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The high-affinity peptidoglycan binding domain of *Pseudomonas* phage endolysin KZ144

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ABSTRACT

The binding affinity of the N-terminal peptidoglycan binding domain of endolysin KZ144 (PBD_{KZ}), originating from *Pseudomonas aeruginosa* bacteriophage ϕKZ, has been examined using a fusion protein of PBD_{KZ} and green fluorescent protein (PBD_{KZ}-GFP). A fluorescence recovery after photobleaching analysis of bound PBD_{KZ}-GFP molecules showed less than 10% fluorescence recovery in the bleached area within 15 min. Surface plasmon resonance analysis confirmed this apparent high binding affinity revealing an equilibrium affinity constant of $2.95 \times 10^7\ M^{-1}$ for the PBD_{KZ}-peptidoglycan interaction. This unique domain, which binds to the peptidoglycan of all tested Gram-negative species, was harnessed to improve the specific activity of the peptidoglycan hydrolase domain KMV36C. The chimeric peptidoglycan hydrolase (PBD_{KZ}-KMV36C) exhibits a threefold higher specific activity than the native catalytic domain (KMV36C). These results demonstrate that the modular assembly of functional domains is a rational approach to improve the specific activity of endolysins from phages infecting Gram-negatives.

Introduction

Endolysins are bacteriophage encoded peptidoglycan hydrolases, produced in phage-infected bacterial cells at the end of the lytic cycle. They degrade the peptidoglycan layer from within, enabling the release of newly formed progeny phage. A common feature of endolysins of phages that infect Gram-positive species is a modular structure, consisting of at least two separate functional domains: an N-terminal catalytic and a C-terminal cell wall binding domain [1]. In contrast, phage endolysins from a Gram-negative background are often globular, single-module enzymes (e.g., endolysins encoded by T4, P22, T7, λ) [2–5]. We recently reported the presence of a peptidoglycan binding domain in endolysin KZ144 of *Pseudomonas aeruginosa* phage ϕKZ [6]. Interestingly, the modular composition of KZ144 is arranged differently compared to other phage endolysins, since the substrate binding activity is located at the N-terminus. In addition, this binding domain of KZ144 recognizes acetylated A1γ chemotype peptidoglycan, which is the common peptidoglycan composition of all Gram-negative species, providing the domain with a much wider binding spectrum compared to the respective domains of endolysins of Gram-positive origin, which often selectively target specific and unique cell wall associated ligands [7–8].

In this study, we evaluate the substrate binding affinity of the peptidoglycan binding domain of KZ144 (PBD_{KZ}) and exploit this protein domain to enhance the specific activity of another *P. aeruginosa* phage encoded peptidoglycan hydrolase.

Material and methods

Recombinant production and purification of PBD_{KZ}-GFP. The plasmid for production of PBD_{KZ}-GFP has been described previously [6]. Expression in Luria Bertani medium was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside in exponentially growing *Escherichia coli* BL21 pLysS cells ($OD_{600nm} = 0.6$) and production was allowed for 4 h at 37 °C. Cells were harvested (3300 g, 15 min, 4 °C) and the pellet was resuspended in 20 mL lysis buffer (10 mM imidazole, 20 mM NaH_2PO_4, 0.5 M NaCl, pH 7.4). This suspension was frozen/thawed four times prior to sonication (3 × 15 s, amplitude 40% on a Vibra CellTM, Sonics, Dandurry, CT, USA) and filtered successively through 0.45 and 0.22 μm Durapore membrane filters (Millipore, Billerica, MA, USA). Purification of the His-tagged fusion protein was performed by a one-step protocol employing metal affinity chromatography (HisTrap HP 1 ml column, GE Healthcare, Buckinghamshire, UK) according to the

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manufacturer's instructions. The wash buffer included 50 mM imidazole.

Confocal scanning fluorescence recovery after photobleaching (CSFRAP). The outer membrane of *P. aeruginosa* PAO1 cells was permeabilized with a chloroform-saturated 0.05 M Tris-buffer (pH 7.7) for 45 min. Purified PBD_{KZ}-GFP (final concentration 5 mM) was added to 100 μl aliquots of these permeabilized cells. The mixture was incubated for 5 min at 20 °C, subsequently spun down (16,060 g, 1 min) and the supernatant discarded. The cell pellet was washed twice in 20 mM NaH_2PO_4–NaOH 0.15 M NaCl pH 7.4. Microscope slides were pre-incubated with a poly-L-lysin solution (Sigma–Aldrich, St. Louis, MO, USA) for immobilization of fluorescently labeled *P. aeruginosa* cells. A #1 cover slide (Waldemar Knittel Glasbearbeitungs-GmbH, Braunschweig, Germany) was placed on top of the microscope slide and the suspended cells were imaged upside down.

Confocal scanning fluorescence recovery after photobleaching (CSFRAP) was performed on a LSM510 system (Carl Zeiss, Jena, Germany). For imaging eGFP, the 488-nm line of an Ar-ion laser (acousto-optical tunable filter 0.5%) was used. Fluorescence of eGFP was captured through a 505-nm long-pass filter on a photomultiplier tube detector. A high-NA objective (C-Apochromat 40×/NA1.2/W) in combination with a 0.9-Airy-unit confocal pinhole was used for optimal brightness and resolution. CSFRAP curves were obtained by illuminating a defined region-of-interest in the cells with a brief high-intensity laser pulse (AOTF 100%) and subsequent monitoring of the average fluorescence intensity (in arbitrary units) in that region as a function of time. The average fluorescence intensity of the entire cell right before the bleaching was used to normalize different FRAP curves. The average fluorescence intensity of the entire cell at each time point was used to account for acquisitional photobleaching.

Surface plasmon resonance analysis. Sensorgrams of binding of the PBD_{KZ}-GFP to the surface of immobilized *P. aeruginosa* PAO1 cells were obtained using surface plasmon resonance (Biacore X, Biacore AB, Uppsala, Sweden), essentially as described earlier [8]. First, the flat carboxymethylated surface of a BIAcore Pioneer C1 chip was coated with PBD_{KZ}-GFP (0.25 mg/ml at a flow rate of 5.0 μl/min; 10 mM sodium acetate pH 5.0), using the amine coupling procedure according to the manufacturer's suggestions. Secondly, autoclaved *P. aeruginosa* cells (3×10^{10} cells/ml) in HBS-T (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween20, pH 7.8) were applied to the surface featuring the covalently bound layers of PBD_{KZ}-GFP (15 μl total volume, flow rate 3 μl/min). This stable 'sandwich' then served as the basis for the actual affinity measurements, which were performed at 25 °C with 100, 250 and 500 nM PDB_{KZ}-GFP fusion protein for 3 min (association phase) and 12 min (dissociation phase), respectively, at a flow rate of 10 μl/min. Every concentration was measured twice. The coatedC1 chip was regenerated between different measurements by washes with stepwise increasing NaCl concentrations (2 M, 3 M, 4 M, 5 M; 15 μl; 10 μl/min). Calculation of kinetic data was performed using the BIAevaluation software (version 3.0; BIAcore).

Production of the chimeric PBD_{KZ}-KMV36C. The chimeric protein PBD_{KZ}-KMV36C consists of PBD_{KZ} (=aa 1–83 of endolysin KZ144) and KMV36C (=aa 737–898 of KMV36) [6,9–10]. Both encoding gene fragments were separately amplified and subsequently directly joined by 'splicing by overlap extension' (SOE) [11]. The final product was cloned in pEXP5-CT/TOPO® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and the sequence was verified. *E. coli* BL21 (DE3) pLysS cells (Invitrogen) were transformed with this construct. Expression and protein purification was performed as described above for PBD_{KZ}-GFP. However, addition of Pefablock® protease inhibitor (Merck, Darmstadt, Germany) was essential to prevent proteolytic cleavage during purification.

Measurement of peptidoglycan hydrolytic activity. An activity saturation curve representing the respective enzymatic activities for different amounts of both KMV36C and PBD_{KZ}-KMV36C was determined using a previously described enzymatic assay [9]. In essence, the measurement of enzymatic activity is based on a linear drop in optical density of the substrate (270 μl) (initial OD_{600nm} of the substrate was around 1.0) upon addition of up to 2.5 μg lytic enzyme (30 μl). The substrate consists of *P. aeruginosa* PAO1 cells from which the outer membrane has been dissolved by incubation with a chloroform-saturated 0.05 M Tris–HCl buffer (pH 7.7) during exactly 45 min. The substrate is washed with and resuspended in the specific enzyme buffer of KMV36C (16.7 mM K_3PO_4–HCl pH 7.0) or KZ144 (87.5 mM K_3PO_4–HCl pH 6.2). Spectrophotometric measurements were conducted using a Bioscreen C Microbiology Reader (Labsystems, Oy, Finland). Measurement details and a standardized calculation method of the enzymatic activities to warrant a maximal reliability are described previously [12].

Results

Mobility analysis of bound PBD_{KZ}-GFP

A fusion protein of PBD_{KZ} and eGFP is uniformly bound to *P. aeruginosa* cell walls which were disposed of their outer membrane, demonstrating the peptidoglycan binding capacity of PBD_{KZ} [6]. To assess and visualize the protein mobility of bound PBD_{KZ}-GFP proteins, a confocal scanning fluorescence recovery after photobleaching (FRAP) analysis was performed (Fig. 1A) [13]. One bacterial pole of the fluorescently labeled cell surface was irreversibly photobleached after which fluorescence recovery due to protein diffusion was monitored to determine the diffusive mobility of the bound molecules. As a control, *E. coli* cells expressing recombinant PBD_{KZ}-GFP in the cytoplasm were included. While unbound PBD_{KZ}-GFP molecules in the *E. coli* cytoplasm were highly mobile showing a complete fluorescence recovery within 300 ms (Fig. 1B), redistribution of PBD_{KZ}-GFP bound to the *P. aeruginosa* peptidoglycan was not significant (less than 10% in 15 min) (Fig. 1C). These results illustrate a strong and almost irreversible immobilization of PBD_{KZ}-GFP on *P. aeruginosa* peptidoglycan.

Real-time kinetic binding analysis of PBD_{KZ}-GFP

To quantify the interaction between PBD_{KZ}-GFP and the *P. aeruginosa* peptidoglycan more precisely, surface plasmon resonance analysis was performed. A triple-layer sandwich setup was used to allow real-time observation of the interaction between the fusion protein PBD_{KZ}-GFP and immobilized Gram-negative cells. In a first step, PBD_{KZ}-GFP was immobilized on a flat C1 sensor chip by covalent amine coupling. Secondly, autoclaved *P. aeruginosa* cells were immobilized on this layer, relying on the unique peptidoglycan binding capacity of these proteins. Finally, free PBD_{KZ}-GFP proteins were applied, allowing observation of the interaction between the immobilized cells and PBD_{KZ}-GFP.

The best fit of the experimental sensorgrams for three different concentrations is a two-state model which describes a 1:1 binding of the PBD_{KZ}-GFP analyte to the immobilized ligand, followed by a conformational change in the complex. It is assumed that the conformationally changed complex can only dissociate through the reverse of the conformational change.

$$A + B \underset{k_{d1}}{\overset{k_{a1}}{\leftrightarrow}} AB \underset{k_{d2}}{\overset{k_{a2}}{\leftrightarrow}} AB^*$$

This model should be taken as an indication rather than direct evidence that a conformational change upon binding is taking