Interactions of Phage P22 Tails with Their Cellular Receptor, *Salmonella* O-Antigen Polysaccharide*

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ABSTRACT Bacteriophage P22 binds to its cell surface receptor, the repetitive O-antigen structure in *Salmonella* lipopolysaccharide, by its six homotrimeric tailspikes. Receptor binding by soluble tailspikes and the receptor-inactivating endorhamnosidase activity of the tailspike protein were studied using octa- and dodecasaccharides comprising two and three O-antigen repeats of *Salmonella enteritidis* and *Salmonella typhimurium* lipopolysaccharides. Wild-type tailspike protein and three mutants (D392N, D395N, and E359Q) with defective endorhamnosidase activity were used. Oligosaccharide binding to all three subunits, measured by a tryptophan fluorescence quench or by fluorescence depolarization of a coumarin label attached to the reducing end of the dodecasaccharide, occurs independently. At 10°C, the binding affinities of all four proteins to oligosaccharides from both bacterial strains are identical within experimental error, and the binding constants for octa- and dodecasaccharides are $1 \times 10^6\ M^{-1}$ and $2 \times 10^6\ M^{-1}$, proving that two O-antigen repeats are sufficient for lipopolysaccharide recognition by the tailspike. Equilibration with the oligosaccharides occurs rapidly, but the endorhamnosidase produces only one cleavage every 100 s at 10°C or about 2 min^{-1} at the bacterial growth temperature. Thus, movement of virions in the lipopolysaccharide layer before DNA injection may involve the release and rebinding of individual tailspikes rather than hydrolysis of the O-antigen.

INTRODUCTION

The first step of a virus infection is the binding of the viral particle to the surface of its host cell. Being an extracellular reaction, the attachment represents an ideal target for agents designed to interfere with the infection (Lentz, 1990). However, very few structural and biophysical data on the interactions between viral adhesins and their host cell receptors are available. The major reason is that both reaction partners, the adhesin as a viral structural or envelope protein and the cellular receptor, a membrane protein, glycoprotein, or glycolipid, are usually insoluble and thus are hardly accessible to solution biophysical methods.

One exception is the attachment apparatus of bacteriophage P22 (Israel et al., 1967). P22 belongs to a large group of phages which, like many animal viruses, use surface carbohydrates as receptors. Its adhesin is the 215-kDa, homotrimeric tailspike protein. Up to six tailspikes are attached at one vertex of the P22 capsid (Fig. 1), but three spikes may be sufficient to form an infectious particle (Israel, 1978). The P22 tailspike recognizes the repetitive O-antigen in the outer membrane lipopolysaccharide of pathogenic *Salmonella* species belonging to serogroups A, B, and D_1. Their O-antigens have the main-chain trisaccharide repeating unit α-D-mannose-(1→4)-α-L-rhamnose-(1→3)-α-D-galactose-(1→4) in common, but differ in the dideoxyhexose substituent at C-3 of the mannose (Fig. 1).

The P22 tailspike possesses endoglycosidase activity, hydrolyzing the O-antigen polysaccharide at the Rha–Gal α(1→3)-glycosidic linkages and producing dimers of the repeating unit as the main product. Receptor-inactivating enzymatic activities are common to polysaccharide-specific bacteriophages (Lindberg, 1977) and have been found in many carbohydrate-specific animal viruses, like influenza, paramyxo, and corona viruses. The O-antigen polysaccharide forms a carbohydrate layer extending about 100 nm from the enterobacterial cell wall. Infection of *Salmonella* by phage P22 is thought to require not only penetration of the polysaccharide layer, but also a surface walk of the phage particle to reach a site suitable for injection of the phage DNA and accompanying proteins. The tailspike endorhamnosidase activity has been implicated in both processes (Lindberg, 1977; Bayer et al., 1980).

During normal infection, some excess tailspike accumulates as a soluble protein, and soluble tailspikes can be purified in large amounts from *Salmonella* infected by mutant P22 defective in capsid assembly and from *Escherichia coli* carrying a recombinant plasmid coding for the tailspike. P22 tailspike protein lacking its amino-terminal 108-residue head-binding domain is fully functional in carbohydrate binding and hydrolysis (Danner et al., 1993). We have recently succeeded in crystallizing this N-terminally shortened tailspike protein, and its x-ray structure has been determined at 2.0 Å resolution (Steinbacher et al., 1994).

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*This paper is dedicated to Prof. Serge N. Timasheff on the occasion of his 70th birthday.

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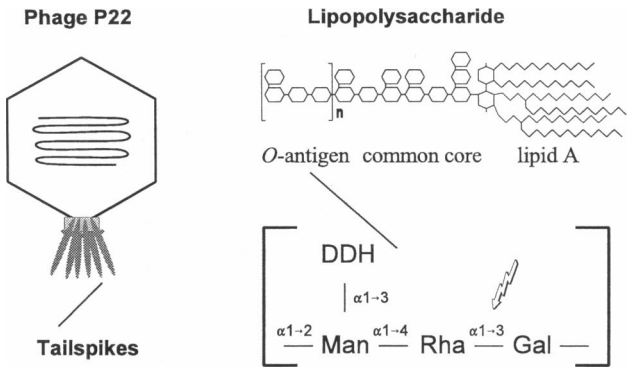

FIGURE 1 Bacteriophage P22 recognizes lipopolysaccharide, its receptor at the surface of the bacterial host cell, with its tailspikes. The phage head binds six homotrimeric tailspikes, which are also accessible as soluble proteins (cf. Fig. 2). Associated with the tailspike protein is a receptor-inactivating enzymatic activity, which cleaves the repetitive *O*-antigen polysaccharide, the outer part of the lipopolysaccharide. The *O*-antigens of *Salmonella* strains susceptible to phage P22 contain around 20 repeats of a common main-chain trisaccharide unit consisting of D-mannose, L-rhamnose (6-deoxymannose), and D-galactose. They differ in the dideoxyhexose (DDH) substituent at C-3 of the mannose, which is abequose (3,6-dideoxygalactose) in *S. typhimurium*, tyvelose (3,6-dideoxymannose) in *S. enteritidis*, and *S. typhi* and paratose (3,6-dideoxyglucose) in *S. paratyphi*. In addition, the *S. typhi O*-antigen carries a glucose substituent at C-4 of the galactose that does not interfere with receptor binding and inactivation by the P22 tailspike. The cleavage site of the endorhamnosidase activity is indicated by the jagged arrow.

The main part of a tailspike subunit is formed by a large right-handed parallel β-helix of 13 complete turns, into which a 63-residue domain of largely irregular structure is inserted between the third and fourth helical turns (Fig. 2). Beyond the β-helices, the carboxy-terminal parts of the three tailspike subunits are highly interdigitated, explaining the thermal stability of the tailspike trimer and its unusual folding pathway with a rate-determining folding reaction at the trimer level (Goldenberg and King, 1982; Seckler et al., 1989; Fuchs et al., 1991).

The cross section of the β-helix is approximately triangular, and a long groove is formed on its solvent-exposed face between the inserted irregular domain and three longer loops contained in turns 5, 7, and 8 of the β-helix. It is in this groove that the polysaccharide binding site of the tailspike adhesin has been identified by x-ray crystallography of tailspike complexes with octasaccharides prepared from *S. enteritidis* and *S. typhimurium* (Steinbacher et al., manuscript submitted for publication). The purpose of the present paper is a biophysical characterization of the interactions between such lipopolysaccharide fragments of defined length and the P22 tailspike endorhamnosidase. Using solution biophysical methods, we have measured binding affinities of native and of fluorescence-labeled octa- and dodecasaccharides from *S. enteritidis* and *S. typhimurium* to wild-type tailspike protein and to mutants with strongly reduced endorhamnosidase activity. We present a quantitative description of the enzymatic reaction catalyzed by the tailspike protein and investigate three active-site carboxylate residues suggested by the x-ray structure as critical in the enzymatic mechanism.

MATERIALS AND METHODS

Materials

The plasmid used for mutagenesis was a derivative of pASK30 with an insert coding for an amino-terminal methionine followed by residues 109 to 666 of P22 tailspike protein, i.e., for the tailspike protein lacking its amino-terminal head-binding domain (Danner et al., 1993). Lipopolysaccharide fragments from *Salmonella thyphimurium* SH4809, serogroup B, O-antigen 4,5,12; and *S. enteritidis* SH1262, serogroup D1, O-antigen 9,12 were purified as described (Eriksson et al., 1979). The strains were from the strain collection at the Division of Clinical Bacteriology, Huddinge University Hospital. The O5 antigen is lost during the process of oligosaccharide preparation. Oligosaccharides were characterized as described by Weintraub et al. (1988) and were quantified by their dry weights. Unless indicated otherwise, *S. enteritidis* oligosaccharides were used in the experiments. 7-Amino-4-methylcoumarin (Amc) and $NaCNBH_3$ were obtained from Aldrich (Steinheim, Germany), acetonitrile was from J. T. Baker (Deventer, England), and maltoheptaose was from Serva (Heidelberg, Germany). Other chemicals were analysis grade, and quartz bidistilled water was used throughout.

Site-directed mutagenesis, protein expression, and purification

Site-directed mutagenesis was carried out essentially as described by Kunkel et al. (1987), with slight variations (Yuckenberg et al., 1991). Expression and purification of amino-terminally shortened tailspike proteins were done as described for the wild-type protein (Steinbacher et al., 1994; Miller, 1995). Their concentrations were determined using a specific absorbance at 278 nm of $A_{1\ mg/ml} = 1.11$ (Danner et al., 1993). Purified protein was stored as a suspension in 40% saturated $(NH_4)_2SO_4$, 50 mM Tris/HCl, pH 8.0.

Labeling of oligosaccharides

Oligosaccharides were chemically labeled at their reducing ends with the fluorophore Amc in a reductive amination reaction, essentially as described by Prakash and Vijai (1983). Oligosaccharide (1 μmol) was dissolved in 500 μl methanol with 3 μmol Amc and 20 μl acetic acid and kept for 1 h at 37°C. After the addition of 5 μmol $NaCNBH_3$ in 500 μl methanol, the mixture was further incubated at 37°C for at least 15 h. The solution was dried in a vacuum centrifuge and redissolved in 50 μl acetonitrile/water (1:1 by volume). Labeled oligosaccharides were purified by reversed-phase high-performance liquid chromatography (HPLC) with an Alltech Econosil C_{18} column (1 × 25 cm, 10-μm particle size) at a flow rate of 2 ml/min. The weaker mobile phase (A) was 0.05% acetic acid, and the stronger mobile phase (B) was 70% (v/v) acetonitrile in 0.05% acetic acid. A gradient of 10% B to 30% B in 60 min was used, and the labeled oligosaccharides were detected by their absorbance at 350 nm. The result of the purification was checked by reversed-phase thin-layer chromatography on RP-18F_{254}s plates from Merck (Darmstadt, Germany). Samples containing labeled oligosaccharides were lyophilized and redissolved in water. The concentration was determined by UV absorbance using an extinction coefficient of 17,000 M^{-1} cm^{-1} at 360 nm (Kanaoka et al., 1985).

Endorhamnosidase assay

To start the enzyme reaction, tailspike (10 μg/ml) and labeled dodecasaccharide were mixed in a total volume of 100 to 400 μl of 60 mM phosphate buffer, pH 7.0. At varied times, 20-μl aliquots were removed and added to

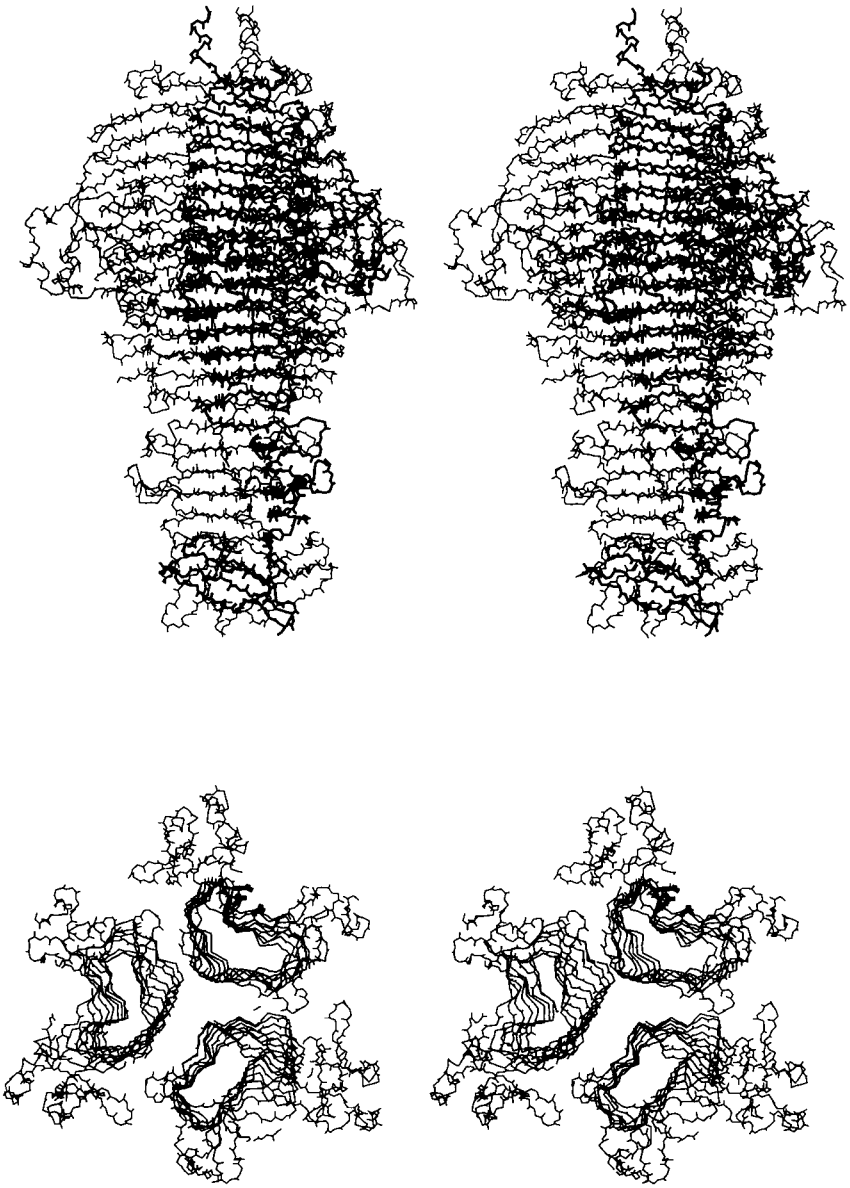

FIGURE 2 Crystal structure of the tailspike protein obtained after removal of the amino-terminal head-binding domain (Steinbacher et al., 1994; Brookhaven Protein Data Base entry 1TSP). A stereo diagram of the complete polypeptide backbone of all three subunits is shown in the top part of the figure. One of the subunits is traced in bold. Note the pronounced groove at the solvent-exposed face of the parallel β-helix. A cross section through the β-helices is shown in the lower part of the figure. The only side chains depicted are those of the three carboxylate residues mutated in the present study (from left to right: E359, D392, D395). They are indicated by bold lines. The tryptophan residues W365 and W391 involved in oligosaccharide binding are located on opposite walls of the groove.

5 μl 0.5 M HCl to stop the reaction. After 2 min, the samples were neutralized with 2.5 μl 0.5 M NaOH. The samples were analyzed by reversed-phase HPLC on a Beckmann ODS column (0.46 × 25 cm, particle size 5 μm) at a flow rate of 1 ml/min. The gradient used was 10% B to 30% B in 20 min, and the Amc label was detected with a Merck Hitachi F1000 fluorescence detector at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Dodecasaccharide and tetrasaccharide peaks were integrated using the program PeakFit (Jandel Scientific), and the ratio of the peak areas was used to calculate the amount of tetrasaccharide produced.

Oligosaccharide binding measured by fluorescence titrations

Protein fluorescence was measured in stirred cells (1.2 ml) employing a Spex Fluoromax spectrofluorimeter with a thermostatted cell holder. The excitation wavelength was 295 nm (spectral bandpass 2 nm) and emission was at 347 nm (bandpass 6 nm), corresponding to the maximum in the fluorescence difference spectrum between native tailspike protein and the tailspike-oligosaccharide complex. The protein concentration was 0.1–0.2 μM tailspike subunits in 60 mM phosphate buffer, pH 7.0; the oligosaccharide concentration was varied between 0.4 μM and 0.1 mM by the addition of 3-μl aliquots of oligosaccharide stock solution. After each addition, the sample was stirred for at least 1 min (no change was detected thereafter). The sample volume was not changed more than 5% over the titration, the protein fluorescence signal was corrected for the dilution, and the change of fluorescence due to absorption of oligosaccharide (inner filter effect) was less than 1%. Assuming one binding site per subunit and no cooperativity between the sites in the trimer, the dissociation constants for oligosaccharide binding were determined by nonlinear least-squares fitting of the data to the equation

$$F = F_0 + \Delta F(E_0 + L_0 + K_D - ((E_0 + L_0 + K_D)^2 - 4L_0E_0)^{1/2})/2E_0,$$

where F is the measured fluorescence, F_0 is the fluorescence of the free protein, ΔF is the change in fluorescence at saturation, E_0 and L_0 are the

total concentrations of protein and oligosaccharide, respectively, and K_D is the dissociation constant. Fits were done by the Marquardt-Levenberg algorithm, as implemented in SigmaPlot (Jandel Scientific).

Binding measurements by fluorescence depolarization

Fluorescence polarization was measured in a Perkin-Elmer MPF-3L spectrofluorimeter with a thermostatted cell holder and the polarization equipment provided by Perkin-Elmer. The excitation wavelength was 360 nm, and emission was recorded at 450 nm with 5-nm and 10-nm spectral bandpass, respectively. D392N mutant protein (3 μM subunits in 60 mM phosphate buffer, pH 7.0) was titrated with 3-μl aliquots of a DodAmc stock solution at 10°C (DodAmc, OctAmc, and TetAmc are *Salmonella* *O*-antigen oligosaccharides of 3, 2, and 1 repeating units labeled at their reducing ends with Amc). Fluorescence intensities with parallel and perpendicular orientation of the polarizers were measured at least 2 min after each addition.

The polarizations and fluorescence efficiencies of the free and the bound DodAmc p_f, Q_f, and p_b, Q_b were determined by extrapolation to infinite protein concentration (Dandliker et al., 1981), as illustrated in Fig. 5. The dissociation constant was determined by nonlinear least-squares fit of the data to the following system of equations resulting from the definition of K_D for ligand binding to independent identical sites, and from equation 8 of Dandliker et al. (1981)

$$L_b = (nE_0 + K_D + L_0 - ((nE_0 + K_D + L_0)^2 - 4nE_0L_0)^{1/2})/2$$

$$W = (Q_f/Q_b)(L_0 - L_b)/L_b)$$

$$p = (Wp_f + p_b)/(W + 1).$$

In these equations, L_b and L_0 are the bound and total concentrations of ligand, respectively; K_D is the dissociation constant; n is the number of independent identical binding sites; E_0 is the total protein concentration; and p is the measured polarization.

RESULTS

Previous biochemical assays for the enzymatic activity associated with bacteriophage tails relied on the liberation of radioactivity from labeled cell envelopes (Iwashita and Kanegasaki, 1976a; Svenson et al., 1979; Berget and Poteete, 1980) or on the determination of reducing ends produced by the glycosidic cleavage of partially purified *Salmonella* polysaccharide (Seckler et al., 1989; Danner et al., 1993). Because we found such assays to be unsuitable for a quantitative analysis of the enzymatic reaction catalyzed by the P22 tailspike protein and a characterization of the catalytic mechanism, we attempted to set up an endorhamnosidase assay based on a homogeneous substrate. The smallest substrate known to be efficiently cleaved by the enzyme, a dodecasaccharide fragment from *Salmonella* lipopolysaccharide purified by gel filtration chromatography (Weintraub et al., 1988), was labeled at its reducing end by reductive amination with the fluorescent dye Amc, and the labeled oligosaccharide was purified by reversed-phase HPLC. Upon incubation with purified P22 tailspike protein, the labeled dodecasaccharide was efficiently cleaved into labeled tetrasaccharide and unlabeled octasaccharide, as identified by reversed-phase thin-layer chromatography (data not shown).

Endorhamnosidase activity of the wild-type protein

For quantitative analysis of DodAmc cleavage, reversed-phase HPLC with fluorescence detection was used (Fig. 3). At a flow rate of 1 ml/min, the HPLC retention times of DodAmc, OctAmc, and TetAmc were 16.8 min, 17.5 min, and 18.1 min, respectively. Fig. 3 *A* shows the elution profiles of pure DodAmc and of a sample from a 15-min incubation at 10°C with purified wild-type protein. As the amino-terminal head-binding domain of the tailspike protein is dispensable for oligosaccharide binding and hydrolysis (Danner et al., 1993), wild-type and point mutant proteins used in this study lack the N-terminal 108 residues that interfere with the crystallographic analysis (Steinbacher et al., 1994). We have not detected any significant differences between full-length and N-terminally shortened proteins in the oligosaccharide hydrolysis and binding assays used. The HPLC peaks of DodAmc and TetAmc were very well separated and could be integrated independently. The fraction of TetAmc was calculated from the peak areas, and the rate of DodAmc hydrolysis was found to be nearly constant up to 10% total turnover (Fig. 3 *B*). Rates determined at increasing substrate concentrations increased in a hyperbolic fashion, and a fit of the data to the Michaelis-Menten equation yielded a K_M of 2.3 ± 0.3 μM and a turnover rate constant of k_{cat} = 0.01 ± 0.002 s^{-1} at 10°C (Fig. 3 *C*). To achieve substrate saturation, the DodAmc concentration was kept above 25 × K_D of the unlabeled dodecasaccharide measured at the respective temperature (cf. below). An Arrhenius plot of the rates measured under these conditions between 2°C and 20°C was close to linear and resulted in an activation energy of 35 ± 5 kJ/mol.

Hydrolytic activity of mutant proteins

Based on the x-ray structure of a *S. enteritidis* octasaccharide complexed to the wild-type protein (Steinbacher et al., manuscript submitted for publication), three candidate active-site carboxylates were selected for site-directed mutagenesis. These residues are Asp392, Asp395, and Glu359. They were replaced by the corresponding amides in the mutants D392N, D395N, and E359Q, respectively. Upon expression in *E. coli*, the mutant proteins folded efficiently and could be purified to homogeneity, as judged by gel electrophoresis with silver staining. None of the mutants showed detectable DodAmc hydrolysis at 10°C. At 37°C and a tailspike subunit concentration of 1.7 μM, significant turnover by the mutant proteins was detected after 1–4 days. Under these conditions, the wild-type protein hydrolyzes 10% of the DodAmc in less than 1 min. Because the mutants bind oligosaccharides with wild-type affinities (cf. below), their turnover numbers could be estimated directly from the observed TetAmc production. They are reduced to about 1/30,000 (D392N), 1/25,000 (D395N), and 1/10,000 (E359Q) of the wild-type rate constant, respectively.

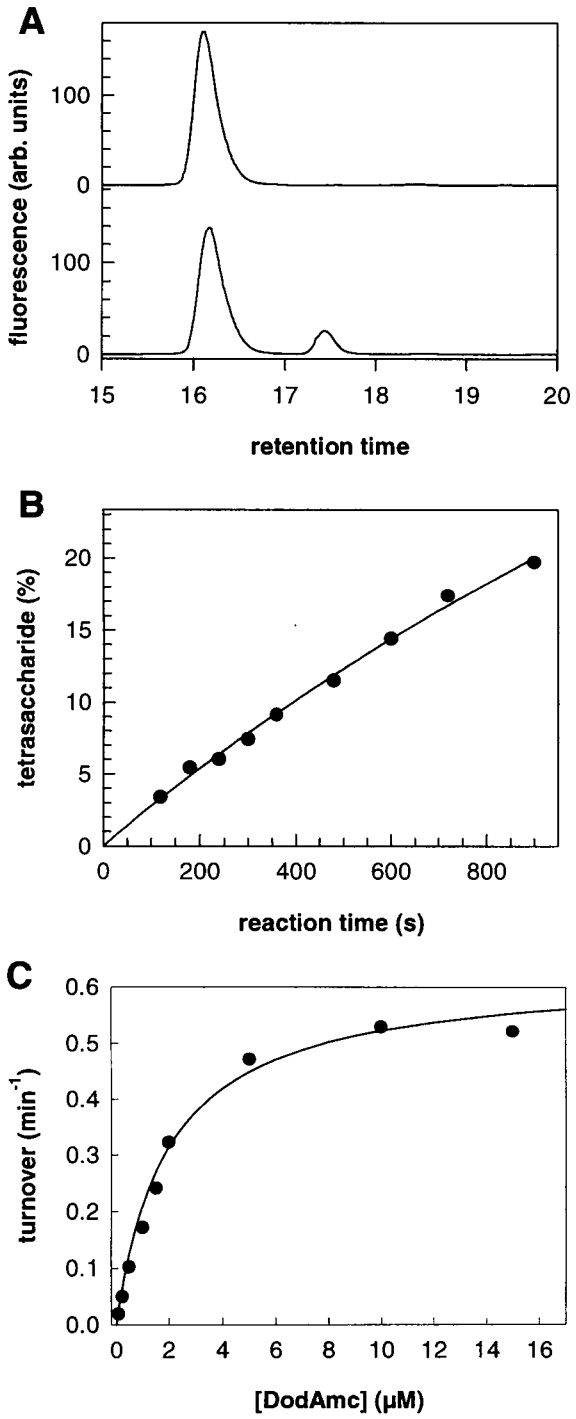

FIGURE 3 P22 tailspike endorhamnosidase activity measured with a defined substrate. (*A*) Reversed-phase HPLC elution profiles of purified DodAmc (15 μM, upper trace) and of a sample that was incubated with purified wild-type tailspike protein (170 nM subunits) for 15 min at 10°C. (*B*) Time course of DodAmc hydrolysis by wild-type tailspike protein (0.5 μM subunits) at 10°C. The substrate concentration was 15 μM; the solid line represents a simulation assuming rapid equilibration with substrate and product, and using turnover number, substrate K_M, and product dissociation constants determined in the present study. The simulation was done with KINSIM (Frieden, 1994). (*C*) Substrate concentration dependence of DodAmc turnover at 10°C. The solid line represents a nonlinear fit to the Michaelis-Menten equation, resulting in $K_M = 2.3$ μM and $k_{cat} = 0.01$ s^{-1}. The tailspike subunit concentration was 170 nM.

Oligosaccharide binding equilibria

Upon addition of purified *Salmonella O*-antigen fragments, the intrinsic fluorescence of the tailspike protein was significantly quenched and blue-shifted (Fig. 4 *A*). At saturation, the reduction in fluorescence intensity was 19 ± 1% for the wild-type and between 13% and 22% for the mutant proteins (Table 1). The fluorescence quench can be rationalized by two out of seven tryptophans of each tailspike subunit being located in the oligosaccharide binding site (Steinbacher et al., manuscript submitted for publication). Both tryptophans (residues 365 and 391) are highly solvent-exposed in the structure of the unliganded protein, and more exposed than any of the other tailspike tryptophans. Thus, quenching of emission from W365 and W391 by the bound oligosaccharide is expected to result in a blue shift concomitant with a reduction in intensity. The observed spectral change allowed us to determine the equilibrium constants for the binding of a number of oligosaccharides to wild-type and mutant tailspike proteins by fluorescence titrations. Example titrations for the wild-type protein with octasaccharides from *S. typhimurium* and *S. enteritidis* are presented in Fig. 4 *B*, together with a maltoheptaose control. As evident from the results of nonlinear regression represented by the solid lines, the titrations were well described by hyperbolic binding to a single class of binding sites. The dissociation constants for both octasaccharides ($K_D = 0.9 \pm 0.1$ μM; cf. Table 1) were identical within experimental error.

The binding affinities to *Salmonella O*-antigen octa- and dodecasaccharide fragments, i.e., to product and substrate of the endorhamnosidase reaction, were measured for the mutant proteins E359Q, D392N, and D395N, which did not hydrolyze significant amounts of dodecasaccharide during the fluorescence titrations. All three mutants bound octasaccharides with wild-type affinity (Table 1), whereas the dissociation constants of octasaccharide complexes with four other point mutants (E359G, W391G, V331A, V331G, data not shown) were 10–100-fold higher. Binding constants for the dodecasaccharides were twofold higher than those of the octasaccharides. Again, oligosaccharides from the two *Salmonella* species bound with equal affinity, and binding by the three mutants was not significantly different.

The temperature dependence of the equilibria was characterized for octa- and dodecasaccharide binding to D392N, and for octasaccharide binding to the wild-type protein (Fig. 4 *C*). Upwardly curved van't Hoff plots were observed in all three cases, indicating a large negative heat capacity change upon binding. The van't Hoff enthalpies for the binding of octa- and dodecasaccharide to D392N at 25°C ($\Delta H_{vH,oct} \approx -68 \pm 1$ kJ/mol and $\Delta H_{vH,dod} \approx -73 \pm 3$ kJ/mol, averages of results obtained in two independent series of binding experiments) were very similar but significantly larger than the value observed for the binding of octasaccharide to wild-type tailspikes ($\Delta H_{vH} \approx -49 \pm 2$ kJ/mol).

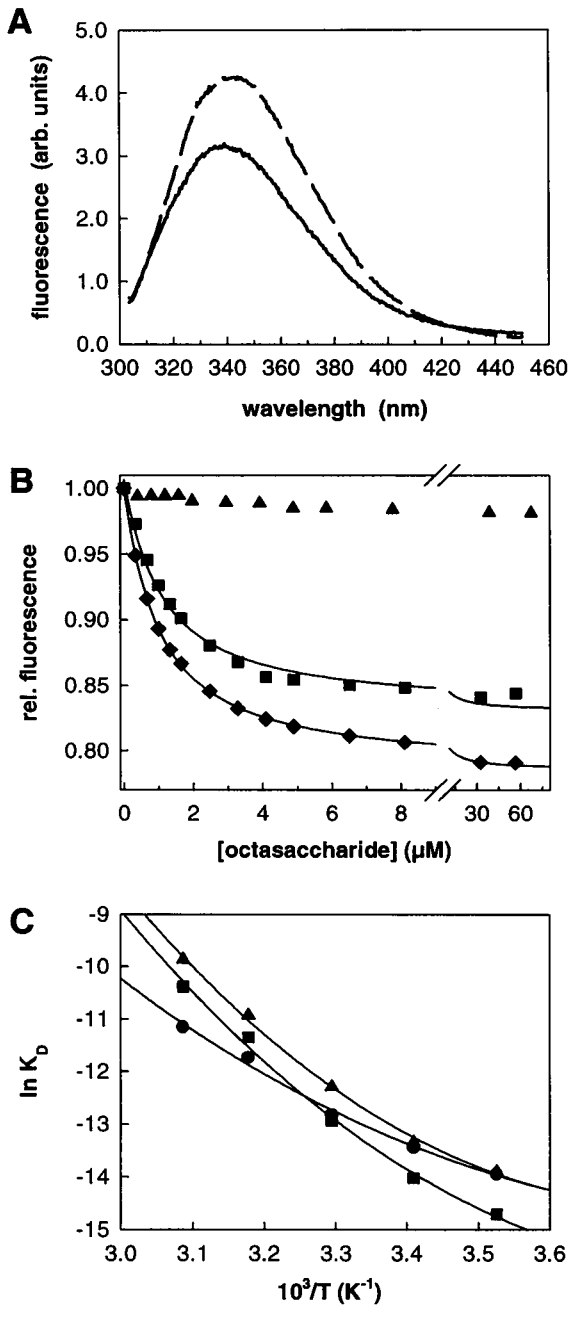

FIGURE 4 Oligosaccharide binding results in a tryptophan fluorescence quench and a blue shift of the emission maximum. (*A*) Fluorescence emission spectra of native tailspike protein (170 nM subunits) in the absence (– – –) and presence (——) of 100 μM octasaccharide from *Salmonella enteritidis O*-antigen. (*B*) Binding titrations of wild-type tailspike with octasaccharides from *S. enteritidis* (■) and *S. typhimurium* (◆) and with maltoheptaose as a control. The solid lines are nonlinear fits assuming independent binding to each subunit in the trimer and resulting in dissociation constants of 0.95 μM and 0.88 μM, respectively. (*C*) Temperature dependence of oligosaccharide binding. Van't Hoff plots of dissociation constants for complexes of wild-type (●) and D392N mutant (▲,■) tailspikes with octasaccharide (●,▲) and dodecasaccharide (■) from *S. enteritidis*. Solid lines represent nonlinear least-squares fits to the equation $\ln K_D = (\Delta H_{bind,25°C} + \Delta C_p (T - 298.3) - T\Delta S_{bind,25°C} + \Delta C_p \ln(T/298.3))/RT$. The fit parameters for ●, ▲, and ■ were −50, −69, and −76 kJ mol^{-1} for $\Delta H_{bind,25°C}$, −57, −124, and −143 J mol^{-1}K^{-1} for $\Delta S_{bind,25°C}$, and −1.05, −2.0, and −1.79 kJ mol^{-1}K^{-1} for ΔC_p.

Binding of the assay substrate and oligosaccharide binding kinetics

As evident from the data presented above, the largely inactive mutants D392N, D395N, and E359Q bound oligosaccharides with identical affinities, and their binding affinities for the octasaccharides were not different from that of the wild-type protein. Thus, the common dissociation constant of their dodecasaccharide complexes (0.4 ± 0.1 μM) appeared likely to reflect the binding affinity of the wild-type endorhamnosidase for the unlabeled substrate. On the other hand, the Michaelis constant of the enzyme for DodAmc was more than fivefold higher at the same temperature. This difference might reflect 1) slow substrate dissociation, 2) a higher dodecasaccharide binding affinity of the mutants, or 3) interference of the fluorescence label with binding. The results of three types of experiments prove the latter to be correct.

First, we attempted to measure the dissociation kinetics of a preformed oligosaccharide-protein complex. When a solution of D392N tailspikes (2 μM subunits), upon equilibration with 5 μM dodecasaccharide (>90% saturation), was diluted 100-fold into a stirred cell at 10°C, no change in fluorescence intensity was observed after the dead time of manual mixing (~5 s), and the fluorescence signal (λ_{ex} = 295 nm, λ_{em} = 347 nm) was that expected for <10% saturation. From this result, lower limits for the dissociation and association rate constants were estimated to $k_{off} = 0.3$ s^{-1} and $k_{on} = 4 \times 10^5$ M^{-1} s^{-1}.

Second, we tried to estimate the binding constant for unlabeled dodecasaccharide to wild-type tailspike protein. Because substrate binding was found to be fast compared to the mixing time and much faster than substrate turnover (k_{cat} = 0.01 s^{-1}), the fluorescence quench upon binding could be determined by adding dodecasaccharide at varying concentrations to samples of wild-type protein and extrapolating the observed fluorescence intensity to the time of mixing. The dissociation constant of the dodecasaccharide complex with wild-type protein so determined was 0.5 ± 0.2 μM.

Third, the binding of DodAmc to D392N mutant protein was measured by fluorescence depolarization (Fig. 5). Quenching of protein fluorescence could not be used for the labeled substrate, because of the high absorbance of the label at the wavelengths of tryptophan absorbance and emission. Upon protein addition, the polarization of the label fluorescence increased from 0.05 to 0.30 at saturation, as determined by extrapolation to infinite protein concentration (Dandliker et al., 1981). Concomitantly, the fluorescence intensity of the label decreased by 10%. A binding curve measured by varying the DodAmc concentration is depicted in Fig. 5. Nonlinear regression resulted in 0.9 DodAmc binding sites per tailspike subunit and a dissociation constant of 2.0 ± 0.3 μM, in agreement with the observed K_M of 2.3 μM.

TABLE 1 Oligosaccharide binding affinities as measured by fluorescence quenching

	Octasaccharide (*S. enteritidis*)			Dodecasaccharide (*S. enteritidis*)		
Mutant	$\Delta F/F_0$ (%)	K_D (μM)	ΔG_b° (kJ/mol)	$\Delta F/F_0$ (%)	K_D (μM)	ΔG_b° (kJ/mol)
wt	19.2 ± 0.8	0.87 ± 0.27	−32.8 ± 1.0	—	—	—
D392N	21.6 ± 0.5	1.05 ± 0.19	−32.4 ± 1.0	20.9 ± 0.3	0.42 ± 0.05	−34.6 ± 0.3
D395N	17.9 ± 0.3	0.83 ± 0.10	−32.9 ± 1.0	17.4 ± 0.2	0.37 ± 0.05	−34.9 ± 0.3
E359Q	13.4 ± 0.2	0.87 ± 0.10	−32.8 ± 1.0	13.7 ± 0.1	0.38 ± 0.04	−34.8 ± 0.3

Titrations of tailspike proteins with oligosaccharides were done at 10°C as described in Materials and Methods. $\Delta F/F_0$ is the fractional decrease in tryptophan fluorescence intensity upon complex formation, K_D is the dissociation constant, and $\Delta G_b^0 = RT \ln K_D$ is the standard free energy of binding.

DISCUSSION

The interactions of the tailspike protein with *Salmonella O*-antigen oligosaccharides at around 10°C can be summarized in a simple scheme:

$$\begin{array}{ccc} \text{Dodecasaccharide} \cdot \text{TSP} & \xrightarrow{k_{cat} \approx 0.01\ s^{-1}} & \text{Octasaccharide} \cdot \text{TSP} \\ \text{fast} \downarrow\uparrow K_D \approx 10^{-6}\ \text{M} & & + \text{Tetrasaccharide} \\ \text{Dodecasaccharide} + \text{TSP} & & \text{fast} \downarrow\uparrow K_D \approx 10^{-6}\ \text{M} \\ & & \text{Octasaccharide} + \text{TSP} \end{array}$$

This model is based on results obtained with wild-type tailspike protein and with three mutant proteins that bind oligosaccharides with wild-type affinity but do not hydrolyze significant amounts over the time course of a binding titration. The binding equilibria for substrate and product are fast compared to the rate of hydrolysis. Equilibration with free oligosaccharide is observed in the dead time of manual mixing, and the K_M for the labeled dodecasaccharide DodAmc in the endorhamnosidase reaction catalyzed by the wild type closely coincides with its K_D determined by fluorescence depolarization with the D392N mutant. The general scheme also holds at the physiological temperature of around 35°C, although both dissociation constants and the catalytic rate are three- to fivefold higher.

As the binding constants and their temperature dependence are similar for octa- and dodecasaccharides, the additional *O*-antigen repeating unit in the dodecasaccharide appears to contribute little to the interactions. This is consistent with the observation that the binding free energy of DodAmc is reduced by less than 1 kcal/mol, although the pyranose ring at the reducing end of the dodecasaccharide is broken and a bulky fluorescence label is added by the chemical modification. Oligosaccharides from *S. enteritidis* and *S. typhimurium*, in which the side-chain dideoxyhexoses are tyvelose (3,6-dideoxymannose) and abequose (3,6-dideoxygalactose), bind to the tailspike with essentially identical affinities, in agreement with the two *Salmonella* species being equally susceptible to infection by bacteriophage P22 (Eriksson et al., 1979). The temperature dependence of the equilibrium constants indicates a large negative heat capacity change upon oligosaccharide binding. Compared to literature values for the binding of monosacccharides to proteins, this value appears unusual, but large heat capacity changes on the order observed here have been reported for the binding of oligosaccharides to antibodies (Zidovetzki et al., 1988; Sigurskjold and Bundle, 1992). A negative heat capacity change is usually taken to indicate a significant reduction of exposed hydrophobic surface area. Accordingly, the large heat capacity change observed for the tailspike-oligosaccharide interaction (1–2 kJ $mol^{-1}K^{-1}$; compare Spolar and Record, 1994) is compatible with most, if not all, of the sugar residues interacting closely with the tailspike protein in the complex with the octasaccharide

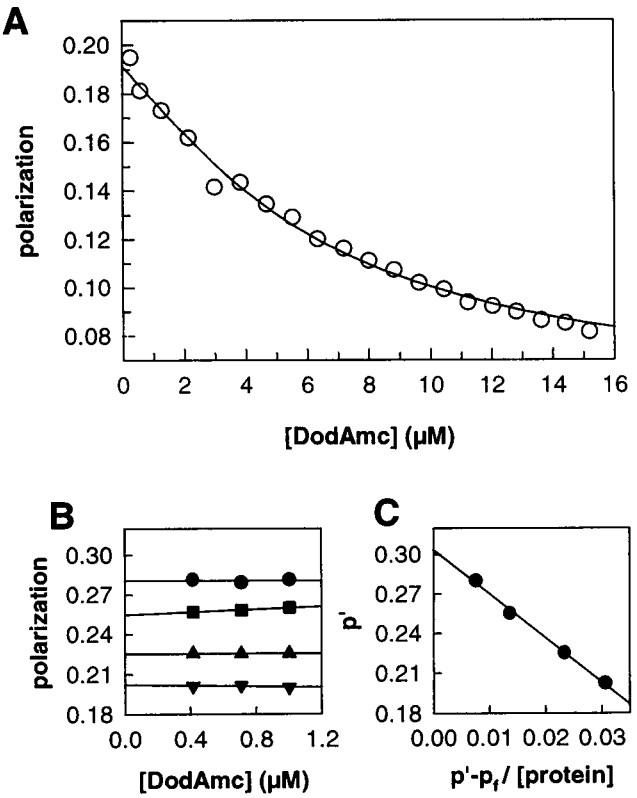

FIGURE 5 Binding of coumarin-labeled dodecasaccharide to D392N tailspike protein. The increase in polarization of the coumarin label upon binding of DodAmc to tailspike protein was used to characterize the binding equilibrium. (*A*) D392N mutant protein was titrated with DodAmc, and the polarization data were evaluated as described in Materials and Methods. The polarization and specific fluorescence of the bound label were determined as illustrated in *B* and *C* for the polarization (Dandliker et al., 1981). Briefly, D392N protein was titrated with DodAmc at tailspike subunit concentrations of 30.5 μM (●), 15.2 μM (■), 7.6 μM (▲), and 5.1 μM (▼). Ordinate intercepts (p') were extrapolated to infinite protein concentration.

(Steinbacher et al., manuscript submitted for publication). Although wild-type and D392N mutant tailspikes bind octasaccharide and dodecasaccharide with essentially identical affinities at 10°C, differences above experimental error were observed between the two proteins in the van't Hoff enthalpies of octasaccharide binding and their temperature dependencies. There is no obvious explanation for this result, and further studies by titration calorimetry and x-ray crystallography will be required to resolve this apparent difference in the mode of binding.

Compared to other endoglycosidases, the P22 tailspike endorhamnosidase has a remarkably low turnover number but a high substrate binding affinity. This may have been expected, as the principal function of the tailspike protein is to attach the bacteriophage to its host's cell surface. Although the bacteriophage particle holds six tailspike trimers, each of which can bind three oligosaccharides, only three or four binding sites appear to be used simultaneously (Israel, 1978). Nevertheless, the observed binding free energies of close to 30 kJ/mol for each site at physiological temperature add up to essentially irreversible attachment. Rapid release and rebinding of individual tailspikes to pairs of *O*-antigen repeats may prevent the phage from becoming stuck at unproductive sites, i.e., locations not suitable for DNA injection.

Even at physiological temperature, the enzyme will cleave only one oligosaccharide every 30 s. What is the significance of the slow endorhamnosidase activity? Endoglycosidase activities of bacteriophage tail particles have been assumed to be required for the lateral movement of phage particles to a DNA injection site or to clear a path through the lipopolysaccharide layer down to the outer membrane of the bacterium (Lindberg, 1977; Bayer et al., 1980). For P22, lateral movement is more likely to occur through receptor relase and rebinding than by its hydrolysis. Phage attachment and DNA uptake require only seconds to very few minutes at physiological temperature, during which the enzymatic activity would hardly be sufficient to cut a clearing into the lipopolysaccharide jungle. Furthermore, the tail structures of *S. anatum* bacteriophage g_{341} are associated with an esterase activity capable of inactivating, but not physically shortening, the receptor *O*-antigen (Iwashita and Kanegasaki, 1976b; Bayer et al., 1980). On the other hand, the receptor-inactivating activities of carbohydrate-specific bacteriophages may be required upon completion of an infectious cycle for detachment of newly assembled viral particles from the host cell debris. Recent studies on the neuraminidases and esterases of influenza viruses indicate that these analogs of the P22 tailspike endorhamnosidase activity, in fact, facilitate the release of viral particles into the supernatant and are dispensible for viral entry (Herrler et al., 1992; Liu et al., 1995; Huberman et al., 1995).

In contrast to earlier bacteriophage endoglycosidase mutants (Berget and Poteete, 1980; Schwarz and Berget, 1989), the new point mutants characterized in this study affect only the inactivation, but not the binding of the phage receptor. They should prove to be valuable tools for defining the function of the P22 tailspike endorhamnosidase activity.

The observed protein fluorescence change upon oligosaccharide binding is likely due to quenching of two tryptophan residues (W365 and W391) that contact the octasaccharide in the x-ray structure of the complex (Steinbacher et al., manuscript submitted for publication). Aromatic residues are a frequent feature of carbohydrate binding sites in proteins (Vyas, 1991). Experimental support for the oligosaccharide contacts to both W365 and W391 being responsible for the fluorescence change comes from the fact that the fluorescence quench upon oligosaccharide binding to a mutant that lacks Trp391 is reduced to about half the amplitude observed for the wild type (B. Schuler and R. Seckler, unpublished data). All seven of the tryptophan residues of the tailspike protein are partially accessible to solvent, but W365 and W391 are among the least buried. They are expected to exhibit a high Stokes' shift, providing an explanation for the observed blue shift of fluorescence emission upon binding of the *O*-antigen fragments.

Although glycosidases are structurally diverse, they are believed to function through common catalytic mechanisms involving at least one carboxylate residue in retaining enzymes and at least two carboxylate residues in inverting enzymes (Koshland, 1953; Withers and Aebershold, 1995). It is not known whether the tailspike endorhamnosidase retains or inverts the configuration during hydrolysis of the glycosidic bond. Three acidic residues (E359, D392, D395) are located near the reducing end of the octasaccharide in the structure of the enzyme-product complex (Steinbacher et al., manuscript submitted for publication). All three appear to be involved in the catalytic mechanism, because the mutations to the corresponding amides resulted in a nearly total loss of activity without significantly affecting the binding of substrate and product. The amide mutants may help to elucidate the catalytic mechanism of this endoglycosidase.

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