Pentosan polysulfate increases affinity between ADAMTS-5 and TIMP-3 through formation of an electrostatically driven trimolecular complex

Linda TROEBERG*[1], Barbara MULLOY†, Peter GHOSH‡, Meng-Huee LEE§, Gillian MURPHY§ and Hideaki NAGASE*

*The Kennedy Institute of Rheumatology, University of Oxford, 65 Aspenlea Road, Hammersmith, London W6 8LH, U.K., †National Institute for Biological Standards and Control, South Mimms, Hertfordshire EN6 3QG, U.K., ‡Proteobioactives Pty Ltd, 27/9 Powells Road, Brookvale, New South Wales 2100, Australia, and §Cambridge Research Institute, Li Ka Shing Centre, Robinson Way, Cambridge CB2 0RE, U.K.

The semi-synthetic sulfated polysaccharide PPS (pentosan polysulfate) increases affinity between the aggrecan-degrading ADAMTSs (adamalysins with thrombospondin motifs) and their endogenous inhibitor, TIMP (tissue inhibitor of metalloproteinases)-3. In the present study we demonstrate that PPS mediates the formation of a high-affinity trimolecular complex with ADAMTS-5 and TIMP-3. A TIMP-3 mutant that lacks extracellular-matrix-binding ability was insensitive to this affinity increase, and truncated forms of ADAMTS-5 that lack the Sp (spacer) domain had reduced PPS-binding ability and sensitivity to the affinity increase. PPS molecules composed of 11 or more saccharide units were 100-fold more effective than those of eight saccharide units, indicating the involvement of extended or multiple protein-interaction sites. The formation of a high-affinity trimolecular complex was completely abolished in the presence of 0.4 M NaCl. These results suggest that PPS enhances the affinity between ADAMTS-5 and TIMP-3 by forming electrostatically driven trimolecular complexes under physiological conditions.

Key words: adamalysin with thrombospondin motifs 5 (ADAMTS-5), aggrecanase, extracellular matrix, glycosaminoglycan, osteoarthritis, tissue inhibitor of metalloproteinases 3 (TIMP-3).

INTRODUCTION

ADAMTS-5 [adamalysin with TS (thrombospondin) motifs 5] is considered to play a key role in the development of OA (osteoarthritis) through its degradation of aggrecan, the major proteoglycan component of the cartilage extracellular matrix [1,2]. As there are currently no disease-modifying therapeutics available to treat OA, there has been considerable interest in developing ADAMTS-5 inhibitors to block aggrecan degradation in cartilage. Synthetic ADAMTS-5 inhibitors [3,4], as well as the endogenous ADAMTS inhibitor TIMP (tissue inhibitor of metalloproteinases)-3 [5], are able to block aggrecan degradation in cartilage explants.

In an alternative approach, we have demonstrated that PPS (pentosan polysulfate), a semi-synthetic molecule manufactured from beechwood hemicellulose by sulfate esterification of the xylopyranose hydroxy groups, is able to block aggrecan degradation by shifting the balance between ADAMTS-5 and TIMP-3 in the cartilage by blocking LRP [LDL (low-density lipoprotein) receptor-related protein]-mediated endocytosis of TIMP-3 by chondrocytes [6]. PPS is also able to directly inhibit the aggrecanolytic activity of ADAMTS-5 and, more importantly, it increases the affinity between ADAMTS-5 and TIMP-3, allowing lower concentrations of TIMP-3 to effectively inhibit ADAMTS-5 [6]. This is of particular importance given that TIMP-3 levels are reduced in OA cartilage [7].

In the present study we investigated the molecular mechanism by which PPS increases the affinity between ADAMTS-5 and TIMP-3, since elucidation of the mechanism of action and of the structural determinants required for PPS activity will enable development of novel chondroprotective agents able to increase the ADAMTS–TIMP-3 affinity with an improved pharmacokinetic profile. Kinetic analysis of the ADAMTS-5–TIMP-3 interaction has become more amenable since the development of fluorescent peptide substrates for the enzyme [8,9]. We used such a substrate to define the regions of ADAMTS-5 and TIMP-3, as well as the minimal size of PPS required for the affinity increase. We have found that forms of ADAMTS-5 and TIMP-3 with reduced ability to bind to PPS were less susceptible to the affinity increase, and demonstrate that the increase in affinity involves the reversible formation of an electrostatically driven trimolecular complex.

MATERIALS AND METHODS

Materials

Sodium PPS was from Bene-PharmaChemie. Recombinant human TIMP-3, N-TIMP-3 (N-terminal domain of TIMP-3) and ADAMTS-5 (full-length and truncated forms) were prepared with a C-terminal FLAG tag, as described previously [9,10]. TIMP-1, MMP (matrix metalloproteinase)-1 catalytic domain, MMP-2 and MMP-3 catalytic domain were prepared as described previously [9]. TIMPs were quantified by titration against a known concentration of the catalytic domain of MMP-3, and ADAMTS-5 and MMPs were quantified by titration against a known concentration of N-TIMP-3 and TIMP-1 respectively. Porcine heparin, M2 anti-FLAG chromatography resin and antibody were from Sigma–Aldrich. Streptavidin–Sepharose was from GE Healthcare.

Fluorescent substrate assays

The activity of ADAMTS-5 was monitored using the fluorescent peptide substrate o-aminobenzoyl-Thr-Glu-Ser-Glu~Ser-Arg-Gly-Ala-Ile-Tyr-(N-3-[2,4-dinitrophenyl]-L-2,3-di-aminopropionyl)-Lys-Lys-NH$_2$ [Abz-TESE~SRGAIY-Dpa-KK] (custom synthesized by Bachem) at a final concentration of 20 μM with an excitation wavelength of 300 nm and an emission wavelength of 430 nm (420 nm cut-off) [9]. All assays were conducted at 37 °C in TNC buffer [50 mM Tris/HCl, 150 mM NaCl, 10 mM CaCl$_2$, 0.05 % Brij-35 and 0.02 % sodium azide). This buffer was prepared with a pH of 7.70 at 20 °C, giving a pH of 7.30 at 37 °C.

Assays were conducted in sealed 0.5 ml microfuge tubes with endpoint fluorescence measured after 18 h using a Gemini microplate spectrofluorometer (Molecular Devices). A series of triplicate endpoint measurements confirmed that the increase in fluorescence was linear over the 18 h assay period, and that ADAMTS-5 and TIMP-3 remained stable over 18 h under these assay conditions. PPS concentrations of up to 10 μM had no effect on ADAMTS-5 hydrolysis of the synthetic substrate.

The MMP-1 catalytic domain, MMP-2 and MMP-3 catalytic domain were assayed as described previously [9].

Measurement of the inhibition constant K_i

K_i values were determined under steady-state kinetic conditions [9,11]. Various forms of ADAMTS-5 (0.5 nM), MMP-1 catalytic domain (1 nM), MMP-2 (0.02 nM) or MMP-3 catalytic domain (1 nM) were incubated with TIMP-3 (0.1–10 nM) in the presence of various concentrations of PPS for 1 h at 37 °C. Steady-state rates of substrate hydrolysis (v_s) were then determined (18 h, 37 °C). The tight-binding equation [11] (eqn 1) was fitted to the data using Prism software (GraphPad).

$$\frac{v_s}{v_o} = 1 - \frac{E_o + I_o + K_{i(app)} - \left[(E_o + I_o + K_{i(app)})^2 - 4E_o K_{i(app)}\right]^{\frac{1}{2}}}{2E_o}$$

(1)

where v_o is steady-state rate of substrate hydrolysis in the absence of TIMP-3, E_o is the total enzyme concentration, I_o is the total inhibitor concentration and $K_{i(app)}$ is the apparent inhibition constant. K_i was calculated by dividing $K_{i(app)}$ by a $(1 + [S]/K_m)$ value of 1.26 [9], assuming a linear competitive mechanism for inhibition of ADAMTS-5 by TIMP-3 in the presence of PPS.

Biotinylation of PPS

Sodium PPS (100 mg) was converted into the TBA (tetrabutyl ammonium) salt by incubating with TBA bromide (100 mg) (Sigma–Aldrich) dissolved in 10 ml of de-ionized water for 4 h at ambient temperature. PPS-TBA was dialysed against de-ionized water for 24 h to remove salt and freeze-dried. PPS-TBA (50 mg, dissolved in 1 ml of DMSO) was mixed with 1,1-carbonyl di-imidazole (28 mg in 0.5 ml of water; Sigma–Aldrich) and heated at 56 °C for 1 h. Biotin hydrazide (47.8 mg; Sigma–Aldrich) was added and the solution was incubated with shaking for 16 h at 45 °C. The biotinylated PPS TBA salt was converted into the sodium salt by addition of excess 4 M NaCl, dialysis against water and freeze-drying. Bio-PPS (biotinylated PPS) was purified by size-exclusion chromatography on a Superdex-200 column (GE Healthcare) equilibrated in 0.25 M NaCl. Column fractions were monitored for PPS using the dimethylmethylene blue assay [12] and for biotin with the 4′-hydroxyazobenzene-2-carboxylic

acid/avidin reagent (Sigma–Aldrich). Fractions positive for PPS and biotin were pooled, desalted and freeze-dried. The purity of Bio-PPS was established by NMR spectroscopy.

C-terminally truncated forms of ADAMTS-5 (100 nM) and TIMP-3 (100 nM) were incubated with or without Bio-PPS (1 μM, calculated using an average molecular mass of 4.7 kDa) (1 h, 37 °C, 50 μl total volume). In the case of ADAMTS5-2, the active-site-directed metalloproteinase inhibitor GM6001 (10 μM; Elastin Products Company) was included to prevent autoproteolysis in the absence of TIMP-3. Proteins bounds to Bio-PPS were recovered by the addition of streptavidin–Sepharose (30 μl of a 50 % slurry equilibrated in TNC buffer for 2 h at 25 °C). After washing the beads (three washes in 200 μl of TNC buffer), bound proteins were eluted with reducing SDS sample buffer (75 μl) and analysed by immunoblotting with an M2 anti-FLAG antibody (Sigma–Aldrich).

Expression and purification of TIMP-3(K26/27/30/76E + R163/K165Q)

The K26/27/30/76E + R163/K165Q mutant of TIMP-3 [13] was recombinantly expressed in HEK (human embryonic kidney)-293 cells using a pCEP4-based expression vector (Invitrogen). This vector was created by PCR using the previously described TIMP-3(K26/27/30/76E + R163/K165Q) expression vector [13] as a template, with the sense primer 5′-CTCGAGTCCCTTTTTTT-TCCACAGGAGCTC*GCCGCCACC*ATGACCCCTTGGCTCG-GGCTCATCGTGCTC-3′ containing an XhoI site (underlined), Kozak consensus sequence (in italics) and TIMP-3 N-terminal sequence, and the antisense primer 5′- CTCGAG*CTAATGGTGA-TGGTGATGGTG*GGGGTCTGTGGCATTGATGATGCTTTTA-TCCGG-3′ containing an XhoI site (underlined), a His$_6$ tag (stop-His-His-His-His-His-His) (in italics) and the C-terminal sequence of TIMP-3. The PCR product was digested with XhoI and ligated into similarly digested pCEP4. Clones with the correct orientation were identified by restriction digestion and sequenced.

HEK-293 cells were transiently transfected with the expression vector using Lipofectamine™ 2000 (Invitrogen) in DMEM (Dulbecco's modified Eagle's medium) containing 10 % (v/v) FBS (fetal bovine serum), penicillin (100 units/ml) and streptomycin (100 units/ml). After 2 days, cells were treated with serum-free DMEM containing 30 mM sodium chlorate (Sigma–Aldrich) [9]. Medium was collected after 3 days and centrifuged to remove cell debris. To reduce the concentration of histidine, the conditioned medium was concentrated 10-fold in a Vivacell 250 filtration unit (5 kDa cut-off; Sartorius) and then diluted 10-fold in TNC buffer. The medium was applied to a 1 ml Ni-NTA (Ni^{2+}-nitrilotriacetate) resin (Qiagen) equilibrated in TNC buffer and the resin was washed in the same buffer to remove unbound proteins. Bound protein was eluted with 300 mM imidazole (Sigma–Aldrich) and extensively dialysed against TNC buffer.

The purity of TIMP-3(K26/27/30/76E + R163/K165Q) was confirmed by reducing SDS/PAGE [14] and immunoblotting using a polyclonal rabbit anti-TIMP-3 antibody (Abcam, catalogue number ab39184). The active concentration of the protein was determined by titration against a known concentration of the catalytic domain of MMP-1.

Solid-phase binding assays for PPS binding

PPS (2.5 μM in PBS) was coated overnight at 25 °C on to heparin-binding plates (BD Life Sciences) [15]. Wells were washed in TNC buffer containing 0.1 % Tween 20 (Sigma–Aldrich) between

each subsequent incubation. Wells were blocked with 0.2 % gelatin in TNC buffer and then incubated with recombinant ADAMTS-5 or TIMP-3 (0.1–40 nM in blocking solution for 3 h at 37 °C). Bound proteins were detected using either an M2 anti-FLAG antibody (Sigma–Aldrich) or a rabbit anti-TIMP-3 antibody (Abcam, catalogue number ab39184, for 3 h at 37 °C), followed by a species-appropriate secondary antibody coupled to horseradish peroxidase (Dako; for 1 h at 37 °C). Hydrolysis of tetramethylbenzidine substrate (KPL) was measured at 450 nm using a BioTek EL-808 absorbance microplate reader.

PPS preparative and analytical size-exclusion chromatography

PPS (40 mg) was applied to Sephadex 30 (GE Healthcare) equilibrated in water at 0.5 ml/min. Fractions (5 ml) were evaluated using the Dimethylmethylene Blue (Sigma–Aldrich) dye assay [12]. The eluate between V_o (15 ml) and V_t (105 ml) was divided into six pools (15 ml each), freeze-dried and reconstituted in a small volume of TNC buffer.

The molecular mass of the parent PPS and preparative size-exclusion chromatography fractions was determined by high-performance gel-permeation chromatography [16]. The chromatographic system TSK SW XL G3000 and G2000 columns (30 mm × 0.7 mm; Anachem) in series with a refractive index detector, was calibrated using the 1st International Reference Preparation Low Molecular Weight Heparin for Molecular Weight Calibration [NIBSC (National Institute for Biological Standards and Control), Potters Bar, U.K., code 90/686]. The average dp (degree of polymerization) of the PPS fractions was determined by dividing their average molecular mass by the molecular mass of a single pentosan sulfate unit (290 Da).

RESULTS

PPS increases the affinity between TIMP-3 and ADAMTS-5

We have previously shown that PPS increases the affinity between TIMP-3 and full-length ADAMTS-5 using recombinant aggrecan interglobular domain as a substrate [6]. We confirmed this observation using Abz-TESE∼SRGAIY-Dpa-KK, a more convenient quenched fluorescent peptide substrate for ADAMTS-5. The rate of hydrolysis of Abz-TESE∼SRGAIY-Dpa-KK by ADAMTS-5 was not directly inhibited by PPS at concentrations up to 10 μM.

In the absence of PPS, a K_i value of 0.56 ± 0.03 nM was determined, in agreement with previous findings [9]. In the presence of 25 nM (0.1 μg/ml) PPS, K_i was too low to be determined, with 0.5 nM ADAMTS-5 completely inhibited by an equimolar amount of TIMP-3 (Figure 1A). The affinity of N-TIMP-3 for full-length ADAMTS-5 was similarly increased by PPS (Figure 1B). Accurate determination of the improved K_i values was not possible, as ADAMTS-5 concentrations below 0.5 nM cannot be reliably assayed with the currently available substrates. Heparin also increased the affinity between TIMP-3 and full-length ADAMTS-5, although 10-fold higher heparin concentrations were required. Other glycosaminoglycans, such as dermatan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate and hyaluronic acid had no effect on affinity at concentrations up to 1 μM.

To determine the specificity of this result, the effect of PPS on TIMP-3 affinity for other metalloproteinases and on ADAMTS-5 affinity for TIMP-1 was assessed. K_i values of 0.43 ± 0.12 nM, 0.16 ± 0.002 nM and 0.90 ± 0.28 nM were determined for TIMP-3 inhibition of the catalytic domain of MMP-1, MMP-2 and the catalytic domain of MMP-3 respectively, in agreement with

previous studies [9,17]. The addition of 25 nM PPS had no effect on TIMP-3 affinity for these MMPs (Figures 1D–1F). PPS slightly improved the ADAMTS-5 affinity for TIMP-1, with the addition of 25 nM PPS reducing the K_i value from 734 ± 0.16 nM to 522 ± 0.24 nM (Figure 1C).

The Sp (spacer) domain of ADAMTS-5 promotes an affinity increase and PPS binding

The domains of ADAMTS-5 required for binding to PPS and for the affinity increase were examined using forms of the enzyme lacking sequential C-terminal non-catalytic domains (Figure 2A).

At concentrations near the K_i of 0.56 ± 0.03 nM, ADAMTS-5 is only partially inhibited by TIMP-3. Thus incubation of 0.5 nM full-length ADAMTS-5 with an equimolar concentration of TIMP-3 reduced enzyme activity to 80 %. PPS increased the affinity between full-length ADAMTS-5 and TIMP-3, such that at PPS concentrations above 10 nM, 0.5 nM full-length ADAMTS-5 (ADAMTS5-1) was completely inhibited by 0.5 nM TIMP-3 (Figure 2B). Deletion of the C-terminal TS domain (ADAMTS5-2) had little effect on susceptibility to the affinity increase, but deletion of the Sp domain (ADAMTS5-3) reduced PPS effectiveness, with EC_{50} increasing from 1 nM to 10 nM (Figure 2B). Further deletion of the CysR (cysteine-rich) domain (ADAMTS5-4) and first TS domain (ADAMTS5-5) had little additional effect on the affinity increase. The effect of the Dis (disintegrin) domain of ADAMTS-5 on the affinity increase could not be assessed as the catalytic domain alone has minimal catalytic activity against the synthetic substrate used.

A solid-phase binding assay was used to evaluate whether the affinity increase correlated with direct binding to PPS. PPS was coated on to multi-well plates [15] and subsequent binding of various forms of ADAMTS-5 was quantified using an antibody against their C-terminal FLAG tag. ADAMTS5-2, lacking the TS-2 domain, bound strongly to immobilized PPS. Binding was much reduced by deletion of the Sp domain (ADAMTS5-3) (Figure 2C), whereas further deletion of the CysR domain (ADAMTS5-4) had little effect. Binding was further reduced by deletion of the TS-1 domain, with binding of ADAMTS5-5 detectable only at high concentrations.

TIMP-3 binding to PPS is required for the affinity increase

A TIMP-3 mutant with low PPS-binding ability was used to assess whether the affinity increase requires TIMP-3 binding to PPS. Lee et al. [13] mutated several positively charged residues of TIMP-3 to generate TIMP-3(K26/27/30/76E + R163/K165Q), which was unable to bind to the extracellular matrix. We postulated that this mutant would also exhibit reduced binding to PPS. We recombinantly expressed and purified the mutant with a C-terminal His_6 tag (Figure 3A), and confirmed it to be an effective ADAMTS inhibitor, with a K_i value of 1.34 ± 0.04 nM for ADAMTS5-2, comparable with that of wild-type TIMP-3.

Binding of TIMP-3(K26/27/30/76E + R163/K165Q) to PPS was evaluated using a solid-phase binding assay as described above. Binding of TIMP-3 and TIMP-3(K26/27/30/76E + R163/K165Q) was quantified using a polyclonal rabbit anti-TIMP-3 antibody which reacts comparably with both proteins (Figure 3A). TIMP-3 bound strongly to the immobilized PPS, whereas TIMP-3(K26/27/30/76E + R163/K165Q) bound only weakly to immobilized PPS (Figure 3C).

Addition of PPS (10 nM) had no effect on the TIMP-3(K26/27/30/76E + R163/K165Q) affinity for ADAMTS5-2
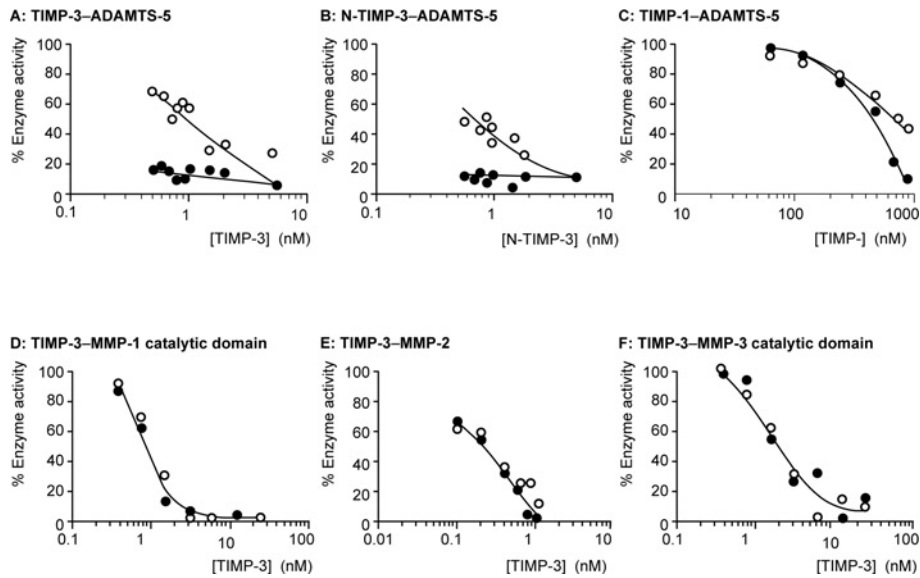
Figure 1 PPS improves the affinity between full-length ADAMTS-5 and TIMP-3

Enzymes were incubated with TIMP-3 in the absence (○) or presence (●) of 25 nM PPS (for 1 h at 37°C) and residual activity was determined. (**A**) TIMP-3 (0.5–5 nM) inhibition of full-length ADAMTS-5 (0.5 nM). (**B**) N-TIMP-3 (0.5–5 nM) inhibition of full-length ADAMTS-5 (0.5 nM). (**C**) TIMP-1 (50–1000 nM) inhibition of ADAMTS-5 (0.5 nM). (**D**) TIMP-3 (0.4–25 nM) inhibition of MMP-1 catalytic domain (1 nM). (**E**) TIMP-3 (0.1–1 nM) inhibition of MMP-2 (0.02 nM). (**F**) TIMP-3 (0.4–25 nM) inhibition of MMP-3 catalytic domain (1 nM). Continuous lines represent the best fit obtained by non-linear regression analysis of the data using eqn (1).
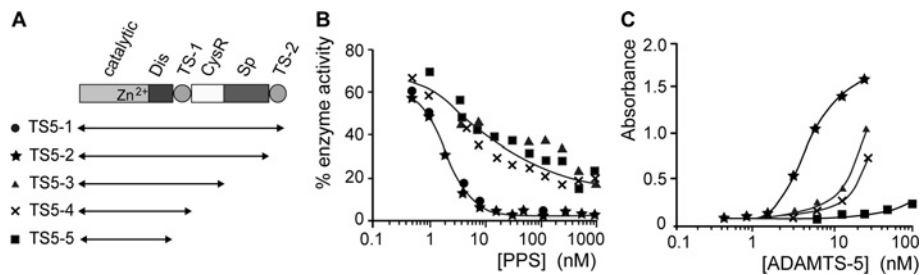
Figure 2 The Sp domain of ADAMTS-5 promotes PPS binding and an affinity increase

(**A**) Schematic representation of ADAMTS-5 forms lacking sequential C-terminal domains. (**B**) Effect of PPS on the affinity between TIMP-3 and different forms of ADAMTS-5. TIMP-3 (0.5 nM) and ADAMTS-5 forms (0.5 nM, symbols as shown in **A**) were incubated with PPS (0.5–1000 nM for 1 h at 37°C) and residual activity against Abz-TESE∼SRGAIY-Dpa-KK was determined (18 h, 37°C). (**C**) PPS (2.5 μM) was immobilized on glycosaminoglyan-binding multi-well plates and wells were then incubated with various forms of ADAMTS-5 (0.4–25 nM, symbols as shown in **A**). Bound ADAMTS-5 was detected using an M2 anti-FLAG antibody.
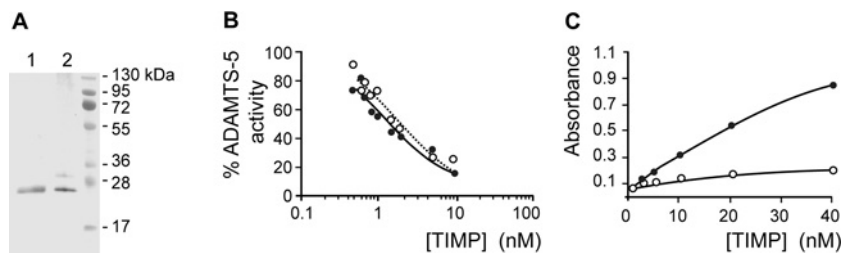
Figure 3 Binding of TIMP-3 to PPS is a prerequisite for the affinity increase

(**A**) Immunoblot showing the comparable reactivity of recombinant TIMP-3 (lane 1, 10 ng) and TIMP-3(K26/27/30/76E + R163/K165Q) (lane 2, 10 ng) with a polyclonal rabbit anti-TIMP-3 antibody. The molecular mass in kDa is indicated. (**B**) TIMP-3(K26/27/30/76E + R163/K165Q) (0.5–10 nM) was incubated with ADAMTS5-2 (0.5 nM) in the absence (○) or in the presence (●) of 25 nM PPS (1 h, 37°C), and residual activity against Abz-TESE∼SRGAIY-Dpa-KK was determined (18 h, 37°C). Continuous and broken lines represent the best fit obtained by non-linear regression analysis of the data using eqn (1). (**C**) PPS (2.5 μM) was immobilized on glycosaminoglyan-binding multi-well plates and subsequent binding of TIMP-3 (●) or TIMP-3(K26/27/30/76E + R163/K165Q) (○) (0.3–40 nM) was detected using a polyclonal rabbit anti-TIMP-3 antibody.

Table 1   Fractions from analytical size-exclusion chromatography

Fraction	Molecular mass (kDa)	Polydispersity	Average dp
Parent PPS	4.7	1.591	16
F1	10.3	1.139	35
F2	7.3	1.139	25
F3	4.8	1.088	16
F4	3.3	1.048	11
F5	2.4	1.221	8.4
F6	2.4	1.216	8.2

(Figure 3B), indicating that TIMP-3 binding to PPS is a pre-requisite for the affinity increase.

Longer PPS molecules are more effective

Our preparation of PPS was eluted as a broad peak on analytical size-exclusion chromatography, indicating that it was made up of heterogeneously sized PPS molecules. We fractionated the preparation on a preparative size-exclusion chromatography Sephadex 30 resin and divided the eluate into six fractions, designated F1–F6. The average molecular masses and dp of the fractions were determined by analytical size-exclusion chromatography and ranged between 10.3 kDa (dp 35) and 2.4 kDa (dp 8) (Table 1).

F1 (10.3 kDa, dp 35), F2 (7.3 kDa, dp 25), F3 (4.8 kDa, dp 16) and F4 (3.3 kDa, dp 11) were equally able to increase ADAMTS-5-2–TIMP-3 affinity (Figure 4A), with concentrations above 10 nM enabling complete inhibition of 0.5 nM ADAMTS5-2 by 0.5 nM TIMP-3. F5 (2.4 kDa, dp 8) and F6 (2.4 kDa, dp 8) were less active, with 100-fold higher concentrations of these fractions required for a comparable increase in ADAMTS5-2–TIMP-3 affinity.

The ability of the various PPS fractions to bind to TIMP-3 and ADAMTS5-2 was determined by solid-phase binding assays. Both TIMP-3 (Figure 4B) and ADAMTS5-2 (Figure 4C) bound strongly to F1, F2, F3 and F4, and bound weakly to F5 and F6.

PPS F5 is equally effective with ADAMTS5-2 and ADAMTS5-5

The ability of short (F5) and long (F1) PPS fractions to increase the ADAMTS5-2–TIMP-3 and ADAMTS5-5–TIMP-3 affinity was compared. F5 was equally active at increasing affinity between TIMP-3 and both forms of the enzyme, whereas F1 was more effective at increasing TIMP-3 affinity for ADAMTS5-2 than for ADAMTS5-5 (Figure 5A).

Formation of trimolecular PPS–ADAMTS-5–TIMP-3 complexes

We next investigated whether the affinity increase involved formation of a stable trimolecular complex using Bio-PPS, which we confirmed was able to increase the ADAMTS-5–TIMP-3 affinity as effectively as unbiotinylated PPS.

TIMP-3 was unable to bind to streptavidin–Sepharose beads, but when incubated with Bio-PPS, TIMP-3 was retained on the beads (Figure 5B), confirming that TIMP-3 interacts directly with PPS. Similarly, ADAMTS5-2 bound to streptavidin–Sepharose beads in the presence of Bio-PPS (Figure 5B). Complexes of ADAMTS5-2 and TIMP-3 formed in the presence of Bio-PPS also bound to streptavidin–Sepharose beads (Figure 5B), indicating that PPS remains associated with the high-affinity ADAMTS5-2–TIMP-3 complex, and does not dissociate after complex formation.

ADAMTS5-5 was not retained on streptavidin–Sepharose beads after incubation with Bio-PPS (Figure 5B), confirming that it has low affinity for PPS as shown in the solid-phase binding assay (Figure 2C). Even upon incubation with TIMP-3, only a small portion of ADAMTS5-5 was retained on streptavidin–Sepharose beads, indicating that PPS–ADAMTS5-5–TIMP-3 complexes are of lower affinity than PPS–ADAMTS5-2–TIMP-3 complexes and dissociate upon washing.

PPS remains effective at high concentrations

PPS may increase the ADAMTS5-2–TIMP-3 affinity by a bridging mechanism, in which its binding to both proteins increases the likelihood of their interaction with each other. If so, then PPS should be less effective at high concentrations, where each of the two proteins is more likely to bind to separate PPS molecules. However, even at a concentration of 50 μM, PPS was able to increase ADAMTS5-2–TIMP-3 affinity to the extent that 0.5 nM ADAMTS5-2 was completely inhibited by 0.5 nM TIMP-3 (Figure 5C). PPS thus retained activity at a 100 000-fold molar excess over ADAMTS-5 and TIMP-3.

PPS effect is electrostatic

The effect of ionic strength on the PPS-mediated increase in ADAMTS-5–TIMP-3 affinity was investigated.

First, the effect of NaCl on the interaction between TIMP-3 and ADAMTS-5 was analysed. Inhibition of ADAMTS5-2 (0.5 nM) by TIMP-3 (0.5 nM) was slightly reduced at NaCl concentrations above 250 mM (Figure 6A, broken line), indicating that there is an electrostatic element to the interaction of these two proteins. In contrast, the inhibition of ADAMTS5-5 by TIMP-3 was not
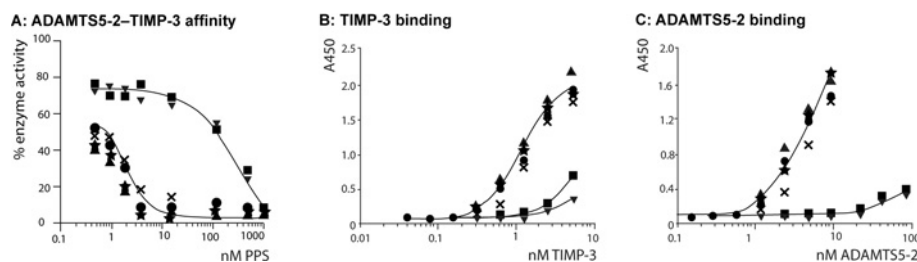
Figure 4   Longer PPS molecules are more effective than short PPS molecules at improving affinity

(**A**) TIMP-3 (0.5 nM) was incubated with ADAMTS5-2 (0.5 nM) and various concentrations of PPS fractions F1–F6 (see Table 1) for 1 h at 37°C. The residual activity against Abz-TESE∼SRGAIY-Dpa-KK was measured. (**B**) PPS fractions F1–F6 (2.5 μM) were immobilized on glycosaminoglyan-binding multi-well plates and wells were then incubated with TIMP-3. Bound TIMP-3 was quantified using an M2 anti-FLAG antibody. TIMP-3 bound strongly to F1 (●, 35 dp), F2 (★, 25 dp), F3 (▲, 16 dp) and F4 (✕, 11 dp), but only weakly to F5 (■, 8 dp) and F6 (▼, 8 dp). (**C**) PPS fractions F1–F6 (2.5 μM) were immobilized on glycosaminoglyan-binding multi-well plates and wells were then incubated with ADAMTS5-2. Bound ADAMTS5-2 was quantified using an M2 anti-FLAG antibody. ADAMTS5-2 bound strongly to F1 (●), F2 (★), F3 (▲) and F4 (✕), and weakly to F5 (■) and F6 (▼).
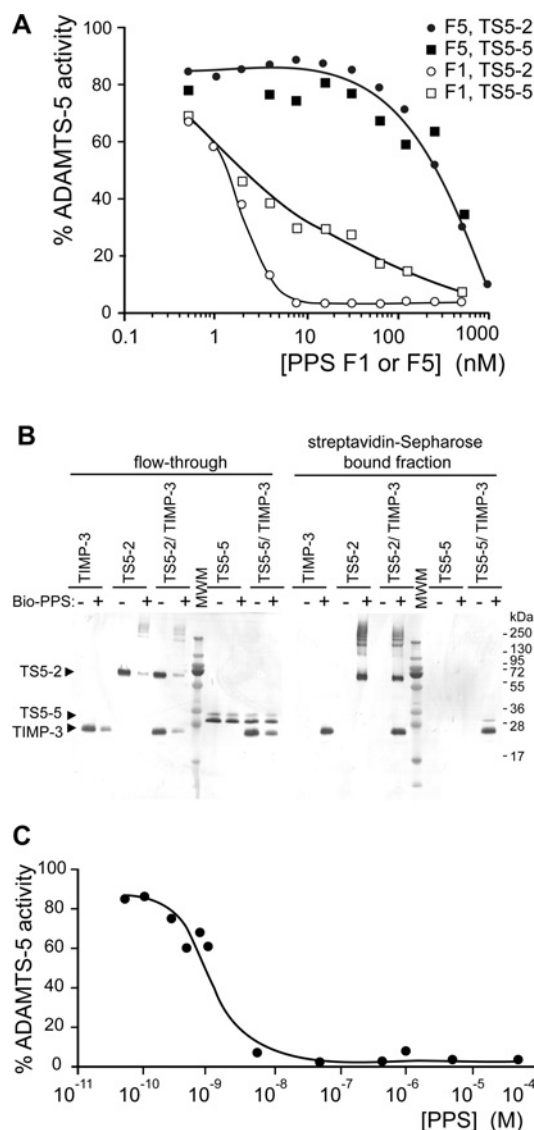
Figure 5   PPS increases affinity by a dual mechanism

(**A**) TIMP-3 (0.5 nM) was incubated with either ADAMTS5-2 or ADAMTS5-5 (0.5 nM) in the presence of PPS size-exclusion chromatography fractions F1 or F5 (0.5–1000 nM, 1 h, 37°C) and residual activity against Abz-TESE~SRGAIY-Dpa-KK was determined (18 h, 37°C). (**B**) TIMP-3 (0.5 nM) was incubated with ADAMTS5-2 or ADAMTS5-5 (100 nM) with or without Bio-PPS (1 μM) (1 h, 37°C). Streptavidin–Sepharose beads were added and bound proteins were recovered in SDS buffer and analysed by immunoblotting with an M2 anti-FLAG antibody. The molecular mass in kDa is indicated. (**C**) ADAMTS5-2 (0.5 nM) was incubated with TIMP-3 (0.5 nM) in the presence of PPS (0.05 nM–50 μM) (1 h, 37°C) and residual activity against Abz-TESE~SRGAIY-Dpa-KK was determined (18 h, 37°C).

Figure 6   The PPS effect is electrostatic

(**A**) TIMP-3 (0.5 nM) was incubated with ADAMTS5-2 (0.5 nM) in the absence (○) or presence (●) of 10 nM PPS and 50–500 mM NaCl (1 h, 37°C), and residual activity against Abz-TESE~SRGAIY-Dpa-KK was determined (18 h, 37°C). (**B**) TIMP-3 (0.5 nM) was incubated with ADAMTS5-5 (0.5 nM) in the absence (○) or presence (●) of 100 nM PPS and 50–500 mM NaCl (1 h, 37°C), and residual activity against Abz-TESE~SRGAIY-Dpa-KK was determined (18 h, 37°C). (**C**) TIMP-3 (0.5 nM) was incubated with ADAMTS5-2 (0.5 nM) and PPS (10 nM) in TNC buffer (containing 250 mM NaCl) at 37°C for times ranging between 1 and 72 h. The concentration of NaCl was then increased to 400 mM and residual activity against Abz-TESE~SRGAIY-Dpa-KK was determined (18 h, 37°C).

affected by NaCl concentrations of up to 500 mM (Figure 6B, broken line), indicating that electrostatic interactions do not play a significant role in the interaction of the TIMP-3 with the catalytic and Dis domain of ADAMTS-5.

The effect of NaCl on PPS–ADAMTS-5–TIMP-3 trimolecular complex formation was then evaluated. NaCl concentrations above 200 mM greatly reduced the effect of PPS on the affinity between ADAMTS5-2 and TIMP-3 (Figure 6A, continuous line) and the affinity between ADAMTS5-5 and TIMP-3 (Figure 6B, continuous line). At NaCl concentrations above 400 mM, PPS was unable to mediate an increase in affinity, indicating that the PPS interaction with both ADAMTS-5–TIMP-3 complexes is largely electrostatic.
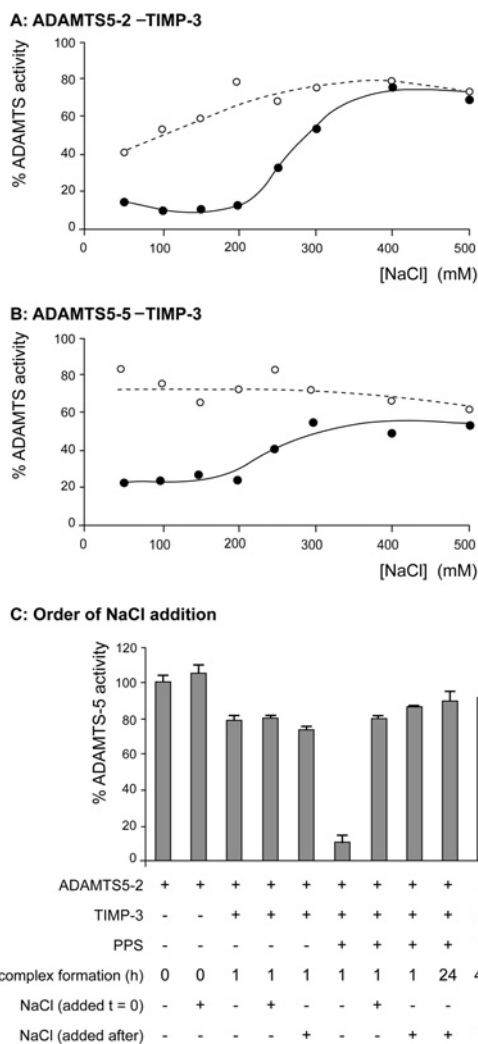
To investigate whether the effect of PPS on affinity was reversible, we compared the effect of adding NaCl before or after formation of the ternary PPS–ADAMTS-5–TIMP-3 complex. When 400 mM NaCl was included during the incubation of 0.5 nM ADAMTS5-2, 0.5 nM TIMP-3 and 10 nM PPS, enhanced affinity of the enzyme–inhibitor complex was not observed (Figure 6C). Similarly, when the salt was added after the PPS–ADAMTS-5–TIMP-3 complex had formed, the enzyme–inhibitor affinity was reduced to that observed in the absence of PPS. A similar reduction in affinity was observed when NaCl was added after 1, 24, 48 or 72 h of complex formation. This indicates that formation of the higher-affinity complex is reversible by dissociation of PPS from the complex in high-salt concentrations.

DISCUSSION

Using *Escherichia coli*-expressed recombinant aggrecan interglobular domain as a substrate, we have previously shown that the semi-synthetic sulfated molecule PPS is able to increase the affinity between TIMP-3 and ADAMTS-5 [6]. At the concentrations used in that study, PPS had no effect on TIMP-3 affinity for MMPs and only slightly increased ADAMTS-5 affinity for TIMP-1, suggesting that PPS uniquely affects the TIMP-3–ADAMTS interaction. Therefore we have further investigated the mechanism of this affinity increase in more detail, and found that the increase in affinity is associated with the formation of a trimolecular PPS–ADAMTS-5–TIMP-3 complex. This rules out the possibility that PPS interacts transiently with the proteins to increase their affinity and then dissociates, as has been shown for the heparin-mediated increase in VEGF (vascular endothelial growth factor)–fibronectin affinity [18]. Furthermore, analysis of truncated and mutant forms of ADAMTS-5 and TIMP-3 indicated that sensitivity to the affinity increase correlates with the ability of both of the proteins to bind to PPS.

The TIMP-3(K26/27/30/76E + R163/K165Q) mutant does not bind to the extracellular matrix [13] and it also has greatly reduced ability to bind to PPS compared with wild-type TIMP-3 (Figure 3C). This mutant is an effective inhibitor of ADAMTS-5, but its affinity for the enzyme was not enhanced by PPS, indicating that binding of TIMP-3 to PPS is a prerequisite for the affinity increase. The mutated amino acids in TIMP-3(K26/27/30/76E + R163/K165Q) form a stretch of basic residues on the surface of the modelled three-dimensional structure of full-length TIMP-3 [13]. This stretch of residues lies on the opposite face of the molecule to the reactive ridge that interacts with metalloproteinases, and extends across both the N- and the C-terminal TIMP-3 domains [13]. We found that the N-TIMP-3 affinity for ADAMTS-5 was increased by PPS, indistinguishably from full-length TIMP-3 (Figure 1B), indicating that the N-terminal domain contains the minimal residues necessary for high-affinity complex formation. However, while N-TIMP-3 has been shown to bind to heparin–Sepharose [19], N-TIMP-3 binding to immobilized heparin or PPS was not detectable in solid-phase binding assays (L. Troeberg and H. Nagase, unpublished work), indicating that the C-terminal domain of TIMP-3 increases the affinity for sulfated glycosaminoglycans, as argued by Lee et al. [13].

Similarly, truncated forms of ADAMTS-5 with reduced PPS binding ability showed decreased sensitivity to the PPS-mediated affinity increase. Deletion of the C-terminal TS-2 domain has no detectable effect on matrix binding or aggrecanolytic activity of ADAMTS-5 [10], and had little or no effect on PPS susceptibility (Figure 2B). The Sp domain has previously been suggested to bind to glycosaminoglycans, as it promotes aggrecan hydrolysis [10] and binding to the extracellular matrix [10]. We found that deletion of the Sp domain (ADAMTS5-3) markedly reduced both direct binding to PPS and susceptibility to the PPS-mediated affinity increase (Figures 2B and 2C). The Sp domain thus contains a major glycosaminoglycan-binding site of ADAMTS-5 (site B in Figure 7). The CysR domain has been shown to promote aggrecan hydrolysis, extracellular matrix binding and susceptibility to exosite inhibition by PPS [6,10], but we found that deletion of this domain (ADAMTS5-4) had little effect on binding to PPS or susceptibility to the PPS-mediated affinity increase. The TS-1 domain is reported to contribute to aggrecan binding and hydrolysis by ADAMTS-4 [20], and deletion of this domain in ADAMTS-5 (ADAMTS5-5) reduced direct binding to PPS, but had little effect on the affinity increase (Figures 2B and 2C). Although ADAMTS5-5 bound weakly to PPS, some binding was still evident at higher concentrations, in agreement with previous findings that this form of the enzyme retains some heparin-binding ability [21]. These data thus indicate that, in addition to the major glycosaminoglycan-binding site on the Sp domain of ADAMTS-5, the TS-1 and catalytic/Dis domains contain additional binding sites (site A in Figure 7). As ADAMTS5-1 and ADAMTS5-2 contain all of these binding sites, they bind to PPS with the highest affinity, and thus require low PPS concentrations for trimolecular complex formation. Truncated forms of ADAMTS-5 that lack the Sp domain bind to PPS with lower affinity and require 100-fold higher PPS concentrations for trimolecular complex formation.

Accurate delineation of the PPS-binding regions of ADAMTS-5 and TIMP-3 is hampered by the lack of three-dimensional crystal structures of both full-length proteins. Electrostatic interactions underpin many glycosaminoglycan–protein interactions [22,23], although non-ionic interactions are also important in some cases, such as interaction of the heparin pentasaccharide with ATIII (antithrombin III) [24]. Formation of the PPS–ADAMTS-5–TIMP-3 complex was prevented by NaCl concentrations above 400 mM (Figure 6), indicating that the complex is largely electrostatic in nature. On the basis of the analysis of 49 heparin-binding domains, Cardin and Weintraub [25] proposed two consensus sequences for protein interaction with glycosaminoglycans. TIMP-3 contains neither the XBBXBX nor XBBBXXBX (where B is a basic residue and X is a hydropathic residue) motif, although it does contain 25 basic residues. ADAMTS-5 contains 86 basic residues, as well as an XBBXBX motif (N[739]KKSKG[744]) in the Sp domain, which may constitute the glycosaminoglycan-binding residues of this domain. Nonetheless, it is more likely that the three-dimensional arrangement of basic residues in ADAMTS-5 and TIMP-3 dictate their interaction with sulfated polysaccharides. Chondroitin 6-sulfate has been shown to increase the TIMP-3 affinity for
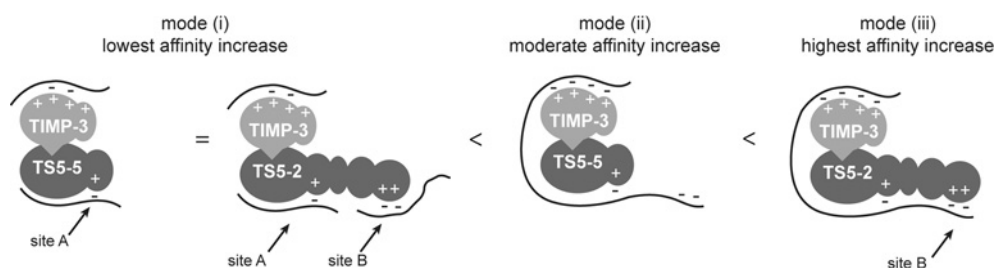

Figure 7   Modes of PPS binding to ADAMTS-5–TIMP-3 complexes

Short PPS chains increase the affinity between TIMP-3 and ADAMTS5-5 or ADAMTS5-2 by altering their interaction with the charged environment [mode (i)]. Longer PPS chains further increase affinity through an increase in the Gibbs free energy of binding brought about by linking the low-affinity binding sites on TIMP-3 and ADAMTS-5 [mode (ii)]. Such longer PPS chains are even more effective with ADAMTS5-2, as their length enables them to interact with multiple positively charged regions of the enzyme, increasing the affinity of the trimolecular complex [mode (iii)].

ADAMTS-4 [8], and we found that heparin (100 nM) was also able to increase the TIMP-3 affinity for ADAMTS-4 and ADAMTS-5 (L. Troeberg and H. Nagase, unpublished work). Heparin has previously been reported to increase the TIMP-3 affinity for two other heparin-binding enzymes, namely MMP-2 and ADAMTS-2 [26,27]. However, in these cases, heparin was shown to be optimally effective at a 100-fold higher concentration than we found to be necessary to increase TIMP-3–ADAMTS-5 and TIMP-3–ADAMTS-4 activity. This suggests that ADAMTS-4 and ADAMTS-5 are particularly sensitive to such effects, possibly due to their high affinity for glycosaminoglycans.

Longer PPS chains were more effective at mediating the affinity increase than short chains (Figure 4A). PPS with an average length of at least 11 saccharide units (F4, dp 11, 3.3 kDa) was 100-fold more effective than PPS of dp 8 (F5 and F6, 2.4 kDa). PPS of dp 35 (F1) was no more effective than dp11 (F4), indicating that 11 saccharide units is sufficient to support high-affinity complex formation. This is in line with previous findings for heparin binding to proteins, with a minimal length of dp 5–6 required for heparin binding to a single protein, and dp 10–12 being sufficient to support simultaneous binding of heparin to two proteins [28–30].

Various other examples of glycosaminoglycan-mediated increases in protein–protein affinity have been characterized. In particular, the heparin-mediated increase in affinity between ATIII and serine proteinases of the coagulation cascade has been extensively studied. Heparin has been found to act in this system in two ways: (i) by a template or bridging mechanism, in which simultaneous binding of heparin to ATIII and the target serine proteinase increases the likelihood of the proteins interacting with each other [31], and (ii) by a conformational change mechanism, in which heparin alters the conformation of ATIII to generate a form of the protein with higher affinity for the target proteinase [24,32]. It is possible to discriminate between these two mechanisms of action by examining the dose-dependence of the glycosaminoglycan activity. If the glycosaminoglycan acts by a conformational change mechanism, then it will be as effective at high as at low concentrations. If, however, the glycosaminoglycan acts by a template mechanism, then it should become less effective at high concentrations, where the proteins are more likely to bind to different glycosaminoglycan molecules. For example, ATIII association with thrombin is increased by heparin concentrations of up to 10^{-7} M, but if heparin concentrations are increased to 10^{-5} M, heparin efficacy is reduced [33,34]. We found that PPS was as effective at 10^{-4} M as it was at 10^{-8} M (Figure 5C), which would appear to indicate that it acts, at least in part, by inducing a conformational change in one or both of the proteins. However, our kinetic analysis is limited by the relatively insensitive substrates currently available to monitor ADAMTS-5 activity, which do not permit accurate determination of the improved K_i. The substrates used to study heparin effects on ATIII inhibition of target proteinases permit measurement of an up to four orders of magnitude increase in the second-order rate constant [33,34], whereas the available ADAMTS substrates permit determination of a less than two orders of magnitude improvement in K_i. It is therefore possible that we were unable to detect a decrease in PPS efficacy at high concentrations. For example, our system is not sensitive enough to discriminate between a 100-fold increase in affinity at 10 nM PPS and a 10-fold increase in affinity at 10 μM PPS. Further investigation using techniques such as NMR and atomic force microscopy is therefore required to see whether PPS alters the ADAMTS-5 and/or TIMP-3 conformation. Although TIMPs are compact molecules that display only limited conformational flexibility, backbone movement of TIMP-1 has been reported upon binding to MMP-3 [35]. Subtle changes in conformation

may be sufficient to increase TIMP-3 affinity for ADAMTSs. Alternatively, the ADAMTSs are multidomain enzymes that are likely to be conformationally flexible [9,36,37]. Allosteric modulation of various other proteinases by glycosaminoglycans and sulfated polysaccharides has been described. For example, binding of glycosaminoglycans to an exosite of cathepsin K alters the conformation of the enzyme, allosterically increasing susceptibility to inhibition by stefin A [38]. Similarly, co-operative binding of PPS to human leucocyte elastase has been shown to modulate its interaction with inhibitors [39].

We found that NaCl concentrations above 250 mM reduced the TIMP-3 affinity for ADAMTS5-2, but had little effect on TIMP-3 affinity for ADAMTS5-5. This indicates that electrostatic interactions contribute to the TIMP-3 interaction with ADAMTS5-2, but do not play a significant role in the TIMP-3 interaction with the catalytic and disintegrin domains (ADAMTS5-5). This mirrors the previous findings of Wayne et al. [8], who found that 100 mM NaCl reduced the TIMP-3 affinity for full-length and Sp-domain truncated ADAMTS-4, but had little effect on the TIMP-3 affinity for forms of the enzyme lacking the CysR domain. Taken together, these data indicate that electrostatic interactions govern TIMP-3 interactions with the C-terminal domains of ADAMTS-4 and ADAMTS-5, but that they are less significant in TIMP-3 interactions with the catalytic and disintegrin domains.

Furthermore, NaCl concentrations above 400 mM abolished the effect of PPS on ADAMTS-5–TIMP-3 affinity, indicating that electrostatic interactions underpin formation of the high-affinity trimolecular complex. Similarly, NaCl reduced the effect of chondroitin 6-sulfate on ADAMTS-4–TIMP-3 affinity [8]. These data indicate that the glycosaminoglycan-mediated increase in TIMP-3 affinity for ADAMTSs is reversible, and that it is driven by electrostatic interactions.

By comparing the effect of long and short chains of PPS on the affinity of ADAMTS5-2 and ADAMTS5-5 for TIMP-3 (Figure 5C), we propose three modes of binding for PPS (Figure 7). Short PPS chains, such as F5 and F6 (dp 8), bind weakly to TIMP-3 and to ADAMTS5-2 and ADAMTS5-5, and are thus only effective at high concentrations [mode (i)]. Longer PPS chains (F1–F4, dp 11–35) are more effective at improving the affinity between TIMP-3 and ADAMTS5-5 as they can simultaneously bind to both proteins, with the affinity of the trimolecular complex being greater than the sum of the individual PPS–protein affinities due to the increase in the Gibbs free energy of binding arising from connection of two low-affinity binding sites [mode (ii)] [40]. These long PPS chains (F1–F4) are even more effective with ADAMTS5-2 than they are with ADAMTS5-5 as their favourable increase in Gibbs energy is further increased by their ability to bind with higher affinity to both TIMP-3 and to the multiple binding sites on ADAMTS5-2 [mode (iii)]. However, the enhancement of TIMP-3–ADAMTS-5 affinity is reversible, regardless of the size of PPS. The exact mechanism by which PPS binding increases affinity is not clear. We postulate that PPS may either induce a reversible conformational changes in one or both proteins, or that binding of short negatively charged PPS molecules alters the interaction of ADAMTS-5 and TIMP-3 with the surrounding solvent due to a shift in molecular surface charge distribution.

AUTHOR CONTRIBUTION

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