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Protecting Gram-negative bacterial cell envelopes from human lysozyme: Interactions with Ivy inhibitor proteins from *Escherichia coli* and *Pseudomonas aeruginosa*☆

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ABSTRACT

Lysozymes play an important role in host defense by degrading peptidoglycan in the cell envelopes of pathogenic bacteria. Several Gram-negative bacteria can evade this mechanism by producing periplasmic proteins that inhibit the enzymatic activity of lysozyme. The *Escherichia coli* inhibitor of vertebrate lysozyme, Ivyc and its *Pseudomonas aeruginosa* homolog, Ivyp1 have been shown to be potent inhibitors of hen egg white lysozyme (HEWL). Since human lysozyme (HL) plays an important role in the innate immune response, we have examined the binding of HL to Ivyc and Ivyp1. Our results show that Ivyp1 is a weaker inhibitor of HL than Ivyc even though they inhibit HEWL with similar potency. Calorimetry experiments confirm that Ivyp1 interacts more weakly with HL than HEWL. Analytical ultracentrifugation studies revealed that Ivyp1 in solution is a monomer and forms a 30 kDa heterodimer with both HL and HEWL, while Ivyc is a homodimer that forms a tetramer with both enzymes. The interaction of Ivyp1 with HL was further characterized by NMR chemical shift perturbation experiments. In addition to the characteristic His-containing Ivy inhibitory loop that binds into the active site of lysozyme, an extended loop (P2) between the final two beta-strands also participates in forming protein–protein interactions. The P2 loop is not conserved in Ivyc and it constitutes a flexible region in Ivyp1 that becomes more rigid in the complex with HL. We conclude that differences in the electrostatic interactions at the binding interface between Ivy inhibitors and distinct lysozymes determine the strength of this interaction. This article is part of a Special Issue entitled: Bacterial Resistance to Antimicrobial Peptides.

1. Introduction

During bacterial infections, lysozymes and proteinaceous lysozyme inhibitors are part of the molecular warfare that is deployed between vertebrate hosts and bacteria. Lysozymes are host defense enzymes that act as a protective barrier against colonization and invasion by bacterial pathogens. They exert their activity by hydrolysing the β-1,4 glycosidic bonds of peptidoglycan, a polysaccharide unique to the bacterial cell wall [1]. The antibacterial activity is stronger against Gram-positive than against Gram-negative bacteria where peptidoglycan accessibility is hindered by the presence of the outer membrane. However, the production of accessory antibacterial proteins which permeabilize the outer membrane, such as lactoferrin, renders also Gram-negative bacteria a lysozyme target [2,3]. Three major lysozyme types have been identified to date and are designated as c-type (chicken or conventional), g-type (goose), and i-type (invertebrate) [4,5]. Although the overall sequence similarity among c-, g-, and i-type lysozymes is limited, their three dimensional structures share significant similarities. The best studied member of c-type lysozymes, the major type of vertebrates, is HEWL (hen egg white lysozyme), the first enzyme to have its structure determined by X-ray crystallography [6]. The enzyme is approximately ellipsoidal in shape, with two structural lobes divided by a large cleft that contains the active site [6–8].

Because of the effectiveness of lysozyme as an antibacterial agent, it is perhaps not surprising that some pathogenic and commensal bacteria have evolved defense strategies to escape from the threat posed by lysozyme. Examples of such mechanisms are the chemical modification of peptidoglycan in the bacterial cell wall [9–11] and the synthesis of a protein named SIC (streptococcal inhibitor of complement) which was shown to bind lysozyme, albeit weakly [12]. More recently two different families of specific inhibitors of c-type lysozymes have been discovered [13,14]. The first identified member of the Ivy (inhibitor of vertebrate lysozyme) family is the product of the *Escherichia coli* ORF gene *ykfE*

Abbreviations: Ivy, inhibitor of vertebrate lysozyme; HEWL, hen egg white lysozyme; HL, human lysozyme; ITC, isothermal titration calorimetry; AUC, analytical ultracentrifugation; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence; LTG, lytic transglycosylase

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[13]. Homologs of the *E. coli* Ivy protein (Ivyc) have been found in at least 35 members of Proteobacteria (Gram-negative bacteria) within the periplasmic compartment, but unexpectedly not in Gram-positive ones [15]. PliC, a periplasmic lysozyme inhibitor of c-type lysozymes originally isolated from *Salmonella enteriditis* and MliC, a membrane-bound lysozyme inhibitor of c-type lysozymes anchored to the luminal face of the outer membranes of *E. coli* and *P. aeruginosa*, represent a second, larger family of c-type lysozyme inhibitors which is predicted to have homologs in at least 52 different genera of Protobacteria and occur in Acidobacteria, Cyanobacteria, and Bacteroides groups [14]. Recently, other protein inhibitors of lysozyme were described for *Moraxella catarrhalis*, suggesting that this bacterial evasion mechanism may be more widespread [16].

The mechanism of inhibition by PliC and MliC has recently been elucidated by analyzing the structures of their complexes with lysozyme [17–21]. The structures of Ivyc and of its evolutionary distant homolog from *P. aeruginosa*, Ivyp1, and their complexes with HEWL have also been determined by X-ray crystallography [15] shedding some light on their mechanism of inhibition. Ivyc is a dimer in the crystal structure. Each monomer of the Ivyc dimer consists of a central β-sheet made up of five antiparallel β-strands flanked by two short α-helices on the convex side and by an amphipatic helix on the concave side. In solution, Ivyc likewise appears to be predominantly dimeric as estimated from gel filtration experiments [13]. The structure reveals that the dimer involves interactions among residues that are located in the C-terminal part of the molecule and are conserved in various *E. coli* strains as well as in homologous amino acid sequences from *Shigella flexneri*, *Klebsiella pneumoniae*, and *Burkholderia cepacia*. However, in other bacteria Ivy seems to be monomeric, and *P. aeruginosa* Ivyp1, which lacks many of the conserved C-terminal residues involved in dimer formation, is a monomeric protein with a similar structure to the Ivyc monomer [15].

The crystallographic structures of the Ivyc–HEWL and Ivyp1–HEWL complexes show that the Ivyp1 monomer interacts with one HEWL molecule while the Ivyc–HEWL crystal unit contains one Ivyc homodimer in complex with two HEWL molecules [15]. Interestingly, in the Ivyc–HEWL complex each HEWL molecule interacts with both Ivyc molecules through two interacting surfaces. The deduced mechanism of lysozyme inhibition by the Ivy molecules suggests that the lysozyme active site becomes occluded by a loop protruding from the Ivy molecules. The sequence of the inhibitory loop (CKPHDC) is strictly conserved among a subset of Ivy homologs even in distantly related bacteria. A disulfide bond linking the two cysteine residues on both sides of this sequence provides some rigidity to this loop.

The inhibitory loop sequence is not conserved in several Ivy paralogs found in the *Pseudomonas* species and is replaced with a more variable sequence flanked by two conserved cysteine residues (CExxDxC). One of these inhibitors, Ivyp2 has been shown to lack the ability to inhibit lysozyme despite their overall sequence similarity. Recently, a new role has been proposed for these paralog sequences [22], namely, the inhibition of lytic transglycosylases (LTG), a group of enzymes responsible for endogenous peptidoglycan lysis. LTGs share the same specificity of cleavage as the lysozymes. Interestingly, Ivyp1 also inhibits LTGs and it was therefore suggested that the inhibition of LTGs may be the true physiological function of the Ivy proteins. However, several recent in vivo studies have confirmed that the Ivy proteins can play a major role in the evasion mechanisms of pathogenic bacteria by inhibiting lysozyme. For example, recent work with *Edwardsiella tarda* [23], *Yersinia pestis* [24], and avian pathogenic *E. coli* [25] showed that Ivy deletion mutants had reduced virulence in a species dependent manner. Moreover, overexpression of Ivy proteins in various *E. coli* organisms gave rise to increased bacterial growth in lysozyme containing media [26]. These studies all reinforce the original work of Deckers et al, who demonstrated by making knockouts and overproducing strains that Ivyc protected *E. coli* against lysozyme [27]. Taken together, it seems clear that the bacterial Ivy proteins are multifunctional and that they can play a role in evading the lysozyme-mediated host response [28].

Ivyc was shown to be a strong inhibitor of HEWL, but inhibited λ-phage lysozyme and goose egg white lysozyme only weakly [13,29]. While K_d values of 1 nM and 25 nM were reported for the inhibition of HEWL by Ivyc and Ivyp1, respectively, no quantitative inhibition data is available for HL (human lysozyme) or any other c-type lysozymes. The structure of HL [30] reveals important differences with respect to HEWL in the residues located at the interface of the HEWL–Ivy complexes, which are expected to affect the strength of interaction and consequently the Ivy inhibitory effect. Moreover because HL has a higher specific activity, and since it plays important roles in the human host-defense in various biofluids, such as milk, saliva, tears, airway epithelium, and blood [31–35], it is important to study the interaction of Ivyc and Ivyp1 with HL in comparison with HEWL. Consequently, in this work, in addition to enzymatic inhibition experiments, we monitored the interaction of these proteins by isothermal titration calorimetry (ITC), analytical ultracentrifugation (AUC), differential scanning calorimetry (DSC), and NMR spectroscopy. Our results show that Ivy proteins differ in their interaction with different c-type lysozymes.

2. Material and methods

2.1. Chemicals and reagents

Unless stated otherwise all reagents were purchased from Sigma-Aldrich (St. Louis, Missouri). Recombinant HL was produced from a synthetic codon-optimised gene in *Pichia pastoris* in our lab (unpublished data) or purchased from Sigma-Aldrich as a recombinant enzyme produced in rice (L-1667). Both enzymes proved to be >95% pure and displayed approximately the same specific activity (data not shown) using the turbidimetric assay described below.

2.2. Cloning of Ivyc and Ivyp1

The 387 bp DNA fragment encoding the mature form of Ivyc and Ivyp1 without its signal peptide was PCR amplified from *E. coli* K12 and *P. aeruginosa* PA14, respectively using Pfu polymerase (Fermentas). The primers used for the PCR amplification were synthesized at the University Core DNA Services, Calgary and the sequences are listed below:

IvycF: 5′-GATATACATATGCAGGATGATTTAACCATTAGCAGC-3′
IvycR: 5′-GTGGTGCTCGAGTTTAAAATTAAAGCCATCCGGATGG-3′
IvypF: 5′-GTTAATCATATGGAGCAGCCGCGGC-3′
IvypR: 5′-CAATAACTCGAGCTTCCAGTTCGGATC-3′.

Restriction sites for NdeI and XhoI were added at each end of the primers and are underlined to allow ligation into the expression vector. The PCR products were first purified using the QIAquick PCR Purification Kit (Qiagen) and then digested with NdeI and XhoI restriction enzymes. The purified digested products were ligated into pET29b (EMD Biosciences) using T4 DNA ligase.

2.3. Expression and purification of Ivyc and Ivyp1

E. coli BL21(DE3) cells transformed with the purified expression plasmids carrying the correct sequence for mature Ivyc and Ivyp1 were grown in LB medium supplemented with 30 μg/mL kanamycin at 37 °C until exponential phase ($OD_{600\ nm}$ approximately 0.7). Overexpression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and the culture was grown for another 3–4 h at 37 °C before the cells were harvested by centrifugation at 6000 rpm for 15 min at 4 °C. The cell pellet was resuspended in Buffer A (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 75 mM imidazole) containing 0.8 mg/mL phenylmethylsufonyl fluoride (PMSF) and lysed by using three passages through a French Pressure Cell Press. The lysate was centrifuged