



X-ray crystal structure of the streptococcal specific phage lysin PlyC

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Edited by Bonnie L Bassler, Howard Hughes Medical Institute, Princeton University, Princeton, NJ, and approved June 22, 2012 (received for review May 18, 2012)

Bacteriophages deploy lysins that degrade the bacterial cell wall and facilitate virus egress from the host. When applied exogenously, these enzymes destroy susceptible microbes and, accordingly, have potential as therapeutic agents. The most potent lysin identified to date is PlyC, an enzyme assembled from two components (PlyCA and PlyCB) that is specific for streptococcal species. Here the structure of the PlyC holoenzyme reveals that a single PlyCA moiety is tethered to a ring-shaped assembly of eight PlyCB molecules. Structure-guided mutagenesis reveals that the bacterial cell wall binding is achieved through a cleft on PlyCB. Unexpectedly, our structural data reveal that PlyCA contains a glycoside hydrolase domain in addition to the previously recognized cysteine, histidine-dependent amidohydrolases/peptidases catalytic domain. The presence of eight cell wall-binding domains together with two catalytic domains may explain the extraordinary potency of the PlyC holoenzyme toward target bacteria.

enzymatic | protein crystallography | peptidoglycan | multimeric protein

Bacteriophage (or phage) typically encode holins and lysins as part of their lytic system to achieve virus exit from the host bacterial cell (1). Holins are responsible for forming pores in the cytoplasmic membrane, following which lysins, having accumulated in the cytoplasm, are responsible for degradation of the peptidoglycan (i.e., cell wall) layer. Damage to this layer results in rapid cell rupture and concomitant virus release through loss of osmotic integrity. These enzymes are also able to rapidly and specifically destroy Gram-positive bacteria when applied exogenously, making the purified lysins functional “inside-out” enzymes (2, 3). There is thus great interest in the development of phage lysins as enzymatic agents to treat antibiotic-resistant bacterial infections (4).

Lysins derived from phage that infect Gram-positive bacteria are generally composed of a single polypeptide consisting of an N-terminal catalytic domain and a C-terminal cell wall binding domain (CBD) held together by a short flexible linker (5). In some rare cases, two to three catalytic domains may be linked to a single binding domain (6, 7). With few exceptions, the catalytic domain is usually represented by one of four families of peptidoglycan hydrolases: *N*-acetylglucosaminidases, *N*-acetylmuramidases (lysozymes), *N*-acetylmuramoyl-L-alanine amidases, and endopeptidases (8). In contrast, the CBDs are markedly divergent and can distinguish discrete epitopes present within the cell wall, typically carbohydrates or teichoic acids, giving rise to the species- or strain- specific activity of a particular lysin. Consequently, it is possible to combine the catalytic domain of one lysin and the CBD of a second lysin to make a chimeric protein with altered specificity or activity (9–11).

The streptococcal C1 phage lysin, PlyC, is the most potent lysin described to date, with a specific activity ~100 fold that of the next most catalytic lysin. Previous research spanning over 50 y has shown that PlyC can rapidly lyse cultures of groups A, C,

and E streptococci in addition to *Streptococcus uberis* and *Streptococcus equi* and has been shown to protect mice from streptococcal challenge (12, 13). PlyC is furthermore unique among the Gram-positive lysins in that it consists of two separate proteins—a single 50-kDa PlyCA subunit that is suggested to form a complex with at least eight 8-kDa PlyCB subunits (14). The two proteins are transcribed from two genes located in a single operon (14). PlyCA is known to contain an active cysteine, histidine-dependent amidohydrolases/peptidase (CHAP) domain, a fold distantly related to the papain-like cysteine-protease family, with Cys₃₃₃ and His₄₂₀ shown to be essential for amidase catalytic activity (14). The PlyCB octamer is suggested to represent the CBD because purified material lacking the PlyCA subunit was able to specifically bind *Streptococcus pyogenes*, *S. uberis*, *S. equi*, and groups C and E streptococci, but not other bacterial species (i.e., *Streptococcus agalactiae*, *Streptococcus mutans*, and *Staphylococcus aureus*) (14).

Although the potent lytic activity of PlyC has been extensively characterized, its structural architecture and mechanism of action has remained unclear. In this study, we have made a pivotal step forward in our understanding of this enzyme by determining the structure and mechanism of PlyC action, including cell wall binding and catalytic activity.

Results

Crystallography and Structure Determination. Initial crystal trials on PlyCB demonstrated that the cell wall-binding subunit crystallized readily, and these crystals diffracted to high resolution. The structure of PlyCB was determined through sulfur single anomalous dispersion phasing (Tables S1 and S2). In contrast, the protein crystallography and structure determination of the PlyC holoenzyme was particularly challenging. More than 500 crystals were screened, and typical diffraction data obtained were limited to ~10 Å. A single native dataset to 3.3 Å was collected from

Author contributions: S.M., A.M.B., V.A.F., J.C.W., and D.C.N. designed research; S.M., A.M.B., M.S.M., J.T.H., D.T.G., R.D.H., Y.S., C.F.R., R.H.P.L., J.C.W., and D.C.N. performed research; D.C.N. contributed new reagents/analytic tools; S.M., A.M.B., M.S.M., J.T.H., D.T.G., R.D.H., Y.S., V.A.F., J.C.W., and D.C.N. analyzed data; and S.M., V.A.F., J.C.W., and D.C.N. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4F87 and 4F88).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1208424109/-DCSupplemental.

a single crystal by using synchrotron radiation (Tables S1 and S2). Attempts to obtain heavy atom derivatives and/or crystals using selenomethionine labeling to acquire experimental phases for PlyC were all unsuccessful. Ultimately, the structure of the octameric 1.4 Å PlyCB ring was used as a molecular replacement probe for the native PlyC dataset. Molecular replacement attempts using CHAP domains failed to identify the location of the CHAP domain in the PlyCA molecules. Preliminary refinement of the molecular replacement model, however, revealed significant regions of positive connective density that clearly did not form part of the PlyCB probe. Accordingly, the structure of PlyCA was determined through iterative rounds of refinement and manual building (as detailed in *Methods*).

Overall Architecture of PlyC Holoenzyme. The 3.3-Å X-ray crystal structure of the PlyC holoenzyme consists of a nine-protein assembly that comprises a single PlyCA catalytic subunit in complex with eight PlyCB molecules (Fig. 1A, Fig. S1, and Movie S1). The PlyCB CBD is arranged in a planar octameric ring that is 80 Å in diameter and 20 Å high (Fig. 1B). One surface of the PlyCB octamer is quite planar, whereas the other is more convex in shape. A similar octameric arrangement of PlyCB monomers was also observed in the 1.4-Å structure of PlyCB alone. The single PlyCA molecule is oriented and positioned entirely on the flat (planar) surface of the PlyCB octamer (Fig. 1A and Fig. S1).

In the PlyCB octamer, each monomer is comprised of a four-stranded β -sheet capped on each side by a short α -helix (Fig. S2). Oligomerization is mediated through strand/helix hydrogen bonding interactions at each interface (Fig. S2). Sequence-based searches reveal that PlyCB contains no significant sequence similarity to any other protein and therefore represents a rare example of a protein family that is apparently defined by a unique family member. Searches using DALI and VAST, however, reveal distant structural similarity between the PlyCB monomer with a functionally uncharacterized *S. mutans* protein (3L9A; rmsd 2.2 Å over 54 C α atoms). We suggest that PlyCB may represent a circular permutation of this fold (Fig. S2), although no pattern of conserved residues common to PlyCB and 3L9A is apparent.

The catalytic PlyCA subunit contains three distinct domains—an N-terminal domain (residues 1–205) that comprises a loose bundle of α -helices (Fig. 1A), a short central helical domain that we term the docking domain (residues 228–286; Fig. 1A), and a C-terminal CHAP domain (residues 309–465; Fig. 1A). Linker regions between the central helical bundle and the two terminal domains showed partial disorder in electron density, consistent with limited proteolysis data that demonstrated that both domains could be readily dissociated from the holoenzyme. Outside the docking domain, a single contact is made with the body of the PlyCB ring. Mutation at this site (PlyCB-Gln₄₆Ala) had no effect on lytic activity (Table S3 and Fig. S3).

The CHAP domain is most closely related to an enzyme from *Staphylococcus saprophyticus* (2K3A) (15) and comprises a small half β -barrel packed against a cluster of two helices. As previously described for related enzymes, the active site (Cys₃₃₃/His₄₂₀) is located in the cleft between these two subdomains (16). Interestingly in the structure, the region linking the N-terminal domain to the central helical structure (linker 1) blocks the CHAP active site (Fig. 2). Rearrangement of this loop would be anticipated to be necessary to expose the CHAP active site.

PlyCB Assembly Localizes Eight Binding Sites for Streptococcal Peptidoglycan. To identify the region of the PlyCB octamer that was involved in cell wall binding, we undertook a mutagenesis study of both surfaces of the ring. Mutation of residues on the flat surface retained WT PlyC assembly, cell-wall binding, and lytic activity (Table S3 and Fig. S3). On the convex surface, we identified a prominent groove in each monomer that was lined by residues Tyr₂₈, Lys₅₉, and Arg₆₆ (Fig. 3, Table S3, and Fig. S3). Mutation of Arg₆₆ completely abolished cell wall binding and PlyC activity (Table S3). Mutation of Tyr₂₈ and Lys₅₉ also had a major impact on the ability of the enzyme to bind the cell wall as well as PlyC activity (Table S3 and Fig. S3). However, none of the mutations interfered with formation of the PlyCB octameric ring or formation of the holoenzyme (Table S3 and Fig. S3). Thus, we suggest that the interface between the cavity formed by the PlyCB C-terminal helix and central sheet forms a binding site for the bacterial cell wall. Presently, it is not clear if the binding

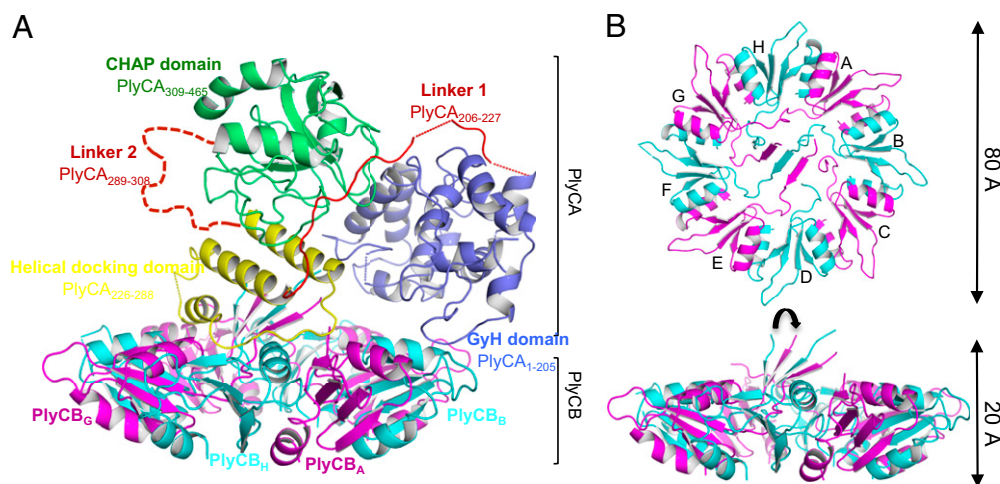


Fig. 1. Structure of PlyC. Two complete PlyC molecules were located within the asymmetric unit with 16 PlyCB molecules (chains A–P) and two PlyCA molecules (chains 1 and 2). Regions of disorder are shown as broken lines. Monomers A–P are essentially identical (average rmsd, 0.14 Å over 61 C α atoms), as are monomers 1 and 2 (average rmsd, 0.86 Å over 460 C α atoms); however, poor electron density was observed for monomers J, K, and L. The cartoons depicted represent PlyCB (A–H) and PlyCA (1). (A) The 3.3-Å X-ray crystal structure of PlyC whereby PlyCB monomers are colored alternately in magenta/cyan and labeled monomers A–H. The PlyCA molecule is colored by domain as indicated. The C α atoms of the model show the N-terminal residues 1–205 in light blue, the disordered linker 1 (residues 206–227) in red, the helical structure (residues 226–288) that docks PlyCA to PlyCB in yellow, the second disordered linker 2 (residues 289–308) in a dashed red line, and the CHAP domain (residues 309–465) in green. Regions of disordered/absent density are depicted by dashed lines. (B) The PlyCB CBD alone colored alternately in magenta/cyan and labeled A–H.

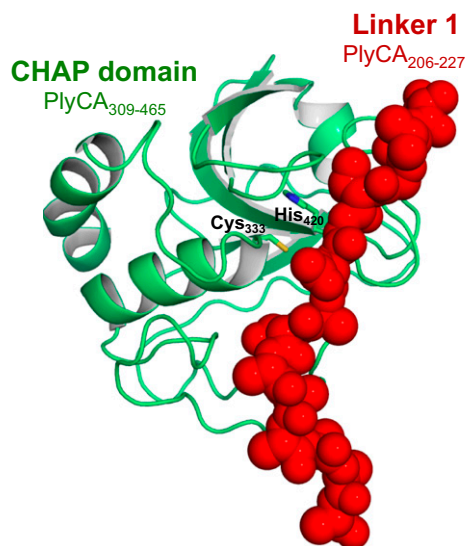


Fig. 2. The active site of the CHAP is occluded by linker region 1 (between the GyH domain and CHAP). Carbon atoms of CHAP catalytic residues Cys333 and His420 are shown in green, and the linker region is shown as red spheres and clearly blocks access to the CHAP active site. Mutation of a linker residue (PlyCA_{S224Q}) had no effect on the activity of the PlyC holoenzymes (Table S3 and Fig. S3).

groove of a single monomer is sufficient to mediate binding the holoenzyme or whether all eight such sites function to coordinate the PlyC lysin onto target bacterial cell walls (Fig. 3).

Catalytic Protein PlyCA and Cell Wall Binding PlyCB Interact Through Unique Protein–Protein Interaction. Despite the high resolution of these data, in the PlyCB octamer alone, the N-terminal region (residues 1–8) of each PlyCB monomer on the planar side is disordered in electron density. In contrast, in the PlyC holoenzyme structure, four of the PlyCB N-termini assemble to form a four-stranded parallel β -sheet (Fig. 1B). The docking domain of PlyCA, which comprises an antiparallel bundle of three α -helices, sits on top of this platform (Fig. 1A). Together, these structures form an exclusively hydrophobic interface (707.5 Å² of buried accessible surface area) and include the majority of interactions between PlyCA and the PlyCB octamer. Sequential deletion of the PlyCB N-terminal residues (Table S3 and Fig. S3) confirms the importance of this interaction, as deletion or mutation of PlyCB residues 3 to 8 results in loss of holoenzyme assembly (Table S3 and Fig. S3). Mutagenesis of the PlyCB-Ile₃ to Ala or Asp also resulted in the loss of holoenzyme assembly, whereas PlyCB-Ile₃Lys resulted in the formation of inclusion bodies (Table S3 and Fig. S3).

PlyCA Has Two Distinct Catalytic Domains Contributing Amidase and Glycosidase Activity to Achieve Cell Lysis. Although the presence of a CHAP domain in PlyCA has been previously reported (14), limited proteolysis of PlyCA indicated that a second globular domain was present in the protein. The holoenzyme structure revealed this domain was present in the N terminus of the protein. VAST and DALI searches show the PlyCA N-terminal domain (residues 1–205) is homologous to class IV family 19 chitinases—a branch of the glycoside hydrolase enzyme superfamily. We therefore have referred to the N-terminal domain of PlyCA as a putative glycosyl hydrolase (GyH) domain. Structural superposition between the PlyCA GyH domain and the top scoring chitinase structure (3CJL) reveals that only a central core of five helices is conserved (Fig. 4). Importantly, however, these data, together with sequence alignments of similar GyH domains

(identified by using PSI-BLAST; Fig. S4), revealed that PlyCA-Glu₇₈, a position conserved in other GyH domains (Fig. S4), corresponds to a key catalytic residues of the family 19 chitinases (Glu₆₈ in 2CJL). Two other absolutely conserved residues cluster in this region—PlyCA-Tyr₇₄ and PlyCA-Asn₈₇ (Fig. S4). Together, we suggest these residues represent a catalytic center.

Family 19 chitinases function to cleave β (1→4) glycosidic bonds of unbranched chains of *N*-acetylglucosamine polymers. As the bacterial peptidoglycan varies in structure from chitin [peptidoglycan is characterized by alternating β (1→4) linked sugars of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) with each MurNAc attached to a short (4- to 5-residue) stem peptide of L- and D-form amino acids], we wanted to ascertain if this domain did indeed act as a glucosaminidase to release free reduced sugars. Notwithstanding any potential glucosaminidase activity associated with the GyH domain, PlyC has been previously shown to have an amidase activity that cleaves between MurNAc and L-alanine of the stem peptide, producing a free N terminus (and releasing amines) (17). Later studies demonstrated that inactivation of the CHAP domain, presumed to be responsible for the amidase activity, significantly reduced the lytic activity of PlyC on streptococcal cells (14). In light of the distinct CHAP and GyH domains revealed by the crystal structure and the potential for multiple catalytic activities, we used two separate biochemical assays to analyze the free sugar (18) and amine (19) release that occurs after PlyC hydrolysis of purified streptococcal peptidoglycan. Purified WT PlyC, PlyCA, and the PlyCB octamer were used as controls. Streptococcal peptidoglycan was the substrate for both assays. For analysis of the contribution of the CHAP domain, we used a catalytic residue mutant PlyCA-Cys₃₃₃Ser (Fig. S5) (14). To analyze the contribution of the GyH domain (and furthermore to test the functional importance of the putative catalytic center) we used four mutations: Glu₇₈Ala, Asn₈₇Ala, His₈₈Ala, and Asn₁₀₄Ala (Fig. S5). Biophysical and gel filtration studies suggested all of the mutations formed PlyC holoenzyme and were properly folded (as detailed in *Methods*).

Our experiments revealed that PlyC indeed possesses significant glycosidase activity (Fig. 5A). We further demonstrated that mutation of putative catalytic residues or surrounding residues in the GyH domain reduces this activity by ~85%. Interestingly, however, even the CHAP active-site KO (Cys333Ser) significantly decreased glycosidase activity suggesting that the CHAP domain itself has intrinsic glycosidase activity, or that it cooperatively interacts with the GyH domain. Finally, we showed that double mutations in the CHAP and GyH domains resulted in total loss of glycosidase activity (Fig. 5A). Next, we performed a similar series of experiments by using an amidase assay (Fig. 5B). Consistent with previous studies (14), we showed that the Cys333Ser mutation resulted in an almost complete loss of amidase function and corresponds to ~99% loss in streptococcal lytic activity. Interestingly, catalytic mutations in the GyH domain also significantly reduced overall amidase activity, but not to the extent of the Cys333Ser mutation. Similar to the glycosidase assay, double mutations to both the CHAP and GyH domains were found to be completely devoid of any measurable amidase activity.

Taken together, our biochemical data reveal that glycosidase and amidase activities exist in the PlyC holoenzyme. Based on our analysis of active-site mutants, homology to characterized domains, and previous studies (14, 17), we suggest the CHAP domain contains the amidase activity, specifically an *N*-acetylmuramoyl-L-alanine amidase activity, and the GyH domain contains the glycosidase activity. As expected, PlyCB was found to be absent of any type of catalytic activity (Fig. 5A and B). However, we note that neither recombinant purified CHAP domain nor full-length PlyCA, which possesses both CHAP and GyH catalytic domains, displayed significant amidase,

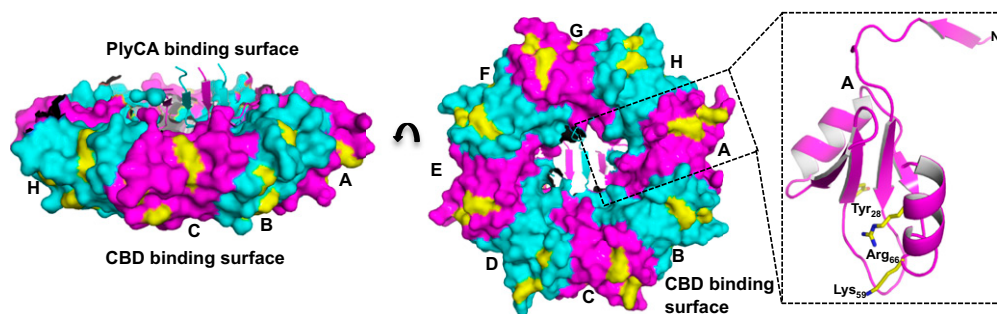


Fig. 3. PlyCB has eight cell wall-binding grooves. The cell wall-binding surface of PlyCB alone (colored as in Fig. 1) shows the residues involved in cell wall binding in yellow. *Inset:* Cartoon depiction of PlyCB monomer A shows cell wall binding residues in yellow sticks as indicated.

glycosidase, or lytic activities (Fig. 5). Any minor activity observed for these constructs is presumably a consequence of random collisions of the catalytic domain(s) with the cell wall in the absence of the PlyCB binding domain. These data serve to further highlight the critical importance of the cell wall binding function of PlyCB to the overall activity of PlyC.

To investigate if catalytic efficiency was a result of cooperative or synergistic activity, we examined the activity of each domain in isolation. First, we constructed two new mutants, PlyC Δ CHAP and PlyC Δ GyH, each of which contain WT PlyCB and PlyCA with the specified catalytic domain deletion. Next, we examined the lytic activity of equimolar amounts (44 nM) of PlyC, PlyCA alone, CHAP alone, PlyC Δ CHAP, and PlyC Δ GyH (Fig. 5C). The two deletion mutants had <1% of WT activity, and complementation with purified CHAP did not restore WT activity. Even when we diluted WT PlyC to 1 nM, it still displayed more than twice the lytic activity of 44 nM PlyC Δ CHAP complemented with 44 nM CHAP (Fig. 5C). We therefore suggest that the unusually high processivity of the PlyC holoenzyme can be explained by synergistic effects resulting from the positioning of both catalytic domains in PlyCA, and in the context of the PlyCB binding domain. The synergy/cooperativity between catalytic domains is further supported by biochemical assays showing a significant decrease in glycosidase activity of the CHAP (i.e., amidase domain) active-site KO and a decrease in amidase activity of the GyH (i.e., glycosidase domain) active-site KOs (Fig. 5A and B).

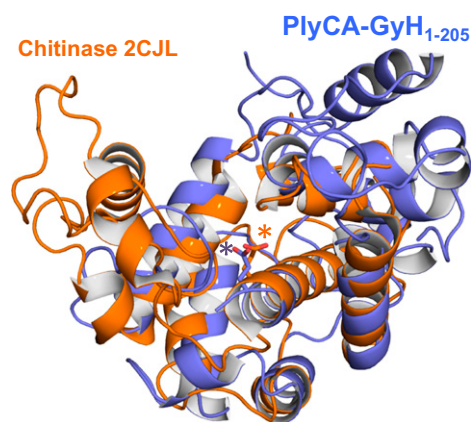


Fig. 4. The PlyCA GyH domain is homologous to class IV chitinases. Superposition of the GyH domain (light blue) with a representative chitinase (orange; 2CJL; rmsd of 3.3 Å over 107 C α atoms). The position of PlyCA GyH (Glu₇₈) (blue asterisk) and the catalytic residue 2CJL (Glu₆₈) (orange asterisk) is shown as a stick.

Discussion

The PlyC lysin is by far the most active lysin described to date, yet it is larger than most lysins by nearly a factor of four and remains the only known lysin composed of distinct subunits. Accordingly, the molecular basis for this potency is interesting from the perspective of the development of novel antibacterial therapeutic agents and molecular dynamics. Here we show through structural and biochemical studies that PlyC contains two functional catalytic domains. This finding was unexpected, as the relationship between the GyH domain and the bacterial chitinases is not detectable through sequence-based searches alone. We further show that PlyC displays amidase and glycosidase activity, and thus can cleave both classes of bond present in the bacterial peptidoglycan. It is notable that several traditional lysins have been identified that likewise contain tandem catalytic subunits. However, when studied at the biochemical level, most of these “extra” domains were found to be silent. For example, the staphylococcal Φ 11 lysin has both D-alanyl-glycyl endopeptidase and N-acetylmuramoyl-L-alanine amidase catalytic domains (6). Although the endopeptidase domain was active by itself (20, 21), the amidase domain was shown to be silent by deletion analysis (21). Likewise, the LysK lysin has an active endopeptidase domain and an inactive amidase domain (22, 23), and the streptococcal λ SA2 lysin contains an active D-glutamyl-L-lysine endopeptidase and an inactive N-acetylglucosaminidase domain (24, 25). Thus, PlyC is one of the first characterized lysins with two active and distinct catalytic domains.

As much as the structural and biological data reveal about the unique properties of PlyC, the question remains, however, why the combination of PlyCB and PlyCA forms such an effective enzyme. Synergy or cooperation between the CHAP and GyH domains may, in part, begin to explain the enhanced lytic activity displayed by PlyC compared with traditional lysins. Significantly, despite possessing amidase and glycosidase activities, a single point mutation to the catalytic center of the CHAP or the GyH domain reduces streptococcal lytic activity by 90% to 99% (Fig. 3 and Table S3). At present, however, it is not known if the two domains act synergistically or cooperatively. Under a synergistic scenario, the two domains could simultaneously cleave the peptidoglycan between GlcNAc and MurNAc, as well as MurNAc and L-alanine, thereby destabilizing both the vertical and horizontal scaffolds of the peptidoglycan. These large defects in the superstructure of the cell wall would lead to rapid bacterial lysis, which would be observed as enhanced activity over a typical lysin. For the cooperative scenario, one domain may be largely silent catalytically, but may function to position the peptidoglycan for optimal hydrolysis by the other catalytic domain. In this way, cooperativity between the two domains may significantly enhance the turnover rate.

Another unique feature of PlyC that stands out in comparison with other lysins is the presence of the octameric cell wall

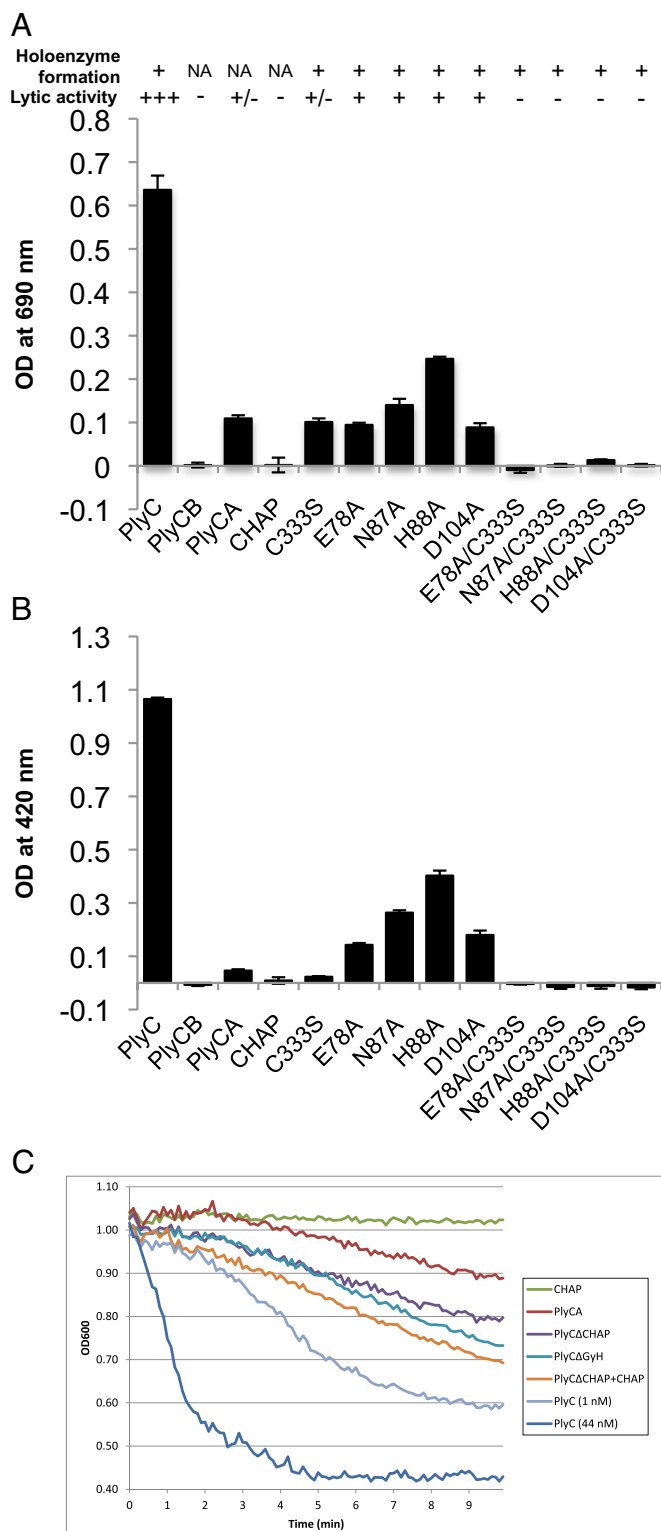


Fig. 5. Biochemical activity of PlyC domains. PlyC, PlyCB alone, PlyCA alone, CHAP, the PlyCA CHAP domain active-site KO (C333S), the four PlyCA GyH mutants with diminished lytic activity (E78A, N87A, H88A, D104A), and the four CHAP/GyH double mutants were tested on purified streptococcal peptidoglycan for (A) glycosidase activity or (B) amidase activity (Methods). Additionally, holoenzyme formation and lytic activity on live streptococcal cells is noted at the top of the figure. +++, WT lytic activity; +, significantly diminished lytic activity ($\leq 10\%$ WT); +/-, negligible lytic activity ($\leq 1\%$ WT), and -, no detectable lytic activity. (C) Additional lytic activity on selected construct. PlyC, CHAP, PlyCA, PlyC Δ CHAP, PlyC Δ GyH, and PlyC Δ CHAP + CHAP

binding subunit, PlyCB. Our data further highlight the crucial functional importance of the PlyCB subunit to direct binding to the streptococcal surface. Although PlyCB itself has no integral enzymatic function, PlyCA is almost devoid of any lytic activity ($<1\%$ of WT) in the absence of PlyCB. Furthermore, our mutational data reveal that PlyCB contains eight potential binding sites for cell wall components. In contrast, most characterized lysins contain only a single CBD. Our structural data do not, however, provide evidence whether all eight binding sites participate simultaneously to coordinate holoenzyme binding to the streptococcal cell wall. Concurrent binding may indicate a very tight, stable interaction between the holoenzyme and cell wall, whereas a consecutive interaction may lead to a more transient interaction. It is worthy to note that similar classes of enzymes have used tight binding domains in combination with flexible catalytic domains to hydrolyze multiple substrate bonds within a range between domains allowed by the linker flexibility. Interestingly, the cellulase family of enzymes, responsible for degrading the insoluble, polysaccharide cellulose, display a similar architecture and mechanism for substrate recognition and cleavage to that which we propose for PlyC. Most cellulases have a modular structure with a catalytic module and a cellulose-binding module. Long, flexible linkers are pivotal for the ability of the enzyme to access the complex surface of cellulose and allow a caterpillar-like displacement to move through the substrate (26, 27). For example, small-angle X-ray scattering studies of the fungal cellulase, Cel45, suggest as many as four cellobiose units on a cellulose chain are within range of the catalytic domain when binding domain is attached to a specific site and the linker is fully extended (26). Appreciably, both catalytic PlyCA domains are connected to the central helical domain (the primary point of contact with PlyCB) by extensive flexible linker sequences, and neither the GyH domain nor the CHAP domain makes substantial contact with the PlyCB ring. Together, these data suggest that the position of the catalytic domains is not spatially restricted by strong interactions with the PlyCB ring. Whether PlyC shares a similar mechanism as put forth for certain cellulases remains to be elucidated. Nonetheless, PlyC is distinctive among known lysin family members for its two functionally active catalytic domains, extreme flexibility between subunits, octameric binding domain, and high processivity. Identification of additional multisubunit lysins may help further define the molecular mechanisms of this new lysin subfamily.

Methods

Biological Methods. Cloning, generation of mutants, and purification techniques. The constructs for pBAD24::plyC, pBAD24::plyCA, and pBAD24::plyCB were previously described (14). Construction of the CHAP, PlyC Δ CHAP and PlyC Δ GyH domain are described in detail in *SI Methods*. Expression of constructs was as previously described (13, 14) with the exception of the CHAP domain (*SI Methods*). Details of the mutagenesis and analysis of mutants are given in *SI Methods*.

Lytic activity and cell wall binding. Deletions, mutants, and subunits were evaluated for lytic activity, proper folding, formation of the holoenzyme (or PlyCB octamer where appropriate), and binding to the streptococcal peptidoglycan. Spectrophotometric lytic assays were performed with an overnight culture of *S. pyogenes* D471 grown at 37 °C in Todd-Hewitt broth supplemented with 1% wt/vol yeast extract. Streptococci were washed in PBS solution (pH 7.4) and resuspended at an OD₆₀₀ of 1.5. In a 96-well plate, 100 μ L of bacteria was mixed with 100 μ L of enzyme (final concentration of 44 nM), and the OD₆₀₀ was measured kinetically on a SpectraMax 190 (Molecular Devices) every 6 s for 10 min. All assays were performed in triplicate.

were all at a final concentration of 44 nM. In addition, PlyC is also shown at 1 nM final concentration to highlight the diminished activity of all non-WT constructs. Plots shown are the average OD reading at each data point from independent experiments, each in triplicate.

To assess cell wall binding, the fluorescent dye Alexa Fluor 488 (Molecular Probes) was conjugated to primary amines of purified (1 mg each) proteins through a tetrafluorophenyl ester. The reaction was quenched by the addition of 100 mM Tris and immediately applied to a PD10 desalting column to separate the labeled protein from unreacted dye. A total of 10 μ g of labeled protein was then incubated with a fresh overnight culture of *S. pyogenes* D471 resuspended in PBS solution. The cells were washed in PBS solution and viewed on a Nikon Eclipse 80i epifluorescent microscope equipped with a Retiga 2000R CCD camera. The degree of fluorescent staining of the cell wall (i.e., binding of the protein) was quantified with the Q Capture Pro or NIS-Elements software packages.

Biochemical assays. The *S. pyogenes* D471 peptidoglycan was purified by a French press method as previously described (7), lyophilized, and used for all biochemical assays. For all assays, purified peptidoglycan was resuspended in PBS solution, pH 7.2, to an OD₅₅₀ of 1.0. WT PlyC or mutants were added at a concentration of 3.5 μ g/mL (30 nM) and allowed to react for with peptidoglycan for 5, 15, 30, or 60 min at 37 °C. All experiments consisted of at least two independent experiments assayed in triplicate. For analysis of reducing sugars released from the peptidoglycan, we used the method of Park and Johnson (28) as modified by Spiro (18). This method is based on the reduction of ferricyanide by sugars in an alkaline solution followed by the formation of Prussian blue (ferric ferrocyanide) upon addition of ferric ions. To determine an increase in free amine groups, we used the trinitrophenylation reaction originally described by Satake et al. (29) and modified by Mokrasch (30).

Structural Biology Methods. Crystallization, X-ray data collection, structure determination, and refinement. Crystals were grown in all cases by using the hanging drop vapor diffusion method, with 1:1 (vol/vol) ratio of protein to mother liquor (0.5 mL well volume). Data were collected by using in-house and synchrotron radiation (Industrial Macromolecular Crystallography Association Collaborative Access Team, Advanced Photon Source, Argonne, IL). The structure of PlyCB was solved by using phases determined using sulfur single

anomalous dispersion phasing using the program AutoSHARP (31). Density modification was applied by using the program SOLOMON followed by automated building by using the program ARP/wARP (32). The refined structure of the octameric PlyCB ring was used as a molecular replacement search probe for the native PlyC dataset using PHASER (33), resulting in two clear solutions. Electron density maps were then calculated using the AutoBUSTER (34) program (*SI Methods*). The initial Fo-Fc map revealed significant unbiased regions of positive connective density that clearly did not form part of PlyCB probe. This strongly indicated a correct molecular replacement solution, and these initial phases were used to manually build the two PlyCA molecules. We noted four short regions of the PlyCA molecule that contained residues identified as Ramachandran outliers (*SI Methods*). Data collection and refinement statistics are detailed in *Tables S1* and *S2*. Further details of structural determination are provided in *SI Methods*.

Figures and structural coordinates. Superpositions were conducted using PDBeFold (35) (<http://www.ebi.ac.uk/msd-srv/ssm/>). Pymol was used to produce all structural representations (<http://www.pymol.org>). The coordinates and structure factors are available from the Protein Data Bank under ID codes 4F87 and 4F88. Raw data and images are available from TARDIS (36) (www.tardis.edu.au).

ACKNOWLEDGMENTS. The authors thank the Industrial Macromolecular Crystallography Association Collaborative Access Team (Advanced Photon Source) for beam time and for technical assistance, Jamie Rossjohn for technical assistance with data collection, Joseph Kotarek for FTIR assistance, and Amanda Altieri for CD assistance. This work was supported in part by US Public Health Service Grant A111822 (to V.A.F.) and US Department of Defense Grant DM102823 (to D.C.N.). S.M. is an Australian Research Council (ARC) Future Fellow. A.M.B. is a National Health and Medical Research Council (NHMRC) Senior Research Fellow. J.C.W. is an ARC Federation Fellow and an NHMRC Honorary Principal Research Fellow.

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