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Evolution in the Amidohydrolase Superfamily: Substrate-Assisted Gain of Function in the E183K Mutant of a Phosphotriesterase-like Metal-Carboxylesterase†

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ABSTRACT: The recent specialization for utilization of pesticides reported for *Pseudomonas diminuta* phosphotriesterase (*p*PTE) strongly suggests that this activity evolved from an enzyme endowed with promiscuous phosphotriesterase activity. Such a putative "generalist" enzyme was recently proposed to be a member of the new phoshotriesterase-like lactonase family (PLL). The promiscuous carboxylesterase and phosphodiesterase activities detected in pPTE and PLLs in turn paved the way for the prediction of the existence in nature of PTE-like enzymes with predominant carboxylesterase or phosphodiesterase activities. An "in silico" analysis of the related *Mesorhizobium loti* ORF MLL7664 and the biochemical characterization demonstrated its prominent carboxylesterase and low phosphotriesterase specificity. On the basis of sequence similarity with the phosphotriesterase homology protein from *Escherichia coli* and the carboxylesterase activity, we called it phosphotriesterase-like carboxylesterase (*Mlo*PLC). The carboxylesterase activity is strictly dependent on divalent cations, and as such *Mlo*PLC is the first phosphotriesterase-like metal-carboxylesterase characterized to date. In related enzymes of the amidohydrolase superfamily either glutamate or carboxylated lysine substitutes for *Mlo*PLC glutamate 183 and the residue appear invariantly involved in maintaining the structural integrity of the binuclear metal center. Accordingly, we changed Glu-183 to lysine or glutamine. All the tested activities were completely abolished in the E183Q mutant, while only a residual phosphotriesterase activity could be detected in the E183K mutant. Surprisingly, in the latter mutant a parallel 650-fold specificity increase in bis-*p*-nitrophenyl-phosphate (B*p*NP-P) was observed, turning *Mlo*PLC from a carboxylesterase into a phosphodiesterase. Chemical, structural, and kinetic data strongly suggested that K183 is not carboxylated and that the gain of the new function is assisted by the substrate.

Organophosphates (OPs)[1] are a large group of synthetic compounds that have appeared on the earth in the last century (*1*). These chemicals range in toxicity from agricultural insecticides to chemical warfare agents, and their persistence in the environment and dangerousness has become an issue of increasing worldwide concern (*2*−*4*). Toxicity is due to the well-known irreversible inhibition of acethylcholinesterase and/or of other activities involved in endocannabinoid signaling, as reported recently (*5*). OPs are toxic to higher organisms but represent a source of carbon and phosphorus for microorganisms, in particular, soil bacteria. In fact, phosphotriesterase enzymes capable of degrading them have been isolated from *P. diminuta* (*p*PTE) (*6*), *Flavobacterium sp.* (*7*), *Agrobacterium radiobacter* (OpdA) (*8*), and *Pseudomonas monteilii* (*9*). The main structural characteristic of these enzymes is a typical "TIM" barrel $(\alpha/\beta)_8$ fold, with a binuclear metal binding site, which makes them members of the amidohydrolase superfamily (*10*−*12*).

Other features that make phosphotriesterases (PTEs) an attractive matter of investigation are their unknown physiological role and intriguing evolutionary origin. OPs—clearly very efficient substrates for some of these enzymes—have been in existence for 60 years, and a naturally occurring PTE substrate has not yet been identified. This enigma has led to the suggestion that *p*PTE enzymatic activity evolved in recent times from a preexisting hydrolase (*1*, *13*). It is quite commonly accepted that promiscuous activities play a key role in the evolution of enzymatic activities, providing a starting point from which new functions may evolve before fixation by gene duplication (*13*−*18*). Conversely, these promiscuous activities may comprise vestiges of the function of the protein from which an enzyme originated (*13*, *14*). Accordingly, in addition to the wide range of OP substrates (including nerve gases) some PTEs were found to hydrolyze, albeit slowly, phosphodiesters, carboxyl esters, and lactones (see Scheme 1 for representative reactions) (*19*−*22*).

A close homologue of *p*PTE is the *Escherichia coli* phosphotriesterase homology protein (*e*PHP), an enzyme with unknown

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[1]Abbreviations: *Mlo*PLC, *Mesorhizobium loti* phosphotriesterase-like carboxylesterase; PHP, phosphotriesterase homology protein; PTE, phosphotriesterase; *p*NP, *p*-nitrophenyl; CE, carboxylesterase; PDE, phosphodiesterase; ORF, open reading frame; OP, organophosphate; TBBL, 5-thiobutyl-γ-butyrolactone; B*p*NP-P, bis-*p*-nitrophenylphosphate PLL, phosphotriesterase-like lactonase.

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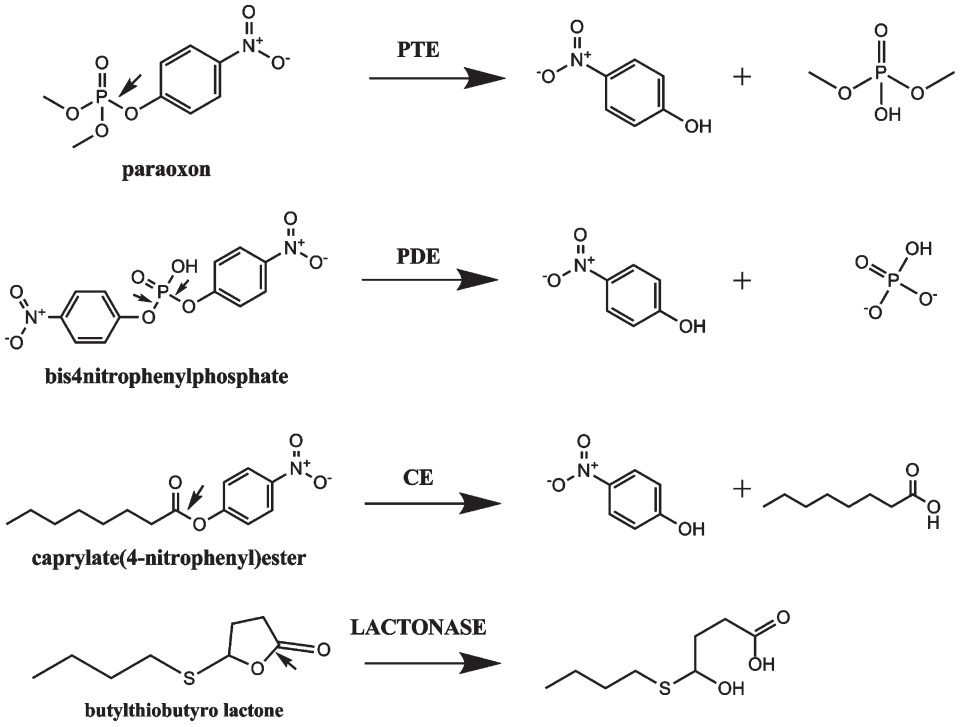
Scheme 1



function, exhibiting 28% sequence identity and 66% sequence similarity to *p*PTE (*23*). Four additional related sequences have been subsequently identified in rat, mouse, mycobacterium, and mycoplasma (*23*−*25*). *p*PTE and *e*PHP possess an essentially identical binuclear (Zn^{2+}) metal center, and with the exception of three surface loops, their overall structures superimpose quite well (*26*). These findings suggest that *e*PHP might have been the ancestor of *p*PTE, or that these enzymes might have diverged from a common ancestor. However, no indication for such evolutionary relationships exists in addition to their structural similarity (*23*, *26*). A weak carboxylesterase (CE; Scheme 1, third reaction) activity recently discovered in *e*PHP seems to support such suggestion (*27*). Afriat and colleagues (2006) went further and provided a nice demonstration of the theory (*28*). A group of proteins with low sequence identity to both *p*PTE and *e*PHP has been identified showing predominant lactonase activity: among these archaeal *Sso*Pox from *S. solfataricus* and *Sac*Pox from *S. acidocaldarius* are endowed with promiscuous paraoxonase activity (*29*−*31*). The recently solved structure of *S. solfataricus Sso*Pox, alone or in complex with a quorum-sensing lactone mimic, has provided a strong structural basis for understanding the lactonase and the promiscuous phosphotriesterase (PTE) molecular mechanisms (*32*). The promiscuous activities of the above phosphotriesterase-like lactonases provide compelling evidence that a few mutations can change dramatically an ancillary activity into the main activity of an enzyme, and the improvement of the promiscuous activity is accompanied by persistence of the main activity (*27*, *33*, *34*). An immediate consequence of the above findings was the prediction of the likely existence in nature of PTE-like enzymes with main CE or phosphodiesterase (PDE) activity. Given the potential use of PTEs and PDEs for the decontamination and bioremediation of pesticides and nerve agents (*3*, *4*, *6*−*9*, *35*, *36*), exploring biodiversity could provide better enzymatic variants than the known ones and will permit us to obtain further insights into the enzyme evolution mechanisms.

Searching the bacterial genomic sequence database, we identified an ORF encoding a putative PTE from *Mesorhizobium loti,* a soil nitrogen-fixing symbiotic proteobacterium (*37*). Here we report the "in silico" identification and biochemical characterization of the recombinant protein and of its mutants E183K and E183Q. The enzyme has been found to be a good CE with promiscuous PTE, PDE, and lactonase activities, and the single mutation E183K turns it into a PDE, while substitution of E183 with glutamine abolishes all the activities. A structural model and kinetic studies has allowed us to propose a working model to explain the observed effects.

MATERIALS AND METHODS

Chemicals. All reagent grade chemicals were from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes were from New England BioLabs (Beverly, MA).

In Silico Analyses. The identification of ORFs encoding putative PTEs was obtained by consulting protein databases UniProt Release 14.1 (Swiss-Prot + TrEMBL) by similarity search, using the program BlastP available at the ExPASy Proteomic Server (http//www.expasy.org). The search was carried out on both "bacterial" (by looking in particular at soil bacteria) and "all" genomes using *p*PTE and *e*PHP as query sequences. With both sequences the program retrieved *M. loti* MLL7664, among the other already known hits. The structural alignment among the *p*PTE, *e*PHP, *Sso*Pox, and MLL7664 sequences was obtained with tools available under the program Swiss PDB Viewer (http//www.expasy.org/spdbv). The alignment was manually adjusted to reduce gaps to a minimum.

Cloning of the MloPLC Gene and Site-Directed Mutagenesis. A 1089-bp fragment containing the entire ORF MLL7664 was amplified using the *M. loti* genomic DNA (kindly provided by Dr. E. J. Patriarca), as template, recombinant Taq DNA polymerase, and oligonucleotides

5′*Mlo*7664 (5′-GGTTGGGTTTCCATATGGATCATTTG-TTCGACAACA-3′) and 3′*Mlo*7664 (5′-ACACACGGAA-TTCTCAGAGGCTCGGCGA-3′) as forward and reverse primers, respectively, in a 25-cycle PCR (1.5 min 92 °C, 1 min 50 °C, 1 min 92 °C). The amplification primer 5′*Mlo*7664 was designed to introduce an *Nde*I restriction site (underlined) upstream of the initiation site, whereas 3′*Mlo*7664 was designed to introduce an *Eco*RI restriction site (underlined) downstream of the stop codon of MLL7664. The PCR product, digested with *Nde*I and *Eco*RI, was ligated into the *Nde*I-*Eco*RI-linearized expression vector pT7-7 (a derivative of pBR322, Stratagene; (*38*, *39*)) to create the pT7-7-*Rlo*7664 construct. The ligation mixture was used to transform *E. coli Top* 10 or BL21(*DE3*) (Invitrogene, CA). Cells were grown at 37 °C in Luria−Bertani (LB) medium containing ampicillin (100 μg/mL). The cloned fragment was completely sequenced to verify that no mutation was introduced during the amplification procedure.

Mutants E183K and E183Q were prepared by PCR mutagenesis. The construct pT7-7-*Rlo*7664 was used as a template for the amplification reaction carried out with a high-fidelity *Pfu*-turbo DNA polymerase (Stratagene), using complementary pairs of mutagenic oligonucleotides (E183K(+) 5′-ATCATCGGC**AAA**-GTCGGCATCAGCAAGG-3′; e183k(-) 5′-TGATGCCGAC-**TTT**GCCGATGATGCCGGCC-3′; E183Q(+) 5′-atcatcggc-**caa**gtcggcatcagcaagg-3′; e183q(−) 5′-tgatgccgac**ttg**gccgatgatgcc-ggcc-3′ (substitution are indicated in bold). PCR reactions were performed as follows: 5 min at 94 °C, followed by 20 cycles of 2 min at 94 °C, 1 min at 50 °C, 5 min at 72 °C. An aliquot of the amplified DNA products were digested with *Dpn*I (60 min at 37 °C) to degrade the templates (methylated DNA). The *E. coli* strain *Top*10 was transformed with the neosynthesized linear plasmid DNAs, and the DNA plasmids were purified from one of the resulting colonies. The whole genes were sequenced in order to exclude unwanted mutations.

Metal Ion Analysis. Ultrapure water 18,2 MΩcm resistivity, obtained from a Milli-Q system (Millipore, Bedford, MA), was used to prepare all the solutions. Nitric acid super pure grade at 69.5%, and multistandard solution (1 mg/L) were obtained from Carlo Erba (Milano, Italy). The water was made metal-free by filtration on Chelex 100 resin (Sigma, St Louis, MO). All glassware used were washed with 30% nitric acid and rinsed with metal-free water. Dialysis tube (Spectra/Por, Spectrum Laboratories, MWCO: 3500) was washed in water metal-free (70 °C) containing 5 mM EDTA three times, and finally rinsed with metal-free water. The protein samples were dialyzed in metal-free 20 mM HEPES pH 8.5 (24 h at 4 °C with a buffer change after 12 h)

For the metal analysis 1 mg of each protein, dissolved in 1 mL 20 mM Hepes/NaOH pH 8.5, was digested with 2 mL of 69.5% HNO_3 in polypropylene volumetric bottles for 2 h at 60 °C. After digestion, samples were allowed to cool to room temperature and brought to a final volume of 10 mL with ultra pure water. The digested samples were processed with an inductively coupled plasma mass spectrometer ICP-MS ELAN DRC-e (Sciex − Perkin-Elmer). The operating conditions were set as follows: power 1.4 kW; plasma flow gas 15 L/min; auxiliary gas flow 1.5 L/min; Scott type nebulizer gas flow 1.0 L/min. The masses of the isotopes for analysis were as follows: ^{55}Mn, ^{66}Zn, ^{60}Ni, ^{24}Mg, ^{114}Cd, ^{44}Ca, ^{54}Fe, and ^{59}Co. The limit of detection was calculated from the three-folds standard deviation of the blank. From the calculated milligrams of metals it was derived the molar ratio metal to protein.

Steady-State Kinetic Measurements and Analysis. The time course of the catalyzed hydrolysis of paraoxon, methyl-paraoxon, *p*NP-hexanoate and bis-*p*NP-phosphate (B*p*NP-P), was monitored essentially as described (*29*, *30*). Standard assays were performed with a Cary double beam spectrophotometer at 50 °C for the wild type enzyme and at 40 °C for the E183K mutant, in a mixture of 20 mM Hepes pH 8.5, containing paraoxon, or methyl-paraoxon or B*p*NP-P (1 mM) or *p*NP-hexanoate (100 μM dissolved in 4% acetonitrile f.c. due to low solubility). The absorption coefficients used for *p*-nitrophenoxide were 21 000 or 20 500 M^{-1} cm^{-1} at 50 or 40 °C (pH 8.5), respectively. Blanks containing all but the enzymes were automatically subtracted.

Initial velocities versus substrate concentration data were analyzed with the GRAFIT program (Grafit Version 3.0, Erithacus Software Ltd., UK). Paraoxon and methyl-paraoxon ranged in concentration from 0.1 to 2.6 mM, *p*NP-hexanoate from 10 to 180 μM, and B*p*NP-P from 0.01 to 2 mM. Assays were carried out in duplicate or triplicate, and the results for the kinetic data were the means of two independent experiments.

The effect of carboxylic acids on the PTE activity of the E183K and E183Q mutants was measured in the standard assay, using paraoxon as substrate. The enzymes were incubated for 1 h at 25 °C in the presence of HCO_3^-, formic acid, or acetic acid, at a final concentration of 50 mM. Only the kinetic parameters for the E183K mutant incubated with formic or acetic acid (50 mM, 1 h at 25 °C) were measured.

The effect of different alkyl-amines on PDE activity of wild type enzyme and E183K mutant was measured by adding three alkyl-amines (methyl-amine, dimethyl-amine, ethyl-amine) at a 50 mM final concentration in the standard assay. In addition, the effect of methyl-amine was investigated in the concentration range from 25 mM to 2 M. Kinetic parameters in the presence of methyl-amine at 1 M final concentration were measured at 50 and 40 °C for wild type and E183K mutant respectively, using bis-*p*NP-phosphate as substrate. Kinetic parameters on TBBL (5-thiobutyl-γ-butyrolactone) were measured as reported (*28*).

Molecular Modeling. Molecular modeling was performed on a Silicon Graphics O2 workstation using the commercial software package Insight II (Biosym/MSI, San Diego, California, USA). The high resolution X-ray crystal structure of ePHP (PDB code: 1BF6) was used as template structure. The alignment was obtained with the tools available under the program Swiss PDB viewer (*40*). Several three-dimensional models were constructed using the Modeler module (*41*) within Insight II. The methodology is based on the satisfaction of spatial restraints that are obtained from an alignment of a target sequence with related 3D structures at high resolution (1.8−2.4 Å), using a conjugate gradient and a molecular dynamics simulated annealing as optimization procedures. A model for the E183K mutant was also obtained starting from the wild type model by using the Homology module of Insight II. The models were analyzed and opportunely minimized using the Swiss PDB viewer. The long loop 1 was excluded from the model. The resulting models were verified using the online software WHATH CHECK, ERRAT and VERIFY 3D on the Structure Analysis and verification Server at the WWW address http://nihserver.mbi.ucla.edu/SAVES/. The models obtained were satisfactory compared to the common parameters used to evaluate the quality of a 3D model. The structures were drawn with Pymol (Innocentive Product, Delano Scientific LLC.).

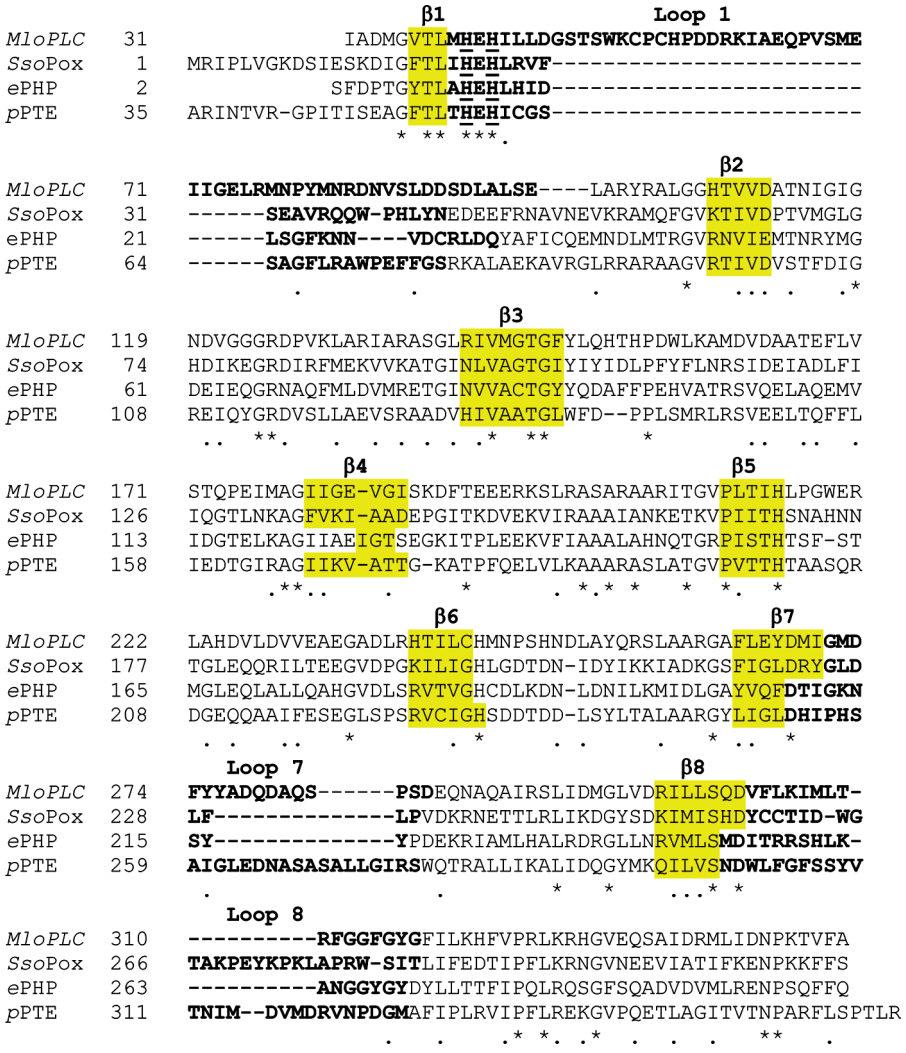


FIGURE 1: Structural multisequence alignment between *p*PTE, *e*PHP, *Sso*Pox, and MLL7664. The *p*PTE, *e*PHP, *Sso*Pox sequences were first aligned by superposing the corresponding structures. Then the MLL7664 sequence was aligned to the others on the basis of secondary structure predictions obtained with SwissPDB Viewer program. Identical residues are marked by an asterisk, and the residues forming the cluster of the binuclear metal center are underlined. The residues that in the 3D structures of *p*PTE and *e*PHP form *loops* 1, 7, and 8 are highlighted in bold. In shadowed yellow are residues of the eight beta strands.

RESULTS AND DISCUSSION

MLL7664 Is a Phosphotriesterase-like Metal-Dependent Carboxylesterase. By searching the full sequenced bacterial genomes with *p*PTE or *e*PHP as query sequences, we retrieved, among soil bacteria, the ORF MLL7664 from *M. loti* displaying 26 and 28% identity, respectively, at the amino acid sequence level. The multiple sequence alignment between MLL7664, *Sso*Pox, *e*PHP and *p*PTE reveals (Figure 1) that MLL7664 differs from *p*PTE in the length of loops 1, 7, and 8 (as defined in ref (*42*)), and in the substitution with glutamate 183 of a conserved lysine present in all PTE and PLL representatives of the amidohydrolase superfamily (*10*, *32*, *42*).

Loops 1, 7, and 8 have been reported to be involved in contacting ligands in the active site of several members of the amidohydrolase family (*42*). Figure 2 shows that this observation holds also for *Sso*Pox in which a short loop 7 and a longer loop 8 form a tunnel accommodating the acyl homoserine lactone analogue (*32*), and also highlights the different conformations of loops 1, 7, and 8 in the three related structures of *e*PHP, *p*PTE, and *Sso*Pox.

The above conserved lysine appears to be always carboxylated from 3D structure analyses and only in the PHP group (group 1 as defined in ref (*28*)) it is substituted with a glutamate; both residues exert the same structural role by bridging the two metal ions at the active site even though, in detail, lysine corresponds to alanine (glycine in ORF MLL7674) plus glutamate (*10*, *26*, *32*). In the features highlighted above, the new hypothetical protein appeared more similar to *e*PHP, but because of a high CE activity (see below), we provisionally dubbed it *Mlo*PLC, namely, *Mesorhizobium loti* phosphotriesterase-like carboxylesterase.

The detailed analyses of whole prokaryotic genomes, using *Mlo*PLC as a template, showed additional related ORFs from *Klebsiella pneumoniae*, *P. syringae* and *R. leguminosarum* having 50−60% sequence identities to *Mlo*PLC, and a very similar *loop* 1. Similarly, sequence searches on whole eukaryotic genomes revealed related ORFs from *Gallus gallus*, *Tetraodon nigroviridis*, *Xenopus laevis*, *Zebrafish*, and *Bos taurus* with sequence identity to *Mlo*PLC ranging between 30 and 36%, but *loop* 1 was more similar to that of hPTER (human phosphotriesterase related).

Since in a recent classification (*28*) *e*PHP has been ascribed with a limited number of closely related proteins to a new group (group I) of the amidohydrolase superfamily, a more specific alignment of *Mlo*PLC was made only with PHP related proteins

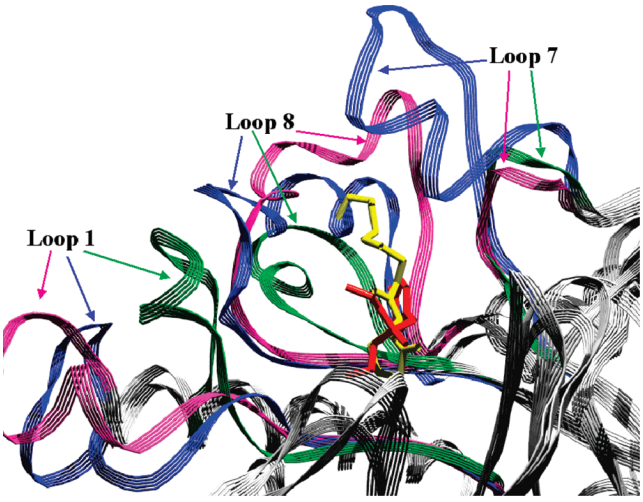


FIGURE 2: Superposition of *p*PTE, *e*PHP, and *Sso*Pox structures. *p*PTE (blue), *e*PHP (green), and *Sso*Pox (magenta) loops 1, 7, and 8 (indicated by arrows) are rendered as ribbons. Active site bound diethylphosphate (*p*PTE) and 5-thiobuthyl-γ-butyrolactone substrate analogue (*Sso*Pox) are shown in red and yellow sticks representation, respectively.

(data not shown). In Figure 4A−C, we focused on the length and composition of *loops* 1, 7, and 8. *Mlo*PLC, Rtox-b (Resiniferatoxin binding from A. *tumefaciens*), *mu*PHP (*murine* PHP), *r*PHP (rat PHP) and *h*PTER possess *loop* 1 of similar length. A detailed analysis of *loop* 1-forming residues of these proteins showed that between the two PHP bacterial enzymes and among the eukaryotic proteins the sequence identity was 32 and 68%, respectively. The sequence identity between the prokaryotic and eukaryotic proteins was less than 10%. This suggests a higher evolutionary divergence in *loop* 1 in Eubacteria with respect to Eukarya that could surmise different biochemical and/or physiological characteristics. Minor differences were observed in the length of *loop* 7: *Mlo*PLC and Rtox-b are identical with six residues less than *p*PTE, whereas deletions of 14, 3, 3, and 3 residues were observed in *e*PHP, *mu*PHP, *r*PHP, and *h*PTER respectively. Finally, all PHP proteins have identical *loop* 8, which are 9-residue shorter than in *p*PTE. Intriguingly, mammalian PTERs (phosphotriesterase-related proteins) belong to this group. These are PHP-like proteins of unknown function that are expressed in proximal tubules of the kidney and found to bind resiniferatoxin, a plant-derived vanilloid which desensitizes certain nociceptive neurons and is therefore of great pharmacological interest (*23*).

The physiological role of ePHP is unknown. When its structure was solved, no activity was found associated with the protein (*26*). Subsequently, a faint arylesterase activity was reported that could be increased by specific mutations (*27*). To shed some light onto the biochemical features of the group I (PHP-like) (*28*), we cloned MLL7664 from *M. loti* genomic DNA and overexpressed the protein in *E. coli* as described in Supporting Information. Preliminary experiments carried out on crude extracts surprisingly revealed that the expressed *Mlo*PLC had high CE activity, in contrast with the related enzyme ePHP (*26*, *27*). A PTE activity was also detected. In both PTE and CE assays, the maximum increase of activity (more than 20-fold) was obtained in the presence of Co^{2+} compared to the metal unsupplemented medium. As detailed in Supporting Information, the protein was purified and characterized together with the E183K mutant (see below). The enzyme was a 40-kDa monomeric protein based on SDS−PAGE and gel-filtration analysis (Figure S1A,B, Supporting Information). The optimal pH and temperature values for both PTE and CE activities were found to be 8.5 and 50 °C, respectively (Figure S2A,B, Supporting Information). These results, together with an identical metal dependency for both activities, suggest that the active site machineries are similar. Analysis of the kinetic parameters (see Table 1) confirmed that *Mlo*PLC represents the first member of the amidohydrolase superfamily to be endowed with metal-dependent CE activity as the principal activity being the specificity constant ($s = k_{cat}/K_M$) 130 000 M^{-1} s^{-1}.

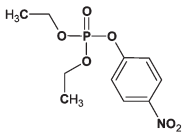
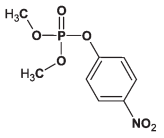
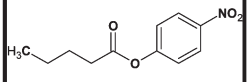
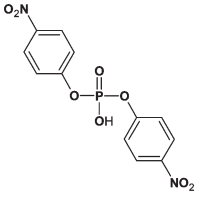
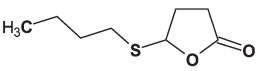
Activities toward *p*NP-esters with acyl chain length from 4 to 8 carbon atoms were tested and the maximum value of k_{cat}/K_M was obtained for *p*NP-hexanoate (data not shown). The CE activity at subsaturing concentration of *p*NP-hexanoate (100 μM) was only slightly inhibited (16%) by paraoxon (500 μM), thus ruling out the presence of contaminating serine-type enzymes, which are generally strongly inhibited by low paraoxon concentration (*43*). No effects on activity were observed with PMSF and physostigmine (data not shown). Both CE and PTE activities were lost after dialysis against a chelating agent (data not shown) suggesting their dependence on the same binuclear metal center (see below). The analysis for the lactonase activity (k_{cat}) showed that it was 7-fold lower with respect to the main (CE) activity, as found in other related enzymes (*27*), and that *s* was significantly lower (288-fold).

Mutant E183K Displays High Phosphodiesterase Activity. The alignment reported in Figure 1 highlights that the residues forming the binuclear metal center in *e*PHP, an enzyme showing a weak CE and no PTE activity (*26*, *27*), are fully conserved in *Mlo*PLC (His-40, His-42, His-215, His-244, Asp-310, and Glu-183). *p*PTE presents the same cluster-forming residues but the ePHP metal-bridging glutamate plus the alanine residue head of it are substituted by the carboxylated K169 (*10*); *Mlo*PLC aligns as ePHP but with glycine in place of alanine. It is worth noting that *p*PTE possesses only a weak esterase activity (*27*), paraoxon being its best substrate ($s = k_{cat}/K_M = 4 \times 10^7$ M^{-1} s^{-1}) (*19*).

In an attempt to verify any possible implication of position 183 in the different substrate specificity, we changed the *Mlo*PLC residue Glu-183 to lysine. To the best of our knowledge this mutation has never been reported for other members of the amidohydrolase superfamily. Mutant E183K was obtained by site-directed mutagenesis (see "Methods"), expressed and purified as described in Supporting Information. Since preliminary experiments showed that the activity was dependent on Cd^{2+}, E183K was prepared at a large scale by adding $CdCl_2$ to the medium (Supporting Information).

Notably, we found that the CE activity in E183K was completely lost and only a weak residual paraoxonase activity could be observed ($k_{cat} = 0.22$ s^{-1}). In contrast, a high PDE activity, not detected in the wild type, was recorded, the optimal pH being the same as the wild type enzyme (pH 8.5; Figure S2A, Supporting Information); the optimal temperature was found to be 40 °C (Figure S2B, Supporting Information). The thermal stability of the mutant was quite similar to that of wild type (Table S1, Supporting Information) ruling out any major conformational change due to the mutation. The differences observed in metal preference and optimal temperature compared to the wild type may instead reflect the electrostatic perturbations imposed by the substitution of E183K in the binuclear metal center and the associated chemical environment.

Table 1: Kinetic Parameters Were Measured at pH 8.5 and 50 or 40 °C for Wild Type and E183K, Respectively[a]

	PTE				CE		PDE		LACTONASE	
	1 		2 		3 		4 		5 	
	wt	E183K	*wt*	E183K	*wt*	E183K	*wt*	E183K	*wt*	E183K
k_{cat} (sec^{-1})	3.0 (±0.4)	0.0097 (±0.0014)	9.0 (±0.9)	0.22 (±0.02)	13.0 (±0.5)	nd	0.060 (±0.006)	4.8 (±0.4)	1.8 (±0.2)	nd
K_M (M)	3.5 (±0.5) x10^{-3}	3.5 (±0.3) x10^{-3}	5.5 (±0.5) x10^{-3}	1.15 (±0.20) x10^{-3}	0.10 (±0.005) x10^{-3}	nd	1.4 (±0.3) x10^{-3}	0.17 (±0.03) x10^{-3}	4.0 (±0.2) x10^{-3}	nd
k_{cat}/K_M ($sec^{-1}M^{-1}$)	857 (±240)	2.8 (±0.6)	1636 (±310)	191 (±50)	130000 (±11000)	nd	43 (±14)	28235 (±5700)	450 (±70)	nd

[a] Paraoxon, (**1**), methyl-paraoxon (**2**), *p*NP-hexanoate (**3**), B*p*NP-P (**4**), and TBBL (**5**) were used as substrates. The results are means of two independent experiments. n.d. = not detectable, means ΔO.D. below the instrument sensitivity after 10 min reaction time.

In order to ascertain if the metals added to the growth medium were effectively incorporated into the recombinant enzymes, we performed the metals determination analyses described in Materials and Methods. For the wild type protein a 2:1 Co^{2+}/protein molar ratio (1.377 μg of Co^{2+} per mg of protein) was obtained with traces amount of Cd^{2+} and Zn^{2+} (0.17 and 0.1 μg per mg respectively). Instead for the E183K protein a 1:1 Cd^{2+}/protein molar ratio (1.150 μg of Cd^{2+} per mg of protein) was found, with a minor amount of Zn^{2+} (0.1 μg per mg).

Kinetic parameters for the wild type and mutant enzyme were measured by focusing on four different activities: PTE on paraoxon and methyl-paraoxon, CE on *p*NP-hexanoate, PDE on B*p*NP-P and lactonase activity measured with the synthetic substrate TBBL (Table 1). For the wild type enzyme the CE activity ($k_{cat} = 13.0 \pm 0.5\ s^{-1}$) has been found to be prominent: a specificity constant of 130 000 $M^{-1}\ s^{-1}$ was measured, which is 3023-, 152-, 79-, and 289-fold higher than the *s* values measured for B*p*NP-P, methyl-paraoxon, paraoxon, and TBBL, respectively. Although substantial activity was observed with methyl-paraoxon ($k_{cat} = 9.0 \pm 0.9\ s^{-1}$) and TBBL ($k_{cat} = 1.8 \pm 0.2\ s^{-1}$), K_M values were quite high; finally activity on B*p*NP-P was 3 orders of magnitude lower ($k_{cat} = 0.06 \pm 0.006\ s^{-1}$). For E183K the substitution had a considerable effect compared to the wild type. Around 300- and 40-fold lower activity was observed on paraoxon and methyl-paraoxon, respectively, whereas the K_M values were unaffected. Strikingly, there was a complete loss of the CE and lactonase activities and an increase of PDE activity, transforming the mutant E183K from a CE into an excellent PDE-like enzyme (PDE specificity from 43 to 28 235 $M^{-1}\ s^{-1}$ in the wild type and mutant, respectively. The PDE activity of mutant was lost after dialysis against a chelating agent (data not shown). The PDE activity at subsaturating concentration of B*p*NP-P (10 μM) was only partially inhibited by methyl-paraoxon (22% with 500 μM), likely due to substrate competition for the same site, as above assumed for the CE activity of wild type enzyme and paraoxon as substrate. In terms of specificity constants with paraoxon and methyl-paraoxon, the decrease was about 300- and 8.5-fold respectively, whereas the increase with B*p*NP-P was about 650-fold compared with the wild type enzyme (Table 1). These results emphasize how a single substitution can transform a promiscuous activity, the PDE activity, into the principal activity of the enzyme, leading to a complete loss of the native CE activity. This result is apparently in contrast with the evolutionary theory for promiscuous activities, which requires that the main activity remains substantially unaltered, at least until a duplication event occurs (*13*−*18*, *27*, *28*, *33*, *34*). However, the very recent report by Patrick and Matsumura (2008) pointed out that sometimes promiscuous activities, although hard to detect, are determinant for organism fitness (*44*). What was unexpected here was the finding that a residue, considered to be "structural" in the sense of being the metal bridging ligand and involved in maintaining the structural integrity of the active site, could determine, if mutated, a considerable change in enzyme chemiospecificity. This opens up some interesting questions about evolution of the binuclear metal center in the amidohydrolase family, the structural role of E/K residues as metal bridging ligands and in general the role of key catalytic residues in enzyme evolution (*45*). The presence of lysine in the more ancient archaeal enzymes (*31*, *32*) seems to suggest that PHP with glutamate plus alanine in place of lysine evolved lately, in concomitance with loops elongation. Since carboxylation of lysine is stabilized by metals and *vice versa* this could have permitted a higher plasticity of the metal binding site and perhaps a sort of rudimentary control of enzyme activity. From this viewpoint, it is interesting that in some nonrelated enzymes in which lysine has a similar role, such as β-lactamases, a direct involvement of carboxylation in enzyme mechanism has been reported (*46*). Moreover, even in the amidohydrolase

superfamily there are interesting examples of enzymes without E/K as the bridging ligand and with a single metal instead (subtype 3; adenosine deaminase (*42*)).

Since detailed analyses in rhizobia have shown the involvement of lactone quorum-sensing molecules in various bacterial behaviors (*47*), we tested *Mlo*PLC and E183K mutant for their lactonase activity. Among several substrates tested, the wild-type enzyme showed only a weak activity on δ-valerolactone (data not shown) and TBBL (Table 1). Therefore, the low activity and the restricted specificity make it unlikely that *Mlo*PLC lactonase activity could be involved in the quorum-quenching of signal molecules, but confirm the promiscuous relationship between CE, lactonase, PTE, and PDE mechanisms. Because *Mlo*PLC has the highest *s* value on esters and significant activity on triglycerides as well (data not shown), it could have a potential role in some esters/lipids catabolic pathways. The analysis of the genome context in PTE-related enzymes showed no clues of a possible function, the contexts being different in each case. Only in the case of *Sso*Pox (*29*) did we notice some ORFs coding for putative esterases/lipases all around the gene. In contrast, around *Mlo*PLC some genes have been found (data not shown) that are annotated as involved in sugars transport and metabolism.

Is Lys-183 Carboxylated in the Mutant Enzyme? A Mechanism to Explain the Change of Substrate Specificity. In a previous work on *p*PTE it has been reported that substitution of Lys-169 (structurally corresponding to *Mlo*PLC E183) into alanine, glycine, glutamate, arginine, or methionine resulted in a significant loss of catalytic activity with respect to phosphotriester substrates (*48*). The loss of activity was partly restored upon inclusion into the assay mixture of short-chain carboxylic acids (*48*, *49*). Therefore, similar experiments were designed for the *Mlo*PLC E183K mutant; in particular we analyzed the chemical rescue of PTE activity with short-chain carboxylic acids (Table 2). An enhancement of activity was observed with all three carboxylic acids tested, but formic acid was the highest activator, with an increase of about 1.6-fold. In contrast, a decrease in PDE activity was observed with all three carboxylic acids. The kinetic parameters of the E183K mutant with paraoxon, in the presence of formic and acetic acids, were calculated and compared to those without incubation (Table 2). Interestingly, the k_{cat} value measured after incubation of the enzyme with the formic acid increased 3.5-fold compared to the sample with no addition; the K_M value instead was reduced about 8-fold, and the effect on specific constant (k_{cat}/K_M) was a 27-fold enhancement. The same effect (10-fold enhancement of k_{cat}/K_M) was observed with acetic acid.

A different work on *p*PTE (*20*) highlighted that the low PDE activity in the wild type enzyme could be increased upon addition of different alkyl-amines into the reaction mixture (k_{cat}/K_M increased up to 200-fold on ethyl-4-nitrophenyl phosphate by addition of 2 M dimethyl-amine). Since the phosphodiester bears a net negative charge, the authors proposed that the observed effect was due to substrate stabilization by the positive charge carried by the alkyl-amine. To test this model, residue Met-317 located nearby the active site was mutated; the analysis of mutants demonstrated that M317K or M317R substitutions, but not M317A, could mimic the alkyl-amine effect, in accordance with the model. It has to be stressed the fact that, in the present study, the mutation to lysine concerned a residue (Glu-183) known to be involved in maintaining the structural integrity of the binuclear metal center from structural analysis of related enzymes (*10*, *26*, *32*) as well as models presented here (Figure 3). From a conceptual point of view, there was no expectation that such mechanism could help to explain the high PDE activity of E183K. Nonetheless, with the aim to thoroughly characterize the new enzymes and to probe the validity of the proposed action mechanism of alkyl-amines for wild type and mutant *Mlo*PLC, we performed experiments similar to those reported by Raushel and co-workers (*20*).

Table 2: Kinetic Parameters of E183K Mutant Were Calculated after 1 h Incubation of the Enzyme at 25 °C without or with Formic or Acetic Acid (50 mM), Using Paraoxon As Substrate[a]

	k_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M (s^{-1} M^{-1})
none	0.0097 ± 0.0014	$3.5 \pm 0.3 \times 10^{-3}$	2.8 ± 0.6
formic acid	0.034 ± 0.003	$0.45 \pm 0.10 \times 10^{-3}$	75 ± 20
acetic acid	0.041 ± 0.004	$1.3 \pm 0.2 \times 10^{-3}$	31 ± 8

[a] The results are means of two independent experiments.

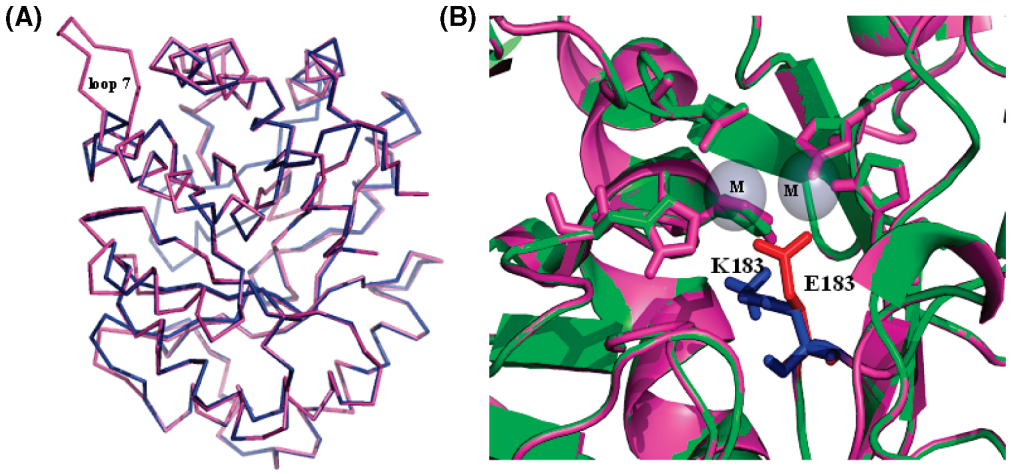


FIGURE 3: Molecular modeling. (A) Superposition of wild type *MloPLC* (magenta) and E183K mutant (green) structures drawn as a cartoon. (B) The active site zoom shows the predicted position of the two metal ions (M; gray spheres), the side chains of residues (in stick representation) forming the binuclear metal cluster (His-42, His-44, His-215, His-244, and Asp-310) and residues Glu-183 and Lys-183 highlighted in red and blue, respectively.

First of all, the effect of different alkyl-amines was evaluated in a preliminary screening (data not shown). For the wild type enzyme in the presence of short alkyl-amines, only a weak increase of PDE activity could be recorded, in contrast with the huge activating effect reported for *p*PTE (*20*). However, similarly to *p*PTE, a decrease of PTE activity was observed. Furthermore, for the E183K mutant the presence of alkyl-amines had a negative effect on PDE activity. The attention was focused on methyl-amine, the greatest activator found for wild type *Mlo*PLC, with the aim to test if the effect on PDE activity was dependent upon methyl-amine concentration; with the wild type enzyme a constant activating effect was observed, with a maximum reached at 1 M, while with E183K mutant, there was always a negative effect on the PDE activity (data not shown). The kinetic parameters obtained in the presence of 1 M methyl-amine are reported in Table 3. For the wild type enzyme an increase of k_{cat} and a decrease in K_M were measured, resulting in a total 3.5-fold enhancement of specificity constant (k_{cat}/K_M) with respect to the value measured in absence of amine. The mutant E183K, in the presence of methyl-amine, had the K_M unaffected with respect to the value in the absence of amine and therefore the negative effect was only on k_{cat}, suggesting that the observed phenomena are related to the active site.

Table 3: Effect of Methyl-amine (1 M) on PDE Activity[a]

	k_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M (M^{-1} s^{-1})
wt	0.103 ± 0.010 (1.7)	$0.65 \pm 0.10 \times 10^{-3}$ (0.5)	158 ± 35 (3.7)
E183K	2.87 ± 0.06 (0.6)	$0.150 \pm 0.010 \times 10^{-3}$ (0.9)	$19.1 \pm 0.6 \times 10^{3}$ (0.7)

[a] The assays were done at 50 and 40 °C for wild type and E183K mutant enzymes, respectively. Results are means of two independent experiments. The ratios between the kinetic parameters measured with or without addition of methyl-amine are reported in parentheses.

This finding and the above-reported effect of carboxylic acids strongly suggest that (i) Lys-183 is not carboxylated, (ii) its positive charge is involved in phosphodiester stabilization in the wild type enzyme, and (iii) the carboxylic acid instead is involved in the stabilization of the binuclear metal center in the mutant. The model also explains well the absence of CE activity in the mutant. In fact, the CE substrate, being uncharged, should not be able to perform the bridging and reconstitution of the metal center. The conclusion that the metal center is impaired in the E183K mutant is also supported by the results of the metal analysis above-reported, revealing that only one metal is present at the active site.

3D models of *Mlo*PLC and E183K constructed by using as reference the ePHP structure (PDB code: 1BP6), and shown in Figure 3, gave provisional support to the above results. The *loop* 1 was excluded from the final model, being too long to be realistically modeled. The rms obtained after superposition of 1080 backbone atoms (Figure 3A) was 0.4 Å. In Figure 3B the superimposed active sites of the wild type and mutant enzymes are depicted, showing the position of K183 compared to the glutamate. It is clear that if Glu-183 is mutated to lysine a clash against the metal cations is expected, even in the absence of lysine carboxylation. As a consequence, the Lys-183 side chain is expected to assume a different conformation, leaving some room underneath the metals, as shown in Figure 5. Because lysine carboxylation and stabilization of the binuclear metal center are thought to be synergic events (*50*), this observation reinforces the idea that lysine is not carboxylated; otherwise, it would interfere more strongly with the structural integrity of the binuclear metal center (Figure 5). The loss of activity observed in other distantly related enzymes, in which the opposite mutation K to E was attempted, has been explained with opposite arguments (*51*).

A

LOOP 1

```
pPTE  : 58 THEHICGS-----------------------------SAGFLRAWPEFFGS
ePHP  : 14 AHEHLHID-----------------------------LSGFKNNVDCRLDQ
MloPLC: 42 MHEHILLDGSTSWKCPCHPDDRKIAEQPVSMEIIGELRMNPYMNRDNVSLDD
Rtox-b: 36 ILNDCRCWWNAPKTAERQYL    AEGFVCMEIIGELRQDPFVNKHNITLDD
muPHP : 29 THEHLTMTFDSFYCPPPPCHEVTS    KEPIMMKNLFWIQK-NPYSHRENL
rPHP  : 29 THEHLTMAFDSFYCPPPPCQEAAS    REPIMMKNLFWIQK-NPYSHQENL
hPTER : 29 THEHLAMTFDCCYCPPPPCQEAIS    KEPIVMKNLYWIQK-NAYSHKENL
```

B

LOOP 7

```
pPTE  :237  GLDHIPHSAIGLEDNASASALLGIRS
ePHP  :207  QFDTIGKNSYY--------------P
MloPLC:266  EYDMIGMDFYYADQDAQSP------S
Rtox-b:259  EYDMIGMDFFYADQQVQCP------S
muPHP :252  EYDLFGTELLNYQ---LSPDIDMPDD
rPHP  :252  EYDLFGTELLNYQ---LSPDIDLPDD
hPTER :252  EYDLFGTELLHYQ---LGPDIDMPDD
```

C

LOOP 8

```
pPTE  :283  LVSNDWLFGFSSYVTNIMDVMDRVNPDGMA
ePHP  :239  MLSMD---------ITRRSHLKANGGYGYD
MloPLC:306  LLSQD---------VFLKIMLTRFGGFGYG
Rtox-b:299  LLSHD---------VFLKMMLTRYGGNGYA
muPHP :294  LMAHD---------IHTKHRLMKYGGHGYE
rPHP  :294  LMAHD---------IHTKHRLMKYGVHGYE
hPTER :294  LVAHD---------IHTKTRLMKYGGHGYE
```

FIGURE 4: Multiple sequence alignment between *p*PTE, *MloPLC*, and other related proteins. Sequences were aligned with the program ClustalW. Residues that in the 3D structure of *p*PTE and ePHP form *loops* 1, 7, and 8 are reported in gray. Loops 1, 7, and 8 from the above cited and other putative bacterial and eukaryotic PHP proteins are drawn in panels A−C, respectively.

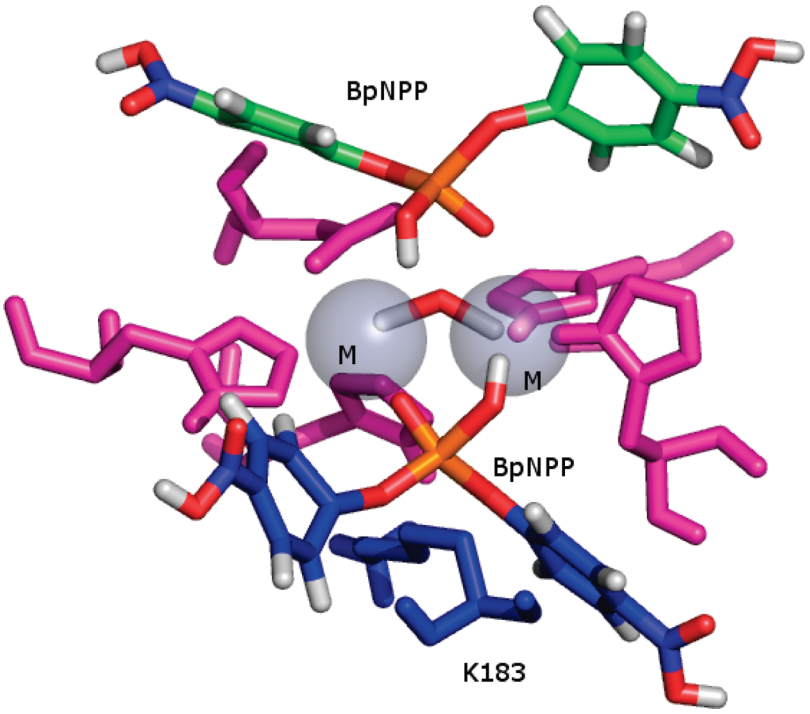


FIGURE 5: Schematic representation of the K183E active site with the proposed role of B*p*NP-P. The orientation of the active site is the same reported in Figure 3B. The "structural" B*p*NP-P is shown in orange and the B*p*NP-P acting as substrate is colored by the atom type. The metals are represented as gray spheres bridged by a water molecule (red stick). The residues contacting the metals are shown in magenta sticks.

Finally, incubation of wild type or mutant enzymes with bicarbonate (data not shown) does not increase activity of both enzymes, in contrast to previously reported data for *p*PTE and other enzymes for which such activation was considered as an indirect proof of lysine carboxylation (*50*, *51*).

Therefore, if Lys-183 is not carboxylated as suggested by several indirect evidence, how is the high PDE activity in the E183K mutant possible, an activity we demonstrated to be metal-dependent? One possibility, schematically depicted in Figure 5, is that the same B*p*NP-P substrate is involved in the stabilization of the binuclear metal center. In other words, B*p*NP-P could perform a chemical rescue of mutation as the carboxylic acid does. The molecular details of how exactly this happens, namely, through the substrate itself or a degradation product, remain to be established. In order to substantiate this hypothesis the following experiment on the mutant E183K was devised: the CE activity that, as previously mentioned, was undetectable in this protein, was measured in the presence of a B*p*NP-P/protein ratio of 1/8. If the model is right, a significant recovery of CE activity should be monitored. As expected, in the presence of 0.1 μM B*p*NP-P and 1.25 μM mutant the CE initial velocity with *p*NP-hexanoate (in the standard CE assay) rose from an undetectable level to 0.030 ± 0.002 s^{-1}! 20-fold less PDE activity (0.0014 s^{-1}) could be detected under those conditions. The model can also explain the mechanism through which the change of substrate specificity from the wild type enzyme to the E183K mutant has been obtained: the presence of the free positive charge of the uncarboxylated Lys-183, analogous to that reported for *p*PTE (*20*), can neutralize the negative charge of the phosphodiester substrate, thus favoring the PDE activity and at the same time interfering negatively on the interaction with uncharged substrates such as phosphotriesters and carboxylesters. To verify if the positive charge of the E183K mutant is free and able to stabilize the negative charge of the phosphodiester substrate, the E183Q mutant protein was designed, produced, and purified. In this mutant all the tested activities (phosphodiesterase, phosphotriesterase, lactonase, and esterase) were missing (data not shown), a datum in agreement with the idea that the metal center cannot be reconstituted and that the lack of a positively charged residue impairs the PDE activity. If the model is right, a significant regain of activity should be monitored when the metal center is reconstituted. As expected, after 1 h incubation of the mutant E183Q in 50 mM HCO_3^- the CE activity with *p*NP-hexanoate as substrate (in the standard CE assay) rose from an undetectable level to 0.040 ± 0.003 s^{-1}, and no PDE activity could be detected under these conditions. However, the same enzyme (incubated 1 h in 50 mM HCO_3^-) when assayed in the presence of 50 mM methyl-amine allowed the rescue of the PDE activity on Bis-*p*NP-phosphate (the activity recorded was 0.067 ± 0.006 s^{-1} in the standard PDE assay).

Overall, the results obtained by (1) mutagenesis, (2) kinetics, and (3) molecular modeling analyses strongly suggest that Lys183 is not carboxylated and not involved in the stabilization of metals, a role that can be fulfilled by a mechanism of substrate-assisted gain of function. Although the gain-of-function mediated by B*p*NPP does not allow the complete recovery of the original (CE) activity to be fulfilled, it however makes explicit the gain of the new function (PDE), also thanks to the presence of the positive charge of the non carboxylated lysine.

Evolutive and Physiological Implications. The finding in *M. loti* of a PTE-like enzyme with predominant CE activity provides a good support to the theory on the enzyme evolution starting from promiscuous activities (*28*, *33*, *34*) in the amidohydrolase superfamily. However, the conversion of *Mlo*PLC into an excellent PDE by means of a single mutation, concomitantly with the complete loss of CE activity, is a rare case of substrate-assisted gain-of-function via stabilization of the binuclear metal center. Different cases of substrate-assisted catalysis (SAC) have been reported previously (for a review see ref (*48*)), some in wild type enzymes and a few in "dead" mutants. One recent example

has been reported in vivo for Src (*52*, *53*). Does an evolutive role of the substrate-assisted gain-of-function exist? Concerning serine-type proteases it has been argued that the catalytic triad could have been originated starting with a dyad of serine and aspartic acid, whereas the histidine could have been provided by the substrate initially (*54*). Our results emphasize how a single substitution transformed a promiscuous activity, the PDE activity, into the principal activity of the enzyme, leading to an apparently complete loss of the native CE activity. This resembles a case of strong negative trade-off through a concave route as described in the paper of Khersonsky et al. (2006), opposite to the convex route via "generalist" intermediates (*34*). In vivo such events should require gene duplication as a necessary prerequisite, since the new function is accompanied by the abrupt loss of the other (*34*). Because a limited rescue of the initial activity mediated by the substrate was also observed (0.2% of initial CE activity), it is tempting to speculate that such transient intermediate can be found in vivo on the route of enzyme evolution paving the way to the appearance of new functionality ahead of gene duplication and further evolution. The above cited work by Patrick and Matsumura (2008) is indicative in this respect (*44*). Additional studies are required to assess if this mechanism is indeed a possibility. These studies could also be of relevance for developing new drugs based on chemical rescue of deleterious mutations, as suggested by recent reports on p53 mutants (*55*).

In conclusion, here we have demonstrated the substrate-assisted in vitro rescue of a function with concomitant change in chemiospecificity. Furthermore, we have probed a structural residue of the active site in a member of the amidohydrolase superfamily by providing the missing complementary experiments to those concerning enzymes having a carboxylated lysine with the same role. These experiments were possible thanks to the high CE activity found in *Mlo*PLC and could be a good starting point to extend these studies to the related eukaryal enzymes.

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SUPPORTING INFORMATION AVAILABLE

(1) *MloPLC* expression and characterization; (2) physical properties of wild type and mutants; (3) dependence from pH and temperature of PTE and CE activity in wild type and mutant. This material is available free of charge via the Internet at http://pubs.acs.org.

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