Published in final edited form as:

Biochemistry. 2019 March 26; 58(12): 1616–1626. doi:10.1021/acs.biochem.9b00031.

Crystal Structure and Conformational Dynamics of *Pyrococcus furiosus* Prolyl Oligopeptidase

Ken Ellis-Guardiola^{3,‡}, Huan Rui^{2,‡}, Ryan L. Beckner³, Poonam Srivastava³, Narayanasami Sukumar^{4,*}, Benoît Roux^{2,3,*}, Jared C. Lewis^{1,*}

¹Department of Chemistry, Indiana University, Bloomington, IN 47405.

²Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637, USA.

³Department of Chemistry, University of Chicago, Chicago, IL 60637, USA.

⁴NE-CAT and Department of Chemistry and Chemical Biology, Cornell University, Building 436E, Argonne National Laboratory, Argonne, IL 60439, USA.

Abstract

Enzymes in the prolyl oligopeptidase family possess unique structures and substrate specificities that are important for their biological activity and for potential biocatalytic applications. The crystal structures of *Pyrococcus furiosus* (*Pfu*) prolyl oligopeptidases (POP) and the corresponding S477C mutant were solved to 1.9 and 2.2 Å resolution, respectively. The wild type enzyme crystallized in an open conformation, indicating that this state is readily accessible, and it contained bound chloride ions and a prolylproline ligand. These structures were used as starting points for molecular dynamics simulations of *Pfu* POP conformational dynamics. The simulations showed that large-scale domain opening and closing occurred spontaneously, providing facile substrate access to the active site. Movement of the loop containing the catalytically essential histidine into a conformation similar to those found in structures with fully-formed catalytic triads also occurred. This movement was modulated by chloride binding, providing a rationale for experimentally observed activation of POP peptidase catalysis by chloride. Thus, the structures and simulations reported in this study, combined with existing biochemical data, provide a number of insights into POP catalysis.

*Corresponding Author jcl3@iu.edu, roux@uchicago.edu, sukumar@ anl.gov.

Present Addresses

K.E.G.: University of California - Los Angeles, Boyer Hall 610, 611 Charles E. Young Drive East, Los Angeles, CA 90095

R.B.: National Institute on Alcohol Abuse and Alcoholism (NIAAA), 5625 Fishers Ln room 3N15, Rockville, MD 20852

‡These authors contributed equally.

ASSOCIATED CONTENT

Uniprot ID for *Pfu* POP

Q51714

Supporting Information

Complete experimental and computational procedures, supplemental tables and figures, and crystallographic data are available. The Supporting Information is available free of charge on the ACS Publications website.

Keywords

prolyl oligopeptidase; protease; serine hydrolase; x-ray crystallography; molecular dynamics simulation

INTRODUCTION

Prolyl oligopeptidases (POPs) hydrolyze small peptide substrates following proline residues.^{1–3} This specificity leads to the involvement of POP family enzymes in a range of important biological processes² and biocatalytic applications⁴. With molecular weights of ~70–80 kDa, POPs are significantly larger than many prototypical proteases like trypsin or subtilisin (25–30 kDa), suggesting that the additional bulk of the former may contribute to their unique substrate specificity.^{3,5} Indeed, structural studies have established that POPs are comprised of a ~30 kDa peptidase domain containing a Ser-His-Asp catalytic triad topped by a ~40 kDa 7-bladed β -propeller domain that restricts substrate access to a large active site cavity.^{3,6–10} Experimental and computational studies have suggested that this domain undergoes large scale conformational changes that regulate substrate access to its active site.

While extensive structural characterization of bacterial and mammalian POPs exists, no structures for archaeal POPs have been reported. Particularly notable in this regard, given the extensive biophysical characterization dedicated to it,^{11–15} is the POP from the hyperthermophilic archaeon, *Pyrococcus furiosus* (*Pfu*). *Pfu* POP is at most 28% identical to previously crystallized POPs, which are up to 44% identical, suggesting that *Pfu* POP could possess properties not present in these enzymes. Indeed, functional differences have been noted and most extensively discussed relative to porcine (*Sus scrofa*) POP. While porcine POP has melting points of 44.6 °C and 52.8 °C (pH 8), for example, *Pfu* POP melts at 91.7 °C and 109.5 °C (pH 8.4)¹⁵ and exhibits maximum activity at 85 °C¹¹. Both enzymes are activated by NaX (X = Cl, Br, etc.), but the nature of their response to [NaX] differs. Finally, while peptidase catalysis by both porcine and *Pfu* POP proceeds via the general mechanism shown in Fig. 1A, substrate entry into the active sites of these enzymes and inter-domain conformational changes associated with this process appear to involve important differences (Fig. 1B).¹⁶ These issues and our interest in biocatalytic applications of *Pfu* POP^{17,18} led us to solve the structures of this enzyme and its S477C mutant. The structures obtained, along with molecular dynamics simulations based on them, comparative analysis of previously reported POP structures, and existing biochemical data, have resolved several previously debated aspects of *Pfu* POP structure and function^{3,13,15}.

MATERIALS AND METHODS

Standard cloning procedures and site directed mutagenesis.

The *Pfu* POP gene, cloned into a pET 11c vector, was obtained from Prof. Harold Schreier (UMBC). Cysteine and alanine mutations were introduced into the gene at position S477 by site directed overlap extension PCR.¹⁹ Two separate PCRs were performed as outlined in the supporting information, each using a perfectly complementary flanking primer (Table S1) at the 5' and 3' end of the sequence and a mutagenic primer. The resulting two overlapping

fragments that contained the base pair substitution were then assembled in a second PCR to give the full-length gene.

PCR amplified fragments and pET11C were digested with NdeI and BamHI enzymes in recommended buffers at 37 °C for 2 hours. Digested DNA was purified by agarose gel extraction before ligation. Ligation reactions were conducted using a molar ratio of 1:3 (plasmid: insert) in 10 µL reaction mix. The reactions were incubated at 16 °C overnight, desalted, and transformed into *E. coli* DH5 cells. Cells were recovered in SOC media for 1 hour at 37 °C and spread onto LB Ampicillin plates (6.25 g LB powder mix, 4 g agar, 250 mL DDI water, 0.1 mg/mL Ampicillin). Plates were incubated at 37 °C overnight, and single colonies that appeared overnight were tested for the desired POP gene by colony PCR. Clones containing the desired insert were used to inoculate LB broth containing 0.10 mg/mL Ampicillin and grown overnight at 37 °C, 250 rpm. Recombinant plasmid DNA from these overnight grown cultures was isolated using miniprep kit from Qiagen (Valencia, CA) and verified via sequencing at the U Chicago sequencing facility using T7 forward and T7 reverse primers (Table S1).

POP expression and purification.

Single colonies of *E. coli* BL21 (DE3) cells harboring either pET11C-POPS477A or pET11C-POPS477C were used to inoculate 5 mL of 2YT/ampicillin. The culture was incubated overnight at 37 °C with constant shaking at 250 rpm. On the following day, 5 mL of the overnight culture was used to inoculate 500 mL of fresh 2YT/ampicillin in a 5 L Erlenmeyer flask. The culture was incubated at 37 °C, 250 rpm, and protein expression was induced by adding 1 mM IPTG when OD₆₀₀ reached 1. The induced culture was incubated for 12 hours, and then the cells were harvested by centrifugation at 4 °C, 3000g, for 20 minutes. Cell pellets were re-suspended in 20 mM phosphate buffer (pH 6.5) and lysed via sonication (40 amplitude, 30 second burst, 20-minute total process). Cell lysate was clarified at 16000g, 4 °C for 30 minutes, and the resulting supernatant was heated at 80 °C for 15 minutes. After cooling, the precipitated proteins were removed by centrifugation (16000g, 4 °C for 30 minutes). The supernatant was further purified by ion-exchange chromatography on Mono Q columns in 20 mM phosphate buffer, pH 6.5, using a linear gradient of 10–30% buffer containing 0.5 M NaCl. Fractions of the purified proteins were collected and the homogeneity of the protein was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Purified protein was buffer exchanged to 10 mM Tris (pH 7.5) and measured by Pierce® BCA Protein Assay Kit as recommended.

POP crystallization.

Crystals of native wild-type and S477C *Pyrococcus furiosus* prolyl oligopeptidase were grown at 20°C using the hanging-drop vapor-diffusion technique. A highly purified (>95%, determined by SDS-PAGE) protein solution of 8 mg/mL (determined by A280) in H₂O was used. Crystallization drops contained 1 µL protein solution with 1 µL of well solution containing 30% PEG 8K and 100 mM Tris•HCl pH 9.0. Diffraction quality crystals were obtained after 4 months of incubation. Crystals were harvested and cryoprotected in a solution of 15% glycerol in well solution. These were then flash frozen in liquid nitrogen

and mounted for data collection. Details regarding data collection and structure refinement are provided in the supporting information.

Molecular dynamics simulations.

Initial configurations for the WT and S477C simulations were taken from the corresponding crystal structures, while the IHBT system was constructed by fusing the covalent inhibitor from a crystal structure of the porcine POP-inhibitor complex (1QFS) to the WT structure. MD simulations were performed for the wildtype (WT), inhibitor ZPR bound (IHBT), and the mutant (S477C) *Pfu* POP enzymes. The chains with a lower B-factor in the crystal structures of the wildtype and the mutant in this report were used to generate the simulation systems (chain B for WT and chain A for S477C). Residual pKa calculations were performed using PROPKA3.1²⁰ and the protonation states of the protonatable residues were assigned based on the calculated pKa values. The wildtype chain B was aligned to other POP crystal structures that are closed with inhibitor bound to model the coordinates of the covalently linked inhibitor ZPR. All the three structures (i.e., WT, IHBT, and S477C) were bathed in 0.15M KCl solution using the *Solvator* Module in CHARMM-GUI²¹. Three replicas for each system were generated by using different random seeds to distribute the ions during the solvation process.

Once the simulation systems were generated, they were subjected to equilibrating simulations at 300 K and 358.15 K. The equilibration took two stages. In the first stage, harmonic positional restraints were imposed on the non-hydrogen atoms in the protein and were gradually released during 675 ps of the simulation. The system was then equilibrated without restraints for an additional 10 ns. The equilibration simulations were performed using OpenMM6.2 simulation package^{21,22} on GeForce GTX 780 Ti GPU processors. After equilibration, the systems were simulated for 1.44 μ s each using the special-purpose supercomputer Anton designed for long time scale simulations²³ with the exception for systems WT_2, WT_3, IHBT_2, and IHBT_3. These systems were simulated for 2.88 μ s each.

The input files for the OpenMM equilibration simulations are taken from CHARMM-GUI *Quick MD Simulator* module²¹. These simulations are performed in an NPT ensemble with the temperature set to either 300 K or 358.15 K and the isotropic pressure set to 1 atm. The Langevin thermostats with a damping coefficient of 1 ps^{-1} are used to keep the temperature constant. The OpenMM package uses a Monte Carlo algorithm to adjust the size of the periodic box, therefore simulating the effect of constant pressure. The van der Waals interactions were smoothly switched off at 10–12 Å by a force switching function²⁴ and the electrostatic interactions were calculated using the particle-mesh Ewald method with a mesh size of ~ 1 Å²⁵.

The production simulations on Anton are performed with the NPT ensemble. The Nosé-Hoover thermostat²⁶ was used to maintain the system temperature. The lengths of all bonds involving hydrogen atoms were constrained using M-SHAKE²⁷. The cutoff of the van der Waals and short-range electrostatic interactions was set to ~ 12 Å as suggested by the guesser script. Long-range electrostatic interactions were evaluated using the k-space Gaussian split Ewald method²⁸ with a $64 \times 64 \times 64$ mesh. The r-RESPA integration method²⁹ was

employed and long-range electrostatics were evaluated every 6 fs. The additive C36 force field was used in all the simulations performed here^{30–33}. The force field parameters for the covalently linked ZPR inhibitor were generated using the GAAMP server³⁴. Additional details regarding simulations of the loop containing H592 and of chloride binding are provided in the supporting information.

RESULTS

Pfu POP Structural Overview

The structures of wild type *Pfu* POP and the corresponding S477C mutant were solved at 1.9 Å and 2.2 Å resolution (Table 1). As observed in previously reported POP structures,² both *Pfu* POP and the S477C mutant possess a two-domain architecture involving a peptidase domain with an α/β -hydrolase fold capped by a 7-bladed β -propeller domain (Fig. 2A–C). The N-terminus of the enzyme (residues 1–47) consists of an α -helical segment that wraps around the C-terminal peptidase domain (residues 367–616). The intermediate segment (residues 48–366) comprises the β -barrel domain. The first and seventh blade of the propeller domain (residues 48–83 and 321–366, respectively) are joined by a number of hydrogen bonding and ion-pairing interactions in addition to hydrophobic interactions between the blade surfaces. The peptidase and β -barrel domains are joined covalently via a hinge region comprised of residues 47–50 and residues 361–367. An inter-domain angle of $\sim 30^\circ$ allows access to a large host cavity at the domain interface. Residues comprising the catalytic triad reside at the peptidase/barrel interface, with S/C477 located centrally within the peptidase domain and D560 and H592 located in flexible loops at the peptidase periphery (Fig. 2D).

Inter-domain Loop Composition and Structure

The composition and conformation of loops at the domain interface of different POPs possess many unique features,³⁵ and notable differences between the loops in these enzymes and *Pfu* POP are like-wise apparent (Fig. S1). Extensive ion pairing is observed between residues located in these loop regions in closed POP structures,^{7–10,13} but only a single inter-domain ion pair is present in the *Pfu* POP crystal structures. The loop containing the catalytic histidine (H592, residues 588–597) possesses a relatively high average B-factor in both structures (46.8/64.0 Å² versus 32.4/45.9 Å² for the full chains of *Pfu* and *S477C*, respectively, Table S1), the density of the main chain and the residues of this loop were apparent in the observed electron density (Fig. 3A, S11). This finding is notable because the histidine loop is disordered in all previously reported structures of POPs that crystallized in their open form^{9,10}. Finally, the loop connecting β -strands 2 and 3 of blade 3 of the POP β -propeller domain (residues 158–169) is significantly shorter than in previously characterized homologues, and it is folded back onto the surface of the β -propeller domain (Fig. 3B). This conformation is enforced by two sequential proline residues (P168 and P169) and a salt bridge between the end of the loop (D164) and the β -propeller domain (R172). This orientation contrasts with other POP structures in which residues near the end of this loop often exhibit hydrogen-bonding and/or salt bridging interactions with the peptidase domain that “latch” the enzyme shut.⁹

Prolylproline and Chloride Binding within *Pfu* POP.

The active site of *Pfu* POP exhibits many features common to other POPs crystallized to date.³ The hydrophobic proline-binding site (S1), comprised of F404, W518, and Y522, is conserved among all previously crystallized POPs,³ and the active-site serine (S477) is predictably positioned within a GX SXGG motif (Fig. 3C)³⁶. Notably, however, both chains of the *Pfu* POP structure contain electron density consistent with a bound prolylproline ligand, albeit with a significantly higher B-factor (58.0 Å²) than the main chains (32.4 Å²). The source of this ligand could not be determined, but we speculate that it could result from POP-catalyzed cleavage of other proteins or itself during the purification process. The orientation of this ligand is similar to proline-based inhibitors in previously reported crystal structures of porcine POP in a closed conformation (Fig. S10).³⁷ In these structures, the proline residue binds in a hydrophobic S1 pocket while the carbonyl of the P1-P2 amide bond forms a hydrogen bond with R643. Similarly, in the *Pfu* POP structure, the C-terminal proline carboxylate of the prolylproline ligand forms an ion pair with the homologous R562 residue.

The *Pfu* POP crystal structures also possess multiple Cl⁻ ions bound within the active site (Fig. 3C, Tris•HCl was present in the crystallization solutions). WT *Pfu* POP contains two Cl⁻ ions bound via opposing arginine residues (R476 and R600) proximal to the active site serine. Each Cl⁻ is coordinated in a bidentate, end-on fashion to one arginine and in a monodentate, side-on fashion to the second with B-factors of 45 and 20 Å², respectively. In the S477C mutant, both of these sites are occupied by Cl⁻ ions with B-factors of 58 and 39 Å², and an additional chloride is also bound in the P1 pocket with a B-factor of 53 Å².

MD Simulations of *Pfu* POP Domain Dynamics

Microsecond MD simulations were carried out to investigate the domain dynamics of *Pfu* POP at 300 K and its optimal temperature for peptidase catalysis (358.15 K). Simulations were conducted in triplicate on wildtype *Pfu* POP (WT), WT containing a covalently linked CbzProProlinal inhibitor (IHBT, Fig. S1), and the inactivated S477C mutant of WT (S477C), all hereafter referred to as “apo” states because they lack a covalently attached cofactor. The simulations show that the apo systems open and close spontaneously as the peptidase and the propeller domains pivot about a hinge region (Fig. 1B). An inter-domain angle (θ) was defined (Fig. 1A, S2) to quantify domain opening, and the time evolution of θ was evaluated at both 300K and 358.15K (Figs. 4 and S3). The open/closed transition occurs at a θ of ~17–23°, and θ ranges from 8–55°. At 358.15 K, the average θ is 20.0° ± 1.8°, 24.4° ± 4.2°, and 23.3° ± 0.9° in the WT, IHBT, and S477C simulations, respectively (Table S2). In general, the high temperature simulations showed slightly increased fluctuation of θ and more open/close transitions.

Inter-domain opening renders the active site solvent-accessible, which is readily visualized in trajectories from the IHBT simulations (Fig. 5A and B). To quantitate this opening, the solvent-accessible area between each propeller blade and the peptidase domain was calculated for the apo systems (Fig. S4). This analysis shows that the largest opening occurs between blade 3 of the propeller domain and the peptidase domain (Tables S3 and S4). In the WT simulations, the average solvent accessible area is 219 ± 74 Å², even at the most

constricted region along the blade 3 opening axis. Large fluctuations in the loop spanning D505-Y522 in the peptidase domain, opposite to blade 3, were also observed (Fig. S5). In contrast, the narrowest opening in the central pore of the propeller domain, which has been proposed as an alternate substrate entry site¹⁰, has a solvent-accessible area of only $93 \pm 24 \text{ \AA}^2$ in the WT simulations. No significant opening between blades 1 and 7 of the propeller domain was observed in any of the simulations.

Histidine Loop Conformational Dynamics

Large-scale domain movements are accompanied by smaller-scale conformational changes throughout the structures examined, but particularly notable are the dynamics of the histidine (H592) loop. In two simulations, the histidine loop achieves a conformation that places H592 within 4 Å from S477, its approximate orientation in crystal structures of POP enzymes in their closed forms (Fig. 6). The process of loop closing primarily involves changes in the ϕ - ψ back-bone dihedral angles (Fig. S6) and sequential interaction of H592 with hydrogen bond acceptors on D94, G117, A118, D119, D561, and R476 (Fig. 7). Histidine loop dynamics were also evaluated starting from a model of *Pfu* POP in the closed form (see supporting information). The similar ϕ/ψ dihedral angles distribution from the trajectories of the engineered His loop and WT systems suggest that similar dynamics are observed from either the open or closed state of *Pfu* POP (Fig. S6).

Effects of Halide Binding on Histidine Loop Conformational Dynamics

As noted above, there are two Cl^- binding sites in the WT *Pfu* POP crystal structure and three in that of the S477C mutant. The sites involving bidentate Cl^- coordination by the guanidinium groups of R476 and R600, respectively, are common to both structures. The bidentate-R600 site has the lowest B-factors in the crystal structures, an intermittent binding to this site is observed in MD simulations. Starting from crystal structures with this site occupied by a Cl^- , both dissociation and association of Cl^- can be observed (Table S6); Cl^- ions bound at other sites dissociate early in the simulations and do not again associate. Interestingly, Cl^- binding to the R476/R600 site has a minor effect on the S477-H592 distance distribution in the WT and the inhibitor bound systems, but not for S477C (Fig. 8). The effect manifests itself as a shift in the distance distribution towards the lower end of the ranges observed (a shorter S477-H592 distance). The Cl^- binding also affects the inter-domain angle but to a lesser extent (Fig. S7).

DISCUSSION

Structural Origins of *Pfu* POP Stability

The stability of *Pfu* POP makes this enzyme a promising candidate for biocatalytic applications, and the crystal structures reported herein provide a number of insights into the origins of its stability. Comparing the molecular weights of *Pfu* POP (70.8 kDa) to those of the other POPs crystallized to date (76–83 kDa) highlights the relatively small size of the former. Aligning the *Pfu* POP sequence to those of other POPs crystallized to date shows that this smaller size results primarily from shortened loop regions between structurally conserved β -sheets in the propeller domain (Fig. S8). The structures of *Pfu* POP and the corresponding S477C mutant also have an average of 0.10–0.11 ion pairs/residue versus

0.061–0.090 for the other (mesophilic) POP enzymes crystalized to date (Table 2). Shortened loop regions and increased ion pair content have both been proposed to contribute to the stability of hyperthermophilic enzymes.^{38,39}

Conformational Dynamics of *Pfu* POP

Despite the stability of *Pfu* POP, earlier kinetic analyses of this enzyme suggested that it might undergo significant conformational changes during catalysis.^{13–15} Both open^{9,10} and closed^{7–10,13} conformations of previous POP crystal structures or homology models can be distinguished by aligning their peptidase domains to that of the *Pfu* POP crystal structures (RMSD = 0.18–1.06 Å², Fig. S8). Using the inter-domain angle (θ) definition described above, closed conformations for previously studied structures have $\theta < 25^\circ$, while θ values of 47° and 58° were reported for open structures. At 29 and 32° , θ values for *Pfu* POP fall between these extremes, suggesting that it crystalized in an intermediate conformation. The single inter-domain ion pair in *Pfu* POP is also intermediate between these enzymes (Table 2), suggesting that ions pairs that could contribute to the stability of the closed form of the enzyme have only partially formed.

While crystal structures show that different domain angles can be achieved in different structures (Fig. S1), and both NMR spectroscopy^{40,41} and MD simulations have been used to study conformational dynamics in individual POPs, none of the previous MD studies reported spontaneous domain opening or closing during simulations^{16,35,42–45}. The microsecond MD simulations conducted herein show, for the first time, spontaneous conversion between the open and closed conformations of POP molecules (Fig. 3 and S3). The peptidase and propeller domains move largely as rigid bodies with important exceptions outlined below. Average θ values are similar to those observed in the crystal structures (Tables 2 and S2), but θ values ranging from 8 – 55° are sampled, consistent with the notion that the 29 or 32° angles observed in the crystal structures are intermediate in nature. The inter-domain ion pairs observed in the crystal structures are observed in all MD simulations, and a negative correlation exists between the number of ion pairs and the inter-domain angle (Fig. S9). The covalently bound ZPR ligand in the IHBT system resulted in slightly larger θ values at 358.15 K, with an average θ of $24.4^\circ \pm 4.2^\circ$ compared to the $20.0^\circ \pm 1.8^\circ$ in the apo structure. These values fall near the upper end of the ~ 17 – 23° θ range that defines the open/closed boundary for *Pfu* POP, which contrasts somewhat with experimental findings for porcine POP that showed inhibitor binding favors the closed form of the enzyme.⁴⁶ This difference could reflect either fundamental differences in how the dynamics of *Pfu* and porcine POP respond to ZPR binding or insufficient simulation time to sample the lowest energy substrate-bound closed state of *Pfu* POP.

Large-scale domain dynamics of POPs have been proposed to be modulated by a so-called “latching loop” (i.e., R158-P169 in *Pfu* POP). Previously reported POP structures show this loop bound to the peptidase domain in closed structures or disordered in open structures.^{7,10} The “latching” action of the loop observed in these structures is primarily mediated through polar and ionic interactions. A homology model of *Pfu* POP built from the porcine structure predicted that this loop would extend to the peptidase domain,¹³ contrary to the conformation observed in the *Pfu* POP crystal structures. The conformation of the “latching

loop” in *Pfu* POP remains relatively constant throughout all MD simulations and makes minimal contact with the hydrolase domain in the closed structures from the simulations. There is no persisting pattern of interactions between the “latching loop” and the hydrolase domain, which could contribute to the apparent favorability of the open form of *Pfu* POP.

The conformation of the histidine (H592) loop also plays a pivotal role in POP peptidase catalysis. In the other POP crystal structures reported to date, the histidine loop was modeled only in closed structures^{7,9} and omitted from open structures due to poor electron density.⁹ This trend holds for the structures of most other POP family enzymes, although the histidine loop of oligopeptidase B from *T. Brucei*⁴⁷ was resolved in the structures of both the open and closed conformations. While the closed form of *Pfu* POP was not observed in any of the crystal structures, overlaying the structures obtained with those for other closed POPs shows that H592 would have to travel 7–12 Å to achieve an orientation similar to the closed structures (Fig. S1). The structure of *Pfu* POP thus offers a rare view⁴⁷ of the position of the catalytic histidine in a POP prior to formation of a competent catalytic triad.

Two approaches were used to examine the conformational dynamics involved in forming active site configurations analogous to those observed in closed POP structures. In the first, triplicate MD simulations were performed on the WT, IHBT, and S477C systems as discussed in the His Loop Conformational Dynamics section above. During these simulations, a number of open-to-closed transitions were observed (Figs. 3 and S3), but only two of these events led to the formation of active site configurations analogous to those observed in the structures of closed, inhibitor-bound POPs (Figs. 9 and S1). The slight structural deviation from the closed structures of these covalently inhibited structures is in part due to the fact that their formation requires bond breakage and formation, including changes in the protonation state of H592. This residue was deprotonated in the beginning of the simulation based on its calculated pKa value of 6.1. The starting structure does not, however, capture the electrostatic environment near H592 when catalytic triad formation is near complete. Indeed, the calculated pKa of the corresponding histidine is raised to around 7 in crystal structures with a fully formed catalytic triad (Fig 9). Nonetheless, competent-like active site configurations were only seen in snapshots of closed POP structures with small open angles, suggesting that domain closing shifts the conformational distribution of the H592 loop to an orientation suitable for catalysis. In the second approach, model structures of *Pfu* POP in its closed form were generated using the H592 loop ϕ/ψ angle information from the crystal structures of POP in closed state. The MD simulations of these model structures without any inhibitors bound show that the H592 loop rapidly moves away from the closed conformation to adopt an orientation similar to that seen in the open conformation. The observed dynamics of these model systems after the H592 loop moves away from the active site are similar to those observed in the WT simulations according to the ϕ/ψ angle distributions for both systems (Fig. S6), providing a clear picture of the large-scale inter-domain opening/closing that occurs in *Pfu* POP.

Effects of *Pfu* POP Dynamics on Peptidase Catalysis

Crystal structures and MD simulations for different POPs suggest that these enzymes undergo large conformational changes, but the extent to which these changes influence POP

catalysis (e.g. substrate binding, formation of a competent active site, etc.) remains an open question. Several findings from previous studies provide key insights into different steps of *Pfu* POP peptidase catalysis (Fig. 1A): 1) The rate-limiting step in peptide hydrolysis differs at lower temperatures (25–40 °C) versus higher temperatures (40–95 °C), according to observed non-linearity in a plot of $\ln(k_{\text{cat}}/T)$ vs $(1/T)$.¹⁵ 2) A 70–80-fold increase in k_1 was observed from 25–55 °C, suggesting a large kinetic barrier for formation of an enzyme-substrate complex at lower temperatures.¹⁵ 3) A plot of k_{cat} versus temperature for hydrolysis of Z-Gly-Pro-pNA was linear between the ranges reported (60–90 °C), indicating that a single elementary step was rate limiting over this range.¹⁵ 4) Peptide hydrolysis at high temperatures (56 and 85 °C) exhibited a solvent isotope effect, implicating a rate-limiting chemical step in this range.^{13,15} 5) Lack of a leaving group effect at high temperatures (55–75 °C) was observed, which, in conjunction with points 3 and 4, suggests that k_3 (chemical hydrolysis of the acyl intermediate) is rate-limiting at high temperatures.¹⁵ 6) A sigmoidal increase in k_{cat}/K_M as a function of $[\text{NaX}]$ ($X = \text{Cl}, \text{Br}, \text{F}$) and no change in k_{cat} was observed for *Pfu* POP up to ~2M NaX, suggesting that halide binding activates *Pfu* POP hydrolase activity (~2–3 fold) via a decrease in K_M .¹³ Thus, while a chemical step is rate limiting at high temperatures, substrate binding, and any conformational changes associated with it, are rate limiting below 40 °C.

Based on these findings, it was previously hypothesized that *Pfu* POP has a stable, closed structure at low temperature, and that the high barrier to substrate binding arises from a high-barrier conformational change between open- and closed-states (Fig. 10A).¹⁵ The crystal structure of *Pfu* POP shows that an open form of the enzyme is in fact readily accessible, that the enzyme possesses several Cl^- binding sites, and that a dipeptide can bind within the open active site (Fig. 3C). MD simulations allowed for observation of the open/closed transition, and the same dynamics are observed in simulations of WT, a model of closed WT, and IHBT (Figs. 4 and S3). These findings are consistent with low barrier binding equilibria involving substrate, Cl^- , and the open and closed forms of *Pfu* POP, in which formation of substrate/ Cl^- -bound open (observed by X-ray crystallography) and closed (observed in MD simulations) forms of the enzyme can be generated. The previously noted rate limiting formation of the ES complex below 40 °C could result not from domain opening but from conversion of a closed, substrate-bound form of the enzyme to one in which the catalytic triad is fully formed and poised for attack of substrate (Fig. 10B)⁴⁸. While such a state is beyond the ability of classical MD simulations to model, conformations resembling those of closed structures with covalent inhibitors bound were observed in simulations of dynamics at 358.15 K but not at 300 K, consistent with the idea that accessing this state is rate limiting at low temperatures.

The *Pfu* POP crystal structures and MD simulations also provide insight into substrate entry into the enzyme (Fig. 10C). Specifically, the $219 \pm 74 \text{ \AA}^2$ opening at the narrowest region between blade 3 and the peptidase domain can accommodate a peptide chain (WT Replica 2 in Table S4). The loop lining the edge of this opening within the peptidase domain (D505-Y522) exhibits large fluctuations in the MD simulations (Fig. S5). Fluctuation of this loop could act to facilitate substrate entry into the POP enzyme interior. While a small opening ($71\text{--}112 \text{ \AA}^2$ at 358.15K, Table S5) is observed at the pore lined by the seven p-blades in the propeller domain, this would only be sufficient for entry of peptides containing small

residues, contrary to the reported substrate scope. No opening is observed between the propeller blades 1 and 7 (b1/b7), so substrate entry from this region is also unlikely. Entry through the much wider opening between the propeller and peptidase domains therefore seems most reasonable for *Pfu* POP.

Finally, the observation of three distinct halide binding sites between WT and S477C *Pfu* POP structures (Fig. 2) is qualitatively consistent with the aforementioned activation by halide.¹³ Halide binding to R476 and R600 in particular provides a structural rationale for the impact of halide concentration on POP activity (Fig. 10D). Forming the catalytic triad in *Pfu* POP requires a large conformational change (Fig. 6) to position H592 between D560 and S477, which move independent of one another. The presence of R476 immediately before to S477 and of R600 in the helix adjacent to the loop containing H592 provides a means to orient the secondary structures containing these residues upon halide binding. By securing the orientation of these secondary structures, the salt bridge helps position S477 and H592 to form the catalytic triad. This hypothesis is consistent with the results of MD simulations showing shorter H592-S477 distances and smaller inter-domain angles (Figs. 3, 6, and S3) when chloride is bound versus when it is not. This mechanistic proposal, which involves active site pre-organization to facilitate substrate binding, would be reflected in K_m , consistent with the kinetic analyses noted above and reminiscent of previous work on angiotensin converting enzyme⁴⁹.

CONCLUSION

The crystal structures of WT *Pfu* POP and its S477C mutant reveal that both enzymes adopt an open structure with an intermediate inter-domain angle relative to other POP enzymes reported to date. MD simulations show that the conformation observed in the crystal structures is indeed intermediate between much larger extremes that can be sampled by the enzyme. The observed dynamics show that substrates can access the *Pfu* POP active site via the inter-domain opening, which lacks a “latch” present in other structures, while other previously proposed openings are much smaller and show no significant opening during MD simulations.

The *Pfu* POP crystal structures provide rare glimpses of the loop containing the catalytic histidine (H592) in an open POP structure. MD simulations show that H592 can access conformations analogous to those observed in previously reported structures of POP-inhibitor complexes. The structure of WT *Pfu* POP also contains a bound prolylproline ligand, showing that substrate-like compounds remain bound in the open form of the enzyme. Up to three bound chloride ions were observed in the different *Pfu* POP structures, and those bound to R476 and R600 provide a rationale for previously observed halide activation involving active site preorganization. MD simulations show that chloride binding at these sites alters both histidine loop conformation and the inter-domain angle to favor a more closed form of the enzyme.

Together, these results resolve a number of questions in the literature regarding native *Pfu* POP structure, inter-domain dynamics, substrate entry, and activation by halide ions. Specifically, our analysis reveals the importance of facile inter-domain opening, rate-limiting

EA formation or hydrolysis, and halide-induced active site preorganization during *Pfu* POP catalysis. We anticipate that these findings will facilitate further development of biocatalytic applications involving *Pfu* POP.^{17,18}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

This work was supported by, or in part by, the U.S. Army Research Laboratory and the U. S. Army Research Office under contract/grant numbers W911NF-14-1-0334 and 66796-LS-RIP (to J.C.L.) and W911NF-18-1-0200 (to J.C.L. and B.R.), and the NSF under CAREER Award CHE-1351991 (to J.C.L.), and The David and Lucile Packard Foundation (to J.C.L.). This work is based on research conducted at the APS on the NE-CAT beamlines, which are supported by a grant from the NIGMS (P41 GM103403) from the NIH. The Pilatus 6M detector on 24-ID-C beam line is funded by a NIH-ORIP HEI grant (S10 RR029205). Use of the APS, an Office of Science User Facility operated for the U.S. DOE Office of Science by Argonne National Laboratory, was supported by the U.S. DOE under Contract No. DE-AC02-06CH11357. This research was supported in part by Lilly Endowment, Inc., through its support for the Indiana University Pervasive Technology Institute, and in part by the Indiana METACyt Initiative. The Indiana METACyt Initiative at IU was also supported in part by Lilly Endowment, Inc.

ABBREVIATIONS

<i>Pfu</i>	pyrococcus furiosus
POP	prolyl oligopeptidase

REFERENCES

- (1). Polgár L The Prolyl Oligopeptidase Family. Cell Mol Life Sci 2002, 59 (2), 349–362. [PubMed: 11915948]
- (2). Szeltner Z; Polgar L Structure, Function and Biological Relevance of Prolyl Oligopeptidase. Curr. Protein Pept. Sci. 2008, 9 (1), 96–107. [PubMed: 18336325]
- (3). Rea D; Fülöp V Structure-Function Properties of Prolyl Oligopeptidase Family Enzymes. Cell Biochem. Biophys. 2006, 44 (3), 349–365. [PubMed: 16679522]
- (4). Ehren J; Govindarajan S; Moron B; Minshull J; Khosla C Protein Engineering of Improved Prolyl Endopeptidases for Celiac Sprue Therapy. Prot. Eng. Des. Sel. 2008, 21 (12), 699–707.
- (5). Walter R; Shlank H; Glass JD; Schwartz IL; Kerenyi TD Leucylglycinamide Released From Oxytocin by Human Uterine Enzyme. Science 1971, 173 (3999), 827–829. [PubMed: 5572174]
- (6). Kaushik S; Sowdhamini R Distribution, Classification, Domain Architectures and Evolution of Prolyl Oligopeptidases in Prokaryotic Lineages. BMC Genomics 2014, 15 (1), 985–13. [PubMed: 25407321]
- (7). Fülöp V; Böcskei Z; Polgar L Prolyl Oligopeptidase: an Unusual B-Propeller Domain Regulates Proteolysis. Cell 1998, 94 (2), 161–170. [PubMed: 9695945]
- (8). Haffner CD; Diaz CJ; Miller AB; Reid RA; Madauss KP; Hassell A; Hanlon MH; Porter DJT; Becherer JD; Carter LH Pyrrolidinyl Pyridone and Pyrazinone Analogues as Potent Inhibitors of Prolyl Oligopeptidase (POP). Bioorg. Med. Chem. Lett. 2008, 18 (15), 4360–4363. [PubMed: 18606544]
- (9). Shan L; Mathews II; Khosla C Structural and Mechanistic Analysis of Two Prolyl Endopeptidases: Role of Interdomain Dynamics in Catalysis and Specificity. Proc. Natl. Acad. Sci. U.S.A. 2005, 102 (10), 3599–3604. [PubMed: 15738423]
- (10). Li M; Chen C; Davies DR; Chiu TK Induced-Fit Mechanism for Prolyl Endopeptidase. J. Biol. Chem. 2010, 285 (28), 21487–21495. [PubMed: 20444688]

- (11). Harwood VJ; Denson JD; Robinson-Bidle KA; Schreier HJ Overexpression and Characterization of a Prolyl Endo-peptidase From the Hyperthermophilic Archaeon *Pyrococcus Furiosus*. *J. Bacteriol.* 1997, 179 (11), 3613–3618. [PubMed: 9171407]
- (12). Harwood VJ; Schreier HJ Prolyl Oligopeptidase From *Pyrococcus Furiosus*. *Meth. Enzymol.* 2001, 330, 445–454. [PubMed: 11210523]
- (13). Harris MN; Madura JD; Ming L-J; Harwood VJ Kinetic and Mechanistic Studies of Prolyl Oligopeptidase From the Hyperthermophile *Pyrococcus Furiosus*. *J. Biol. Chem.* 2001, 276 (22), 19310–19317. [PubMed: 11278687]
- (14). Juhasz T; Szeltner Z; Polgar L Properties of the Prolyl Oligopeptidase Homologue From *Pyrococcus Furiosus*. *FEBS Letters* 2006, 580 (14), 3493–3497. [PubMed: 16714022]
- (15). Juhasz T; Szeltner Z; Polgar L Truncated Prolyl Oligopeptidase From *Pyrococcus Furiosus*. *Proteins* 2007, 69 (3), 633–643. [PubMed: 17623862]
- (16). Kaushik S; Etchebest C; Sowdhamini R Decoding the Structural Events in Substrate-Gating Mechanism of Eukaryotic Prolyl Oligopeptidase Using Normal Mode Analysis and Molecular Dynamics Simulations. *Proteins* 2014, 82 (7), 1428–1443. [PubMed: 24500901]
- (17). Yang H; Swartz AM; Park H-J; Srivastava P; Ellis-Guardiola K; Upp DM; Lee G; Belsare K; Gu Y; Zhang C; Moellering RE; Lewis JC Evolving Artificial Metalloenzymes via Random Mutagenesis. *Nat. Chem.* 2018, 10 (3), 318–324. [PubMed: 29461523]
- (18). Gu Y; Guardiola KE; Srivastava P; Lewis JC Preparation, Characterization, and Oxygenase Activity of a Photocatalytic Artificial Enzyme. *Chem Bio Chem* 2015, 16 (13), 1880–1883.
- (19). Heckman KL; Pease LR Gene Splicing and Mutagenesis by PCR-Driven Overlap Extension. *Nat Protoc* 2007, 2 (4), 924–932. [PubMed: 17446874]
- (20). Olsson MHM; Søndergaard CR; Rostkowski M; Jensen JH. PROPKA3: Consistent Treatment of Internal and Surface Residues in Empirical pKa Predictions. *J. Chem. Theory Comput.* 2010, 7 (2), 525–537.
- (21). Jo S; Kim T; Iyer VG; Im W CHARMM-GUI: a Web-Based Graphical User Interface for CHARMM. *J. Comput. Chem.* 2008, 29 (11), 1859–1865. [PubMed: 18351591]
- (22). Eastman P; Friedrichs MS; Chodera JD; Radmer RJ; Bruns CM; Ku JP; Beauchamp KA; Lane TJ; Wang L-P; Shukla D; Tye T; Houston M; Stich T; Klein C; Shirts MR; Pande VS OpenMM 4: a Reusable, Extensible, Hardware Independent Library for High Performance Molecular Simulation. *J. Chem. Theory Comput.* 2012, 9 (1), 461–469. [PubMed: 23316124]
- (23). Shaw DE; Dror RO; Salmon JK.; Grossman J; Mackenzie KM; Bank JA; Young C; Deneroff MM; Batson B; Bowers KJ. Proceedings of the Conference on High Performance Computing Networking Storage and Analysis; Acm: 2009.
- (24). Steinbach PJ; Brooks BR New Spherical-Cutoff Methods for Long-Range Forces in Macromolecular Simulation. *J. Comput. Chem.* 1994, 15 (7), 667–683.
- (25). Essmann U; Perera L; Berkowitz ML; Darden T; Lee H; Pedersen LG A Smooth Particle Mesh Ewald Method. *The Journal of Chemical Physics* 1995, 103 (19), 8577–8593.
- (26). Martyna GJ; Klein ML; Tuckerman M Nose-Hoover Chains: the Canonical Ensemble via Continuous Dynamics. *The Journal of Chemical Physics* 1992, 97 (4), 2635–2643.
- (27). Kräutler V; van Gunsteren WF; Hünenberger PH A Fast SHAKE Algorithm to Solve Distance Constraint Equations for Small Molecules in Molecular Dynamics Simulations. *J. Comput. Chem.* 2001, 22 (5), 501–508.
- (28). Shan Y; Klepeis JL; Eastwood MP; Dror R O.; Shaw, D. E. Gaussian Split Ewald: a Fast Ewald Mesh Method for Molecular Simulation. *The Journal of Chemical Physics* 2005, 122 (5), 054101–054114.
- (29). Tuckerman M; Berne BJ; Martyna GJ Reversible Multiple Time Scale Molecular Dynamics. *The Journal of Chemical Physics* 1992, 97 (3), 1990–2001.
- (30). Best RB; Zhu X; Shim J; Lopes PEM; Mittal J; Feig M; MacKerell AD Jr. Optimization of the Additive CHARMM All-Atom Protein Force Field Targeting Improved Sampling of the Backbone Φ , Ψ and Side-Chain χ_1 and χ_2 Dihedral Angles. *J. Chem. Theory Comput.* 2012, 8 (9), 3257–3273. [PubMed: 23341755]

- (31). Jorgensen WL; Chandrasekhar J; Madura JD; Impey RW; Klein ML Comparison of Simple Potential Functions for Simulating Liquid Water. *The Journal of Chemical Physics* 1983, 79 (2), 926–935.
- (32). Klauda JB; Venable RM; Freites JA; O'Connor JW; Tobias DJ; Mondragon-Ramirez C; Vorobyov I; MacKerell AD Jr; Pastor RW. Update of the CHARMM All-Atom Additive Force Field for Lipids: Validation on Six Lipid Types. *J Phys Chem B* 2010, 114 (23), 7830–7843. [PubMed: 20496934]
- (33). MacKerell AD Jr.; Bashford D; Bellott M; Dunbrack RL Jr; Evanseck JD; Field MJ; Fischer S; Gao J; Guo H; Ha S; Joseph-McCarthy D; Kuchnir L; Kuczera K; Lau FTK; Mattos C; Michnick S; Ngo T; Nguyen DT; Prodhom B; Reiher WE; Roux B; Schlenkrich M; Smith JC; Stote R; Straub J; Watanabe M; Wiorkiewicz-Kuczera J; Yin D; Karplus M All-Atom Empirical Potential for Molecular Modeling and Dynamics Studies of Proteins. *J Phys Chem B* 1998, 102 (18), 3586–3616. [PubMed: 24889800]
- (34). Huang L; Roux B Automated Force Field Parameterization for Nonpolarizable and Polarizable Atomic Models Based on Ab Initio Target Data. *J. Chem. Theory Comput.* 2013, 9 (8), 3543–3556.
- (35). Szeltner Z; Juhasz T; Szamosi I; Rea D; Fülöp V; Modos K; Juliano L; Polgar L *Biochimica Et Biophysica Acta. BBA-Proteins and Proteomics* 2013, 1834 (1), 98–111. [PubMed: 22940581]
- (36). Ollis DL; Cheah E; Cygler M; Dijkstra B; Frolov F; Franken SM; Harel M; Remington SJ; Silman I; Schrag J; Sussman JL; Verschueren KHG; Goldman A The A/B Hydrolase Fold. *Prot. Eng. Des. Sel.* 1992, 5 (3), 197–211.
- (37). Fülöp V; Szeltner Z; Renner V; Polgar L Structures of Prolyl Oligopeptidase Substrate/Inhibitor Complexes. Use of Inhibitor Binding for Titration of the Catalytic Histidine Residue. *J. Biol. Chem.* 2001, 276 (2), 1262–1266. [PubMed: 11031266]
- (38). Vieille C; Zeikus GJ Hyperthermophilic Enzymes: Sources, Uses, and Molecular Mechanisms for Thermostability. *Microbiol Mol Biol R* 2001, 65 (1), 1–43.
- (39). Sterpone F; Melchionna S Thermophilic Proteins: Insight and Perspective From in Silico Experiments. *Chem. Soc. Rev.* 2012, 41 (5), 1665–1676. [PubMed: 21975514]
- (40). Kichik N; Tarrago T; Claasen B; Gairi M; Millet O; Giralt E 15N Relaxation NMR Studies of Prolyl Oligopeptidase, an 80 kDa Enzyme, Reveal a Pre-Existing Equilibrium Between Different Conformational States. 2011, 12 (18), 2737–2739.
- (41). Tarrago T; Claasen B; Kichik N; Rodriguez-Mias RA; Gairi M; Giralt E A Cost-Effective Labeling Strategy for the NMR Study of Large Proteins: Selective 15N-Labeling of the Tryptophan Side Chains of Prolyl Oligopeptidase. *Chem Bio-Chem* 2009, 10 (17), 2736–2739. [PubMed: 19798721]
- (42). Kaszuba K; Rog T; Danne R; Canning P; Fülöp V; Juhasz T; Szeltner Z; Pierre JFS; Garcia-Horsman A; Männistö PT; Karttunen M; Hokkanen J; Bunker A Molecular Dynamics, Crystallography and Mutagenesis Studies on the Substrate Gating Mechanism of Prolyl Oligopeptidase. *Biochimie* 2012, 94 (6), 1398–1411. [PubMed: 22484394]
- (43). Kaushik S; Sowdhamini R Structural Analysis of Prolyl Oligopeptidases Using Molecular Docking and Dynamics: Insights Into Conformational Changes and Ligand Binding. *PLoS ONE* 2011, 6 (11), e26251.
- (44). St-Pierre J-F; Karttunen M; Mousseau N; Róg T; Bunker A Use of Umbrella Sampling to Calculate the Entrance/Exit Pathway for Z-Pro-Prolinal Inhibitor in Prolyl Oligopeptidase. *J. Chem. Theory Comput.* 2011, 7 (6), 1583–1594. [PubMed: 26596426]
- (45). Kotev M; Lecina D; Tarragó T; Giralt E; Guallar V Unveiling Prolyl Oligopeptidase Ligand Migration by Comprehensive Computational Techniques. *Biophys J* 2015, 108 (1), 116–125.
- (46). López A; Herranz-Trillo F; Kotev M; Gain M; Guallar V; Bernadó P; Millet O; Tarragó T; Giralt E Active-Site-Directed Inhibitors of Prolyl Oligopeptidase Abolish Its Conformational Dynamics. *ChemBioChem* 2016, 17 (10), 913–917. [PubMed: 26918396]
- (47). Canning P; Rea D; Morty RE; Fülöp V. Crystal Structures of Trypanosoma Brucei Oligopeptidase B Broaden the Paradigm of Catalytic Regulation in Prolyl Oligopeptidase Family Enzymes. *PLoS ONE* 2013, 8 (11), e79349.

- (48). Smith AJT; Mueller R; Toscano MD; Kast P; Hellinga HW; Hilvert D; Houk KN Structural Reorganization and Preorganization in Enzyme Active Sites: Comparisons of Experimental and Theoretically Ideal Active Site Geometries in the Multistep Serine Esterase Reaction Cycle. *J. Am. Chem.Soc.* 2008, 130 (46), 15361–15373. [PubMed: 18939839]
- (49). Liu X; Fernandez M; Wouters MA; Heyberger S; Husain A Arg1098 Is Critical for the Chloride Dependence of Human Angiotensin I-Converting Enzyme C-Domain Catalytic Activity. *J. Biol. Chem.* 2001, 276 (36), 33518–33525. [PubMed: 11432860]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

A) General scheme for peptidase catalysis involving an enzyme (E) and a peptide substrate (S) to generate two product peptides (P_1 and P_2) via enzyme-substrate (ES) and enzyme-acyl (EA) intermediates. B) Potential domain opening and closing during *Pfu* POP peptidase catalysis.

Figure 2.

A-C) Annotated ribbon diagram of *Pfu* POP showing inter-domain angle (θ), blade (b1–7) and hinge locations, and key active site residues and Cl[−] ions. D) expanded view of POP active site showing residues in the catalytic triad, bound chloride ions, and the Pro-Pro ligand (omitted in A-C for clarity).

Figure 3. Electron density (contoured at 1.0 σ) for key features in the PfU POP crystal structure, including: A) the loop containing His592, B) the “latch loop”, C) a bound prolylproline ligand and bound chloride ions.

Figure 4.

The open angle and its distribution in the MD simulations at 300 K (red) and 358.15 K (green) in replica 2 of the WT, IHBT, and S477C systems. The yellow banner indicates the intermediate open angle values between the open and the closed states.

Figure 5.

The cartoon and surface representation showing the largest opening between propeller (light grey) blade b3 and the peptidase domain (dark grey). The snapshots are taken from the covalently-linked inhibitor simulation IHBT_3 to illustrate (A) the closed state and (B) the open, substrate-accessible state enzyme. The bottom panel shows a surface presentation of the same snapshot. The covalently bound inhibitor is colored green.

Figure 6.

The time evolution of the S477 and H592 distance, $D_{S477-H592}$. The hydroxyl oxygen (OG) in S477 and the imidazole amide hydrogen (HE2) are used to define the distance.

Figure 7. Sequential steps in the H-bond network H592 uses to get into the catalytic site (A-F). The contributing residues are shown in cyan sticks. The H592 look is in green and the residues H592 and S477 are colored in yellow.

Figure 8.

The impact on S477-H592 distance from the binding of Cl^- to the R600 site. The site is occupied when the Cl^- is within 1.5 Å to the crystal binding site of this Cl^- . The transparent, filled curves show the normalized population (P_{norm}) distributions of $D_{\text{S477-H592}}$ computed from the entire trajectory at 358.15 K. The thick, colored lines are the P_{norm} distributions of $D_{\text{S477-H592}}$ drawn from when Cl^- is bound to the R600 site. The three replicas are shown in red, green, and blue.

Figure 9. Overlay of closed crystal structures with a simulation snapshot with a closed H592 loop. Important residues are shown with sticks. The pKa values of the residues that correspond to H592 are 6.6, 6.6/6.9, 7.2, and 6.9 for 1QFS, 2BKL, 3IVM, and 4HVT. The pKa values are calculated using the program PROPKA31²⁰.

Figure 10.

Conceptual summary of updated models for A) the inter-domain angle of the *Pfu* POP, B) the rate limiting step of *Pfu* POP peptidase catalysis, C) substrate entry into *Pfu* POP, and D) effects of chloride binding within *Pfu* POP (S = substrate).

Table 1.**X-ray Diffraction Data Collection and Refinement Statistics^a**

	<i>Pfu</i> POP (5T88)	S477C (6CAN)
<i>Data collection</i>		
wavelength (Å)	0.98	0.98
space group	P2 ₁	P2 ₁
unit cell dimensions		
a, b, c (Å)	55.5, 176.8, 57.9	56.3, 178.8, 59.3
α, β, γ (°)	90, 106.0, 90	90, 104.4, 90
res. limit (Å)	50–1.9 (1.97–1.9)	100–2.2 (2.28–2.2)
<i>I</i> /Sigma(<i>I</i>)	12.3 (1.5)	9.5(1.2)
R _{pim} (%)	5.9(48.3)	7.1(44.0)
highest shell resol. CC ^{1/2} (%)	57.5	53.0
completeness (%)	99.9(100.0)	93.7(74.6)
redundancy	9.7 (9.3)	4.5(3.5)
<i>Refinement</i>		
resolution range (Å)	47.1 – 1.9	19.5 – 2.2
R _{work} (%)	19.3	19.7
R _{free} (%)	24.7	25.1
R (working + test) (%)	19.4	19.7
no. of reflections	83441	53366
<i>Model</i>		
no. of amino acids	1232	1232
no. of H ₂ O mol.	946	842
no. Cl [−] ions	4	6
no. residues:		
in generously allowed region	12	13
in disallowed regions	0	0
<i>Stereochemical ideality</i>		
bonds (Å)	0.01	0.01
angles (°)	1.44	1.24
dihedral angles (°)	11.39	16.92
planarity (Å)	0.01	0.00

^aValue in parentheses are for the outer shell.

Table 2.

Structural data for previously crystalized POPs.

POP ^a	MW ^b	Additional Residues ^c		Ion Pairs ^d		Inter domain Angle (°) ^d
		Peptidase domain	Propeller domain	Per residue	Inter-domain	
<i>Pfu</i>	70824	-	-	0.11	1	29
<i>S477C</i>	70840	-	-	0.11	0	32
<i>Pfu_mod</i>	70824	-	-	0.084	5	19
<i>M. xanthus</i>	76848	14	28	0.086	5	21
<i>R. typhi</i>	83074	27	54	0.061	3	23
<i>S. scrofa</i>	80770	27	40	0.076	4	22
<i>H. sapiens</i>	80700	27	40	0.061	3	22
<i>A. caviae_c</i>	76467	19	24	0.078	2	25
<i>A. caviae_o</i>				0.070	0	47
<i>S. capsulata</i>	78435	11	34	0.064	0	58

^a *Pfu* chain B from 5T88; *Pfu S477C* chain B from 6CAN; *Pfu_mod* from a previously reported homology model; *M. xanthus* chain A from 2BKL; *R. typhi* from 4HVT; *S. Scrofa* from 1QFS; *H. sapiens* from 3DDU; *A. caviae_c* from 3IVM; *A. caviae_o* from 3IUJ; *S. capsulata* from 1YR2.

^b Determined using the ExpASY server.

^c relative to *Pfu*.

^d Determined using VMD or UCSF Chimera as described in the SI.