PLASMALOGEN BIOSYNTHESIS IN A CELL-FREE SYSTEM. ENZYMIC DESATURATION OF 1-*O*-ALKYL (2-ACYL) GLYCEROPHOSPHORYL ETHANOLAMINE

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1. Introduction

It has been demonstrated by numerous experiments *in vivo* that *O*-alkyl glycerol lipids are precursors of *O*-alk-1′-enyl glycerol lipids [1–13]. In our earlier studies on the biosynthesis of plasmalogens we obtained evidence that the intact alkyl acyl glycerophosphoryl ethanolamine is converted to alk-1′-enyl acyl glycerophosphoryl ethanolamine (plasmalogen), probably by a desaturase reaction [12, 13]. Only recently did Wykle et al. [14] succeed in developing an *in vitro* system capable of synthesizing plasmalogens from long chain alcohols and dihydroxy acetone phosphate. The system required the cofactors CoA, ATP, Mg^{2+} and NADP for optimal activity.

The experiments described in this report were aimed at studying the final step in the reaction sequence during plasmalogen biosynthesis, i.e. the enzymic desaturation at carbons 1 and 2 of the alkyl moiety of alkyl (acyl) glycerophosphoryl ethanolamine (alkyl acyl GPE). The microsomal fraction from hamster small intestine was found to contain an enzyme system which converts alkyl acyl GPE or alkyl GPE to 1-*O*-alk-1′-enyl acyl GPE. The reaction requires molecular oxygen and NADPH or NADH and is inhibited by cyanide. Unlike the oxidative cleavage of free alkyl glycerol ethers, which is stimulated by 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine, desaturation of alkyl (acyl) GPE is inhibited by this coenzyme.

2. Materials and methods

1-*O*-(9,10-$^3H_2$-octadecyl) 2-octadecenoyl *sn*-glycerol-3-phosphoryl ethanolamine (specific activity 10^7 counts/min/μmole) and the 2-lyso-derivative were synthesized as described earlier [13].

The synthesis of 1-*O*-(9,10-$^3H_2$-octadecyl) *sn*-glycerol of the same specific activity has been described [4]. 1-^{14}C-stearic acid and 1-^{14}C-stearyl alcohol were purchased from Amersham, England. NADH, NADP, CoA, ATP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Boehringer, Mannheim, 2-amino-4-hydroxy-6,7-dimethyl pteridine came from Schuchardt, München. It was reduced to the tetrahydro pteridine with H_2/Pt in aqueous HCl [15]. Adult gold hamsters of either sex were used. Microsomes were prepared from intestinal mucosa scrapings as described by Rao and Johnston [16]. Substrates (330 nmoles/ml) were dispersed in water containing 0.1% Tween 80 by ultrasonication. Incubation conditions are described in the table. The incubations were terminated by the addition of 12 ml chloroform–methanol (1:2, v/v) and lipids were extracted by the method of Bligh and Dyer [17]. Phosphatidyl ethanolamine prepared from rat brain was added as a carrier. The incorporation of radioactivity into aldehydogenic groups of plasmalogens was determined by two dimensional reaction TLC [18]. Chloroform–methanol–water (65:25:4, v/v/v) was employed as the developing solvent for the first chromatography. The plates were then thoroughly dried in a stream of nitrogen and exposed to HCl vapors for 10 min; excess HCl was removed in a stream of nitrogen and the plate was developed in the second direction using petroleum ether–ether (95:5, v/v) as the developing solvent. Spots corresponding to free aldehydes released from ethanolamine phospholipids were visualized by exposure to iodine vapor and scraped into counting vials for determination of radioactivity. Alternatively, total

lipids were subjected to column chromatography in order to remove neutral lipids including free aldehydes. Neutral lipids were eluted with chloroform, phospholipids with chloroform–methanol 3:2. BF_3-catalyzed methanolysis of the phospholipids yielded fatty acid methyl esters and dimethyl acetals which were separated by TLC [19]. The radioactivity associated with dimethyl acetals was approximately 15% higher than that associated with aldehydes recovered after two dimensional reaction TLC. Aldehydes released from ethanolamine plasmalogens by HCl treatment were identified by TLC analysis. In addition, they were reduced with $LiAlH_4$ and the resulting alcohols were acetylated; after purification by TLC, aldehydes, alcohols and alkyl acetates had identical specific radioactivities. The identity of alk-1-enyl acyl GPE was further proven by the fact that no labeled aldehydes were released from phosphatidyl ethanolamine when the lipid extract was hydrogenated over Pt catalyst prior to the two dimensional reaction TLC.

3. Results and discussion

When 1-*O*-(9,10-$^3H_2$-octadecyl) 2-octadecenoyl glycerol-3-phosphoryl ethanolamine was incubated with microsomes from hamster small intestine a low but reproducible incorporation of radioactivity into aldehydogenic groups of plasmalogens of the phosphatidyl ethanolamine fraction was observed (see table). Identification of labeled plasmalogens was based on the analysis of various derivatives as described in the Methods section. Even higher plasmalogen biosynthesis occurred when alkyl GPE was employed as the substrate. Utilization of the latter was only slightly dependent on the addition of ATP and CoA, indicating that an energy-independent acylation of the lyso alkoxy phosphatidyl ethanolamine occurred during incubation. Addition of free octadecyl glycerol ether or octadecanol had no influence on plasmalogen formation, indicating that neither compound may be regarded as an intermediate in this reaction. This assumption is confirmed by the negligible incorporation of radioactivity into plasmalogens from labeled octadecanol, stearic acid or octadecyl glycerol ether (see table). These data strongly confirm our earlier findings that alkyl acyl GPE or alkyl GPE is the immediate plasmalogen precursor [12, 13]. This hypothesis, based on *in vivo* studies, has also been proposed by other authors [6, 11, 20].

Studying plasmalogen biosynthesis *in vitro*, Wykle et al. [14] obtained optimal incorporation of radioactivity from 1-^{14}C-hexadecanol when NADP was added as a cofactor in addition to the cofactors (CoA and ATP) required for the formation of the alkyl glycerol bond. This result is in contradiction to our finding that desaturation of the alkyl glycerol bond depends on the presence of reduced nucleotides (NADPH or NADH). We also found that the reaction requires molecular oxygen, as does the oxidative cleavage of alkyl glycerol ethers [21]. However, whereas the latter reaction is stimulated by 2-amino-4-hydroxy-6,7-dimethyl tetrahydro pteridine, plasmalogen formation is rather inhibited.

The observed differences in the utilization of alkyl acyl GPE and alkyl GPE in plasmalogen biosynthesis (see table) may be explained by the assumption that alkyl GPE is the immediate plasmalogen precursor. However, one could also speculate that the lyso alkyl GPE is more readily bound to the microsomes and acylated prior to the desaturation. The evidently higher production of fatty acids from alkyl GPE than from alkyl acyl GPE may be due to the fact that substantial amounts of the alkyl GPE undergo hydrolytic cleavage of the glycerol-P bond, giving rise to the formation of the readily oxidizable free alkyl glycerol ether. Alkyl acyl GPE is more resistant against the phosphohydrolase (unpublished results) and is obviously not attacked by the glycerol ether oxidizing enzyme. These results, together with the hypothesis of Tietz et al. [21] that plasmalogens are not intermediates in the oxidative cleavage of alkyl glycerol ethers, would suggest that different enzymes are involved in the oxidative degradation of free alkyl glycerol ethers and the desaturation of alkyl (acyl) glycerol phospholipids.

It is interesting to note that both desaturation and oxidative cleavage of the alkyl glycerol bond are inhibited by cyanide. Oshino et al. [22] have shown that fatty acid desaturation is sensitive to cyanide. *O*-alkyl (acyl) glycerophospholipid desaturase and fatty acid desaturase therefore have much in common: both enzyme systems produce cis-double bonds [23], both have the same requirements of oxygen and reduced pyridine nucleotides (NADPH or NADH) and both are inhibited by cyanide. More data will be

Table
Biosynthesis of plasmalogens in microsomes of the hamster small intestinal mucosa.

	Substrate	Incubation system	Product formed (pmoles)	
			Plasmalogens[d]	Fatty acids[e]
1	^{3}H-alkyl acyl GPE[a]	complete[c]	157	1000
2	^{3}H-alkyl GPE[b]	complete	228	3000
3	^{3}H-alkyl GPE[b]	minus CoA, ATP	157	
4	^{3}H-alkyl GPE[b]	minus G-6-P, GDPH	22	
5	^{3}H-alkyl GPE[b]	as 4, plus NADH	225	
6	^{3}H-alkyl GPE[b]	minus O_2, under He	15	240
7	^{3}H-alkyl GPE[b]	plus 10^{-3} M KCN	41	300
8	^{3}H-alkyl GPE[b]	complete + 10^{-3} M 2-amino-4-hydroxy-6,7-dimethyl-tetra-hydropteridine	100	9000
9	^{3}H-alkyl GPE[b]	plus 100 nmoles 1-octadecyl *sn*-glycerol	230	
10	^{3}H-alkyl GPE[b]	plus 100 nmoles octadecanol	225	
11	^{3}H-alkyl GPE[b]	complete, except microsomes were boiled for 10 min	0	
12	1-^{14}C-stearic acid	as 5	0	
13	1-^{14}C-octadecanol	as 5	0	
14	1-*O*-(9,10-$^3H_2$)-octadecyl *sn*-glycerol	complete	60	6000

[a] 1-*O*-(9,10-$^3H_2$-octadecyl)2-octadecenoyl *sn*-glycero-3-phosphoryl ethanolamine.
[b] 1-*O*-(9,10-$^3H_2$-octadecyl) *sn*-glycero-3-phosphoryl ethanolamine.
[c] The complete system contained in a final volume of 3 ml: 100 nmoles of the respective substrate (10^6 counts/min), ATP (10 mM), CoA (0.3 mM), GSH (2 mM), NADP (1.2 mM), glucose-6-phosphate (G-6-P, 7 mM), glucose-6-phosphate dehydrogenase (GPDH, 25 μg), Mg^{2+} (4 mM), 0.1 M phosphate buffer, pH 7.1, NaF (12 mM), microsomes (15 mg protein). Flasks were incubated for 3 hr at 37°.
[d] Calculated from ^{3}H associated with aldehydes released from phosphatidyl ethanolamine by HCl treatment.
[e] Calculated from ^{3}H radioactivity of fatty acid methyl esters formed by BF_3-catalyzed methanolysis of total lipids.

necessary to decide whether alkyl (acyl) glycerophospholipid desaturase involves cytochrome b_5 as does fatty acid desaturase.

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