*Proc. Natl. Acad. Sci. USA*
Vol. 93, pp. 10584–10588, October 1996
Biochemistry

# Crystal structure of phage P22 tailspike protein complexed with *Salmonella* sp. O-antigen receptors

(endoglycosidase/hemagglutinin/neuraminidase/virus/infection)

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**ABSTRACT** **The *O*-antigenic repeating units of lipopolysaccharides from *Salmonella* serogroups A, B, and D1 serve as receptors for the phage P22 tailspike protein, which also has receptor destroying endoglycosidase (endorhamnosidase) activity, integrating the functions of both hemagglutinin and neuraminidase in influenza virus. Crystal structures of the tailspike protein in complex with oligosaccharides, comprising two O-antigenic repeating units from *Salmonella typhimurium*, *Salmonella enteritidis*, and *Salmonella typhi 253Ty* were determined at 1.8 Å resolution. The active-site topology with Asp-392, Asp-395, and Glu-359 as catalytic residues was identified. Kinetics of binding and cleavage suggest a role of the receptor destroying endorhamnosidase activity primarily for detachment of newly assembled phages.**

The infection of *Salmonella* by phage P22 starts with the recognition of the O-antigenic repeating units of the cell surface lipopolysaccharide by the homotrimeric tailspike protein (TSP), which is present in six copies on the attachment apparatus (1, 2). Phages that use lipopolysaccharide as receptors are faced with the chemical diversity of the O-antigenic repeats, which differ in carbohydrate composition and stereochemistry of the O-glycosidic linkage. In addition, microheterogeneity concerning the number of repeating units and additional modifications as acetylation or glucosylation known as form variation is an important feature of lipopolysaccharide (3–5). P22 has adapted to *Salmonella* serotypes A, B, and D1 (Fig. 1), sharing a common trisaccharide repeating unit α-D-mannose-(1,4)-α-L-rhamnose-(1,3)-α-D-galactose for the O-antigen but differing in their branching carbohydrate moieties, a 3,6-dideoxyhexose α-(1,3)-linked to D-mannose. Dideoxyhexoses are paratose (serogroup A), abequose (serotype B), or tyvelose (serotype D1) and reflect the correlation of serotype classification and chemical structure of the O-antigenic repeats of *Salmonella* (6, 7). In addition, P22 tolerates the O-antigen $12_2$, which shows an α-(1,4)-linked D-glucose at D-galactose as in *Salmonella typhi 253Ty* (8). In the phage–host interaction, the dideoxyhexose can be viewed as a wobble position that allows for some flexibility in a specific interaction.

Receptor destroying enzymatic activities are well known for viruses that use carbohydrates as receptors. Influenza A and B virus and paramyxovirus have neuraminidases releasing terminal *N*-acetylmuraminic acid from glycoproteins and glycolipids (9), whereas influenza virus C and some coronaviruses that recognize an O-acetylated sialic acid epitope have a sialate 9-O-acetylesterase, removing an acetyl group (10, 11). Endoglycosidase or acetylesterase activities have also been demonstrated for a large number of phages acting on encapsulated Gram-negative bacteria like *Escherichia coli*, *Salmonella*, or *Klebsiella* (12). TSP possesses receptor destroying endorhamnosidase activity cleaving the α(1,3)-O-glycosidic bond between rhamnose and galactose of the O-antigenic repeats (13, 14). To elucidate the structural basis of both variable and specific aspects of the interaction of phage P22 with its O-antigen receptor and the receptor destroying endoglycosidase activity, we have analyzed the crystal structures of three O-antigen fragments comprising two repeating units in complex with the TSP.

## MATERIALS AND METHODS

**Olígosaccharide Preparation.** Lipopolysaccharide fragments from *Salmonella typhimurium SH4809*, serogroup B, O-antigen 4,5,12; *Salmonella enteritidis SH1262*, serogroup D1, O-antigen 9,12; and *S. typhi 253Ty*, serogroup D1, O-antigen $09,12_2$ were purified as in ref. 15. 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 in ref. 8 and were quantified by dry weights.

**Crystallization.** Recombinant shortened TSP (residues 109–666) was crystallized from 1.0 M $(NH_4)_2SO_4$ in 0.1 M sodium phosphate (pH 10.0) at 4°C as in ref. 16. Soaking experiments were performed with 2 mM oligosaccharide in 1 M $Na_2SO_4$ in 0.1 M Tris·HCl (pH 7.5) at 20°C for 4 days. For data collection, the soaked crystals were harvested directly from the drop.

**Data Collection and Refinement.** Diffraction data were collected on a MAR Research image-plate system with CuKα x-rays generated by a Rigaku RU200 rotating anode generator operating at 5.4 kW with a graphite monochromator. Data collection was performed at 16°C. Data were processed using the MOSFLM package (17) and were scaled and reduced using ROTAVATA/AGROVATA and TRUNCATE of the CCP4 (1994) package (18). Oligosaccharide fragments were fitted into |Fo|-|Fc| electron density maps contoured at 3 σ with FRODO (19). After refinement with the program X-PLOR (20) 2|Fo|-|Fc| maps were used for final interpretation of the carbohydrate conformation. For the protein parameters derived by Engh and Huber (21) and for the carbohydrate parameters derived by Weis, as distributed with X-PLOR (20) were used, respectively. Solvent accessible surface areas for bound carbohydrates were calculated with X-PLOR (20) using a probe radius of 1.4 Å.

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Abbreviation: TSP, tailspike protein.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (references 1TSP, 1TYU, 1TYV, 1TYW, and 1TYX).

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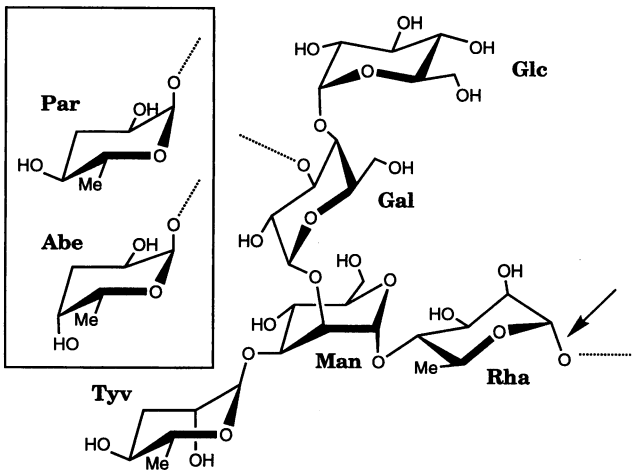


FIG. 1. Chemical structure of *Salmonella* O-antigenic repeating units. 3,6-dideoxyhexoses (paratose, serotype A; abequose, serotype B; or tyvelose, serotype D1) are α-(1,3)-linked to Man of the repeating unit trisaccharide α-Man-(1,4)-α-L-rhamnose-(1,3)-α-Gal. O-antigen $12_2$ (serotype D1) has randomly α-(1,4)-linked Glc. The arrow marks the cleavage site.

## RESULTS AND DISCUSSION

**Structural Features of TSP.** TSP lacking the N-terminal head binding domain has an unusual three-dimensional structure (ref. 16; Fig. 2). Each polypeptide chain of the homotrimer forms distinct segments. These differ significantly in the mode of interaction on the trimer level. In the central part, each subunit is composed of a right-handed parallel β-helix of 13 complete turns. The right-handed β-helix motif is also present in pectate lyase C from *Erwinia chrysathemi* and related enzymes (23, 24), in alkaline protease from *Pseudomonas aeruginosa* (25), and the *Bordetella pertussis* virulence factor P.69 pertactin (26). A left-handed β-helix was found in UDP-*N*-acetylglucosamine acyltransferase from *E. coli* (27) and in carbonic anhydrase from *Methanosarcina thermophila* (28). A common function is not apparent, except for the binding of and cleavage of carbohydrates in TSP and pectate lyase.

**Binding Site of Receptor Fragments.** The binding site is located in the central part of the β-helix, where a long, richly structured cleft is formed by a 60-residue insertion on one side and three smaller insertions of 5–25 residues on the other side (Fig. 3). The cleft is between approximately 80 and 100 Å apart from the C terminus the protein, which is most distant from the phage head. The binding cleft is 21 Å long, 8–13 Å wide, and accommodates all eight carbohydrate residues of two repeating units, well defined by electron density (Table 1; Fig. 4). All principal features of carbohydrate–protein interaction (29) are present in the binding of O-antigenic repeats to TSP. These include stacking of aromatic side chains with pyranose rings in a hydrophobic manner, H-bonds to polar and ionic side chains, and substitution of weakly bound water molecules by hydroxyl groups of the carbohydrate. Upon binding, only ≈39-43% of the surface area remains accessible to solvent compared with the free oligosaccharides (1332 $Å^2$ to 568 $Å^2$ for *S. typhimurium*, from 1340 $Å^2$ to 571 $Å^2$ for *S. enteritidis*, and from 1411 $Å^2$ to 546 $Å^2$ for *S. typhi 253Ty*, respectively), reflecting an extensive contact surface. The carbohydrate backbone forms an arch complementary to an elevation formed by Trp-365, Lys-363, and Glu-359 in the center of the binding site. Flat depressions on both sides harbor the dideoxyhexose of repeating unit 2 and the terminal rhamnose of repeating unit 1, respectively. The elevation is also the most narrow site and is only between 8 and 9 Å wide. Here Man1, Gal1, and Rha2 make van der Waals interactions, tightly nestling against Trp-365 on one side, flanked by Val-236 and Ser-237 on the other. Complementarity in shape in this region is probably a

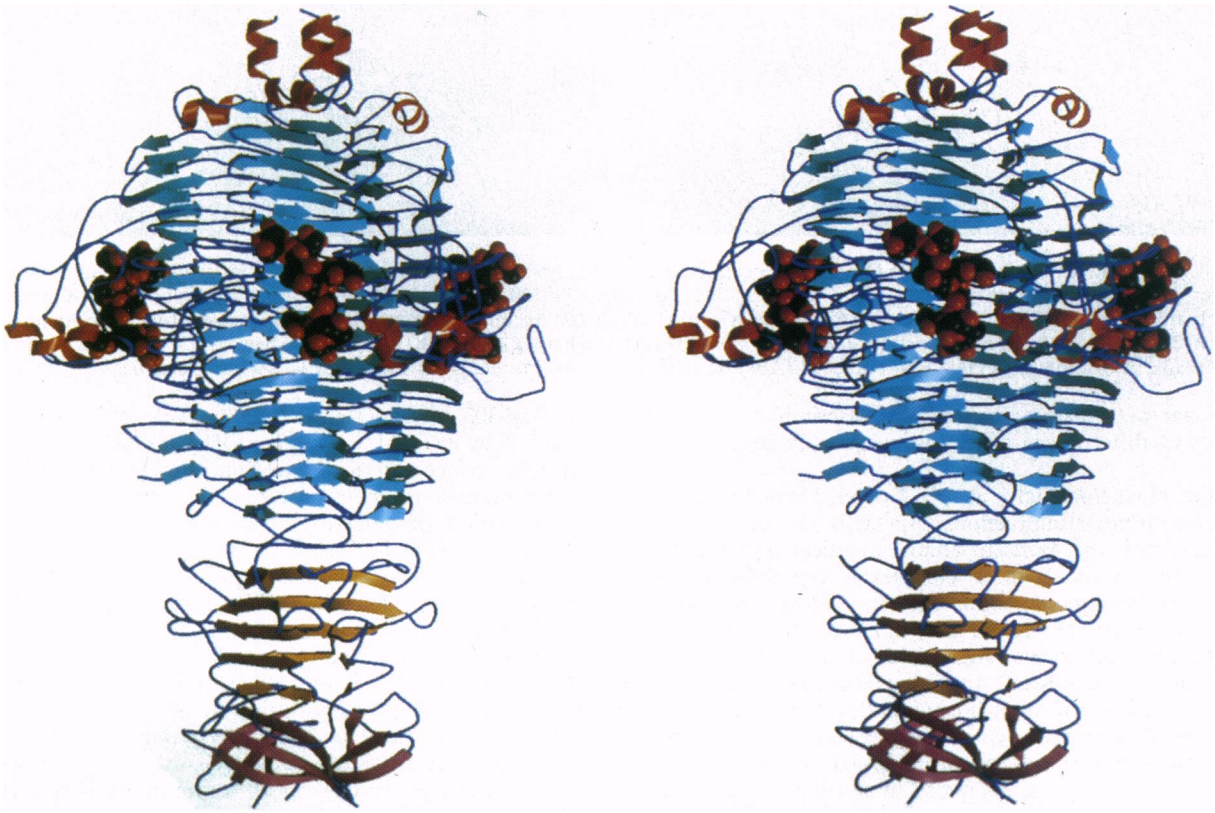

FIG. 2. Ribbon diagram of the TSP trimer. The N termini are on top, pointing to the phage head, and the C termini are on the bottom. The nonasaccharide from *S. typhi 253Ty* O-antigen is shown as balls. The figure was prepared with MOLSCRIPT (22).

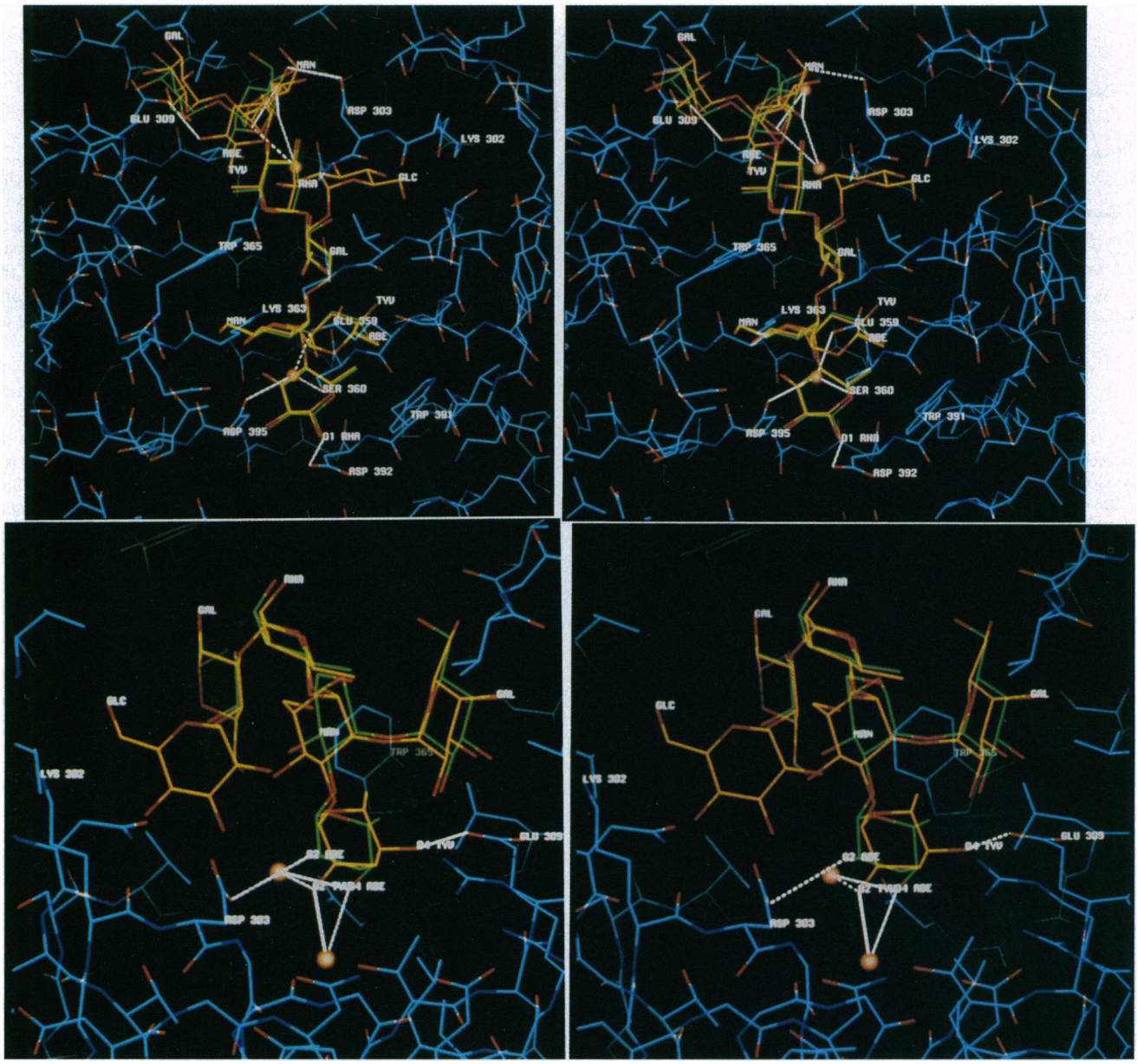


FIG. 3. (*A*) O-antigen binding site. Two O-antigen repeating units from *S. typhimurium* (green) and *S. typhi 253Ty* (yellow) are superimposed. The lower part shows the active site topology with Asp-392, Asp-395, Glu-359, and a potential catalytic water molecule. *S. typhi 253Ty* nonasaccharide shows an additional glucose due to O-antigen form variation. (*B*) Accommodation of 3,6-dideoxyhexoses from *S. typhimurium* (Abe) and *S. typhi 253Ty* (Tyv) O-antigens by positional wobbling. Tyv OH4 is H-bonded to Glu-309 (2.7 Å), whereas abequose OH2 forms a longer H-bond to Asp-303 (3.2 Å). Contacts of OH2 (Tyv) or OH4 (Abe), respectively, to the protein are mediated by two water molecules.

determinant of substrate binding and discriminates for O-antigens with different stereochemistry in backbone glycosidic bonds.

**Multiple Host Specificity and O-Antigen Form Variation.** Together with polar interactions, especially for the terminal rhamnose Rha1, this leads to almost identical positions for repeating unit 1 in all three complexes, especially as the dideoxyhexose here points to the solvent, making only van der Waals contacts with its methyl group to Val-240 and Ser-237. In contrast, torsions in the range of 15–20° around the glycosidic bonds between Rha2 and Man2 or Abe2 and Tyv2, respectively, and Man2 allow positional deviations for repeating unit 2 for different serotypes. This is necessary, because the dideoxyhexose here faces the protein surface directly. The different dideoxyhexoses, abequose in serotype B (*S. typhimurium*) and tyvelose in serotype D1 (*S. enteritidis*) are accommodated by a slight positional wobbling between two acidic residues, Asp-303 and Glu-309. Abequose is H-bonded to Asp-303 by its OH2, whereas tyvelose is H-bonded to Glu-309 by its OH4, with either OH4 or OH2 pointing to the protein surface and making H-bonds to two water molecules, which remain in their position (Fig. 3*B*). Random $\alpha$-1,4-glucosylation of galactose is known as form variation of O-antigens (5) as in *S. typhi 253Ty* (8). Soaking experiments with the respective O-antigen fragments revealed an additional subsite (Fig. 3). Here Lys-302 has to change its side-chain conformation to open the subsite. This is the only major rearrangement observed upon carbohydrate binding. The remaining carbohydrate residues do not change their position significantly, compared with the *S. enteritidis* octasaccharide. All interactions lead to binding constants of 1 to 2 $\times$ $10^6$ liters/mol for octa- and dodecasaccharides (30), indicating an unusually strong protein–carbohydrate interaction (31).

**O-Antigen Binding to Antibodies.** Cell surface lipopolysaccharides are not only the target for bacteriophages but also the major antigen upon gastrointestinal infection with *Salmo-*

Table 1. Data collection and refinement statistics

| Parameter | TSP native | *S. typhimurium* | *S. enteritidis* | *S. typhi 253Ty* |
|---|---|---|---|---|
| Data collection (to 1.8 Å) | | | | |
| Total observations, no. | 203,785 | 232,552 | 276,996 | 258,313 |
| Unique reflections, no. | 53,872 | 54,496 | 51,290 | 53,243 |
| Completeness* (%) | 98.5 (96.4) | 99.5 (99.6) | 93.5 (36.5) | 97.7 (93.4) |
| $R_{merge}$† (%) | 5.5 (27.5) | 6.5 (30.8) | 6.1 (29.3) | 6.4 (27.4) |
| Refinement (8.0 to 1.8 Å) | | | | |
| Reflections, no. | 53,375 | 53,883 | 50,712 | 52,553 |
| Protein atoms, no. ($B_{av}$, Å$^2$) | 4115 (15.2) | 4115 (14.5) | 4115 (13.2) | 4115 (13.2) |
| Water atoms, no. ($B_{av}$, Å$^2$) | 225 (26.5) | 209 (27.0) | 209 (26.2) | 209 (25.6) |
| Carbohydrate atoms, no. ($B_{av}$, Å$^2$) | — | 83 (24.7) | 83 (23.8) | 94 (30.7) |
| Rms bonds, Å | 0.009 | 0.009 | 0.009 | 0.009 |
| Rms angles, degrees | 1.6 | 1.6 | 1.6 | 1.6 |
| *R* factor, % | 18.1 | 18.3 | 18.4 | 18.3 |

*$\infty$ – 1.80 Å (1.85 – 1.80 Å)

†$R_{merge} = \Sigma_h\Sigma_i(|I(h,i) - <I(h)>|)/\Sigma_h\Sigma_i I(h,i)$, where $I(h,i)$ is the intensity value of the $i$th measurement of $h$ and $<I(h)>$ the corresponding mean value of $h$ for all $i$ measurements of $h$; the summation is over all measurements. 8.0 – 1.80 Å (1.85 – 1.80 Å).

*nella*. In contrast to the interaction with TSP, the branching dideoxyhexose is the main target for the recognition of the O-antigenic repeats by antibodies. It therefore defines the serotype of *Salmonella* (6). Crystal structures of *S. typhimurium* O-antigen oligosaccharides in complex with Fv and Fab fragments of the anti-carbohydrate antibody Se155-4 are known (32, 33). There, the branching abequose is recognized by a highly complementary surface of a pocket 8 Å deep and 7 Å wide, characterized by the overwhelming presence of aromatic residues. H-bonds are only formed to a single buried water molecule and to the NH group of a Trp side chain and a backbone NH group, but not to charged or polar residues. As typical for antibodies, the recognized epitope is rather small with specific interactions, resulting in an association constant of $2 \times 10^5$ liter/mol, lower than that of TSP (30–32). To keep the host range as broad as possible for phage P22, an attachment protein could either recognize only small constant parts of the receptor or have alternative interactions with the varying components. The wobbling mechanism observed in TSP allows a larger binding site compensated by alternative interactions.

**Active Site Topology.** The subsite for the terminal rhamnose Rha1 harbors the active-site residues (Fig. 3*A*). Rha1 fits the electron density best in a distorted boat conformation, which is often observed for carbohydrates bound to an active site. The C1 hydroxyl group, which is clearly present in the $\alpha$-configuration, as in the substrate is H-bonded to Asp-392 with a distance of 2.7 Å. A water molecule W-219, which is tightly bound also in the absence of the product, is located between Rha1 and the protein surface with an angle of 130° for W-219-Rha1C1-Rha1O1 and a distance of 2.9 Å between Rha1C1 and W-219. The water molecule W-219 is H-bonded to two acidic residues, Asp-395 and Glu-359, and Ser-360 with distances of 2.8, 2.7, and 2.6 Å, respectively. Glu-359 is involved in a salt bridge with Lys-363, both residues shielded from the solvent by the bound octasaccharide. Prompted by the reported studies, Asp-392, Asp-395, and Glu-359 were individually replaced by Asn or Gln, respectively. This results in a 10,000- to 30,000-fold reduction of enzymatic activity, although all three mutants bind the octasaccharide product and the dodecasaccharide substrate with wild-type affinity (30).

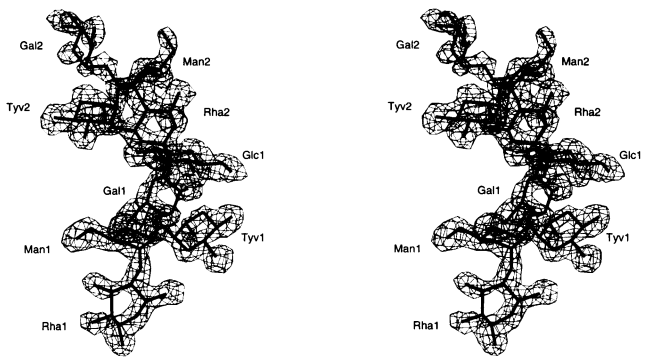


FIG. 4. Final 2Fo-Fc electron density for *S. typhi 253Ty* nonasaccharide at 1.8 Å resolution contoured at 1 $\sigma$. Repeating unit 1 at the reducing end is located proximal and repeating unit 2 is located distal to the active site.

Glycosidases either invert or retain the configuration of the anomeric carbon atom of the cleaved glycosidic bond. Although inverting and retaining enzymes share structural features with two carboxylic acids on opposite sides of a substrate binding cleft, the average distance between the carboxylic acids is quite different, ≈4.5-5.5 Å for retaining and 9.0–10 Å for inverting enzymes (34, 35). It was proposed that the greater distance for inverting enzymes arises from the necessity to accommodate both the substrate and a nucleophilic water molecule activated by the general base between the carboxylic acids. It is not known to which class of enzymes the P22 endorhamnosidase belongs. As Asp-392 is H-bonded to the $OH_1$ group of Rha1 of the bound product, it is likely to serve as general acid. The mean distances of the four carboxylic oxygen atoms are 5.8 Å (Asp-392, Asp-395), 8.2 Å (Asp-392, Glu-359), and 7.1 Å (Asp-395, Glu-359). The average distance between Asp-392 and Asp-395 of 5.8 Å is quite near to the distance range observed for retaining enzymes using a double displacement mechanism. In this case, Asp-392 would serve as general acid, as it is H-bonded to $OH_1$ of Rha1 and Asp-395 as an intermediate nucleophile. No potential general base is present in the opposite side of Rha1. On the other hand, the steric situation is ideal for a direct attack of the water molecule W-219 on C1 of Rha1, which additionally does not require significant changes in the position of the substrate compared with the bound product. Thus, the structural results also allow an inverting mechanism with Asp-392 as general acid and W-219 as nucleophile activated by the general bases Asp-395 and Glu-359, which directly attacks the anomeric C1 of the rhamnose.

**Possible Functions of Receptor Destroying Activity.** The TSP endorhamnosidase cleaves only two bonds per minute at physiological temperatures of ≈35°C (30). This activity is certainly too low to give TSP the function of a drill, considering the short time of the infection process. Because O-antigen binding and dissociation occur rapidly (30), it is also not likely to contribute to significant lateral mobility, which might be necessary to find a potential second receptor, that finally allows DNA injection. Eventually, however, as the active site

is located proximal to the cell surface, O-antigen cleavage results in a release of the phage from the lipopolysaccharide compatible with a liberating function of the endorhamnosidase. This suggests that the receptor destroying function of TSP is particularly important for detachment of newly assembled phages from the cell debris after host cell lysis. Analogous findings have been reported for influenza type A virus (36) and human parainfluenza 3 virus (37), where binding and receptor destroying activity are either separated on hemagglutinin and neuraminidase, or combined in one protein, the hemagglutinin–neuraminidase, respectively. In these cases, loss or reduction of receptor destroying neuraminidase activity does not influence viral entry but blocks multicycle infections due to a shortage in virus particles or results in a delay of virus release. Compared with TSP, much smaller epitopes that can be attached to various cell surface compounds are recognized by binding regions on the tips of the analogous influenza virus hemagglutinin (38, 39) and neuraminidase (40, 41). But in both cases, the receptor binding and receptor destroying activities probably play similar roles. Point mutations of active site residues that bind the receptor with affinities comparable to wild-type TSP allow further *in vivo* studies to elucidate the precise role of the endorhamnosidase activity during the infection process.

Long circulating mutants of phage P22 have recently been demonstrated as antibacterial agent against *S. typhimurium CRM3* infections in a mouse model (42). In this approach, the therapeutic efficiency depends on the host range of the phage, determined by the interaction between the TSP and the O-antigen.

We thank A. v. d. Locht and P. Hof for helpful discussions.

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