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ORIGINAL PAPER

A Spectrophotometric Microtiterplate Assay to Determine the Transphosphatidylation Potential of Phospholipase D

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Abstract In addition to the hydrolysis of the terminal phosphate ester bond in glycerophospholipids, phospholipases D (PLDs) are able to catalyze the exchange of the polar head group. The biocatalytic potential of PLDs strongly depends on the ratio of the transphosphatidylation to hydrolysis rate which, therefore, is an important criterion in the screening for efficient PLDs from natural sources or combinatorial DNA libraries. Here, we present a fast spectrophotometric assay that allows the determination of the rate of both hydrolysis and transphosphatidylation of PLD-containing solutions including cell extracts in one microtiterplate. The assay is based on the reaction of phosphatidylcholine solubilized in Triton X-100 micelles with ethanolamine and the determination of phosphatidic acid (PA) and choline. PA is determined via Fe(III) complexation and represents hydrolysis, while choline is determined by the conventional choline oxidase/peroxidase assay and yields the total conversion. The difference between both values corresponds to the transphosphatidylation product. The method is suitable for measuring reactions rates as well as product yields after defined time periods. As shown for *E. coli* cells expressing PLD from cabbage, the assay can be applied to extracts of cells grown and lysed in microtiterplates.

Keywords Cabbage · Choline oxidase · High-throughput screening · Iron(III) salicylate · Phosphatidic acid · Phospholipase D · Phospholipid transformation · Poppy · *Streptomyces* · Transphosphatidylation

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Introduction

Phospholipase D (PLD), E.C. 3.1.4.4, obtained from plants or microorganisms has been used as a biocatalytic tool in the laboratory and on an industrial scale for many years [1]. Its great importance in biocatalysis is based on the unique property of PLD to catalyze the transesterification of glycerophospholipids with appropriate alcohols as exemplified in Fig. 1 for the reaction of phosphatidylcholine (PC) with ethanolamine. This enzymatically mediated head-group exchange in phospholipids known as transphosphatidylation is an alternative to the mostly difficult chemical synthesis of phospholipids. It is used for the large-scale production of several glycerophospholipids from PC, especially if the desired lipid cannot be obtained from natural sources. The choline moiety can be replaced by a great variety of hydroxylated compounds, and even sterically challenging biologically active molecules such as nucleosides can be used as acceptor alcohols [2].

Transphosphatidylation, however, proceeds in competition to the hydrolysis of the phospholipid (Fig. 1) yielding phosphatidic acid (PA).

For the biocatalytic efficiency, the transphosphatidylation rate (v_T) related to the hydrolysis rate (v_H), which is called the transphosphatidylation potential (v_T/v_H), or to the total transformation rate ($v_T + v_H$), which is called the transphosphatidylation selectivity ($v_T/(v_T + v_H)$), respectively, are decisive parameters. In addition to the acceptor alcohol and the reaction medium, these parameters mainly depend on the PLD source [3]. However, although a great variety of PLDs are known, the enzymes from only a few sources have been exploited for biocatalytical purposes. For industrial applications PLDs from selected *Streptomyces* strains are preferred due to the high yields of the transphosphatidylation product and the ready accessibility

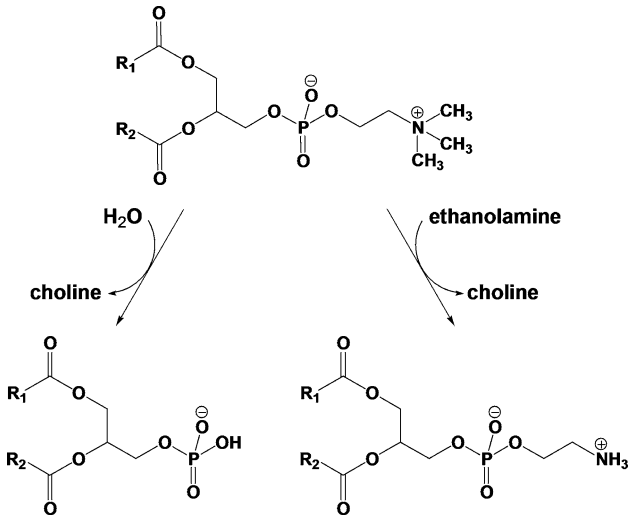

Fig. 1 Reaction scheme of PLD-catalyzed phospholipid transformations. In the presence of an acceptor alcohol such as ethanolamine, PC is transphosphatidylated (*right*) in competition with its hydrolysis (*left*). In both reactions choline is liberated

of the enzyme from bacterial culture media. In contrast to the bacterial enzymes, PLDs from plant sources are usually characterized by much higher production of the undesirable by-product PA. Nevertheless, they are beneficial in the introduction of certain alcohols such as *myo*-inositol [4]. The further progress in the application of enzyme-catalyzed phospholipid transformation will strongly depend on the availability of new PLD variants with high transphosphatidylation potentials. In addition to screening for new microbial and plant PLD sources, the techniques of directed evolution [5] present a challenge for finding and optimizing new PLD variants. The lack of an appropriate assay, which would allow the screening of a large number of mutants for high transphosphatidylation activity, seems to be the main limiting factor for exploiting methods such as random mutagenesis.

The most common method for the analysis of phospholipid transphosphatidylation is high performance thin layer chromatography (HPTLC), which is based on the separation of PC, PA and the transphosphatidylation product on silica with subsequent phospholipid staining and densitometric evaluation of the different phospholipid bands [6]. The head-group-specific interaction of phospholipids with silica matrices also makes possible the analysis of the reaction products by HPLC [7]. Even conductimetric measurements have been applied for the examination of transphosphatidylation reactions [8]. However, all these methods are not appropriate for high-throughput screenings because they require special equipment and are time-consuming. The transphosphatidylation of the synthetic substrate phosphatidyl-4-nitrophenol with ethanol [9] can be easily observed by measuring the release of 4-nitrophenol spectrophotometrically, but this method can only be applied to a few bacterial PLDs which do not show detectable hydrolysis in the presence of the substrate alcohol.

In the present paper, we describe a simple spectrophotometric assay, in which the transphosphatidylation potential as well as the transphosphatidylation selectivity of PLD-containing solutions, including cell lysates, can be determined on the microtiterplate scale. This assay is based on the spectrophotometric determination of PA, released from PC/Triton X-100 micelles, via the replacement of salicylate from the colored Fe(III)-salicylate complex (FeSal) as described recently [10] and the subsequent determination of liberated choline by the well-known choline oxidase reaction. In the presence of ethanolamine as acceptor alcohol, v_H can be determined from the increase of PA with time, while the determination of choline as a function of time yields the sum of v_T and v_H, which allows the calculation of v_T/v_H and $v_T/(v_T + v_H)$, respectively.

Experimental Procedures

Materials

PC (98%), PA (98%) and phosphatidylethanolamine (PE) (98%), all from soybean (*Glycine max*), were products of LIPOID GmbH, Germany. Choline oxidase from *Alcaligenes* sp., horseradish peroxidase, PLD from *Streptomyces* sp. type VII (sPLD), 4-aminoantipyrine and choline chloride were obtained from Sigma, USA. Phenol, water-free glycerol, 2-morpholinoethanesulfonic acid (MES) and 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) were received from Merck, Germany. Lysozyme from chicken egg white, ampicillin and boric acid were from Serva, Germany, and DNase I, RNase A, Triton X-100 and ethylenediaminetetraacetic acid (EDTA) were from Applichem, Germany. Ethanolamine and hydroxylamine hydrochloride were purchased from Fluka, Germany, and tryptone and yeast extract from Difco, USA. All other chemicals were of the highest purity commercially available.

Production and Purification of PLD Isoenzymes

α-Type PLD2 from white cabbage (cPLD) was produced and purified as described by Stumpe et al. [11]. PLD1 from opium poppy (pPLD) was obtained according to Lerchner et al. [12]. The determination of protein concentrations was performed by the Micro bicinchoninic acid protein assay kit from Pierce, following the manufacturer's instructions.

Cell Cultivation and Lysis

E. coli BL21 (DE3) cells containing the construct cpld2pRSET5a were grown in 100 µl LB-medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl, 100 µg ml^{-1} ampicillin) in microtiterplate wells at 15 °C and 650 rpm [13]. After 4 days, cells were harvested using a microtiterplate centrifuge at 3,220×*g* for 10 min. The cell pellet was suspended in 100 µl of 25% (w/v) sucrose, 10 mM Triton X-100, 10 mM EDTA, pH 8.0, and shaken for 10 min at 800 rpm. Then the cells were centrifuged at 3,220*g* for 10 min and treated for 20 min with 30 µl of lysis reagent (5 mM HEPES/NaOH, pH 7.5, 2 mM $MgCl_2$, 10 mM Triton X-100, 1.5 mg ml^{-1} lysozyme, 10 µg ml^{-1} RNase A, 15 µg ml^{-1} DNase I) under shaking (800 rpm) at 25 °C. After lysis, 90 µl of buffer (135 mM MES/NaOH, pH 5.6, 80 mM $CaCl_2$) containing the acceptor alcohol (267 mM ethanolamine/HCl, pH 5.6) for the subsequent phospholipid transformation were added. The cell debris was sedimented by centrifugation at 3,220×*g* for 10 min, and the supernatant (40 µl) containing the PLD activity was transferred to another microtiterplate for the subsequent phospholipid transformation with PA and choline determination.

Phospholipid Transformation

Reactions were performed in microtiterplate scale (80 µl) and started by the addition of PLD-containing solution or cell extract (40 µl) to the substrate solution (40 µl). The final mixtures contained 5 mM PC/5 mM Triton X-100 micelles and 100 mM ethanolamine/HCl, pH 5.6, in 100 mM MES/NaOH, pH 5.6, 60 mM $CaCl_2$. In the experiments with pure enzymes, the PLD concentrations were 0.4 (cPLD), 17.2 (pPLD) and 3.6 (sPLD) µg ml^{-1}, respectively. The PC/Triton X-100 micelles were prepared from evaporated chloroform solutions of PC as described in [10]. The reactions were performed with shaking at 400 rpm and 30 °C for defined time intervals and stopped by the FeSal reagent for PA determination as described in the following section. All experiments were performed in triplicate.

PA Determination by FeSal

The concentration of PA in microtiterplate scale was determined with a modification based on [10]. After phospholipid transformation (see above), 100 µl of FeSal solution (2 mM $FeCl_3$, 12 mM sodium salicylate, 1 M HCOOH/NaOH, pH 4.0) were added to the samples (80 µl). After equilibriation for 5 min at room temperature, the absorbance of the samples was measured at 490 nm yielding the PA concentration by means of a calibration curve obtained using PA/PC mixtures (total phospholipid concentration: 5 mM) in the same medium as used in the phospholipid transformation.

Enzymatic Choline Determination

After PA determination, the samples (180 µl) were decolored by the addition of 120 µl of neutralization buffer (600 mM boric acid/NaOH, 50 mM EDTA, pH 10.3). Choline determination was performed using a modification of the method described in [14]. The reaction was started by the addition of 20 µl of a mixture containing 5 mM 4-aminoantipyrine, 10 mM phenol, and 8.5% (v/v) glycerol in 50 mM boric acid/NaOH, pH 8.3, and 20 µl of enzyme solution containing 0.75 U ml^{-1} choline oxidase, 15 µg ml^{-1} horseradish peroxidase, and 8.5% (v/v) glycerol in 50 mM boric acid/NaOH, pH 8.3. In the measurements of *E. coli* extracts, the enzyme solution additionally contained 0.25 mM hydroxylamine hydrochloride to inhibit endogenous catalases [15]. The increase in absorbance at 490 nm was recorded between 3 and 15 min incubation at 23 °C. From the linear progress curves the choline concentration was determined by means of standard curves taken with mixtures containing *x* mM choline chloride, *x* mM PA, (5–*x*) mM PC and all other reagents used in the PA and choline determination steps except PLD. The activity of choline oxidase was sensitive to storage and was, therefore, precisely dosed in each run. In contrast, horseradish peroxidase could be used on the basis of protein concentration because it was stable and applied in excess.

Activity Assay for Choline Oxidase

The activity of choline oxidase (10 µg ml^{-1}) was determined in a reaction mixture containing 0.1 M choline chloride, 0.1 mg ml^{-1} horseradish peroxidase, 0.5 mM 4-aminoantipyrine, 1 mM phenol, 0.85% (v/v) glycerol in 100 mM boric acid/NaOH, pH 8.3. Measurements were performed in microtiterplates at 23 °C. Production of quinoneimine dye was measured continuously within 8 min at 490 nm. For calculation of reaction rates, a standard curve taken with H_2O_2 (0–60 µM) in the absence of choline oxidase and choline chloride was used. One unit (U) represents the amount of enzyme which liberates 1 µmol of H_2O_2 in 1 min.

Results and Discussion

Establishment of the Assay

The FeSal method recently developed for the spectrophotometric quantification of PA [10] was used to devise an

assay for the fast determination of both the hydrolytic and transphosphatidylation activities of PLD in the same microtiterplate. In this assay, the most common substrate PC was converted in a micellar reaction system in the presence of ethanolamine, which proved to be a good substrate for PLD-catalyzed transphosphatidylation [16]. In the first analytical step, v_H is determined by the measurement of PA formation from PC, while in the second step the sum of v_H and v_T is determined by the measurement of the choline liberation by the conventional spectrophotometric choline oxidase/peroxidase method. In the latter coupled enzymatic step, choline is oxidized by choline oxidase forming H_2O_2 which is subsequently used for the peroxidase-mediated production of a quinoneimine dye from 4-aminoantipyrine and phenol [14]. The difference between the two experimental values (rate of choline formation−rate of PA formation) yields v_T. As the development of the present assay was aimed at future high-throughput application in screening of PLD-containing mutant libraries, a check for its compatibility with cell cultivation and cell lysis conditions was included in the assay development.

In Fig. 2, the scheme of the assay including the cell cultivation and cell lysis steps, which may precede the analyses in another microtiterplate, is depicted. The establishment of the cell cultivation and lysis in microtiterplate scale, exemplified on transformed *E. coli* cells containing the DNA of cPLD, included experiments with varying volumes of growth medium (100–200 µl), periods of growth time (0–96 h), concentrations of EDTA (5–50 mM) and sucrose (0–25%, w/v) as well as experiments with varying incubation times (0–45 min) for cell disruption (results not shown). Moreover, the analytical reactions were adapted to the special conditions. It was shown that none of the reaction components in cell lysis and phospholipid transformation disturbed the PA determination by the FeSal assay. In contrast, the concentration of choline oxidase in the subsequent choline determination had to be carefully optimized because the ethanolamine used as acceptor alcohol in the PLD-catalyzed phospholipid transformation showed a certain inhibitory effect on choline oxidase. Moreover, the concentrations of hydroxylamine added as inhibitor of endogenous catalases and the concentrations of the reagents phenol and 4-aminoantipyrine were varied to check their influence on the results and to choose optimum conditions. In the section "Experimental Procedures" the optimized protocols are given. While the PA determination by the FeSal assay was performed in end point mode, the enzymatic choline determination was performed in kinetic mode. In this way PA concentration could be determined up to 3.5 mM, corresponding to 70% hydrolysis of PC, and choline could be determined up to 5 mM, corresponding to 100% conversion of PC. Figure 3a, b show the corresponding standard

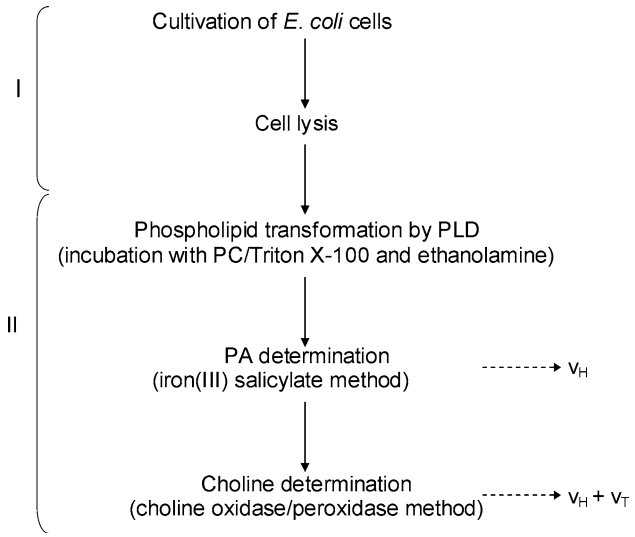

Fig. 2 Scheme of the procedure for the determination of the transphosphatidylation potential of PLD expressed in *E. coli* cells. Cell cultivation and lysis are performed in microtiterplate I, while phospholipid transformation and analytics are performed in microtiterplate II

curves. These curves could be well-fitted to hyperbolic functions from which PA and choline concentrations of unknown samples can easily be determined.

Application of the Assay to Purified PLDs from Different Sources

The designed assay was applied for comparing the transphosphatidylation and hydrolysis of PC with ethanolamine by three different PLD enzymes originating from cabbage (cPLD), poppy (pPLD), and *Streptomyces* sp. (sPLD). While the two plant enzymes were recombinantly produced in *E. coli* and purified to homogeneity as previously described [11, 12], the bacterial sPLD is a commercial product often used in phospholipid transformations. Figure 4a–c demonstrates the conversion of PC by the three enzymes as a function of time. While the formation of PA and the formation of choline, corresponding to the formation of PA + PE, are directly measured by the assay, the data representing the formation of PE result from the difference of the directly determined data. To confirm the identity of the results with those obtained by conventional methods, the cPLD-catalyzed conversion of PC was additionally followed by means of HPTLC [6, 10] (Fig. 4a).

Table 1 summarizes the initial rates v_H and v_T derived from the progress curves and the relative transphosphatidylation parameters v_T/v_H and $v_T/(v_T + v_H)$. For comparison, the initial rate of hydrolysis was also determined in the absence of an acceptor alcohol. In these experiments, all reaction conditions were the same as described in the "Experimental Procedures" but ethanolamine was omitted.

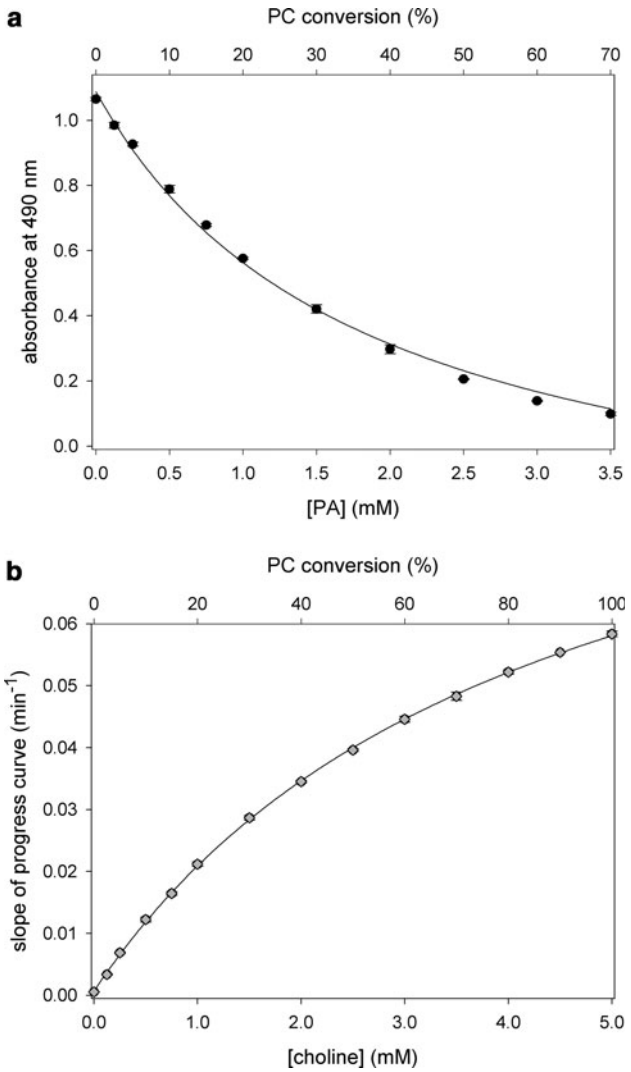

Fig. 3 Standard curves for PA (**a**) and choline (**b**) determination. Definite amounts of PA or choline were added to PLD-free reaction mixtures as used for phospholipid transformation and determined as described in the "Experimental Procedures"

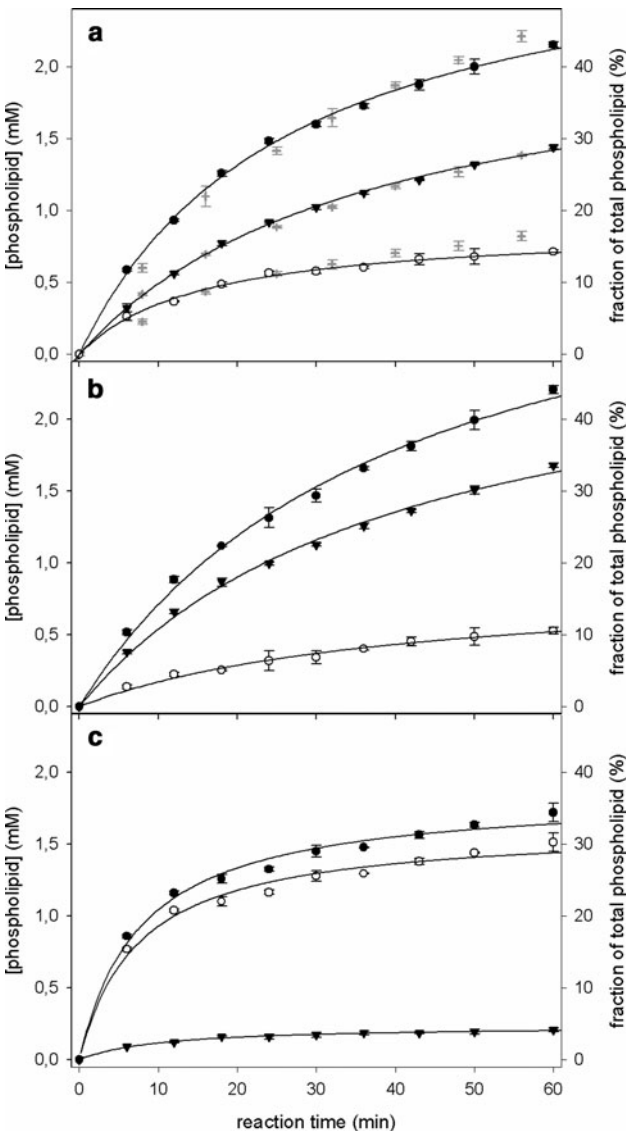

Fig. 4 Phospholipid transformation by cPLD (**a**), pPLD (**b**) and sPLD (**c**). Reactions were performed with PC/Triton X-100 vesicles and ethanolamine in the presence of the purified enzymes as described in the "Experimental Procedures". *Filled circles* Choline released (= PA + PE), *inverted filled triangles* PA, *open circles* PE, calculated. Progress curves were fitted according to a hyperbolic function. The data marked by a *plus sign* show corresponding data determined by HPTLC as described previously [6, 10]

The results show that the reaction rates are strongly dependent on the source of the PLD used, which is in accordance with other literature reports [3, 16–18]. cPLD shows the highest rates of phospholipid conversion, while the rates for the enzyme from poppy, although the amino acid sequence shows 87% similarity [12], are lower by at least one order of magnitude. The values for sPLD with <10% identity to the plant PLDs [19] are intermediate. Above all, the results demonstrate great differences in the transphosphatidylation potentials and the transphosphatidylation selectivities, respectively. Here sPLD is highly superior to cPLD and even more so to pPLD. In all cases, the initial rates of PA formation in the transphosphatidylation reactions are very similar compared to the hydrolysis reaction in the absence of ethanolamine, indicating that hydrolysis is unaffected by the presence of the acceptor alcohol.

The transphosphatidylation potentials of PLDs tested here indicate similar trends as determined previously in two-phase reaction systems containing organic solvents by HPTLC [3, 12, 16], showing that the aqueous micellar reaction system used in this assay is highly appropriate to characterize the transphosphatidylation properties. Even the relatively low ethanolamine concentration compared to the acceptor alcohol concentrations used in other studies

Table 1 Initial rates of transphosphatidylation (v_T) and hydrolysis (v_H) in the reaction of PC with ethanolamine catalyzed by different PLD enzymes

Enzyme	v_T (μmol min^{-1} mg^{-1})	v_H (μmol min^{-1} mg^{-1})	v_T/v_H	$v_T/(v_T + v_H)$	$v°_H$ (μmol min^{-1} mg^{-1})
cPLD	70.55 ± 2.07	79.33 ± 2.13	0.89 ± 0.04	0.47 ± 0.07	82.30 ± 0.98
pPLD	0.62 ± 0.08	1.99 ± 0.04	0.31 ± 0.04	0.24 ± 0.02	1.65 ± 0.02
sPLD	30.44 ± 1.24	3.93 ± 0.18	7.75 ± 0.47	0.89 ± 0.09	4.04 ± 0.09

The reactions were performed and analyzed as described in the Experimental Procedures. $v°_H$ gives the initial rate of hydrolysis in the absence of ethanolamine

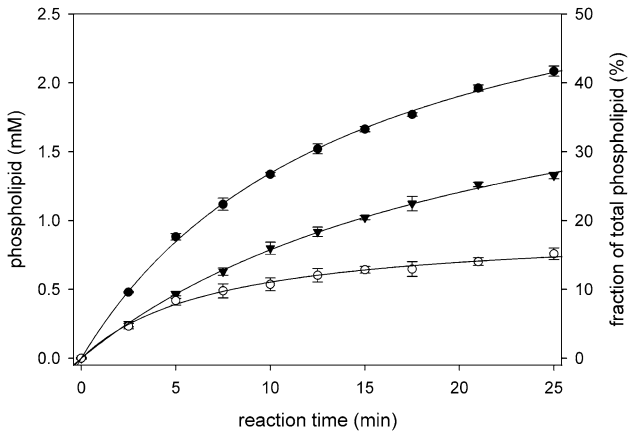

Fig. 5 Phospholipid transformation by cell extract. Reactions were performed with PC/Triton X-100 vesicles and ethanolamine in the presence of cell extract as described in the "Experimental Procedures". *Filled circles* Choline released (= PA + PE), *inverted filled triangles* PA, *open circles* PE, calculated. Progress curves were fitted according to a hyperbolic function

[3, 20] is sufficient to yield respectable transphosphatidylation rates.

Application of the Assay to Cell Lysates

E. coli cells overexpressing cPLD were grown and disrupted by a chemoenzymatic lysis in a microtiterplate as described in the "Experimental Procedures". The PLD-containing extracts were transferred to a second microtiterplate where the phospholipid transformation was performed. Figure 5 demonstrates the progress of the reaction. The rate of hydrolysis reaction was 0.91 ± 0.05 μmol min^{-1} ml^{-1}, and transphosphatidylation proceeded with 1.07 ± 0.03 μmol min^{-1} ml^{-1}. The transphosphatidylation potential (0.85 ± 0.05) and the transphosphatidylation selectivity (0.54 ± 0.02) were similar to the parameters of purified cPLD (Table 1), which verifies the quality of the method. In high-throughput experiments, one time point in the initial period of reaction (e.g. 10 min) should be sufficient to estimate the transphosphatidylation potential. Therefore, 96 enzyme variants can be grown per 96-well microtiterplate and subsequently analyzed in a second microtiterplate.

Conclusions

The method presented is the first spectrophotometric assay that allows the determination of the transphosphatidylation activity relative to the competing hydrolytic activity of PLD in one microtiterplate. As demonstrated for *E. coli* cells expressing cPLD, the assay can be combined with a cell growth and lysis procedure in a second upstream microtiterplate, which provides the basis for the high-throughput application in screening metagenomic or combinatorial DNA libraries encoding PLD variants.

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