Carbohydrate binding of *Salmonella* phage P22 tailspike protein and its role during host cell infection

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Abstract

TSPs (tailspike proteins) are essential infection organelles of bacteriophage P22. Upon infection, P22TSP binds to and cleaves the O-antigen moiety of the LPS (lipopolysaccharide) of its *Salmonella* host. To elucidate the role of TSP during infection, we have studied binding to oligosaccharides and polysaccharides of *Salmonella enterica* Typhimurium and Enteritidis *in vitro*. P22TSP is a trimeric β-helical protein with a carbohydrate-binding site on each subunit. Octasaccharide O-antigen fragments bind to P22TSP with micromolar dissociation constants. Moreover, P22TSP is an endorhamnosidase and cleaves the host O-antigen. Catalytic residues lie at the periphery of the high-affinity binding site, which enables unproductive binding modes, resulting in slow hydrolysis. However, the role of this hydrolysis function during infection remains unclear. Binding of polysaccharide to P22TSP is of high avidity with slow dissociation rates when compared with oligosaccharides. *In vivo*, the infection of *Salmonella* with phage P22 can be completely inhibited by the addition of LPS, indicating that binding of phage to its host via TSP is an essential step for infection.

Introduction

The outer membrane of Gram-negative bacteria builds an effective barrier against host immune systems and macromolecules such as antibiotics [1]. Protection mainly comes from LPS (lipopolysaccharide) located at the outermost membrane leaflet. Bacteriophages, however, can overcome this obstacle without destroying the cell. Upon infection, these fascinating molecular machines recognize specific receptors on bacterial surfaces that subsequently trigger DNA ejection from the phage. Although some tailed phages such as T4 or T7 bind to core saccharides of LPS before contacting a protein receptor for DNA release [2], others are restricted to the hosts of which they specifically recognize the O-antigen, i.e. the *Salmonella* phages P22, ε15 or ε34 [3]. These phages also have glycanase activity and shorten the length of the O-antigen during infection. The purpose of O-antigen hydrolysis during the infection process so far remains obscure and different functions have been discussed. Enzymatic cleavage of polysaccharide might facilitate access to the membrane and to a secondary receptor [3,4]. Also, phages hydrolysing their receptor would be able to dissociate and rebind and could thus move on the cell surface to find a good position for infection [5]. Additionally, it was proposed that the hydrolysis function could be important for release of newly synthesized phages upon cell lysis from cell debris, such as in influenza virus [6]. We have intensively studied the TSP (tailspike protein) of *Salmonella* phage P22, a double-stranded DNA phage with a short, non-contractile tail, and its role in the infection process. Up to six TSPs are attached to the phage tail and recognize the host cell O-antigen [7]. However, so far it remains unknown how the carbohydrate binding event leads to DNA ejection into the host. Our results indicate that a multivalent lectin-like fixation of phage on the cell surface via TSP is an essential step during the infection process.

In *vitro* oligosaccharide-binding studies with P22TSP

The TSP of bacteriophage P22 recognizes and cleaves the O-antigen moiety of the LPS of *Salmonella enterica* spp. [8]. One O-antigen repeat unit has the composition α-D-Galp-(1-4)α-D-Manp-(1-4)α-L-Rhap-(1-3) and varies in the substitution of the mannose with a dideoxyhexose substituent in different serotypes [9]. P22TSP is a trimer composed of three right-handed β-helices oligomerized via a C-terminal trimerization domain (Figure 1A). It has endorhamnosidase activity and cleaves the glycosidic linkage of the rhamnopyranoside producing octasaccharides of two repeat units [10,11]. Two aspartic acid residues and one glutamic acid residue make up the active site, and mutants have a strongly reduced enzymatic activity [8]. The octasaccharide-binding site is located on the solvent-exposed groove formed by the β-helix [12] (Figure 1B). Dideoxyhexose substituents of three different serotypes are recognized and enable phage activity on an extended...
host range. Interaction of P22TSP with oligosaccharides of defined composition was intensively studied using a protein fluorescence quench upon binding or isothermal titration calorimetry [8,13]. It was shown that a minimum of two repeat units, corresponding to an octasaccharide, are required for high-affinity binding and a free enthalpy yield of approx.
30 kJ/mol per binding site. Oligosaccharides of three repeat units bind with similar affinities, indicating that no additional high-affinity binding sites were present. A large negative binding enthalpy upon binding was observed, which strongly depended on temperature, together with a large heat-capacity change. Given good enthalpy–entropy compensation, it was concluded that binding to P22TSP is enthalpically driven due to hydrophobic interactions between sugar and protein. Stopped-flow measurements showed the binding equilibrium to be highly dynamic. By contrast, hydrolysis rates of fluorescently labelled dodecasaccharides were also measured and were small compared with the dissociation rates [8]. For cleavage of a dodecasaccharide at 10°C, $k_{\text{cat}}$ is 0.01 s$^{-1}$, indicating that, even at physiological temperatures, hydrolysis is slow compared with oligosaccharide binding. This is substantiated by the special architecture of the active site where the catalytic residues lie at the end of a high-affinity binding groove (Figure 1B). Therefore the smallest hydrolysis products obtained of polysaccharide digests with P22TSP are octasaccharides corresponding to two repeat units. They cannot be cleaved further, due to a lack of high-affinity binding sites beneath the active-site residues. Moreover, from this architecture an unproductive binding mode for hydrolysis products results in a reduction of polysaccharide hydrolysis. Hydrolysis products that are multiples of O-antigen repeats have also been found for other phages, i.e. coliphage Ω8 [14] or Shigella phage Sf6 [15], indicating that this is a particular feature of phage glycanases active on O-antigen. These findings illustrate that P22TSP counterbalances two features in its O-antigen binding site, a lectin-like carbohydrate-binding function and a glycosidase activity. As discussed below, both are essential for the phage infection process.

In vitro polysaccharide-binding studies with P22TSP
The natural receptor of bacteriophage P22 is the polysaccharide moiety of the LPS of the *Salmonella* host [3]. However, polysaccharides are a polydisperse mixture of different chain lengths, which prevents quantification of molar binding affinities. When polysaccharide purified from *S. enterica* Typhimurium was added to P22TSP, tryptophan fluorescence was quenched, analogous to oligosaccharide binding [8] (Figure 2A). To avoid polysaccharide cleavage, the active-site mutant P22TSP D392N was used, which binds octasaccharides with similar affinities to the wild-type but has a 1/30,000 reduced turnover rate constant for cleavage [8]. We measured fluorescence kinetics of polysaccharide
Figure 2 | Binding of P22TSP to polysaccharide

The preparation of O-antigen polysaccharide has been described previously [9]. Enzymatically inactive mutant P22TSP D392N [8] was incubated with polysaccharide of S. enterica Typhimurium. (A) Protein fluorescence spectra (λ_{ex} = 295 nm) of 0.17 μM P22TSP D392N with 0.06 mg/ml polysaccharide (broken line) or without polysaccharide (continuous line). (B) Kinetic trace of change in intrinsic tryptophan fluorescence of P22TSP D392N (k_{em} = 350 nm) at 10°C (open circles). Relaxation to binding equilibrium could best be described with a biexponential model with k_{1} = 0.262 ± 0.017 s^{-1} and k_{2} = 0.013 ± 0.001 s^{-1} (solid line). (C) Apparent rate constants k_{app} were determined at different polysaccharide (PS) concentrations as illustrated in (B). The determination of the dissociation rate constant from a linear plot of k_{app} against polysaccharide (PS) concentration has been described [13]. Accordingly, the ordinate intercept of the linear regression yields a k_{diss} of 0.081 ± 0.006 s^{-1}. Error bars show the S.D. of three independent experiments for each k_{app}.

binding to P22TSP at different concentrations with manual mixing to quantify polysaccharide affinity of P22TSP (Figure 2B). Relaxation to binding equilibrium takes place in a time scale of approx. 2 min. The data was fitted to a biexponential model. The fast phase yielded apparent rate constants, k_{app}, for the initial binding event, whereas the much slower phase was independent of polysaccharide concentration. Apparent rate constants, k_{app}, were determined at different concentrations of polysaccharide. From this, a dissociation rate constant, k_{diss}, for polysaccharide of 0.08 s^{-1} was calculated (Figure 2C), which indicates strong binding. The dissociation rate constant for an octasaccharide of S. enterica Enteritidis was determined previously in stopped-flow experiments to be 0.25 s^{-1} [13]. This means that the oligosaccharide dissociates approx. 3-fold faster than the polysaccharide. The P22TSP trimer has three identical independent binding sites with affinity for octasaccharide O-antigen repeats. Polysaccharide can hence be regarded as a multivalent ligand which binds to P22TSP with high avidity. Moreover, only the high-affinity binding sites add to P22TSP avidity. If further low-affinity carbohydrate-binding sites were present in P22TSP, the difference of dissociation rates between polysaccharide and octasaccharide would be more pronounced.

Role of polysaccharide during P22 phage infection in vivo

Different roles of P22TSP during the infection could be imagined from our binding experiments. Unambiguously, phages need P22TSP to recognize their hosts by detecting the correct serotype. Moreover, via the O-antigen, phage P22TSP might sense the presence of prophages. These cause serotype conversions, i.e. glucosylations or acetylations of O-antigens, so that TSPs no longer can bind to or cleave and that result in an effective infection barrier [16,17]. Moreover, it is reasonable that phages have to be fixed and positioned on the cell surface in order to inject their DNA properly. To show that strong binding is a prerequisite for infection, we incubated P22 phages with LPS for different times before infection of S. enterica Typhimurium (Figure 3). After 90 s of LPS incubation, infectivity decreased and was only half of the initial value. This time scale is in good agreement with the kinetics of polysaccharide binding to P22TSP as described above. After 100 min, no more infective phage could be detected. Hence the polysaccharide moieties of LPS have bound to the phages and blocked the binding sites on the TSP so that it cannot position on the host cell membrane for infection. However, we cannot exclude additional interactions of LPS core sugars or lipid A moiety with the phages. Our results clearly indicate that for proper infection, phage P22 has to bind the Salmonella host O-antigen with its TSP. For this they recognize stretches of two repeating units on the polysaccharide. Each phage has up to six TSPs [7] and hence can bind to the host cell membrane with high avidity. Still, more information is needed to draw a complete picture of the infection process, especially about the role of the endorhamnosidase activity. It might on the one hand help the phage to get closer to the membrane to sense a secondary receptor. On the other hand, enzymatic cleavage of LPS would be advantageous when newly synthesized phages have to dissociate from LPS on cell debris after lysis of the host bacterium [5]. TSP is able to cleave O-antigen on LPS, so why would phages not simply release themselves
Figure 3 | Inhibition of phage P22 in vivo plaque forming by LPS

The plaque-forming assay and the preparation of LPS have been described previously [9,18]. P22 phages (8 × 10⁴) were incubated with 2.5 μg/ml purified S. enterica Typhimurium LPS (●) or buffer (Δ) for different time periods before plating on S. enterica Typhimurium. Plaques were counted after overnight incubation at 37°C.

from LPS and subsequently infect Salmonella, in contrast with our observations in the plaque-forming assay where phages are inactivated? Experiments with oligosaccharides as described above showed that binding equilibriums are fast compared with hydrolysis. Hence, one might speculate that after fixation of phage on the LPS membrane via O-antigen binding, an additional event takes place that would be able to start DNA release. However, new experimental setups are needed to detect this. In the future, these might help to explain how carbohydrate recognition events direct the following steps of the infection mechanism.

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