

Chapter 2

Biosynthesis of Plasmalogens in Brain

2.1 General Considerations and Distribution of Plasmalogens in Brain

Plasmalogens account for the major portion of the ethanolamine glycerophospholipids in the adult human brain (50%), but the brain of newborn babies has low levels (7% of total phospholipids mass) (Horrocks and Sharma, 1982). Levels of ethanolamine plasmalogen (PlsEtn) increase rapidly during the intense period of myelination and ethanolamine glycerophospholipids of myelin sheath contain up to 70% PlsEtn. An eight-fold increase in PlsEtn levels per gram of brain tissue occurs in white matter during first year of life so that PlsEtn accounts for 20% of the glycerophospholipid mass and 70% of the ethanolamine glycerophospholipids (Balakrishnan et al., 1961). At that time, myelination is rapid. The highest level of myelin is between 30 and 40 years of age (Toews and Horrocks, 1976). In human brain, there is a steep rise in PlsEtn content, followed by a further rise up to 30–40 years of age. This is followed by a decline of PlsEtn levels during normal aging. At 70 years of age, the levels of PlsEtn are 18% less than at 40 years of age (Rouser and Yamamoto, 1968; Horrocks et al., 1981). In chicks, there is a marked increase in plasmalogen levels in synaptosomes during the first 3 days after hatching (Getz et al., 1968). Collectively, these studies suggest that plasmalogens are major glycerophospholipids in brain tissue. Their metabolism may be involved in signal transduction processes associated with neural cell functions such as synaptogenesis, myelination, and ion transport (Farooqui and Horrocks, 2001).

Plasmalogens impart membranes with different biophysical properties such as phase transition temperature, bilayer thickness, acyl chain packing free volume, and lateral domain. The perpendicular orientation of the *sn*-2 acyl chain at the membrane surface and the lack of a carbonyl group at the *sn*-1 position in plasmalogens affect the hydrophilicity of the head group, resulting in stronger intermolecular hydrogen bonding between the head groups (Lohner, 1996). These properties allow PlsEtns to adopt the inverse hexagonal phase and may be responsible for a different membrane potential compared with other glycerophospholipids (Lohner, 1996). This property affects lipid packing, fluidity, and interaction with neural membrane receptors and ion channels. In cellular membranes and lipoproteins,

plasmalogens account for 15–20% and 5% of all phospholipids, respectively (Nagan and Zoeller, 2001; Engelmann et al., 1994). PlsEtn and PlsCho are the two major plasmalogen species found in mammalian cell membranes. In most cells, PlsEtns exceed the choline plasmalogens by 10-fold, with the exception of cardiac and skeletal muscle where choline plasmalogen dominates. The level of plasmalogens in brain tissue depends on the degree of myelination and increases rapidly during myelinogenesis (Horrocks, 1972; Horrocks and Sharma, 1982). Factors that modulate the levels of plasmalogens in neurons, astrocytes, and oligodendrocytes during myelination and aging remain unknown.

2.2 Biosynthesis of Plasmalogens

The enzymes for plasmalogen biosynthesis have not been purified and characterized from brain tissue. The reasons for this lack of information on the purification and characterization of plasmalogen biosynthesizing enzymes from brain are not known. However, the low activity of plasmalogen synthesizing enzymes, complex, laborious, and time consuming assays for determining activities, and the heterogeneity and complex organization of brain tissue may be responsible for the lack of information. Several investigators have reviewed the biosynthesis of plasmalogens in nonneural tissues (Fig. 2.1) (Horrocks and Sharma, 1982; Lee, 1998; Nagan and Zoeller, 2001; Murphy, 2001; Brites et al., 2004). The starting metabolite for plasmalogen biosynthesis is dihydroxyacetone phosphate from glycolysis, which is used to form the glycerol backbone of the plasmalogen. The biosynthesis of plasmalogens is initiated in peroxisomes and completed in the endoplasmic reticulum. Thus, the first three enzymes of plasmalogen biosynthesis, dihydroxyacetone phosphate acyltransferase, alkyl dihydroxyacetone phosphate synthase, and acyl/alkyl dihydroxyacetone reductase, are located in peroxisomes. The endoplasmic reticulum contains the other enzymes, namely 1-alkyl-*sn*-GroP acyltransferase, 1-alkyl-2-acyl-*sn*-GroP phosphohydrolase, and 1-alkyl-2-acyl-*sn*-Gro: CDP-choline (CDP-ethanolamine) choline (ethanolamine) phosphotransferase.

The rate-limiting step for plasmalogen biosynthesis has not been identified. However, it is proposed that regulation point lies downstream from first three steps (Nagan and Zoeller, 2001). This suggestion is based on the incorporation of 1-*O*-[9'-(1''-pyrenyl)]nonyl-*sn*-glycerol (pAG), a fluorescent ether lipid with a pyrene moiety covalently attached at the alkyl chain terminus (Zheng et al., 2006) (Fig. 2.2). CHO-K1 and NRel-4 cells take up this ω -pyrene-labeled 1-*O*-alkyl-*sn*-glycerol. NRel-4 cells are a variant defective in dihydroxyacetone phosphate acyltransferase. Treatment of CHO-K1 and NRel-4 cells results in the incorporation of pAG into ethanolamine and choline phospholipids as well as into a neutral lipid fraction tentatively identified as alkyldiacylglycerols. NRel-4 cells incorporate more fluorescence in the phospholipid fraction than CHO-K1, specifically in the ethanolamine phospholipids. Analysis of the fluorescent lipids demonstrates that 93% of the pAG is taken up by glycerolipids with the intact ether bond. Although

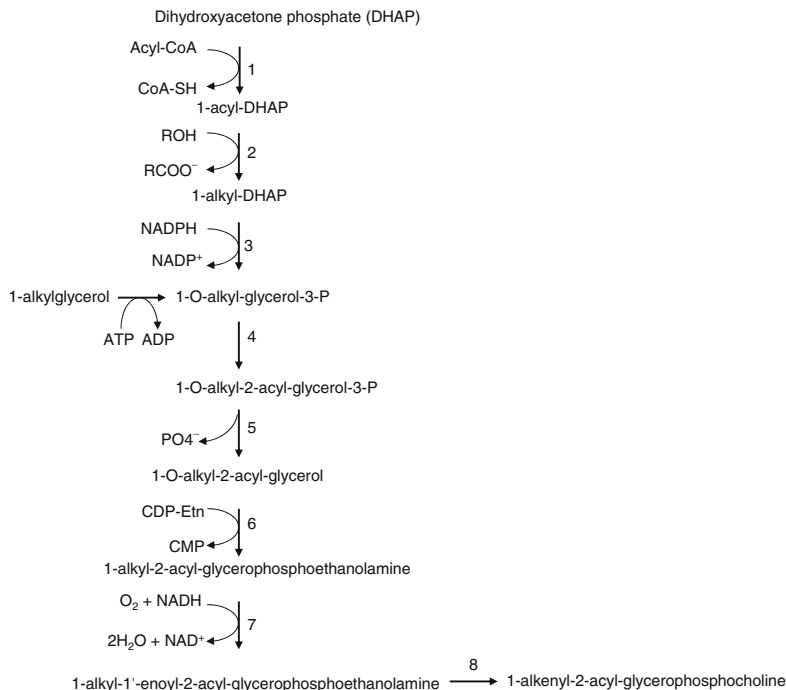


Fig. 2.1 Biosynthesis of plasmalogens in mammalian tissues. (1) Dihydroxyacetone phosphate acyltransferase, (2) 1-acyl dihydroxyacetone phosphate synthase, (3) 1-acyl/alkyl dihydroxyacetone phosphate reductase, (4) 1-alkyl-*sn*-glycerophosphate acyltransferase, (5) 1-alkyl-2-acylglycerophosphate phosphohydrolase, (6) CDP-ethanolamine transferase, (7) 1-alkyl-2-acyl-*sn*-glycerophosphoethanolamine desaturase, and methyltransferases and base-exchange enzymes.

the addition of 20 μ M 1-*O*-hexadecyl-*sn*-glycerol (HG) (Fig. 2.2) to the medium fully restores PlsEtn biosynthesis in NRel-4 cells, pAG only partially restores PlsEtn synthesis (Zheng et al., 2006). Both pAG and HG inhibit CHO-K1 and NRel-4 cell growth. The molecular mechanism associated with this inhibitory process remains unknown (Zheng et al., 2006). Incubation of cells with pAG followed by irradiation with long-wavelength (>300 nm) ultraviolet light produces cytotoxicity due to the generation of reactive oxygen species such as singlet oxygen. NRel-4 cells exhibit an increase in sensitivity to UV light compared with CHO-K1 cells. This photodynamic cytotoxicity approach can be used to select for mutants that are defective in downstream steps in ether lipid biosynthesis (Zheng et al., 2006).

Dihydroxyacetone phosphate acyltransferase may be a crucial enzyme for plasmalogen biosynthesis, but it is not a rate-limiting step for plasmalogen synthesis (Nagan and Zoeller, 2001). Sphinganine is an effective donor of the 1-alkenyl chain of plasmalogens (Stoffel et al., 1970). Most fatty alcohols from sphinganine predominantly incorporate into choline plasmalogens and only a little into PlsEtns. The reason

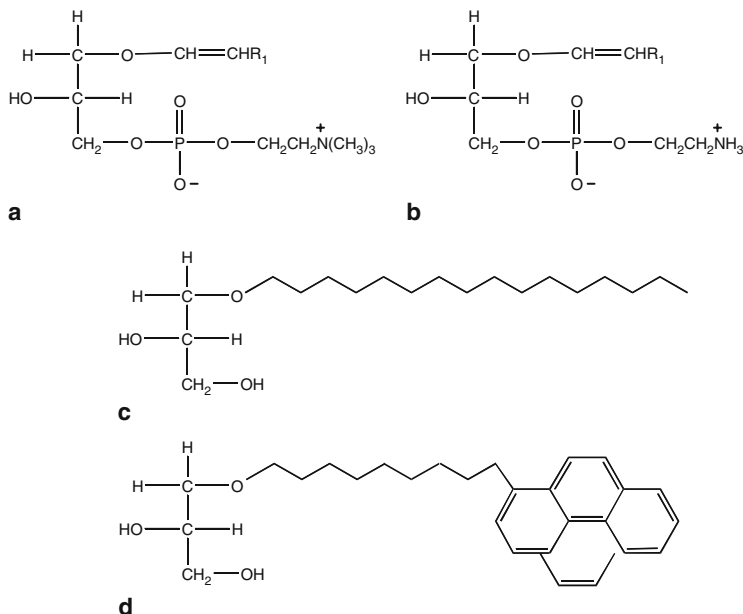


Fig. 2.2 Chemical structures of lyso-choline plasmalogen (a), lyso-ethanolamine plasmalogen (b), 1-*O*-hexadecyl-*sn*-glycerol (c), and 1-*O*-[9'-(1''-pyrenyl)]nonyl-*sn*-glycerol (pAG) (d).

for the preferential incorporation of fatty alcohols from sphinganine into choline plasmalogens remains unknown. Detailed investigations are required on this topic.

2.2.1 Dihydroxyacetone Phosphate Acyltransferase

This enzyme catalyzes the esterification of the free hydroxyl group of dihydroxyacetone phosphate by utilizing long chain (>C10) acyl CoA to form acyl dihydroxyacetone phosphate. This enzyme is associated with peroxisomal membranes. It was purified from rat and guinea pig livers and human placenta with multiple column chromatographic procedures (Jones and Hajra, 1983; Webber and Hajra, 1993; Ofman and Wanders, 1994; Causeret et al., 1997). The purified enzyme has molecular mass of 69 kDa and migrates as a single band on SDS-polyacrylamide gel electrophoresis, but gel filtration studies indicate a molecular mass of 90 kDa (Webber and Hajra, 1993). The purified enzyme has a K_m value of 70 μ M and a V_{max} value of 4 μ mol acyl dihydroxyacetone phosphate formed per minute per milligram of protein (Table 2.1) Free coenzyme A inhibits the acyltransferase reaction with an inhibition constant (K_i) of approximately 0.76 mM. Dihydroxyacetone phosphate acyltransferase is resistant to inhibition

Table 2.1 Kinetic properties of enzymes associated with plasmalogen biosynthesis.

Enzyme	pH optimum	K_m value (μM)	V_{\max} (nmol/min/mg)	Reference
DHAP-AT	7.6	70	4,000	(Webber and Hajra, 1993)
DHAP-S	7.5	68	42.80	(Zomer et al., 1993)
Acyl/alkyl dihydroxyacetone reductase	7.5	21	67,000	(Datta et al., 1990)
1-Alkyl-2-acyl- <i>sn</i> -GroP phosphohydrolase	6.0–6.5	–	813	(Jamal et al., 1991)
CDP-ethanolamine:DAG ethanolaminephosphotransferase	8.0	0.57	252	(Mancini et al., 1999)
Plasmanylethanolamine desaturase	7.1			(Paltauf, 1994; Zheng et al., 2006)
Alkylglycerol phosphotransferase				(Zheng et al., 2006)

Table 2.2 Localization and molecular weights of enzymes associated with plasmalogen biosynthesis.

Enzyme	Localization	Molecular mass (kDa)	Reference
Dihydroxyacetone phosphate acyltransferase	Peroxisome	77	(Ofman et al., 1998)
Alkyl dihydroxyacetone phosphate synthase	Peroxisome	65	(Zomer et al., 1993)
Acyl/alkyl dihydroxyacetone reductase	Peroxisome	60	(Datta et al., 1990)
Alkylglycerophosphate acyltransferase	Endoplasmic reticulum	–	(Stamps et al., 1997)
Alkylacylglycerophosphate phosphohydrolase	Endoplasmic reticulum	–	–
CDP-ethanolamine:diacylglycerol ethanolaminephosphotransferase	Endoplasmic reticulum	38	(Mancini et al., 1999)
Cyanide-sensitive $\Delta 1$ -alkyl desaturase	–	–	(Paltauf, 1994)

by *N*-ethylmaleimide, a sulfhydryl group blocking agent, and utilizes only dihydroxyacetone phosphate as the acyl group acceptor.

Human placental dihydroxyacetone phosphate acyltransferase was purified using octyl-Sepharose CL-4B, Hydroxyapatite HTP, and CM-Sepharose CL-6B column chromatographic procedures along with PBE 94 chromatofocusing and TSK G3000 SW size exclusion chromatography. The purified enzyme has an isoelectric point of 5.1–5.3. The molecular mass of native enzyme is 60–80 kDa as calculated from HPLC size exclusion chromatography (Table 2.2). SDS-PAGE indicates a molecular mass of 65 kDa.

The cDNA for dihydroxyacetone phosphate acyltransferase was cloned (Thai et al., 1997). The nucleotide-derived amino acid sequence revealed a protein consisting of 680 amino acid residues of molecular mass, 77 kDa containing a C-terminal type 1

peroxisomal targeting signal (PTS). Monospecific antibodies prepared against this polypeptide efficiently immunoprecipitate dihydroxyacetone phosphate acyltransferase activity from solubilized peroxisomal preparations, thus confirming that the cloned cDNA codes for dihydroxyacetone phosphate acyltransferase (Thai et al., 1997). Using the amino acid sequence of human acyl-CoA:dihydroxyacetone phosphate acyltransferase as bait to screen the database of expressed sequence tags (dbEST), several partial mouse cDNA clones showing high identity have been identified (Ofman and Wanders, 1994; Ofman et al., 1999). Primers were selected based on the dbEST sequences and used for amplification of this transcript from cDNA prepared from mouse skin fibroblasts. The complete nucleotide sequence has revealed an open reading frame of 2,034 bp encoding a protein consisting of 678 amino acids with a molecular mass of 77 kDa. The deduced amino acid sequence shows high identity (80%) with human dihydroxyacetone phosphate acyltransferase and also suggests a typical peroxisomal targeting signal type 1 (PTS1) at its extreme carboxy-terminus (alanine–lysine–leucine). Definitive evidence that this cDNA indeed codes for dihydroxyacetone phosphate acyltransferase is obtained by heterologous expression in the yeast *Saccharomyces cerevisiae* (Ofman et al., 1999). Northern blot analysis indicates high expression of dihydroxyacetone phosphate acyltransferase especially in mouse heart, liver, and testis.

Dihydroxyacetone phosphate acyltransferase is absolutely required for the synthesis of plasmalogen but its activity is not a limiting factor for plasmalogen synthesis in CHO cells (Liu et al., 2005). Earlier studies indicate that acylation of dihydroxyacetone phosphate is important for the biosynthesis of nonether glycerolipids (Hajra et al., 2000), but recent studies have clearly shown that dihydroxyacetone phosphate acyltransferase does not contribute to the synthesis of diacyl glycerolipids. Acylated dihydroxyacetone phosphate is also synthesized by a NEM-sensitive microsomal dihydroxyacetone phosphate acyltransferase activity (Schlossman and Bell, 1977). This enzyme does not contribute to plasmalogen synthesis but may be involved in the synthesis of nonether glycerolipids (Liu et al., 2005). These authors isolated a fibroblast-like cell line CHO-K1 that is deficient in plasmalogens due to the loss of various steps of plasmalogen biosynthesis pathways (Nagan et al., 1997; Nagan et al., 1998). The mutant CHO cell line NRel-4 has markedly reduced plasmalogen levels because of decreased dihydroxyacetone phosphate acyltransferase activity. The lower activity of this enzyme is due to reduced levels of the message for dihydroxyacetone phosphate acyltransferase. Expression of the dihydroxyacetone phosphate acyltransferase gene in NRel-4 cells results in the restoration of plasmalogen biosynthesis suggesting that this enzyme is essential for plasmalogen synthesis (Liu et al., 2005).

The gene for dihydroxyacetone phosphate acyltransferase is located on chromosome 1q42.12–43. It spans approximately 28 kb and consists of 16 exons and 15 introns (Ofman et al., 2001). In brain, the mRNA for dihydroxyacetone phosphate acyltransferase is mainly localized in white matter peroxisomes. This expression is weak compared to intense expression in liver peroxisomes (André et al., 2005b). The blood–brain barrier prevents the transport of plasmalogens to the brain. The expression of dihydroxyacetone phosphate acyltransferase may be lower than in

liver, but is sufficient to maintain a plasmalogen pool necessary for structural and metabolic activities in brain tissue (André et al., 2005b).

Dihydroxyacetone phosphate acyltransferase is highly expressed in the inner segment of photoreceptors and in the retinal pigment epithelium (RPE), suggesting two distinct sites for plasmalogen biosynthesis (Acar et al., 2007). PlsEtn is the main class of plasmalogens in both neural retina and RPE (28–29% of the total ethanolamine glycerophospholipids). Compared to other tissues, photoreceptors and the RPE monolayer contain a greater proportion of octadecanal in the *sn*-1 position of plasmenylethanolamine. The RPE monolayer is located in a highly oxygenated environment and is exposed to high levels of visible light. Therefore, it is at risk for oxidative damage (Cai et al., 2000; Acar et al., 2007). In the RPE monolayer, PlsEtns may be involved in protection against oxidative stress (Acar et al., 2007).

2.2.2 Alkyl Dihydroxyacetone Phosphate Synthase

This enzyme replaces the acyl chain in acyl dihydroxyacetone phosphate with a long-chain fatty alcohol to form 1-alkyl-*sn*-glycero-3-phosphate (Paltauf, 1994). The fatty alcohol may be supplied either from dietary intake or by the reduction of long-chain acyl-CoA through the action of an acyl-CoA reductase. Alkyl dihydroxyacetone phosphate synthase is localized in peroxisomes. It was solubilized from an enriched peroxisome fraction with Triton X-100 and potassium chloride. The solubilized enzyme was purified by chromatography on QAE-Sephadex, Matrex Red, phosphocellulose, and Concanavalin A. SDS-polyacrylamide gel electrophoresis of alkyl dihydroxyacetone phosphate synthase indicates a molecular mass of 65 kDa. Chromatofocusing studies indicate an isoelectric point of pH 5.9. The pH optimum of alkyl dihydroxyacetone phosphate synthase is between pH 7 and 8. The purified enzyme has a specific activity of 350 nmol/min/mg protein corresponding to a purification of at least 13,000-fold (Zomer et al., 1993). Recombinant alkyl dihydroxyacetone phosphate synthase from guinea pig liver follows ping-pong rather than a sequential reaction mechanism (de Vet et al., 1999).

N-ethylmaleimide, *p*-bromophenacylbromide, and 2,4-dinitrofluorobenzene irreversibly inhibit the alkyl dihydroxyacetone phosphate synthase activity. Saturating concentrations of palmitoyl dihydroxyacetone phosphate protect the enzyme from inactivation. The rate of inactivation of the enzyme by *p*-bromophenacylbromide depends upon pH and is highest under alkaline conditions. Collectively, these results suggest the involvement of cysteine, histidine, and lysine residues in the reaction catalyzed by alkyl dihydroxyacetone phosphate synthase. The divalent cations Mg²⁺, Zn²⁺, and Mn²⁺ inhibit the enzymic activity, whereas Ca²⁺ has no effect. Mutational analysis indicates that histidine 617 is an essential amino acid for catalytic activity: replacement of this residue by alanine results in complete loss of enzymic activity (de Vet et al., 1999). A recombinant enzyme after the deletion

of five C-terminal amino acids shows no activity, indicating the importance of the C-terminus for catalytic activity.

Although the reaction catalyzed by alkyl dihydroxyacetone phosphate synthase is not a net redox sensitive reaction, the amino acid sequence of the enzyme indicates the presence of a flavin adenine dinucleotide (FAD)-binding domain (de Vet et al., 2000). On the basis of fluorescence properties and UV-visible absorption spectra, alkyl dihydroxyacetone phosphate synthase contains an essential FAD molecule that acts as a cofactor. The FAD participates directly in catalysis. During incubation of the enzyme with the substrate, palmitoyl dihydroxyacetone phosphate, the flavin moiety is reduced, indicating that in this initial step the substrate is oxidized (de Vet et al., 2000). Stopped flow assay studies show that the reduction of the flavin moiety is a monophasic process yielding an oxygen-stable, reduced-enzyme species. Upon addition of hexadecanol to the reduced enzyme species, the flavin moiety is efficiently reoxidized. Thus the collective evidence suggests that FAD participates in the reaction catalyzed by alkyl dihydroxyacetone phosphate synthase (de Vet et al., 2000).

From amino acid sequence information, cDNAs encoding alkyl dihydroxyacetone phosphate synthase have been cloned from both guinea pig and human liver. In both cases, the enzyme is synthesized as a precursor protein with a N-terminal cleavable presequence containing a PTS type 2 (de Vet and van den Bosch, 2000). Human fibroblasts derived from Zellweger syndrome and rhizomelic chondrodysplasia punctata patients contain much lower levels of the enzyme protein (see Chap. 6).

Radiation inactivation experiments were used to determine the in situ functional size of dihydroxyacetone phosphate acyltransferase and alkyl dihydroxyacetone phosphate synthase. Alkyl dihydroxyacetone phosphate synthase displays single exponential decay when enzymic activity and immunoreactive protein levels are measured with target sizes of 79 kDa and 78 kDa, respectively. Dihydroxyacetone phosphate acyltransferase activity is increased at lower doses and decays upon further irradiation with an apparent target size of 62 kDa. These data indicate that the functional unit sizes for both enzymes in situ are represented by single polypeptide chains (Biermann et al., 1998). After cross-linking, alkyl dihydroxyacetone phosphate synthase can be detected in a 210-kDa complex together with dihydroxyacetone phosphate acyltransferase. Both enzymes are located entirely on the luminal side of the peroxisomal membrane (Biermann et al., 1999). Coimmunoprecipitation studies confirm that the two enzymes interact with each other in a heterotrimeric complex. Furthermore, alkyl dihydroxyacetone phosphate synthase also forms a homotrimeric complex in the absence of dihydroxyacetone phosphate acyltransferase as observed by immunoblot analysis after cross-linking experiments with either dihydroxyacetone phosphate acyltransferase deficient human fibroblast homogenates or recombinant (His)6-tagged alkyl dihydroxyacetone phosphate synthase. In summary, alkyl dihydroxyacetone phosphate synthase interacts selectively with dihydroxyacetone phosphate acyltransferase in a heterotrimeric complex and in the absence of dihydroxyacetone phosphate acyltransferase can also form a homotrimeric complex (Biermann et al., 1999).

2.2.3 *Acyl/alkyl Dihydroxyacetone Phosphate Reductase*

In the presence of NADPH, acyl/alkyl dihydroxyacetone phosphate reductase reduces alkyl dihydroxyacetone phosphate at the *sn*-2 position to generate 1-alkyl 2-lyso-*sn*-glycero-3-phosphate, the ether-linked analog of lyso-phosphatidic acid. In guinea pig and rat liver, acyl/alkyl dihydroxyacetone phosphate reductase is localized in peroxisomal and microsomal fractions (Ghosh and Hajra, 1986). From the distribution of marker enzymes, about two-thirds of the acyl/alkyl dihydroxyacetone phosphate reductase activity is present in peroxisomes with the rest in microsomes. The properties of this enzyme in peroxisomes and microsomes are similar with respect to heat inactivation, pH optima, sensitivity to trypsin, and inhibition by NADP⁺ and acyl CoA. The enzymic activity in peroxisomes and microsomes from mouse liver is increased to the same extent by chronically feeding clofibrate, a hypolipidemic drug. The kinetic properties of this enzyme in these two different organelles are also similar. From these results, the same enzyme is present in two different subcellular compartments of liver.

Acyl/alkyl dihydroxyacetone phosphate reductase was purified from pig liver peroxisomes using multiple column chromatographic procedures (Datta et al., 1990). The purified enzyme migrates as a single band on SDS-polyacrylamide gel electrophoresis with an apparent molecular weight of 60kDa. The molecular weight of the native enzyme is estimated to be 75kDa by size exclusion chromatography. The protein is very hydrophobic. It contains 27% hydrophobic amino acids and so it requires strong detergents to solubilize the enzymic activity. The K_m value of the purified enzyme for hexadecyl dihydroxyacetone phosphate is 21 μ M, and the V_{max} value in the presence of 0.07 mM NADPH is 67 μ mol/min/mg protein (Datta et al., 1990). The turnover number (K_{cat}), after correcting for the isotope effect of the cosubstrate NADP³H, was 6,000 mol/min/mol of enzyme, assuming the enzyme has a molecular weight of 60kDa. The purified enzyme also uses palmitoyl dihydroxyacetone phosphate as a substrate with a K_m value of 15.4 μ M and a V_{max} of 75 μ mol/min/mg protein. Palmitoyl dihydroxyacetone phosphate competitively inhibits the reduction of hexadecyl dihydroxyacetone phosphate, indicating that the same enzyme catalyzes the reduction of both acyl dihydroxyacetone phosphate and alkyl dihydroxyacetone phosphate. NADH can substitute for NADPH, but the K_m of the enzyme for NADH (1.7 mM) is much higher than that for NADPH (20 μ M). The purified enzyme is competitively (against NADPH) inhibited by NADP⁺ and palmitoyl-CoA. The enzyme is stable on storage at 4°C in the presence of NADPH and dithiothreitol (Datta et al., 1990).

Enzymes catalyzing subsequent steps of plasmalogen biosynthesis are localized in the endoplasmic reticulum and may not be identical to those involved in diacyl glycerophospholipid synthesis (Lee, 1998). These enzymes include alkyl-GP acyltransferase (1-alkyl-*sn*-GroP acyltransferase), alkylacyl-GP phosphohydrolase I (1-alkyl-2-acyl-*sn*-GroP phosphohydrolase), 1-alkyl-2-acyl-*sn*-Gro:CDP-choline (CDP-ethanolamine) choline (ethanolamine) phosphotransferase, and plasmanylethanolamine desaturase.

2.2.4 *Alkylglycerophosphate Acyltransferase*

This enzyme is also known as lysophosphatidate acyltransferase (LPAAT). It catalyzes the transfer of acyl group from acyl-CoA to alkyl-lysophosphatidate. Triton X-100 and bovine serum albumin stimulate it. Alkyl-GP acyltransferase is stereospecific. Thus only the 1-alkyl-*sn*-glycero-3-phosphate isomer, and not other optical enantiomers, is active with brain alkyl-GP acyltransferase. Substrate specificity studies of the brain acyltransferase activity for different acyl-CoA species (16:0, 18:0, 18:2, 20:4, 22:4, 22:6) indicate selectivity dependent on the alkylglycerophosphate concentration. At low 1-alkyl-*sn*-glycero-3-phosphate concentrations, the enzyme prefers polyunsaturated acyl-CoA species to saturated species. Based on specific activity and kinetic parameters towards a series of acyl-CoA donors, alkyl-GP acyltransferase may be different from acyl-GP (lysophosphatidic acid) acyltransferase (Fleming and Hajra, 1977).

Two human LPAAT have been cloned, LPAAT- α and LPAAT- β (West et al., 1997). Human LPAATs resemble (48% identical) yeast and bacterial enzymes in their amino acid sequences. This enzyme is encoded by a gene located on chromosome 9p34.3 (Aguado and Campbell, 1998). Overexpression of these two cDNAs in mammalian cells leads to increased LPAAT activity in cell-free extracts. This correlates with enhancement of transcription and synthesis of tumor necrosis factor- α and interleukin-6 from cells upon stimulation with interleukin-1 β . LPAAT overexpression may amplify cellular signaling responses from cytokines (West et al., 1997). LPAAT has not been purified from mammalian sources.

2.2.5 *Alkylacyl Glycerophosphate Phosphohydrolase*

This enzyme hydrolyzes the phosphate group from alkylacyl glycerophosphate. This enzyme has not been purified and characterized from either neural or nonneural sources. It is not known whether alkylacyl glycerophosphate phosphohydrolase is different from well-characterized diacyl glycerophosphate phosphohydrolase (Lee, 1998). Diacyl glycerophosphate phosphohydrolase was partially purified from rat liver. Gel filtration of rat liver cytosol on Bio-Gel A-5m results in four peaks (Ide and Nakazawa, 1985). All show activity with either phosphatidate bound to microsomal membranes (PAmb) or phosphatidate dispersed in sonicated microsomal lipids (PAaq) as the substrate. A major part of the PAmb phosphohydrolase activity (52%) is eluted in a peak with an apparent molecular mass of 500kDa in which the PAaq phosphohydrolase activity is very low. A major PAaq phosphohydrolase activity peak (48%) is obtained in the void volume, in which the PAaq phosphohydrolase activity is higher than the PAmb phosphohydrolase activity. The addition of 0.075% Tween 20 to the elution buffer results in one peak with molecular mass of 500kDa. Phosphatidate phosphohydrolase is a Mg^{2+} -dependent enzyme that is inhibited by *N*-ethylmaleimide. It is involved in phospholipase D-mediated signal transduction processes.

2.2.6 *CDP-Ethanolamine: Diacylglycerol Ethanolaminephosphotransferase*

This enzyme catalyzes the transfer of the phosphoethanolamine head group from CDP-ethanolamine to alkylacylglycerols. It has been purified from bovine liver microsomes to homogeneity with multiple column chromatographic procedures (Mancini et al., 1999). The purification method is based on the high hydrophobicity of the protein whose charged sites appear to be masked from interaction with the chromatographic stationary phase when membranes are solubilized with an excess of nonionic detergent. The purified enzyme migrates as a single band on SDS-polyacrylamide gel electrophoresis with molecular mass of 38 kDa and has both ethanolaminephosphotransferase and cholinephosphotransferase activities. Collective evidence based upon kinetic studies suggests that both activities are Mn^{2+} -dependent and that the same catalytic site is involved in cholinephosphotransferase and ethanolaminephosphotransferase reactions (Mancini et al., 1999). Mg^{2+} -dependent CDP-choline:diacylglycerol cholinephosphotransferase (EC 2.7.8.2) activity is completely inactivated during the solubilization and purification steps (Mancini et al., 1999).

The conversion of 1-alkyl-2-acyl-*sn*-GroPEtn to 1-alk-1'-enyl-2-acyl-*sn*-GroPEtn (ethanolamine plasmalogen) is carried out by a cytochrome b5-dependent microsomal electron transport system. This system consists of cytochrome b5, NADH:cytochrome b5 reductase, and cyanide-sensitive Δ^1 -alkyl desaturase (Snyder et al., 1985). Choline plasmalogens are synthesized from PlsEtns by polar-head group modifications by a base-exchange enzyme or *N*-methyltransferases (Paltauf, 1994; Horrocks et al., 1986; Lee, 1998; Mozzi et al., 1989). Enzymes that catalyze the last step of PlsEtn synthesis are not fully characterized and so more studies are required on the isolation and characterization of these enzymes (Nagan and Zoeller, 2001; Lee, 1998).

Plasmalogens can also be synthesized from alkylglycerols, bypassing the first three steps through the action of a kinase, ATP:1-alkyl-*sn*-glycerol phosphotransferase (alkylglycerol kinase). The product 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate enters the synthesizing cycle after the reductase step (Nagan and Zoeller, 2001). This pathway represents a salvage pathway for plasmalogen biosynthesis from partially degraded plasmalogens and alkylacyl glycerophospholipids. In another pathway, peroxisomes utilize acetyl-CoA, a product of β -oxidation, and tetradecanoyl-CoA to form hexadecanol, which condenses with 1-acyl dihydroxyacetone phosphate to generate 1-alkyl dihydroxyacetone phosphate. This reaction is catalyzed by 1-alkyl dihydroxyacetone phosphate synthase (Whitehouse, 1997).

Chemical synthesis of 1-*O*-[9'-(1''-pyrenyl)]nonyl-*sn*-glycerol (pAG), a fluorescent ether lipid with a pyrene moiety covalently attached at the alkyl chain terminus was reported (Zheng et al., 2006) (Fig. 2.2). This ω -pyrene-labeled 1-*O*-alkyl-*sn*-glycerol is taken up by CHO-K1 and NRel-4 (a variant that is defective in dihydroxyacetone phosphate dehydrogenase) cells. Treatment of CHO-K1 and NRel-4 cells results in the incorporation of pAG into ethanolamine and choline

glycerophospholipids as well as a neutral lipid fraction tentatively identified as alkyldiacylglycerols. NRel-4 cells incorporate more fluorescence in the phospholipid fraction than CHO-K1, specifically in the ethanolamine glycerophospholipids. Analysis of the fluorescent lipids demonstrates that 93% of the pAG is taken up by glycerolipids with the intact ether bond. Although the addition of 20 μ M HG to the medium fully restores PlsEtn biosynthesis in NRel-4 cells, pAG only partially restores PlsEtn synthesis (Zheng et al., 2006). Incubation of cells with pAG followed by irradiation with long-wavelength (>300 nm) ultraviolet light produces cytotoxicity. NRel-4 cells exhibit an increase in sensitivity to UV light compared with CHO-K1 cells. It is proposed that this photodynamic cytotoxicity approach can be used to select for mutants that are defective in downstream steps in ether lipid biosynthesis.

2.3 Plasmalogen Synthesizing Enzymes During Brain Development

Plasmalogens are a major constituent of the myelin sheath. The levels of PlsEtn in brain tissue depend on the degree of myelination (Horrocks and Sharma, 1982). Highly unsaturated fatty acids including docosahexaenoic acid (DHA) are in brain plasmalogens. Plasmalogens are in lipid raft microdomains isolated from myelin (Rodemer et al., 2003). Initial accumulation of plasmalogen occurs in 5- and 7-days-old rat brain followed by rapid accumulation between 10 and 17 days after birth (Korey and Orchen, 1959; Wells and Dittmer, 1967). The specific activity of NADPH:alkyl dihydroxyacetone phosphate oxidoreductase is highest in microsomes from 5-days-old rat brain (El Bassiouni et al., 1975), indicating that the increased synthesis of a key intermediate used for plasmalogen synthesis occurs earlier than the main burst of galactosylcerebroside synthesis for incorporation into myelin. Collective evidence suggests that the concentration of PlsEtn increases rapidly during the intense period of myelination. Ethanolamine glycerophospholipids from the myelin sheath contain up to 70% PlsEtn. An eightfold increase in PlsEtn levels (per gram of brain tissue) occurs in human white matter during the first year of life so that PlsEtn accounts for 20% of the glycerophospholipid mass (70% of the ethanolamine glycerophospholipids). Developmental studies of human brain indicate that there is a steep rise in PlsEtn content, followed by a further rise up to 30–40 years of age. This is followed by a decline of PlsEtn levels and myelin during normal aging. At 70 years of age, the levels of PlsEtn are 18% less than that at 40 years of age (Rouser and Yamamoto, 1968; Horrocks et al., 1981). Besides brain, a decline in plasmalogen level has been observed in other tissues in normal aging and in some pathologic conditions. A negative correlation of age with serum plasmalogen-derived hexadecanal dimethylacetal (16:0 DMA) or octadecanal dimethylacetal (18:0 DMA) is observed in healthy adults (Brosche, 2001). The DMAs are formed during process of methylation of fatty acids from the aldehydes bound at the *sn*-1 position of plasmalogens. Data from 118 elderly subjects (57–94 years of age)

indicate that the highest 16:0 DMA values are found in hypercholesterolemic subjects. Furthermore, there is a negative correlation between serum triacylglycerols and plasmalogen-derived 16:0 DMA ($n=118$) suggesting a relationship between low DMA values and elevated triacylglycerol levels.

2.4 Topology and Distribution of Plasmalogens and Enzymes Synthesizing Plasmalogens

As stated earlier, dihydroxyacetone phosphate acyltransferase, alkyl dihydroxyacetone phosphate synthase, and acyl/alkyl dihydroxyacetone reductase are located in peroxisomes. Dihydroxyacetone phosphate acyltransferase and alkyl dihydroxyacetone phosphate synthase are intraperoxisomal proteins facing the peroxisomal lumen, whereas acyl-CoA reductase and acyl/alkyl dihydroxyacetone reductase are located on the side of the peroxisomal membrane facing the cytosol (Brites et al., 2004). This topology of plasmalogen synthesizing enzymes indicates that the substrates of the dihydroxyacetone phosphate (acyl-CoA and dihydroxyacetone phosphate) should either be transported from cytosol into peroxisomes or synthesized inside peroxisomes. Free fatty acid generated during the plasmalogen biosynthesis pathway should be reactivated to its CoA-ester form to be a substrate for the dihydroxyacetone phosphate acyltransferase reaction. Peroxisomes contain an acyl-CoA synthase facing the peroxisomal lumen (Brites et al., 2004). This acyl-CoA is the very long chain acyl-CoA synthase. ATP, which is required for the synthetase reaction, comes from cytosol in exchange for AMP. This transport requires PMP34, a peroxisomal adenine nucleotide transporter that belongs to a family of mitochondrial solute carrier family of transporters (Visser et al., 2002). Dihydroxyacetone phosphate, the other reactant of the reaction catalyzed by dihydroxyacetone phosphate acyltransferase, is either transported from the cytosol into peroxisomes or generated in the peroxisomal matrix by glycerol-3-phosphate dehydrogenase (Hajra et al., 2000). The peroxisomal localization of dihydroxyacetone phosphate acyltransferase and alkyl dihydroxyacetone phosphate synthase is not only important for enzymic activity but also for the stability of these enzymes (Brites et al., 2004). Dihydroxyacetone phosphate acyltransferase and alkyl dihydroxyacetone phosphate synthase are known to interact and form a heterotrimeric complex, but the active functional units of these enzymes are monomers (Biermann et al., 1998). The heterotrimeric complex may regulate plasmalogen biosynthesis by modulating and facilitating substrate channeling (Brites et al., 2004).

In myelin, PlsEtns are predominantly localized in the inner leaflet (Kirschner and Ganser, 1982). Also, in red blood cell membranes, PlsEtns are found predominantly in the inner leaflet (Marinetti and Crain, 1978). Thus at equilibrium, 79% of PlsEtns are located in the inner leaflet. In contrast, the inner leaflet has only 20% of the choline plasmalogens. Thus in the red blood cell membrane, the asymmetric distribution of plasmalogens is similar to that of diacyl glycerophospholipids.

2.5 Plasmalogens in Lipid Rafts

Membranes contain submicron-sized domains called lipid rafts. These lipid rafts are enriched in cholesterol, sphingolipids, and plasmalogens (Pike et al., 2001). They also contain proteins such as the GPI-anchored proteins. Lipid rafts do not have a characteristic morphology but their occurrence in membranes compartmentalizes cellular processes. Lipid rafts can be stabilized to form larger platforms through protein–protein and protein–lipid interactions. Lipid rafts play an important role in intracellular protein transport, membrane fusion, and platforms for signal transduction processes in which sphingolipids and plasmalogens provide second messengers (Suzuki, 2002). They also serve as platforms for cell surface antigens and adhesion molecules that are crucial for cell activation, polarization, and signaling. In neurons, lipid rafts have been found in dendrites where they sustain a variety of postsynaptic protein complexes. Changes in plasmalogen levels in pathological conditions result in alterations in the composition of lipid rafts causing abnormalities in signal transduction processes. On the basis of the importance of plasmalogens in model membranes, it is proposed that plasmalogens not only provide second messengers but also contribute to signal transduction efficiency (Farooqui and Horrocks, 2004; Wanders and Waterham, 2006).

2.6 Plasmalogens in the Nucleus

Plasmalogens are also found in the nucleus where they are associated with chromatin (Albi et al., 2004). Although the role of plasmalogens in the nucleus is not fully understood, based on the occurrence of plasmalogen-selective phospholipase A_2 (Farooqui et al., 2004) and phospholipase C (Albi et al., 2004) in the nucleus, it is proposed that plasmalogens may be associated with neural cell proliferation, differentiation, and regulation of cell cycle. Plasmalogen-derived lipid mediators mediate these processes. The molecular mechanism involved in neural cell proliferation and differentiation is not fully understood, but plasmalogen-derived lipid mediators may be involved. These lipid mediators are generated through the stimulation of plasmalogen-selective PLA_2 (Antony et al., 2003; Farooqui et al., 2004) and PLC (Albi and Magni, 2004). Stimulation of these enzymes produces arachidonic acid and alkylacyl glycerols. Both metabolites stimulate protein kinase C and enhance the generation of other lipid mediators such as eicosanoids and platelet-activating factor.

Ischemic/reperfusion injury of rat hearts results in a 50% loss of myocytic nuclear choline and ethanolamine glycerophospholipids when compared with perfused hearts of controls. The loss of nuclear choline and ethanolamine glycerophospholipids during reperfusion of ischemic myocardium is partially reversed by the PlsCho- PLA_2 inhibitor, bromoenol lactone (Williams et al., 2000). This suggests that the loss of nuclear phospholipids during ischemia/reperfusion is mediated, in part, through the activation of PlsCho- PLA_2 . Western blotting studies on isolated

nuclei from ischemic hearts indicate that PlsCho-PLA₂ is translocated to the nucleus after myocardial ischemia (Williams et al., 2000). Collective evidence suggests that the nuclear phospholipid mass decreases after myocardial ischemia by a mechanism that involves, at least in part, phospholipolysis mediated by PlsCho-PLA₂.

2.7 Factors Affecting Plasmalogen Biosynthesis in Brain

Plasmalogen biosynthesis is affected by endogenous and exogenous factors such as diet, age, and genetic factors (Paltauf, 1994). A deficiency of n-3 fatty acids in the diet may result in plasmalogen deficiency and abnormal signal transduction processes in neural membranes (Farooqui and Horrocks, 2001). Dietary supplements of fish oil ethyl esters reduce the arachidonate-containing species of PlsEtns, whereas molecular species having 20:5(n-3), 22:6(n-3), and/or 22:5(n-3) acyl groups are increased in the spleen, lung, and kidneys (Blank et al., 1994). In testicular tissue from rats fed with fish oil diets, the molecular species of PlsEtns containing 22:5 (n-6) acyl groups are also reduced. An increase of PlsEtns with 18:1 alk-1-enyl moieties paired with highly unsaturated *sn*-2 acyl groups are found in the tissues of rats fed with fish oil plus selachyl alcohol diacetate supplements (Blank et al., 1994). Collective evidence suggests that supplementation of n-3 fatty acid normalizes signal transduction processes and many functions of the brain, liver, heart tissues, and reproductive organs. The incorporation of n-3 fatty acids in various mammalian tissues significantly modulates arachidonic acid metabolism by inhibiting the production of eicosanoids (Horrocks and Farooqui, 2004).

Ageing modulates the activity of dihydroxyacetone phosphate acyltransferase, the first enzyme of plasmalogen biosynthesis. Although diet has no effect on dihydroxyacetone phosphate acyltransferase, the aging process influences its activity. Littermates from two generation of n-3-deficient rats were fed an equilibrated diet containing either α -linolenic acid alone or with two doses of DHA. After weaning, or 3, 9, or 21 months of diet, rat brains were used for the determination of enzymic activity and plasmalogen levels. Dihydroxyacetone phosphate acyltransferase activity and plasmalogen levels are markedly increased in rat brain at 3 months when compared with age-matched brains from weaning. The enzymic activity is significantly decreased (30% and 40%) from 3 months to 9 and 21 months, respectively. In senescence-accelerated R1 mice, the PlsEtn content reached a maximal level at 5 months and then decreased from 5 to 9 months (André et al., 2006a). No age-dependent changes were observed in brain plasmalogen contents in senescence-accelerated P8 mice. In another study, levels and acyl compositions of PlsEtn, PtdEtn, and PtdSer were determined in the frontal cortex and hippocampus from 2 and 18-month-old rats (Favrelière et al., 2000). In 18-month-old rats, the fatty acid compositions of these three glycerophospholipids show an increase of monounsaturated fatty acid (18:1 n-9 and 20:1 n-9) and a decrease in polyunsaturated fatty acid (PUFAs), essentially DHA. DHA is markedly decreased in hippocampus PtdEtn at

18 months. Both DHA and arachidonic acid are considerably lower in frontal cortex PlsEtn. Hippocampus and frontal cortex undergo specific age-induced modifications in PlsEtn and PtdEtn acyl composition. It is proposed that decreased plasmalogen levels in aged rat brain may be not only due to decreased dihydroxyacetone phosphate acyltransferase activity, but also due to increased activity of plasmalogen-selective phospholipase A₂ (André et al., 2006b; André et al., 2005a).

Administration of *myo*-inositol (*myo*-Ins) increases the levels of plasmalogens in the brain tissue (Pettegrew et al., 2001). *myo*-Ins is an important organic osmolyte that is found in brain, retina, and kidney (Nonaka et al., 1999). Its concentration increases in brain and cerebrospinal fluid with age. Its levels in cerebrospinal fluid may be an indicator of brain atrophy (Chang et al., 1996). The mechanism of action of *myo*-Ins is not fully elucidated. However, significant information is available about its biological roles. *myo*-Ins is metabolized to PtdIns, which makes up a small, but very significant, component of neural membranes. PtdIns is metabolized to PtdIns-4,5-*P*₂, a key intermediate in biological signaling. The possible benefit of *myo*-Ins in the management of depression, panic attacks, and obsessive-compulsive behavior may be explained by the role of *myo*-Ins as a second-messenger precursor (Patel et al., 2006; Shaldubina et al., 2007).

Acute administration of *myo*-Ins plus [2-¹³C]ethanolamine ([2-¹³C]Etn) significantly elevates levels of PlsEtns in whole rat brain (Hoffman-Kuczynski and Reo, 2004). This increase in PlsEtns is localized to specialized brain areas. Thus cerebellum is the brain area most affected by the *myo*-Ins plus Etn administration (Hoffman-Kuczynski and Reo, 2005). Surprisingly, earlier studies demonstrated that administration of *myo*-Ins alone minimally affects cerebellum (Patishi et al., 1996; Kofman et al., 1998). Collectively these studies suggest that the administration of *myo*-Ins plus Etn increases the ability of cerebellum to synthesize new PlsEtn molecules. The molecular mechanism of *myo*-Ins-mediated PlsEtn synthesis is still not known, but it is proposed that *myo*-inositol catabolism through the pentose phosphate cycle generates 2 mol of NADPH. This increase in NADPH level may be associated with increased PlsEtn synthesis in rat brain. An elevated PlsEtn/PtdEtn ratio can lead to tighter neural membrane packing. This may affect membrane dynamics and induce alterations in fluidity and permeability. The synthesis of new PlsEtns may be important due to its potential role as a cellular antioxidant (Hoffman-Kuczynski and Reo, 2005).

2.8 Conclusion

Plasmalogens are major constituents of neural membranes. Plasmalogen synthesizing enzymes are localized in peroxisomes, mitochondria, and endoplasmic reticulum. Although some plasmalogen synthesizing enzymes have been purified by multiple column chromatographic procedures from liver, but their purification from brain tissue has not been achieved. Two major reasons may be responsible for the lack of isolation and characterization of enzymes synthesizing brain plasmalogens. First, the

activities of these enzymes in brain tissue are quite low when compared with other lipid synthesizing enzymes. Second, assays for determining activities are complex, laborious, and time consuming.

Dihydroxyacetone phosphate acyltransferase catalyzes the acylation of dihydroxyacetone phosphate with acyl-CoA with formation of 1-acyl dihydroxyacetone phosphate. The acyl group in this intermediate is then replaced by a long-chain alcohol that provides the oxygen for the ether linkage in the reaction catalyzed by alkyl dihydroxyacetone phosphate synthase. The carbonyl function in alkyl dihydroxyacetone phosphate is then reduced and acylated to give an alkyl analog of phosphatidic acid. This intermediate is dephosphorylated prior to introduction of the phosphocholine or phosphoethanolamine group (Lee, 1998; Nagan and Zoeller, 2001; Murphy, 2001). The rate-limiting step for plasmalogen biosynthesis has not been identified but dihydroxyacetone phosphate acyltransferase is essential for plasmalogen synthesis. Collective evidence suggests that the synthesis of PlsEtns is a very complex process that initially requires the participation of peroxisomal enzymes, followed by contributions from mitochondrial and endoplasmic reticulum enzymes.

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