ENTRANCE OF GLYCEROL INTO PLASMALOGENS OF SOME STRICTLY ANAEROBIC BACTERIA AND PROTOZOA

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1. Introduction

The pathways involved in the biosynthesis of ether lipids in mammalian tissues have been largely elucidated [1–3]. Of paramount importance for our current understanding of these pathways was the observation by Wykle and Snyder [4] and by Hajra [5] that dihydroxyacetone phosphate, or rather acyldihydroxyacetone phosphate, is the obligate precursor of the glycerol portion of alkyl- and alk-1-enyl lipids. 1-Alk-1′-enyl-2-acyl phosphoglycerides (plasmalogens) occur in abundant amounts in many strictly anaerobic bacteria [6–8] and protozoa [9]. Our knowledge of the mechanisms operating in the formation of plasmalogens in these strictly anaerobic micro-organisms is, however, still very scarce [6].

Hill and Lands [10] followed the incorporation of [2-^{3}H]- and [3-^{14}C]glycerol into the total 1,2-diacyl- and 1-alk-1′-enyl-2-acyl phosphoglyceride fractions of *Clostridium butyricum*. The ^{3}H at position 2 of the glycerol was not labilized upon incorporation of glycerol into plasmalogens. Actually, the ^{3}H/^{14}C ratio of the plasmalogens was found to be higher than that of the glycerol supplied to the medium. From this observation Hill and Lands concluded that in *C. butyricum* glycerol does not enter into plasmalogens via dihydroxyacetone phosphate as had been observed for mammalian tissues [4,5]. However, the approach of Hill and Lands [10] was criticized by Manning and Brindley [11]. These authors provided evidence that, at least for the liver, a true estimate of the relative importance of the incorporation of glycerol via dihydroxyacetone phosphate can only be obtained by comparing the isotopic ratios of phosphoglycerides with that of the intracellular *sn*-glycerol-3-phosphate and not with that of the glycerol substrate as done by Hill and Lands [10]. In the present paper we report the results obtained from studies in which *Megasphaera elsdenii* or *Veillonella parvula*, both strictly anaerobic bacteria, and *Isotricha prostoma* or *Dasytricha ruminantium*, both strictly anaerobic protozoa, were grown or incubated (protozoa) in the presence of a combination of [1,3-^{3}H]- or [2-^{3}H] glycerol and either [2-^{14}C]glycerol or $^{32}P_i$. Comparison of the ^{3}H/^{14}C or ^{3}H/^{32}P ratios of the various plasmalogens and diacyl phosphoglycerides with the isotopic ratios of *sn*-glycerol-3-phosphate permitted the conclusion that in these strictly anaerobic bacteria and protozoa glycerol enters into diacyl phosphoglycerides and into their plasmalogen analogues via a common pool which is not dihydroxyacetone phosphate but rather *sn*-glycerol-3-phosphate.

2. Material and methods

2.1. *Micro-organisms and culture conditions*

M. elsdenii B 159 (ATCC 17752) was generously provided by Dr M. P. Bryant (University of Illinois, Urbana, USA) and *V. parvula* (ATCC 10790) was obtained from the American Type Culture Collection (Rockville, Maryland, USA). These strictly anaerobic bacteria were grown in media of which the composition has been described in details previously [8,12]. Suspensions of *I. prostoma* and *D. ruminantium* were prepared exactly as described by Prins and Prast [13]. Microscopical examination showed that

the preparations were free of other ciliates. The protoza were incubated in an anaerobic rumen fluid-medium described by Clarke and Hungate [14]. Aliquots (8.8 ml) of the medium were dispensed under anerobic conditions into roll-tubes (150 × 16 mm) and autoclaved. Subsequently, the following substances were added by injection through the rubber stoppers: 0.05 ml of a 5% (w/v) solution of ampicillin (Penbritin®, Heppignies, Belgium), 0.15 ml of a solution of cysteine · HCl (3%, w/v) and finally 1.0 ml of the inoculum suspension. The tubes were placed on a reciprocating shaker (5-cm stroke, 150 strokes per min) at 39°C.

2.2. *Experiments with labelled substrates*

Ten-ml cultures of *M. elsdenii* or *V. parvula* were grown until an absorbance of 0.15 was reached. At this point the following combinations of radioactive substrates were added: 100 μCi; [1,3-^{3}H]glycerol plus 50 μCi; $^{32}P_i$; 100 μCi; [2-^{3}H]glycerol plus 50 μCi; $^{32}P_i$; 100 μCi [1,3-^{3}H]glycerol plus 20 μCi; [2-^{14}C] glycerol and 100 μCi; [2-^{3}H]glycerol plus 20 μCi; [2-^{14}C]glycerol. In addition, 0.5 μmol non-labelled glycerol were added to each incubation. The labelled glycerols were all obtained from the Radiochemical Centre in Amersham (Great Britain) and the $^{32}P_i$ from Philips Duphar, Petten (The Netherlands). After the appropriate time of incubation in the presence of the labelled substrates the cells were collected by filtration through Gelman Metrical Alpha-6 filters (pore size 0.45 μm) and the lipids extracted exactly as described by Van Golde et al. [15], except that no HCl was included in the chloroform–methanol mixtures. In some experiments cultures of *M. elsdenii* were pulsed for 2 min with mixtures of [2-^{14}C]glycerol and either [1,3-^{3}H]- or [2-^{3}H]glycerol after which not only the lipids but also the intracellular *sn*-glycerol-3-phosphate were extracted from the cells [15]. Similar experiments with [1,3-^{3}H]- or [2-^{3}H]glycerol and [2-^{14}C]glycerol were carried out with the anaerobic protozoa *I. prostoma* and *D. ruminantium.*

2.3. *Analytical procedures*

Separation of the diacylphosphoglycerides and their plasmalogen analogues was carried out via two-dimensional silica thin-layer chromatography. Before development in the second direction the alk-1-enyl moieties of the plasmalogens were released by treatment with a 12% (v/v) solution of conc. HCl in methanol as described by Singh et al. [16]. The details of this procedure have been published previously [12]. The various lipid components were detected by staining with iodine and radiochromatogram scanning and subsequently assayed for radioactivity by means of liquid scintillation counting using a Packard Tricarb model 2425 B. The various lipids were also subjected to mild alkaline hydrolysis following the method of Dawson [17] as modified by Chang and Kennedy [18]. *sn*-Glycerol-3-phosphate was isolated from the aqueous extracts of the micro-organisms by ion-exchange chromatography on Dowex-formate using a linear gradient of 0–4 M formic acid as eluent [19], followed by paper-chromatography using 1 M ammonium acetate–ethanol (1:2, v/v) as developing solvent [12].

3. Results

The phosphoglyceride composition of *M. elsdenii* and, particularly, the uncommon accumulation of diacyl phosphatidylserine and its plasmalogen analogue, have been documented extensively in a previous paper [12]. Table 1 shows the results obtained after the growth of *M. elsdenii* from O.D. 0.15 until late logarithmic phase in the presence of $^{32}P_i$ and either [1,3-^{3}H] or [2-^{3}H]glycerol. Since under these conditions the [^{3}H]glycerols may also incorporate into the apolar side-chains of the phosphoglycerides, the $^3H/^{32}P$ ratios in table 1 were determined specifically for the glycerol portion of the phosphoglycerides. Interesting is the comparison of the $^3H/^{32}P$ ratios after growth in the presence of [1,3-^{3}H]glycerol (first column of table 1) with those found in the presence of [2-^{3}H]glycerol (second column). If glycerol would incorporate into the plasmalogens via acyldihydroxyacetone phosphate, as has been shown for mammalian tissues [4,5], one would expect the $^3H/^{32}P$ ratio to be 0 in case of [2-^{3}H]glycerol as substrate. Instead however, even higher $^3H/^{32}P$ ratios are found if [2-^{3}H]glycerol is used. This enrichment of [2-^{3}H]- over [1-^{3}H]glycerol in the phosphoglycerides (column 3) is probably due to an isotope effect possibly at the level of glycerol-phosphate dehydrogenase as found by Manning and

Table 1
Enrichment of [2-^{3}H]- over [1,3-^{3}H]glycerol in the glycerol portion of the phosphoglycerides of *M. elsdenii* after growth of the organism in the presence of either [1,3-^{3}H]- or [2-^{3}H]glycerol and $^{32}P_i$ until late logarithmic phase

	$^3H/^{32}P$ [1,3-^{3}H]glycerol plus $^{32}P_i$	[2-^{3}H]glycerol plus $^{32}P_i$	Enrichment of [2-^{3}H] over [1,3-^{3}H]
Diacylphosphatidylserine	0.8	2.3	2.9
Diacylphosphatidylethanolamine	1.3	3.8	2.9
Serine plasmalogen	1.1	3.5	3.2
Ethanolamine plasmalogen	1.7	5.3	3.1

Results are expressed as isotopic ratios.

Brindley [11] in rat liver. Important, however, is the observation that the enrichment of 2-^{3}H over 1,3-^{3}H is equal for the diacyl phosphoglycerides and their plasmalogen analogues. This strongly suggests that in this organism glycerol enters into diacylphosphoglycerides and plasmalogens via a common precursor.

Similar results were obtained when *M. elsdenii* was incubated with [2-^{14}C]glycerol and either [1,3-^{3}H]- or [2-^{3}H]glycerol (table 2). In the short incubation period employed in this experiment the incorporation of the labelled glycerols was confined to the glycerol backbone of the phosphoglycerides as was checked by mild alkaline and acid hydrolysis of the phosphoglycerides. As could be expected, [1,3-^{3}H]- and [2-^{14}C]glycerol incorporated in about equal amounts into the phosphoglycerides of *M. elsdenii*. However, [2-^{3}H]glycerol was again enriched by a factor of 3 over [1,3-^{3}H]glycerol and again this enrichment factor was found to be similar for the diacylphosphoglycerides and their plasmalogen derivatives, strongly suggesting a common precursor for these two classes of phosphoglycerides. Similar data were obtained for another strictly anaerobic bacterium, *V. parvula* (data not presented). It is possible that the enrichment of [2-^{3}H]- over [1,3-^{3}H]glycerol in the phosphoglycerides is preceded by a much higher enrichment in the intracellular *sn*-glycerol-3-phosphate as has been observed in rat liver by Manning and Brindley [11]. In that case dihydroxyacetone phosphate could still be an important precursor for the bacterial plasmalogens. Therefore, an experiment was conducted in which *M. elsdenii* was incubated with [2-^{14}C]glycerol and either [1,3-^{3}H]- or [2-^{3}H]glycerol for only 2 min

Table 2
Enrichment of [2-^{3}H]- over [1,3-^{3}H]glycerol in the phosphoglycerides of *M. elsdenii* after incubation with either [2-^{3}H]- or [1,3-^{3}H]glycerol and [2-^{14}C]glycerol for 30 min

	$^3H/^{14}C$ [1,3-^{3}H]glycerol plus [2-^{14}C]glycerol	[2-^{3}H]glycerol plus [2-^{14}C]glycerol	Enrichment of [2-^{3}H] over [1,3-^{3}H]
Diacylphosphatidylserine	1.0	2.9	2.9
Diacylphosphatidylethanolamine	0.9	3.2	3.6
Serine plasmalogen	1.0	3.1	3.1
Ethanolamine plasmalogen	0.9	3.1	3.4

(Corrected for the $^3H/^{14}C$ ratio in the original glycerol mixture.)
Results are expressed as isotopic ratios.

Table 3
Enrichment of [2-^{3}H]- over [1,3-^{3}H]glycerol in *sn*-glycerol-3-phosphate and phosphoglycerides of *M. elsdenii* after a 2 min. pulse with either [2-^{3}H]- or [1,3-^{3}H]glycerol and [2-^{14}C]glycerol

	^{3}H/^{14}C [1,3-^{3}H]glycerol plus [2-^{14}C]glycerol	[2-^{3}H]glycerol plus [2-^{14}C]glycerol	Enrichment of [2-^{3}H] over [1,3-^{3}H]
Diacylphosphatidylserine	1.1	3.3	3.0
Serine plasmalogen	0.8	2.6	3.3
sn-Glycerol-3-phosphate	1.0	3.4	3.4

(Corrected for the ^{3}H/^{14}C ratio in the original glycerol mixture.)
Results are expressed as isotopic ratios.

in order to isolate a significantly labelled *sn*-glycerol-3-phosphate. The results shown in table 3 clearly demonstrate that the enrichment of [2-^{3}H]glycerol in diacylphosphatidylserine and its plasmalogen analogue is very similar to that in *sn*-glycerol-3-phosphate. This observation provides unambiguous evidence that glycerol enters into diacyl phosphatidylserine and serine plasmalogen via a common precursor which is *sn*-glycerol-3-phosphate. In this short incubation period phosphatidylethanolamine did not become sufficiently labelled to determine an accurate isotopic ratio.

Table 4 presents the results of similar studies with the strictly anaerobic protozoon *I. prostoma*. The contribution of bacteria present in the protozoal suspensions (less than 1 per 1000 protozoa) to the processes studied here can be neglected as discussed previously [13]. Also in *I. prostoma* [2-^{3}H]glycerol was enriched over [1,3-^{3}H]glycerol in phosphatidylcholine, phosphatidylethanolamine and ethanolamine plasmalogen, major phosphoglycerides in this organism [20]. Again a similar enrichment factor was found in the intracellular *sn*-glycerol-3-phosphate. Similar results (not presented) were obtained for another anaerobic protozoon *D. ruminantium*. These data provide strong evidence that also in these anaerobic protozoa glycerol enters into diacyl phosphoglycerides and plasmalogens via a common intermediate which is *sn*-glycerol-3-phosphate rather than dihydroxyacetone phosphate.

4. Discussion

Incubation of *M. elsdenii* in the presence of $^{32}P_i$ or [2-^{14}C]glycerol and either [1,3-^{3}H]- or [2-^{3}H]glycerol (tables 1 and 2) shows that [2-^{3}H]glycerol is enriched over [1,3-^{3}H]glycerol in all individual phos-

Table 4
Enrichment of [2-^{3}H]- over [1,3-^{3}H]glycerol in *sn*-glycerol-3-phosphate and some of the major phosphoglycerides of *I. prostoma* after a 10 min pulse with either [2-^{3}H]- or [1,3-^{3}H]glycerol and [2-^{14}C]glycerol

	[1,3-^{3}H]glycerol plus [2-^{14}C]glycerol	[2-^{3}H]glycerol plus [2-^{14}C]glycerol	Enrichment of [2-^{3}H] over [1,3-^{3}H]
Diacylphosphatidylcholine	1.3	4.2	3.2
Diacylphosphatidylethanolamine	1.1	4.4	4.0
Ethanolamine plasmalogen	0.7	2.4	3.4
sn-Glycerol-3-phosphate	1.1	3.6	3.3

(Corrected for the ^{3}H/^{14}C ratio in the original glycerol mixture.)
Results are expressed as isotopic ratios.

phoglycerides of this organism. An enrichment of [2-^{3}H]- over [3-^{14}C]glycerol in the total diacylphosphoglyceride and plasmalogen fraction of *C. butyricum* had been reported by Hill and Lands [10]. However, the conclusion of these authors that this enrichment ruled out the possibility of glycerol entry into plasmalogens via dihydroxyacetone phosphate was at best preliminary, since it could be that the enrichment in the phosphoglycerides was preceded by a much higher enrichment in *sn*-glycerol-3-phosphate as was actually shown to be the case in rat liver [11]. The results presented here in tables 1 and 2 do show that there is a common precursor for diacylphosphoglycerides and their plasmalogen analogs because of the similar enrichment of [2-^{3}H]glycerol over [1,3-^{3}H]glycerol in both classes of phosphoglycerides. The fact that this enrichment is also similar in the intracellular *sn*-glycerol-3-phosphate provides conclusive evidence that in these strictly anaerobic bacteria glycerol enters into all phosphoglycerides, including the plasmalogens, via *sn*-glycerol-3-phosphate rather than dihydroxyacetone phosphate as occurs in mammalian tissues [4,5].

Very interesting is the finding that anaerobic protozoa apparently utilize the same pathway for incorporation of glycerol into plasmalogens as anaerobic bacteria. This suggests that rather than having different mechanisms for the synthesis of plasmalogens in eukaryotic and prokaryotic organisms, the differences are really between aerobic and anaerobic organisms. More work remains to be done to clarify the mechanisms involved in the conversion of *sn*-glycerol-3-phosphate into plasmalogens in these strictly anaerobic organisms.

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