 c.p.m. of [^{32}P]) and ceramide (100 μg) in a total volume of 1 ml. Prior to the addition of enzyme, the lipid substrates were sonicated in a sonic bath to assist dispersion. The incubation was at 25°C for 4 h, under shaking conditions, and was terminated by the addition of chloroform–methanol (2 : 1, v/v) (5 ml) followed by vigorous mixing. A boiled enzyme blank was processed in all incubations. The organic phase was evaporated to dryness and an alkali-stable lipid fraction obtained by the method of Steiner et al. [14].

3. Results and discussion

3.1. *The phosphoinositides of A. niger*

The identification of phosphoinositide 1 as phosphatidyl inositol was shown by the following procedures. A sample of the pure lipid was deacylated and the water-soluble deacylation products chromatographed on Whatman No. 1 paper in the system 2-propanol-aq. NH_3 (sp. gr. 0.88) (2 : 1, v/v) alongside authentic glycerylphosphorylinositol from yeast, descending for 3 days. All label ran as glycerylphosphorylinositol. Acid hydrolysis (2 h) of a sample of the pure lipid, followed by paper chromatography of the water-soluble material in the system 1-butanol–pyridine–water (6 : 4 : 3, v/v/v), alongside authentic inositol, showed all [^{3}H] label migrating as inositol. T.l.c. in chloroform–methanol–4M NH_3 (9 : 7 : 2, v/v/v) (System 1) of a small volume of the lipid solution, followed by autoradiography, showed a single spot corresponding to authentic phosphatidyl inositol.

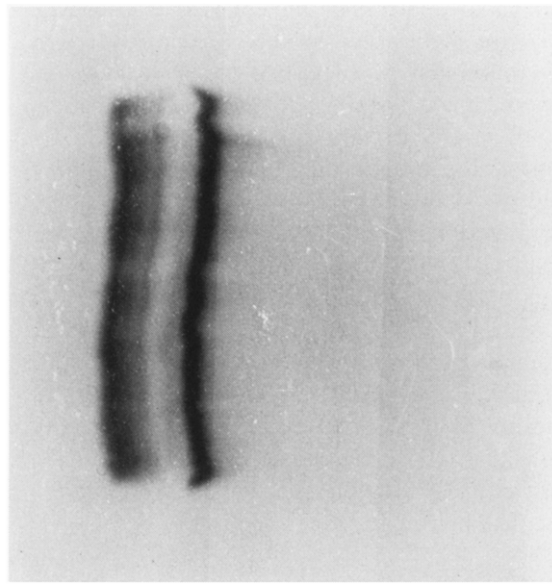

Fig.1. The distribution of the labeled phosphosphingolipids of *A. niger* on t.l.c.. A labeled phosphosphingolipid preparation was obtained from *A. niger* as described in the text and subjected to preparative t.l.c. in chloroform–methanol–4M NH_3 (9 : 7 : 2, v/v/v). The plate was developed by autoradiography (12 h exposure).

The amalgamated fractions of phosphoinositide 2 were evaporated to dryness and an alkali-stable fraction obtained by the method of Steiner et al. [14]. This material (containing 90% of the [^{32}P] counts) was subjected to preparative t.l.c. in System 1. Two bands were detected by autoradiography (fig.1) and were eluted individually. The lower band (2 mg) contained 0.6×10^7 c.p.m. of [^{32}P] and 0.24×10^7 c.p.m. of [^{3}H] while the upper band (4.1 mg) had 0.9×10^7 c.p.m. of [^{32}P] and 0.31×10^7 c.p.m. of [^{3}H].

Methanolysis of 0.5 mg of the upper band material, followed by addition of internal standard (mannitol, 0.2 μmol) and g.l.c. analysis showed the presence of a small amount of mannose, and of inositol. Acid hydrolysis (24 h) of 0.5 mg of the material showed no increase in the amount of inositol released. The inositol : sugar molar ratio of the lipid was 10 : 1, and this alkali-stable lipid was consequently identified as principally ceramide-phosphoinositol, possibly

contaminated by a small amount of the mannosylated derivative. Methanolysis of 0.5 mg of lower band lipid followed by g.l.c. analysis gave inositol and mannose in a molar ratio of 4 : 1, again, methanolysis was effective in causing the liberation of the lipid inositol. The isotope ratios ^{32}P : ^{3}H in both lipid bands were close to each other and to that of the phosphatidyl inositol prepared in the same experiment (see earlier). The isotope ratio of the slower band, combined with the high inositol : sugar molar ratio, may point to the existence in *A. niger* of the ceramide-di (phosphoinositol) described from other fungal sources [14,15]. The facile liberation of innositol from a phosphodiester linkage by mild methanolysis may be a consequence of the availability of a free *cis*-hydroxyl in the inositol ring of the described lipids.

3.2. *The biosynthesis of the ceramide-phosphoinositol of A. niger*

Incubation of microsomal extracts of *A. niger* with [^{32}P] phosphatidyl [^{3}H] inositol, for 4 h, caused the conversion of both labels to water-soluble materials which were chacterised by paper chromatography (1-butanol–pyridine–water; 6 : 4 : 3, v/v/v, descending) as [^{32}P]P_i and [^{3}H]inositol. It was concluded that any biosynthetic activity present in the membrane fraction was masked by the breakdown of the added substrate. Incubation of soluble protein fractions of *A. niger* with [^{32}P]phosphatidyl [^{3}H]inositol (10^4 c.p.m. of [^{32}P] and 7.7×10^3 c.p.m. of [^{3}H]) for 4 h at 25° resulted in label incorporation into alkali-stable lipid, with preservation of the ^{32}P : ^{3}H count ratio (table 1). This activity was enhanced by the addition of *A. niger* ceramide containing 2-hydroxy fatty acids; 2,3-dihydroxy fatty acid-containing ceramides inhibited activity. The label incorporation was low (typically 5% per mg protein). T.l.c. (System 1) of the labeled lipid extract alongside labeled ceramide-phosphoinositol (see earlier), followed by scintillation counting of 0.5 cm portions of track, showed that the lipid synthesised in vitro co-chromatographed with ceramide-phosphoinositol.

The variation in the extent of the biosynthetic reaction with time was studied. Soluble protein fractions from *A. niger* were incubated with [^{32}P] phosphatidyl [^{3}H]inositol (10^4 c.p.m. of [^{32}P]) and 2-hydroxy fatty acid-containing ceramide (100 μg), for varying periods, and the alkali-stable labeled lipid characterised as ceramide-phosphoinositol. Maximum label incorporation was obtained after 4 h of incubation, the decrease in incorporation after this time presumably reflected endogenous degradation processes.

Table 1
The incorporation of label from [^{32}P]phosphatidyl [^{3}H]inositol into ceramidephosphoinositol

Tube	Ceramide present	[^{32}P] into CerPl	[^{3}H] into CerPl
1	–	120	104
2	α OHcer	560	440[a]
3	α,β OHcer	32	10

[a] The decrease in the ratio [^{32}P]/[^{3}H] in the phosphatidylinositol is due to decay of [^{32}P] over an approximate two-week period from its initial isolation

A soluble protein fraction of *A. niger* (1.2 mg protein/ml) was incubated with [^{32}P]phosphatidyl [^{3}H]inositol (10^4 c.p.m. of [^{32}P] and 7.7×10^3 c.p.m. of [^{3}H]) at 25°C for 4 h. In some cases, ceramide (100 μg) was also present in the mixture. Some 0.8 mg protein (0.7 ml) was added to each tube and the total incubation volume was 1 ml. The alkali-stable lipid extract was isolated and characterised as described in the text. An enzyme blank yielded no detectable alkali-stable lipid counts. Each result below is an average of the results from three tubes; the individual values for each tube differed by less than 7%. α OHcer, 2-hydroxy fatty acid-containing ceramide; α,β OHcer, 2,3-dihydroxy fatty acid-containing ceramide; CerPl, ceramide-phosphoinositol.

The evidence quoted supports the idea that ceramidephosphoinositol biosynthesis proceeds as follows:

ceramide (containing 2-hydroxy fatty acid) + phosphatidyl inositol → ceramide-phosphoinositol + diglyceride.

This description of ceramide-phosphoinositol (and possibly also ceramide-di(phosphoinositol)) in *A. niger* extends the results of Brennan and Roe [5,16] who have detected sugar-containing phosphosphingolipids in this organism. The demonstration, in vitro, of the biosynthesis of ceramide-phosphoinositol via inositol-phosphate donation from phosphatidyl inositol to ceramide, confirms the results of Angus and Lester [6] and may assist in the full description of the biosynthesis of the complex glycosylated phosphosphingolipids.

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