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Enzymatic Modification of Heparan Sulfate on a Biochip Promotes Its Interaction with Antithrombin III

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A heparan sulfate glycosaminoglycan chain, biotinylated at its reducing-end, was bound to a streptavidin-coated biochip. Surface plasmon resonance spectroscopy showed a low affinity interaction with antithrombin III (ATIII) when it was flowed over a surface containing heparan sulfate. ATIII bound tightly with high affinity when the same surface was enzymatically modified to using 3-*O*-sulfotransferase isoform 1 (3-OST-1) in the presence of 3′-phosphoadenosine 5′-phosphosulfate (PAPS). The 3-OST-1 enzyme is involved in heparan sulfate biosynthesis and introduces a critical 3-*O*-sulfo group into this glycosaminoglycan affording the appropriate pentasaccharide sequence capable of high affinity binding to ATIII. This experiment demonstrates the specific structural modification of a glycosaminoglycan bound to a biochip using a biosynthetic enzyme, suggesting a new approach to rapid screening glycosaminoglycan-protein interactions.

Antithrombin III (AT III) is a member of the serine proteinase inhibitor (Serpin) superfamily and is the principal inhibitor of thrombin and factor Xa, serine proteinases involved in the blood coagulation cascade. Heparin, a polysaccharide based anticoagulant, works by binding tightly to ATIII causing it to undergo a conformational change exposing a reactive loop on which the serine proteinases can act (1). Action of thrombin or factor Xa on this reactive loop in ATIII results in the formation of a 1:1 covalent complex inactivating the serine proteinase. The rate of complex formation is dramatically enhanced through the interaction of ATIII with glycosaminoglycans containing a specific pentasaccharide sequence (2). In addition to pharmaceutical heparin, an anticoagulant heparan sulfate found on the endothelium also contains this pentasaccharide sequence (3).

Heparan sulfate (HS) is initially biosynthesized as a copolymer of glucuronic acid and *N*-acetylated glucosamine by D-glucuronyl and *N*-acetyl-D-glucosaminyltransferase (4), followed by various modifications. These modifications include C_5-epimerization of glucuronic acid to form iduronic acid residue, 2-*O*-sulfation of iduronic and glucuronic acid, *N*-deacetylation and *N*-sulfation of glucosamine as well as 6-*O*-sulfation and 3-*O*-sulfation of glucosamine. Several enzymes that are responsible for the biosynthesis of heparan sulfate have been cloned (5–10). In addition, heparan sulfate *N*-deacetylase/*N*-sulfotransferase, 3-*O*-sulfotransferase and 6-*O*-sulfotransferase are present in multiple isoforms, each isoform is believed to recognize the saccharide sequence around the modification site to generate specific sulfated saccharide sequences (11–15). HS modified by 3-*O*-sulfotransferase isoform 1 (3-OST-1) binds to antithrombin to contain anticoagulant activity (16), whereas the HS modified by 3-*O*-sulfotransferase isoform 3 (3-OST-3) binds to herpes simplex 1 envelope glycoprotein D to serve as an entry receptor for herpes simplex virus 1 infection (17).

ATIII-binding HS (HS^{act}) contains defined ATIII-binding sites with the sequence of -GlcNS(or Ac)6S-GlcA-GlcNS3S(±6S)-IdoA2S-GlcNS6S- (where GlcN is glucosamine, IdoA is iduronic acid, GlcA is glucuronic acid, S is sulfate and Ac is acetate) (15, 16). The 3-*O*-sulfation of glucosamine to generate GlcNS3S(±6S) is a critical modification that results in the formation of HS^{act} (20). 3-OST-1 (EC 2.8.2.23) is the critical enzyme that forms the ATIII binding site (10, 16). Using purified 3-OST-1 enzyme to study the synthesis of HS^{act} *in vitro*, we had previously found that there were six ATIII-binding sites in a single HS^{act} chain. These results suggest that the synthesis of HS^{act} is a highly organized process and requires a specific biosynthetic

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pathway (21). Besides the important role of 3-OST-1 modification in increasing the binding of HS to ATIII, it is worthwhile to note that ATIII-binding HS also binds to fibroblast growth factor-2 receptor (22). However, it remains to be determined whether fibroblast growth factor-2 receptor and ATIII bind to identical saccharide sequences.

One of the most important considerations in understanding heparan sulfate-related biological functions is to link the saccharide structural specificity with a particular biological process. We have been focusing our attention on developing a high-throughput approach to screen the proteins that may bind to a heparan sulfate containing a specific saccharide sequence. Surface plasmon resonance (SPR) is a sensitive, real-time, optical measurement of ligand binding at the surface of a biosensor chip. This study uses SPR to examine ATIII interaction to a biochip containing HS and 3-OST-1 modified heparan sulfate (HS^{act}). Differences in the binding kinetics and dissociation constant of ATIII for surfaces containing both HS and HS^{act} are reported.

MATERIALS AND METHODS

Materials. Unlabeled 3′-phosphoadenosine 5′-phosphosulfate (PAPS), chondroitin sulfate C, Mops, Mes Chaps, Triton X-100, ADP-agaroses concanavalin A-Sepharose 4B and protamine chloride were purchased from Sigma (St. Louis, MO). Human ATIII is from Cutter Biological (Berkeley, CA). Heparin-Toyopearl was from TosoHaas (Montgomeryville, PA). Peptidoglycan heparan sulfate was prepared from bovine kidney (23) following the procedure for preparing heparan sulfate proteoglycan (24). Succinimidyl-6-(biotinamido) hexanoato (NHS-LC-biotin) and BSA were from Pierce, (Rockford, IL). Dialysis tubing was from Spectrum Medical (Los Angeles, CA). SPR measurements were performed on a BIAcore 2000 (Biacore, Uppsala, Sweden) operated using the version software. SPR buffers and streptavidin sensor chip were from Biacore.

Expression of 3-OST-1. Mouse 3-OST-1 in pcDNA3 (7) was digested with XmaI and XhoI and cloned into an XmaI/XhoI cleaved pBluescript vector (from Stratagene). The resultant construct was digested with XhoI, and partially digested with BamH1. The fragment was then cloned into BamH1/XhoI cleaved pMelBacA vector (from Invitrogen). The product and the anticipated reading frame of the expression plasimid were confirmed by DNA sequencing analysis (Nucleic Acid/Protein Research Core Facility, Children's Hospital of Philadelphia). Recombinant virus containing mouse 3-OST-1 was obtained by using MaxBac 2.0 Baculovirus kit (from Invitrogen).

Expression of mouse 3-OST-1 enzyme was achieved by infecting exponentially growing SF9 cells as described previously for the expression of 3-OST-3A (25). Briefly, fast growing SF9 cells (3 to 4 $\times$ 10^7 cells/T175 flask) were infected with 25 μl (2 to 4 $\times$ 10^7 plaque form units/ml) of recombinant viral stock solution. The cell medium (40 ml/T175 flask) was changed to serum-free medium (from Invitrogen) 48 h after infection. The medium was harvested every 24 h for 7 days. The harvested medium was centrifuged at 1000*g* for 15 min, and Chaps was added to a final concentration of 0.6%. This solution was frozen in liquid nitrogen and stored at -80°C for subsequent purification.

Purification of 3-OST-1. Purification of 3-OST-1 from serum-free medium was carried out on heparin-toyopearl gel and 3′,5′-ADP-agarose at 4°C. The harvested medium was mixed with Tris–HCl to a final concentration of 10 mM, adjusted pH to 8 with 1 N sodium hydroxide. The solution was centrifuged at 3,000*g* for 15 min to remove precipitates, and the supernatant was mixed equal volume of a buffer containing 10 mM Tris, pH 8, and 2% glycerol. The diluted medium (approximately 2 L) was loaded onto a heparin-toyopearl gel (1 $\times$ 10 cm, TosoHaas) at a flow rate of 4 ml/min. The column was then washed with 80 ml of a buffer containing 150 mM sodium chloride, 10 mM Tris, and 0.6% Chaps, pH 8. The enzyme was eluted with 30 ml of a buffer containing 500 mM sodium chloride, 10 mM Tris, and 0.6% Chaps, pH 8. The eluate was dialyzed against a buffer containing 25 mM Mops, 50 mM NaCl, 1% glycerol and 0.6% Chaps, pH 7 using a 12,000 to 14,000 MWCO dialysis membrane. The dialyzed material was loaded onto a 3′,5′-ADP-agarose column (1 ml) at a flow rate of 0.2 ml/min. The column was then washed with 10 ml of a buffer containing 50 mM sodium chloride 25 mM Mops, 1% glycerol, and 0.6% Chaps, pH 7. The enzyme was eluted from the column by a linear gradient of sodium chloride from 50 mM to 1000 mM in 60 min in a buffer containing 25 mM Mops, 1% glycerol, and 0.6% Chaps, pH 7 at a flow rate of 0.2 ml/min.

Assay of 3-OST-1 activity. The procedures for determining the activity of 3-OST-1 have been published previously (16). Briefly, in a 50 μl-standard reaction mixture, it contains 100,000 cpm of $[^{35}S]HS^{inact}$ (isolated from $[^{35}S]Na_2SO_4$-metabolically labeled wild type CHO cells), 0.5 mM PAPS, 50 mM Mes, pH 7.0, 10 mM $MnCl_2$, 5 mM $MgCl_2$, 75 μg/ml protamine, 0.4 mg/ml chondroitin sulfate C, 0.12 mg/ml bovine serum albumin, and 1% Triton X-100 (v/v). The reaction was incubated at 37°C for 20 min, and deactivated by heating to 80°C for 10 min, mixed with water (60 μl), and centrifuged at 2000*g* for 10 min to remove insoluble matters. The supernatant was added to 50 μl of 10 mM Tris–HCl, pH 7.5, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 150 mM NaCl, 0.0004% Triton X-100 (v/v), 0.02% NaN_3 (w/v) (ConA gel buffer) containing 0.1 mg/ml antithrombin and 60 μg/ml dextran sulfate. The reaction mixture was incubated at 24°C for 30 min, and 120 μl of the mixture was applied to a 200 μl-column of Con A-Sepharose 4B equilibrated with Con A gel buffer. The gel was incubated at 24°C for 15 min, washed with 2 ml of Con A gel buffer, and eluted with 750 μl of 1 M NaCl in Con A gel buffer. The eluate was mixed with scintillation fluid to determine the amount of radioactivity to calculated the percentage of $[^{35}S]HS$ bound to antithrombin/ConA gel. The percentage of HS^{act} is linearly related to the amount of 3-OST-1 activity in the reaction.

Biotinylation of peptidoglycan heparan sulfate. The *N*-succinimidal ester of biotin was reacted with the free amino groups of the peptide side-chain of peptidoglycan HS as follows. Peptidoglycan HS (10 mg, 0.71 μM) was dissolved in 1 ml of 50 mM sodium bicarbonate at pH 8.3 and incubated with NHS-LC-biotin (2 mg, 4.3 μmol) dissolved in 10 μl of dimethylformamide at 4°C for 2 h. The reaction mixture was exhaustively dialyzed (3500 MWCO) to remove unreacted biotin. Following dialysis, the biotinylated peptidoglycan HS was freeze-dried and stored at -20°C.

Immobilization of biotinylated peptidoglycan heparan sulfate onto biochip. A streptavidin sensor chip was pre-treated with 5 μl injections of 50 mM NaOH in 1 M NaCl, to remove any non-specifically bound contaminants. The sensor chip contains four lanes, and each can be modified differently. HS was bound to two of the four lanes. Biotinylated HS (5 μl 10 μg/ml) in HBS running buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA adjusted to pH 7.4, containing 0.005% (v/v) Tween-20 surfactant) was flowed, (5 μl/min) over the surface followed by a 10 μl injection of 2 M NaCl. The last two lanes in the sensor chip that are not exposed to biotinylated HS serve as control lanes to determine the contribution of non-specific interaction between streptavidin and ATIII. The surface of the biochip (all lanes) was cleaned by injecting 10 μl of running buffer containing 1 M NaCl.

Modification of biochip by 3-OST-1. The 3-OST-1 (1 μl, 70 ng) was dissolved in a buffer containing 50 mM MES, 0.1 mg/ml bovine serum albumin, 5 mM $MgCl_2$, 10 mM $MnCl_2$, 75 μg/ml protamine chloride, 1% Triton X-100, and 0.5 mM PAPS, which was added just before making an injection on the HS surface. The above enzyme

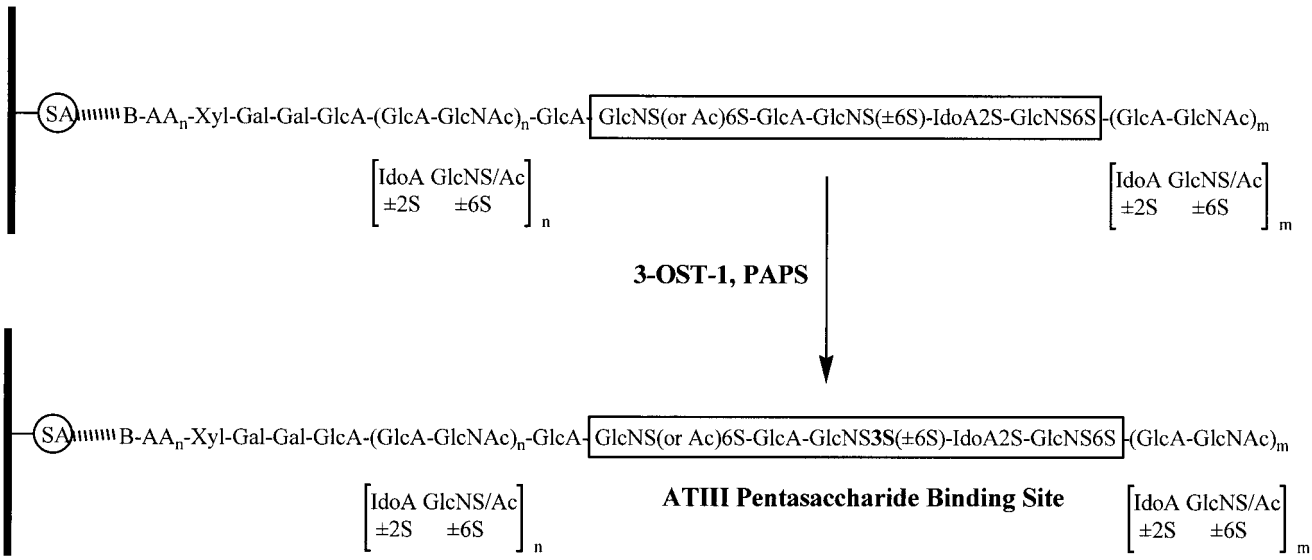

FIG. 1. A HS chain immobilized on the surface of a sensor chip is treated with 3-OST-1 and PAPS resulting in the introduction of a 3-*O*-sulfo group and the formation of an ATIII pentasaccharide binding site. Minor structural variants are shown in square brackets below each chain. SA, streptavidin; B, biotin; AA, amino acid; n + m, ~40.

solution (15 μl) was then injected for four times into the first lane, at a flow of 1 μl/min and at a temperature of 28°C, to ensure the maximum modification of HS on the biochip surface (modified HS). After each enzyme injection the surface was cleaned using a 10 μl injection of running buffer containing 1 M NaCl. The enzymatic reaction terminated and the modified HS surface in the first lane was cleaned with running buffer (HBS).

Kinetic measurement of ATIII interaction with unmodified and 3-OST-1 modified heparan sulfate. A 15 μl injection of ATIII (at concentrations ranging from 0.18 to 1.08 μM) was made at a flow rate of 5 μl/min to each of the four lanes in the sensor chip. After injection of the sample, the same buffer (without added protein) was flowed past the sensor chip surface to allow dissociation. After a suitable dissociation phase the sensor surface was regenerated for the next protein sample by injecting 10 μl of 2 M NaCl. In each experiment surface plasmon resonance in response units (RUs) was monitored as a function of time (sensogram) at 25°C. The data were fit to a simple biomolecular reaction model using the BIA Evaluation software from BIACORE (The New version 3.0.2, 1999).

RESULTS

The chemistry used for immobilization of biotinylated HS on the streptavidin surface is shown in Fig. 1. Biotinylated HS was injected multiple times over a sensor chip until a constant SPR response was observed. The immobilization of biotinylated HS was confirmed by the observation of a 330 RU increase in the sensor chip (Fig. 2A). The biotinylated HS was stably immobilized on the sensor surface, as >90% of the reactivity remained even after 10 regeneration steps using 2 M NaCl.

The modification of HS on the sensor surface was carried out by injecting 3-OST-1 in buffer containing PAPS (Fig. 1). After injecting the enzyme solution containing PAPS a response of 5500 RU was observed

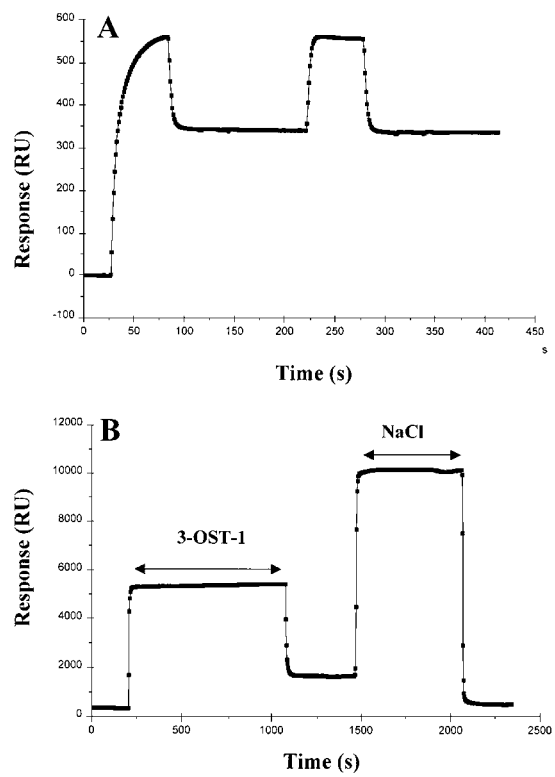

FIG. 2. Sensograms showing the modification of the HS containing biochip. (A) Sensogram of the immobilization of biotinylated peptidoglycan heparan sulfate onto a streptavidin containing biochip. Two injections of HS are shown. (B) Sensogram of the injection of 3-OST-1 and PAPS over the HS biochip. During this process a 3-OST-1 catalyzed reaction between HS and PAPS results in the introduction of a 3-*O*-sulfo group into the HS (Fig. 1). After the injection of 3-OST-1 and PAPS the surface was cleaned up with a 5 μl injection of 1 M NaCl.

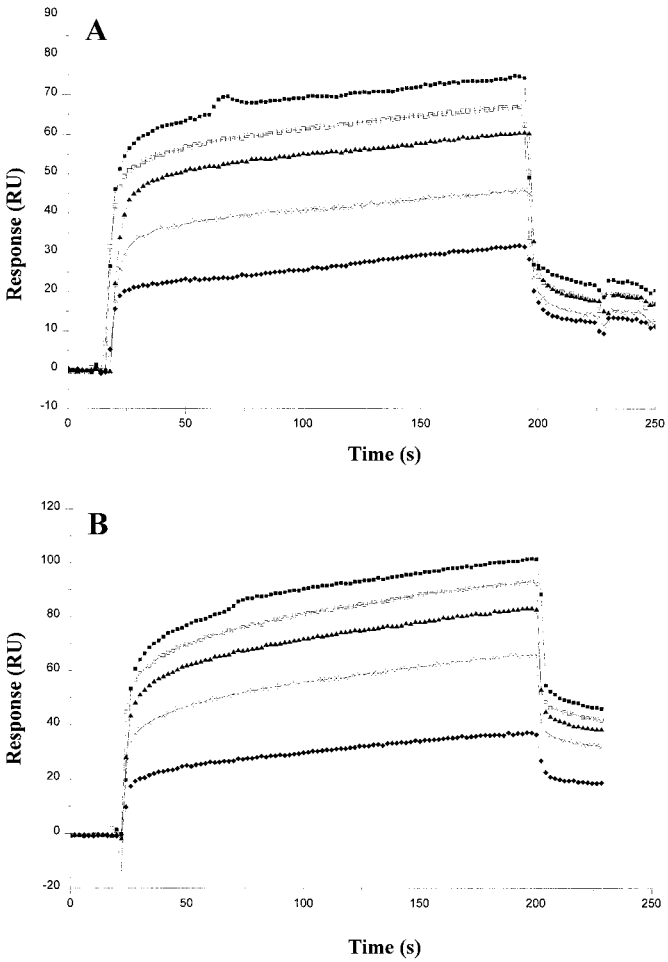

FIG. 3. Sensograms showing the interaction of ATIII with the unmodified HS surface (A) and with the 3-OST-1 modified HS surface (B).

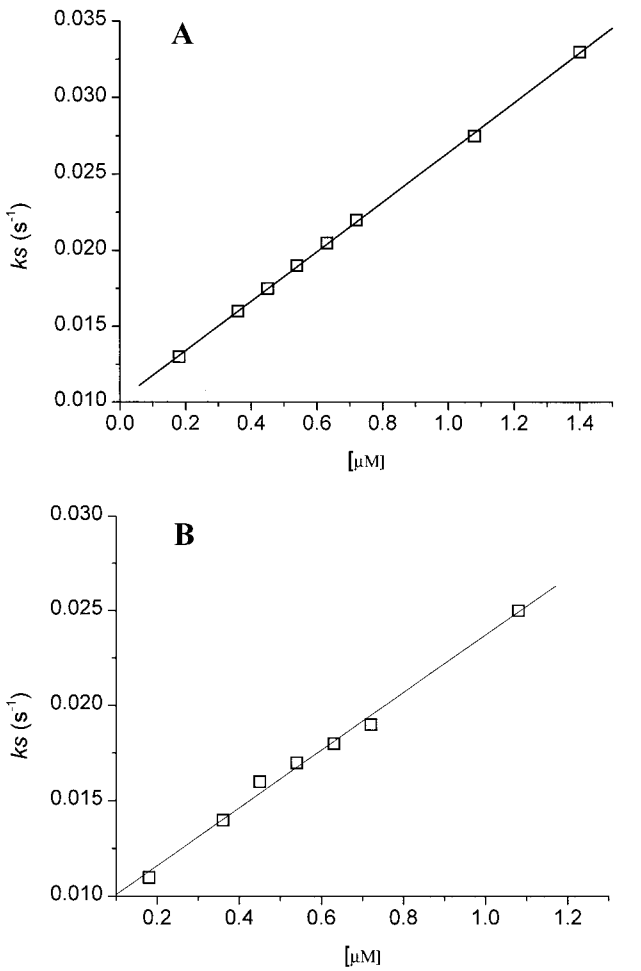

FIG. 4. A plot of the observed association rate, k_s, as a function of the concentration of injected ATIII is shown for: the unmodified HS surface (A) and with the 3-OST-1 modified HS surface (B). The slope of these plots gives k_{on} (Table 1).

showing that 3-OST-1 interacts with the HS on the surface of the chip (Fig. 2B). The bound 3-OST-1 and residual PAPS were removed from the surface by washing with NaCl and the signal returned to a baseline value (Fig. 2B).

Sensograms for the interaction of ATIII with 3-OST-1 modified and unmodified HS are shown in Figs. 3A and 3B. ATIII showed a small non-specific interaction with the underlying streptavidin surface (not shown) so that the control lanes were used to subtract the contribution of this interaction. The initial part (0–20 s) of these sensogram curves represents a buffer flowing past the sensor surface. The second and rising part of the curves (22–60 s) corresponds to the response of the sensor surface following the injection of ATIII. The final portion of the curves (195–210 s) corresponds to the dissociation of bound ATIII as the buffer flows past the sensor surface again. The R_{max} values, reflecting the maximum amount of bound ATIII, increased from 70 to 100 RU (at 1.08 μM concentration) following 3-OST-1 modification of the HS on the biosensor surface, demonstrating that the introduction of a 3-*O*-sulfo group into HS, increased the amount of surface bound ATIII.

The ATIII binding curves for the unmodified and 3-OST-1 modified HS were fitted to an A + B = AB model and analyzed by linear transformation. A plot of k_s versus ATIII concentration from the association phase (Figs. 4A and 4B) yielded the association rate constants (k_{on}) for unmodified and 3-OST-1 modified HS (Table 1). The dissociation rate constants (k_{off}) were

TABLE 1

SPR Data for Interaction of ATIII with Biochips Containing Unmodified and 3-OST-1 Modified HS

Heparan sulfate	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (nM)
Unmodified	1.7×10^4	2×10^{-3}	118
3-OST-1 modified	2.7×10^4	3×10^{-4}	11

obtained from the direct analysis of the dissociation phase of the sensograms (Figs. 4A and 4B, Table 1). The binding of AT-III to modified HS is characterized the dissociation constants K_d, where $K_d = k_{off}/k_{on}$. Modification of HS by 3-OST-1 increased its binding affinity (K_d) for ATIII by 11-fold.

DISCUSSION

Biological chips (biochips), such as those used in microarrays for oligonucleotide-chip technology in functional genomics (26, 27) and sensor chips used for screening hybridomas or phage display libraries (28, 29), represent important tools for modern biochemistry and clinical chemistry. Biochips have been used primarily to study oligonucleotide–oligonucleotide, oligonucleotide–protein, and protein–protein interactions. There are few examples of biochip technology being applied to the study of carbohydrate-protein interactions. The highly specific interaction between ATIII and a specific pentasaccharide sequence was selected for this study. A carbohydrate biochip was prepared and modified enzymatically to examine a carbohydrate-protein interaction.

The biochip in SPR is divided into discrete lanes, corresponding to a microarray, solutions of binding partners are passed over the biosensor surface and interactions are measured. HS was immobilized to two lanes of the biosensor chip through the very strong, non-covalent biotin-streptavidin interaction (30). This attachment was through the reducing-end of the glycosaminoglycan chain, resembling its natural attachment in a proteoglycan (30) and optimally exposing the glycosaminoglycan chain to the solution phase containing its protein-binding partner (31). One limitation of streptavidin chips is the non-specific interactions between streptavidin and many proteins, which must be compensated for through the use of control lanes, as done in the current study. ATIII showed some interaction with unmodified HS (Table 1). This interaction was expected, as HS is known to have a nonspecific electrostatic interaction with ATIII, known as low affinity binding (32). Furthermore, the HS peptidoglycan used in these experiments contained a small number (>3% of the chains bound an ATIII affinity column) of chains containing ATIII binding sites (HS^{act}) that would be expected to demonstrate high affinity binding. Next, 3-OST-1 in the presence of PAPS was used to modify the immobilized HS to form HS^{act}. While this modification resulted in only a slight increase in the k_{on}, it significantly decreased k_{off}, resulting in an 11-fold decrease in K_d affording a value comparable to that measured by other methods (33). The conformational change in ATIII, which takes place after binding to HS^{act} (1) may strengthen this interaction. This effect may explain the significant decrease in k_{off} observed on 3-OST-1 modification of HS.

The application of 3-OST-1 in the modification of an HS containing biochip suggests that other recently isolated enzymes, involved in glycosaminoglycan biosynthesis (4–16) might be similarly used to prepare HS chains containing very specific structural modifications. This approach opens the possibility of preparing glycosaminoglycan biochips containing structural microarrays for the screening of glycosaminoglycan-binding proteins. Such screening methods might be useful for biochemists investigating the specificity of such interactions and clinical chemists interested in using changes in heparin binding proteins for diagnostic applications.

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