Polysaccharide Structural Features That Are Critical for the Binding of Sulfated Fucans to Bindin, the Adhesive Protein from Sea Urchin Sperm*

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We have investigated the structural features of sulfated fucose-containing polysaccharides which are responsible for their selective binding to Strongylocentrotus purpuratus bindin. The data presented demonstrate that the sulfate esters and a molecular weight in excess of approximately 15,000 are required for high affinity binding of the fucans to bindin. Desulfation destroys the binding activity of the fucans, which can be fully restored by chemical resulfation. Sulfated fucans are common components of the cell surfaces of sea urchin sperm as previously described (1). Several lines of evidence suggest that the complementary egg surface ligands for bindin are high molecular weight, sulfated, fucose-containing glycoconjugates. Isolated "receptors" have been characterized as containing substantial amounts of carbohydrate and esterified sulfate (2, 3). Sulfated fucose-containing polysaccharides have been isolated after Pronase digestion of the egg surface which selectively bind to bindin particles, inhibit the bindin-dependent agglutination of eggs (4, 5), and inhibit fertilization (3). Sulfated fucans are common components of the cell surfaces of marine animals, including the egg jelly layer and vitelline layer in several species of sea urchins (3, 5-8) and eggs of the ascidians (9) and the ascidian Ciona (10, 11). Sulfated fucans also occur in the extracellular matrix of the sea urchin embryo (12) and adult sea cucumber (13).

Other sulfated fucose-containing polysaccharides such as fucoidan and the egg jelly fucan also bind to bindin with high affinity (14). Fucoidan has a composition similar to the fucan purified from the egg surface by affinity chromatography on bindin. Fucoidan and the egg surface fucan have identical bindin-binding activities. The interaction of bindin with sulfated fucans is specific, since other sulfated polysaccharides such as the glycosaminoglycans heparin and chondroitin sulfate show a range of affinities that are 10- to 1000-fold lower than those observed for sulfated fucans (14). Sulfate esters appear to be important for the interaction of bindin and fucoidan, since solvolytic desulfation results in the inactivation of fucoidan (14). This interpretation, however, is complicated by the fact that the desulfation procedure also results in some degree of depolymerization of the polysaccharide.

In this report, we have further investigated the polysaccharide structural features which are important for the binding of sulfated fucans to bindin. Here we report that sulfate esters and high molecular weight (more than approximately 15,000) are requirements for the high affinity interaction of sulfated fucans with bindin. We suggest that the role of the polysaccharide backbone in the selective binding of sulfated fucans to bindin is to determine the correct spatial orientation of the sulfate groups to match the corresponding structure of the active binding site of bindin.

EXPERIMENTAL PROCEDURES

Materials

All reagents and materials were obtained from Sigma with the following exceptions: chlorosulfonic acid and phthalic acid was from Aldrich, fucoidan was obtained from Pfaltz and Bauer (Stamford, CT), triethylamine was from Pierce Chemical Co., DEAE-cellulose (Cellex D) was from Bio-Rad, and o-toluidine blue was from Fluka (Ronkonkoma, NY). Bindin was isolated from Strongylocentrotus purpuratus sperm as previously described (1).

Analysis of Polysaccharides

HPLC. Analysis of the size distribution of polysaccharides and oligosaccharides was done by high performance gel filtration chromatography. A Waters Protein Pak 125 column with a 6000A pump and a U6K injector was used. The column was equilibrated in 0.1 M ammonium formate (pH 6.2) at a flow rate of 0.5 to 1.0 ml/min. Samples (30-300 µg of fucose equivalents) were adjusted to 0.1 M.
ammonium formate, clarified by centrifugation, and injected onto the column. Fractions (0.25 to 0.7 ml) were collected and assayed for sugar by the phenol/H₂SO₄ method (15). Small scale assays (1 ml total volume) were heated in a boiling water bath for 4 to 7 min after acid addition to obtain full color yield. The column was calibrated with various size dextran and dextran sulfate fractions and heparin.

Metachromatic assay—A spectrophotometric assay for charge density of the polysaccharides was adapted from previous work which was done to determine surface charge of heparin (16). This assay is based on the observation that the absorption maximum of 0-toluidine blue shifts from blue to red in the presence of polyanions in low ionic strength aqueous solutions. This metachromatic shift is due to the stacking of the cationic dye molecules along the polyanion. Five nanograms of toluidine blue was added to a series of test tubes containing from 0.01 to 500 ng of polysaccharide in 0.2 ml of H₂O. After 5 min, 1.0 ml of 10 mM sodium phosphate buffer, pH 7.0, was added with mixing. The metachromatic shift of weakly interacting polysaccharides was often dissipated upon dilution. Absorption spectra were recorded from 650 to 450 nm. The metachromatic shift titration point is reproducible for a given polysaccharide and was used as a means of comparing the apparent surface charge of the fucans. The 50% point was defined as the sugar concentration at which the dye’s absorbance at the nonshifted wavelength (628 nm) decreased to one-half of its original value in the absence of polyanions. Valid titration points were reached only if there was an indication of metachromatic shift by the appearance of a new peak at 520 ± 30 nm.

Sulfate Determination—The esterified sulfate content of the polysaccharides was determined by ion chromatography (17) after polarysis (18). Typically, a sample containing 0.5-50 μg of monosaccharide equivalents and 2-50 nmol of sulfated equivalents was mixed with 1 μl of 0.1 M NaOH and dried in a 20-mm tube which was previously cleaned with nitric acid. The samples were pyrolyzed by heating for 6 s in a Fisher burner flame, allowed to cool, and resuspended in 75 μl of water. The samples were clarified by centrifugation, and duplicate injections of this solution were analyzed on a Vydac 300C4column. The column was eluted with 1.5 mM phthalic acid (adulterant) to pH 8.0 at a flow rate of 1 ml/min. Detection was accomplished by indirect photometry at 230 nm, 0.05 absorbance units at full scale. Peak areas were integrated and the amount of sulfate was determined by external standard calibration using a Dynamic Solutions Application data acquisition system.

Purification of Egg Jelly Fucans

The large molecular weight sulfated fucans were purified from other smaller and less negatively charged components in the egg coat jelly (8). The crude egg jelly from S. purpuratus eggs was β-eliminated to degrade the egg jelly sialoglycoprotein and remove any O-linked protein. After resuspension of crude egg jelly in water, the solution was adjusted to 0.1 M NaOH with 10 M NaOH, and NaBH₄ (10 times the mass of crude egg jelly) was added. After incubation of the reaction mixture for 6-24 h at 37°C, the pH was adjusted to pH 5 with glacial acetic acid and dialyzed against distilled water. The contents of the dialysis bag were adjusted to 10 mM NaCl, 0.15 M NaCl, and 1 M NaCl, applied to a 46 × 10 cm column of DEAE-cellulose equilibrated in the same buffer. A step gradient from 0.25 to 1 M NaCl was used to elute the sulfated fucans from other egg jelly components. The eluant was tested for sugar by the phenol/H₂SO₄ assay, and fractions with fucose were pooled (approximately 0.5 M NaCl), dialyzed, and concentrated. Further chromatography on Sepharose CL-4B in 0.1 M ammonium formate, pH 7.4, yielded a white large molecular weight polysaccharide. Pronase digestion was also done on some aliquots of fucan to remove residual protein. Pronase (Behring Diagnostics) at 0.2 mg/ml in 20 mM Tris, pH 8, with 0.15 M NaCl and 4 mM CaCl₂ was incubated at room temperature with egg jelly preparations for 12 to 24 h and the fucan was recovered by gel filtration on the Sepharose CL-4B column described above.

Purification of Fucoidan

Fucoidan was β-eliminated and Pronase-digested to remove contaminating protein components as described above and then purified by gel filtration on Sephadex G-100.

Solvolysis Desulfation of Polysaccharides

Polysaccharides were desulfated by solvolysis as described (19). The various sulfated polysaccharides were first exchanged from the Na⁺ to the pyridinium salt with a Dowex 60 column equilibrated in 0.1 M aqueous pyridine. The column eluant was lyophilized thoroughly and redissolved with vortexing in dry dimethyl sulfoxide/pyridine (5:1) mixture at concentrations of 5-15 μg/ml. The desulfation reaction was conducted in a screw-cap tube containing 4 A molecular sieves. The tube was flushed with nitrogen, sealed, and then incubated in a boiling water bath for 9 h. The cooled reaction mixture was then blown under nitrogen, and fractionated by gel filtration on Sephadex G-50 and lyophilized.

Acid Hydrolysis of Polysaccharides

Mild partial acid hydrolysis was performed on fucans in 0.1 M HCl at 100°C at a sugar concentration of 0.1 mg/ml of fucose equivalents in 0.1 M HCl and 0.1 M NaCl, pH 8.0. After 5 min, 1.0 ml of TBS and 0.1 ml of distilled water were added and the sample was refolded to its original volume. The sample was then added to the reaction mixture containing 50 μg of sulfated polysaccharide in 0.2 ml of Tris-buffered saline (TBS, 0.15 M NaCl, pH 8.0). After 5 min, 50-500 ng of radioiodinated fluorescein-derivatized fucoidan (1000-5000 cpm/pg) in 0.05 ml of TBS was added and allowed to incubate for an additional 8 min. The tube was then filled with 5 ml of TBS and filtered rapidly through a glass fiber filter (Whatman GF/C). Two more times were done and the filters were collected for γ counting.

In experiments measuring the binding of 125I-fucoidan to DEAE-cellulose or polysyline (M, = 18,000), 10 μg of DEAE-cellulose or 100 ng of radioiodinated fluorescein-derivatized fucoidan (1000-5000 cpm/pg) in 0.05 ml of TBS was added and allowed to incubate for an additional 8 min. The tube was then filled with 5 ml of TBS and filtered rapidly through a glass fiber filter (Whatman GF/C). Two more times were done and the filters were collected for γ counting.

Filter Binding Assay for Fucan/Bindin Interaction

Competition of the binding of the 125I-fucoidan to bindin particles was used to determine the affinity of the various fucan fractions for bindin as previously described (14). Briefly, 2 to 10 μg of bindin was mixed with 0 to 1000 μg of polysaccharide in 0.2 ml of TBS-buffered saline (TBS, 0.15 M NaCl, pH 8.0). After 5 min, 50-500 ng of radioiodinated fluorescein-derivatized fucoidan (1000-5000 cpm/pg) in 0.05 ml of TBS was added and allowed to incubate for an additional 8 min. The tube was then filled with 5 ml of TBS and filtered rapidly through a glass fiber filter (Whatman GF/C). Two more times were done and the filters were collected for γ counting.

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Asay for the Agglutination of Eggs by Bindin

The egg agglutination assay was performed as previously described (4). The inhibition of egg agglutination by sulfated polysaccharides was determined as described (14).
RESULTS

Effect of Desulfation and Chemical Resulfation on Fucan Binding—Previous studies have indicated that after solvolysis desulfated fucoidan is a very poor inhibitor of 125I-fucoidan binding to bindin particles (14). However, the desulfation procedure results in some concomitant depolymerization of the polysaccharide as determined by HPLC gel filtration analysis. The molecular weight of fucoidan before solvolysis is approximately 100,000 (14, 23) and runs in the void volume ($V_0$) of a Protein Pak 125 column (Fig. 1). The desulfation reaction produces various size fragments of fucoidan, which elute over the entire fractionation volume of the column (Fig. 1). Two size classes of oligosaccharides accumulate: one that runs slightly before the included volume, suggesting an average molecular weight of 3,000, and a larger polysaccharide that is slightly included in the fractionation volume of the Protein Pak column, corresponding to a molecular weight of approximately 40,000. The sulfated fucoidan from egg jelly (Fig. 1) is also depolymerized during solvolytic desulfation (Table I). The apparent surface charge of desulfated fucoidan and egg jelly fucan are greatly reduced, as measured by the toluidine blue metachromatic shift titration point. This result is consistent with the decrease in sulfate ester content measured for fucoidan and egg jelly fucan (Table I).

After chemical resulfation of desulfated fucoidan with chlorosulfonic acid (CISO$_3$:fucose (100:1), 20 °C), the sulfate content is restored to values comparable to or slightly higher than the starting material (Table I). The chemical resulfation reaction does not detectably change the gel filtration profile of the fucan preparations: fucoidan still runs in the $V_0$ with no release of smaller molecular weight sugars, and desulfated fucoidan’s 2 peaks elute at the same positions: slightly after the $V_0$ and immediately before the $V_1$ (data not shown).

After chemical resulfation, the larger molecular weight desulfated fucoidan fraction has about the same inhibitory activity in the 125I-fucoidan filter binding assay as the original fucoidan from which the desulfation products were derived (Table I). The smaller fragment remains inactive after chemical resulfation (data not shown). Chemical sulfation of the starting material (fucoidan not previously desulfated) results in a slight increase in sulfate content and stimulation of inhibitory activity as compared to fucoidan (Table I). The extent of reactivation by chemical sulfation appears to be proportional to the extent of resulfation since the fucoidan preparation with an intermediate charge density displayed an intermediate inhibitory activity (Table I).

Molecular Weight Dependence of Fucan Binding Activity—The finding that the small fucoidan fragments remained inactive even after chemical resulfation suggests that large size may be important for the binding of sulfated fucans to bindin. However, fucoidan is a heteropolysaccharide and the different size fractions resulting from hydrolysis have different carbohydrate compositions. We further investigated the size dependence of fucan binding with fragments of the S. purpuratus egg jelly fucan. The egg jelly fucan is a homopolysaccharide and it exhibits a more random fragmentation pattern than fucoidan following solvolytic desulfation or mild acid hydrolysis. The solvolysis and mild acid hydrolysis products of the egg jelly fucan elute as a broad peak on the Protein Pak 125 column, with the majority of the sugar in the molecular weight range of about 18,000 to 4,000 (data not shown).

The desulfated fucan preparation was chemically resulfated and subfractionated into three size classes of fragments by gel filtration on Sephadex G-50 (Fig. 2). The molecular weight distribution of these fractions was analyzed by rechromatography on the Protein Pak 125 column (Fig. 3A). The elution profiles of the three fractions have roughly Gaussian distributions and give estimates of the average molecular weight of the three fractions of $M_1$, 15,000, 10,000, and 4,000, respectively. The starting fucan ran in the $V_0$ of the Protein Pak 125 column as expected for a polysaccharide with a molecular weight in excess of 1 million (Fig. 3A). The relative affinity of the fragments for bindin was determined by measuring their ability to compete for the binding of 125I-fucoidan to bindin particles in the filter binding assay. After resulfation, only the largest desulfated egg jelly fucan fractions ($M_1 > 15,000$) exhibit high levels of binding activity. The largest resulfated fraction ($M_1 = 15,000$) is approximately 4-fold less active than the starting material (Fig. 3B). The intermediate fraction ($M_1 = 10,000$) and the smallest fraction ($M_1 = 4,000$) are 2 and 3 orders of magnitude, respectively, less active than the starting material (Fig. 3B).

Similar results were obtained with fragments of the fucan from S. purpuratus generated by mild acid hydrolysis. The fucan was incubated in 0.1 N HCl at 100 °C for 2 min. The S.

* C. G. Glabe, unpublished data.
Binding of Sulfated Fucans to Bindin

Characterization of fucans and fucan derivatives

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Toluidine blue metachromat ic titation point</th>
<th>50% inhibition of fucoidan binding</th>
<th>Molecular weight</th>
<th>Degree of sulfation</th>
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*Expressed as percent of starting material.
ND, not determined. The sulfate content of these fractions could not be accurately determined. They were resulfated in the same reaction mixture as the higher molecular weight fractions and therefore should have similar sulfate contents.

purpuratus hydrolys products were chemically sulfated as described above and subfractionated into three size classes by gel filtration on Sephadex G-50 (Fig. 2). The size distribution of the three fractions was analyzed by rechromatography on the Protein Pak 125 column giving estimates of the average molecular weight of 13,000, 8,000 and 4,000, respectively (Fig. 4A). Once again, only the largest fraction (Mw = 15,000) displayed high activity in inhibiting the binding of 125I-fucoidan to bindin. It is only about 2-fold less active than the starting material, whereas the smaller fractions are 100-fold less active (Fig. 4B). These results, together with those obtained from the fragments produced by solvolysis, suggest that a decrease in molecular weight from 13,000 to about 8,000 is accompanied by a 100-fold decrease in binding affinity.

Effect of Ionic Strength on Fucoidan Binding—Since the previous results suggest that the binding of sulfated fucans to bindin require the presence of negatively charged sulfate esters, we investigated the effect of salt concentration on binding of fucoidan to bindin. Many simple polyanions bind strongly to anion exchange resins at low ionic strength and are displaced at higher salt concentrations. This type of binding is observed for the binding of 125I-fucoidan to polylysine and DEAE-cellulose (Fig. 5). Fucoidan binding is maximal at 0 and 20 mM NaCl (5 mM Tris, pH 8.0), respectively. Fucoidan binding to polylysine is inhibited by 50% at 0.5 M NaCl and binding to DEAE is inhibited by 50% at 0.2 M NaCl. In contrast, the ionic strength dependence for the binding of 125I-fucoidan to bindin is very different: at low ionic strength (5 mM Tris) there is no detectable binding (Fig. 5). The extent of binding increases rapidly with added salt reaching a maximum between 0.2 and 0.5 M NaCl and then decreases slowly from 0.5 to 2.0 M NaCl. Fucoidan binding to bindin is half-maximal at 1.2 M NaCl. This finding suggests that the binding of fucans to bindin is not a simple ionic interaction and that other forces such as hydrogen bonding may help stabilize the interaction between the fucan and bindin.

Effect of Size and Charge Density on the Binding of Other
weakly to bindin. This has been interpreted as a lectin-like polysaccharides, such as the glycosaminoglycans heparin, chondroitin sulfate, and dermatan sulfate, which have a charge density similar to that of the sulfated fucans, bind only weakly to bindin. This has been interpreted as a lectin-like specificity of bindin for polysaccharides containing fucose (14). Our finding that the size and charge density of the fucan fragments is critical for high affinity binding to bindin raises the question of to what extent these parameters are responsible for the observed polysaccharide specificity of bindin. The size distributions of heparin, chondroitin sulfate, and dextran sulfate fractions were also analyzed on the Protein Pak 125 column, and their relative affinities for bindin were determined. The apparent molecular weights determined for heparin and chondroitin sulfate were 15,000 and 20,000, respectively (Table II), consistent with values reported in the literature (24). The observed affinities of heparin and chondroitin sulfate are 2 to 3 orders of magnitude lower than the fucan fragments of similar size (Table II). Thus, the relatively low affinity of these glycosaminoglycans for bindin cannot be explained solely on the basis of their size.

A sulfated galactan (λ-carrageenan) from *Gigartina manoca*pus, polyvinyl sulfate (Mr = 100,000) and dextran sulfate fractions (Mr = 500,000 and 5,000) were also tested (Table II). Although the sulfated galactan has about the same size and charge density as the fucans (25), it has a very low affinity for bindin. The dextran sulfate fractions have substantially higher charge densities than the fucans. The Mr = 500,000 dextran sulfate fraction exhibits a high affinity for bindin as previously reported (3). Although binding activity of the fucans depends critically on the size of the fucan, the Mr = 5,000 dextran sulfate is only 2-fold less active than the Mr = 500,000 dextran sulfate at 0.15 M ionic strength (Table II). The small sulfated dextran is much more active than a similarly sized sulfated fucan fragment at 0.15 M ionic strength. At the seawater ionic strength of 0.54 M NaCl, however, the low molecular weight dextran sulfate is 133-fold less active than at 0.15 M NaCl. In contrast, the binding activity of the Mr = 10,000 fragment of egg jelly decreases only 10-fold 0.15 to 0.54 M NaCl.

Polyvinyl sulfate has the highest affinity for bindin of any polymer tested (Table II). It also has the highest charge density. The binding of polyvinyl sulfate is also unaffected by the salt concentration in the range of 0.15 to 0.54 M NaCl. Thus, it appears that the presence of a high density of sulfate esters on a high molecular weight polymer is sufficient for high affinity binding to bindin and that other structural features of the polysaccharide are not critical requirements for binding.

In order to test the biological relevance of the binding of polyvinyl sulfate to bindin, we determined its effect on the bindin-mediated agglutination of sea urchin eggs. The IC50 for the inhibition of bindin-mediated egg agglutination by polyvinyl sulfate was determined to be 1.5 μg/ml as compared to 7.5 μg/ml for fucoidan.

**Discussion**

This study indicates that the molecular weight and degree of sulfation of the fucans are critical determinants of the high affinity binding of fucans to bindin. Removal of 80% of the sulfate esters from fucoidan results in a 400-fold increase in the concentration of fucan required to inhibit the binding of 125I-fucoidan by 50% (IC50). Removal of only 35% of the sulfate esters from the egg jelly fucan results in a greater than 400-fold increase of the IC50. This finding suggests that the solvolyis-sensitive fraction of sulfate residues are critical for the binding of fucans to bindin. These critical sulfate residues may be located on the equatorial C-4 position of the fucans, although this remains to be demonstrated. Fucose 4-sulfate has been isolated from both fucoidan (26) and egg jelly fucan (27) after mild acid hydrolysis. For fucoidan, it has been

**Fig. 4. Characterization of the molecular weight and binding activity of egg jelly fucan fragments prepared by mild acid hydrolysis.** A, high performance gel filtration analysis of resulfated egg jelly mild acid fragments. Δ, resulfated fucan IA, 73 μg; □, resulfated fucan IB, 73 μg; ○, resulfated fucan IC, 85 μg. B, inhibition of 125I-fucoidan (approximately 30 ng) was added for each data point. The maximum specific binding was 17,500 ± 500 cpm. The nonspecific background was 1,950 ± 50 cpm. ●, starting egg jelly. ■, resulfated fucan IA. ▲, resulfated fucan IB. ○, resulfated fucan IC.

**Fig. 5. Ionic strength dependence of the binding of 125I-fucoidan to bindin, polylysine, and DEAE-cellulose.** The binding of 125I-fucoidan to bindin, polylysine, or DEAE-cellulose was determined in 5 mM Tris, pH 8, containing 0–2 M NaCl, 150,000 cpm of 125I-fucoidan (approximately 20 ng) was added for each data point. The maximum specific binding was 14,700 cpm for bindin, 42,000 cpm for polylysine, and 47,000 cpm for DEAE-cellulose. The nonspecific background increased linearly from 1,100 cpm at 0 M NaCl to 5,400 cpm at 2 M NaCl. ●, bindin. □, polylysine. Δ, DEAE-cellulose.

Sulfated Polysaccharides and Polyanions—Previous studies have shown that the binding of sulfated polysaccharides to bindin displays a fairly high degree of specificity (14). Other polysaccharides, such as the glycosaminoglycans heparin, chondroitin sulfate, and dermatan sulfate, which have a charge density similar to that of the sulfated fucans, bind only weakly to bindin. This has been interpreted as a lectin-like
Binding of Sulfated Fucans to Bindin

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Binding activity relative to fucoidan In 0.15 M NaCl</th>
<th>Binding activity relative to fucoidan In 0.54 M NaCl</th>
<th>Molecular weight</th>
<th>Charge density (electrostatic charge per monomer)</th>
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<tr>
<td>Fucoidan</td>
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<td>1.0</td>
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<td>0.3</td>
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<tr>
<td>Hyaluronic acid</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>10⁶</td>
<td>0.5</td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>&gt;4,500</td>
<td>&gt;4,500</td>
<td>20,000-25,000</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Estimated that 90% of the sulfate esters are located on the C-4 position on the basis of their stability to alkaline hydrolysis (20). In the S. purpuratus egg jelly fucan used in these studies, 40% of the sulfate esters are believed to be on the equatorial C-4 position based on infrared spectroscopy (28). This value correlates well with the observed 35-44% of sulfate esters that are sensitive to solvolysis (Table I) and suggests that the axial C-2 or C-3 position sulfate esters may be resistant to solvolysis. Previous studies have shown that the axial sulfate esters on the C-2 position of iduronic acid in heparin are resistant to solvolysis (29).

Treatment of the desulfated fucans with chlorosulfonic acid resulfates the fucans to levels comparable to or slightly higher than the starting material. This reestablishes the high affinity binding of fucan fragments larger than approximately M₉ = 10,000. Although we have not established the position at which the sulfate esters are linked, previous studies of chemical sulfation of fucose with chlorosulfonic acid have shown that rate of sulfation of the equatorial C-4 hydroxyl is much higher than the axial C-2 and C-3 positions (22).

Three different size classes of fragments were prepared from the egg jelly fucan by gel filtration after solvolysis or mild acid hydrolysis. We observed a 10-fold increase in the IC₅₀ for each M₉ = 5,000 decrease in size from 15,000 to 5,000 with the fragments produced by solvolysis. The mild acid hydrolysis fragments exhibit a 76-fold increase in the IC₅₀ as the size decreases from 13,000 to 8,000. This indicates that there is a large decrease in the binding of fucans as the degree of polymerization decreases from 60 to 20 fucose residues. This may be a reflection of a possible multivalent nature of the binding site on bindin for the fucans. A minimum of 40 fucose residues may be an indication of a requirement to span two or more active sites on the surface of the bindin particle. The requirement for large size may also be a reflection of the fact that the binding mechanism involves a large number of weak interactions.

Similar requirements for high molecular weight have been observed for the binding of heparin to fibronectin (30), endothelial cells (31), and the antithrombin III-thrombin-heparin ternary complex (32). The IC₅₀ of dextran sulfate M₉ = 500,000 for the binding of von Willebrand factor to sulfated glycoplodies is 100-fold lower than that observed for M₉ = 5,000 dextran sulfate (33). In contrast, the monovalent binding of heparin to antithrombin III (34-37), heparin cofactor II (38), and platelet factor 4 (39) exhibit a smaller minimum size requirement of approximately 8, 12, and 16 monosaccharide residues, respectively, for high affinity binding. The binding of low molecular weight (M₉ = 3,900) hyaluronic acid oligosaccharides to fibrinogen is only 50% less active than high molecular weight (M₉ = 800,000) hyaluronic acid (40).

We have extended previous observations on the polysaccharide selectivity of bindin and investigated how the structural determinants for high affinity binding contribute to the observed polysaccharide specificity. Our results demonstrate that the sulfated fucans have affinities that are at least 2 orders of magnitude higher than any of the other naturally occurring polysaccharides tested. The glycosaminoglycans, heparin, chondroitin sulfate, and hyaluronic acid have charge densities that are comparable to or higher than the sulfated fucans, and yet they display relatively low affinities for bindin. Although we found that large size is necessary for fucan binding, this cannot explain the low affinity of the glycosaminoglycans. Heparin (M₉ = 15,000) and chondroitin sulfate (M₉ = 20,000) are comparable in size to fucan fragments which have affinities for bindin that are 2 orders of magnitude higher. In addition, we found that a sulfated galactan (x-carrageenan) from G. mastocarpus, which has a charge density and size that is comparable to fucoidan, has an affinity for bindin that is 3 orders of magnitude lower than the sulfated fucans. This polysaccharide is predominantly a repeated β1-3-α1-4 linked galactose disaccharide sulfated at the C-2 positions (41). This result demonstrates that the active site of bindin can also distinguish between other polysaccharides which are not glycosaminoglycans.

Synthetic sulfated polymers such as dextran sulfate and polyvinyl sulfate have charge densities higher than the sulfated fucans and also have a high affinity for bindin. We found that salt concentration has a much more dramatic effect on low molecular weight than high molecular weight polysaccharides. For example, low molecular weight (M₉ = 5,000) dextran has an IC₅₀ that is approximately 200-fold higher than that observed for the high molecular weight (M₉ = 500,000) dextran in 0.54 M NaCl. However, at 0.15 M NaCl, the IC₅₀ for the low molecular weight dextran sulfate is only 2-fold higher than that of the high molecular weight dextran sulfate. This finding can explain the observed differences in the relative affinity of dextran sulfate for bindin reported by different groups (2, 14).

Although the finding that the negatively charged sulfate groups are critical for fucan binding may seem to indicate that the interaction of bindin and the sulfate groups is electrostatic, other results argue that the binding mechanism is more complex. Conventional electrostatic interactions governed by Coulomb’s Law demonstrate a linear reduction in interaction as the salt concentration is raised. Bindin, however, maintains a high level of activity over a range of 0.1 to 0.6 M NaCl. On the other hand, the interaction of fucan with model polycations such as DEAE-cellulose and polylysine are effectively and linearly disrupted by increasing salt concentration over the same range. The binding of fucoidan to bindin
is half-maximal at a NaCl concentration of 1.2 M. In contrast, the binding of fucoidan to DRAE-cellulose is half-maximal at 0.2 M NaCl and to polysylne is half-maximal at 0.5 M NaCl. This stability of the fucan-bindin complex at high ionic strength may be important for their function in their natural environment, sea water. The binding of fucoidan to bindin and model polycations is also very different at low salt concentrations. At low salt concentrations (5 mM Tris, pH 8.0), there is no detectable binding of the fucan to bindin, whereas the binding of the fucan to polysylne is maximal under these same conditions.

It is also possible that the sulfate groups interact with the protein by hydrogen bonding. Recent structural analysis of the sulfate binding protein of Salmonella typhimurium indicate that sulfate is bound solely through hydrogen bonding. There are no basic groups within range of the sulfate binding site (42). Ionization of the sulfate diazon is suppressed by resonance and a series of seven coordinated hydrogen bonds are made with the polypeptide. Since hydrogen bonds are very sensitive to the spatial alignment of the dipoles, the orientation of the sulfate groups could determine the affinity of polysaccharide binding. A more detailed description of the molecular basis for the selective binding of fucans to bindin must await further structural characterization of the fucans and the active site of bindin.

 Previous studies have suggested that the molecular mechanism for the selective binding of glycoconjugates by bindin may be due to a lectin-like recognition of saccharides (14). Bindin satisfies the definition of a lectin as a protein that agglutinates red blood cells and is inhibited by specific saccharides (14). Our results indicate that this interpretation may be an oversimplification, since polyvinyl sulfate also has a high affinity for bindin. This suggests that the polysaccharide backbone may not contribute directly to the binding mechanism by making hydrogen bonds or van der Waals interactions with the bindin polypeptide. Since the position of the sulfate esters on the polysaccharide appears to be important for high affinity binding, it seems likely that the precise three-dimensional location of the sulfate groups may be critical for determining binding affinity. The role of the polysaccharide backbone may be to determine the specificity of binding by providing a correct spatial orientation of the sulfate groups. The very high sulfate content of dextran sulfate and polyvinyl sulfate may make it more likely that a sulfate group is in a favorable location for binding. This suggests that the location of the sulfate residues is more important than the charge density per se. Critical sulfate residues have been identified which are important in determining the high affinity binding of other sulfated polysaccharides to proteins. Sulfation of the C-6 position of glucosamine residue 2 and the C-3 position of glucosamine residue 4 are critical determinants of the binding of the heparin octasaccharide to antithrombin III (37).

It is interesting to note that there are several other adhesive proteins that display a selective affinity for fucoidan. These include laminin, thrombospondin, and von Willebrand factor (32). Fucoidan is the most potent inhibitor of the binding of laminin and thrombospondin to sulfated glycolipids. The IC50 of fucoidan for laminin binding is 150-fold lower than heparin and the IC50 for thrombospondin is 30-fold lower than heparin. Dextran sulfate (Mw = 500,000) is the most potent inhibitor of von Willebrand factor binding, followed by fucoidan. It has been suggested that the spatial orientation of sulfate esters may also be a major determinant of the selectivity of sulfated polysaccharide binding to these adhesive proteins. Thus, bindin may represent one member of a class of adhesive proteins for which the spatial orientation of sulfate esters may play a role in determining the specificity of the adhesive protein-polysaccharide interaction.

REFERENCES