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Factors Affecting Binding of Galacto Ligands to *Actinomyces viscosus* Lectin

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The specificity requirements for the binding of *Actinomyces viscosus* T14V were examined by testing simple sugars, oligopeptides, and glycoproteins as inhibitors of the aggregation of glycoprotein-coated latex beads and washed *A. viscosus* cells. Lactose was the most inhibitory simple sugar; D-fucose and D-galactose were equally inhibitory, methyl-α-D-fucoside was slightly less inhibitory, and L-fucose and raffinose were not inhibitory. The concentration of galactose residues required for 50% inhibition of aggregation was 15 times higher in the form of lactose than in the form of asialoglycoprotein, suggesting an enhancement of lectin binding when galactose residues are clustered. However, when the inhibitory power of bi-, tri-, and tetraantennary asialooligopeptides of α_1-acid glycoprotein was compared with that of equivalent concentrations of galactose in the form of lactose, the biantennary form was slightly less effective than lactose, the triantennary form was approximately as effective as lactose, and the tetraantennary form was slightly more effective than lactose. Steric interference may prevent this type of clustering from enhancing lectin binding. The O-linked asialooligopeptides of asialofetuin were 10 times more inhibitory than an equivalent concentration of galactose in the form of N-linked asialooligopeptides. Thus, galactose β-1→3 linked to *N*-acetylgalactosamine exhibits greater specificity for the *A. viscosus* lectin than does galactose β-1→4 linked to *N*-acetylglucosamine. These results, taken together with previously reported data, are consistent with a lectin of low affinity, binding enhanced by multivalency, and specificity for β-linked galactose.

Actinomyces viscosus has been closely associated with periodontal disease (2) and root caries (16). It is capable of adhering to other oral-colonizing bacteria, such as streptococci (3, 5), and to pellicle-coated hydroxyapatite (6, 21). A related species, *A. naeslundii*, binds to oral epithelial cells (11). The binding of *A. viscosus* to coated hydroxyapatite is mediated by type-1 fimbriae on the bacteria (7), and some researchers have proposed that this type of binding involves hydrophobic interactions (23). Other modes of binding are probably mediated by type-2 fimbriae on the bacteria, which exhibit a lectin-like property (4, 22). For example, *A. viscosus* agglutinates erythrocytes in a neuraminidase-dependent, lactose-inhibitable manner (8) in which terminal sialic acid residues are first removed, thus exposing galactose (Gal) residues, which then bind to the bacterial lectin. Using a latex bead aggregation system, Heeb et al. (15) demonstrated that the *A. viscosus* lectin was clearly specific for asialoglycoproteins terminating in β-linked Gal or α-linked *N*-acetylgalactosamine (GalNAc). Other details of specificity requirements were elucidated, but differences in aggregation rates with different ligands raised questions about the importance of the linkage and identity of the penultimate sugar residue of the asialoglycoprotein and the importance of the clustering of Gal termini. The present study addresses these questions and extends the definition of the specificity of the *A. viscosus* lectin. Glycopeptides of fetuin and α_1-acid glycoprotein were tested for the first time in this system. These model glycoproteins contain carbohydrate structures similar to those found on many cell surfaces and in glycoproteins included in dental pellicle. A comparison of the binding of the respective oligopeptides allows the first serious assessment of structural elements of biological surfaces as potential ligands for *A. viscosus* lectin interaction.

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MATERIALS AND METHODS

Materials. *A. viscosus* T14V was obtained from B. Hammond, University of Pennsylvania School of Dentistry, Philadelphia, Pa. α_1-Acid glycoprotein was a gift from Milan Wickerhauser, American Red Cross Blood Research Laboratories, Bethesda, Md. Bovine submaxillary mucin was donated by Gilbert Ashwell, National Institutes of Health, Bethesda, Md. Crystalline bovine serum albumin was obtained from Miles Laboratories, Inc., and pronase was from Kaken Chemical Co. Galactose dehydrogenase and NAD were from Boehringer Mannheim Biochemicals, and Bio-Gel beads were from Bio-Rad Laboratories. Gal and mannose were from Fisher Scientific Co.; raffinose and fucose were from Pfansteil. Other sugars, latex beads, buffers, ovalbumin, fetuin type IV, *N*-acetylneuraminic acid (NeuAc), and *Clostridium perfringens* neuraminidase type IX were obtained from Sigma Chemical Co. DE-52 cellulose was from Whatman, Inc.

The aggregation system. The latex bead aggregation assay was previously described and characterized in detail (15). Briefly, 0.5 mg of glycoproteins per ml were adsorbed to latex beads (1:50 [vol/vol] in Tris-glycine saline (7.3 g of glycine and 10 g of NaCl per liter) (pH 8.2) containing 0.1 mM $CaCl_2$ and 0.02% NaN_3. Before use, the beads were washed in buffer with 1 mg of BSA bovine serum albumin per ml substituted for the glycoprotein. The beads were suspended in a round cuvette and adjusted to an A_{650} of approximately 0.9 with stirring. Inhibitors were added as noted. Bacteria were then added in a small volume to a final concentration of 1.5×10^8 cells per ml, and the progress of aggregation was monitored with a recording spectrophotometer.

The concentration of an inhibitor needed to produce a given degree of inhibition of aggregation varied with the batch of cells, the growth conditions, and the age of the harvested and stored cells. Cells stored for months at 4°C demonstrated little or no loss of ability to aggregate glycoprotein-coated beads. However, the aggregation became more sensitive to inhibitors with time. All comparisons within a group of inhibitors made here or in previous work (15) were made with the same batch of cells within a short time, usually 1 or 2 days.

Preparations of glycopeptides. Fetuin (1 g) was dissolved in 40 ml of 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid) buffer (pH 7.6)–10 mM $CaCl_2$–0.5% toluene. Pronase was added in 10-mg portions at 0, 12, 24, 48, and 72 h of incubation at 37°C. Ninhydrin tests (26) were performed at each of those times to estimate the extent of digestion. The mixture was chromatographed in 0.1 M pyridine acetate (pH 5.2) on a column (2.5 by 85 cm) of Bio-Gel P-4, and tubes were assayed for neutral sugars (10), sialic acid (25), and ninhydrin-positive material. After comparison with the data of Spiro and Bhoyroo (28) and with the position of molecular weight markers, fractions tentatively designated as N-linked oligopeptides (fraction A), O-linked oligopeptides (fraction C), and mixtures of the two (fraction B) were pooled and lyophilized. Pool B, containing the mixture, was rechromatographed on the same P-4 column (after washing) and additional N- and O-linked fractions were separately pooled. The O-linked fraction (fraction C) was chromatographed on a DE-52 cellulose column (1.7 by 83 cm). The applied material was washed on the column with 120 ml of 0.01 M pyridine acetate buffer (pH 5.2) and then eluted with an 840-ml linear gradient of 0.01 to 0.11 M pyridine acetate. Most of the ninhydrin-positive material was eluted in the initial wash, and a sharp peak of material containing sialic acid and neutral sugars eluted halfway through the gradient. This peak was lyophilized. Samples of each of the above fractions were tested to confirm the identity of O- or N-linked material. Sugars were hydrolyzed for 5 h in 2 N H_2SO_4 under vacuum at 100°C (27) and then cooled and neutralized with barium carbonate. The supernatant after centrifugation was passed through a small column of Amberlite to remove charged materials. The effluent was concentrated by evaporation in vacuo and chromatographed on paper in pyridine-ethyl acetate-water (1:3.6:1.15, top layer), along with standards of Gal and mannose. The chromatogram was developed by silver nitrate dip (1). Glycopeptides of α_1-acid glycoprotein were prepared by methods very similar to those employed for fetuin, as described in detail elsewhere (Ann Marini, Ph.D. dissertation, Georgetown University, Washington, D.C., 1978).

Other methods. Gal concentrations in neutralized sugar hydrolysates were determined by a spectrophotometric assay, using NAD and Gal dehydrogenase (12). To remove sialic acid, glycoproteins were treated with neuraminidase (0.3 U/10 mg glycoprotein) for 2 h at 37°C. Sialic acid was measured (25) to establish that 76 to 100% of the available residues had been removed. For experiments in which latex beads were coated with mixtures of native glycoproteins and asialoglycoproteins, sialic acid was removed by treatment at 80°C for 1 h in 0.1 N H_2SO_4 followed by neutralization.

RESULTS

Inhibition of aggregation by simple sugars. An aggregation assay in which latex beads were coated with model asialoglycoproteins and aggregated with washed cells of *A. viscosus* T14V has been previously described and characterized (15). The initial rate of aggregation was followed by monitoring the decrease in light scattering (decrease in absorbance) with time. When beads were coated with defined mixtures of asialofetuin and native fetuin, the rate of aggregation increased in proportion to the asialofetuin concentration (Fig. 1). D-Gal and D-fucose were equally effective in inhibiting aggregation, indicating that recognition by *A. viscosus* lectin does not require a hydroxyl group at the number 6 carbon of the sugar. D-Fucose is inhibitory, but L-fucose is not, indicating strict stereospecificity. Raffinose, a trisaccharide terminating in an α-1→6-linked Gal, is not inhibitory, demonstrating that Gal is not recognized in that linkage. Previous studies had revealed that various sugars inhibited aggregation in the following order, beginning with the most effective: lactose, methyl-β-D-galactopyranoside, D-Gal, GalNAc, and methyl-α-D-galactopyranoside. Glucose, sucrose, glycerol and *N*-acetylglucosamine (GlcNAc) were not inhibitory. We again found that Gal in β-linkage (lactose) was a more effective inhibitor than D-Gal or D-fucose, and that D-fucose in α-linkage was slightly less effective than those sugars.

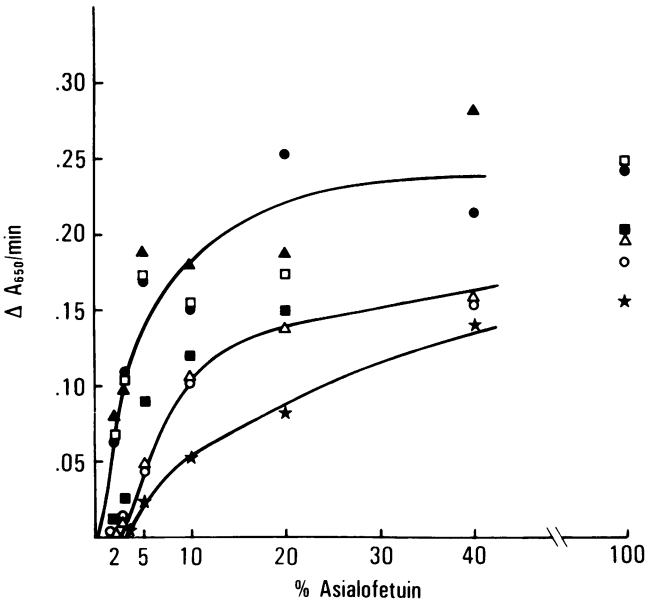

FIG. 1. Effect of ligand density on the rate of aggregation of fetuin-coated beads by *A. viscosus* T14V in the presence and absence of various sugars. Beads were coated with various mixtures of asialofetuin and native fetuin. Each type of bead was tested for rate of aggregation (ΔA/min) in the standard assay. Symbols: ●, control with no added sugar; △, 10 mM D-Gal; ★, 1 mM lactose; ▲, 10 mM raffinose; ○, 10 mM D-fucose; □, 10 mM L-fucose; and ■, 10 mM methyl-α-D-fucoside.

Inhibition of aggregation by asialoglycoproteins. Asialoglycoproteins were tested for their ability to inhibit the aggregation of asialofetuin-saturated latex beads by *A. viscosus* (Table 1). Asialofetuin, asialo-α_1-acid glycoprotein, and asialomucin were roughly equivalent in their ability to inhibit aggregation. These findings are consistent with the previous observation (15) that glycoproteins with terminal carbohydrates of either β-linked D-Gal or α-linked GalNAc serve equally well as bead receptors for the *A. viscosus* lectin. By comparing the concentration of Gal in the form of asialoglycoprotein needed for 50% inhibition with the concentration of Gal in the form of lactose needed for 50% inhibition, we found that the asialoglycoprotein is about 15 times as effective as the lactose. This difference represents a

TABLE 1. Inhibition by asialoglycoproteins and lactose of the aggregation of asialofetuin-coated beads by *A. viscosus* T14V

Ligand	Concn	% Inhibition
Asialofetuin	0.1 mg/ml	35
Asialofetuin	3.0 mg/ml[a]	54
Asialo-α_1-acid glycoprotein	0.1 mg/ml	30
Asialo-α_1-acid glycoprotein	1.0 mg/ml	34
Asialo-α_1-acid glycoprotein	10.0 mg/ml	64
Asialobovine submaxillary mucin	1.0 mg/ml	36
Bovine serum albumin	1.0 mg/ml	0
Ovalbumin	1.0 mg/ml	0
Lactose	5 mM	14
Lactose	10 mM	53
Lactose	20 mM	87
Lactose	200 mM	100

[a] 0.056 mM asialofetuin, 0.67 mM in galactose termini.

significant enhancement in apparent affinity for the *A. viscosus* lectin and probably indicates that a multivalent interaction is involved.

Preparation of fetuin oligopeptides. Previous studies (15) revealed significant differences in aggregation of *A. viscosus* between beads coated with asialofetuin and beads coated with asialo-α_1-acid glycoprotein, even when the number of Gal termini was approximately the same. To better assess the reason for these differences, oligopeptides were prepared from these two model glycoproteins.

Pronase-digested fetuin was fractionated on Bio-Gel P-4 as described above, and peaks A, B, and C of sialic acid-containing oligopeptides were separately pooled in order of decreasing molecular size. Pool B was rechromatographed on Bio-Gel P-4, as in Fig. 2. Pool C from the first Bio-Gel column was rechromatographed on DE-52 cellulose as described above. When portions of the various fractions were hydrolyzed, neutralized, treated with Amberlite, and chromatographed on paper, pools A and B-1 were identified as N-linked, containing Gal and mannose as neutral sugars. Pools C, B-2, and B-3 were identified as O-linked, containing only galactose as a neutral sugar. Fraction B-1 was used in the competition experiments to follow, since its elution position from the Bio-Gel P-4 column relative to the position of molecular weight markers precluded the possibility that it could contain peptide fragments with more than one N-linked oligosaccharide group on the same chain. Fractions B-3 and C, based on elution position, must contain only one O-linked oligosaccharide group per peptide fragment.

Preparation of α_1-acid glycoprotein oligopeptides. Similar steps were used to prepare oligopeptides from α_1-acid glycoprotein, which contains only N-linked oligosaccharides that are bi-, tri-, and tetraantennary. The ion exchange step is shown in Fig. 3. Peaks P-2, P-3, and P-4 were analyzed and contained two, three, and four sialic acid residues per asparagine residue, respectively, and only traces of other amino acids. To ensure that sialic acid residues had not been lost, thus leading to an erroneous ratio, the ratio of sialic acid to Gal was measured and found to be 0.9 to 1.1. Samples were then desialylated and used as inhibitors.

Inhibition of aggregation of oligopeptides. The N- and O-linked oligopeptides from asialofetuin were evaluated as inhibitors of aggregation by using *A. viscosus* cells and latex beads with various concentrations of asialofetuin ligand (Fig. 4). One mole of triantennary N-linked oligopeptide (fraction B-1) was roughly equivalent in inhibitory power to three moles of lactose. Each Gal terminus, β-1→4 linked to GlcNAc, is therefore approximately as effective in inhibition as a Gal residue β-1→4 linked to glucose. On the other hand, the O-linked oligopeptides (fraction C) inhibited aggregation to approximately the same degree as 10 times the equivalent concentration of Gal in the form of lactose. The same relative inhibitory power was observed for fractions B-2 and B-3, which were also O-linked oligopeptides. If the B-2 fraction (a fraction of higher molecular weight than B-3) contained any fragments with two O-linked oligosaccharides on the same polypeptide backbone, they did not exert any additional inhibitory effect. The data suggest that either the β-1→3 linkage or the second sugar (GalNAc) of the O-linked structure is more easily recognized (better fits the lectin binding site) than a β-1→4 linkage to GlcNAc or glucose.

The bi-, tri-, and tetraantennary oligopeptides of asialo-α_1-acid glycoprotein (Fig. 5) were all equally effective as inhibitors of aggregation. The biantennary structure (P-2) is somewhat less effective than the equivalent concentration of Gal in the form of lactose, the triantennary structure (P-3) is approximately as effective as lactose or the triantennary structure of asialofetuin, and the tetraantennary structure (P-4) is somewhat more effective than lactose. In a separate experiment performed with a different preparation on a different date (data not shown), the inhibition curve for the tetraantennary fraction diluted to 1:2 relative to lactose nearly coincided with the lactose inhibition curve (i.e., this fraction was approximately twice as effective as lactose).

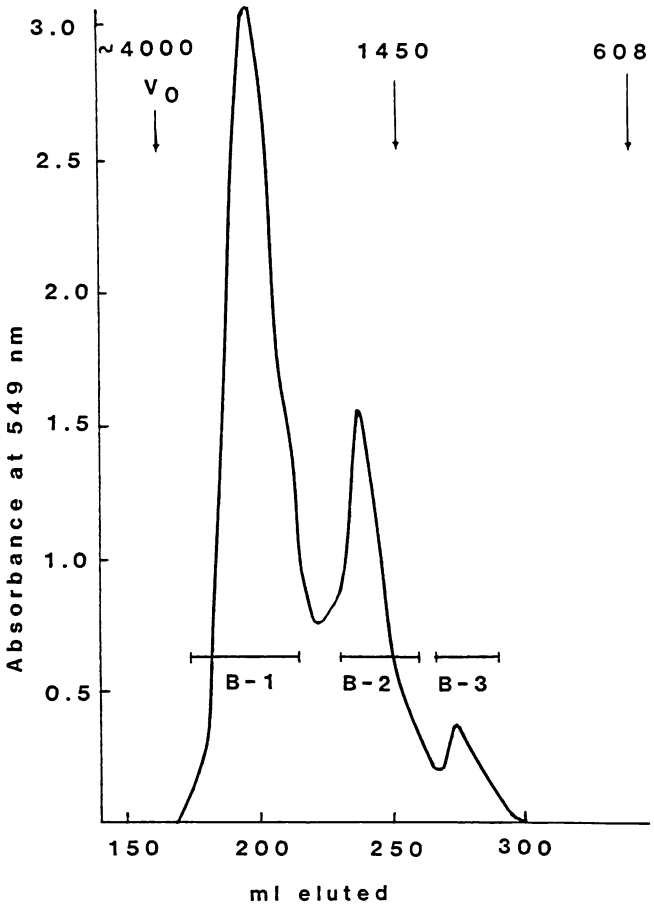

FIG. 2. Rechromatography of pronase-digested fetuin peak B on Bio-Gel P-4. Column size was 2.5 by 85 cm. The buffer was 0.1 M pyridine-acetate (pH 5.2). Volume applied was 5.3 ml. Fractions were 5 ml each. Absorbance at 549 nm: sialic acid determination (25) after acid hydrolysis of aliquots of each fraction.

We concluded that the multiple Gal residues of the branched structures can bind to the lectin binding sites simultaneously but that there is probably very little enhancement of binding affinity due to the multiple termini compared with that of gal termini binding at separate locations on the polypeptide chain.

DISCUSSION

The latex bead aggregation with *A. viscosus* allows the use of well-defined ligands, which can be chemically or enzymatically modified (15). These properties were previously used to great advantage to clearly demonstrate neuraminidase-dependent, lactose-inhibitable aggregation when the glycoproteins adsorbed to the beads possess β-linked galactose or α-linked GalNAc residues which are exposed after the action of neuraminidase. When beads were coated with defined mixtures of asialofetuin and native fetuin, the rate of aggregation with washed *A. viscosus* cells was found to increase in proportion to asialofetuin concentration. The initial rate data have the appearance of classical chemical kinetic data for a saturable system. When similar experiments were performed with the same cells and mixtures of asialo-α_1-acid glycoprotein and native α_1-acid glycoprotein, however, a threshold was observed with respect to minimum concentration of asialoglycoprotein required for aggregation to occur. The differences in the aggregation data with the two different glycoproteins was not due to the differences in numbers of Gal termini per bead, since these were determined to be nearly the same. The present data suggest that the differences may be due to the enhanced ability of Gal β-1→3 GalNAc termini to bind to the *A. viscosus* lectin compared with that of Gal β-1→4 GlcNAc. Asialofetuin contains three of the former group and nine of the latter group per molecule. Asialo -α_1-acid glycoprotein contains 15 of the latter group only per molecule. In the experiments presented here, Gal β-1→3 GalNAc was 10 times as effective as Gal β-1→4 GlcNAc as an inhibitor of aggregation (Fig. 4). F. McIntire has reported independently that Gal β-1→3 GalNAc is the most effective inhibitor tested in the coaggregation between *A. viscosus* or *A. naeslundii* and *Streptococcus sanguis* 34 (18). Numerous examples of this structure have been found in both glycoproteins (13, 14) and glycolipids (29). In many cases, one or both of the sugars is substituted with α-2→3- or α-2→6-linked NeUAc, and in some cases, α-1→2-linked fucose.

Beads coated with [^{3}H]NeuAc^7 fetuin and then treated with limiting amounts of neuraminidase did not aggregate until about 10% of the Gal residues were exposed (15). This was in contrast to the experiments just discussed, in which no threshold was observed with beads coated with various mixtures of asialofetuin and fetuin. The Gal residues exposed after limited neuraminidase treatment of the [^{3}H]NeuAc^7 fetuin beads would be less clustered than in the other experiments, and this fact is one possible reason for the differences between the results of the two experiments. In the present work, we found that the type of clustering of Gal residues which occurs in multiantennary N-linked oli-

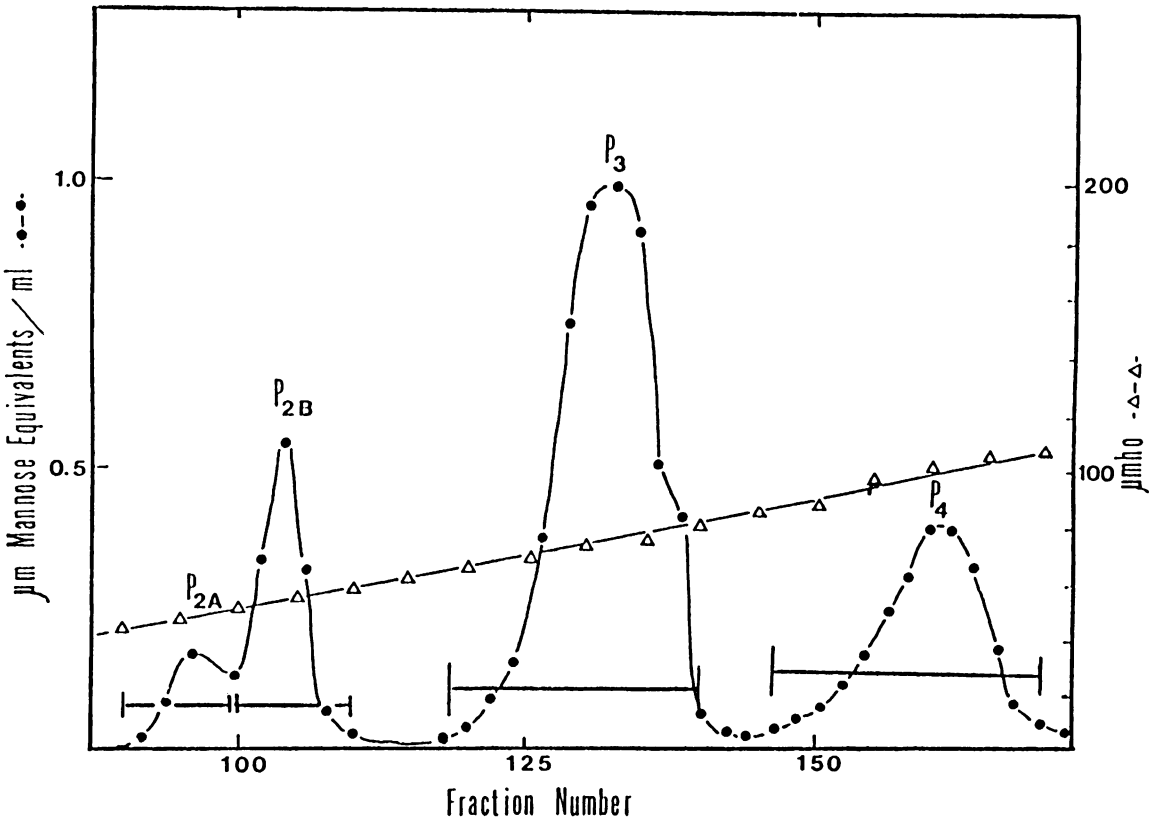

FIG. 3. Chromatography of pronase-digested α_1-acid glycoprotein on DEAE-cellulose. The digest was chromatographed by molecular sieving in a manner similar to that described for asialofetuin oligopeptides. The major fraction containing sialic acids and neutral sugars was chromatographed on DE-52 cellulose with a gradient of 0.005 to 0.3 M pyridine acetate (pH 5.2, conductivity 5.3 to 183 μS [μmho]). Fraction size was 8.7 ml.

gosaccharide structures resulted in little enhancement of binding to the *A. viscosus* lectin (Fig. 5). The tetraantennary structure was only about twice as effective in inhibition of aggregation as the equivalent concentration of lactose. (In contrast, multiantennary glycopeptides and synthetic oligosaccharides were sometimes orders of magnitude more effective in recognition by the hepatic binding protein on hepatocytes [17].) Clustered Gal residues on the same branched oligosaccharide probably have less flexibility for multiple binding to the *A. viscosus* lectin than do Gal residues on different oligosaccharide chains that are part of the same molecule. This latter type of clustering may partially explain the differences between the two fetuin experiments. The increased ability of asialoglycoproteins to inhibit aggregation (as compared to that of equivalent concentrations of lactose) (Table 1) supports this explanation.

Another explanation for the differences between the two fetuin experiments lies in the specificity of the neuraminidase. Most neuraminidases, including that of *C. perfringens*, prefer α-2→3 linkages to α-2→6 linkages (9), and Newcastle disease virus has strict specificity for α-2→3 (20). For example, cleavage of α-2→3 would preferentially expose more Gal β-1→3 GalNAc groups than Gal β-1→4 GlcNAc groups in fetuin (13a). In the experiments reported here we have demonstrated the enhanced binding of the β-1→3-linked structures to the *A. viscosus* lectin; however, one must consider that in many cases α-2→6-linked NeuAc is substituted for the GalNAc residues, and this substitution may obstruct binding.

Using simple sugars as inhibitors of aggregation (Fig. 1), we have confirmed and extended previous findings (15). The strict stereospecificity of lectin binding was demonstrated by the effectiveness of D-fucose, but not L-fucose, as an inhibitor. A similar finding was reported for inhibition of coaggregation between *A. viscosus* and *S. sanguis* (19). The lectin does not require a hydroxyl group in the C-6 position of the sugar, since D-fucose is as effective as D-Gal in inhibition. However, the conversion of the tetrahedral C-6 group of Gal to a planar aldehydo form by the action of Gal oxidase abolished the aggregation of asialoglycoprotein-coated beads, and aggregation could be restored by reduction with borohydride (15). The lack of response of the aggregation assay to raffinose indicates that α-1→6-linked Gal is not recognized by the *A. viscosus* lectin. Compared to Gal alone, an α linkage to Gal or fucose is unfavorable for binding to the lectin (raffinose, methyl-α-D-fucoside, or methyl-α-D-galactoside), and β linkage is more favorable for such binding (lactose, methyl-β-galactoside, or the complex oligopeptides). On the other hand, asialomucin, terminating in α-linked GalNAc, serves as a good bead ligand. Like the hepatic binding protein (24), *A. viscosus* lectin is capable of recognizing both β-linked Gal and α-linked GalNAc. The possibility of two distinct lectins in *A. viscosus* is discounted by the fact that lactose inhibits the aggregation of asialomucin-coated beads with approximately the same effectiveness with which it inhibits aggregation of asialofetuin beads (data not shown), and asialomucin inhibits the aggregation of asialofetuin-coated beads about as effectively as asialo-α_1-acid glycoprotein (Table 1). In addition, only one antigen has been found to be associated with lectin activity in *A. viscosus* (4, 22).

The present study used simple sugars and naturally occurring carbohydrate oligomers which are known to exist on many cell surfaces and in dental pellicle. The data presented here and in previous studies are consistent with the concept

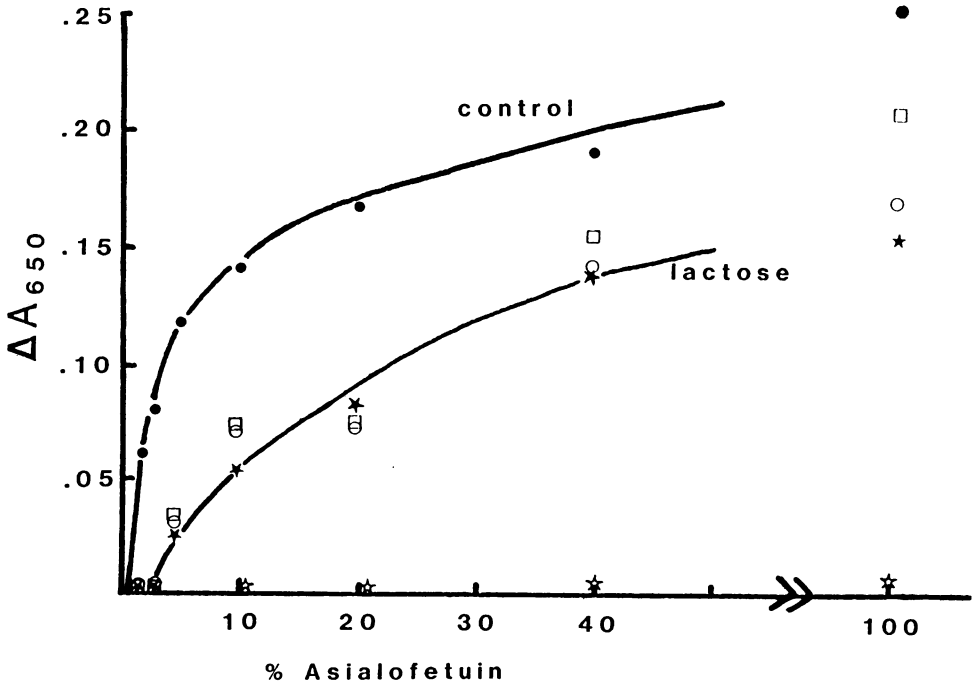

FIG. 4. Inhibition by asialooligopeptides from fetuin of *A. viscosus*-induced aggregation of latex beads coated with various mixtures of fetuin and asialofetuin. Symbols: ●, control data points with no inhibitor; ★, 0.5 mM lactose data points; □, N-linked oligopeptide of fetuin (fraction B-1) at 0.17 or 0.5 mM in Gal residues; ☆, O-linked oligopeptide of fetuin (fraction C) at 0.5 mM, or 0.5 mM in Gal residues; ○, O-linked oligopeptide of fetuin at 0.05 mM. Data points taken for the O-linked oligopeptide fractions B-2 and B-3 (data not shown) were very similar to those for fraction C.

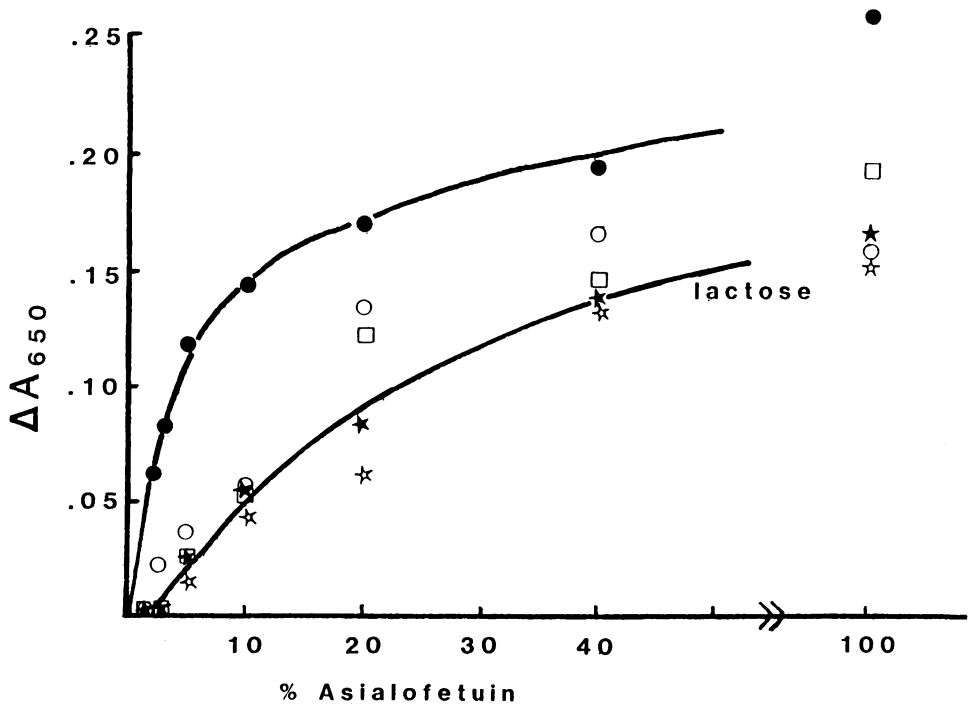

FIG. 5. Inhibition by asialooligopeptides of α_1-acid glycoprotein of *A. viscosus*-induced aggregation of latex beads coated with various mixtures of fetuin and asialofetuin. Symbols: ○, control data points; ★, 0.5 mM lactose data points; ○, biantennary oligopeptide fraction P-2; □, triantennary oligopeptide fraction P-3; ☆, tetraantennary oligopeptide fraction P-4. All inhibitors were tested at a concentration equivalent to 0.5 mM in Gal residues.

that *A. viscosus* lectin binding depends on the interactions of numerous low-affinity sites. Cisar (3) has suggested that this type of binding may have developed because salivary asialoglycoproteins present a host defense mechanism which can be more easily overcome by multiple low-affinity sites than by a smaller number of high-affinity sites. With low-affinity sites, many lectin binding sites could escape blocking by soluble asialoglycoproteins. Attachment to host tissues or to previously colonized organisms would still be possible, provided a sufficient number of lectin sites are available for binding.

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