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On the effects of site-specific mutations on activity and expression of the *Streptomyces* PMF phospholipase D

Claudio Fedeli, Giacomo Carrea, Daniela Monti *

Istituto di Chimica del Riconoscimento Molecolare, C.N.R., Via Mario Bianco 9, 20131 Milan, Italy

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Abstract

The effects of specific mutations on the hydrolytic activity and heterologous expression levels of Streptomyces PMF phospholipase D (PLD–PMF), an enzyme industrially employed for glycerophospholipid (GPL) modification, have been studied by using natural GPLs and synthetic chromogenic derivatives (*p*-nitrophenyl derivatives) as substrates. Specifically, the essential role of the histidines (H167 and H440) and lysines (K169 and K442) of the two highly conserved $HXKX_4DX_6G(G/S)X(D/N)$ (HKD) domains for catalysis was shown by isolation and characterization of the corresponding enzyme variants, whose activity was completely lost without any significant structural modification. The abolishment of the phospholipase D (PLD) activity strongly influenced the expression levels in *Escherichia coli*, with recovery yields 5–10 times higher than with the wild-type enzyme, thus indicating that citotoxicity for this host is indeed strictly correlated to enzyme activity. On the contrary, enzyme variants where the conserved aspartic acids (D174 and D447) of the two HKD domains were replaced with asparagine residues showed a decreased but still detectable PLD activity. The hypothesis that these conserved residues might play a structural role is supported by evidence of net superficial charge modifications observed during enzyme variants purification.

Keywords: Phospholipase D; Streptomyces PMF; Site-directed mutagenesis; HKD Domain; Heterologous expression

1. Introduction

Phospholipase D (PLD; EC 3.1.4.4) catalyzes the hydrolysis of phospholipids, e.g., phosphatidylcholine (PC), into phosphatidic acid (PA) and the corresponding alcohol. In addition to this hydrolytic activity, PLD also catalyzes the competing reaction of transesterification (transphosphatidylation) in the presence of different alcohols to form new phospholipids [1,2]. PLD activities have been detected in simple to complex organisms from viruses and bacteria to yeast, plants, and mammals. When compared with their eukaryotic counterparts, prokaryotic PLDs show a broader substrate specificity and a marked preference towards the catalysis of transphosphatidylation reactions in the respect of hydrolytic transformations. As a consequence, these enzymes have been exploited as biocatalysts for the synthesis of modified glycerophospholipids (GPLs) of interest for the pharmaceutical and food industries, starting from the low cost natural phosphatidylcholine [3,4].

Moreover, it has been recently shown that bacterial PLDs are part of a superfamily of proteins, the so-called PLD superfamily [5,6], which includes PLDs from different sources, phosphatidylserine and cardiolipin synthases, bacterial endonucleases, and a human tyrosyl-DNA phosphodiesterase [1,7]. Beyond their functional similarity (all these enzymes recognize phosphodiester substrates and catalyze their cleavage with water or different alcohols acting as nucleophiles), the members of the PLD superfamily share the presence, usually in two copies, of the signature $HXKX_4DX_6G(G/S)X(D/N)$ motif, named the HKD motif, and have been suggested to be structurally and mechanistically related. Since prokaryotic PLDs are substantially smaller than the eukaryotic enzymes, they are particularly suitable as models for a deeper understanding of the common catalytic mechanism and of the role of conserved residues.

Recently, the gene coding for the *Streptomyces* PMF PLD (PLD–PMF) [8], an enzyme industrially employed for GPL modification whose 3-D structure has been solved by X-ray analyses [9], has been cloned and expressed in *Escherichia coli*, affording a soluble and active recombinant PLD, suitable for structural and functional studies [10].

* Corresponding author. Tel.: +39 02 28500038; fax: +39 02 28901239.
E-mail address: daniela.monti@icrm.cnr.it (D. Monti).

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A general reaction mechanism was lately deduced from the X-rays analyses of PLD–PMF crystals soaked in short-chain substrates and products [11]. The proceeding of the phosphoryl transfer reaction through an S_N2-type associative pathway and the formation of a pentacoordinate intermediate, specifically a phosphohistidine intermediate between the product (PA) and the conserved histidine of the N-terminal HKD domain (H167, numbering according to [10]), was shown by short-time soaking experiments using dibutyryl phosphatidylcholine as a substrate. These results support the hypothesis that H167 acts as the nucleophile which attacks the phosphorus atom of the substrate phospholipid, whereas the conserved histidine of the C-terminal HKD domain (H440) seems to play a role as hydrogen donor for the releasing of the leaving group (e.g., choline) and in the activation of the entering nucleophile (water or a different alcohol).

The role of the other conserved residues of the HKD motif is not fully cleared yet. The results obtained with different members of the PLD superfamily suggest that the conserved lysines can be implicated in substrate binding [9,12–14], as well as in transition state stabilization [15], whereas a structural role has been hypothesized for the aspartate residues [9,12].

In this study, we used site-directed mutagenesis to evaluate the effect of amino acids substitutions in the HKD domains of PLD–PMF on enzyme hydrolytic activity. Moreover, since it has been suggested that the low levels of production of PLDs from Streptomycetes in *E. coli* cells are caused by enzyme toxicity for this host [10,16,17], the effects of enzyme inactivation by site-directed mutagenesis on the expression levels have been investigated.

2. Experimental

2.1. Bacterial strains and plasmids

E. coli BL21(DE3)pLysE and pET27b(+) were purchased from Novagen (Merck KGaA, Darmstadt, Germany). The expression vector, pETPLD, was produced by in frame cloning of the DNA fragment encoding the mature phospholipase D from *Streptomyces* sp. strain PMF (PLD–PMF) downstream of the *Erwinia carotovora pelB* leader sequence of pET27b(+), as previously described [10].

2.2. Site-directed mutagenesis

All the mutations of the PLD–PMF gene were performed according to the protocol of the QuikChange II® XL Site-Directed Mutagenesis Kit (Qiagen) using pETPLD as template, *PfuUltra* polymerase for amplification, and the following primer pairs (all purchased from MWG Biotech, mutagenic triplets are in bold): 5′-GCGTTCTCCTGGAAC**AAC**TCCAAGATCCTCGTG-3′ and 3′-CGCAAGAGGACCTTG**TTG**AGGTTCTAGGAGCAC-5′ (H167N-PLD); 5′-CACCCGTACGCGCAG**AAC**CACAAGCTGGTCTCC-3′ and 3′-GTGGGCATGCGCGTC**TTG**GTGTTCGACCAGAGG-5′ (H440N-PLD); 5′-TCCTGGAACCACTCC**TCG**ATCCTCGTGGTCGAC-3′ and 3′-AGGACCTTGGTGAGG**AGC**TAGGAGCACCAGCTG-5′ (K169S-PLD); 5′-TACGCGCAGCACCAC**AGC**CTGGTCTCCGTCGAC-3′ and 3′-ATGCGCGTCGTGGTG**TCG**GACCAGAGGCAGCTG-5′ (K442S-PLD); 5′-TCCTCGTGGTC**AAC**GGCCAGTCGGC-3′ and 3′-AGGAGCACCAG**TTG**CCGGTCAGCCG-5′ (D174N-PLD); 5′-GCTGGTCTCCGTC**AAC**AGCTCCACGTTCT-3′ and 3′-CGACCAGAGGCAG**TTG**TCGAGGTGCAAGA-5′ (D447N-PLD). Mutations were confirmed by dideoxy DNA sequencing of the double-stranded plasmids using the DYEnamic ET Dye Terminator Cycle Sequencing System with Thermo Sequenase II DNA Polymerase and the automated MegaBACE 1000 96-capillary sequencer (Amersham Biosciences). For each mutation, the entire gene insert coding for mature PLD–PMF (1518 bp) was sequenced to ensure that no second site mutations were present, and none were found.

2.3. Heterologous expression of wild-type and mutant PLDs in E. coli

Freshly transformed *E. coli* BL21(DE3)pLysE harboring pETPLD or mutagenized plasmids was cultured in 50 ml of LB medium containing 50 μg/ml kanamycin and 25 μg/ml chloramphenicol at 37 °C and 220 rpm until $OD_{600} = 1$. The culture was used to inoculate 500 ml of fresh medium and growth of recombinant *E. coli* was carried out at 30 °C and 220 rpm until $OD_{600} = 1$. Isopropyl-β-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM and the culture was kept at 25 °C for 24 h. Wild-type and mutant PLDs production was monitored by collecting culture samples at scheduled times and separating cells and medium by centrifugation (1 min at 13,000 rpm and 4 °C). Total cell extracts were obtained by cell lysis and protein solubilization in the presence of Sample Buffer 1X (SB1X = 62.5 mM Tris–HCl buffer, pH 6.8, 10% v/v glycerol, 2% w/v SDS, 0.001% w/v bromophenol blue, 5% (v/v) β-mercaptoethanol) for 10 min at 100 °C. PLD activity assays with the synthetic substrate phosphatidyl *p*-nitrophenol (PpNP) were carried out on the culture medium samples immediately after centrifugation. For detection of PLD released in the culture medium, the latter was stored frozen at −20 °C until proteins were concentrated 20-fold by 10% (v/v) TCA precipitation and resuspension of the pellets in adequate volumes of SB1X for subsequent immunoblotting analyses.

2.4. Purification of wild-type and mutant PLDs from E. coli culture supernatants

Secretion in the culture medium of both wild-type and mutant PLDs produced by recombinant *E. coli* cells was expected to occur after post-translational removal of the PelB leader peptide. Wild-type PLD–PMF was purified after heterologous expression in *E. coli* as previously described [10]. Mutant PLDs were purified from 500 ml of the corresponding culture supernatants collected by centrifugation 24 h after IPTG induction. The enzyme variants were precipitated by adding $(NH_4)_2SO_4$ (65% saturation), resuspended in 10 mM sodium acetate buffer, pH 6.0, and extensively dialyzed against the same buffer. The dialyzed solu-

tion (20 ml) was centrifuged to remove precipitated materials and applied to a Fractogel TSK CM-650 (M) (Merck) column (16 mm × 140 mm), previously equilibrated with 10 mM sodium acetate buffer, pH 6.0, at a flow rate of 1 ml/min. After loading and washing the column with the equilibration buffer, the bound proteins were eluted by a linear gradient from 0 to 0.5 M NaCl in the same buffer in 2 h at 1 ml/min. Dot blot analyses were carried out on the fractions (3 ml) collected during the gradient. H167N-PLD (1.5 mg), K169S-PLD (2.5 mg), H440N-PLD (2 mg) and K442S-PLD (2.2 mg) were eluted at 0.25 M NaCl, dialyzed against Tris–HCl buffer, pH 8.0, and lyophilized. D174N-PLD and D447N-PLD were detected among the unbound proteins by dot blot analyses and not further purified.

2.5. Enzyme and protein assays

PLD activity during expression experiments and purification was determined using the synthetic phosphatidyl *p*-nitrophenol substrate [18]. 30 μl of a 10 mg/ml solution of PpNP in 0.1 M Tris–HCl buffer, pH 8.0, containing 10% (v/v) Triton X-100, were diluted with 0.1 M Tris–HCl buffer, pH 8.0, to a final volume of 1 ml. Production of *p*-nitrophenol after addition of enzyme solution (10–100 μl) was monitored spectrophotometrically at 405 nm and 30 °C ($\varepsilon_{405\,nm} = 18450\,M^{-1}\,cm^{-1}$). Homogenous PLDs were also assayed using the natural substrate, phosphatidylcholine, in the spectrophotometric coupled assay described in [19]. Five to fifty microliter of enzyme solution were mixed to 135 μl of phosphatidylcholine solution (12.5 mg/ml in 0.1 M Tris–HCl buffer, pH 8.0, containing 5% (v/v) Triton X-100), 70 μl of choline oxidase solution (50 U/ml in 0.1 M Tris–HCl buffer, pH 8.0), 70 μl of peroxidase solution (100 U/ml in 0.1 M Tris–HCl buffer, pH 8.0), 35 μl of $CaCl_2$ solution (100 mM in 0.1 M Tris–HCl buffer, pH 8.0), and 700 μl of 4-aminoantipyrine (4-APA)/phenol reagent. The 4-APA/phenol reagent was freshly prepared by mixing 2 ml of phenol solution (2 mg/ml in 0.1 M Tris–HCl buffer, pH 8.0), 2 ml of 4-APA solution (3 mg/ml in 0.1 M Tris–HCl buffer, pH 8.0) and 21 ml of 0.1 M Tris–HCl buffer, pH 8.0. The reaction course was monitored spectrophotometrically at 550 nm and 37 °C ($\varepsilon_{550\,nm} = 6000\,M^{-1}\,cm^{-1}$).

Protein concentration was determined using the Bio-Rad Protein Assay according to Bradford [20], using bovine serum albumine as a standard.

2.6. Electrophoretic and immunoblotting analyses

SDS-PAGE was performed on 10% polyacrylamide gels according to Laemmli [21] and proteins were stained with 0.25% (w/v) Coomassie Blue R-250 solution in 50% (v/v) ethanol and 10% (v/v) acetic acid. Molecular mass under denaturing conditions was determined using standard proteins from Bio-Rad (myosin, 200.000 Da; β-galactosidase, 116.250 Da; phosphorylase b, 97.400 Da; serum albumin, 66.200 Da; ovalbumin, 45.000 Da; carbonic anhydrase, 31.000 Da; trypsin inhibitor, 21.500 Da).

Western blotting was performed using Novablot kit and Multiphor II Electrophoresis Unit (Amersham Biosciences). A Hybond ECL nitrocellulose membrane (Amersham Biosciences) was used for protein transfer using a constant current value of 0.8 mA/cm^2 for 3 h. Before electrophoretic separation, culture media samples were concentrated by adding 0.1 volume of 100% (w/v) TCA to 1 ml of culture medium, resuspending the pellet into 50 μl of SB1X and incubating at 100 °C for 3 min. Cell extracts were prepared by resuspension of cell pellets into adequate amounts of SB1X and boiling for 3 min. Anti-wild-type-PLD antibody binding to nitrocellulose membrane was detected with alkaline phosphatase-conjugated anti-rabbit goat IgGs (Sigma) as a second antibody and using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium as substrates [22]. Dot blot analyses were performed with the same procedure by directly spotting the samples (1 μl) onto nitrocellulose membrane.

2.7. Circular dichroism

CD spectra were recorded on a Jasco J600 spectropolarimeter interfaced to a personal computer for data collection and manipulation. Wavelength scans (190–250 nm) were carried out at 25 °C with wild-type and mutant protein samples (0.1 mg/ml) dissolved in 20 mM potassium phosphate buffer, pH 7.0, in a 0.1 cm-path length quartz cuvette. All protein spectra were baseline-corrected by subtracting buffer spectra.

3. Results and discussion

We have recently developed an heterologous expression system of *Streptomyces* PMF *pld* gene using *E. coli* as a host [10], which was shown to afford a soluble and active PLD with physical-chemical and catalytic properties identical to those of the wild-type protein. Specifically, the region of the DNA frag-

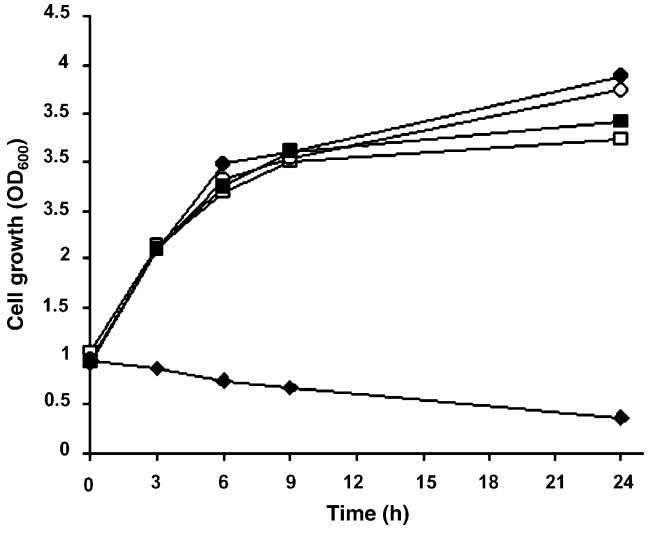

Fig. 1. Cell growth in *E. coli* BL21(DE3)pLysE during the expression of *Streptomyces* PMF wild-type PLD (♦), and of the H167N-PLD (□), K169S-PLD (■), H440N-PLD (○), K442S-PLD (●) enzyme variants. The recombinant strains were cultured at 30 °C until OD_{600} was 1.0, then production of wild-type and mutant PLDs was induced (time = 0) and the cultures were kept at 25 °C and 220 rpm for 24 h.

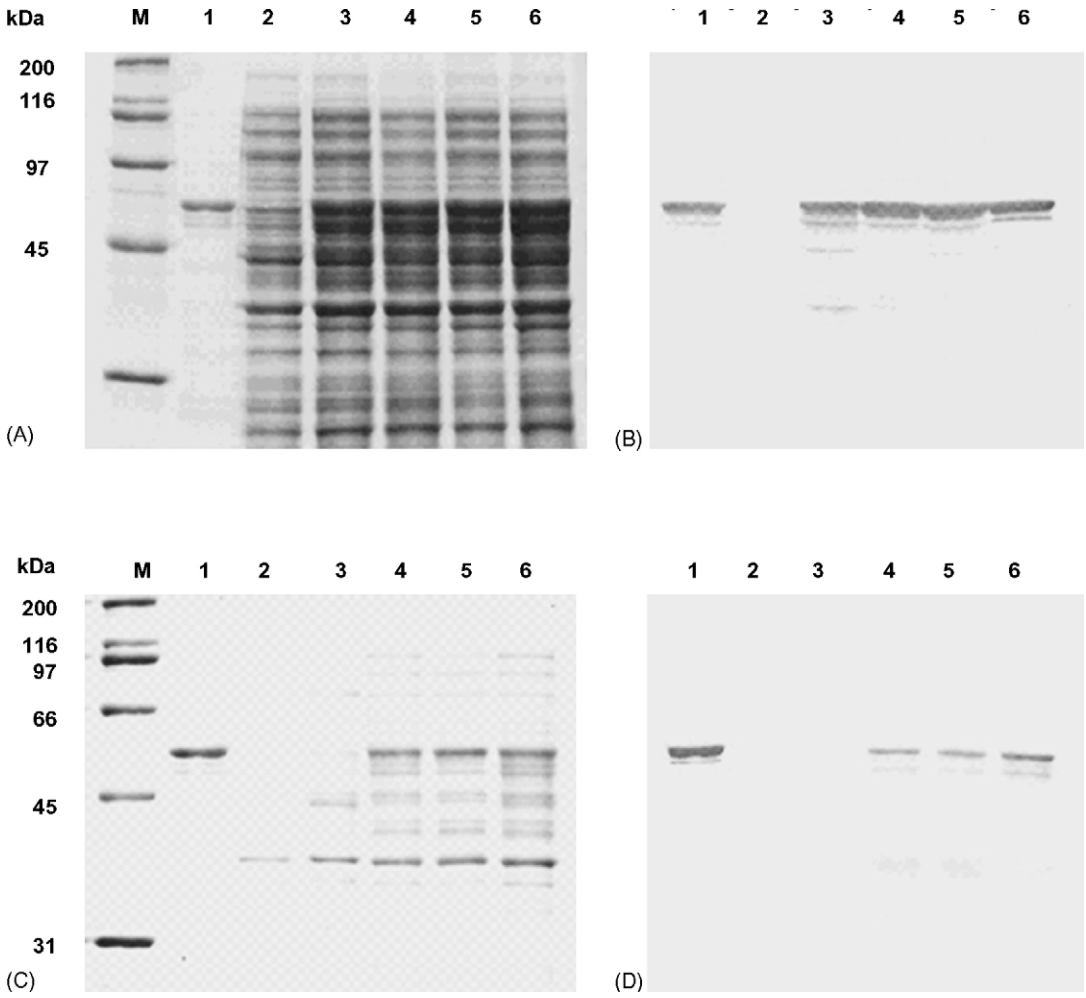

Fig. 2. SDS–PAGE (A, C) and Western blot analyses (B, D) against anti-wild-type-PLD serum of culture of *E. coli* BL21(DE3)pLysE during the expression of K169S-PLD. (A) and (B) cell extracts; (C) and (D) concentrated media samples. In each panel: (M), molecular mass markers (mass in thousand); (1), wild-type PLD–PMF, (2–6), cells and media samples taken at 0, 3, 6, 9 and 24 h, respectively, after induction with IPTG. Almost identical profiles were observed during the expression of H167N-PLD, H440N-PLD and K442S-PLD.

ment encoding the mature *Streptomyces* PMF PLD protein was cloned into the *Nco*I-*Eco*RI sites of the pET27b(+) vector, yielding pETPLD. In this construct, the sequence encoding the mature *Streptomyces* PMF PLD sequence was fused at the C-terminus of the *Erwinia carotovora pelB* leader sequence and, as expected, recombinant *Streptomyces* PMF PLD was found to be secreted into the culture medium after post-translational removal of the PelB leader peptide [10].

Since, at present, *Streptomyces* PMF PLD is the only phospholipase D whose three-dimensional structure has been solved by crystallographic analyses, the availability of a simple system for its modification, production and purification can help in understanding of the molecular properties of PLDs and related enzymes and exploiting their biocatalytic potential.

In this work, we employed this expression system for the generation of variants of the enzyme by site-directed mutagenesis of the *pld* gene, specifically addressing the highly conserved histidines, lysines and aspartic acids of the two HKD domains. On the basis of previous structural and mechanistical studies carried out on different members of the PLD superfamily [7,13,14], histidines of the first or second conserved motif were replaced with asparagine (H167N and H440N, respectively), lysines were substituted with serine (K169S and K442S) and the aspartic acids with asparagine (D174N and D447N).

Expression of mutant proteins was performed using freshly transformed *E. coli* BL21(DE3)pLysE cells harboring the corresponding mutated plasmid and inducing enzyme expression with 0.1 mM IPTG. After induction, the cultures were grown at 25 °C for about 24 h and, at scheduled times, samples were taken and cell growth and PLD activity release in the culture broth were monitored. Moreover, protein production and partitioning between cells and culture medium was evaluated by western blot analyses of cell extracts and concentrated culture medium using anti-wild-type PLD antibodies.

Expression of PLDs mutated in the histidines and lysines (H167N-PLD, K169S-PLD, H440N-PLD and K442S-PLD) showed almost identical profiles both during protein production and subsequent analyses. As shown in Fig. 1, whereas a significant decrease of cell growth rate was observed after IPTG addition (time = 0) during the expression of wild-type PLD–PMF, *E. coli* cells maintained viability during the expression of these four mutant proteins.

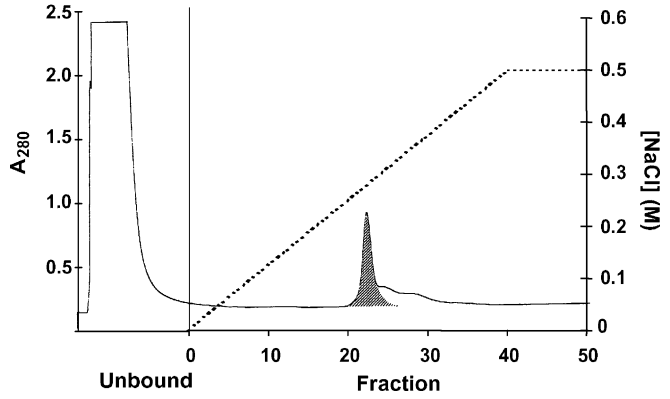

Fig. 3. Separation of K169S-PLD (dashed peak) from total culture supernatant proteins (solid line) by cation-exchange chromatography in relation to NaCl gradient (dotted line). Elution of the inactive PLD–PMF variant was detected by dot blot analyses (see Section 2). Almost identical profiles were observed during the purification of H167N-PLD, H440N-PLD and K442S-PLD.

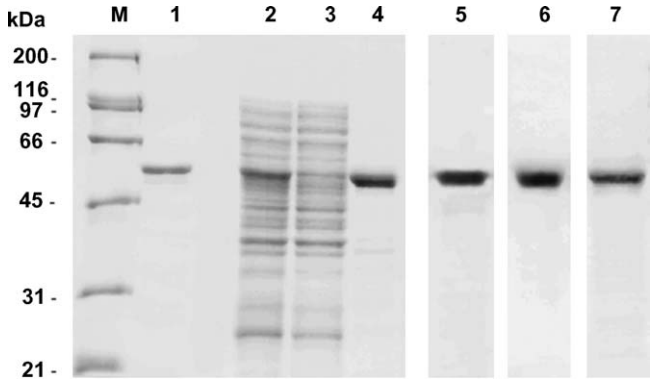

Fig. 4. Purification of PLD–PMF variants (specifically K169S-PLD, H167N-PLD, H440N-PLD and K442S-PLD) monitored by SDS–PAGE: (M), molecular mass markers (mass in thousand); (1), purified wild-type PLD–PMF; (2), K169S-PLD culture supernatant proteins after 65 % $(NH_4)_2SO_4$ precipitation and dialysis; (3), unbound proteins after K169S-PLD culture supernatant proteins loading on CM, pH 6.0; (4), purified K169S-PLD after CM, pH 6.0; (5), purified H167N-PLD; (6), purified H440N-PLD; (7), purified K442S-PLD.

The lack of toxic effect on host cells was accompanied by the loss of PLD activity released in the medium, as assayed on culture broth samples collected at various times using the synthetic substrate phosphatidyl *p*-nitrophenol.

The production and localization of the inactive variants H167N-PLD, K169S-PLD, H440N-PLD and K442S-PLD was shown by SDS–PAGE and Western blot analyses against anti-wild-type-PLD serum of cell extracts and concentrated media samples. Fig. 2 shows the results obtained during the expression of K169S-PLD and almost identical profiles were observed during the expression of H167N-PLD, H440N-PLD and K442S-PLD. Expression was efficiently induced after IPTG addition, leading to intracellular accumulation (Fig. 2A–B) and secretion in the culture medium (Fig. 2C–D) of the mutated proteins. The production levels of the PLD variants in cell extracts and culture media were significantly higher than those observed for wild-type PLD–PMF, being detected also by Coomassie Blue staining of SDS-PAGE gels and not only by Western blot analyses as in the case of the active protein.

H167N-PLD, K169S-PLD, H440N-PLD and K442S-PLD were recovered from culture medium (0.5 l each) by ammonium sulfate precipitation and, thanks to the higher expression levels and low host lysis, purified to homogeneity by one single cation-exchange chromatographic step. Fig. 3 shows the efficient separation of K169S-PLD from total culture supernatant proteins and similar chromatographic profiles were observed during the purification of H167N-PLD, H440N-PLD and K442S-PLD. As the mutated PLDs were completely inactive towards the commonly used synthetic substrate PnNP, elution was detected by dot blot analyses on fractions collected during the purification and confirmed by subsequent SDS-PAGE analyses of the purified proteins (Fig. 4, lanes 4–7).

The recovered yields of mutant proteins (3–5 mg/l culture, see Section 2) confirmed the significant improvement of the expression level of these inactive variants in comparison with wild-type PLD–PMF (0.5 mg/l culture) and are in agreement with our preliminary results obtained during the optimization of the expression system [10], which suggested a relationship between PLD activity and host cell viability. Nonetheless, as also in the case of these inactive variants the amount of synthesized PLD reached a plateau in a few hours after induction, both at the intracellular level and in the culture medium, it is likely that a different mechanism(s), for instance protein accumulation at periplasmic level, can be involved in limiting enzyme over expression.

As reported for other members of the PLD superfamily [7,13,14], these mutations were expected to specifically affect the enzyme catalytic performances without significant effects on the overall protein structure. Our results confirm these hypotheses. The chromatographic behavior of these enzyme variants on cation-exchange resins was indistinguishable from that of wild-type PLD–PMF, thus indicating that mutations did not affect the surface net charge. Moreover, conservation of the secondary structure was confirmed by circular dichroism analyses of purified wild-type and mutated PLDs (Fig. 5).

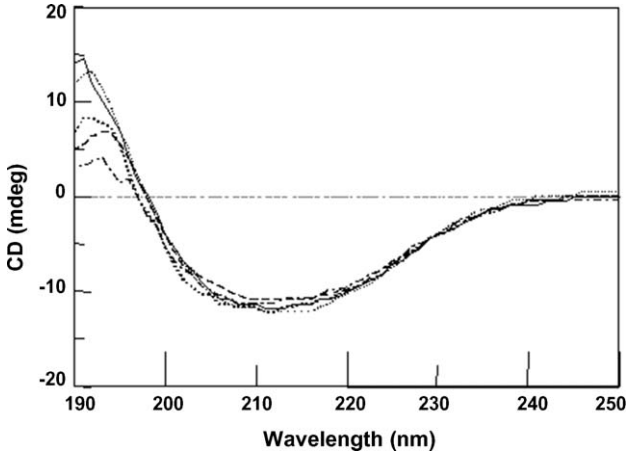

Fig. 5. CD analysis of purified PLD–PMF variants (specifically K169S-PLD, H167N-PLD, H440N-PLD and K442S-PLD). (—), wild-type PLD–PMF; (▪▪▪▪▪), K169S-PLD; (- - -), H167N-PLD; (· · ·), H440N-PLD; (–·–), K442S-PLD. All protein spectra were baseline-corrected by subtracting buffer spectra (see Section 2).

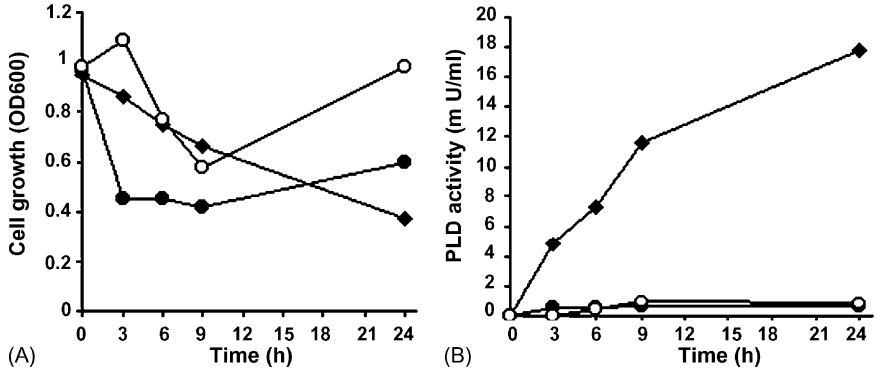

Fig. 6. Cell growth (A) and PLD activity released in the medium (B) in *E. coli* BL21(DE3)pLysE during the expression of wild-type PLD–PMF (♦), and of D174N-PLD (●) and D447N-PLD (◯) enzyme variants. The recombinant strains were cultured at 30 °C until OD_{600} was 1.0, then production of wild-type and mutant PLDs was induced (time = 0) and the cultures were kept at 25 °C and 220 rpm for 24 h. Activity assays were performed using the synthetic substrate PpNP as described in Section 2.

Hydrolytic activity of purified H167N-PLD, K169S-PLD, H440N-PLD and K442S-PLD was evaluated using phosphatidylcholine as the substrate, by monitoring the rate of formation of choline with a coupled choline oxidase-peroxidase spectrophotometric assay. All these substitutions resulted in the complete loss of the phosphodiesterase activity on the natural substrate, even when using a purified protein amount one hundred times higher than with the wild-type protein. The complete enzyme inactivation after substitution of these highly conserved residues indicates an essential role for catalysis not only for the histidine residues, which are supposed to act in the reaction mechanism as the initial nucleophile (H167) and a general acid-base (H440), respectively [11], but also for the lysine residues (K169 and K442). In fact, if the role of these conserved lysines in substrate binding has been early suggested by crystal structure analyses [9,12], the deleterious effect of K169S and K442S substitutions suggests that they could be also implicated in the catalytic mechanism, e.g., in transition state stabilization, as recently described for another member of the PLD superfamily, the human tyrosyl-DNA phosphodiesterase [15].

Concerning the expression of D174N-PLD and D447N-PLD, a highly toxic effect on *E. coli* cells, which were no more viable and underwent lysis, was observed after IPTG addition (Fig. 6A). Surprisingly, this toxic effect, which was comparable to that related to wild-type PLD production, was not accompanied by a corresponding release of enzymatic activity in the culture medium, whose levels were 30–40 times lower than those observed during the expression of PLD–PMF (Fig. 6B).

Although D174N-PLD and D447N-PLD could be detected, after ammonium sulfate precipitation of culture medium proteins, both by dot blot analyses and PLD activity assays, we failed in their purification by cation-exchange chromatography from the abundant *E. coli* proteins released as a consequence of cell lysis. In fact, these two enzyme variants did not bind to the carboxymethyl resin and were recovered during the washing step with the equilibration buffer. This significant change of the chromatographic behavior of D174N-PLD and D447N-PLD in respect of wild-type PLD–PMF, as well as of the first set of mutants (H167N-PLD, K169S-PLD, H440N-PLD and K442S-PLD), suggests the possible occurrence, in these cases, of tertiary structure modifications with consequent effects on protein surface net charges. This is in agreement with previous structural studies of PLDs and of other members of the PLD superfamily, which showed that the aspartate residues of the HKD motifs are located far from the active site, forming a series of stabilizing hydrogen bonds with surrounding amino acids [9,12]. Moreover, the fact that mutants at D174 and D447 positions maintained, albeit at a lower extent, their phosphodiesterase activity, is a further confirmation that these residues are not essential for catalysis.

4. Conclusions

The expression system of *Streptomyces* PMF *pld* gene in *E. coli* is suitable for the generation of variants of the enzyme by site-directed mutagenesis of the *pld* gene on the basis of modeling studies. Moreover, the exploitation of specific anti-wild-type PLD antibodies permits the recognition and the study of mutants with low or no hydrolytic activity, such as those presented herein. Further investigations by mutagenesis techniques and structural analyses may provide new insights into the biological functions and biocatalytic potential of phospholipases D and related enzymes.

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