Cooperative activity of phospholipid-*N*-methyltransferases localized in different membranes

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Abstract The possibility that the phospholipid-N-methyltransferases from yeast are capable of acting upon a phospholipid substrate, localized in a different membrane than in which the enzymes reside ('*trans*-catalysis' hypothesis), was investigated using *cho2* and *opi3* gene disruptant strains, which are defective in phosphatidylethanolamine transferase (PEMT) and phospholipid methyltransferase (PLMT), respectively. When cell homogenates or microsomes of the two disruptant strains are mixed, the combined methyltransferase activity, measured as the incorporation of [^{3}H]methyl label from *S*-adenosyl methionine, exceeds that expected based on the separate activities of PEMT and PLMT. The increased incorporation implies that monomethylphosphatidylethanolamine generated by PEMT becomes available for PLMT, as evidenced by increased synthesis of dimethylphosphatidylethanolamine and phosphatidylcholine. The kinetics of the cooperativity suggest a collision-based process, enabling either transport of substrate or '*trans*-catalysis'.

Key words: Phosphatidylcholine biosynthesis; Phosphatidylethanolamine methyltransferase; Phospholipid methyltransferase; Microsome; Endoplasmic reticulum; Yeast

1. Introduction

Phosphatidylcholine (PC) is a major phospholipid found in membranes of eukaryotic cells. In yeast it accounts for 40–50% of the total membrane phospholipids [1]. As in higher eukaryotes, PC is synthesized via two distinct pathways in yeast, either via the triple methylation of phosphatidylethanolamine (PE) or via the cytidine diphosphate (CDP)-choline (Kennedy) pathway (for a recent review, see [2]). In yeast, the methylation of PE is the primary pathway of biosynthesis of PC when cells are grown in the absence of choline, whereas the CDP-choline pathway is considered to be an auxiliary route since it requires exogenous choline for net PC synthesis [3]. PE undergoes three sequential methylations using *S*-adenosyl-L-methionine (SAM) as the methyl donor, with monomethyl PE (PMME) and dimethyl PE (PDME) formed as intermediates [4]. Two enzymes are responsible for the reactions, PE methyltransferase (PEMT) catalyzes the first methylation reaction and phospholipid methyltransferase (PLMT) catalyzes the second and the third [5–7].

The genes encoding PEMT and PLMT are *CHO2* and *OPI3*, respectively, which were cloned by complementation of mutants defective in the corresponding methylation reactions [5–7]. The genes are regulated at the level of transcription in response to the soluble phospholipid precursors, inositol and choline, as well as to growth phase (for a recent review, see [8]). The double mutant (*opi3 cho2*) has no phospholipid methylation activity and displays a stringent choline auxotrophy [5–9]. The single mutants are not choline auxotrophs [6,7,9]. When grown in the absence of choline, the *opi3* mutant accumulates PMME and exhibits low or undetectable levels of PC [7,10] while *cho2* mutants accumulate PE and have low levels of PC [6,10]. The residual synthesis of PC in the *cho2* mutants is due to the ability of the *OPI3* gene product to catalyze, although at a low rate, the first methylation of PE to PMME [6,10]. This ability is also reflected in the fact that the *OPI3* gene, when present in multiple copies, suppresses the phospholipid methylation defect of a *cho2* mutation [9]. The *CHO2* and *OPI3* genes encode proteins with assumed molecular weights of 101 and 25 kDa, respectively, which were predicted to be integral membrane proteins based on hydropathy plots of the amino acid sequences [5]. The enzymes have been localized to the endoplasmic reticulum (ER) [11,12].

In several enzymological studies, using membrane extracts from strains carrying *opi3* and *cho2* disruption mutations, the phospholipid substrates were added in the form of sonicated lipid suspensions [5,10,13]. Both enzymes exhibited saturation kinetics when the concentration of the phospholipid substrate was varied while the concentration of SAM was kept constant, and vice versa [13]. However, it remains unclear how the phospholipid substrates become available to the methyltransferase enzymes localized in the microsomal membranes. Possible explanations are transport of the substrates to the microsomes or fusion of the vesicles with the microsomal membranes. An alternative explanation would be that the methyltransferase enzymes located in the ER can act upon a substrate in another ('*trans*') membrane.

Subcellular fractions from strains deficient in PEMT or PLMT offer a simple model system that allows us to investigate the possibility of '*trans*'-methylation. It was found that

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Abbreviations: CDP, cytidine diphosphate; (HP)-TLC, (high performance)-thin layer chromatography; PC, phosphatidylcholine; PDME, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine; PEMT, PE methyltransferase; PLMT, phospholipid methyltransferase; PMME, phosphatidylmonomethylethanolamine; PNMT, phospholipid-*N*-methyltransferase; SAM, *S*-adenosyl-L-methionine

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upon mixing of membranes from *opi3* and *cho2* strains the incorporation of label increased and that more of the methylated PE derivatives were formed than expected based on the activity of each enzyme in the separate fractions. This indicates that PEMT and PLMT localized in different microsomal membranes are capable of cooperating. The findings will be discussed in the light of the '*trans*-catalysis' hypothesis.

2. Materials and methods

2.1. Materials

SAM was purchased from Sigma (St. Louis, MO, USA). The radiochemical S-adenosyl-L-[methyl-3H]-methionine ([^{3}H]SAM, 15 Ci/mmol) was obtained from Amersham (Amersham, UK). Silicagel 60 high performance-thin layer chromatography (HP-TLC) plates were from Merck (Darmstadt, Germany). Yeast extract was obtained from Sigma. Zymolyase was supplied by Seikagaku (Japan). All other chemicals were of analytical grade.

2.2. Subcellular fractionation

The yeast strains listed in Table 1 were grown aerobically to late log at 30°C in semi-synthetic lactate medium [15] supplemented with 1 mM choline and adenine, histidine, leucine, tryptophan, and uracil at a concentration of 20 mg/l each, except for strain D273-10B, which was grown without supplements. Spheroplasts were prepared using zymolyase as described previously [15] and homogenized using a Dounce homogenizer in a buffer containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.0, and 0.6 M sorbitol (D buffer), to which 1 mM PMSF and 0.2% (w/v) bovine serum albumin (BSA) were added. Crude mitochondria were isolated using D buffer and were further purified by nycodenz gradient centrifugation as reported [16], with some minor changes. The nycodenz gradient was made in D buffer and consisted of a layer of 18% (w/v) nycodenz overlaid with a 13.5% (w/v) nycodenz layer. Microsomes were isolated as the $32\,500\times g$ pellet of a $20\,200\times g$ post-mitochondrial supernatant, as described [17], with the exception that D buffer was used for all resuspension and wash steps. The subcellular fractions, all in D buffer, were snap-frozen in liquid N_2 and stored at −80°C.

2.3. Measurement of methyltransferase activity and product composition

The activity of the phospholipid-*N*-methyltransferases (PNMTs), expressed as nmol SAM metabolized into chloroform soluble material per min per mg protein, was determined by following the methylation of endogenous PE in the cellular fractions in the presence of 0.5 mM [^{3}H]SAM (10 000 dpm/nmol) and 50 mM Tris–HCl, pH 8.0 (based on [13]). To prevent enhancement of membrane fusion and aggregation by divalent cations, Mg^{2+} was omitted from the incubation mixture, as in our in vitro system, addition of Mg^{2+} did not increase PNMT enzyme activities (data not shown). Incubations were carried out at 30°C in a final volume of 100 μl and ended by adding 475 μl of a mixture of chloroform, methanol and 0.5 M HCl (6:12:1, v/v/v). This was followed by lipid extraction [18] and liquid scintillation counting of the dried lipid extracts. The incorporation of label was linear with time up to at least 10 min, and with protein concentration at least up to 0.25 mg/ml (determined using microsomes from strain D273-10B, data not shown). To determine product compositions, [^{3}H]SAM was used at 100 000 dpm/nmol to increase the sensitivity of the analysis, and the lipid extracts were analyzed by HP-TLC, using chloroform/methanol/25% ammonia (71:25:4, v/v/v) as eluent. The radioactive spots on the TLC plate were quantified by a Berthold Automatic TLC linear analyzer (Wildbad, Germany). The product composition is presented as the percentage of ^{3}H label incorporated into PMME, PDME, and PC, together set at 100%, without correcting for the different number of methyl groups in each methylated PE derivative.

2.4. Other methods

Protein concentrations were measured using the BCA method (Pierce) with 0.1% (w/v) SDS added and BSA as a standard. Phospholipids were extracted according to the method of Bligh and Dyer [18]. Phosphorus was determined by the method of Fiske and Subbarow [19] after destruction with perchloric acid. Phospholipid compositions were determined by two-dimensional TLC analysis of lipid extracts corresponding to 350 nmol of phospholipid phosphorus as described [20].

3. Results

3.1. Characterization of methyltransferase activities and product compositions

The specific activity of the methyltransferases in the cellular homogenates of the *opi3* and *cho2* knock-out strains and the congenic wild-type was determined (Table 2). A high methyltransferase activity was found in the wild-type strain, whereas no phospholipid methylation activity was found in the double knock-out (*opi3 cho2*). The low residual methylation activity in the double knock-out was attributed to sterol methylation based on TLC analysis (not shown). The methylation activity of the *opi3* and *cho2* single knock-out strains is reduced compared to the wild-type strain. The specific activity of the strain lacking the PEMT enzyme (*cho2*) is lower than that of the strain lacking the enzyme catalyzing the second and third methylation (*opi3*), which is explained by differences in substrate availability for each enzyme. The substrate for the first methylation, PE, is present in high amounts, whereas, due to the absence of PEMT in the *cho2* mutant, the substrates are lacking for the second and third methylation. The abundance of PE and the virtual absence of PMME and PDME were confirmed by phospholipid analysis. Similar phospholipid compositions were found for homogenates of the different strains under the growth conditions employed, i.e. in the presence of choline, with PE and PC constituting approximately 30% and 40%, respectively, of the total phospholipids, and PMME and PDME not detected (data not shown). This indicates that the concentrations of endogenous substrates are similar in all strains and excludes that differences in phospholipid compositions are the cause of differences in enzyme activities between strains. The virtual lack of the preferred substrates for the second methyltransferase in the *cho2* mutant implies that this enzyme is to some extent capable of using PE as a substrate, as was found before [5,6].

Subcellular fractionation shows that, in all three strains, the activity is highly enriched in the microsomal fraction, compared to the homogenate, and is depleted from the mitochondria after gradient purification (Table 2), in agreement with the ER localization of both methyltransferase enzyme activities [11,12].

The products of the methylation reactions in microsomes from the wild-type and single disruption strains were analyzed by TLC (Table 3). The microsomal fractions produce similar relative amounts of methylated PE derivatives as the corresponding homogenates (data not shown). For the wild-type strain, PC is the principal product, with minor amounts of

Table 1
Genotypes of strains employed in this study

Strain	Designated as	Genotype
SH921[a]	(congenic) wild-type	*MATα his3 leu2 ura3 ade2*
SH922[a]	*opi3 cho2*	*MATα his3 leu2 ura3 ade2 opi3::URA3 cho2::LEU2*
SH414[a]	*opi3*	*MATa his3 leu2 ura3 ade2 trp1 can1 opi3::URA3*
SH458[a]	*cho2*	*MATα his3 leu2 ura3 cho2::LEU2*
D273-10B	wild-type	*MATα*

[a][14].

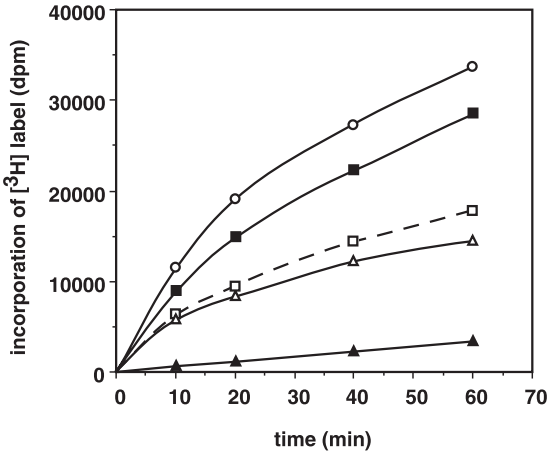

Fig. 1. A mixture of *opi3* and *cho2* homogenates incorporates more [^{3}H]methyl label into lipids than the separate homogenates taken together. The incorporation of ^{3}H label into chloroform soluble material was determined after incubation of homogenate samples (100 μg on protein basis) with [^{3}H]SAM at 30°C for the times indicated. Results from a typical experiment are shown, with the incorporation into wild-type (○), *opi3* (△), *cho2* (▲), a 1:1 mixture of *opi3* and *cho2* homogenates (■, 100 μg of each), and the incorporation expected in such a mixture, calculated as the sum of the separate incorporations (□), depicted.

PMME and PDME. The *opi3* strain, which only contains PEMT, produces PMME as major product, as reported before for *opi3* strains [5,6,9,13], although some conversion to PDME was observed. For the *cho2* strain, which only has PLMT, the major product is PC, as reported before for *cho2* strains [5,6,9]. The overall product composition in this strain is similar to that in the wild-type, including the presence of some PMME. These results demonstrate that the substrate specificity of both methyltransferase enzymes is not absolute since both are able to utilize the non-preferred substrates, although with a much lower efficiency, in agreement with literature [5,6].

3.2. Cooperativity between PEMT and PLMT activities

To get a first indication whether the methyltransferases are capable of converting substrates provided in another membrane, homogenates from the single knock-out strains were mixed and the incorporation of [^{3}H]methyl label and the composition of the products formed were monitored. If PLMT present in membranes from the *cho2* disruptant has access to PMME produced by membranes from the *opi3* disruptant, this might result in higher production of PDME and PC, since

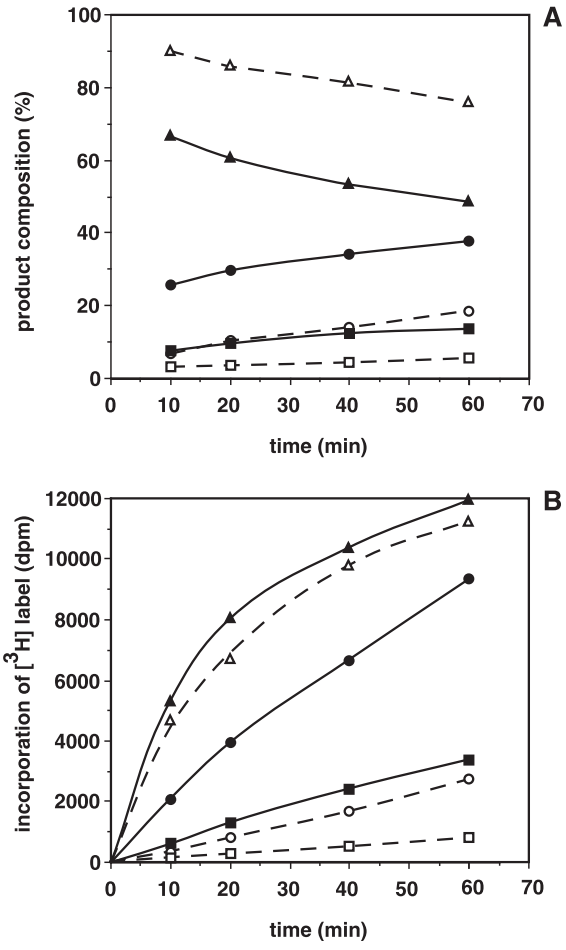

Fig. 2. Effect of mixing *opi3* and *cho2* homogenates on the distribution of [^{3}H]methyl label over the methylated phospholipids. The products formed by the methylation reactions with [^{3}H]SAM during incubation at 30°C for the times indicated were determined by TLC analysis. A typical experiment is shown. The closed symbols and continuous lines represent the values measured in a 1:1 mixture of *opi3* and *cho2* homogenates (100 μg of each, on protein basis) as calculated from the incorporation of ^{3}H label (see Fig. 1) and the corresponding TLC data. The open symbols and dotted lines represent the values expected in such a mixture, as calculated from the incorporation of ^{3}H label (see Fig. 1) and the product composition measured in separate incubations of the two homogenates. A: The product composition is expressed as the % of ^{3}H label incorporated into PMME (▲, △), PDME (■, □), and PC (●, ○), together set at 100%; B: the data shown in (A) are expressed as the amount of radioactivity incorporated in each of the lipids shown.

Table 2
Specific activities of PNMT in homogenates and distribution over subcellular fractions

Strain	Specific activity in homogenate (nmol/min/mg)	Relative specific activity			
		H	CM	M	μ
WT	1.01 ± 0.22	1.0	1.5 ± 0.4	0.46 ± 0.19	4.3 ± 0.6
opi3 cho2	0.019 ± 0.011	1.0	n.d.[a]	n.d.	n.d.
opi3	0.47 ± 0.17	1.0	0.86 ± 0.21	0.34 ± 0.08	2.01 ± 0.27
cho2	0.059 ± 0.014	1.0	1.88 ± 0.05	0.72 ± 0.10	5.5 ± 0.3

The specific activity of the combined methyltransferase enzymes was measured in subcellular fractions. Of the homogenates (H), crude mitochondria (CM) and purified mitochondria (M), 50 μg protein was used, and 10 μg protein of the microsomal fraction (μ) was used. The average (± variation) of two independent preparations is shown. The relative specific activities were calculated using the specific activity in the corresponding homogenates.
[a]n.d., not determined.

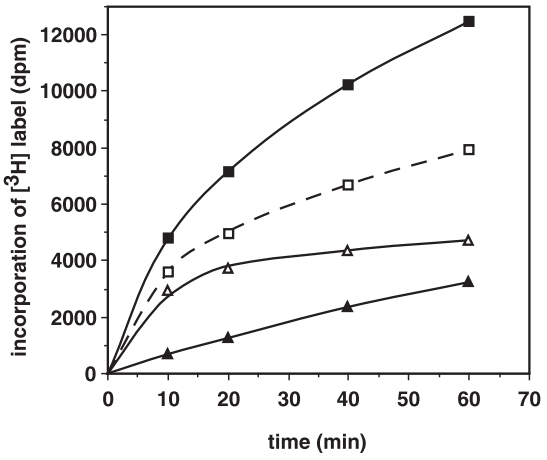

Fig. 3. A mixture of *opi3* and *cho2* microsomes incorporates more [^{3}H]methyl label into lipids than the separate microsomes taken together. The incorporation of ^{3}H label into chloroform soluble material was determined after incubation of microsomal samples (25 μg on protein basis) with [^{3}H]SAM at 30°C for the times indicated. Results from a typical experiment are shown, with the incorporation into *opi3* (△), *cho2* (▲), a 1:1 mixture of *opi3* and *cho2* microsomes (■, 25 μg of each), and the incorporation expected in such a mixture, calculated as the sum of the separate incorporations (□), depicted.

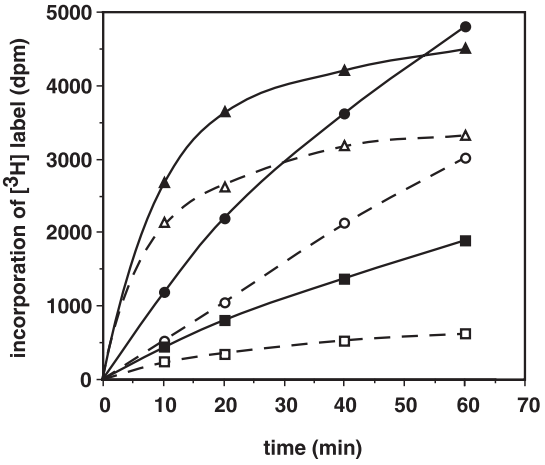

Fig. 4. Effect of mixing *cho2* and *opi3* microsomes on the incorporation of ^{3}H label into the lipid products. The products formed by the methylation reactions with [^{3}H]SAM during incubation at 30°C for the times indicated were determined by TLC analysis. A typical experiment is shown. The closed symbols and continuous lines represent the values measured in a 1:1 mixture of *opi3* and *cho2* microsomes (25 μg of each, on protein basis) as calculated from the incorporation of ^{3}H label and the corresponding TLC data. The open symbols and dotted lines represent the values expected in such a mixture, as calculated from the incorporation of ^{3}H label and product composition measured in separate incubations. The data shown are expressed as the amount of radioactivity incorporated into PMME (▲, △), PDME (■, □), and PC (●, ○).

the amount of PMME substrate for PLMT is limiting in the absence of PEMT. Fig. 1 shows the time course of the incorporation of [^{3}H]methyl label into a 1:1 mixture of homogenates of both single methyltransferase mutants (100 μg protein each) compared to that in the separate homogenates (100 μg protein each). The combined activity exceeds that expected based upon the separate activities of PEMT and PLMT in 100 μg homogenate of each. At all time points, the amount of radioactivity incorporated in the mixture exceeds the sum of the amounts of radioactivity incorporated in the separate homogenates by some 50%, and approaches the amount of label incorporated into 100 μg of the wild-type homogenate. The increased incorporation was not due to the mere presence of extra microsomal membranes, since the amount of radioactivity, incorporated into a mixture of 100 μg of *cho2* homogenate and 100 μg homogenate of the double knock-out (*cho2 opi3*), corresponded exactly to the sum of the amounts of radioactivity, incorporated into the separate samples, whereas the increase upon mixing *opi3* and *cho2 opi3* homogenates did not exceed 10% (data not shown). Thus, it appears that, in a mixture of *opi3* and *cho2* homogenates, the substrate generated by the first methyltransferase enzyme becomes available for the second enzyme, leading to higher incorporation of the [^{3}H]methyl label.

Table 3
Products of the PNMT enzymes in microsomes

Strain	PMME (%)	PDME (%)	PC (%)
WT	1.8	11.7	86.5
opi3	91.9	8.2	0
cho2	1.9	7.7	90.5

The products formed by the methylation reactions with [^{3}H]SAM were determined by TLC analysis after a 10 min incubation at 30°C using 25 μg of microsomal protein. The product composition is expressed as the % of ^{3}H label incorporated into PMME, PDME, and PC, together set at 100%.

Fig. 2A shows that the composition of the products formed by the mixture of homogenates from the single mutants is shifted towards a higher PC content compared to the product composition calculated based on the incorporation of label and product compositions of the separate homogenates. This is even more apparent when the data are presented as the amount of radioactivity in each methylated phospholipid (Fig. 2B). While the amount of PMME is hardly affected upon mixing the single mutant homogenates, a large increase is observed in the amounts of PC and PDME produced, reflecting an increased substrate availability for PLMT. Increased synthesis of PDME and PC while the amount of the intermediate PMME is not reduced implies enhanced synthesis of PMME. This suggests that in the absence of the PLMT enzyme there might be some product inhibition of PEMT or that the substrate availability for the first methyltransferase enzyme is also higher in the mixture.

To investigate whether soluble factors or membranes other than microsomal membranes are of importance for the increased substrate availability, the possible cooperativity of the methyltransferases was examined in mixtures of isolated microsomes from the single deletion mutants. The incorporation of label from [^{3}H]SAM into 1:1 mixtures of microsomes of both single methyltransferase mutants was elevated compared to the incorporation into the separate microsomal fractions at different time points (Fig. 3). The cooperative effect observed in the mixture of the microsomal fractions is of similar magnitude as that in the homogenate mixture. The change in product formation is depicted in Fig. 4 with the data expressed as the absolute amount of each methylated PE derivative. The amount of PMME product is elevated in the mixture compared to the separate incubations, in accor-

dance with the suggestion of increased substrate availability for PEMT or relief of product inhibition (because of dilution into a larger product matrix). The substrate availability for the second methyltransferase enzyme is increased in the mixtures judged from the fact that the amounts of PDME and PC formed are highly increased, although to a lesser extent compared to the homogenate mixtures (cf. Figs. 2B and 4). This could be due to a dependence of the exact product composition on the relative amounts of activity of each enzyme present in the mixture. These differ between microsomes and homogenates, due to different enrichments of PEMT and PLMT enzyme in the microsomal fractions compared to the homogenates (a factor of two to three in the *opi3* strain vs. a factor of five to six in the *cho2* strain, see Table 2). Thus, the PEMT/PLMT ratio will be lower in the 1:1 microsome mixture than in the 1:1 homogenate mixture. Therefore, we investigated the effect of varying the ratio of the microsomes from the mutant strains on the product composition and incorporation of label into each of the methylated PE derivatives. Indeed, as shown in Fig. 5, decreasing the relative amount of microsomes containing PLMT results in a higher increase in the relative amount of PC and PDME produced, compared to the calculated sum of the separate incubations, than for equal amounts of both types of microsomes. This indicates that the cooperative effect occurring in microsomes is similar to that found in whole cell homogenates.

4. Discussion

The aim of the present study was to investigate the possibility that the PNMTs act upon phospholipid substrates localized in a different membrane than the one in which the enzymes reside (the so-called '*trans*-catalysis' hypothesis). We have established a cooperative effect of the PNMTs localized in different membranes.

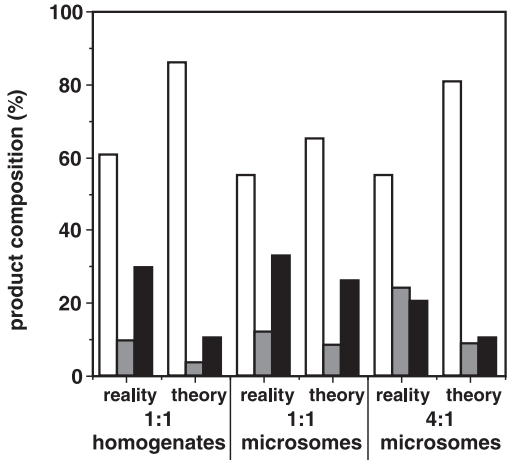

Fig. 5. Effect of varying the ratio of PEMT and PLMT on the product composition. The product compositions, measured in mixtures of *opi3* or *cho2* homogenates or microsomes (reality), are compared to the expected product compositions of such mixtures (theory), as calculated from the incorporation of ^{3}H label and the product composition measured in separate incubations. A 1:1 ratio corresponds to 100 μg samples for homogenates and to 25 μg samples for microsomes of each strain on protein basis. A 4:1 ratio corresponds to 20 and 5 μg microsomal samples on protein basis, of the *opi3* and *cho2* strains, respectively. The product composition is expressed as the % of ^{3}H label incorporated into PMME (white bars), PDME (gray bars), and PC (black bars), together set at 100%. The products formed by the methylation reactions with [^{3}H]SAM were determined by TLC analysis after a 20 min incubation at 30°C.

Subcellular fractions were isolated from *opi3* and *cho2* gene disruptant strains. As was found before for the combined activity of the PNMTs [11,12], both PEMT and PLMT were enriched in the microsomal fraction, although to different extents. The relative enrichment of PEMT in the microsomal fraction is lower than that of PLMT, while the relative enrichment of the combined PNMTs in the microsomal fraction of the wild-type strain is intermediate between the two. This result could indicate a slight difference in localization of both methyltransferase enzymes, as was proposed before based on the appearance of methylation intermediates in the Golgi and the presence of a C-terminal ER retention signal in PLMT but not in PEMT [21].

Upon mixing of homogenates of the single *cho2* and *opi3* knock-out strains, the combined methyltransferase activity, measured as the incorporation of label from [^{3}H]SAM, exceeds that expected based on the separate activities. A similar cooperativity of the PEMT and PLMT enzymes was found in a mixture of isolated microsomes, excluding the involvement of soluble factors or membranes other than microsomal membranes in the phenomenon. These results indicate that the substrate generated by PEMT becomes available for PLMT, for which enzyme the amount of phospholipid substrate is otherwise limiting the incorporation of label from [^{3}H]SAM. Analysis of the product composition revealed increased levels of PC and PDME which provided further support for increased substrate availability for PLMT in the mixture. PMME was also synthesized in increased quantities, suggesting either that product inhibition of PEMT occurs in the absence of PLMT activity or that the substrate availability for PEMT is also increased in the mixture.

The exact product composition was found to depend on the relative amount of the activity of each enzyme in the mixture. Decreasing the relative amount of PLMT (*cho2* microsomes) resulted in a higher increase in the relative amount of PC and PDME produced with respect to the amounts calculated as the sum of the separate incubations. Furthermore, it was found that the incorporation of label increased to a similar extent (approximately 50%) relative to what was expected based on the separate levels of incorporation in microsomes for ratios varying from 1:1 to 1:9 (*opi3*:*cho2*, PEMT:PLMT) while keeping the total protein concentration constant (data not shown). These results indicate that the formation of PMME is most likely the rate-limiting step in the cooperative effect.

There are several possibilities to explain the observed increase in substrate availability. It could be due to '*trans*'-action of the methyltransferase enzymes, to fusion of membranes or to transport of substrates between membranes. It is very unlikely that fusion of microsomes is involved based on literature data. Fusion of yeast ER membranes in vitro is a process that requires ATP and that even under optimized conditions does not exceed 20% after 1 h of incubation [22]. In our in vitro system, ATP is not present. Furthermore, since after 10 min already a similar relative increase in the incorporation of substrate can be observed as at later times, the cooperativity must be due to a very fast and efficient process. A collision-based process resulting either in transport of sub-

strate or in '*trans*-catalysis' thus seems to be more plausible. In favor of the latter explanation, it can be noted that a bacterial PLMT enzyme has been identified that appears to be a soluble protein [23].

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