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Factor H Is a Dermatan Sulfate–Binding Protein: Identification of a Dermatan Sulfate–Mediated Protease That Cleaves Factor H

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Dermatan sulfate mediates the blood coagulation cascade by binding to heparin cofactor II and potentiating the antithrombin activity. In order to explore another function of dermatan sulfate, a dermatan sulfate affinity column was prepared from biotinylated dermatan sulfate and Streptavidin Sepharose. When human plasma was applied on the dermatan sulfate column, factor H was bound and cleaved. The cleavage products, a 30-kDa N-terminal fragment and a 120-kDa fragment, were eluted from the column with 500 mM NaCl and detected after Western blotting with anti–factor H. The bond between the tandem arginine residues in the sixth domain of factor H was cleaved. When purified factor H was applied on the column, the factor H was not cleaved and was recovered from the column as an intact 150-kDa fraction. The finding that dermatan sulfate–mediated cleavage of factor H was inhibited by (*p*-amidinophenyl) methanesulfonyl fluoride, but not *N*-ethylmaleimide or EDTA, indicates that a serine protease in the plasma was activated on the dermatan sulfate column and factor H was cleaved without intervention of the plasma protease inhibitors. Amidase activity was detected in the effluent from the dermatan sulfate column but was abolished by pretreatment of the plasma with dermatan sulfate. Therefore, dermatan sulfate participates in the activation of a protease as well as having the protease inhibitory action.

Key words: complement protein, dermatan sulfate column, human plasma, proteolysis.

Abbreviations: APMSF, (*p*-amidinophenyl) methanesulfonyl fluoride; BCIP, 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt; CBB, Coomassie Brilliant Blue; CHCA, α-cyano-4-hydroxycinnamic acid; DS, dermatan sulfate; FH, factor H; FHR, factor H related protein; HC II, heparin cofactor II; NBT, Nitro Blue Tetrazolium; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; PMF, protein mass fingerprinting; SCR, short consensus repeat; TB, 20 mM Tris-HCl pH 7.5; TBS, TB containing 150 mM NaCl.

Dermatan sulfate (DS) is a sulfated glycosaminoglycan that is a constituent of various proteoglycans present on the cell surface and in the extracellular matrix (*1–3*). DS is composed of iduronic acid and *N*-acetyl galactosamine. The iduronic acid present in DS differentiates it from chondroitin sulfates (*4*). Iduronic acid also exists in heparan sulfate and heparin, which are involved in biological events including cell growth, cell division and blood coagulation (*5–8*). While there is a large body of knowledge regarding the physiological roles of heparin and heparan sulfate, only a few studies have investigated the biological functions of DS (*9–11*). The plasma proteins that interact with DS include heparin cofactor II and apolipoproteins B and E (*12–16*). Unlike antithrombin III and protease nexin-1, heparin cofactor II interacts with DS as well as heparin (*17*, *18*). Among the human plasma proteins that bound to the DS affinity column, we have identified fragments of factor H (FH). FH is a multidomain, multifunctional protein, which regulates the complement cascade alternative pathway and participates as a cofactor of factor I in the inactivation of C3b (*19*, *20*). FH is a heparin-binding protein with three distinct binding sites (*21–23*). While human plasma contains a significant amount of FH, cleaved products cannot be detected. However, cleavage of plasma-derived FH could be initiated by the action of a serine protease that is activated by DS. This report describes the unique action of DS in mediating the activation of the protease responsible for the cleavage of FH.

EXPERIMENTAL PROCEDURES

Materials—DS from pig skin was the product of Seikagaku Corporation (Tokyo, Japan). HiTrap *N*-hydroxysuccinimide (NHS)–activated and HiTrap Streptavidin columns were the products of Amersham Pharmacia Biotech Inc. (Picataway, NJ, USA). EZ-Link Sulfo-NHS-LC-Biotin and ImmunoPure HABA were the products of Pierce (Rockford, IL, USA). Adipic acid dihydrazide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, BSA and donkey anti–sheep IgG alkaline phosphatase conjugate were purchased from Sigma (St. Louis, MO, USA). Nitro Blue Tetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP), (*p*-amidinophenyl)methanesulfonyl fluoride (APMSF), DTT and iodoacetic acid were obtained from Wako (Osaka, Japan). Sheep anti–human FH was the product of The Binding Site Limited (Birmingham, UK). Human heparin cofac-

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tor II (HC II) and rabbit anti–human HC II were the products of TechnoClone G.m.b.H. (Vienna, Austria) and BioPur AG (Bubendorf, Switzerland), respectively. Benzoyl-L-arginine *p*-nitroanilide and succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide were the products of Peptide Institute, Inc. (Osaka, Japan). The following chromogenic substrates (Testzymes) were obtained from KabiVitrum AB (Stockholm, Sweden): S-2222 (isoleucyl-glutamyl-glutamyl-arginine *p*-nitroanilide for Xa), S-2238 (phenylalanyl-pipecolyl-arginine *p*-nitroanilide for prothrombin), S-2266 (valyl-leucyl-arginine *p*-nitroanilide for tissue kallikrein), S-2302 (prolyl-phenylalanyl-arginine *p*-nitroanilide for plasma kallikrein), and S-2444 (glutamyl-glycyl-arginine *p*-nitroanilide for urokinase). Copper stain kit for reversible visualization of protein bands on SDS-PAGE was the product of Bio-Rad Laboratories (Hercules, CA, USA). Attoprep MF for the extraction of proteins from SDS-PAGE gel was the product of Atto Corporation (Tokyo, Japan). Immobilon P, ZipTip C18 and C4 were obtained from Millipore Corporation (Bedford, MA, USA). α-Cyano-4-hydroxycinnamic acid (CHCA), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and standard peptides (angiotensin I and the fragments of adrenocorticotrophic hormone) for the calibration of protein mass fingerprinting (PMF) measurement were purchased from Applied Biosystems (Foster City, CA, USA).

Preparation of DS Affinity Columns—DS-N affinity column: The HiTrap NHS-activated column (1 ml in bed volume) was washed with 6 ml of ice cooled 1 mM HCl, and 2 mg of DS in 1 ml of 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 (coupling buffer) was immediately injected into it. The column was then kept for 30 min at room temperature. Unreacted DS was washed out with 3 ml of the coupling buffer. The column was washed with 6 ml of 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 (Buffer A), and 6 ml of 0.5 M NaCl in 0.1 M acetate buffer, pH 4 (Buffer B). The column was then equilibrated with Buffer A for 30 min to inactivate the remaining NHS groups. The column was washed in succession with 6 ml each of Buffer B, Buffer A, Buffer B and phosphate-buffered saline (PBS). The flow rate during the affinity column preparation was maintained below 1 ml/min or 1 drop/2 s.

bDS-N affinity column: Introduction of biotin with EZ-Link Sulfo-NHS-LC-Biotin to the amino group of the residual core peptide of DS was described previously (*24*), and 2.97 mg of the lyophilized product (bDS-N) was obtained from 4 mg of DS. The biotinylated sample was dissolved in 0.8 ml of PBS and the biotin content was measured using avidin-HABA according to the manufacturer's protocol. The incorporation of biotin per mol of disaccharide unit of DS was 0.043 mol. The HiTrap Streptavidin column (1 ml in bed volume) was washed with 10 ml of PBS and treated with the biotinylated ligand solution at a flow rate of less than 1 ml/min. After 30 min at room temperature, the unbound ligand was removed, and the column was washed with 10 ml of PBS. No biotin was detected in the breakthrough fraction, indicating that the most of the biotinylated ligand was bound to the column.

bDS-C affinity column: The conversion of the carboxyl group of DS to hydrazide derivative with adipic dihydrazide followed by biotinylation with EZ-Link Sulfo-NHS-LC-Biotin was described (*24*), and 3.87 mg of the lyophilized product (bCS-C) was obtained from 4 mg of DS. The biotin affinity column using the HiTrap Streptavidin column was prepared as above.

Application of Human Plasma to the DS Affinity Column—An aliquot (50–250 μl) of expired human plasma was diluted 10-fold with 20 mM Tris-HCl pH 7.5 (TB) and loaded onto the DS affinity column equilibrated with TB. The column was then eluted successively with TB (1.5 ml × 5), 50 mM NaCl in TB (1.5 ml × 5), 150 mM NaCl in TB (1 ml × 5) and 1M NaCl in TB (1 ml × 4). In another experiment, the column was eluted with TB (1.5 ml × 5), 50 mM NaCl in TB (1.5 ml × 5), 500 mM NaCl in TB (1 ml × 4) and 2 M NaCl in TB (1 ml × 3). An aliquot (10 μl) of the respective eluates was subjected to SDS-PAGE (10% gel) under reducing conditions, then blotted onto a PVDF membrane. The membrane was blocked with 3% BSA, treated with sheep anti–human FH followed by rabbit anti–sheep IgG conjugated with alkaline phosphatase, then visualized with BCIP and NBT. For the N-terminal amino acid sequencing of the samples, the membrane was stained with Coomassie Brilliant Blue R-250 (CBB R-250) and the blot was sequenced with a 494-cLC sequencer (Applied Biosystems, Tokyo, Japan).

The DS-bound fraction eluted with 500 mM NaCl was treated with 100 mM DTT in 50 mM $NaHCO_3$ at 56°C for 1 h followed by 50 mM iodoacetic acid for 1 h at room temperature in the dark. The excess reagents were removed by gel filtration and the reduced/carboxymethylated sample was analyzed on the DS affinity column under the same running conditions as above.

Preparation of FH and FHR from Plasma—Expired human plasma (20 ml) was dialyzed against 1 liter of TB containing 150 mM NaCl (TBS), and half of the dialyzed plasma was loaded onto a HiLoad 26/60 Superdex 200 column equilibrated with TBS at a flow rate of 1 ml/min. The flow-through fraction (100 ml) was discarded, then fractions of 2 ml were collected. An aliquot (2 μl) of every third fraction was electrophoresed on SDS-PAGE and subjected to Western blotting with anti-FH. The fractions containing FH (150-kDa band) and factor H related proteins (FHRs, 37- and 43-kDa bands) were respectively combined and concentrated using a membrane filter (Ultrafree, Millipore). The other half of the plasma sample was also chromatographed as above and the FH and FHR fractions were respectively combined. The FH fractions from two gel filtrations were combined (98.1 mg in 6 ml) and subjected to another HiLoad column preparation under the same running conditions as above. The combined FH fraction (41.4 mg in 24 ml) from the third gel filtration was dialyzed against 2 liters of TB and loaded at a flow rate of 1 ml/min onto a Q Sepharose column (bed volume 12 ml) equilibrated with TB. The column was eluted with a linear gradient of NaCl from 0 to 500 mM (80 ml each in TB), and fractions of 2 ml were collected. The fractions rich in FH were combined (16 mg) and loaded at a flow rate of 1 ml/min without buffer change onto a hydroxyapatite column (bed volume 8 ml) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl. The column was eluted with a gradient of 10 mM to 200 mM phosphate buffer (80 ml each), and fractions of 2 ml were collected. The fractions con-

taining FH were combined (2.7 mg) and applied to the bDS-N affinity column to remove minor impurities.

Preparation of FHR of the combined fraction (185 mg) from the HiLoad column was carried out with Q-Sepharose and hydroxyapatite columns as above, and the two forms of FHR were copurified. Because large amount of proteins that disturbed the sequencing of FHR were not removed by the above chromatographies, the bDS-N affinity column was used for the final step of the purification.

Substrate Specificity of the DS-Mediated Proteinase—To the fractions (50 μl) from the DS affinity column of diluted plasma sample eluted with various concentrations of NaCl in TB were added 500 μl of TBS and 5 μl of chromogenic substrates, and the increase in the absorbance at 410 nm was measured upon incubation at 37°C (*25*). Benzoyl-L-arginine *p*-nitroanilide and succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide were dissolved in H_2O to 100 mM, and Testzymes were dissolved in N-methyl-2-pyrolidone to 20 mM.

Preparation of Native and Proteolyzed FH from SDS-PAGE Gel—Human plasma (500 μl) was diluted with 4.5 ml of TB and centrifuged at 3,000 rpm for 10 min to remove the insoluble debris. The diluted plasma was loaded on a bDS-N affinity column and eluted in succession with 10 ml of TB, 8 ml of 50 mM NaCl in TB, 4 ml of 500 mM NaCl in TB, and 2 ml of 2 M NaCl in TB. Aliquots of 2 ml of each fraction were analyzed by Western blotting with anti-FH and the 500 mM NaCl eluate was concentrated and applied to Superose 12 on FPLC. Fractions containing native and proteolyzed FH were subjected to preparative SDS-PAGE under reducing conditions. After electrophoresis, the gel was stained using a copper stain kit, and unstained protein bands corresponding to FH and 120-kDa and 30-kDa fragments of FH were excised. The gel was homogenized in the SDS-PAGE sample buffer containing β-mercaptoethanol, and the protein was extracted by centrifugation using Attoprep MF.

IAsys Affinity Sensor Analysis—The binding of FH and the fragments was conducted on the IAsys AUTO+Advantage apparatus (Affinity Sensors Ltd., Cambridge, UK) at 22°C using DS immobilized on a biotin cuvette (Affinity Sensors Ltd.). The sensor surface of a biotin cuvette was equilibrated with PBS/0.05% Tween 20 (PBST) and 20 μl of streptavidin (2 mg /ml) was deposited for 15 min, and 20 μl of bDS-N (1 mg/ml) was added for 15 min after washing with PBST and 2 M NaCl. The responses of streptavidin and bDS-N depositions were 1,160 and 83 arc seconds, respectively. Before applying FH, immobilization of DS to the cuvette was tested using human plasma and BSA. Human plasma (1 μl in 50 μl of TB), which contains DS-binding proteins such as heparin cofactor ll and apolipoprotein B, gave a response of 267 arc seconds, while BSA (5 μg or 1.5 μM) gave a response of 15 arc seconds, which diminished upon changing the sample solution to TB and resulted in a rectangular sensorgram, indicating no binding of BSA to DS. The binding of FH to immobilized DS was investigated at four different concentrations from 0.093 to 0.371 μM in 50 μl of TBS. The association phase was monitored for 3 min, then the sample was replaced with 50 μl of TBS, and the dissociation process was followed for 3 min before washing with 2 M NaCl. The binding experiments of the 120- and 30-kDa fragments of FH on the IAsys apparatus were carried out as above except that higher molar concentrations were used for the 30-kDa fragment. The binding of HC ll to DS was also examined in TBS from 0.069 to 0.274 μM. The association phase of the sensorgram was analyzed with FASTfit software (Affinity Sensors Ltd.), and the association and dissociation rate constants, ka and kd, respectively, were calculated. The ratio of kd to ka represents the dissociation equilibrium constant, K_D.

In-Gel Digestion of FH—The fractions of FH and the fragments separated by SDS-PAGE followed by staining with CBB R-250 were excised from the gel, and the pieces of gel were destained by successive washing in 250 μl of 50% acetonitrile in 25 mM NH_4HCO_3 and then dehydrated with acetonitrile. The gels were incubated with 100 μl of 10 mM DTT in 50 mM NH_4HCO_3 for 1 h at 56°C. After a rinse with 50 mM NH_4HCO_3, the gels were treated with 100 μl of 50 mM iodoacetic acid in 50 mM NH_4HCO_3 for 45 min in the dark. The gels were washed with H_2O and dehydrated with acetonitrile. Porcine trypsin (Promega, Madison, USA) was diluted with 50 mM NH_4HCO_3, and a 20-μl aliquot (50 ng) was added to the gel, which were then incubated for 15 h at 37°C. The digested samples were extracted three times with 50 μl of 5% trifluoroacetic acid in 50% acetonitrile, and the combined extracts were concentrated to less than 10 μl by use of a Speed-Vac. The extracts were purified with ZipTip C18, and 0.5-μl aliquots were mixed with an equal volume of 1% CHCA on the sample board for the MALDI-TOF MS. The undigested protein samples were purified with ZipTip C4 and mixed with 1% sinapinic acid.

MALDI-TOF MS Analysis—Mass spectra of FH and trypsin-digested peptides of FH were measured with a Voyager-DE STR Biospectrometry Workstation (Applied Biosystems Japan Ltd., Tokyo, Japan). A reflector mode was applied for the in-gel digested samples, and a linear mode was used for the protein samples. The samples were mixed with either CHCA or sinapinic acid, and the laser irradiation was repeated until the appropriate intensity of the mass peaks was obtained. BSA and trypsinogen were used as external standards for mass calibration of the protein samples. PMF was carried out by Mascot (Matrix Science, London, UK), and NCBInr was used to seach the database of protein.

RESULTS

DS Affinity Column—Three types of DS affinity columns (DS-N column, bDS-N column and bDS-C column) were prepared. When DS was treated with the NHS-activated column, DS was covalently bound to the Sepharose gel matrix through the terminal amino group of the remaining core peptide residue (DS-N). Streptavidin mediated chromatography was used for the other two DS affinity columns. Biotin was introduced to either the uronic acid or the core peptide of DS (*24*). The iduronic acid of DS was converted to the hydrazide derivative, followed by modification with EZ-Link Sulfo-NHS-LC-Biotin (bDS-C). The terminal amino group of the core peptide of DS was directly modified with the biotinylation reagent (bDS-N). DS in the bDS-N column bound to the Streptavidin column at a single site, while DS in the

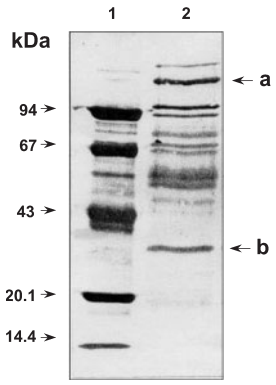

Fig. 1. **SDS-PAGE of DS column bound fraction.** Human plasma (0.5 ml) was diluted with 1 ml of TB and applied on a bDS-N column. The column was washed with 50 mM NaCl followed by elution with 1M NaCl. The bound fraction was dialyzed against 50 mM NaCl and rechromatographed on the DS column. The bound fraction eluted with 500 mM NaCl after washing with 50 mM NaCl was subjected to SDS-PAGE on 10% gel under reducing conditions. The proteins were blotted to a PVDF membrane and stained with CBB R-250. Lane 1, molecular mass marker proteins (Amersham Pharmacia); lane 2, DS column bound fraction (15 μg). The 120-kDa (a) and 30-kDa (b) bands were identified as FH fragments.

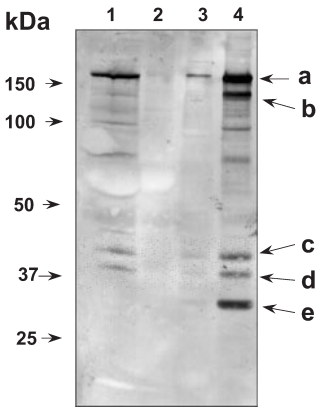

Fig. 2. **SDS-PAGE of DS column eluate.** Human plasma (0.25 ml) was diluted with TB (2.25 ml) and loaded on a bDS-N column. The column was eluted stepwise with increasing NaCl solutions. Lane 1, intact plasma (0.3 μl); lane 2, unbound fraction (6 μg); lane 3, 50 mM NaCl eluate (7 μg); lane 4, 500 mM NaCl eluate (5 μg). After electrophoresis on a 10% gel and blotting, the membrane was treated with anti-FH and visualized as described in the text. a, FH; b, C-terminal 120-kDa fragment of FH; c, FHR-1β; d, FHR-1α; e, N-terminal 30-kDa fragment of FH.

bDS-C column bound to the column at several sites. The binding capacity of the DS affinity columns was investigated by loading diluted plasma and washing away the unbound proteins with TB. The bound proteins were eluted by sequential washes with 50 mM, 500 mM, and 2 M NaCl. Very few proteins were eluted with 2 M NaCl. Elution with 150 mM and 1 M NaCl after 50 mM NaCl washing gave similar comparable amounts of protein. When the amounts of the bound fractions eluted with 500 mM NaCl from the three DS affinity columns were compared, the bDS-N showed the highest binding capacity (9.6% of the total protein), followed by DS-N (6.5%) and bDS-C (3.6%). The columns that attached DS through the core peptide had higher binding capacities than those attaching DS using uronic acid. Therefore, subsequent experiments were done with the bDS-N column.

Cleavage of FH—After charging of the DS column with human plasma, elution, and analysis of eluate by SDS-PAGE, several distinct bands were observed (Fig. 1). The N-terminal amino acids of some of the bands were sequenced. The sequence of the 120-kDa band (a) was Arg-Pro-Tyr-Phe-Pro-Val-Ala-Val-Gly-Lys-Tyr-Tyr-Ser-Tyr-Tyr-, and that of the 30-kDa (b) was Glu-Asp-X-Asn-Glu-Leu-Pro-Pro-Arg-Arg-, where X indicates an unidentified residue. An unequivocal sequence was not obtained from any of the other bands. The N-terminal sequence of the 30-kDa fragment corresponded to that of human complement FH, whereas the 120-kDa band represented the sequence beginning at the 324th residue of FH (*19*). To further investigate the behavior of FH on the DS column, effluents from the column after washes with TB, 50 mM NaCl and 500 mM NaCl were subjected to Western blotting (Fig. 2). Unfractionated human plasma was examined in the immunoblots in the same experiment Three major bands (150 kDa, 120 kDa and 30 kDa) and two faint bands (43 kDa and 37 kDa) were observed in the 500 mM NaCl eluate, but a 150-kDa band corresponding to intact FH and two faint bands around 40 kDa appeared in the intact plasma sample, indicating that FH was cleaved during the DS column application. FH has three heparin-binding sites, but none are present on the 30 kDa N-terminal region (*22*). The cleavage of the 6th domain was not sufficient to isolate the 30-kDa and the 120-kDa proteins due to the disulfide bonds within the domain. Therefore, the DS column-bound fraction was reduced with DTT, treated with iodoacetic acid and then rechromatographed on a second DS column. The bound fraction of the unreduced sample eluted with 500 mM NaCl from the second column showed bands similar to those that were detected after the initial elution (Fig. 3, lane 1). The reduced and carboxymethylated sample that was also eluted with 500 mM NaCl showed a pattern similar to the unreduced sample, although the bands were blurred (Fig. 3, lane 2).

In order to clarify the mechanism of the cleavage of FH, we attempted to purify FH from plasma. Conventional procedures such as gel filtration, ion exchange and hydroxyapatite columns could not remove the contaminating haptoglobin from FH. The purification of undegraded FH was achieved using the DS column at the final step. The faint bands of 43 kDa and 37 kDa (Fig. 2, c and d, respectively) that cross-reacted with anti-FH were purified from the plasma. The 43- and 37-kDa bands were identified as FHR-1β and FHR-1α, respectively (*26*, *27*) as determined by both the identical N-terminal sequence (Glu-Ala-Thr-Phe-X-Asp-Phe-Pro-Lys-Ile-) and molecular weights.

DS-Mediated Proteolysis—Because a protease in the plasma may be responsible for the generation of the 30-kDa fragment from FH, the effect of protease inhibitors in the eluents was investigated. The addition of a serine protease inhibitor such as APMSF (1 mM) to the eluent throughout the chromatography prevented the cleavage of FH (Fig. 4, lane 3). However, EDTA (5 mM), a metalloprotease inhibitor, and *N*-ethylmaleimide (5 mM), a thiol

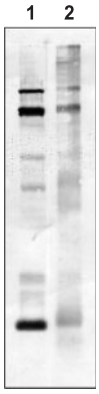

Fig. 3. **Rechromatography of DS bound fractions.** The DS column-bound fraction (750 μg) was reduced and carboxymethylated as described in the text and analyzed on a bDS-N column. The unreduced sample was also subjected to the second DS column. An aliquot (10 μl) of the fraction eluted with 500 mM NaCl from the second DS column was subjected to SDS-PAGE followed by Western blotting with anti-FH as described in the text. Lane 1, unreduced sample (1.8 μg); lane 2, reduced and carboxymethylated sample (1.4 μg).

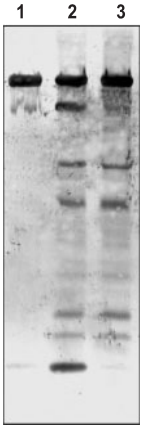

Fig. 4. **Effect of protease inhibitors.** Purified FH (74 μg) and human plasma (0.1 ml) diluted with TB (0.9 ml) in the presence or absence of a protease inhibitor were loaded on a bDS-N column and eluted with TB, 50 mM NaCl and 500 mM NaCl. An aliquot of the 500 mM NaCl eluates was analyzed on SDS-PAGE followed by Western blotting with anti-FH as described in the text. Lane 1, FH (0.75 μg); lane 2, plasma (3 μg); lane 3, plasma eluted in the presence of 1 mM APMSF (2.8 μg).

protease inhibitor, had no effect. As shown in Fig. 4, lane 1, purified FH was not cleaved, indicating that the plasma components were necessary for FH cleavage on the DS column. The effect of the serine protease inhibitor was investigated with or without APMSF in the eluents. When the column was washed with TB containing the inhibitor, the cleavage of FH was observed. However, addition of the inhibitor to the 50 mM or 500 mM NaCl elution buffer prevented the appearance of the 30-kDa band (Fig. 5, lanes 2 and 3). Because it was evident that a serine protease was activated on the DS affinity column, the effect of DS in solution on the cleavage of FH was studied. When diluted plasma pretreated with 10 μg of DS was applied to a DS column, the eluate with 50 mM or 500 mM NaCl buffer did not contain the 30-kDa fragment (data not shown).

Fig. 5. **Effect of APMSF in the eluent.** Human plasma (0.1 ml) was diluted with TB (0.9 ml) and loaded on a bDS-N column. APMSF (1 mM) was added to the eluent solution. An aliquot (3–4 μg) of 500 mM NaCl eluate was analyzed on SDS-PAGE followed by Western blotting with anti-FH as described in the text. Lane 1, TB containing APMSF; lane 2, TB containing APMSF and 50 mM NaCl; lane 3, TB containing APMSF and 500 mM NaCl.

Since the DS-mediated protease cleaved the peptide bond between tandem arginine residues, amidase activity of the effluents was measured with the chromogenic substrates of trypsin and elastase. The 500 mM NaCl eluate from the DS column hydrolyzed benzoyl-L-arginine *p*-nitroanilide but not succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide (data not shown). To investigate the specificity of the activated protease, arginine-specific chromogenic substrates (Testzymes) were tested. Figure 6 shows the amidase activity of the 500 mM NaCl eluate from the DS column after application of human plasma and washing with TB and 50 mM NaCl according to the method of Gallimore and Friberger (*25*). The plasma kallikrein substrate (S-2302) was found to be most active, followed by the tissue kallikrein substrate (S-2266). Accordingly, the amidase activity of the intact plasma and the DS-pretreated plasma eluted from the DS column was compared with S-2302. As shown in Fig. 7, most of the amidase activity of the plasma was present in the 500 mM NaCl eluate. However, there was very little activity in any of the fractions from plasma that were pretreated with DS. The absence of the amidase activity reflected the prevention of the FH cleavage by the plasma treated with DS.

Interaction of DS with the Native and Proteolyzed FH—The real-time interaction of FH and its fragments with DS was investigated by use of the IAsys, which uses the optical phenomenon of an evanescent field and a resonant mirror detector. HC ll interacts with DS as well as haparin and facilitates the inhibition of thrombin (*12*, *13*). Therefore, HC ll was included in the IAsys analysis for comparison. The sensorgrams of FH, the 30-kDa and 120-kDa fragments, and HC ll on the DS immobilized cuvette of the IAsys are shown in Fig. 8. The graduating intensity of the 30-kDa fragment, as well as that of the FH and the 120-kDa fragments, indicated the dose-dependent increase in the response. The dissociation equilibrium constants obtained from the association phase of the sensorgrams are 3.15×10^{-7} M, 2.99×10^{-7} M, 7.58×10^{-7} M and 3.91×10^{-7} M for FH, the 120-kDa fragment, the 30-kDa fragment and HC ll, respectively. These

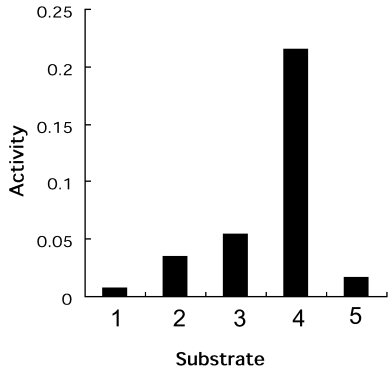

Fig. 6. **Substrate specificity of a DS mediated protease.** An aliquot (50 μl) of the 500 mM NaCl eluate from a bDS-N column was incubated with 5 μl of each chromogenic substrate in TBS (0.5 ml) for 30 min at 37°C. The activity indicates the increase in the absorbance at 410 nm. Column 1, S-2222; column 2, S-2238; column 3, S-2266; column 4, S-2302; column 5; S-2444.

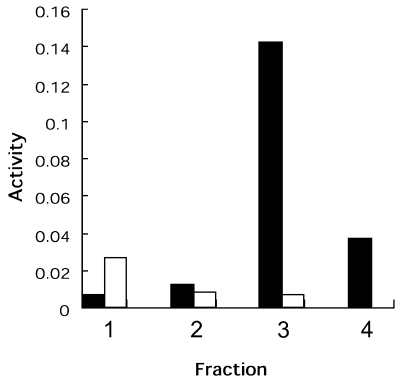

Fig. 7. **Amidase activity of bDS-N column eluates and the effect of DS pretreatment.** Human plasma (50 μl) was diluted with TB (450 μl) with or without 25 μg of DS and applied on a bDS-N column. A 50-μl aliquot of the eluate was incubated with 5 μl of S-2302 in TBS (0.5 ml) for 20 min at 37°C. The activity indicates the increase in the absorbance at 410 nm. Column 1, flow-through fraction; column 2, 50 mM NaCl eluate; column 3, 500 mM NaCl eluate; column 4, 2 M NaCl eluate. Solid bars, the plasma was diluted with TB; open bars, the plasma was pretreated with DS.

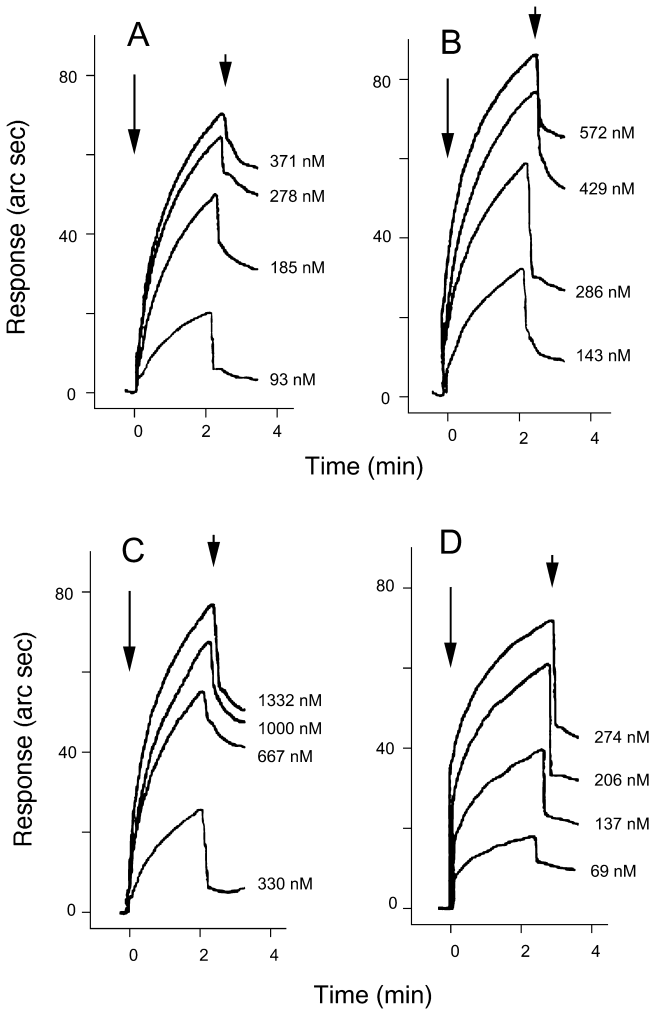

Fig. 8. **Interactions of DS with native and proteolyzed FH by the IAsys.** To the DS-immobilized cuvette was added the indicated concentration of FH (A), 120 kDa fragment of FH (B), 30 kDa fragment of FH (C) and HC ll (D) as described in the text. The long arrows indicate the addition of the samples and the short arrows indicate the change of the samples to TBS.

data clearly show that the DS-binding site exists between domains 1 and 6.

Mass Spectrometry of Native and Proteolyzed FH—The molecular weights of the 30-kDa and 120-kDa fragments of FH, as well as the intact form of FH, were determined by MALDI-TOF MS. The cleaved fragments were separated by SDS-PAGE under reducing conditions and extracted from the gel following reverse staining with copper ion. The monoprotonated form of the 30-kDa fragment showed an average molecular mass of 36,411.4. The 120-kDa fragment showed a rather broad peak with a molecular mass of 116,875.5. The intact form of FH gave two distinct peaks at 152,888.9 and 76,992.8, which corresponded to the mono- and di-protonated forms, respectively.

The cleavage site of FH by DS-mediated protease determined by the N-terminal sequencing of the 120-kDa fragment was confirmed by PMF. The sequences of the peptide derived by the trypsin digestion of FH, and the 30-kDa and 120-kDa fragments were investigated around the cleavage site at residues 323–324. The intact form of FH showed mass peaks corresponding to amino acids 314–323, 324–333 and 314–333. The 120-kDa fragment also showed the same peaks, although the most intense peak of 1,133.7 corresponded to amino acids 324–333. The peaks derived from the 120 kDa fragment were dispersed from 314 to the C- terminus. The 30-kDa fragment, on the contrary, showed peaks at amino acids 291–301, 302–313 and 291–313, and the mass peak of residues 314–323 was not detected. These data show that the initial cleavage was at residues 313–314 (K-H), rather than 323–324 (R-R), and the release of the 314–323 fragment followed the cleavage.

DISCUSSION

In this study, we report the binding of human plasma proteins to DS affinity columns. DS was immobilized

through either the carboxy group of iduronic acid or the amino group of the core protein, and the latter resulted in a higher binding capacity. Moreover, the immobilization of DS using the streptavidin-biotin binding was more effective than the covalent linkage for the retention of the plasma proteins. The bound proteins eluted with 150 mM or 500 mM NaCl after washing with 50 mM NaCl were identified as FH fragments. FH is a 150-kDa glycoprotein and a regulator of the alternative pathway of the complement (*28*). FH consists of 20 repeats of a characteristic 60–amino-acid domain designated as the short consensus repeat (SCR). Each of the repeated 60–amino-acid domains has two conserved disulfide bonds. When several functional SCR domains of FH were characterized, the cofactor activity was located in SCRs 1–4, the C3 binding site in SCRs 1–4, 8–14 and 19–20, and the streptococcal M-protein binding site in SCR 7 (*20*). The heparin binding site in FH has been confirmed to reside in domains 7, 13 and 20 by a heparin agarose affinity column using various recombinant FH proteins (*21–23*). The recombinant protein having the SCR domains 1–5 and another recombinant protein with the domains from 1–6 and 8–9 had little heparin-binding activity.

The domains 1–6 derived by trypsin digestion of FH did not bind to the heparin affinity column (*22*). However, the present results using the DS affinity column and IAsys on a DS immobilized cuvette revealed the presence of the DS-binding site in the SCR domains 1–6. We have demonstrated the binding of the diluted plasma to the DS column in TB containing 50 mM NaCl, not 150 mM NaCl. Most of the bound proteins were eluted with 150 mM NaCl. FH and its fragments were included in this eluate, and HC ll, which is confirmed to bind to DS and potentiate the thrombin inhibition (*12*, *13*), was also eluted with 150 mM NaCl (data not shown). Moreover, previous binding experiments of FH and recombinant proteins to a heparin affinity column were conducted in 75 mM NaCl in 2.5 mM sodium vernal pH 7.4 (*21*) or in 50 mM NaCl in phosphate buffer, pH 7.4 (*22*, *23*). On the other hand, IAsys experiments of FH and its fragments as well as HC ll were carried out in TBS. Our results show that the dissociation constant of FH and immobilized DS was 315 nM, a comparable value to that of HC ll. The dissociation constants quantified by IAsys following interaction with midkine were reported to be 61.6 nM for chondroitin sulfate E and 204 nM for heparin (*29*); and those quantified with Biacore equipment following interaction with immobilized C3b were 130 nM for FHR-3 and 340 nM for FHR-4 by Hellwage and colleagues (*30*). The dissociation constant of the 30-kDa fragment of FH with DS was 758 nM, comparable to that of FHR-4 with C3b. Because our experiments employed DS, discrepancies in the results of the binding assays may be attributed to differences between glycosaminoglycans. The previous investigations on the heparin binding of FH were carried out using an affinity column. The use of optical phenomena such as an evanescent field or surface plasmon resonance to study the kinetics of heparin binding by FH has not been reported. As the biosensor system has higher sensitivity than the affinity column and detects a wider range of interactions, the presence of the 4th heparin binding domain in the N-terminal region of FH is not excluded. The precise domain of FH that interacts with DS was not identified in our experiments, but our data, based on sequence comparison with domains 7, 13, and 20, pointed to domain 5. A cluster of basic amino acids is found in the one-third of the N-terminus that has the heparin-interacting domains. The sequence of RIKHR was also in a similar region in the domain 5.

The N-terminal sequence of the 150-kDa band in the intact plasma sample that was blotted to the membrane corresponded to a mature form of FH, while the 42-and 37-kDa bands showed the identical N-terminal sequence and were confirmed to be the two glycosylation isoforms of FHR-1. FHR-1 consists of five domains of SCR, and SCR-5 shows 97% amino acid identity with SCR-20 of FH, which accounts for the capacity of FHR-1 to bind DS as well as heparin (*27*, *31*). Other homologous proteins of FHR-1, such as FHR-2, FHR-3, FHR-4 and FHR-5, are present in the human plasma and have a domain homologous to the heparin-binding site (SCR20) of FH (*32–36*). Therefore, these FHR proteins would bind to the DS column, but we have not detected them in the eluate.

PMF analysis revealed that the tryptic digest of the 30-kDa fragment of FH included 39% of the N-terminal region of FH, and the digest of the 120-kDa fragment included 30% of the C-terminal region. However, in-depth analysis of the peptides showed that the decapeptide (314–323) that was expected to be the C-terminal of the 30-kDa fragment, was present in the 120-kDa fragment. Because the intensity of the mass peak of 314–323 peptide was less than that of the 324–333 peptide, the initial cleavage site appeared to be 313–314. Following the cleavage at 313–314, most of the N-terminal decapeptide of the 120-kDa fragment was removed by a protease.

Evidence for the DS-mediated activation of a protease and the subsequent cleavage of FH is as follows. Fragments of FH were detected in the eluate from the DS column but not in the intact plasma (Fig. 2). The cleavage of FH occurred between the tandem arginine bonds and was prevented by a serine protease inhibitor, such as APMSF (Fig. 4). Without the plasma component, purified FH was not cleaved on the DS column (Fig. 4) The pretreatment of the plasma with DS prevented the subsequent decomposition of FH on the DS column, indicating that the activated protease was inactivated by the inhibitors in the plasma. Finally, amidase activity was detected in the eluent from the DS column following application of the plasma (Figs. 6 and 7). These findings suggest that the inhibitors were eluted from the DS column before the protease was activated. If the plasma protease inhibitors are eliminated prior to the activation of the protease, FH would be cleaved in the solution. When methylamine was added to the plasma to inactivate α-2-macroglobulin, a protease inhibitor having broad specificity (*37*), and then the plasma was treated with DS, the cleavage of FH in the solution was not observed (data not shown). A more specific protease inhibitor may be responsible for the inactivation of the DS-mediated protease.

Trypsinization of purified FH has been investigated by several groups (*21*, *38–40*). The tandem arginine bonds in SCR 6 represented the primary tryptic cleavage site. This is the site that is cleaved by the DS-mediated protease, but trypsin cleaved additional sites of the 120-kDa fragment, resulting in products of 66, 48, and 18 kDa. The DS-mediated protease activity was more specific than

that of trypsin, since additional cleavage was not detected after prolonged treatment. Because the 500 mM NaCl eluate from the DS column showed the plasma kallikrein activity (Fig. 6), the effect of plasma kallikrein on FH was investigated. However, plasma kallikrein did not cleave intact FH, and DS did not affect the activity. Okada et al. reported that low molecular weight heparin enhanced the amidase activity of human plasma several-fold using a chromogenic substrate for thrombin (*41*). The partially purified protease cleaved both tissue-type and urokinase-type plasminogen activators, and low molecular heparin increased the activity. Further, the activity was not inhibited by antibodies against plasminogen, factor Xll or plasma kallikrein (*41*). Prevention of FH cleavage by APMSF in the 50 mM NaCl, as well as the 500 mM NaCl, eluent raises the question of whether the protease in the 500 mM NaCl eluate that was specific for plasma kallikrein was the same as the one activated by DS. It is likely that DS had activated a protease eluted with 50 mM NaCl, and in turn the protease either directly or indirectly activated the plasma kallikrein like protease in the 500 mM NaCl eluent. Therefore, a cascade reaction, common to blood coagulation systems, might be occurring. Glycosaminoglycans such as heparin and DS play an important role in potentiating protease inhibitors in the coagulation systems (*5*, *6*, *11*). If DS directly or indirectly activates the protease in the coagulation systems, DS would participate in both the coagulation and anticoagulation systems. Further work will be needed to determine whether the protease in 500 mM NaCl eluate was responsible for FH cleavage. Studies on the physiological significance and mechanism of the DS-mediated activation of the protease will provide insight into a new function of glycosaminoglycans.

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