PEGylating a bacteriophage endolysin inhibits its bactericidal activity

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Abstract

Bacteriophage endolysins (lysins) bind to a cell wall substrate and cleave peptidoglycan, resulting in hypotonic lysis of the phage-infected bacteria. When purified lysins are added externally to Gram-positive bacteria they mediate rapid death by the same mechanism. For this reason, novel therapeutic strategies have been developed using such enzybiotics. However, like other proteins introduced into mammalian organisms, they are quickly cleared from systemic circulation. PEGylation has been used successfully to increase the in vivo half-life of many biological molecules and was therefore applied to Cpl-1, a lysin specific for S. pneumoniae. Cysteine-specific PEGylation with either PEG 10K or 40K was achieved on Cpl-1 mutants, each containing an additional cysteine residue at different locations. To the best of our knowledge, this is the first report of the PEGylation of bacteriophage lysin. Compared to the native enzyme, none of the PEGylated conjugates retained significant in vitro anti-pneumococcal lytic activity that would have justified further in vivo studies. Since the anti-microbial activity of the mutant enzymes used in this study was not affected by the introduction of the cysteine residue, our results implied that the presence of the PEG molecule was responsible for the inhibition. As most endolysins exhibit a similar modular structure, we believe that our work emphasizes the inability to improve the in vivo half-life of this class of enzybiotics using a cysteine-specific PEGylation strategy.

Keywords: Bacteriophage, S. pneumoniae, Cpl-1, PEGylation, Endolysin, Enzybiotic

Introduction

Streptococcus pneumoniae is the first cause of otitis media and a common cause of sinusitis, community-acquired pneumonia, bacteremia, and meningitis (Jacobs, 2004.). Antibiotic misuse and overuse has progressively selected for resistance against major drug classes, and treatment failures are widely reported (Fuller and Low, 2005.; Klugman, 2002.). This justifies the search for new drugs with different mechanisms of action. The bacteriolytic action of bacteriophage lysins enables the release of phage progeny from the bacterial sacculus. Purified pneumococcal phage lysin Cpl-1 has been used to successfully treat pneumococcal sepsis, endocarditis, meningitis, and pneumonia in rodent models (Entenza et al., 2005.; Grandgirard et al., 2008.; Loeffler et al., 2003.). However, due to its short circulating half-life (~20.5 minutes) (Loeffler et al., 2003.), optimal efficacy requires repeated injections or continuous infusion (Entenza et al., 2005.). We recently showed that pre-dimerization of Cpl-1, which doubles the molecular weight of the enzyme, decreased its plasma clearance by a factor of ten (Resch et al., 2011.). PEGylation (Veronese and Pasut, 2005,) was shown to extend even more so the serum half-life of interferon-α2b from minutes to hours (Ramon et al., 2005,) and of lysostaphin from 1 to 24 h (Walsh et al., 2003,). Here we mono-PEGylated (Gaberc-Porekar et al., 2008; Walsh et al., 2003) Cpl-1 at various cysteine residues and determined the anti-pneumococcal activity of the resulting conjugates.

Materials and methods

Reagents

Plasmid mini-prep kits were bought from Qiagen (Valencia, CA, USA). The QuickChange II Site-Directed Mutagenesis Kit was purchased from Stratagene (Cedar Creek, TX, USA). Mutagenic primers were obtained from Fischer Biotechnology (Pittsburgh, PA, USA) and DNA sequencing reactions were performed by Genewiz.
(South Plain, NJ, USA). DEAE-Sepharose, HiLoad 16/60 SuperdexTM 200 prep grade column, and PD-10 desalting columns were obtained from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, USA). Amicon Ultra centrifugal units Ultracel 30K were from Millipore (Car- rightwahill, Co. Cork, Ireland). Chemically competent Escherichia coli (E. coli) Max Efficiency DH5α cells and NuPAGE 4-12% Bis-Tris Gels were from Invitrogen (Carlsbad, CA, USA). Poly-ethylene glycol maleimide MW 10 kDa (PEG 10) and Y-shape poly-ethylene glycol maleimide MW 40 kDa (PEG 40) were purchased from Jenkem Technology (Allen, TX, USA). All other chemi- cals were from Sigma-Aldrich (Saint Louis, MO, USA).

Choosing PEGylation sites
In the present study, seven mutants previously described elsewhere as showing comparable antimicrobial activity to parent Cpl-1 were included (Resch et al., 2011). The mutants are as follows: Cpl-1C45S;D149C, Cpl-1C45S;D149C, Cpl-1C45S;G216C, Cpl-1C45S;D256C, Cpl-1C45S;N214C, Cpl-1C45S;S269C, and Cpl-1C45S;D194C (Table 1). A previous study of Cpl-1 mutants is available from the Genbank database with the accession number NC_001825.

Table 1 List of mutagenic primers used in site-directed mutagenesis experiments

| Cpl-1 mutant | Forward mutagenic primer | Reverse mutagenic primer |
|--------------|--------------------------|--------------------------|
| Cpl-1C45S    | 5'-CGA CCT ATT TAA ACC CTA GCT TGT CTC CTC AAG TGG AGC AGT CAA ACC C-3' | 5'-GGG TTT GAC TGG TCC ACT TGA GCA GAC AAG CTA GGG TTT AAA TAG GTC G-3' |
| Cpl-1C45S;D149C | 5'-GTT TCT TCA CAA CGT GCC GAC TAT GTG CTA TAA AAT CGA GTA CTG CG-3' | 5'-CGT AGT CCA ATA TAA CGTATT TAA CGC ACA TAG GCA CGT TGT CAA GGA AAA AC-3' |
| Cpl-1C45S;G216C | 5'-GTT AGA CGA TGA AGA AAG CTG CAA GCC AAA GAC CGC TGG A-3' | 5'-TCC AGC GGT CTT TGG CTT GCA GTC TCC TTC ATC GTC TAA C-3' |
| Cpl-1C45S;D256C | 5'-GGG TGG TGG TTC ATG CCA TGG AAT GGC AGT TTC TCA TAA A-3' | 5'-TAA GGA AAA CTG CCA TTG CAT CTT CAT CTA ACC CAC CC-3' |
| Cpl-1C45S;S269C | 5'-GTG TGT TGG TCC CAT AAG AAA CAA TGG CAT TTT CCC TT-3' | 5'-AAG GGA AAC TGC AAAT TGT TGG TGC TGA ACC ACC AC-3' |
| Cpl-1C45S;D194C | 5'-AAA TGG TAC TAC CTC AAG TGG TGC GGA GTA ATG GCC AC-3' | 5'-GTC GCC ATT GCG CGG TTG CAC TTG AAG TAG TCA CAT TT-3' |
| Cpl-1C45S;D256C | 5'-GTT GGG TGG TGC GGT AGT ATT TAA GGG AC-3' | 5'-GTC CAT ATA ATA CCA CTC GCA CCC GAC TAG CAC CCA AC-3' |
| Cpl-1C45S;D324C | 5'-ACA CAA ACG GAG AGC TTG CAT GCA TTC CAA GGT CTA CGA AAG-3' | 5'-CTT TGG TCA AAG TGG GTG TGG CAT GA CTC CTT TGG TGT-3' |

Mutated positions are underlined.

Production and purification of Cpl-1 mutants
The production and purification of all proteins followed a protocol that has already been described for Cpl-1 (Loeffler and Fischetti, 2003,) and Cpl-1 mutants (Resch et al., 2011). Briefly, E. coli DH5α cells were grown in Luria-Broth (LB) for 16 h aerobically at 37°C with agitation at 250 rpm. The cultures were diluted 10X (vol/vol) and allowed to grow for an additional 5 h in the same conditions. Protein expression was induced by the addition of 2% (w/v) lactose to the cultures. 16 h later, cells were pelleted, resuspended in phosphate buffer 50 mM, pH 7.4 (enzyme buffer), and sonicated on ice (three cycles of 30 sec at 70% power, Sonoplus, Bandelin Elec- tronics, Berlin, Germany). Cell debris was pelleted by centrifugation (1 h at 4°C and 15,000 rpm) and superna- tants were treated with 20 units (20 U) of DNase I for 16 h at 4°C. 0.45 μm filtered supernatants were applied to a DEAE-Sepharose fast flow column previously equili- brated with enzyme buffer. Following a wash step with enzyme buffer containing 1 M NaCl, the enzymes were eluted with enzyme buffer containing 10% (w/v) choline.
After extensive dialysis (cutoff 30,000 kDa) against enzyme buffer, the purified enzymes were concentrated using Ultracel 30K centrifugal filters and stored at -20°C.

**PEGylation of Cpl-1 mutants**

Purified mutant enzymes were reduced for 30 min at room temperature (RT) in enzyme buffer containing 10 mM dithiotreitol (DTT), and desalted on PD-10 columns previously equilibrated with enzyme buffer. Protein concentrations were adjusted to 1 mg/ml and either PEG maleimide MW 10,000 kDa (PEG 10K) or Y-shaped PEG Maleimide MW 40,000 kDa (PEG 40K) was added (1/25 and 1/10 mol protein/mol PEG for PEG 10K and 40K, respectively). After a 15 min. incubation period at RT with constant gentle agitation, the excess of unbound PEG was removed by applying the mixtures to a DEAE-Sepharose column previously equilibrated with enzyme buffer. PEGylated conjugates and residual fractions of non-PEGylated enzymes were eluted with enzyme buffer containing 10% (w/v) choline, and then purified by gel filtration on a HiLoad 16/60 Superdex™ 200 prep grade column pre-equilibrated in enzyme buffer. Fractions containing the purified PEGylated enzymes were pooled, concentrated using Ultracel 30K centrifugal filters and stored at -20°C until further use.

**In vitro killing assay**

The killing assay was performed using *S. pneumoniae* strain DCC1490 (serotype 14) obtained from A. Tomasz and has been described elsewhere (Loeffler and Fischetti, 2003; Loeffler et al., 2001). Briefly, DCC1490 was grown to log-phase in aerobic conditions without agitation (OD595 nm of 0.3) in brain heart infusion (BHI) at 37°C. After centrifugation and re-suspension of DCC1490 in enzyme buffer at a concentration of 10⁹ cfu/ml, serial dilutions of enzymes were added to the cells. Reaction kinetics were obtained by measuring the decrease of the OD595 nm at 37°C over a period of 28 min. in a EL808 microplates reader (Biotek Instruments Gmbh, Luzern, Switzerland).

**Results**

As previously reported (Resch et al., 2011), Cpl-1\(^{C45S;D194C}\) generated the expected 37 kDa band plus a 74 kDa band on non-reducing SDS-PAGE (Figure 1, lane 2). The 74 kDa band vanished upon reduction with 10 mM DTT (Figure 1, lane 3) and therefore corresponded to a dimer. Indeed, dimerization was likely due to cysteine cross-bridging, thus indirectly indicating that the de novo introduced cysteines were properly exposed. A similar migration pattern was observed with all mutants in this study (data not shown). The seven fully active mutants (Resch et al., 2011) were further PEGylated. Figure 1 depicts a representative PEGylation experiment with PEG 40K. As determined by ImageJ (Abramoff et al., 2004), a small fraction of enzyme (3-12%, depending on the mutant), was not PEGylated (Figure 1, lane 5 for Cpl-1\(^{C45S;D194C}\)). After gel filtration, fractions containing highly pure PEGylated conjugates were recovered (Figure 1, lane 9 and 10 for Cpl-1\(^{C45S;D194C}\)) and pooled. The seven PEGylated conjugates lost 100% of their activity in the *in vitro* killing assay (data not shown), suggesting that the bulky effect of the PEG 40K molecule drastically interfered with enzyme function.

We reasoned that smaller adducts would be less detrimental to the enzyme, and therefore repeated the experiments with PEG 10K. Figure 2 depicts a representative PEGylation experiment with PEG 10K. This PEGylation reaction was also incomplete with 15-20% of residual non-PEGylated enzyme remaining in the mixture (Figure 2, lane 2 for Cpl-1\(^{C45S;D194C}\)). Following gel filtration, fractions containing highly pure PEG 10K conjugates (Figure 2, lane 6, 7 and 8 for Cpl-1\(^{C45S;D194C}\)) were separated from fractions containing non-PEGylated enzymes (Figure 2, lane 11 and 12 for Cpl-1\(^{C45S;D194C}\)) and pooled. As for PEG 40K conjugates, none of the PEG 10K conjugates retained significant *in vitro* antimicrobial activity when tested in the *in vitro* killing assay (data not shown). The reduced electrophoretic migration of the PEG conjugates (ca.120 kDa instead of...
residues totally abrogated it. This might be related to the complex structure and mode of action of the enzyme, which makes it susceptible to bulky adducts. Cpl-1 has a C-terminal domain that mediates binding to choline in the cell wall for adequate positioning of the N-terminal catalytic domain to cleave its substrate (Diaz et al., 1990,; Perez-Dorado et al., 2007,). Optimal positioning may also depend on enzyme C-terminus dimerization and catalytic activation of the autolysin LytA, an important virulence factor in Streptococcus pneumoniae (Romero et al., 2007).

Susceptibility to PEG-related hindrance is supported by the fact that PEGylation on the hinge region (C194) inhibited activity, in spite of the fact that this region is independent of both the binding and active domains. Adding a bulky adduct to this location is thought to affect flexibility of the hinge and interfere with optimal orientation of the enzyme into the wall.

The present results do not preclude that PEGylation at other sites or with different types of PEG could possibly extend Cpl-1 half-life with less detrimental effect on its bactericidal activity. However, we believe that this work highlights the fact that cysteine-specific PEGylation could be unsuitable for a large set of enzymatics with a similar architecture.

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Competing interests
The authors declare that they have no competing interests.

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Figure 2 Non-reducing SDS-PAGE of Cpl-1C45S,D194C PEGylated with PEG 10K. Protein ladder (lanes 1 and 3); Cpl-1C45S,D194C PEGylated with PEG 10K and purified on a DEAE-sepharose column (lane 2); further purification of Cpl-1C45S,D194C PEGylated with PEG 10K on a HiLoad 16/60 Superdex column (lane 4 to 12). Fractions 6, 7, and 8 were pooled and further used in the in vitro killing assay. Residual non-PEGylated Cpl-1C45S,D194C is shown (lane 11 and 12).
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