A phospholipase D (PLD) superfamily was recently identified that contains proteins of highly diverse functions with the conserved motif HXKKX,DX,X,G(G/S). The superfamily includes a bacterial nuclease, human and plant PLD enzymes, cardiolipin synthases, phosphatidylserine synthases, and the murine toxin from *Yersinia pestis* (Ymt). Ymt is particularly effective as a prototype for family members containing two conserved motifs, because it is smaller than many other two-domain superfamilies, and it can be overexpressed. Large quantities of pure recombinant Ymt allowed the formation of diffraction-quality crystals for x-ray structure determination. Dimeric Ymt was shown to have PLD-like activity as demonstrated by the hydrolysis of phosphatidylcholine. Ymt also used bis(para-nitrophenol) phosphate as a substrate. Using these substrates, the amino acids essential for Ymt function were determined. Specifically, substitution of histidine or lysine in the conserved motifs reduced the turnover rate of bis(para-nitrophenol) phosphate by a factor of $10^4$ and phospholipid turnover to an undetectable level. The role of the conserved residues in catalysis was further defined by the isolation of a radiolabeled phosphenzyme intermediate, which identified a conserved histidine residue as the nucleophile in the catalytic reaction. Based on these data, a unifying two-step catalytic mechanism is proposed for this diverse family of enzymes.

Human phospholipase D (PLD)$^1$ is an effector in multiple signaling cascades. Activity of this lipid-modifying enzyme is, therefore, tightly regulated by multiple activators including small guanine nucleotide-binding proteins from the ADP-ribosylation factor and Rho families, phosphatidylinositol 4,5-bisphosphate, and protein kinase C (reviewed in Ref. 1). Human PLD1 catalyzes the hydrolysis of phosphatidylcholine to generate phosphatic acid and choline. Phosphatic acid can be further hydrolyzed to lysophosphatic acid by phospholipase A$_2$ or to diacylglycerol by phosphatic acid phosphohydrolase. PLD can also catalyze a transphosphatidylation reaction in which an alcohol (usually ethanol) substitutes for water in the reaction, resulting in the formation of the alcohol derivative of phosphatic acid (typically phosphatidylethanol); this assay is commonly employed to measure PLD activity (2).

Other lipid-modifying enzymes such as cardiolipin and phosphatidylserine synthases also catalyze transphosphatidylation reactions.

PLD is one of a group of enzymes with diverse functions that has been referred to as the PLD superfamily (3–5). All members contain a conserved motif, HXKKX,DX,X,G(G/S). Members of this family that contain a single copy of the conserved motif include a bacterial endonuclease (Nuc) and a helicase-like protein from *Escherichia coli* (6–7). Most other family members contain two copies of the conserved motif (HXKKX,DX,X,G(G/S)), including human and plant PLD enzymes (3, 8), cardiolipin synthase (9–10), phosphatidylserine synthase (11), and a murine toxin from *Yersinia pestis* (12). This signature motif is not, however, absolutely conserved, and variation exists in many of the conserved residues in the motif (4, 13).

The *Y. pestis* murine toxin (Ymt) is encoded on a 110-kilobase plasmid that is unique to the bacterium which causes the plague (12). *Y. pestis* is transferred between mammalian hosts via a flea vector. As a known determinant for plague virulence, Ymt is believed to play a role in *Y. pestis* pathogenesis, both in the rodent host and the flea vector (14–15). For example, Ymt may contribute to the more rapid death of mice injected with *Y. pestis*, as compared with mice injected with other *Yersinia* species (16). In addition, Ymt is required for *Y. pestis* survival in the flea.$^2$

Ymt is a 586-amino acid protein with two conserved motifs, HXKKX,DX,X,G(G/S). It is similar to human PLD in having two of the superfamily signature motifs, but it is substantially smaller than the 1074-residue human PLD protein. The similarities in the two conserved motifs suggested that Ymt and PLD might utilize a common catalytic mechanism. Finally, an understanding of the activity and mechanism of Ymt would afford a rational approach for exploring the role of Ymt as a virulence factor for the plague bacterium, *Y. pestis*.

We have, therefore, investigated the physical properties, function, and catalytic mechanism of the cloned toxin as a prototype for superfamily enzymes with two conserved motifs. In this report, we demonstrate that Ymt has PLD-like activity as assessed by the hydrolysis of phosphatidylcholine, phos-

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$^1$ The abbreviations used are: PLD, phospholipase D; Ymt, *Y. pestis* murine toxin; PAGE, polyacrylamide gel electrophoresis; pNPP, para-nitrophenyl phosphate; pNP, para-nitrophenolate; PI, 1,2-di-O-lysino-3-O-phosphatidylglycerol; PA, 1,2-di-O-dipalmitoyl-sn-glycero-3-phosphatidylcholine.

$^2$ B. J. Hinnebusch, unpublished observations.
phatidyldiethanolamine, and phosphatidylserine as well as the transphosphatidylidation reaction involving phosphatidylcholine. In addition, we have identified artificial substrates for Ymt to explore the catalytic mechanism. These substrates have allowed us to demonstrate that the invariant histidine and lysine residues found in the conserved motifs of all superfamily members are essential for catalysis. We have also shown that the catalysis by Ymt proceeds via a phosphoenzyme intermediate. Based on our findings, we propose a unifying, two-step mechanism for substrate hydrolysis by dual-domain PLD superfamily members. The expression of large concentrations of recombinant Ymt also allowed us to crystallize this protein, setting the stage for structural analysis of this member of the PLD superfamily.

**EXPERIMENTAL PROCEDURES**

**Materials**

Bis(p-nitrophenyl) phosphate (bis-pNPP) and Sephadex G-100 were purchased from Sigma. Immobilon P membrane was obtained from Millipore. Nickel nitrotri-acetic acid-agarose was purchased from Qiagen. 32P-labeled inorganic phosphate (6000 Ci/mmol), l-α-dipalmitylcholine-methyl-3H-phosphatidylcholine (50 Ci/mmol), l-α-[myo-inositol-2-3H]phosphatidylglycerol (PI; 11 Ci/mmol); [inositol-2-3H]phosphatidylcholine, 4,6-bisphosphate (37 Ci/mmol), l-α-dipalmitoyl-[2-palmitoyl,9,10-3H]phosphatidylcholine (89 Ci/mmol), and l-α-dipalmitoylglycerol-U-14C phosphatidic acid (PA; 100–200 Ci/mmol) were purchased from Amersham. D3-dipalmitoyl-[2-14C]serine (54 mCi/mmol) were purchased from Amersham. Silica Gel 60 thin-layer chromatography plates were utilized in conjunction. All mutations were verified by sequencing.

**Construction of Expression Plasmids**

Wild-type Ymt was amplified from the 110-kb plasmid from the KIM strain of *Y. pestis* provided by J. Hinnebusch. The 5′ primer incorporated a restriction site for *NdeI* (GTCATCGATATGCCTCAAATAAATAATACTCA), and the 3′ primer added a *SalI* restriction site and a polyclonitide tag (GAGGTCGCTAATGGATATTGTGGCA). The PCR product was ligated into the *NdeI*/*SalI* sites of a modified pT7–7 expression vector, which contains the lac operator (Ref. 17, provided by J. C. Clemens). The entire coding sequence was verified by sequencing. Using the wild-type Ymt construct as a template, the following mutations were made using the Transformer™ site-directed mutagenesis kit (CLONTECH): H188N, H224N, K190S, K362S, D195N, D195E, D249N, D249E, S359A, and S359T. For mutants with changes in two conserved residues, primers for both substitutions were designed from the modified pT7–7 expression vector, which contains the *lac* operator (Ref. 11825). All mutagenesis reactions were verified by sequencing.

**Expression and Purification of Ymt**

The pT7–7 Ymt construct was used to transform Novobact D33 (Novagen) *E. coli* by standard methods. Single colonies were used to inoculate 1 liter of 2× YT medium containing ampicillin (100 μg/ml). Cultures were grown overnight at 37 °C to an optical density (600 nm) of 0.5–0.9, induced by the addition of isopropyl β-thiogalactoside to a final concentration of 0.4 mM, and grown for an additional 4 h at 25 °C. The cells were harvested by centrifugation at 5000 × g for 5 min, and the resulting pellets were frozen at −70 °C. Pellets were thawed at 0 °C by three passages through a French press at 12000 psi. Cellular debris was removed by centrifugation at 20,000 × g for 20 min at 4 °C. The clarified supernatant was incubated with gentle rotation for 1 h at 4 °C with 12 ml of nickel nitrotri-acetic acid-agarose that had been equilibrated in low salt buffer (30 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol). The slurry was poured into a 50-ml column and washed with high salt wash buffer (30 mM Tris, pH 6.8, 300 mM NaCl, 10% glycerol). Ymt was eluted with a gradient of 0–200 mM imidazole. Samples of fractions were subjected to SDS-PAGE, and the gel was stained with Coomassie blue. The protein bands were collected and concentrated using a Centriprep-30 filtration unit (Amicon), adjusted to 50% glycerol, and stored at −20 °C. The final product was >99% pure by SDS-PAGE.

**Crystallization of Ymt**

Full-length Ymt was diluted to 5 mg/ml in 10 mM NaCl, 5 mM Tris, pH 7.4, and screened for crystallization conditions. Crystals (0.01 × 0.02 × 0.04 mm) formed in condition #33 (2.0 mM ammonium formate and 100 mM Hepes, pH 7.5) of the Hampton Research Crystal Screen II. Larger crystals (0.1 × 0.1 × 0.1 mm) of full-length Ymt were grown by slow evaporation. Crystals grown in 1.35–1.6 M ammonium formate, 1% polyethylene glycol 200, 100 mM Hepes, pH 7.5, were placed in equilibrated 10-μl hanging drops containing 5 μl of protein solution, 15 mg/ml, and 5 μl of 1.5 M ammonium formate containing 100 mM Hepes, pH 7.5, and allowed to grow for 21 days at 22 °C. The macromolecular crystals were frozen in 20% glycerol, 1.5 M ammonium formate, 100 mM Hepes, pH 7.5, and diffraction data were collected on a Rigaku RU300 rotating anode equipped with a RAXIS IV detector.

**Physical Characterization of Ymt**

UV spectra of wild-type and mutant enzymes were recorded over a wavelength range of 240–310 nm at 25 °C with a Perkin-Elmer 16 spectrophotometer in assay buffer (25 mM Tris, 25 mM Bis-Tris, 50 mM acetate, pH 6.0). The final concentration of all proteins was 0.250 mg/ml in a final volume of 0.5 ml. Circular dichroism (CD) spectra were recorded using a Jasco J-710 spectropolarimeter at 25 °C over a wavelength range of 178–260 nm. The final concentration of each protein was 0.1 mg/ml in distilled water. Size exclusion chromatography was used to determine the oligomeric state of purified recombinant Ymt. Ymt (100 μg) was injected onto a Superose 6 column (Amersham Pharmacia Biotech) using 30 mM Tris, pH 7.4, 150 mM NaCl as the mobile phase at a flow rate of 0.5 ml/min. Protein elution was monitored by absorbance at 280 nm.

The concentration of the purified protein was quantified by the University of Michigan Protein Core Facility (amino acid analysis performed on a Perkin-Elmer Applied Biosystems model 420H hydrolyzer/derivatizer and 130A Separation System). Amino terminal sequence analysis was performed using a Perkin-Elmer Applied Biosystems 494 sequenator.

**Artificial Substrate Hydrolysis**

All assays were performed at 30 °C in assay buffer pH 6.0. For substrate screening, 1 μM of wild-type Ymt was incubated with bis[para-nitrophenyl] phosphate (2–20 mM), para-nitrophenyl phenyl phosphate (2–100 mM), thymidine 5′-monophosphate para-nitrophenyl ester (2–50 mM), para-nitrophenyl phosphate (2–100 mM), or para-nitrophenyl phosphorylcholine (2–50 mM). Stock solutions of each substrate were made in assay buffer, adjusted to pH 6.0, diluted, and stored at 30 °C until use. For mutant enzyme assays, Ymt was incubated with 20 mM bis-pNPP in assay buffer at 30 °C. Final enzyme concentrations were as follows: 1 μM for wild-type Ymt, 10 μM for Ymt mutants with single amino acid substitutions, and 50 μM for Ymt with two amino acid substitutions. Product release (para-nitrophenolate) was monitored via the absorbance at 405 nm. Initial rates of enzyme-catalyzed hydrolysis were determined from continuous measurements taken over 3 min for wild-type Ymt or discontinuous readings taken over 2 h for Ymt mutants. Rates were calculated using the molar extinction coefficient for pNP, 18,000 M⁻¹ cm⁻¹, and corrected for pNP ionization (18). All reactions were linear for the indicated time, and rates were corrected for spontaneous substrate hydrolysis.

**Physiological Substrate Hydrolysis**

**Nuclease Assay—**The nuclease assay was performed as described previously (19). Briefly, calf thymus DNA (7.5 μg) was incubated with 0–3 μg of Nuc or Ymt for 20 min at 37 °C in assay buffer with 100 μM EDTA. Samples were resolved using a 1% agarose gel, and DNA was visualized under a UV light source.

**Phosphatidylcholine Hydrolysis—**Phosphatidylcholine vesicles were prepared by mixing l-α-dipalmitoyl-[2-palmitoyl,9,10-3H] phosphatidylcholine (89 Ci/mmol) with unlabeled phosphatidylcholine to give a final concentration of 342 μM phosphatidylcholine (0.12 μCi/μmol). 10 μM of wild-type or mutant enzyme was incubated with 25 μl of vesicles for 1 h in a final volume of 150 μl. Lipids were extracted from the mixture by the addition of chloroform:methanol:acetic acid (50:50:0.5). The organic phase was dried under nitrogen, dissolved in chloroform: methanol:acetic acid (90:10:10), and subjected to thin-layer chromatography on a silica gel 60 plate in the solvent used for solubilization. Plates were sprayed with En3Hance® (NEN Life Science Products), and radiolabeled products were detected by autoradiography. Radiolabeled phosphatidic acid was used as a standard.

**Phospholipid Headgroup Release Assay—**All assays were performed...
in 50 mM Tris, pH 7.4, 80 mM KCl at 37 °C for 1 h. Headgroup-labeled phosphatidylcholine, phosphatidylethanolamine, and phosphatidyl-serine (as sonicated vesicles; final concentration, 10 μM) were incubated with wild-type Ymt (final concentration, 5 μM) in 1 μl for 1 h. The entire reaction was extracted in 1 ml of chloroform:methanol:conc. 1 N NH4OH (1:2:0.5). A portion of the aqueous phase was subjected to scintillation counting.

Transphosphatidylation Assay—To measure phosphatidic acid formation and transphosphatidylation, sonicated phosphatidylcholine vesicles were prepared from chain-labeled 1,2-dipalmitoyl-[2-3H]phosphatidylcholine and unlabeled dipalmitoylphosphatidy- cholines. Wild-type or mutant enzyme (final concentration, 10 μM) was incubated with 25 μl of vesicles for 1 h in a final volume of 150 μl Tris with or without ethanol (final concentration, 0.5%). Lipids were extracted from the reaction by the addition of chloroform:methanol:acetic acid (50:50:0.5). The lower phase was dried under nitrogen, dissolved in 1 ml of chloroform:methanol:conc. 1 N HCl. A portion of the upper, aqueous phase was subjected to scintillation counting.

Phosphoamino Acid Analysis

Ymt was labeled as described above and subjected to SDS-PAGE using a 10% gel. Slices of acrylamide containing labeled Ymt were excised and submerged in 3 N KOH at 105 °C for 5 h. The resulting hydrolysate was diluted 400-fold with water containing internal standards. Phosphoamino acids were separated by ion-exchange chromatography (21). O-Phthalaldehyde was added to the eluate, and the resulting fluorescence was detected on-line (21). Radioactivity was quantified by liquid scintillation counting. Phospholysine and phosphohistidine were synthesized as described previously (21). All other standards were purchased from Sigma.

RESULTS

Purification, Enzymatic Activity, and Crystallization—Ymt is a member of the PLD superfamily which, like human and plant PLD, contains two conserved motifs, HXXK/DX_{x}G(G/S) (Fig. 1A). The sequence for Ymt previously reported by Cheremanov et al. (12) contained three potential initiation methionine residues at positions 1, 42, and 56. We attempted to express recombinant protein using each of these residues as initiation codons. However, recombinant proteins starting with Met-42 and Met-56 were insoluble. Therefore, we utilized Met-1 as the initiation methionine for the protein used in our studies. Using this construct, Ymt containing a carboxyl-terminal hexahistidine “affinity tag” was overexpressed and isolated using a single-step procedure to ≥99% purity (Fig. 1B). Each liter of culture yielded 10–25 mg of purified Ymt, and amino-terminal sequencing of the recombinant protein revealed the predicted sequence, Met-Leu-Gln-Ile-Asp-Asn.

Ymt has no known native substrates and no well defined catalytic activity. The PLD superfamily members include nucleases, lipid-synthesizing enzymes, and lipid-degrading enzymes. The one feature of all the substrates utilized by this enzyme was that they contained a hydrolysable phosphate diester. Therefore, we explored nucleic acids and phospholipids as potential Ymt substrates. Ymt failed to hydrolyze DNA (data not shown); however, Ymt did hydrolyze phosphatidylethanol- amine and to a lesser extent, phosphatidylcholine and phosphatidylserine in a headgroup release assay (Fig. 2). In a separate headgroup release assay, phosphatidylglycerol and phosphatidylinositol 4,5-bisphosphate (at 75 and 42 μM, respec-
tively, the highest concentration possible in the assay), only 0.2 pmol of headgroup was released from phosphatidylinositol/h with Ymt, whereas phosphatidylinositol 4,5-bisphosphate was not cleaved. Upon TLC analysis, it was clear that Ymt cleaved the terminal phosphodiester bond of L-α-dipalmitoyl[2-palmitoyl-9,10-3H]phosphatidylcholine to form phosphatidic acid and, in the presence of 0.5% ethanol, the transphosphatidylation product, phosphatidylethanol (data not shown). Although Ymt displays PLD characteristics, hydrolysis of phosphatidylcholine in either reaction appears not to be stimulated by recombinant human ADP-ribosylation factor 1 (ARF1), phosphatidylinositol 4,5-bisphosphate, or sodium oleate (data not shown).

Because end point lipid hydrolysis assays provide only a qualitative assessment of Ymt activity, we explored artificial substrates to facilitate quantitation of Ymt catalytic activity.

**Multiple phosphomonoester and phosphodiester substrates were screened as potential Ymt substrates (See “Experimental Procedures”). The best artificial substrate tested was the phosphodiester, bis-pNPP (Fig. 3). Ymt hydrolyzed bis-pNPP to pNP and pNPP. pNPP is not a substrate for Ymt; thus, this enzyme is a phosphodiesterase and will not function as a phosphatase on phosphate monoesters.**

Hydrolysis of bis-pNPP by Ymt can be measured in a continuous assay and was found to be linear over 3 min at several substrate concentrations (Fig. 3A). When initial velocities were plotted against the substrate concentrations, the reaction did not appear to follow Michaelis-Menton kinetics (Fig. 3B). Limiting solubility (26 mM) of bis-pNPP prohibited determination of the kinetic parameters for hydrolysis. The artificial substrate does, however, afford a quantitative measure of activity and has a number of advantages over the end point assay employing phosphatidylcholine.

The large quantities of pure Ymt allowed us to search for crystallization conditions for this protein. Ymt formed small rod-like crystals in 2.0 M ammonium formate and 100 mM Hepes, pH 7.5, and diffraction-quality crystals were obtained by macroseeding (Fig. 4). The macroseeded crystals frozen in a solution containing 20% glycerol, 1.5 M ammonium formate, and 100 mM Hepes, pH 7.5, diffracted to 3.5 Å resolution. These conditions provide the necessary starting point for determining the structure of Ymt by x-ray crystallography.

**Mutagenesis of Amino Acids Located in the Two Conserved Motifs—**

Recently, Sung et al. (22) found that site-directed mutagenesis of amino acids in the conserved motifs of human PLD1 resulted in a marked loss of PLD activity. These investigations employed human PLD produced by transiently transfected COS-7 cells. The limited quantity of enzyme obtained from cell lysates prohibited a detailed analysis of the role of these residues in PLD catalysis. To define the role of the conserved residues in the two Ymt signature motifs, histidines in the first and/or second conserved motif of Ymt were replaced...
with asparagine by site-directed mutagenesis (H188N, H524N, and H188N/H539N). Using similar methodology, lysines were substituted with serine (K190S, K526S, K190S/K526S), and the aspartic acids were replaced with asparaginic or glutamic acid (D195N, D531N, D195N/D531N, or D/E). In addition, a conserved serine in the second motif was replaced by threonine or alanine (S539T or S539A, Table I). Although substitution of the conserved aspartic acid in either domain rendered the enzyme insoluble, all other mutations yielded soluble proteins that were purified to homogeneity (Fig. 5).

As the mutations may potentially disrupt secondary structure, the structure of each mutated enzyme was evaluated using UV absorbance, CD, and size-exclusion chromatography. The UV and CD spectra of the Ymt mutants were identical to that of wild-type Ymt suggesting that replacement of residues in the conserved motifs did not disrupt the secondary structure of this enzyme (data not shown). In addition, the oligomeric state of the Ymt was assessed using size-exclusion chromatography. Amino acid analysis as well as the cDNA sequence predicted a protein of 61.1 kDa which is consistent with the size determined by SDS-PAGE (63 kDa, Fig. 1B). However, all proteins eluted from a Superose 6 size-exclusion column at an apparent molecular mass of 125 kDa, which suggests that wild-type and mutant Ymt exist as dimers (data not shown).

In an effort to functionally evaluate these mutant enzymes, we employed the physiological and artificial substrates for wild-type Ymt, phosphatidylcholine and bis-pNPP, respectively. Replacement of both conserved histidines or lysines reduced phosphatidylcholine hydrolysis by Ymt to an undetectable level (data not shown). Artificial substrate hydrolysis was also markedly reduced as a result of substitutions in the conserved motifs (Table I). Substitution of the asparagine for histidine in the first conserved motif (H188N) reduced the turnover rate of bis-pNPP 164-fold. Similarly, mutagenesis of the histidine in the second conserved motif (H524N) reduced the rate of hydrolysis 470-fold. Replacement of both conserved histidines in the signature motifs resulted in a multiplicative functional loss and reduced the turnover rate 16,400-fold. Mutagenesis of Ser-539 in Ymt catalysis using the artificial substrate, bis-pNPP, Mutagenesis of Ser-639 in Ymt to threonine resulted in a 2-fold reduction in the rate of bis-pNPP hydrolysis, and replacement of this amino acid with alanine reduced the rate by 12-fold. We conclude that the histidines and lysines in the conserved motifs are critical for Ymt function and the conserved serine in the second motif serves a nonessential role in catalysis.

![Figure 4: Crystal of Ymt](image)

**TABLE I**

| Relative Specific Activity | % Activity |
|---------------------------|------------|
| **H188N** | H K K S | 0.06 x 10^-3 | 0.008 |
| **H524N** | N N | 0.65 x 10^-3 | 0.008 |
| **H188N/H524N** | N N | 2.30 x 10^-3 | 0.21 |
| **K190S** | S | 4.4 x 10^-3 | 0.28 |
| **K526S** | S | 2.1 x 10^-3 | 0.16 |
| **K190S/K526S** | S | 0.95 x 10^-3 | 0.066 |
| **S539T** | T | 0.67 x 10^-3 | 0.86 |
| **S539A** | A | 9.2 x 10^-3 | 0.03 |

*Activity is based on a subsaturating substrate concentration (20 mM), n = 3.

**Wild type.**

Numerous examples of two-domain proteins where the domains function independently have been described. Two examples of this class of proteins include the receptor protein tyrosine phosphatases (PTPases), LCA (leukocyte common antigen) and LAR (LCA-related molecule). These receptors contain two highly homologous cytoplasmic domains that appear to function independently (23). For example, mutagenesis of key catalytic residues in the first cytoplasmic domain of either PTPase abolishes more than 99% of the PTPase activity, whereas analogous mutations in the second cytoplasmic domain had little or no effect on activity.

Unlike the functionally independent domains of the receptor protein tyrosine phosphatases, the domains of the Ymt dimer appear to display functional dependence. Substitution of a single conserved residue in either domain reduced the catalytic activity by more than 99%. Several models were developed to explain these data. Fig. 6 depicts three possible dimer configurations for mutant Ymt with a single substitution in one domain. In model A, the conserved motif in domain I of one monomer would associate with the conserved motif in domain II of another monomer. Substitution of a single histidine in either domain will affect the catalytic activity at both active sites in the dimer, resulting in a nearly complete functional loss (i.e., >99%). Model C shows the monomers associating via the linker regions between the domains. The active sites of the dimer in model C are composed of residues found in the first...
indicated for each monomer. Conserved motif, and domains labeled represents a mutant Ymt dimer with a substitution in the conserved histidine from different domains act in concert to form active sites. Homodimers form between Ymt monomers such that histidines from like-domains interact to form active sites. Ymt monomers associate through linker regions between domains I and II such that intramolecular associations between catalytic residues are formed.

We attempted to further characterize the enzyme intermediate by determining the amino acid in Ymt that formed the enzyme-phosphate linkage (25–26). To evaluate the stability of the phosphoenzyme, the labeled Ymt phosphoprotein was transferred to Immobilon P membrane. Radiolabeled Ymt bound to the membrane was stable under alkaline conditions (1 M NaOH) but labile on exposure to acid (1 M HCl) or 1 M hydroxylamine (Fig. 8B, top panel). Membranes containing the Ymt phosphoprotein were stained with Coomassie Blue after incubation to verify that the protein remained bound (Fig. 8B, bottom panel). The stability of the phosphoprotein was also examined over a wide range of pH conditions, and the resulting hydrolysis curve paralleled that of phosphohistidine (Fig. 8C, 27, 28). Finally, we identified the labeled amino acid directly by ion-exchange chromatography after complete alkaline hydrolysis of the labeled intermediate (Fig. 9). The only radiolabeled amino acid detected in the hydrolysate co-eluted with phosphohistidine, with the remainder of the radiolabel being inorganic phosphate. Collectively, these data suggest that Ymt catalysis proceeds via a single-step displacement mechanism where no intermediate is formed, i.e. Ymt acts as a general base (Base-Ymt) to extract a proton from a nucleophile water molecule. In B, the reaction proceeds as a two-step mechanism where Ymt serves as the nucleophile (Nuc-Ymt). A phosphoenzyme intermediate is formed and subsequently broken down by hydrolysis.

![Fig. 6. Three possible Ymt dimer configurations.](image)

Each model represents a mutant Ymt dimer with a substitution in the conserved histidine in one domain. Domains labeled H contain a histidine in the conserved motif, and domains labeled N have an asparagine substituted for the conserved histidine. The amino (N) and carboxy (C) termini are indicated for each monomer. A, two Ymt monomers associate to form heterodimers such that histidines from different domains act in concert to form active sites. B, homodimers form between Ymt monomers such that histidines from like-domains interact to form active sites. C, Ymt monomers associate through linker regions between domains I and II such that intramolecular associations between catalytic residues are formed.

**Dissection of the Mechanism of Ymt Catalysis**—There are at least two possible mechanisms by which Ymt could accomplish substrate turnover. Ymt catalysis could proceed via a simple displacement reaction that does not involve the formation of an enzyme intermediate (Fig. 7A), or Ymt catalysis could be accomplished by a two-step reaction involving the formation and breakdown of a covalent intermediate (Fig. 7B). To discriminate between these possibilities, we took advantage of the fact that a number of phosphohydrolases have been shown to catalyze phosphate (oxygen)-water exchange (24). We asked if Ymt would similarly catalyze phosphate (oxygen)-water exchange using 32P-inorganic phosphate. If the reaction pathway shown in Fig. 7A is used by Ymt, no incorporation of label would be seen in the enzyme. If, however, the mechanism shown in Fig. 7B is employed, then it might be possible to “trap” 32P-labeled phosphate covalently bound to the enzyme.

Ymt was incubated at pH 4.5 with 32P-labeled phosphate. Rapid denaturation and analysis by SDS-PAGE demonstrated that 32P-labeled phosphate was covalently bound to Ymt (Fig. 8A). Substitution of the conserved serine (S539T and S539A) in Ymt resulted in a reduction in the amount of 32P-labeled protein. Substitution of a single conserved histidine (H188N, Fig. 8A) or lysine (data not shown) residue in the conserved motif of Ymt, however, prohibited the formation of a covalent phosphoprotein. These data argue that although the conserved histidines and lysines in the signature motifs are essential for phosphoenzyme formation, the conserved serine is not.

We attempted to further characterize the enzyme intermediate by determining the amino acid in Ymt that formed the enzyme-phosphate linkage (25–26). To evaluate the stability of
DISCUSSION

We report for the first time the physiochemical properties, crystallization, a potential native substrate, and the mechanism of catalysis for this dual-domain PLD superfamily enzyme. We demonstrate that for this PLD superfamily member, each signature motif (H4X6G(S)) is required for catalysis. Rudge et al. recently determined that the conserved lysine in the second motif in the yeast PLD, SPO14, is essential for PLD function in meiosis (29). Consistent with these findings, substitution of any one of these residues in either conserved motif of Ymt markedly reduced enzymatic activity. Further, mutagenesis of both histidine or lysine residues in the active site resulted in a multiplicative functional loss. Our findings are also consistent with the recent observations of Xie et al. (30), who demonstrated that co-expression of the amino- and carboxyl-terminal domains of rat brain PLD resulted in active proteins, whereas expression of either domain alone in COS cells produced no catalytic activity. The conclusions from these studies by Exton and coworkers (1) is that the two halves of PLD can associate, and that this may be essential to bring the two HKD domains together to form an active site.

Sung et al. (22) suggested a model for PLD catalysis in which the conserved serine (Ser-539 in Ymt) functions as the nucleophile. The model was based on assays of cell lysates from PLD-transfected COS-7 cells. We propose a catalytic mechanism where the conserved histidine in one domain serves as the nucleophile. The conserved histidine from a second domain (His-B) acts as a general acid to protonate the leaving group, which facilitates the formation of a covalent phosphoenzyme intermediate. The free histidine (His-B) then functions as a general base to activate water by proton abstraction. The intermediate is subsequently hydrolyzed by the activated water molecule.

Ymt active site resulted in a multiplicative functional loss. Our findings are also consistent with the recent observations of Xie et al. (30), who demonstrated that co-expression of the amino- and carboxyl-terminal domains of rat brain PLD resulted in active proteins, whereas expression of either domain alone in COS cells produced no catalytic activity. The conclusions from these studies by Exton and coworkers (1) is that the two halves of PLD can associate, and that this may be essential to bring the two HKD domains together to form an active site.

Sung et al. (22) suggested a model for PLD catalysis in which the conserved serine (Ser-539 in Ymt) functions as the nucleophile in catalysis. The model was based on assays of cell lysates from PLD-transfected COS-7 cells. We propose a catalytic mechanism where the conserved histidine in one domain serves as the nucleophile.
as the nucleophile, and the histidine from another domain facilitates the release of the leaving group by protonation. The role of histidine as the nucleophile in catalysis is supported by biochemical studies of both Ymt and the single domain PLD superfamily member, Nuc (31). Mutation of the single conserved histidine in Nuc or both conserved histidines in Ymt reduced the rate of artificial substrate hydrolysis by a factor of 105. In contrast, mutagenesis of Ser-539 in Ymt reduced the rate of bis-pNPP hydrolysis only by a factor of 10. In addition, substitution of the conserved serines in Ymt or Nuc had little impact on intermediate formation, whereas mutagenesis of any single conserved histidine abolished intermediate formation. The chemical properties of the enzyme intermediate were also consistent with histidine and not with serine. Finally, phosphoamino acid analysis identified phosphohistidine as the only radiolabeled amino acid in the labeled enzyme intermediate.

Our data demonstrate that Ymt catalysis proceeds via a two-step mechanism involving the formation and breakdown of a phosphoenzyme intermediate. It is known that the single-domain family member, Nuc, also utilizes a two-step catalytic mechanism (29). In addition, hydrolysis of substrates by cage PLD and E. coli phosphatidylerine synthase (two members of the PLD superfamily) proceeds with retention of configuration, suggesting a reaction mechanism involving two steps: the formation and breakdown of an enzyme intermediate (32–33).

Although the crystal structure of this dual-domain PLD superfamily member has not been completed, the structure of the single-domain superfamily enzyme, Nuc, has been solved (34). Enzymes in the PLD superfamily are likely to share both a common catalytic mechanism and common structural features. The structure of Nuc provides an indication of how conserved residues in Ymt might function. For example, in the structure of Nuc complexed with the competitive inhibitor, tattagase, histidine residues from two monomers of Nuc are coordinated to the tattagase ion. These residues are properly positioned to serve as the nucleophile and the general acid in catalysis. The conserved lysine residues also interact with the tattagase ion. It was concluded from the structure that the lysine residues bind and stabilize the negatively charged oxygen atom(s) on the phosphate diester. The biochemical analysis of Ymt described in this study is consistent with the structural data from Nuc. The catalytic role of the residues in the conserved motifs is likely to be the same not only for Ymt and Nuc but for other PLD superfamily members as well. Although the proteins in the superfamily have diverse physiological functions and substrates that range from nucleic acids to phospholipids, our data suggest that these enzymes are likely to utilize a common catalytic mechanism in which a phosphohistidine enzyme intermediate is formed and subsequently hydrolyzed in a two-step reaction.

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