Modulation of the proteolytic activity of the complement protease C1s by polyanions: implications for polyanion-mediated acceleration of interaction between C1s and SERPING1


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The complement system plays crucial roles in the immune system, but incorrect regulation causes inflammation and targeting of self-tissue, leading to diseases such as systemic lupus erythematosus, rheumatoid arthritis and age-related macular degeneration. In vivo, the initiating complexes of the classical complement and lectin pathways are controlled by SERPING1 [(C1 inhibitor) serpin peptidase inhibitor, clade G, member 1], which inactivates the components C1s and MASP-2 (mannan-binding lectin serine peptidase 2). GAGs (glycosaminoglycan) and DXS (dextran sulfate) are able to significantly accelerate SERPING1-mediated inactivation of C1s, the key effector enzyme of the classical C1 complex, although the mechanism is poorly understood. In the present study we have shown that C1s can bind to DXS and heparin and that these polyanions enhanced C1s proteolytic activity at low concentrations and inhibited it at higher concentrations. The recent determination of the crystal structure of SERPING1 has given rise to the hypothesis that both the serpin (serine protease inhibitor)–polyanion and protease–polyanion interactions might be required to accelerate the association rate of SERPING1 and C1s. To determine what proportion of the acceleration was due to protease–polyanion interactions, a chimaeric mutant of α1-antitrypsin containing the P1–P4 residues from the SERPING1 RCL (reactive-centre loop) was produced. Like SERPING1, this molecule is able to effectively inhibit C1s, but is unable to bind polyanions. DXS exerted a biphasic effect on the association rate of C1s which correlated strongly with the effect of DXS on C1s proteolytic activity. Thus, whereas polyanions are able to bind C1s and modulate its activity, polyanion interactions with SERPING1 must also play a vital role in the mechanism by which these cofactors accelerate the C1s–SERPING1 reaction.

Key words: C1 inhibitor, complement, glycosaminoglycan (GAG), protease, SERPING1.

INTRODUCTION

The complement system in blood plasma is a major effector of innate immunity, playing a pivotal role in inflammation, and combating infection; conversely, it is also associated with inflammatory disease. The complement system has three activation pathways, classical, lectin and alternative, which, although initiated via different mechanisms, all converge with the formation of a C3 convertase complex (either C4b2a or C3bBb). Subsequent events include the release of the inflammatory species C3a and C5a, opsonization of pathogens and ultimately, formation of the membrane attack complex (reviewed in [1]).

The classical pathway is initiated by the C1 complex, which comprises a C1q hexamer complexed with a zymogenic (C1r), (C1s)2 heterotetramer. When this complex encounters antigen–antibody aggregates, a conformational change in C1q occurs, leading to distortion and autoactivation of zymogenic C1r [2]. Activated C1r is subsequently able to cleave zymogenic C1s into an activated, disulfide-linked, two-chain form [2], and this proteolytically competent species cleaves C2 and C4, leading to the generation of the C3 convertase [3].

Complement activity is regulated by many processes, two of the most important being interaction of heparin with complement proteins and inhibition of complement proteases by SERPING1 [(C1 inhibitor) serpin peptidase inhibitor, clade G, member 1; also known as plasma protease C1 inhibitor], a member of the serpin (serine protease inhibitor) family [4,5]. SERPING1 irreversibly inhibits C1r and C1s [2,6]. MASP-2 (mannan-binding lectin serine peptidase 2, the C1s orthologue in the lectin pathway) and MASP-1 (mannan-binding lectin serine peptidase 1) [7], as well as modulating complement activation via pathways unrelated to protease inhibition [8]. Heparin modulates complement activity, at numerous points of the cascade, by binding to various proteins from all three complement pathways [9–13]. However, its primary effect is to downregulate complement activation by potentiation of SERPING1 function [10,14,15]; heparin is the most potent endogenous species with this ability [14]. Other non-physiological polyanions, including

Abbreviations used: α1- (P1–P4), α1-antitrypsin with the P1–P4 residues replaced by the corresponding residues of SERPING1; Abz, 2-aminobenzoyl; AFB, alternative fluorescence buffer; AMC, aminomethylcoumarin; CSA, chondroitin sulphate A; CSC, chondroitin sulphate C; DMF, dimethylformamide; Dnp(Lys), dinitrophenyl lysine; DS, dermatan sulfate; DXS, dextran sulfate; DXS10k, DXS of average-molecular-mass 10 kDa; DXS50k, DXS of average-molecular-mass 500 kDa; DXS5k, DXS of average-molecular-mass 5 kDa; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; FAB, fluorescence assay buffer; fC1a, factor C1a; GAG, glycosaminoglycan; MASP-1, mannan-binding lectin serine peptidase 1; MASP-2, mannan-binding lectin serine peptidase 2; PSP, polysulfated polysaccharide; RB, resuspension buffer; RCL, reactive-centre loop; Serpin, serine protease inhibitor; SI, stoichiometry of inhibition; SERPING1, (C1 inhibitor) serpin peptidase inhibitor, clade G, member 1; Z, benzyloxycarbonyl.

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PSPs (polysulfated polysaccharides) and DXS (dextran sulfate) also potentiate SERPING1 function, with the high-molecular-mass DXS exerting the highest reported effect [14,16].

The crucial role of SERPING1 in complement regulation is reflected in its association with various disease states, including the potentially fatal disease, hereditary angioedema [17], and age-related macular degeneration [18], which is the leading cause of blindness in the elderly in the developed world. SERPING1 is also able to provide protection against LPS (lipopolysaccharide)-induced sepsis and cytotoxicity, both during and after organ transplant [19,20]. Enhancing the inhibitory capacity of SERPING1 with polyanions therefore represents a potential means of reducing the complement-mediated tissue damage seen in inflammatory diseases.

The mechanism by which polyanions are able to potentiate SERPING1 function remains unclear. Preliminary work has determined that two known mechanisms of serpin potentiation, allostery and bridging, cannot account for potentiation of the C1s–SERPING1 reaction by polyanions (see Supplementary material at http://www.BiochemJ.org/bj/422/bj4220295add.htm).

As a result, we considered the possibility that interactions between the protease and GAGs (glycosaminoglycans) may be responsible for potentiation. This was in part on the basis that in our previous work [22–24] we found that the rate of inhibition of a cysteine protease, cathepsin V, by the nuclear serpin MENT (myeloid and erythroid nuclear termination stage-specific protein), was increased by DNA. In this system, much of the effect of the polyanion occurred via an effect on the protease rather than the serpin.

In order to do this, we first characterized the effect of polyanion–C1s interactions on the proteolytic activity of C1s, against both peptide and protein substrates. Following this, we engineered a chimaeric serpin, comprising the main body of SERPING1 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1; also known as alpha1-antitrypsin] with the P1–P4 residues of the RCL (reactive-centre loop) substituted for the corresponding residues from SERPING1. This chimaeric serpin was able to react with, and inhibit C1s, yet was unable to bind polyanions. As a result, it allowed us to determine the contribution of the C1s–polyanion interaction to the overall acceleration of the C1s–SERPING1 reaction.

**EXPERIMENTAL**

Materials

The following fluorescence-quenched and coumarin substrates were custom synthesised by Auspep at greater than 80 % purity: C2 P4–P4’ substrate, 2Abz (2-aminobenzoyl)-SLGR-KIQI-Dnp(Lys) (2,4-dinitrophenyl lysine)-NH2; C4 P4–P4’ substrate, 2Abz-GLQR-ALEI-Dnp(Lys)-NH2; SERPING1 P4–P4’ substrate, 2Abz-SVAR-LLL-Dnp(Lys)-NH2; C2 P1–P1 substrate, Z(benzyloxycarbonyl)-SLGR-AMC (amino-4-methylcoumarin); C4 P4–P4’ substrate, Z-GLQR-AMC; and SERPING1 P4–P4’ substrate, Z-SVAR-AMC. C2 P1–P1 coumarin substrate Boc (t-butoxycarbonyl)-GLR-AMC was from Bachem. Note that the nomenclature for residues within the RCL is based on that outlined by Schechter and Berger [24a] for the substrates of proteases. The residues are numbered from the scissile bond (P1′–P1) as follows: P4–P4’–P3–P3’–P2–P2’–P1–P1’–P4–P4’–P3–P3’–P2–P2’–P1–P1’–...

Human C1s (activated, two chain form), C1 complex, SERPING1, C2 and C4 and lung heparin were all purchased from Calbiochem. DXS43k (DXS sodium salt of average-molecular-mass 36–50 kDa), CSA (chondroitin sulfate A) and DS (dermatan sulfate) were purchased from MP Biomedicals.

DXS5k (DXS sodium salt of average-molecular-mass 5 kDa) was purchased from Fluka, DXS10k and DXS500k (DXS of average-molecular-mass 10 kDa and 500 kDa respectively), porcine mucosal heparin sodium salt (average-molecular-mass approx. 18 kDa), low-molecular-mass mucosal heparin (average 5 kDa), heparan sulfate fast-moving-fraction sodium salt and CSC (chondroitin sulfate C) were purchased from Sigma–Aldrich. N-desulfated heparin was prepared using the method described previously in [25]. The pyridinium salt form of heparin was treated with dimethyl sulfoxide/water (95:5; v/v) for 1.5 h at 50 °C. The N-desulfated heparin was acetylated with acetic anhydride as described in [26]. Totally desulfated heparin was prepared as described in [27] by treatment of the pyridinium salt form with dimethyl sulfoxide/water (9:1; v/v) for 2 h at 100 °C. 2-O-desulfated heparin was prepared by hydrolysis with N-methyl-N-(trimethylsilyl)trifluoroacetamide as described in [29]. Porcine mucosal heparin was decarboxylated by treatment with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and subsequent sodium borohydride reduction as described previously in [30]. All other reagents not specified above were obtained at the highest purity commercially available.

Cloning, expression, and purification of the chimaeric alpha1-antitrypsin-P4–P1 mutant

A chimaeric mutant, called α1-(P4–P1) and comprising α1-antitrypsin with the P1–P4 residues of the RCL replaced by the corresponding residues of the SERPING1 RCL, was produced essentially according to the method of Hopkins et al. [31]. Briefly, a construct containing Pittsburgh α1-antitrypsin (M358R) in the pTERMET vector was used as a template for site-directed mutagenesis with the following primers: sense, 5′–GGG GGG GAT AGA TCT GGC TAC GCT CTC−3′; antisense, 5′–GGG GGG GAT AGA TCT GGC TAC GCT CTC−3′.

The resulting construct was transfected into Escherichia coli BL21(DE3) cells, and protein expression was induced at 37 °C with 0.5 mM IPTG (isopropyl β-D-thiogalactoside), followed by the addition of rifampicin (1 mM) 30 min after induction. Cells were harvested after 4 h by centrifugation at 6000 g for 20 min and stored at −80 °C in RB (resuspension buffer; 50 mM Tris/HCl, pH 7.8, containing 100 mM NaCl and 10 mM EDTA).

Cell lysis was achieved by thawing at room temperature (22 °C), incubation with 167 μM PMSF and 1 mg lysozyme for 30 min, followed by sonication and centrifugation of the lysate at 8000 g for 20 min. The pellet was resuspended in RB and incubated with DNaseI for 15 min at room temperature, after which the insoluble material was sonicated a second time, washed four times with RB containing 0.5 % (v/v) Triton X-100, and finally resuspended in RB before centrifugation at 6000 g for 20 min. The inclusion body was then denatured in 50 mM Tris/HCl, pH 7.8, containing 6 M GuHCl (guanidine hydrochloride) and 100 mM DTT (dithiothreitol), and was refolded by drop-wise addition to > 100 × volume of 50 mM Tris/HCl, pH 7.8, containing 5 mM DTT overnight at 4 °C. Following this, the refolded protein solution was centrifuged at 10000 g for 30 min to remove all precipitated material.

The refolded protein was loaded onto a Q-Sepharose column (GE Healthcare) equilibrated with 50 mM Tris/HCl, pH 7.8, containing 50 mM NaCl, and eluted with a 50–350 mM salt gradient. Protein-containing fractions were pooled and ammonium sulfate was slowly added, with constant stirring, to the pooled protein fractions, yielding a final concentration of 2 M. 

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This was then loaded onto a Hi-Trap™ phenyl-Sepharose column (GE Healthcare) equilibrated with 2 M ammonium sulfate, washed with 2× column volumes of buffer and eluted with a salt gradient from 2 M to 0 M ammonium sulfate. Protein-containing fractions were assayed for inhibitory activity against kallikrein, and analysed by SDS/PAGE, revealing a final purity of > 90%.

**EMSA (electrophoretic mobility-shift assays)**

A fixed quantity of protein (2 μg) was incubated with various GAG concentrations in FAB [fluorescence assay buffer; 0.05 M Tris/HCl, pH 7.4, containing 0.15 M NaCl, 0.2% PEG [poly-(ethylene glycol)] 8000 and 0.02% NaN₃] at 37°C for 20 min, electrophoresed on an agarose/1× TAE (Tris/acetate/EDTA) gel (0.8% gel) at 80 V for 90 min at 4°C, and stained with Coomassie Brilliant Blue. Gels were imaged using a Typhoon Trio variable mode imager (GE Healthcare; excitation wavelength λ = 623 nm with no emission filters) and protein levels were quantified using ImageQuant software (GE Healthcare). Non-linear regression was performed using GraphPad Prism version 4.0 (GraphPad Software), with binding curves fit to the data according to the equation:

\[
(I/I_0) = (I/I_0)_{max} + [(I/I_0)_{max} - (I/I_0)] \times \log_{10} ([GAG]^d / [K_{H}^d + \log_{10} ([GAG]^d)]
\]

where \((I/I_0)\) is the ratio of intensities of the sample to the protein-only control, \(K_{H}\) is the binding constant and \(H\) is the Hill slope (variable). Curves were normalized to give an \((I/I_0)_{max}\) value of 1.

**Effects of GAGs and DXS on C1s activity**

Assays were carried out in FAB at 37°C using either C1s (10 μg/ml) or C1 complex (5 μg/ml) in the presence of a range of GAG and DXS dilutions, with final substrate concentrations of 25 μM for fluorescence quenched substrates and 50 μM for coumarin substrates. The rate of increase of fluorescence was measured on a BMG Labtech fluorescent plate reader using excitation and emission wavelengths of 320 nm and 420 nm respectively for the fluorescence quenched substrates, and 370 nm and 460 nm for the coumarin substrates. The C1 complex was activated by incubation at 37°C for 30 min [32], and C1 complex assays were performed in the presence of CaCl₂ (150 μM).

**Determination of kinetic constants for substrate cleavage**

The initial reaction rate was determined as above using fluorescence quenched P₂–P₄ substrates, with the final concentrations ranging from 2–50 μM substrate, in the presence of two concentrations (one stimulatory, one inhibitory) of each polyanion. In order to determine steady-state reaction constants (V_{max} and K_{0.5}), nonlinear regression analysis was performed using the GraphPad Prism Version 3.0. Curves were fitted to the data according to the following equation:

\[
V = (V_{max} \times X^H) / (K_{H}^d + X^H)
\]

which describes the dependence of initial enzyme velocity on substrate concentration for an enzyme displaying positive cooperativity, where \(X\) is the substrate concentration, \(V_{max}\) is the maximal reaction velocity, \(K_{0.5}\) is the half saturation constant, and \(H\) is the Hill slope.

**Analysis of C2 protein cleavage**

C1s (2.5 nM) was incubated in FAB at 37°C with C2 (0.5 μM), either in the presence of DXS5k (100 nM or 100 μM) or no polyanion. The reaction was stopped at a variable time points (up to 20 min) by addition of reducing SDS/PAGE loading buffer and samples were subjected to SDS/PAGE (12.5% gel).

**Characterization of inhibitory behaviour of α1-(P₂–P₄)**

SI (stoichiometry of inhibition) was determined by residual C1s activity or by SDS/PAGE analysis. The active concentration of C1s was first determined by titration against fully active wild-type SERPING1 of known concentration. C1s was titrated against an equal volume of α1-(P₂–P₄) mutant at various different concentrations to give a range of molar ratios (0.25–2.0), as well as protease-only and protease-free controls. The samples were then incubated at 37°C for over five half-lives of reaction time (90 min) and the activity against Boc-LGR-AMC (50 μM) was assayed as above.

For SI determination by SDS/PAGE C1s (~2 μg) was titrated against various amounts of α1-(P₂–P₄) to give molar serpin/protease ratios of 0.25–2.0, as well as protease-only and serpin-only controls. Samples were incubated at 37°C for over five half-lives of reaction (90 min) and analysed by SDS/PAGE (12.5% gel) and visualized with Coomassie Brilliant Blue. Assays were performed in, and reagents diluted with, AFB (alternative fluorescence buffer; 50 mM Tris/HCl, pH 7.4, containing 0.1 M NaCl, 0.05% Tween 20 and 0.02% NaN₃,).

**Effect of DXS5k on the kinetics of inhibition of C1s by α1-(P₂–P₄)**

Discontinuous kinetic assays were performed by incubating α1-(P₂–P₄) solution (final concentration 20 nM) with a range of DXS5k concentrations at 37°C and adding pre-warmed C1s (2 μM) at various time points. Boc-LGR-AMC (200 μM) was added and the residual activity measured as for the peptide cleavage kinetic studies. A plot of the natural logarithm of the residual proteolytic activity against incubation time yielded k_{obs}, the pseudo-first order rate constant, as the magnitude of the gradient. In turn, k_{obs} was used to provide an estimate of the association rate constant, k_{as}, according to the relationship k_{as} = (k_{obs}/[I]). All kinetic assays were performed in AFB and a correction was made for background substrate hydrolysis.

**RESULTS AND DISCUSSION**

Poylanions play key roles in modulating physiological protease–serpin systems in vivo [33], including those involving SERPING1 [14,15]. However, the exact mechanism by which polyanions influence the C1s–SERPING1 reaction had not been determined, with current data indicating it is distinct from the bridging and allosteric mechanisms known to operate in other systems (see Supplementary material at http://www.BiochemJ.org/bj/422/bj4220295add.htm). Two recent reports [22,34] have respectively introduced polyanion–protease interactions or a charge sandwich mechanism as means of acceleration of serpin–protease reactions. These led us to investigate how important protease–polyanion interactions were with respect to the C1s–SERPING1 system.

**Binding of C1s to synthetic and endogenous polyanions**

All DXS species caused an increase in the mobility of C1s (relative to a C1s-only control) under native conditions on EMSAs,
This was achieved using six different preparations of mucosal groups and the carboxylate group for C1s binding (Figure 1B) and to determine the relative importance of each of the sulfate C1s molecule binds per polyanion chain. The average polyanion chain length, suggesting that more than one indicates that binding-affinity increases as a function of increasing DXS43k and DXS 500k respectively (Figure 1A). These results also suggest that 6-O-sulfation of the glucosamine moiety of heparin is crucial for heparin binding to C1s and that 2-O-sulfation also appears to play an important role. Furthermore, our observation that CSA and CSC cannot bind C1s suggests that the configuration of the uronic acid moiety (which is iduronic as opposed to glucuronic) is a key determinant of polyanion–C1s binding.

Having confirmed that polyanions are able to bind C1s, we investigated whether binding of DXS5k or heparin to C1s caused conformational changes in the protein, by examining changes in the tryptophan fluorescence of the enzyme following addition of polyanion. The results indicated that C1s does not undergo a significant conformational change in the presence of either DXS or heparin (results not shown).

**Effect of polyanions on C1s proteolytic activity**

The activity of C1s against the C2 P→P peptide substrate was determined over a range of DXS5k concentrations (Figure 2). The effect exerted by DXS was concentration-dependent and biphasic, with enzyme activity increased up to 1.7-fold at lower DXS5k concentrations (100 nM), but inhibited at DXS5k concentrations above 10 μM. The maximum downregulation of C1s activity was a 6.4-fold decrease, observed in the presence of 1 mM DXS5k. The dependence of the initial velocity of C1s cleavage of the C2 P→P peptide substrate on the substrate concentration followed a sigmoidal relationship, both in the absence and presence of DXS (Figure 2). The presence of an activating concentration of DXS5k (100 nM) caused a 3-fold decrease in V max when compared with C1s alone. This contributed to a 2-fold higher specificity value (V max/K m) than that for C1s alone. Further studies with DXS10k, DXS43k and DXS500k gave similar results, which are summarised in Table 2. These results show that as the average molecular mass of the DXS chains increased, the concentration required to either stimulate or inhibit C1s activity decreased. The endogenous polyanion, heparin, also affected C1s activity (against the same substrate), with all forms exerting the same biphasic effect on C1s activity as found with DXS (Table 2).
Table 1 Kinetic constants for the proteolytic activity of C1s against a synthetic substrate corresponding to the P_{4-}P_{4} residues of the physiological substrate C2

<table>
<thead>
<tr>
<th>DXS5k</th>
<th>K_{S1} (μM)</th>
<th>V_{max} (nM/min)</th>
<th>Relative specificity (V_{max}/K_{S1}) (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DXS5k</td>
<td>35.4 ± 3.4</td>
<td>311 ± 19.2</td>
<td>1.00</td>
</tr>
<tr>
<td>100 nM</td>
<td>20.1 ± 1.2</td>
<td>377 ± 14.2</td>
<td>2.13</td>
</tr>
<tr>
<td>100 μM</td>
<td>12.4 ± 0.8</td>
<td>839.0 ± 4.7</td>
<td>0.76</td>
</tr>
</tbody>
</table>

The table shows the kinetic parameters (± S.E.M.) in the absence of polyanions, and the presence of both stimulatory (100 nM) and inhibitory (100 μM) concentrations of DXS5k. Assays were performed in duplicate and the S.E.M. was less than 10 % in all cases.

**Figure 2** Effect of DXS5k on kinetics of C1s substrate cleavage

(A) Initial velocity of substrate cleavage by C1s in the presence of various DXS5k concentrations. (B) Kinetic behaviour of cleavage in the absence of DXS5k (○), the presence of a stimulatory concentration (100 nM) of DXS5k (□) and the presence of an inhibitory concentration of DXS5k (△). In both cases, activity was measured in duplicate and data points display means ± S.E.M. Graphs are representative of at least two independent experiments. AFU; arbitrary fluorescence units.

The effects of DXS5k on C1s activity shown above were not just applicable to the cleavage of the C2 P_{4-}P_{4} substrate. Cleavage of a range of C1s substrates of different lengths (C2 P_{4-}P_{4}, C4 P_{3-}P_{4}, C4 P_{3-}P_{4}, SERPING1 P_{4-}P_{4} and SERPING1 P_{4-}P_{4}) in the presence of DXS yielded similar effects (results not shown). Again, the main effect on the kinetic constants was through V_{max} rather than K_{S1}. However, these effects were not observed with the shortest substrate used, the C2 P_{4-}P_{4} sequence. The difference in behaviour between the C2 P_{4-}P_{4} and C2 P_{3-}P_{4} substrate, which differ by only one residue, may shed light on the mechanism by which polyanions exert their effect on C1s activity. One possible explanation is that there may be two polyanion-binding sites on C1s, a high-affinity stimulatory site, and a low-affinity inhibitory site. Analysis of the active site cleft of C1s (from structure 1ELV in the Protein Data Bank [37]) shows that the electrostatic surface near the S_{1} pocket is positively charged, thereby defining it as a putative polyanion-binding site. The consequence of polyanion binding in this region would probably be obstruction of the S_{1} pocket, thereby affecting substrates longer than three residues. This would also explain why several other groups failed to see a similar effect of heparin on C1s activity when using short synthetic peptides [16,38,39]. One argument against this interpretation is that, although it is consistent with the effects on activity, the binding data revealed only one major shift in protein mobility, implying only one binding site. However, given that the high-affinity binding site appears to affect activity at very low polysaccharide concentrations, the C1s mobility shift associated with high-affinity binding may be below the sensitivity range of the EMSA. For this reason, the observation of only one distinct mobility shift in the data above is not necessarily inconsistent with the presence of more than one polyanion-binding site on C1s.

**Effect of polyanions on the proteolytic activity of the C1 complex**

The effect of DXS5k on the kinetic parameters for cleavage of the C2 P_{4-}P_{4} substrate by the C1 complex, the physiological context for C1s, was investigated. Two concentrations of DXS5k, one stimulatory and one inhibitory to C1s activity, were used, and assays were conducted in the presence of CaCl_{2} (150 μM) in order to ensure correct assembly and function of the C1 complex. Similar trends were seen as for C1s alone (results not shown), which suggests that DXS5k has the ability to modulate the activity of C1s in the C1 complex in a concentration-dependent manner. These observations were consistent with previous reports stating that polyanions affect the rate of inhibition of C1s as part of the C1 complex [40]. This strongly suggests that the behaviour of isolated C1s is likely to bear relevance to its behaviour when present in the C1 complex.

**Effect of DXS5k on the rate of cleavage of C2 protein by C1s**

We next considered the results thus far obtained in relation to a physiological substrate of C1s by examining cleavage of full-length C2 protein, as analysed by SDS/PAGE, in both the presence and absence of DXS5k (Figure 3). In the absence of polyanion, incubation of C1s with C2 gave rise to a cleavage product that was detectable after 1 min, with the reaction nearing completion after 45 min. When 100 nM DXS5k was present, the cleavage was...
contrast with our results that show polyanions do not affect the 
proteolytic activity of trypsin, thrombin and plasmin [43], as well 
[42]. There is also evidence that heparin is able to modulate the 
availability of human leucocyte elastase in a dose-dependent manner 
polyanionic glycoprotein, mucin, is able to inhibit the proteolytic 
activity of C1s, as well as causing non-Michaelis–Menten kinetic behaviour. This is in 
...al to either DXS5k or heparin (Figure 4). After this, we 
verified that the chimaeric mutant could indeed inhibit C1s as 
the structure of our mutant is intermediate between wild-type 
SERPING1 and the human protein, which is the major structural 
difference between the two chimaeric serpin mutants, although contributions from the different residues at P1 and P2 (valine and alanine respectively) cannot be excluded.

Binding of a chimaeric α1-antitrypsin mutant to polyanions and inhibition of C1s

Given that both C1s and SERPING1 bind polyanions, acceleration of inhibition may be mediated via C1s–polyanion interactions, SERPING1–polyanion interactions or a combination of both interactions. We isolated the contribution of the protease–polyanion interaction, compared with the overall effect, by using a chimaeric mutant. The mutant comprised α1-antitrypsin with the P2–P1 residues of the RCL replaced by the P2–P1 residues of the SERPING1 RCL, called α1-(P2–P1). This approach was based on an experiment reported by Sulikowski et al. [44], where replacement of the P2–P1 residues of the RCL of α1-antitrypsin with the sequence LGR resulted in the production of a reasonably effective C1s inhibitor. Our rationale was to produce a chimaeric mutant that would be able to react with C1s, yet be incapable of 

Effect of DXS5k on the kinetics of inhibition of C1s by α1-(P2–P1)

A discontinuous assay under pseudo first-order conditions over a 
range of DXS5k concentrations was performed and kass value 
plotted as a function of DXS5k concentration (Figure 5). This graph revealed a trend reminiscent of the response exerted by DXS5k on C1s proteolytic activity against full-length C2 protein. The primary effect was a decrease in kass at higher DXS5k concentrations (a maximum 3.3-fold decrease at 10−3 M DXS5k).
The gradient, was used to estimate the second-order rate constant \( k_{ass} \) using the relationship in the absence of polyanions. The pseudo-first order rate constant \( k_{obs} \), the magnitude of relevant context, it is worth noting that inhibition of C1s. To put the former finding into a physiologically-

interactions are crucial for acceleration of SERPING1-mediated interactions on the classical pathway of complement. Firstly, polyanions dir-

the results reported here demonstrate two effects of polyanions on the classical pathway of complement. Firstly, polyanions directly inhibit C1s activity, and secondly, polyanion–protease interactions are crucial for acceleration of SERPING1-mediated inhibition of C1s. To put the former finding into a physiologically-relevant context, it is worth noting that in vivo mammalian cell surfaces are covered in proteoglycans, many of which contain polyanionic GAGs (e.g. heparan sulfate) [45a]. The results in the present study predict that these endogenous polyanions would exert the same effects on C1s activity as the polyanions investigated here, with inhibitory effects on activity being dominant. Providing the GAG concentration on the cell surface is high enough, our data suggest that complement activation would be downregulated, thereby protecting self-tissues during immune surveillance. This mechanism of direct complement downregulation by polyanions would probably operate alongside polyanion-mediated potentiation of SERPING1 function, given that the two are not mutually exclusive. The direct mechanism of complement downregulation postulated above is likely to be most important in controlling basal levels of complement proteolytic activity when there is little or no SERPING1 present. However, when large amounts of SERPING1 are present, the dominant mechanism by which complement activity is downregulated is almost certainly through SERPING1 potentiation. An interesting point to note is that pathogenic molecular surfaces, especially on bacteria, are non-sulfated [45a], and would hence be unable to protect themselves from complement-mediated killing via either direct C1s inhibition or SERPING1 potentiation.

Our results concerning the effect of polyanions on the rate of the C1s–SERPING1 reaction generally agree with the charge neutralization mechanism recently proposed by Beinrohr et al. [34]. A key finding here is that when the serpin–polyanion interaction is removed from the ternary C1s/SERPING1/polyanion system, the acceleration of the rate of interaction is lost, confirming that polyanion–serpin interactions are indeed critical for SERPING1 potentiation. However, it should be noted that the proposed mechanism would also involve neutralization of positive charge on the protease surface, and as such, protease–polyanion interactions probably play a part in the potentiation. A recent study, investigating the effect of protease–polyanion interactions in the reactions of FXa with both antithrombin and SERPING1 [46], showed that basic residues in the 170-loop of FXa constitute a heparin binding site, with the Arg[171] residue being most important for heparin-mediated acceleration of the FXa–SERPING1 reaction. Mutation of this arginine residue to alanine reduced the heparin-mediated potentiation of the reaction approx. 3-fold, but did not abrogate the effect of heparin altogether. This suggests that, in the case of the FXa–SERPING1 reaction, charge neutralization of both the serpin and protease are crucial for potentiation.

Our results also hold relevance for potential therapeutic strategies for the treatment of HAE (hereditary angioedema) and myocardial infarction, where modulation of SERPING1 is emerging as a significant option for treatment [47]. Previous work has identified DXS5k as a candidate for the pharmacological manipulation of complement activation via the potentiation of SERPING1 [14], as this polyanion is unable to activate the contact coagulation system and has 3-fold lower affinity for antithrombin than heparin [48]. Consequently DXS5k does not have an enhancing effect on antithrombin-mediated inhibition of thrombin and factor Xa [49]. In addition, DXS5k is more effective in potentiating SERPING1-mediated inactivation of complement than heparin [14], making it a desirable pharmacological candidate. However, although DXS lacks antithrombotic activity, its charged nature may lead to other complications, such as thrombocytopenia due to aggregation of cell surface receptors.

More generally, care should be taken when applying these results to a physiological setting, as the affinity of heparin for C1s is higher than its affinity for SERPING1. As a result, heparin may preferentially bind to the protease (or indeed with other proteins) rather than SERPING1 in vivo. As a result, validation of the effect of endogenous polyanions, such as heparin, on the C1s–SERPING1 interaction in vivo remains an important area of study.

**General discussion**

The results reported here demonstrate two effects of polyanions on the classical pathway of complement. Firstly, polyanions directly inhibit C1s activity, and secondly, polyanion–protease interactions are crucial for acceleration of SERPING1-mediated inhibition of C1s. To put the former finding into a physiologically-relevant context, it is worth noting that in vivo mammalian cell surfaces are covered in proteoglycans, many of which contain polyanionic GAGs (e.g. heparan sulfate) [45a]. The results in the present study predict that these endogenous polyanions would exert the same effects on C1s activity as the polyanions investigated here, with inhibitory effects on activity being dominant. Providing the GAG concentration on the cell surface is high enough, our data suggest that complement activation would be downregulated, thereby protecting self-tissues during immune surveillance. This mechanism of direct complement downregulation by polyanions would probably operate alongside polyanion-mediated potentiation of SERPING1 function, given that the two are not mutually exclusive. The direct mechanism of complement downregulation postulated above is likely to be most important in controlling basal levels of complement proteolytic activity when there is little or no SERPING1 present. However, when large amounts of SERPING1 are present, the dominant mechanism by which complement activity is downregulated is almost certainly through SERPING1 potentiation. An interesting point to note is that pathogenic molecular surfaces, especially on bacteria, are non-sulfated [45a], and would hence be unable to protect themselves from complement-mediated killing via either direct C1s inhibition or SERPING1 potentiation.

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**AUTHOR CONTRIBUTION**

Thomas Murray-Rust conducted the binding studies between all proteins and different forms of polyanions, oversaw testing of the effect of polyanions on the association between serpins and C1s, and wrote an early draft that formed the basis of the manuscript. Felicity Kerr carried out the work elucidating the effects of polyanions on C1s activity and wrote an early draft that formed the basis of the manuscript. Adele Thomas oversaw work on the testing of the chimaeric
REFERENCES

6 Arlaud, G. J., Reboul, A., Sim, R. B. and Colomb, M. G. (1979) Interaction of serpin and carried out work on the effects of polyanions on the association between C1s and serpins. Tina Wu made the mutant serpin, purified it and carried out initial testing of its ability to inhibit C1s. Tang Yongqing carried out the final determination of the effect of polyanions on the association between serpins and C1s. Poh Ong oversaw the construction of the chimeraic serpin and its testing. Noetlene Quinsey oversaw the initial phases of all experimental work and helped interpret data and write the early drafts of the manuscript. James Whistock aided structural interpretation of results and contributed to writing of the manuscript. Ineke Wagenaar-Bos helped conceive the original experiments, aided in interpretation of results and contributed to writing of the manuscript. Craig Freeman provided physiological polyanions and their variants, helped interpret experimental results and contributed to writing of the manuscript. Robert Pike conceived many of the initial experiments, guided all experimental work and polished numerous drafts of the manuscript.

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15 Sahu, A. and Pangburn, M. K. (1993) Identification of multiple sites of interaction in heparins with the C1-inhibitor serpin and carried out work on the effects of polyanions on the association between C1s and serpins. Tina Wu made the mutant serpin, purified it and carried out initial testing of its ability to inhibit C1s. Tang Yongqing carried out the final determination of the effect of polyanions on the association between serpins and C1s. Poh Ong oversaw the construction of the chimeraic serpin and its testing. Noetlene Quinsey oversaw the initial phases of all experimental work and helped interpret data and write the early drafts of the manuscript. James Whistock aided structural interpretation of results and contributed to writing of the manuscript. Ineke Wagenaar-Bos helped conceive the original experiments, aided in interpretation of results and contributed to writing of the manuscript. Craig Freeman provided physiological polyanions and their variants, helped interpret experimental results and contributed to writing of the manuscript. Robert Pike conceived many of the initial experiments, guided all experimental work and polished numerous drafts of the manuscript.
Effect of polyanions on the activity and inhibition of complement C1s


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SUPPLEMENTARY ONLINE DATA

Modulation of the proteolytic activity of the complement protease C1s by polyanions: implications for polyanion-mediated acceleration of interaction between C1s and SERPING1


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SUPPLEMENTARY EXPERIMENTAL

Plasma SERPING1 (Cetor) was obtained from Sanquin, C1s was purchased from Calbiochem, heparin was purchased from Leo Pharma (Breda, The Netherlands), and the chromogenic substrate S-2314 (D-Val-Ser-Arg-p-nitroanilide) was obtained from Chromogenix.

SUPPLEMENTARY RESULTS AND DISCUSSION

A classic template mechanism cannot explain potentiation

The binding of both serpin and protease to GAGs (as demonstrated in the main section of this paper) is a hallmark of the classical template mechanism. When this mechanism is operative, as in the case of the thrombin–antithrombin interaction [1], a plot of the association rate between the serpin and protease against GAG concentration typically yields a bell-shaped curve [2]. A plot of residual protease activity against heparin concentration would therefore yield an inverse bell-shaped curve.

There is a clear enhancement of SERPING1 function with increasing heparin concentration (Figure S1), as demonstrated by the reduced residual C1s activity. This effect cannot be due to direct heparin-mediated inhibition of C1s, as the substrate used was shorter than four residues. However, the curve is not an inverse bell-shape, indicating that a classic template mechanism is unlikely to fully account for potentiation of SERPING1 inhibitory activity.

REFERENCES


Figure S1 The effect of heparin on the SERPING1–C1s reaction

C1s (2 nM) was incubated with SERPING1 (2 nM) and chromogenic substrate S-2314 (3.5 mM) over a range of heparin concentrations, and the substrate conversion after 6 h determined by measuring the absorbance at 405 nm. The graph is representative of two independent experiments, which gave similar results.

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