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Molecular Insights into Enzymes of Membrane Bilayer Assembly

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Biological membranes physically delineate cellular boundaries and intracellular compartments and mediate a diverse set of vital complex cellular functions ranging from their own synthesis to transmembrane signaling. A central effort of molecular and cellular biology is to achieve a fundamental understanding of how the lipid and protein constituents of the membrane assemble and interact to realize this diverse spectrum of function. The biochemical approach has simplified this formidable problem by independently considering the concurrent processes of membrane lipid assembly and membrane protein biosynthesis and incorporation into the bilayer.

The perception of the phospholipid bilayer as a simple static barrier in which proteins mediate function has been supplanted by a recognition of complexity in both composition and dynamics. Membrane lipids exhibit a bewildering chemical heterogeneity, with over 1000 distinct chemical species known to occur in eukaryotic cells (Raetz, 1986). Phospholipids comprise the majority of membrane lipids and are a diverse group of amphipathic molecules which differ according to their charged head groups and the composition of their fatty acyl/alkyl chains.

Membrane lipids also exhibit topographic complexity. The principal organelle of lipid biosynthesis is the endoplasmic reticulum where phospholipids are produced asymmetrically with respect to the transverse plane of the membrane (Bell et al., 1981) (see Figure 1). Some steps of synthesis are compartmentalized in mitochondria and peroxisomes (Bishop & Bell, 1988a). The distribution of phospholipids from their site of synthesis involves a series of complex steps which result in asymmetric phospholipid compositions of opposing leaflets of the bilayer and organelles/membranes of distinct lipid composition (Bishop & Bell, 1988a). The relatively recent and unexpected finding that lipids and their metabolites function as second messengers and bioregulators (Exton, 1990; Majerus et al., 1988; Bishop & Bell, 1988b; Hanahan, 1986) has established a functional complexity that may explain their chemical diversity. This has necessitated new perspectives in terms of functional pools or reservoirs of bioactive lipid molecules. The metabolism of these specialized lipids imposes additional structural and dynamic complexity upon the functioning membrane.

A dynamic conceptual framework of membrane assembly was developed to account for the chemical, topographic, and functional complexities of the membrane. Four discrete processes facilitate a conceptual understanding of this fundamental problem: (1) *lipid biosynthesis*; (2) *transmembrane movement*, required as a consequence of the asymmetric synthesis of most lipid products; (3) *lipid transport*, which distributes newly synthesized lipids from their site of synthesis to other cellular membranes; (4) *bilayer maintenance*, involving turnover and remodeling of lipid molecules and the generation and attenuation of intramembrane lipid signals. Within this conceptual framework of membrane assembly, the enzymes of lipid biosynthesis perform critical functions. They are uniquely poised to generate and maintain the chemical diversity of the bilayer and, thus, are expected to exert major regulatory influence on the amounts and types of lipid molecules produced.

Most phospholipid biosynthetic enzymes are integral membrane proteins and must be incorporated into and function in the membrane. Thus, in-depth analysis of these enzymes promises understanding of the early steps of membrane biogenesis, the structure and function of membrane proteins, and the regulation of membrane lipid synthesis. This paper focuses on recent molecular insights into the enzymes of phospholipid biosynthesis. Later steps in membrane biogenesis including transmembrane movement (Bishop & Bell, 1988a), lipid transport (van Meer, 1989; Sleight, 1987), and membrane maintenance (van Meer, 1989; Dawidowicz, 1987) have been recently reviewed elsewhere.

Enzymes of Membrane Biosynthesis

The pathways of glycerophospholipid biosynthesis are shown in Figure 1. Where relevant, differences between the pathways in *Escherichia coli*, *Saccharomyces cerevisiae*, and higher

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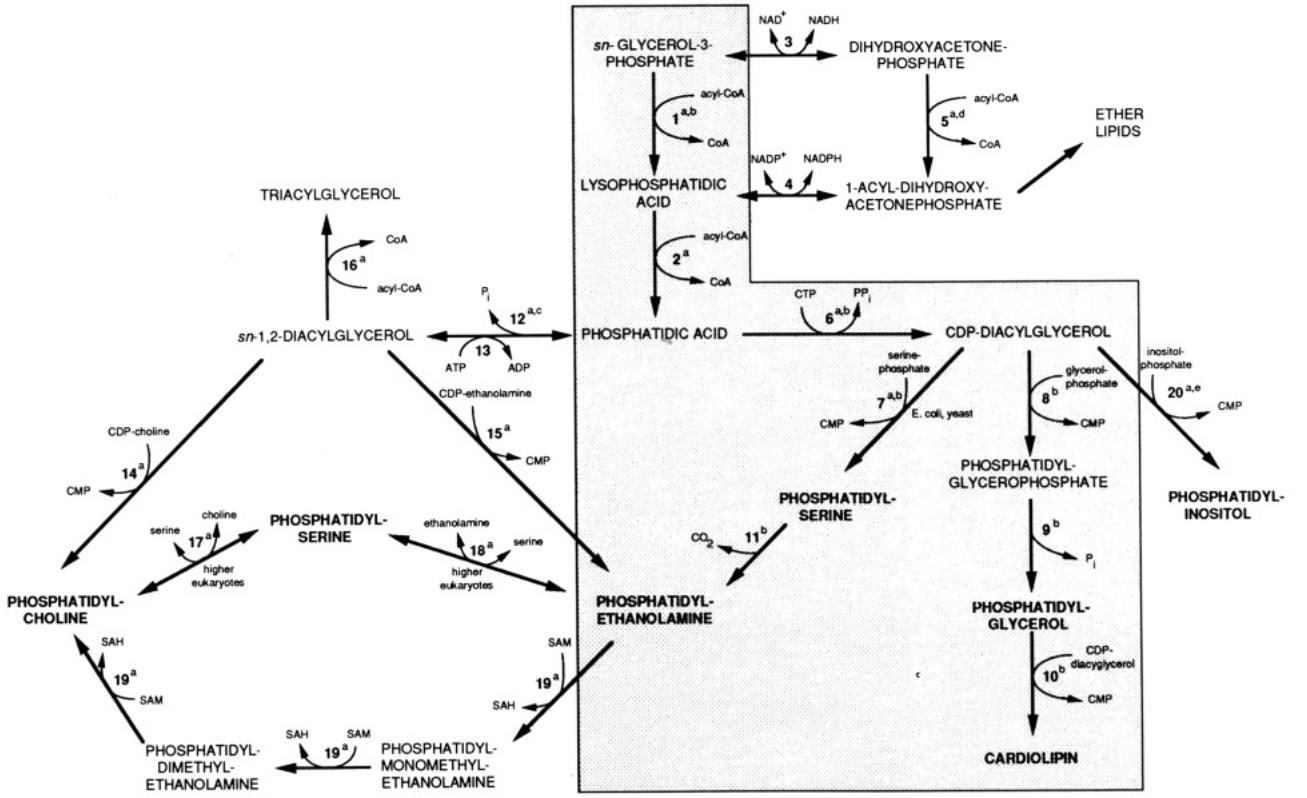

FIGURE 1: Pathways of glycerophospholipid biosynthesis. The major metabolic conversions of glycerophospholipid biosynthesis are indicated. Pathways common to *E. coli*, yeast, and higher eukaryotes are enclosed in the shaded area. The remaining reactions occur only in eukaryotes except as indicated. Enzymes catalyzing these conversions are as follows: (1) *sn*-glycero-3-phosphate acyltransferase (EC 2.3.1.15); (2) lysophosphatidic acid acyltransferase (EC 2.3.1.0); (3) dihydroxyacetonephosphate oxidoreductase; (4) acyl-dihydroxyacetonephosphate oxidoreductase (EC 1.1.1.101); (5) dihydroxyacetonephosphate acyltransferase (EC 2.3.1.42; (6) CDP-diacylglycerol synthase (EC 2.7.7.41); (7) phosphatidylserine synthase (EC 2.7.8.8); (8) phosphatidylglycerophosphate synthase (EC 2.7.8.5) (9) phosphatidylglycerophosphate phosphatase (EC 3.1.3.27); (10) cardiolipin synthase; (11) phosphatidylserine decarboxylase; (12) phosphatidic acid phosphatase (EC 3.1.3.4); (13) diacylglycerol kinase (EC 2.7.107); (14) diacylglycerol cholinephosphotransferase (EC 2.7.8.2); (15) diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1); (16) diacylglycerol acyltransferase (EC 2.3.1.20); (17) phosphatidylcholine:serine *O*-phosphatidyltransferase; (18) phosphatidylethanolamine:serine *O*-phosphatidyltransferase; (19) phosphatidylethanolamine *N*-methyltransferase (EC 2.1.17); (20) phosphatidylinositol synthase (EC 2.7.8.11). Subcellular locations of eukaryotic enzyme activities are indicated as follows: (a) endoplasmic reticulum; (b) mitochondria; (c) cytosol; (d) peroxisomes; (e) plasma membrane (Bishop & Bell, 1988; Kuchler et al., 1986).

eukaryotes are indicated. Since the pioneering delineation of these pathways by Kennedy and co-workers (Kennedy, 1962), considerable effort has been applied to the characterization of these enzymes. Earlier reviews (Bishop & Bell, 1988a; Esko & Raetz, 1983; Bell & Coleman, 1980) cover the descriptive enzymological foundation of this field.

The molecular characterization of the enzymes of phospholipid biosynthesis has sought to address the number of enzymes/isoenzymes catalyzing each step, the subcellular location of each enzyme, their biochemical properties, the structural features of the enzymes and how they confer function, and mechanisms of local regulation.[1] Significant experimental obstacles inherent to membrane-associated enzymes have impeded progress in these studies. Over the past decade, new experimental approaches have provided solutions to these problems. Theoretical and experimental advancements pertaining to the properties of detergents and detergent micelles containing lipid molecules (Helenius et al., 1979; Moller et al., 1986; Lichtenberg et al., 1983; Womack et al., 1983; Neugebauer, 1990) have permitted new mixed-micellar assay methods[2] to be developed. Successful purification strategies for these membrane-bound enzymes using detergent solubilization have been applied. Genetic and molecular genetic methods have permitted specific correlation between gene products and enzymatic activities, produced gene sequence data from which gene product primary structures have been inferred, and facilitated purification with genetically engineered overproducing strains.

[1] A complete discussion of regulatory mechanisms is precluded by space considerations. Substrate availability, posttranslational modification, and complex physiologic and genetic coordinate regulation of enzyme expression are all documented; the latter has been largely elucidated in yeast and has recently been reviewed (Carman & Henry, 1989).

[2] Conceptually, mixed micellar assays for membrane-bound enzymes exploit the property of detergents to associate into monodisperse aggregates (micelles) of relatively uniform size at concentrations above their critical micellar concentrations. The inclusion of low concentrations of neutral or amphipathic lipids into detergent micelles does not greatly disrupt micellar structure and results in solubilization of these molecules homogeneously into the micellar phase. The concentration of added lipids in the mixed micellar phase is customarily expressed as moles of lipid per moles of detergents × 100 (mol %). To a first approximation, the average number of lipid molecules present in a mixed micelle may be estimated from the detergent aggregation number (monomers per micelle) and the mol % concentration of the lipid. For example, 0.7 mol % lipid in Triton X-100 (aggregation number 140) mixed micelles corresponds to approximately one lipid molecule per micelle. Similarly, one lipid molecule per micelle requires approximately 1.2 mol % lipid in octyl β-glucoside (aggregation number 84) mixed micelles. Mixed micelles can effectively solubilize membrane-bound enzymes and constitute a surface of known composition on which the enzyme can function, facilitating presentation of hydrophobic and amphipathic substrates and cofactors to enzymes in a physically defined system. Endogenous lipids are also subject to surface dilution in the mixed micellar assay and, thus, can be effectively eliminated.

Table I: Purified Phospholipid Biosynthetic Enzymes: Biochemical Properties

enzyme	purified apparent M_r	lipid cofactor requirements	other cofactor requirements	reaction mechanism	ref
E. coli					
CDP-DG synthase	27000	Triton X-100	Mg^{2+}	sequential	Sparrow & Raetz, 1985
PGP synthase	24000	Triton X-100	Mg^{2+}		Hirabayashi et al., 1971
glycerol-P acyltransferase	83000	phospholipid	Mg^{2+}		Green et al., 1981, 1983
DG kinase	14000	multiple lipids	Mg^{2+} MgATP		Loomis et al., 1985; Walsh & Bell, 1986a,b
PS decarboxylase	30000 (β); 15000 (α)[a]	Triton X-100	pyruvate		Li & Dowhan, 1974; Dowhan et al., 1974
PS synthase[b]	54000	Triton X-100, phospholipid		ping-pong	Larson & Dowhan, 1976
S. cerevisiae					
PS synthase	23000	Triton X-100, phospholipids	Mg^{2+}	sequential bi-bi	Bae-Lee & Carman, 1984, 1990
CDP-DG synthase	56000; 54000[c]	Triton X-100	Mg^{2+}	sequential bi-bi	Kelley & Carman, 1987
PI synthase	34000	Triton X-100, phospholipids	Mg^{2+}		Fischl & Carman, 1983
PA phosphatase	91000	Triton X-100	Mg^{2+}		Lin & Carman, 1989
mammalian					
PE *N*-methyltransferase	18000	phospholipid		ordered bi-bi	Ridgway & Vance, 1987, 1988b
DG kinase (rat brain, membrane)	150000	anionic detergents, phospholipids			Kato & Takenawa, 1990
DG kinase (rat brain, cytosolic)	110000	anionic detergents, phospholipids			Kato & Takenawa, 1990
DG kinase (pig brain, cytosolic)	78000	deoxycholate, phospholipids			Kanoh et al., 1983

[a] The *E. coli* PS decarboxylase consists of two subunits present in approximately equimolar ratios. [b] The PS synthase from *Bacillus licheniformus* has also been purified and has an apparent M_r of 53K, exhibits Triton X-100 dependence, and has a sequential bi-bi reaction mechanism (Dutt & Dowhan, 1985). [c] The yeast CDP-DG synthase consists of two subunits as assessed by denaturing gel electrophoresis. The native enzyme is a complex of M_r 114000 as determined by radiation inactivation.

Table II: Phospholipid Biosynthetic Enzymes: Genetic and Molecular Genetic Information

gene	gene product	open reading frame (amino acids)	predicted M_r ($\times 10^{-3}$)	predicted transmembrane segments	ref
E. coli					
plsB	glycerol-P acyltransferase	806	91.3	0	Lightner et al., 1983
cds	CDP-DG synthase	250	27.6	4	Icho et al., 1985
pss	PS synthase	ns[a]			Raetz, 1986
psd	PS decarboxylase	322	35.9[b]	1	Li & Dowhan, 1988
pgsA	PGP synthase	216	24.8	5	Gopalakrishnan et al., 1986
pgpA	PGP phosphatase	167	19.4	0	Icho, 1988
cls	CL synthase	ns			Raetz, 1988
dgk	DG kinase	122	13.2	3	Lightner et al., 1983
S. cerevisiae					
CPT1	DG cholinephosphotransferase	407	46.3	7	Hjelmstad & Bell, 1990
EPT1	DG ethanolamine phosphotransferase	391	44.5	7	Hjelmstad & Bell, 1991a
PSS (*CHO1*)[c]	PS synthase	276	30.8[d]	4	Nikawa et al., 1987a; Kiyono et al., 1987
PEM1	PE *N*-methyltransferase	869	101.2	5	Kodaki & Yamashita, 1987
PEM2	PE *N*-methyltransferase	206	23.2	3	Kodaki & Yamashita, 1987
mammalian (porcine) cDNA	DG kinase	734	82.6	0	Sakane et al., 1990

[a] ns not sequenced. [b] The *E. coli* PS decarboxylase is synthesized as proenzyme with subsequent processing into α (M_r = 7.3K) and β (M_r = 28.6K) subunits. [c] Structural gene mutants in the yeast PS synthase have been independently isolated by two laboratories (Carman & Henry, 1989). The *PSS* and *CHO1* genes are identical as demonstrated by DNA sequencing. [d] The yeast PS synthase is synthesized as a proenzyme and subsequently processed to a M_r 23K polypeptide.

The genetics and biochemistry of phospholipid biosynthesis have been most extensively studied in *E. coli* (Raetz, 1986) and in the yeast *S. cerevisiae* (Carman & Henry, 1989). The development of molecular tools in higher eukaryotic organisms is just beginning (Bishop & Bell, 1988a). A significant number of phospholipid biosynthetic enzymes have now been purified and are listed in Table I. Mutants in many structural genes encoding these enzymes have been isolated, and these have facilitated the cloning and sequencing of a number of structural genes. The genetic and molecular genetic tools now available are summarized in Table II. Molecular insights into the structure, function, and regulation of these enzymes are just

beginning to emerge as a dividend of the development of these tools and advancements in enzymologic studies.

Basic Enzymologic Issues

Number of Enzymes and Their Substrate Specificities. The question of whether multiple or single enzymes catalyze a given reaction is frequently encountered in phospholipid metabolism since lipid substrates are generally comprised of chemically heterogeneous species. The recognition of specialized second messenger (Exton, 1990; Majerus et al., 1988; Bishop & Bell, 1988b) and bioactive extracellular (Hanahan, 1986) lipid molecules has renewed interest in this area. These specific pools of lipid species seem to require independent enzymatic machinery for their generation (Woodard et al., 1987; Francescangeli & Goracci, 1989) and attenuation (MacDonald et al., 1988b). In a broader sense, these observations are pertinent in that they suggest that enzyme pluarality may be necessary for the generation of chemically defined product pools. Additional situations in which the number of enzymes involved is relevant include sequential and parallel reactions utilizing common substrates, metabolic branch points involving chemically similar divergent reactions, and the presence of enzyme activity in two or more subcellular fractions (see Figure 1). The development of genetic and molecular genetic systems has proved particularly powerful in resolving these issues.

As shown in Figure 1, lysophosphatidic acid may be synthesized by two routes in eukaryotic cells: acylation of *sn*-glycerol 3-phosphate (glycerol-P)[3] and acylation of dihydroxyacetone phosphate (DHAP) followed by reduction of acyl-DHAP. Considerable controversy has surrounded elucidation of the number of acyltransferases catalyzing these reactions. Using the yeast system, it has been established that a single enzyme catalyzes both reactions (Tillman & Bell, 1986). Mutants defective in glycerol-P acyltransferase activity were isolated with a colony autoradiographic screening assay. Alterations in DHAP acyltransferase activities closely paralleled the corresponding defects in the glycerol-P acyltransferase activities and responded similarly to the effects of a number of differentiating conditions. Thus, these parallel acylation reactions appear to be catalyzed by a single enzyme in yeast.

The yeast diacylglycerol (DG) cholinephosphotransferase activity is catalyzed by two enzymes, the *CPT1* and *EPT1* gene products (Hjelmstad & Bell, 1987, 1988, 1991c). The isolation of structural gene mutants using colony autoradiographic assays has facilitated the cloning of both genes and the generation of null mutants. By use of *cpt1* and *ept1* null mutants, the cholinephosphotransferase activity attributable to each gene product has been studied independently by mixed-micellar assays (Hjelmstad & Bell, 1991b). The cholinephosphotransferase activities of the two enzymes were distinguishable on the basis of their dioleoylglycerol substrate dependencies, activation by Mg^{2+}, and CMP inhibition profiles. In contrast, their CDP-choline dependencies and phospholipid cofactor activation properties were identical.

The yeast ethanolaminephosphotransferase activity appears to be primarily attributable to the *EPT1* gene product (Hjelmstad & Bell, 1988). Thus, these studies supported the conclusions of enzymological studies in higher eukaryotes which indicated that two aminoalcoholphosphotransferases were present (Esko & Raetz, 1983; Bell & Coleman, 1980). However, these results were somewhat surprising in that a strict division of substrate specificity between the two enzymes was not seen. The in vitro results would indicate that phosphatidylcholine (PC) is synthesized by both of these enzymes whereas phosphatidylethanolamine (PE) is synthesized solely by the *EPT1* gene product. Preliminary experiments indicate that PC synthesis in vivo via the CDP-choline pathway also proceeds with contributions from both enzymes (S. C. Morash, R. H. Hjelmstad, and R. M. Bell, unpublished results).

Using a similar genetic and biochemical approach, Yamashita and co-workers have shown that the first step of the sequential methylation of PE to PC is catalyzed by two enzymes, the *PEM1* and *PEM2* gene products (Kodaki & Yamashita, 1989, 1984). The PE *N*-methyltransferase activities due to each gene product could be distinguished on the basis of pH optima and their K_M values for *S*-adenosyl-L-methionine. The PE *N*-methyltransferase activity due to the *PEM2* gene product, which also catalyzes the second and third methylation reactions, was quantitatively lower than that due to the *PEM1* gene product. Thus, the first methylation step of PE appears to be catalyzed by two enzymes, while the second and third methylation steps are catalyzed exclusively by one enzyme. When these data are coupled to the conclusions derived from study of the choline- and ethanolaminephosphotransferases described above, an interrelationship emerges between the de novo synthesis of PC and PE via the CDP-choline and CDP-ethanolamine pathway and the conversion of PE to PC by sequential methylation. The significance of the involvement of two enzymes with variable substrate specificities in each pathway remains to be elucidated. Multiple enzyme reactions may permit the synthesis of functionally specialized products or create chemically defined product pools on the basis of substrate specificity. An additional molecular rationale which could explain the presence of two enzymes catalyzing single conversions pertains to the potential for differential regulation. Multienzyme reactions may serve to control the distribution between phospholipid classes.

Like the yeast *PEM2* gene product, the PE *N*-methyltransferase purified from rat liver (Ridgway & Vance, 1987) catalyzes all three sequential methylation reactions in the conversion of PE to PC. An enzyme analogous to the yeast *PEM1* gene product has not been detected in higher eukaryotes; the possibility that such an enzyme has been retained in evolution cannot currently be excluded.

The yeast phosphatidylserine (PS) synthase activity is present in both the endoplasmic reticulum and mitochondria (Kuchler et al., 1986). The availability of the cloned *CHO1* gene, which encodes the PS synthase (Letts et al., 1982), and methods for purification of the enzyme (Bae-Lee & Carman, 1984) facilitated experiments which provided definitive evidence that both activities are attributable to a single enzyme (Kohlwein et al., 1988). Specifically, enzyme from both subcellular fractions was immunoreactive with antiserum raised against a hybrid protein containing *CHO1* gene product sequences, and immunoreactive and enzymatically active protein copurified. Thus, at least for the yeast PS synthase, distinct subcellular localization of enzyme activity is achieved by targeting a single enzyme to two separate sites.

Lipid Substrate Specificity. The overall diversity of phospholipids at the level of fatty acyl chains is incorporated into lipid substrates early in phospholipid synthesis. Thus, most subsequent reactions utilize a chemically heterogeneous pool

[3] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PGP, phosphatidylglycerophosphate; DG, *sn*-1,2-diacylglycerol; PA, phosphatidic acid; PMME, phosphatidyl-*N*-monomethylethanolamine; PDME, phosphatidyl-*N*,*N*-dimethylethanolamine; glycerol-P, *sn*-glycerol 3-phosphate; DHAP, dihydroxyacetone phosphate.

of lipid substrates. Interest in lipid substrates of the phospholipid biosynthetic enzymes has focused on evaluating the degree to which specificity for these substrates influences the chemical composition of reaction products and the eventual composition of the membrane. In general, studies have revealed a broad specificity of most enzymes for the acyl chain length and degree of fatty acyl unsaturation of their lipid substrates (Esko & Raetz, 1983; Bell & Coleman, 1980). A few enzyme activities are relatively selective (Esko & Raetz, 1983).

The development of molecular tools and improved assay techniques now make it possible to systematically study the lipid substrate specificities of these enzymes in depth. Recently, a structure/function analysis of the lipid substrate specificity of the *E. coli* DG kinase has been performed with a large number of DG analogues (Walsh et al., 1990). Kinetic analysis using a number of modifications of the fatty acyl chains and the *sn*-2 ester group demonstrated that these analogues affected primarily the apparent K_M and had only small effects on the V_{max}. Significantly, none of these analogues were inhibitors of DG kinase. However, analogues bearing structural modification at the *sn*-1 ester and *sn*-3-methylene positions of the molecule exibited altered V_{max} values and comparatively small effects on K_M values. These analogues were frequently found to be DG kinase inhibitors. The results were interpreted to suggest a role of acyl chains of the substrate in orientating the molecule within the micelle and, by extension, the membrane, for presentation to the active site. In contrast, the *sn*-1 ester and *sn*-3-methylene groups were suggested to interact with the active site. These studies seem to provide a molecular explanation for the observed broad specificity of DG kinase for its lipid substrate (Schneider & Kennedy, 1976; Sandermann et al., 1982).

The specificity of the purified PE *N*-methyltransferase from rat liver for molecular species of PE has also been investigated (Ridgway & Vance, 1988a). The purified enzyme, like the enzyme from microsomal sources, synthesized PC which had a distribution of molecular species similar to the mole percent distribution of molecular species in the substrate. Similarly, when phosphatidyl-*N*-monomethylethanolamine (PMME) and phosphatidyl-*N*,*N*-dimethylethanolamine (PDME) were methylated by the pure enzyme, the products synthesized showed a distribution of labeled molecular species similar to that of the starting substrates. When synthetic PEs were provided as substrates, the pure methyltransferase showed higher rates of methylation with more unsaturated species while long chain saturated species were not methylated by the enzyme. Interestingly, rates of methylation of saturated and monounsaturated PE were enhanced when polyunsaturated-rich microsomal PC was included in the mixed micelles at high mole percent. In contrast to the in vitro results, in vivo pulse labeling studies revealed that the methyltransferase exhibits limited selectivity, particularly for 1-palmitoyl-2-docosahexaenoyl-PE. This discrepancy between in vitro and in vivo results underscores the potential role for the microenvironment of the enzyme to influence the catalytic activity of the enzyme, a mechanism further explored under Lipid Cofactor Requirements.

The concept of enrichment of specific molecular species in product pools on the basis of substrate specificity has recently been extended by studies of DG kinase activity in Swiss 3T3 cells. Mammalian tissues appear to have two different types of DG kinase, a cytosolic enzyme and a membrane-bound enzyme (Kato & Takenawa, 1990; MacDonald et al., 1988a,b) (see Table I). Studies of the membrane-bound DG kinase in Swiss 3T3 cells (MacDonald et al., 1988b) indicate that this enzyme selectively phosphorylates 1-stearoyl-2-arachidonyl-DG relative to the cytosolic enzyme. It was proposed that this substrate-specific DG kinase functions on a specific DG pool which is generated in cell membranes in response to hormones which stimulate phosphatidylinositol (PI) turnover. Attenuation of second messenger DG has been attributed to phosphorylation by DG kinase (Bishop et al., 1986). The specificity of the membrane-bound DG kinase for arachidonyl-DGs may result in the formation of arachidonyl-enriched species of PI (MacDonald et al., 1988b).

Clearly, the study of lipid substrate specificities of enzymes of phospholipid synthesis is required to delineate the mechanism by which these enzymes contribute to the molecular species distribution of the lipid bilayer. These studies must seek not only to define the intrinsic lipid substrate specificity of the enzymes involved but also to address the mechanisms by which the substrate specificity is modulated in response to the lipid environment. The potential for modulation of substrate specificity in vivo and the relationship of this phenomenon to biochemically definable protein–lipid interactions may redefine the way in which substrate specificity has been viewed. The catalytic activity of these enzymes is perhaps best viewed in terms of dynamic selectivity, which is responsive to complex mechanisms of local regulation.

Lipid Cofactor Requirements

Many of the enzymes of phospholipid biosynthesis are known to be activated by lipids and/or detergents (Table I), a phenomenon frequently observed for membrane-associated enzymes (Moller et al., 1986). The lipid dependency of these enzymes is of considerable interest not only in the context of the general implications for lipid–protein interactions but in view of the dual role of lipid molecules as substrates/products and modulators of activity. The latter situation raises important questions as to the number and types of lipid interaction sites these enzymes possess and the relationship between substrate and activator binding sites. More importantly, the potential regulatory function which could be achieved through lipid modulation of the phospholipid biosynthetic enzymes is intriguing. Despite the early recognition of the lipid and/or detergent dependencies of these enzymes as solubilization and purification were attempted, it is relatively recent that in-depth analysis of lipid activation has been undertaken. These studies indicate that (1) lipid activation involves either a few activator molecules, presumably interacting with specific sites, or a large number of molecules which form a "lipid boundary" and (2) lipid activation may represent an important mechanism of local regulation.

Lipid Activator Requirements Involving Small Numbers of Molecules. The *E. coli* DG kinase currently represents the most extensively studied lipid-dependent membrane-bound enzyme of phospholipid metabolism. Early observations that the purified enzyme required the addition of lipids for activity (Bohnenberger & Sandermann, 1983) established that a broad spectrum of lipid molecules including phospholipids, fatty acids, and neutral lipids could restore the activity of the enzyme in cosonicated mixtures. Subsequently, the reconstitution of DG kinase activity in a mixed micellar assay containing octyl β-glucoside, cardiolipin (CL), and DG was reported (Loomis et al., 1985; Walsh & Bell, 1986a). This system facilitated the in-depth analysis of the requirement of DG kinase for a lipid activator (Walsh & Bell, 1986a,b).

Initial studies indicated that a lipid cofactor was not absolutely required (Walsh & Bell, 1986a). However, the observation that the *sn*-1,2-dioleoylglycerol substrate dependence

was highly cooperative in the absence of a lipid activator suggested that the substrate could also serve as lipid activator (Walsh & Bell, 1986b). This was established by structural dissociation of the substrate and cofactor functions of the lipid substrate. Specifically, *sn*-1,3-dioleoylglycerol was shown to be a lipid activator but not a substrate, and *sn*-1,2-dioctanoylglycerol served as a substrate but not an activator. In this manner, an absolute lipid cofactor requirement for DG kinase could be demonstrated with *sn*-1,2-dioctanoylglycerol (Walsh & Bell, 1986b).

Detailed study of the kinetic properties of activation of DG kinase by its lipid cofactor revealed several interesting features. Since half-maximal activation was observed at low mole percent activator, it was concluded that lipid activation involved interaction with a small number of activator molecules[2] (Walsh & Bell, 1986a). Lipid activation was highly cooperative (Walsh & Bell, 1986b), a commonly observed feature of lipid-dependent enzymes (Sandermann, 1986). This most likely represents a need for several cofactor molecules. Kinetic analysis of dioleoylglycerol, MgATP, and free Mg^{2+} dependencies as a function of lipid activator concentration suggested that lipid activation precedes the interaction with substrates and the free Mg^{2+} cofactor (Walsh & Bell, 1986b). Evidence that lipid cofactors induce a conformational change in the enzyme was derived from a strong correlation between catalytic activation and protection from substrate-dependent thermal inactivation (Walsh & Bell, 1986a,b).

The yeast choline- (*CPT1* gene product) and ethanolamine- (*EPT1* gene product) phosphotransferases also exhibit absolute requirements for lipid activators (Hjelmstad & Bell, 1991b). The lipid activator requirement of each enzyme was suggested by the presence of an activator substance contained in the enzyme preparation. Effective dispersion of this activator into the micellar phase was achieved at low enzyme concentrations, and enzyme activity became completely dependent on exogenous phospholipid. Like *E. coli* DG kinase lipid activation, half-maximal activation of the *CPT1* and *EPT1* gene products occurred at low mole fractions of phospholipid, suggesting that a small number of molecules are required.

In some cases, lipid activation involving small numbers of activator molecules cannot be demonstrated to be absolute. Mixed-micellar assays of the membrane-bound DG kinase activity from Swiss 3T3 cells revealed greater than 2-fold activation by phospholipids (MacDonald et al., 1988b). Long-chain DGs did not exhibit cooperative concentration dependencies, and short-chain (didecanoylglycerol) substrates supported activity in the absence of additional lipids, suggesting that DGs were not serving as activators in this system. The role of detergent or endogenous lipids in partial activation was not evaluated.

The purified yeast PI and PS synthases also exhibit partial lipid activation in Triton X-100 mixed micelles containing phospholipid (Bae-Lee & Carman, 1990). In the absence of nonsubstrate lipids, both of these enzymes are known to require the nonionic detergent Triton X-100 for optimal activity (Fischl & Carman, 1983; Bae-Lee & Carman, 1984), but the potential contribution of detergent in satisfying lipid cofactor requirements for these enzymes has not been explored. The possibility that the substrate CDP-DG also serves to satisfy the lipid cofactor requirements for these enzymes is unlikely since substrate dependencies do not show cooperativity. The PS synthase activity exhibited marked dependence on added phospholipid. Phosphatidic acid (PA) served as a potent activator, giving 5.5-fold stimulation with an apparent activation constant far less than 1 mol %. Several phospholipids slightly stimulated PI synthase activity (maximally 1.4-fold).

Lipid Activation Involving a "Lipid Boundary". In contrast to the activation of some enzymes by a small number of cofactor molecules, other enzymes appear to require interaction with a large number of phospholipid molecules. This distinct class of lipid activation is customarily interpreted in terms of a lipid boundary model (Sandermann, 1986; Cortes et al., 1982) and is exemplified by the *E. coli* glycerol-P acyltransferase (Scheideler & Bell, 1989) and the rat liver PE *N*-methyltransferase (Ridgway & Vance, 1988b). The substrate dependencies of all three of the sequential methylation reactions catalyzed by the purified rat liver PE *N*-methyltransferase were reported to exhibit marked cooperativity when assayed in Triton X-100–mixed micelles (Ridgway & Vance, 1987), suggesting a requirement for a lipid activator that could be satisfied by substrates. Hill coefficients determined for substrate dependencies were converted to near unity by the addition of 30–40 mol % of the nonsubstrate phospholipid PC (Ridgway & Vance, 1988b). At high concentrations of phospholipids in detergents, micellar structures are replaced by lamellar structures (Lichtenberg et al., 1983). However, it was established that PE *N*-methyltransferase functioned in a monodisperse population of detergent micelles containing lipid substrate, lipid activator, and enzyme (Ridgway & Vance, 1988b). This permitted the inference that the enzyme requires a large number of phospholipid molecules localized in a boundary layer rather than a bilayer structure of phospholipid.

Structural Requirements and Potential Regulatory Role of Lipid Activation. The structural specificity of the lipid cofactor requirement of *E. coli* DG kinase was explored by assessing the ability of a large number of chemically defined lipid molecules to support enzyme activation with the nonactivator substrate dioctanoylglycerol. Lipid activation was not stereospecific (Walsh & Bell, 1986a), and a broad structural specificity was observed (Walsh & Bell, 1986b). Phospholipids, sulfolipids, neutral lipids, and detergents (but not octyl β-glucoside) supported activation to varying degrees. The demonstration that detergents can satisfy lipid cofactor requirements may explain previously reported detergent requirements for a number of enzymes of phospholipid metabolism (Table I), suggesting that reevaluation of possible lipid activator requirements for these enzymes is warranted. In all cases, activation was cooperative. Molecules containing more alkyl/acyl chains were better activators, anionic lipids were better activators than neutral lipids, and cationic lipids supported activation poorly. A striking dependence on alkyl/acyl chain length was noted, poorer activation being observed with decreasing chain length in homologous series of compounds. This latter observation may be related to interaction of the activator with transmembrane structures of the enzyme (see Enzyme Structure–Function). Phospholipid activation of the DG kinase from Swiss 3T3 cells showed head-group specificity but little acyl chain selectivity (MacDonald et al., 1988b).

The yeast choline- and ethanolaminephosphotransferases exhibited competition for activation between PC and PE, indicating a degree of structural specificity for the phospholipid head group in the activator–protein interaction (Hjelmstad & Bell, 1991b). Interestingly, the cholinephosphotransferase activity of both the *CPT1* and *EPT1* gene products exhibited similar properties with respect to phospholipid activation while the ethanolaminephosphotransferase activity of the *EPT1* gene product differed significantly in the structural specificity of activation. This interdependency between CDP-amino alcohol specificity and phospholipid activation suggested that interaction between the enzyme and its lipid cofactor may influence

substrate specificity. This observation has potential implications for regulation in the native membrane where lipid cofactor binding site occupancy would be expected to dynamically reflect the microenvironment of the enzyme.

Additional evidence for a regulatory role for lipid activation has been obtained in mixed-micellar and reconstitution studies of purified yeast PS synthase (Hromy & Carmon, 1986; Bae-Lee & Carman, 1990). Phospholipid activation of this enzyme exhibited dramatic structural specificity for PA when a series of naturally occurring phospholipids were examined (Bae-Lee & Carman, 1990). Since PA is a minor membrane phospholipid situated at a branchpoint between alternate pathways of PE and PC synthesis (see Figure 1), stimulation of PS synthase by PA may serve as a regulatory mechanism favoring synthesis of PE and PC via the PS synthase pathway. The latter pathway is known to predominate in yeast under a variety of physiologic conditions (Bae-Lee & Carman, 1990). The activity of this enzyme is also modulated by the composition of phospholipid vesicles into which it is reconstituted (Fischl et al., 1986; Hromy & Carman, 1986). While the nature of the interaction through which this modulation is achieved cannot be addressed by kinetic studies in phospholipid vesicles, this system does facilitate direct interpretation of in vitro results in terms of the membrane environment of the native enzyme. Significantly, the PI/PS ratio of phospholipid vesicles modulated PS synthase activity in a fashion which could, in part, account for alterations in PS synthase activity observed under physiological conditions which influence the proportion of these lipids in the membrane (Hromy & Carman, 1984).

Enzyme Structure–Function

The ultimate goal of the molecular characterization of the enzymes of phospholipid biosynthesis is to understand their structure and function. Far less is known about the structures of membrane proteins than of soluble proteins, and the predictive methods commonly used for soluble proteins are of unproven reliability when applied to membrane proteins. Nonetheless, preliminary insight into the structure–function relationships for these enzymes is emerging. Amino acid sequences readily available from molecular genetic studies are being exploited to construct predictive models which seek to define the number and location of membrane-spanning segments of the protein (Engelman et al., 1986). Such membrane-spanning segments define the two-dimensional structure of the protein with respect to the transverse plane of the membrane and partition predicted hydrophilic stretches between the two hydrophilic surfaces of the bilayer. The predictive value of these methods has proven reliable (Wang et al., 1989; Dohlman et al., 1987; Boyd et al., 1987), although pitfalls and examples of discrepancies exist (Jennings, 1989).

Transmembrane Topography and Functional Asymmetry. Enzymological studies of the topography of the phospholipid biosynthetic enzymes with respect to the transverse plane of the membrane in microsomal vesicles suggested that the active sites of many enzymes were asymmetrically disposed on the cytoplasmic surface (Ballas & Bell, 1980, 1981; Bell et al., 1981). Understanding the molecular basis for asymmetrical phospholipid synthesis is essentially a problem in transmembrane topography of the enzymes involved. To date, no experimentally determined topographical structures for these enzymes have been reported. However, hydropathic profiles of many enzymes have been reported (Table II), permitting preliminary assignment of transmembrane topography. Such analysis for several eukaryotic gene products has suggested a striking asymmetry of hydrophilic domains with respect to the transverse plane of the membrane which is imposed by predicted transmembrane segments. The yeast PS synthase is predicted to possess four C-terminal, closely spaced transmembrane segments and a large N-terminal hydrophilic domain which would localize entirely to one face of the membrane (Nikawa et al., 1987a). A bitopic membrane topography is predicted for the yeast PI synthase (Nikawa et al., 1987b). The two internally located predicted transmembrane segments in this protein would localize a large N-terminal portion of the polypeptide as well as a smaller C-terminal portion of the protein on one face of the membrane, while a small internal hydrophilic stretch would be located on the opposite face. The yeast *CPT1* and *EPT1* gene products have highly homologous amino acid sequences and are predicted to have virtually identical, polytopic transmembrane topographies which could confer a striking asymmetric distribution of hydrophilic regions with respect to the transverse plane of the membrane (Hjelmstad & Bell, 1990, 1991a). For each of these yeast proteins, the predicted structural asymmetry suggests a basis for the localization of the enzymes' active sites on the cytoplasmic surface.

Other Roles for Multiple Transmembrane Domains. Hydropathy analysis of the amino acid sequences derived from a number of *E. coli* phospholipid biosynthetic structural genes has also been performed (Table II). A variety of topographic arrangements have been predicted from these studies, ranging from no transmembrane segments (Lightner et al., 1983; Icho, 1988) and one transmembrane segment (Li & Dowhan, 1988) to complex polytopic configurations (Icho et al., 1985; Gopalakrishnan et al., 1986; Loomis et al., 1985; Lightner et al., 1983) (Table II). Thus, this method of analysis predicts multiple transmembrane segments for the majority of phospholipid biosynthetic enzymes studied. Since anchoring of the protein in the membrane theoretically would require only a single transmembrane segment, the potential functional roles of additional transmembrane segments is intriguing. Clearly, multiple transmembrane segments would confer a broad repertoire of lipid–protein interactions to the enzymes. These interactions may be involved in binding of the hydrophobic portions of lipid substrates and positioning of the hydrophilic components of amphipathic substrates in the active site. The recognition of one or more specific lipid activator binding sites for several proteins (see above) has implicated transmembrane segments, in mediating this lipid–protein interaction. These interactions may fundamentally underlie the responsiveness of the enzymes to the membrane environment. In addition, functions not presently attributed to these enzymes could possibly be mediated by their transmembrane domains. For example, multiple transmembrane domains as well as luminal hydrophilic domains of these enzymes may participate in transmembrane transport of product molecules (phospholipid "flip-flop") (Bishop & Bell, 1985; Kawashima & Bell, 1987; Backer & Dawidowicz, 1987). This possibility would provide a simple strategy by which phospholipid synthesis and transmembrane transport could be integrated.

Protein Homologies and Enzyme Structure–Function. Additional molecular insight into the structure–function of phospholipid biosynthetic enzymes has relied upon regional protein homology analysis. A functional interpretation of identified protein homologies is facilitated when these homologies involve proteins for which biochemical correlations to structural features have been established. In addition to homologies involving well-characterized soluble proteins, homologies between the phospholipid enzymes themselves have provided insight into structurally significant regions. In some

cases, this approach complements and extends inferences based on the predicted transmembrane topography of these proteins.

Protein homology analysis has been most extensively applied to the yeast choline- and ethanolaminephosphotransferases (Hjelmstad & Bell, 1990, 1991a). These two enzymes exhibit 54% amino acid sequence identity and have highly similar predicted transmembrane topographies (see above). Overlapping sets of protein homologies to these enzymes were identified. A short internal region of the cholinephosphotransferase which was predicted to correspond to a short hydrophilic domain by membrane topography analysis was found to exhibit highly significant homology to the acetylcholine receptor (Hjelmstad & Bell, 1990). Significantly, the region of the acetylcholine receptor sequence involved in this homology has been shown to functionally participate in α-bungarotoxin binding and responsiveness to acetylcholine (Neumann et al., 1986; Mishinia et al., 1985). This analysis suggests that the homologous region of the cholinephosphotransferase is involved in choline binding. This same region of the ethanolaminephosphotransferase constitutes a focal point of sequence divergence between these otherwise highly homologous proteins. These two phosphotransferases differ significantly in their utilization of a series of CDP-amino alcohol substrates (Hjelmstad & Bell, 1991b), suggesting that divergence in this region may confer differences in substrate specificity.

Homology between the cholinephosphotransferase and phosphoglycerol kinase and a related homology between the ethanolaminephosphotransferase and glyceraldehyde-3-phosphate dehydrogenase were highly significant (Hjelmstad & Bell, 1990, 1991a). Since all four proteins catalyze reactions involving phosphorylated glycerol derivatives, these homologies may reflect conserved structural features involved in binding this common moiety (Hjelmstad & Bell, 1991a). The location of these homologies on the linear amino acid sequences of the choline- and ethanolaminephosphotransferases corresponded to the main hydrophilic regions of both proteins. Identification of a cryptic homology between the cholinephosphotransferase and the glyceraldehyde-3-phosphate dehydrogenase sequences was uncovered, exploiting the relationship between the two phosphotransferases, and precisely correlated to the nicotinamide portion of the structurally defined dinucleotide binding site (Rossman et al., 1975). The sequences proposed to participate in the formation of the mononucleotide binding site are assembled from discontinuous portions of the cholinephosphotransferase sequence. Similar sequences were identified in the ethanolaminephosphotransferase (Hjelmstad & Bell, 1991a).

Five distinct cytidine diphosphate utilizing enzymes including the yeast cholinephosphotransferase (Hjelmstad & Bell, 1990), ethanolaminephosphotransferase (Hjelmstad & Bell, 1991a), PI synthase (Nikawa et al., 1987b), and PS synthase (Kiyono et al., 1987) and the *E. coli* phosphatidylglycerolphosphate (PGP) synthase (Gopalakrishnan et al., 1986) contain short hydrophilic regions which exhibit a high degree of homology. The substrates recognized by these enzymes and the chemical reactions they catalyze are similar (Hjelmstad & Bell, 1990), suggesting that homology between them reflects conservation of a structural element or elements in the active site. Interestingly, the location of this homologous region of the choline- and ethanolaminephosphotransferases also maps to the main hydrophilic region as predicted by topographic analysis (Hjelmstad & Bell, 1990, 1991a).

The identification of protein homologies between individual phospholipid biosynthetic enzymes has implications for the broader question of the species relatedness of these enzymes and their evolution. Unfortunately, few examples permitting comparison exist. The relationship between a number of cytidine diphosphate utilizing yeast enzymes and the *E. coli* PGP synthase described above suggests conservation from *E. coli* to yeast. However, striking dissimilarities between the *E. coli* (Loomis et al., 1985; Lightner et al., 1983) and mammalian DG kinases (Kato & Takenawa, 1990; Sakane et al., 1990) suggest major evolutionary divergence. Similarities between the yeast *PEM2* gene product (Kodaki & Yamashita, 1987, 1990) and the rat liver PE *N*-methyltransferase (Ridgway & Vance, 1988b, 1987) include similar protein molecular weights and analogous substrates specificities. Significant homologies between the yeast choline- and ethanolaminephosphotransferases (Hjelmstad & Bell, 1991a) and between the two yeast PE *N*-methyltransferases (Kodaki & Yamashita, 1987) strongly suggest relationships in protein evolution.

Concluding Statement

Significant advancements in the study of the enzymes of phospholipid metabolism have built upon a foundation of progress in protein purification, molecular genetics, and new methods of enzymological analysis. The combined biochemical and genetic approach has overcome formidable obstacles unique to the study of membrane-bound enzymes and has begun to yield new information which definitively addresses basic enzymological and structural issues relevant to these proteins. Previously inaccessible problems such as the role of lipid–protein interactions in enzyme regulation have been explored. A refined conceptualization of these enzymes has emerged from these early efforts to understand their intramembrane structure and how they interact with substrates and other lipids.

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Articles

Transbilayer Transport of Phosphatidic Acid in Response to Transmembrane pH Gradients†

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ABSTRACT: Preliminary studies have shown that asymmetric transbilayer distributions of phosphatidic acid (PA) can be induced by transmembrane pH gradients (ΔpH) in large unilamellar vesicles [Hope et al. (1989) *Biochemistry 28*, 4181–4187]. Here the mechanism of PA transport is examined employing TNS as a fluorescent probe of lipid asymmetry. It is shown that the kinetics of PA transport are consistent with the transport of the uncharged (protonated) form. Transport of the neutral form can be rapid, exhibiting half-times for transbilayer transport of approximately 25 s at 45 °C. It is also shown that PA transport is associated with a large activation energy (28 kcal/mol) similar to that observed for phosphatidylglycerol. The maximum induced transbilayer asymmetry of PA corresponded to approximately 95% on the inner monolayer for vesicles containing 5 mol % PA.

It is generally accepted that many biological membranes exhibit asymmetric transbilayer distributions of lipid (Op den Kamp, 1979). However, the reasons for such asymmetry and the means by which it is generated and maintained remain relatively obscure. In the case of phosphatidylethanolamine (PE) and phosphatidylserine (PS), there is strong evidence for a protein-dependent transport mechanism which results in translocation of these lipids to the inner monolayer of plasma membranes such as the erythrocyte (Zachowski et al., 1986; Seigneuret & Devaux, 1984; Connor & Schroit, 1988) although a protein exhibiting such activity has yet to be isolated. Alternatively, in protein-free liposomal systems, asymmetry of lipids such as PE, PS, and phosphatidylglycerol (PG), in mixed phospholipid systems with phosphatidylcholine (PC), has been observed for small (sonicated) vesicles (Lentz et al., 1980; Massari et al., 1978). However, it is generally agreed that these asymmetries are related to curvature effects arising from the small radius of the sonicated vesicles, and have limited implications for lipid asymmetry in biological membranes.

Research in this laboratory has been focused on the influence of ion gradients, particularly pH gradients, on the transbilayer distributions of lipids in large unilamellar vesicles (LUVs).[1] It has been shown for lipids which are simple weak bases or weak acids such as stearylamine or fatty acids that the presence of a pH gradient (ΔpH) can dramatically affect equilibrium transbilayer distributions (Hope & Cullis, 1987). The presence of a ΔpH (inside acidic) results in the rapid migration of stearylamine to the inner monolayer of LUVs, for example, whereas oleic acid migrates to the inner monolayer in LUVs exhibiting a basic interior. Phospholipids which are weak acids can exhibit similar behavior. In the case of PG, for example, the presence of a ΔpH (interior basic) results in the migration of PG to the inner monolayer (Hope et al., 1989); however, the rate of PG transport is considerably slower than observed for oleic acid. A more detailed kinetic analysis (Redelmeier et al., 1990) of PG transport in response to ΔpH reveals that PG is transported in the neutral (protonated) form which can exhibit half-times for transbilayer movement on the order of seconds.

A preliminary study of the influence of ΔpH on the transbilayer distribution of phosphatidic acid (PA) revealed that PA can also be accumulated into the inner monolayer of LUVs with a basic interior (Hope et al., 1989). However, a quantitative analysis of this transport was precluded by the lack of an appropriate assay for PA asymmetry. In this investigation, we have developed a fluorescent assay for PA asymmetry utilizing the probe 2-(*p*-toluidinyl)naphthalene-6-sulfonic acid (TNS), which reports on the surface potential of membranes (McLaughlin & Haray, 1976; Eisenberg et al., 1979; Searle & Barber, 1979). This assay should be of general utility for detecting asymmetry of acidic lipids in LUV systems. In the case of DOPA, we show that ΔpH-induced transport

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[1] Abbreviations: LUV, large unilamellar vesicle; MLV, multilamellar vesicle; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; pyranine, 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt; TNS, 2-(*p*-toluidinyl)naphthalene-6-sulfonic acid, sodium salt.

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