An Active Site Water Network in the Plasminogen Activator Pla from Yersinia pestis

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SUMMARY

The plasminogen activator Pla from Yersinia pestis is an outer membrane protease (omptin) that is important for the virulence of plague. Here, we present the high-resolution crystal structure of wild-type, enzymatically active Pla at 1.9 Å. The structure shows a water molecule located between active site residues D84 and H208, which likely corresponds to the nucleophilic water. A number of other water molecules are present in the active site, linking residues important for enzymatic activity. The R211 side-chain in loop L4 is close to the nucleophilic water and possibly involved in the stabilization of the oxy-anion intermediate. Subtle conformational changes of H208 result from the binding of lipopolysaccharide to the outside of the barrel, explaining the unusual dependence of omptins on lipopolysaccharide for activity. The Pla structure suggests a model for the interaction with plasminogen substrate and provides a more detailed understanding of the catalytic mechanism of omptin proteases.

INTRODUCTION

Yersinia pestis, the causative agent of plague, evolved recently from a much less virulent species, Yersinia pseudotuberculosis (Bercouvier and Mollaret, 1984). Y. pestis is transmitted primarily by the bite of infected fleas. Due to the rapidly ingested but minute blood meal taken by fleas, and because Y. pestis infection can be readily suppressed by adaptive immunity (Degen et al., 2007), efficient transmission requires that the bacteria quickly reach high densities in the circulation. Y. pestis utilizes several mechanisms, both active and passive, to suppress and evade innate immune responses. Some of these are shared with Y. pseudotuberculosis, while others are not. One important adaptation unique to Y. pestis is the production of an outer membrane protease known as Pla. Specific inactivation of this protease results in a million-fold increase in LD₅₀ for mice infected subcutaneously (Sodeinde et al., 1992; Sebbane et al. 2006) and also greatly reduces the virulence of Y. pestis following pneumonic infection (Latham et al. 2007; Agar et al. 2009). Pla has been shown to degrade several potentially important mammalian proteins in vitro (Yun and Morrissey, 2009; Suomalainen et al., 2007). In addition, it has been shown to act as both an adhesin and invasin, mediating tight binding to fibronectin and promoting uptake of the bacteria by nonphagocytic cells in culture (Suomalainen et al., 2007; Cowan et al. 2000). However, the only activity that has been strongly associated with virulence is the cleavage (activation) of plasminogen by Pla to generate plasmin (Degen et al., 2007; Beesely et al., 1967), an important blood protease that degrades many blood plasma proteins, most notably fibrin. Plasmin activity promotes dissemination of Y. pestis from the primary infection site by inhibiting microabscess formation (Degen et al., 2007; Sodeinde et al., 1992).

Pla belongs to a unique family of integral outer membrane proteases known as omptins, which are widely distributed within the family Enterobacteriaceae. Although omptins share high sequence identity (50%–75%), their biological functions range from a possible housekeeping role in Escherichia coli (Haiko et al., 2009) to the important contribution to virulence in plague described above. A medium resolution structure of only one omptin, E. coli OmpT (50% identical to Pla) crystallized from refolded, inactive protein, has been reported (Vandeputte-Rutten et al., 2001). Although OmpT was originally classified as a serine protease (Kramer et al., 2000a), mutagenesis experiments and the crystal structure were inconsistent with this mechanism (Vandeputte-Rutten et al., 2001; Kramer et al., 2001). Furthermore, inhibitors of the major classes of proteases are ineffective against omptins (Sugimura and Nishihara, 1988), complicating the development of Pla-based therapeutics.

Molecular dynamics studies suggested a novel catalytic mechanism for omptins in which a water molecule is responsible for nucleophilic attack on the substrate (Baaden and Sansom, 2004). While consistent with mutagenesis data, no direct evidence for this mechanism exists. Here, we report high-resolution crystal structures of wild-type Pla protease obtained from enzymatically active crystals in order to obtain detailed information about the catalytic mechanism and to understand the structural basis of substrate specificity of omptin family members.

RESULTS AND DISCUSSION

Description of the Pla Structure

Crystals of wild-type Pla and the inactive Pla mutant D86A were obtained using the detergent C₈E₄. Remarkably, after overnight
Dialysis against C₈E₄ the Pla proteins elute as a broad peak close to the void volume on analytical gel filtration chromatography columns (see Figure S1 available online; compare with protein purified in OG). This surprising result shows that crystals of (membrane) proteins can form from high-molecular weight, heterogeneous protein aggregates.

An initial model for wild-type Pla was generated from phases obtained from a single anomalous dispersion (SAD) data set of an osmium derivative (Table 1; Figure S2). This model was used to solve two structures of wild-type Pla at high-resolution (1.9 Å and 2.3 Å) by molecular replacement. Pla is approximately 70 Å long and forms a narrow β-barrel with an elliptical cross-section. The barrel consists of 10 antiparallel β-strands connected by short periplasmic turns and five extracellular loops (Figure 1). Residues E252–S269 in loop L5 are not visible in the wild-type Pla structure, possibly due to autocatalytic activity (Kukkonen et al. 2001) of Pla at residue K261. The entire Pla molecule is visible in the structure of the active site mutant D86A, solved at a resolution of 2.55 Å (Table 1). The D86A mutant has very low activity (Figures 2A and 2B) and therefore does not undergo autocatalysis. The structures of wild-type Pla and the D86A mutant are very similar, with rmsd values of 0.45 Å (all atoms). In addition, and as expected from the high sequence homology (Figure 1C), the overall structure of Pla is similar to that of OmpT, with an rmsd of 1.1 Å.

### Active Site Water Molecules

The catalytic site residues D84, D86, D206, and H208 are conserved in all omptins (Hritonenko and Stathopoulos, 2007) and are located in a cleft on the extracellular surface of the β-barrel (Figures 1, 3A, and 3B). D84/D86 form a couple and are located on one side of the barrel, whereas D206/H208 form the other couple, located on the opposite side of the barrel (Figures 3A and 3B). The minimum distance between both active site couples is between D84 and H208 (4.7 Å). A water molecule (W1) is present between the D84 carboxyl group and the H208 N3 atom (Figures 3A and 3B). This water molecule most likely corresponds to the catalytic nucleophile that was previously proposed based on molecular dynamics studies of OmpT (Baaden and Sansom, 2004). The D206/H208 couple likely forms a catalytic dyad that activates the nucleophilic water molecule for attack on the carbonyl carbon of the scissile peptide bond. While there are other enzymes that utilize a catalytic dyad for activity (e.g., phospholipase A₂) (Scott et al., 1990), the presence of a catalytic dyad in a protease is unique to omptins. The crucial importance of D206/H208 for catalysis is supported by

<table>
<thead>
<tr>
<th>Table 1. Data Collection, Phasing, and Refinement Statistics for Wild-Type Pla and the Inactive D86A Mutant</th>
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<tbody>
<tr>
<td><strong>Wild-Type Pla</strong></td>
</tr>
<tr>
<td>OsCl₅ (APS ID-23B)</td>
</tr>
<tr>
<td><strong>Data collection</strong></td>
</tr>
<tr>
<td>Space group</td>
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<tr>
<td>Cell dimensions (a, b, c)</td>
</tr>
<tr>
<td>Resolution (Å)</td>
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<td>I/σI</td>
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<td>Completeness (%)</td>
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<tr>
<td><strong>Phasing</strong></td>
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<tr>
<td>Number of sites</td>
</tr>
<tr>
<td>SOLVE Z-score/FOM</td>
</tr>
<tr>
<td>SHARP Pp/FOMcen, acen</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
</tr>
<tr>
<td>Resolution (Å)</td>
</tr>
<tr>
<td>Number of reflections</td>
</tr>
<tr>
<td>Rwork/Rfree (%)</td>
</tr>
<tr>
<td>Rmsds</td>
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<tr>
<td>Bonds/angles</td>
</tr>
<tr>
<td>Number of atoms</td>
</tr>
<tr>
<td>Protein/detergent/water</td>
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<tr>
<td>Ramachandran plot</td>
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Pp, phasing power.

⁴ Values in parentheses are for the highest resolution shell.

b Rwork = Σ|Fo–Fc|/ΣFo. Rfree is the cross-validation of R-factor, with ~5% of the total reflections omitted in model refinement.
density as water molecules is supported by the lower resolution structure (2.3 Å) of wild-type Pla. Here, density is present only for the W1 nucleophilic water and one additional water molecule (Figure S3), separated by ~3.6 Å.

Pla also has a water molecule (W2) located between D84 and D86 (Figures 3A and 3B; Figure S3), which is reminiscent of aspartic proteases (Brik and Wong, 2003). However, mutagenesis studies for Pla show that mutations of D84/D86 result in proteins that still have some residual activity (Kukkonen et al., 2001) (Figures 2A and 2B), suggesting a supporting rather than a central role for these residues in catalysis. This notion is supported by the pH-activity profiles for Pla (Figure 2C) and OmpT (Kramer et al., 2000b), showing that omptins, unlike aspartic proteases, are inactive below pH 5.0. We therefore propose that omptins should not be classified as aspartic proteases (Suomalainen et al., 2007). D84 and D86 likely perform important structural roles, by coordinating the nucleophilic water molecule either directly (D84) or indirectly via W2 (the distance between W1 and W2 is 2.8 Å) (Figure 3B). In addition to the active site residues, S99 and H101 are two residues located close to the active site that have also been shown to be important for omptin activity (Kramer et al., 2000a). The Pla structure provides an explanation for the importance of S99 and H101, since they coordinate the active site residues D84 and D86 via bridging water molecules.

A Model for the Interaction of Pla with Plasminogen Substrate

Pla cleaves the R561-V562 bond within the sequence PGVVGG located in a surface-exposed loop of plasminogen (Sodeinde et al., 1992; Wang et al., 1998). Placing the scissile peptide bond approximately at the position of the nucleophilic water molecule, the distribution of residues within the active site cleft strongly suggests that the arginine side chain of the substrate will bind in a deep, negatively charged pocket formed by Pla residues E29, D204, D206, and E217 (Figure 4). The two valine residues C-terminal to the scissile bond will likely bind in the shallow, hydrophobic pocket located on the other side of the plane formed by the active site residues D84, H208, and the nucleophilic water molecule (Figure 4).

Another Pla residue that is likely to be important for interaction with the plasminogen substrate is R211, which is located at the tip of loop L4 and points inward to the active site. The structure of OmpT did not give any clues as to the location of the analogous residue K217 since it was disordered, presumably as a result of the several mutations introduced to aid crystallization (G216K/K217G). The arginine side chain in Pla is only ~6 Å away from the nucleophilic water molecule and within hydrogen bonding distance (3.2 Å) of one of the additional active site waters (Figure 3). The location of the arginine side chain makes this residue very likely to interact with the plasminogen substrate. We propose that the positive charge on the arginine side chain could play a role in the stabilization of the oxyanion intermediate during catalysis, analogous to the role of calcium in phospholipase A2 (Scott et al., 1990). Consistent with this hypothesis, substitution of R211 for alanine results in low levels of plasminogen cleavage (Figure 2B). Remarkably, the conservative mutant R211K is also a poor plasminogen activator (Kukkonen et al., 2001) (Figure 2B), indicating that the interaction of R211 with the substrate is highly specific.

mutagenesis studies (Kramer et al., 2001; Kukkonen et al., 2001) (Figure 2B) and by the pH-activity profile, which suggests involvement of a histidine residue in catalysis (Figure 2C). In the 1.9 Å structure, additional electron density is present close to W1, which we have modeled and refined as two additional active site water molecules (Figure 3A). The short distances between these three water molecules (2.1–2.2 Å) (Figure 3B) suggest the presence of a mixed water model analogous to that observed in, e.g., urease (Pearson et al., 2000). The assignment of this

Figure 1. Overall Structure of Y. pestis Wild-Type Pla
(A and B) Backbone representation viewed from the side (90° rotated in B), with β strands colored blue, loop 1 salmon, loop 2 olive, loop 3 cyan, loop 4 lime, and loop 5 purple. Cytoplasmic turns are colored wheat. The active site residues D84, D86, D206, and H208 (yellow carbons, blue nitrogens, and red oxygens), as well as R211 (green carbons), are shown as stick models. The residues of the putative LPS binding site are shown as magenta stick models. The modeled CxE detergent molecules close to the LPS binding site are colored orange. All figures were made using PYMOL (DeLano, W.L. The PyMOL Molecular Graphics System, 2002, DeLano Scientific, Palo Alto, CA, USA).

(C) Sequence alignment of Pla and OmpT with the observed secondary structures. Identical residues (red), active site residues and catalytic residues (blue) and R211(Pla)/K217(OmpT) (green) are indicated. Pla secondary structure elements (loops in specific colors, β strands in blue, and cytoplasmic turns in wheat) are shown on top. Extracellular loops are labeled L1–L5 and transmembrane β strands are labeled S1–S10, with the dark blue regions indicating the parts that are embedded in the outer membrane.
Activation of OmpTins by Lipopolysaccharide

OmpTins require LPS for enzymatic activity (Kukkonen et al., 2001, 2004; Kramer et al., 2002) by a mechanism that is unclear. Based on the structure of the OM protein FhuA with bound LPS (Ferguson et al., 1998), it has been proposed that ompTins have an LPS binding site composed of the residues Y134, E136, R138, and R171 (numbering for Pla). The previously determined crystal structure of E. coli OmpT at 2.6 Å resolution (Vandeputte-Rutten

Figure 2. Plasminogen Activation

(A) Activity of β-OG purified wild-type Pla (Pla), wild-type Pla isolated from crystals (Pla Crystals), β-OG purified D86A mutant (D86A), and D86A mutant isolated from crystals (D86A Crystals).

(B) Activity of membrane-bound wild-type Pla and active site residue Pla mutants. The activity of D84A and H208N mutants were at background levels.

(C) pH optimum for plasminogen activation. Plasminogen activation values of membrane-bound (□) and β-OG (■) purified wild-type Pla were determined at a pH range 4.5–8.5. All plasminogen activation values in (A), (B), and (C) correspond to specific activities of proteins and are the mean of three independent measurements carried out with at least two different preparations of purified proteins or membranes. The error bars represent the standard errors (SEM).
et al., 2001) was obtained from protein refolded from inclusion bodies in the absence of LPS and therefore corresponds to inactive protein. In contrast, the Pla protein used for crystallization was purified from the bacterial OM, using LDAO for extraction and the first gel filtration column (Experimental Procedures). Remarkably, while the purified wild-type protein used for crystallization was only \( \frac{1}{2} \) active, wild-type Pla reisolated from the crystals had full activity (Figure 2A). The low overall activity of our purified protein is likely due to the extensive removal of LPS from the protein by LDAO. Indeed, when LPS or variants thereof are added to inactive, LDAO-purified Pla, reactivation of the protein occurs (Figure 5A). The inactivating effect of LDAO on Pla is exhibited by many detergents, but not by C8E4 or OG (Figure 5B). The fact that the protein in the crystals is fully active could be explained by assuming that the crystals are formed by a small population of LPS-bound Pla molecules that exists in our preparation. This notion is supported by the fact that there are five striking, parallel tubes of density close to the

**Figure 3. The Active Site of Wild-Type Pla Contains a Network of Water Molecules**

(A) Stereoview of the active site from the extracellular side. The active site residues are shown as stick models in green, whereas the other residues that are important for activity are shown in yellow. Water molecules are shown as red crosses. Fo-Fc densities obtained by simulated annealing omit maps are shown as a blue mesh. The nucleophilic water molecule W1, as well as the W2 water molecule between D84 and D86, is highlighted in boldface.

(B) Schematic diagram showing the hydrogen bonding network of water molecules and amino acids in the active site. Bond lengths of all H-bonds are shown in blue.

**Figure 4. Putative Model for the Interaction of Pla with Plasminogen**

(A) Surface view from the extracellular side, showing the charge distribution across the active site cleft. The putative binding sites for plasminogen residues are indicated. The position of R211 is indicated.

(B) Stereoview of the active site cleft, with approximately the same orientation as in (A), showing residues E29, D204, D206, and E217 of the negatively charged binding pocket as magenta stick models, the active site residues D84 and H208 as yellow stick models, and residues S99, H101, R211, Y148, Y150, F159, V175, I166, and M210 as green stick models (oxygens red, nitrogens blue). The location of the nucleophilic water is indicated with an asterisk. (A) was generated using the ABSP plug-in within PYMOL.
Figure 5. Mechanism of Pla Activation by LPS

(A) Effect of LPS on Pla activity. Plasminogen activation by Pla was measured in the presence of KDO2-Lipid A (5:1; KDO2-Lipid A:Pla molar ratio), E. coli LPS (8:1; LPS:Pla molar ratio) and Y. pestis LPS (2.5:1; LPS:Pla molar ratio), after inactivation of wild-type Pla by LDAO (Pla LDAO). The activity of β-OG purified wild-type Pla (Pla) is shown as a positive control.

(B) Effect of detergents on plasminogen activation by Pla. Plasminogen activation by membrane-bound Pla was measured in the absence of any detergent (control) or after incubation with the detergents indicated in the figure. Plasminogen activation values in (A) and (B) correspond to specific activities of proteins. All values are means of three independent measurements of activity with at least two different preparations of membranes. Error bars represent the standard error (SEM).

(C) Closeup side view of the barrel of wild-type Pla, with the residues in the putative LPS binding site shown as yellow stick models (nitrogens blue, oxygens red). Bound acyl chains in the vicinity of the LPS binding site that may belong to a partially ordered bound LPS molecule are shown in magenta, with Fo-Fc densities obtained by simulated annealing omit maps shown as a blue mesh.

(D) Closeup of the superimposed active sites of active Pla (yellow) and inactive OmpT (blue), showing the conformational shift for strand S7 and H208 away from the active site in OmpT.

Table 2. LPS Detection in Wild-Type Pla Preparations and Crystals

<table>
<thead>
<tr>
<th>Protein</th>
<th>LPS:Protein Molar Ratio</th>
<th>SE (%)</th>
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<tbody>
<tr>
<td>Pla-OG</td>
<td>3.00</td>
<td>0.05</td>
</tr>
<tr>
<td>Pla-LDAO</td>
<td>0.06</td>
<td>0.0012</td>
</tr>
<tr>
<td>Pla Crystals</td>
<td>1.02</td>
<td>0.07</td>
</tr>
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</table>

All values are the mean of three independent measurements. Pla-OG, wild-type Pla purified in OG; Pla-LDAO, wild-type Pla purified in LDAO.
et al., 2001), OmpT was converted into a reasonably efficient plasminogen activator (~25% Pla activity) by substituting K217 in loop L4 with the corresponding arginine residue present in Pla (R211; Figure 1C), combined with the removal of D213/ P214 (absent in Pla). The additional introduction of Pla loop L3 in this OmpT mutant results in a hybrid protein with 90% activity of wild-type Pla (Kukkonen et al., 2001). Thus, loops L3 and L4 are important contributors toward substrate specificity, with R211 in L4 being the single most important residue. A sequence comparison between OmpT and Pla shows that the tips of loops L3 and L4 are indeed among the most divergent areas between Pla and OmpT, with the Pla loops being two and four residues shorter, respectively, than the corresponding loops in OmpT (Figure 1C). The L3 and L4 loops form the entrance to the active site and are very likely to be involved in plasminogen binding. Steric hindrance by the longer OmpT loops is therefore likely to be an important reason for the poor plasminogen activating capability of OmpT. Pla likely binds with high affinity to plasminogen (K_m for plasminogen activation ~120 nM), suggesting that differences in the interaction surface (such as those existing between Pla and OmpT) may therefore have a large effect on the activity of the protease toward plasminogen. Unfortunately, the very low activity of OmpT toward plasminogen does not allow determination of Michaelis constants for plasminogen activation by OmpT.

In summary, the current high-resolution crystal structure of wild-type Pla provides a detailed view of the active site of an ompalin protease, highlighting the importance of water molecules for catalytic activity, as well as identifying an unusual network of water molecules linking the active site residues that explains previous mutagenesis data. The protein within the crystals is catalytically active, and a comparison with inactive OmpT suggests that LPS activates omptins by inducing a subtle conformational change for strand S7 including the active site residue H208. Due to its surface localization and critical importance in both bubonic and pneumonic plague (Sodeinde et al., 1992; Sebbane et al., 2006; Lathem et al., 2007; Agar et al., 2009), Pla is a potential target for development of novel therapeutics, as are the omptins of other pathogenic enterobacteria. Exploration of this potential and understanding of the catalytic mechanism employed by this unique protease family will be greatly aided by the availability of high-resolution Pla structures.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Membrane Preparation of Proteins

The pla gene from Y. pestis and ompT from E. coli were cloned into the E. coli expression vector pB22 (Guzman et al., 1995; Van den Berg et al., 2004) with a C-terminal hexa-histidine tag for purification. Active site residue mutations were made by using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). DNA sequencing was performed at CFAR DNA sequencing facility (UMass Medical School, Worcester, MA). C43 cells (Miroux and Walker, 1996) were transformed with Pla-pB22. The cells were grown to OD_600 ~1.0 at 37°C and then induced with 0.2% arabinose at 20°C overnight. Cells were harvested by centrifugation at 4500 rpm for 20 min (Beckman Coulter, J6-MC). Cell pellets were resuspended in 10 mM Tris-Cl, 50 mM NaCl (pH 8.0), and cells were lysed by sonication (3 × 40 s intervals) (Branson Digital Sonifier). Unbroken cells and inclusion cells were removed by centrifugation at 9000 rpm for 20 min (Beckman L8-70M ultracentrifuge). Total membranes were obtained by centrifugation at 40,000 rpm for 40 min (45 Ti rotor; Beckman L8-70M ultracentrifuge). Membranes were homogenized in 10 mM Tris, 50 mM NaCl (pH 8.0) and kept at –80°C. Pla and OmpT amounts in total membranes was determined by comparison of the band intensities in Western blots with that of purified Pla and OmpT using the spot densit analysis program (Alphalager 2200), using serial dilutions to avoid saturation of the band intensities. In Western blots the C terminus histidine tag was detected using Penta-His HRP conjugate (QiAGEN, Germantown, MD). Detergent-purified Pla and OmpT protein concentrations were determined by the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Structure 18, 809–818, July 14, 2010 ©2010 Elsevier Ltd All rights reserved 815
Purification of Pla for Crystallization

Total membranes of C43 cells overexpressing Pla were diluted to 6 mg/ml in TSB (20 mM Tris, 300 mM NaCl, 10% glycerol [pH 8.0]). Membranes were solubilized by stirring in TSB with 1% LDAO (Anatrace, Maumee, OH) for 1 hour at 4°C. Solubilized membranes were centrifuged at 40,000 rpm for 30 min. The membrane extract was applied to a 10 ml nickel column. The column was washed with 10 column volumes (CV) of TSB containing 0.2% LDAO and 15 mM imidazole. Pla was eluted with 3 CV TSB containing 0.2% LDAO and 250 mM imidazole. Pla was further purified by gel filtration chromatography using 10 mM Tris, 50 mM NaCl, 0.05% LDAO (pH 8.0). Purified protein was concentrated to ~10 mg/ml and then dialyzed (50 kDa molecular weight cutoff; Spectrapor) overnight against 10 mM Tris, 100 mM LiCl, 0.4% C8E4 (Anatrace) [pH 8.0]. The Pla mutant D86A was purified as described above and dialyzed against 10 mM Na-acetate, 100 mM LiCl, 0.4% C8E4 (pH 5.0).

Purification of Active Pla and OmpT

Pla and OmpT-containing total membranes were diluted to ~6 mg/ml in 10 mM Tris-Ci, 150 mM NaCl (pH 8.0). Membranes were solubilized in 1% OG (β-octyl glucoside; Anatra) at 65°C for 30 min followed by centrifugation at 40,000 rpm for 30 min to remove precipitates and unsolubilized membranes. The membrane extract was applied to a 5 ml nickel column. The column was washed with 5 CV 10 mM Tris-Ci, 150 mM NaCl (pH 8.0), 1% OG, 15 mM imidazole. The protein was eluted with 3 CV 10 mM Tris-Ci, 50 mM NaCl (pH 8.0), 1% OG. The proteins were concentrated to 5 mg/ml and dialyzed against 10 mM Tris-Ci, 50 mM NaCl, 1% OG (pH 8.0). Protein quantification was carried out using the BCA assay (Thermo Scientific).

Crystallization of Pla and Structure Determination

Wild-type Pla was diluted to 6 mg/ml in 10 mM Tris, 100 mM LiCl, 0.4% C8E4. Crystals were obtained by hanging drop vapor diffusion at 22°C by adding 1 μl of protein solution to 1 μl of mother liquor containing 27% PEG 400, 0.1 M LiSO4, 0.1 M Li-citrate [pH 4.0] (final pH 4.8 after addition of protein). Zeppelin-shaped crystals appeared between 2 days to 1 week and reached a maximum size of ~300 microns in the longest direction. They belong to space group P6122 (Table 1) and contain one Pla molecule per asymmetric unit (Vm ~3.3 Å3/Da, corresponding to ~77% solvent content). A heavy atom derivative was prepared by adding 5 mM OsCl3 to the crystal drop for 2 hours, resulting in a yellow-brown color of the crystals. Interestingly, the soaking of osmium (and platinum; data not shown) into the crystals resulted in an inability resulting in a yellow-brown color of the crystals. Interestingly, the soaking of osmium (and platinum; data not shown) into the crystals resulted in an inability to scale the data in P6122. Apparently the soaking resulted in a change of space group, which is now C2221 (three molecules per asymmetric unit; Vm 77% solvent content). A heavy atom derivative was prepared by adding 0.8 Å3/Da, corresponding to ~77% solvent content). As shown in Figure S2, the osmium atoms are bound on the surface of the protein and may be involved in lattice contacts, providing an explanation for the subtle change in space group (the packing of the molecules is virtually identical between the two space groups). Crystals were flash-frozen in liquid nitrogen directly from the mother liquor. D86A mutant Pla crystals were obtained via the same method, by adding 0.8 Å3/Da of protein (8 mg/ml) to 1 μl of mother liquor containing 16% PEG 400, 0.1 M Li-citrate (pH 3.5). The cube-shaped crystals appeared after about 1 week and grew to a maximum size of ~150 microns. They belong to space group P21, and contain four Pla molecules in the asymmetric unit (Vm ~3.4 Å3/Da, corresponding to ~64% solvent content). These crystals were flash-frozen from crystallization solution containing 30% PEG400.

Diffraction data were collected at APS beamlines ID-23B/D and NSLS beamlines X25 and X6A, and were processed with HKL2000 (Otwinowski and Minor, 1997) or XDS (Kabsch, 1993). A highly redundant SAND data set at the osmium peak wavelength (1.13 Å) was collected and used for phasing by SOLVE (Terwilliger and Berendzen, 1999). Three clear sites (one per Pla molecule) were found and refined in SHARP (Bricogne et al., 2003). The resulting density-modified maps were of excellent quality and allowed automated model building of ~50% of the asymmetric unit by REVEOLVE (Terwilliger, 2000). The model (without waters and detergents) was completed by manual building within COOT (Emsley and Cowtan, 2004) and subjected to a single round of refinement within PHENIX (Adams et al., 2002). This model was used as the search model to solve the structures of wild-type Pla and the D86A mutant by molecular replacement using Phaser (Storoni et al., 2004). All structures were refined within PHENIX, using a protocol that included TLS refinement. Simulated annealing Fo-Fc omit maps (Figures 3A and 5C) were made in CNS1.2 (Brüger et al., 1998), using a start temperature of 1000K and cooling steps of 25K.

Pla and OmpT Activity Assays

Activity measurement of purified proteins was carried out with 2.5 μg Pla and 800 nM plasminogen in 50 mM Tris-Acetate, 50 mM MES (pH 6.5) buffer in a total volume of 100 μl. Pla and plasminogen were incubated at room temperature for 15 min, after which the reaction pH was changed to pH 8.0 by addition of 90 μl 1M Tris (pH 8.0). Chromogenic substrate S2250 (Sigma, St. Louis, MO) was added to the reaction mixture to a final concentration of 5 μM. The absorbance change at 405 nm was followed for 30 min (TECAN; Saffie, Männedorf, Switzerland). Background activity of reagents or proteins was subtracted from all measured activities.

For activity determination of Pla crystals, several large crystals were collected from the mother liquor using 0.1–0.2 mm loops and washed three times by serial transfers into 2 μl mother liquor aliquots. The washed crystals were redissolved in 10 mM Tris, 50 mM NaCl (pH 8.0), 0.4% C8E4. The amount of protein within the crystals was determined both by BCA assay and spot densitometry analysis of protein bands run on SDS-PAGE NuPAGE 4%–12% Bis-Tris Gel; Invitrogen, Carlsbad, CA) gels. The single crystal (approximately 300 microns in size) that was used for structure determination was also collected and analyzed in the activity assay. The activity of Pla from crystals was determined as described above except that in these measurements 0.09–0.25 μg of Pla was used.

The determination of Pla activity in membranes was carried out as described above except that 30 μg total membranes were used in the assay. Activities of E. coli total membranes obtained from cells transformed with the empty plasmid were subtracted from all membrane activities. Activities of active site mutants were normalized according to the expression levels, which differed by only about 15%. In detergent inhibition assays, total membranes (8 mg/ml protein) were incubated with any of 1% (final concentration) OG, Tween-20, C8E4, C6E6, DM, DDM, DHPC (di-heptanoyl-phosphatidylcholine), or LDAO for 10 min at room temperature and 5 μl was transferred into the activity assay mixture.

For reactivation of pla by Kdo2–LipidA and LPS, Pla was diluted to 0.1 mg/ml using 10 mM Tris, 50 mM NaCl, 1% OG (pH 8.0). The protein was inactivated by addition of 0.25% LDAO and incubation at room temperature for 10 min. The minimum inhibitory concentration of LDAO that would inactivate the protein by more than 90% was determined by titration of protein with different amounts of LDAO. Subsequently 5 μl of inactivated Pla was added to the activity assay mixture. Reactivation was performed by titration of inactive Pla with Kdo2–LipidA, E. coli LPS or Y. pestis LPS.

Determination of the pH-Activity Profile

The pH profile was determined by measuring the activity of OG-purified Pla or total membranes containing Pla using two different buffer systems ranging from pH 4.5 to 9.5. The first buffer system used was 50 mM Tris-acetate, 50 mM MES, and the second buffer system was 50 mM Tris-Ci and 50 mM Bis-Tris. Both systems yielded identical results. In Figure 2C the results obtained using the Tris-MES buffer system are shown.

LPS Detection Assay

LPS detection in purified Pla proteins extracted with either OG or LDAO and in Pla crystals was carried out by Limulus Amoebocyte Lysate (LAL) endotoxin assay kit (GenScript, Piscataway, NJ) according to the manufacturer’s protocols. Pla crystals for assay were obtained and washed as described in the “Pla and OmpT activity assays” section. For the assays 50 pg of purified protein was used. LPS to protein molar ratios were calculated considering that 1 ng endotoxin standard corresponds to 10 enzyme units of activity, and by assuming an average molecular weight of LPS of 10,000 (Erridge et al., 2002).

ACCESSION NUMBERS

Coordinates and structure factors were deposited in the Protein Data Bank with the following PDB ID codes: wild-type Pla Native 1, 2X55; wild-type Pla Native 2, 2X66; and Pla mutant D86A, 2X4M.
Supplemental Information

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.str.2010.03.013.

Acknowledgments

We thank Debra Tow for help in crystal data collection and data processing. We thank the staff of APS beamlines ID-23B/D and NSLS beamlines X25 and X6A for their assistance during data collection. We also thank the RapiData 2009 instructors (rapid data collection and structure solving) at the NSLS for their guidance in and help with the collection of preliminary data of Pla crystals.

Received: January 26, 2010
Revised: March 22, 2010
Accepted: March 31, 2010
Published: July 13, 2010

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