Biochimica et Biophysica Acta 1820 (2012) 56–63

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Regular article

Potentiation of C1-esterase inhibitor by heparin and interactions with C1s protease as assessed by surface plasmon resonance

Mohsen Rajabi [a], Evi Struble [b], Zhaohua Zhou [c], Elena Karnaukhova [a,*,1]

[a] Laboratory of Biochemistry and Vascular Biology, Division of Hematology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, 20892 USA
[b] Laboratory of Plasma Derivatives, Division of Hematology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, 20892 USA
[c] Division of Monoclonal Antibodies, Center for Drugs Evaluation and Research, Food and Drug Administration, Bethesda, MD, 20892 USA

ARTICLE INFO

Article history:
Received 7 June 2011
Received in revised form 30 September 2011
Accepted 14 October 2011
Available online 25 October 2011

Keywords:
C1-esterase inhibitor
C1s
Heparin
Surface plasmon resonance

ABSTRACT

Background: Human C1-esterase inhibitor (C1-INH) is a multifunctional plasma protein with a wide range of inhibitory and non-inhibitory properties, mainly recognized as a key down-regulator of the complement and contact cascades. The potentiation of C1-INH by heparin and other glycosaminoglycans (GAGs) regulates a broad spectrum of C1-INH activities *in vivo* both in normal and disease states.
Scope of research: We have studied the potentiation of human C1-INH by heparin using Surface Plasmon Resonance (SPR), circular dichroism (CD) and a functional assay. To advance a SPR for multiple-unit interaction studies of C1-INH we have developed a novel (consecutive double capture) approach exploring different immobilization and layout.
Major conclusions: Our SPR experiments conducted in three different design versions showed marked acceleration in C1-INH interactions with complement protease C1s as a result of potentiation of C1-INH by heparin (from 5- to 11-fold increase of the association rate). Far-UV CD studies suggested that heparin binding did not alter C1-INH secondary structure. Functional assay using chromogenic substrate confirmed that heparin does not affect the amidolytic activity of C1s, but does accelerate its consumption due to C1-INH potentiation.
General significance: This is the first report that directly demonstrates a significant acceleration of the C1-INH interactions with C1s due to heparin by using a consecutive double capture SPR approach. The results of this study may be useful for further C-INH therapeutic development, ultimately for the enhancement of current C1-INH replacement therapies.

Published by Elsevier B.V.

1. Introduction

Human C1-INH is an acute-phase plasma protein that belongs to the serpin superfamily, the largest class of serine protease inhibitors that also includes antithrombin (ATIII), α_1-proteinase inhibitor (α_1-PI) and many other proteins that share a high structural similarity and regulate diverse physiological systems [1,2]. C1-INH is a multifunctional plasma protein with a wide range of inhibitory and non-inhibitory properties. It is mainly recognized as a major down-regulator of the complement and contact (kallikrein–kinin) amplification cascades [3]. Physiological level of functional C1-INH in plasma is ~240 μg/mL (~3 μM), but may double during inflammation [4]. The importance of C1-INH is underlined by its deficiency which is considered a cause of the hereditary angioedema (HAE) [5,6]. Whereas most C1-INH research still focuses on understanding the mechanism and treatment of HAE, the physiological and pharmacological activities of C1-INH are much broader. In plasma C1-INH is shared by several proteases of the complement, contact, coagulation and fibrinolytic systems that are functionally closely related. Therefore, a lack of the functional C1-INH in plasma not only triggers an inappropriate activation of the kallikrein–kinin pathway leading to a high level production of bradykinin and to angioedema [7,8], but has also a profound effect on the complement cascade and other systems. It is known that *in vivo* activities of many serpins including ATIII and C1-INH are modulated by GAGs, particularly by heparin, [9–11]. In turn, the pharmacological activity of heparin is mediated by ATIII, C1-INH and other serpins. C1-INH is the only known regulator of an early stage of the complement activation due to inhibition of the C1r and C1s proteases of the first component of complement system. Since 1975, when it was first reported that C1-INH binding to GAGs may increase its binding to the complement proteins C1s and C1r [12, 13],

Abbreviations: Anti-C1-INH ab, anti-C1-esterase inhibitor antibody; α_1-PI, α_1-proteinase inhibitor; ATIII, antithrombin; C1-INH, C1-esterase inhibitor; CD, circular dichroism; DMSO, dimethyl sulfoxide; GAG, glycosaminoglycan; L/P, ligand-to-protein molar ratio; PBS, phosphate buffer saline; RCL, reactive center loop; SA, Streptavidin; SPR, Surface Plasmon Resonance

* Corresponding author at: Division of Hematology, Center for Biologics Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, National Institutes of Health Building 29, Bethesda, Maryland 20892, USA. Tel.: +1 301 402 4635; fax: +1 301 402 2780.
E-mail address: elena.karnaukhova@fda.hhs.gov (E. Karnaukhova).

[1] The opinions and assertions herein are the scientific views of the authors and are not to be construed as policy of the United States Food and Drug Administration.

0304-4165/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.bbagen.2011.10.008

the potentiation of C1-INH by heparin has been under intensive research [14–16].

Investigation of C1-INH potentiation by heparin is essential for a better understanding of the mechanism of C1-INH potentiation. Moreover, it is important for a regulation of the C1-INH potentiation as it may result in an accelerated consumption of the activated C1-INH by multiple proteases, thus leading to a deficiency of the functional C1-INH level which is essential for an accurate regulation of the complement and contact pathways. It is also important for the development of tools that may enhance the efficiency of the currently available pharmaceutical preparations of C1-INH (*e.g.*, inhibitory activity of ATIII is being enhanced up to 4000-fold by heparin [17,18].

SPR has been previously used to study the binding of complement proteins to heparin [19, 20], however, to our knowledge this is a first report in which the interactions of C1-INH with C1s and the role of heparin in the enhancement of these interactions have been demonstrated by SPR. We have developed a unique double capture SPR method to investigate the C1-INH binding to C1s with and without heparin. To visualize the effect of each individual interactant, we have immobilized C1s, C1-INH and heparin one at a time to further understand the role of the heparin in the enhancement of C1-INH to C1s binding. The data presented here for three different SPR experimental designs consistently showed a strongly marked (from 5- to 11-fold) increase of the association rate between C1-INH and C1s due to the potentiation of C1-INH by heparin. The SPR data for the reference experiments without heparin are in agreement with earlier reports on the kinetics of C1-INH with C1s as assessed by functional assay [21].

2. Materials and methods

2.1. Materials

Complement C1-INH and activated C1s were obtained from EMD chemicals USA (Gibbstown, NJ). Anti-Human C1s was purchased from American Research Products, Inc. (Belmont, MA). Porcine intestinal heparin (sodium salt) and biotinylated heparin were from Sigma (St. Louis, MO). Sensor chip SA (Streptavidin) and sensor chip CM5, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), ethanolamine–HCl, HBS-P buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% surfactant P20), Phosphate buffer saline, 10× (PBS) (0.1 M phosphate buffer with 27 mM KCl and 1.37 M NaCl, pH 7.4), Acetate buffer (pH 5.0), Glycine (pH 2.5), NaOH (50 mM), NaCl (4 M) $MgCl_2$ (5 M) and deionized water were from GE Healthcare (Piscataway, NJ). Technochrom® C1-INH test kit was from Technoclone GmbH (Vienna, Austria).

2.2. Methods

2.2.1. Immobilization of C1s on CM5 chip

C1s in sodium acetate 3 μM (pH 5) was immobilized on the surface of CM5 sensor chip using amine coupling. Approximately 6000 RU of C1s was immobilized on CM5 chip surface. The surface was then blocked by injecting 1 M ethanolamine. 50 mM NaOH was injected to wash off non-covalently bound C1s. Flow cell 1 was similarly treated with buffer in the absence of C1s (control).

2.2.2. Immobilization of anti-human C1-INH on CM5 chip

Anti-human C1-INH was diluted in 10 mM sodium acetate, pH 5.0 to the concentration 250 μg/mL and immobilized on sensor chip CM5 using amine coupling. Approximately 10,000 RU of the antibody was immobilized on CM5 chip surface. The surface was then blocked by injecting 1 M ethanolamine. 50 mM NaOH was injected to wash off non-covalently bound antibody. Flow cell 1 was similarly treated with buffer in the absence of the antibody (control).

2.2.3. Immobilization of biotinylated heparin on SA chip

A sensor chip SA was pretreated with three injections, 5 μL each, of 50 mM NaOH in 1 M NaCl, to remove any nonspecifically bound contaminants. A 20-μL injection of biotinylated heparin (500 μg/mL) in HBS-P running buffer (flow rate, 10 μL/min) was made in flow cell 2, followed by a 10-μL injection of 2 M NaCl. Flow cell 1 was similarly treated with buffer in the absence of biotinylated heparin (control). Approximately 66 RU of biotinylated heparin was immobilized in flow cell 2.

2.2.4. SPR assessment of the binding kinetics

Biacore 3000 instrument (GE Healthcare) was programmed to conduct SPR experiments and kinetic analysis. The sensorgram was recorded as a plot of binding response (resonance unit) versus time. All the sensorgrams were processed using the double referencing method to eliminate the nonspecific binding from background contribution and buffer artifacts by subtracting signals from the reference flow cell and from buffer blank injections [22]. The data for all binding proteins were analyzed and fitted to 1:1 Langmuir binding using the Biaevaluation software, version 4.1.1, supplied by GE Healthcare.

2.2.5. C1s/C1-INH binding kinetics with and without heparin using immobilized C1s

C1-INH and C1-INH incubated with heparin in PBS buffer (pH 7.4) were injected separately over C1s-immobilized CM5 surface (prepared as described in Section 2.2.1) and reference flow cells for 3 min at a flow rate of 30 μL/min over a range of concentrations. At the end of the sample injection, the running buffer (PBS, pH 7.4) was flowed for 5 min over the sensor surface to allow dissociation.

2.2.6. C1s/C1-INH binding kinetics with and without heparin using immobilized anti-human C1-INH antibody

A double capture kinetics method was used to study the interaction of C1-INH with C1s in the absence and presence of heparin using anti-human C1-INH antibody immobilized on CM5 chip as described above in Section 2.2.2. Briefly, C1-INH at 6 μM was first injected over the surface for 3 min. Approximately 60 RU of C1-INH was captured. For heparin-related experiment, heparin at 50 mg/mL was then injected for 3 min, resulting in 30 RU of captured heparin on the surface. 90 μL of C1s over the range of concentration was injected. At the end of the sample injection, the running buffer (PBS, pH 7.4) was flowed for 5 min over the sensor surface to allow dissociation. Finally, the surface was regenerated using 2 M $MgCl_2$ and Glycine (pH 2.5) for 30 s.

2.2.7. C1s/C1-INH binding kinetics using immobilized heparin

A single capture method was used to study the binding of C1-INH with C1s on SA chip with immobilized heparin (as described in Section 2.2.3). Briefly, C1-INH at 1 μM was injected over the heparin surface for 3 min. Approximately 300 RU of C1-INH was captured. 90 μL of C1s over the range of concentrations was injected. At the end of the sample injection, the running buffer (PBS, pH 7.4) was flowed for 5 min over the sensor surface to allow dissociation. The surface was regenerated using 2 M $MgCl_2$, 2 M NaCl and 5 mM NaOH for 1 min. A direct kinetics between heparin and C1-INH was also performed, in which 90 μL C1-INH over the range of concentration was injected on heparin surface then the running buffer was flowed for 5 min to allow dissociation. The surface was regenerated using the same conditions as in the single capture method. For a direct kinetics between heparin and C1s, 90 μl of C1s over the range of concentrations was injected on heparin surface then the running buffer was flowed for 5 min to allow dissociation. The surface was regenerated using 1 M NaCl for 30 s.

2.2.8. SPR data analysis

Resonance signals were corrected for nonspecific binding by subtracting the background of the control flow cell. Binding isotherms were analyzed and binding constants K_D and K_A were calculated

using the manufacturer supplied software, Biaevaluation version 4.1.1. A good fit was obtained when a simple bimolecular interaction model ($A + B \rightleftarrows AB$) (1:1 Langmuir) was applied. Visual inspection of the fitted curves overlaid on the sensorgrams showed the goodness of the fit; the model also generated chi2 (χ^2) value less than 1% of Rmax that also indicates a good fit (Table 1).

2.2.9. C1-INH functional assay

Chromogenic determination of the residual activity of C1s in the mixtures with C1-INH alone, heparin alone, and C1-INH/heparin complex was performed *in duplicate* using chromogenic substrate C_2H_5CO-Lys (ε-Cbo)-Gly-Arg-pNA, C1s and buffers of the Technochrom® C1-INH test kit (Technoclone GmbH, Vienna, Austria) as follows. Lyophylized C1-INH was pre-diluted to a ~0.4 μM stock solution (approximately 0.1 U/mL) by using kit sample buffer A. Further dilutions were performed to obtain a range of C1-INH concentrations of 4.8 nM, 3.2 nM, 2.4 nM, 1.5 nM and 0. A 12.5 μM aliquot of each C1-INH diluted solution was loaded onto the 96-well plate in four columns. Two columns were uploaded with 12.5 μL of buffer A to serve as a control; two other columns were uploaded with 12.5 μL of 15 nM heparin stock solution. After 5 s mixing on the plate-reader shaker, the plate was incubated for 30 min at 25 °C followed by adding 12.5 μL of the C1s 6 nM stock solution in the reaction buffer from the kit simultaneously to all wells of the four columns. After 5 s mixing on the shaker, the plate was incubated at 37 °C for 5 min. The chromogenic substrate was added simultaneously over the plate, and the continuous kinetics was monitored at 405 nm for 6 min, followed by the end-point measurement. The reading from the wells containing only buffer and chromogenic substrate was subtracted as a background from the actual reading. The wells containing only C1s and C1s/ heparin were monitored in the same experiment to evaluate the effect of heparin on amidolytic activity of C1s alone.

2.2.10. CD measurements

The far-UV CD spectra were recorded between 180 and 260 nm on a Jasco J-810 Spectropolarimeter (JASCO Co., Japan) at 25 ± 0.2 °C in a rectangular quartz cuvette with 0.05 cm path length. The scan speed was 100 nm/min, bandwidth was 1.0 nm, and resolution was 0.2 nm. All spectra were accumulated in triplicate. Protein concentration in all samples was 4.5 μM in PBS, pH 7.4. Heparin concentrations in the titration set varied from average 2.25 to 27 μM, thus providing the ligand-to-protein ratio from 0.5 to 6 by adding 0.5 μL of the heparin stock solution (10 mg/mL which corresponds to ~550 μM). Two forms of heparin were used for the CD titration experiments: unfractionated pharmaceutical heparin (average MW 15,000) in water for injections (WFI) and the same stock solution after repeated centrifugation using Amicon 3 K filtration (14,000 rpm, 30 min). An ellipticity of CD spectra is expressed in millidegrees (mdeg). The baseline was subtracted by running PBS as a blank. The CD spectra of C1-INH with and without heparin were converted into Δε and analyzed for the percentage of secondary structure elements using CDPro/CONTIN (SP43).

2.2.11. Structural assessments

Molecular coordinates of three serpins, namely ATIII [23], α_1-PI [24] and C1-INH [25] were obtained from the Protein Data Bank. The three dimensional structures were compared and aligned using DALI pairwise comparison web server (http://ekhidna.biocenter.helsinki.fi/dali_lite/start) [26]. The overlapped structures were then visualized using PyMOL (The PyMOL Molecular Graphics System, Version 1.1r1, Schrödinger, LLC) and the global alignment was manually and automatically improved, if necessary.

3. Results

The results from the binding experiments of C1-INH to C1s showed that heparin enhanced the affinity of C1-INH to C1s. The binding kinetics of C1-INH to C1s, using three different surfaces: (a) immobilized C1s on CM5 chip, (b) immobilized anti-human C1-INH on CM5 surface, and (c) immobilized heparin on SA surface are reported in detail below.

3.1. Interaction of C1-INH with immobilized C1s

As shown in Fig. 1A, C1-INH bound to immobilized C1s with relatively fast k_a ($1.02\times10^3\,M^{-1}\,s^{-1}$) and slow k_d ($3.46\times10^{-5}\,s^{-1}$) with K_D of 3.4×10^{-8} M. Incubation of C1-INH with heparin resulted in a tighter binding with C1s as evidenced by a lower k_d ($6.63\times10^{-8}\,s^{-1}$) and three orders of magnitude decrease in K_D (Fig. 1B, Table 1).

3.2. Interaction of C1-INH with C1s on the immobilized antibody against C1-INH

Fig. 2A,B shows the sensorgrams from the double vs. single capture binding kinetics of C1-INH with C1s. The interaction of C1-INH bound to the immobilized antibody with C1s in the presence of heparin showed a faster "on" rate compared to the one without heparin. The binding affinity of C1-INH to C1s was stronger in the presence of heparin as evidenced by a decrease in K_D by one order of magnitude (Table 1).

3.3. Interaction of C1-INH with C1s on the immobilized heparin

Fig.3 presents the sensorgrams for the binding of C1-INH to heparin surface (A) and further binding of C1s (B). The reference flow cell responses were subtracted from that of the active cell to correct for nonspecific binding. Equilibrium constants (K_D) for the interaction of C1-INH with heparin and further with C1s were 2.87×10^{-7} M and 7.17×10^{-8} M, respectively (Table 1). The sensorgrams shown in Fig. 3C correspond to the binding of C1s to heparin surface. The K_D value for the interaction of C1s with heparin was determined as 4.88×10^{-7} M (Table 1). When the C1s captured by the immobilized heparin was used for the interaction with C1-INH at various concentrations, there was no measurable binding.

3.4. CD analysis

Fig. 4 and Table 2 provide the far-UV CD assessment for the C1-INH samples (protein concentration 4.5 μM) with various amounts of heparin (from 0 to a total of 27 μM, which corresponds to ~6-fold molar excess of heparin over the protein). The far-UV CD spectrum of the initial C1-INH shows the intensive negative Cotton Effect at 208 nm and less expressed negative band around 220 nm, which together with a strong positive peak at 194 nm are indicative for α-helical structure with β-sheet elements that is in line with crystal structure data known for serpins. The raw (unsmoothed) spectral data for C1-INH samples without heparin (4.5 μM in PBS, pH 7.4) and with various amounts of heparin were performed for the estimation of structural elements using CDPro CONTIN (SP43). The estimates of α-helix, β-sheet, turns and the remainder are listed in Table 2. For C1-INH alone, it resulted in ~29.5% of α-helix (total of regular and distorted), ~23.7% of β-sheet (total of regular and distorted), ~20.9% of β-turns, and ~25% of unordered structure. With an increasing amount of heparin in C1-INH sample, the CD spectra do not change much, *i.e.*, either practically coincide with the initial C1-INH spectrum, or show some minor changes around 194 nm per increasing concentration of heparin (Fig. 4 shows only selected spectra for clarity). Table 2 shows the actual results (derived from the unsmoothed CD spectra) for the C1-INH/heparin titration, which reflect rather minor alterations (not more than 4%) in the C1-INH secondary structure upon heparin binding.

3.5. Functional assay

A plausible impact of heparin on the inhibitory activity of C1-INH and on amidolytic activity of C1s [27,28] was evaluated by using a

Table 1
Kinetic rate constants and equilibrium binding constants for the interactions of C1-INH and C1s with immobilized C1s, C1-INH and Heparin.

Ligand Analyte	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D(M)[a]	R_{max} (RU)	χ^2
C1s (C1s surface)[b]					
C1-INH	1.02×10^{3}	3.46×10^{-5}	3.4×10^{-8}	142	0.791
C1-INH with Heparin	1.09×10^{4}	2.48×10^{-7}	2.27×10^{-11}	159	2.29
C1-INH (antibody surface)[c]					
C1s	3.46×10^{3}	1.09×10^{-3}	3.15×10^{-7}	7.78	0.288
C1s after Heparin	1.75×10^{4}	4.92×10^{-4}	2.82×10^{-8}	30.7	3.66
Heparin (heparin surface)[d]					
C1-INH	2.09×10^{3}	6.01×10^{-4}	2.87×10^{-7}	23.3	0.258
C1-INH (heparin surface)[e]					
C1s	5.51×10^{4}	3.95×10^{-3}	7.17×10^{-8}	174	17.2
Heparin (Heparin surface)[f]					
C1s	3.13×10^{3}	1.53×10^{-3}	4.88×10^{-7}	136	32.5

[a]All the kinetics parameter, Rmax and χ^2 were analyzed using the Biaevaluation software, version 4.1.1. The data were fitted using 1:1 Langmuir binding model ($A + B \rightleftarrows AB$); the K_D values are not considered to be absolute representatives.
[b]Data correspond to the setup described in Materials and methods, Section 2.2.5.
[c]As described in Materials and methods, Section 2.2.6.
[d,e,f]As described in Materials and methods, Section 2.2.7.

plate-based version of Technochrom® C1-INH assay. According to our data (Fig. 5A) the amidolytic activity of C1s with heparin (black triangles) was essentially the same as that of the reference C1s without heparin (red circles). For the mixtures of C1s/C1-INH with and without heparin, the amidolytic activity of the residual C1s in the samples C1s/C1-INH with heparin (blue rhombs) shows clearly lower than that of the C1s/C1-INH without heparin (black rhombs). The impact of the heparin concentration (5 nM to 500 nM) in the mixtures of C1s/C1-INH on the observed absorbance at 405 nm was rather similar

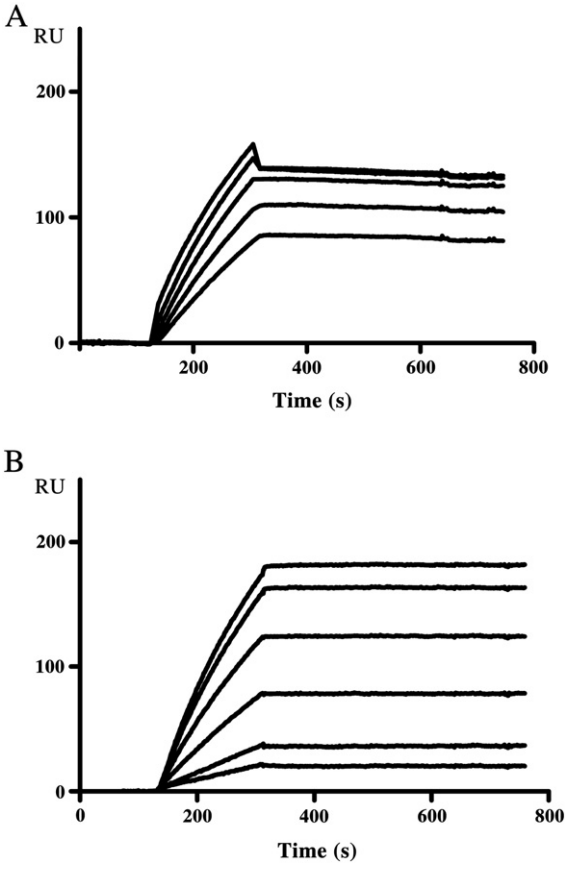

Fig. 1. SPR analysis of the binding of C1-INH to C1s immobilized on the surface, as described in Section 2.2.1. (A) Sensorgrams for the interaction of C1s with C1-INH; the curves shown correspond to C1-INH concentrations of 6, 3, 1.5, 0.75 and 0.375 μM (from upper to lower curve, respectively). (B) Sensorgrams for the interaction of C1s with C-INH pre-incubated with 1 μM heparin; the C1-INH concentrations are 6, 3, 1.5, 0.75, 0.375 and 0.1875 μM.

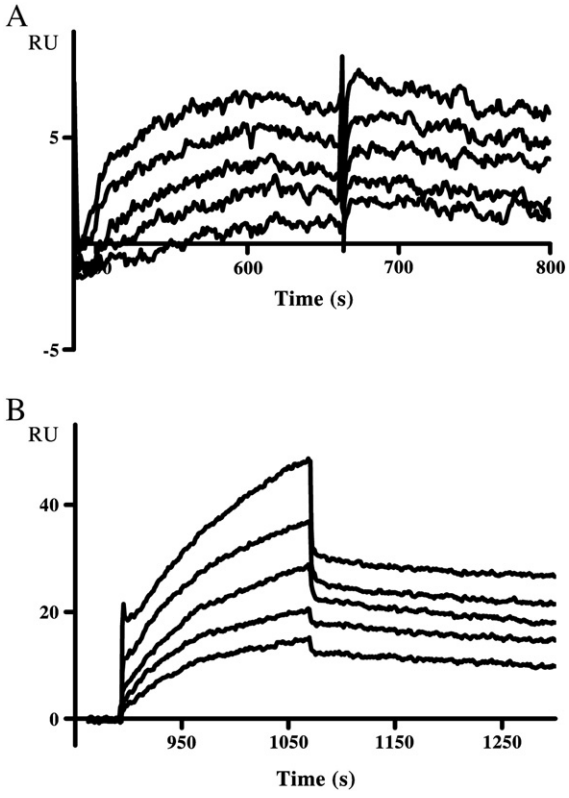

Fig. 2. SPR analysis of the binding of C1s to C1-INH captured by the immobilized antibody against C1-INH surface as described in Section 2.2.2. (A) Sensorgrams for the C1s interaction with captured C1-INH; the curves shown correspond to C1s concentrations of 6, 3, 1.5, 0.75 and 0.375 μM (from upper to lower curve, respectively). (B) Sensorgrams for the C1s interaction with captured C1-INH and heparin (2.7 mM); the C1s concentrations are 1.5, 0.75, 0.375, 0.1875 and 0.094 μM.

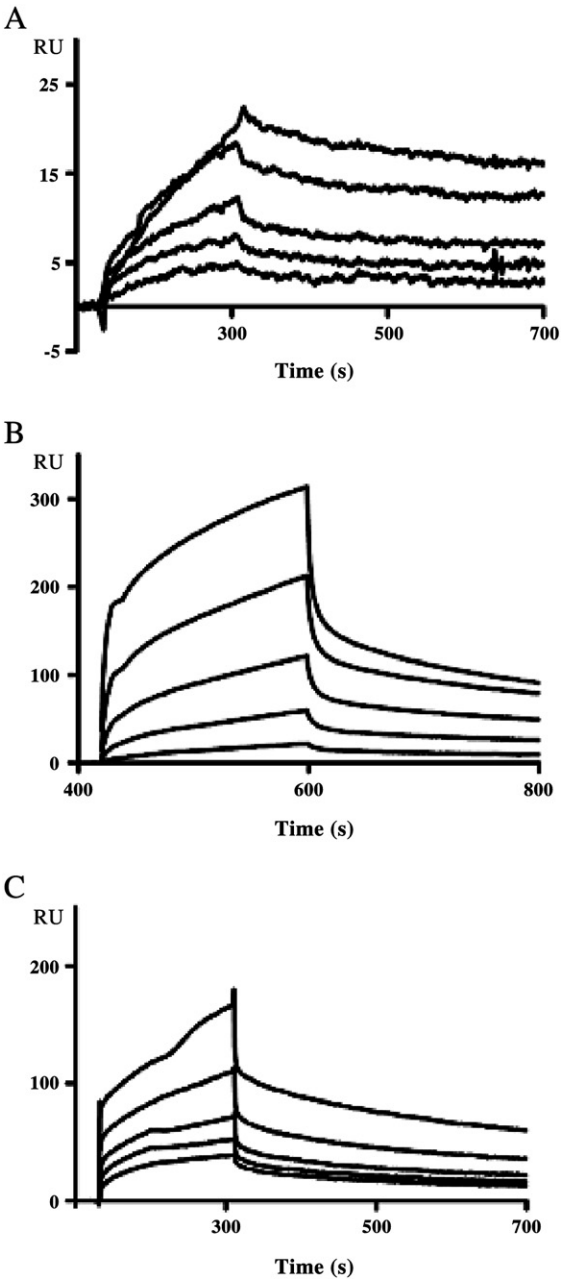

Fig. 3. SPR analysis of the binding of C1-INH with C1s on the heparin immobilized surface as described in Section 2.2.3. (A) Sensorgrams for the C1-INH binding to heparin surface; the curves shown correspond to C1-INH concentrations of 6, 3, 1.5, 0.75 and 0.375 μM (from upper to lower curve, respectively). (B) Sensorgrams for the C1s binding to C1-INH; the C1s concentrations are 3, 1.5, 0.75, 0.375 and 0.1875 μM. (C) Sensorgrams for the C1s binding to heparin surface; the C1s concentrations are the same as in panel (B).

to that reported earlier [29]. Fig. 5B demonstrates an impact of the heparin concentrations on the observed absorbance at 405 nm that is related to the residual activity of the C1s in the mixtures with C1-INH that were pre-incubated with various amounts of heparin. The absorbance minimum at ~500 nM of heparin corresponds to the lowest residual amount of C1s in the mixtures with C1-INH/ heparin, and thus, to the highest level of C1-INH potentiation. Interestingly, the impact of higher heparin concentrations (from 550 nM to 1 μM, Fig. 5B) on the residual activity of C1s is less significant than that observed for heparin in the range of physiologically relevant concentrations (~55 nM to 500 nM). The reason for that is unclear, although similar results were obtained in two independent experiments and close to earlier published data [29].

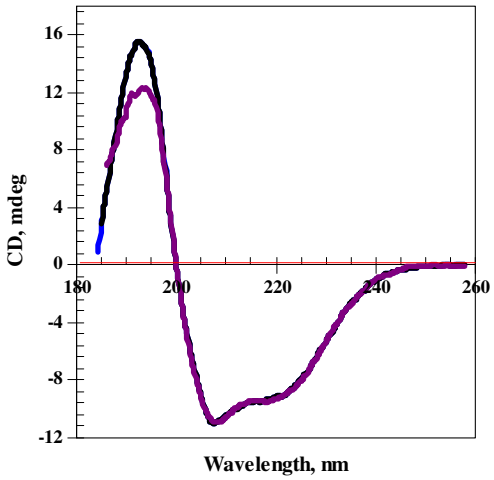

Fig. 4. CD spectra of C1-INH (4.5 μM in PBS, pH 7.4) alone (black trace), and in the mixtures with heparin: 4.5 μM heparin (blue trace overlaying black trace) and 27 μM heparin (purple trace).

4. Discussion

4.1. SPR assessment

This study was designed to examine the nature of C1-INH potentiation by heparin and its subsequent binding to C1s by SPR. Analysis of binding kinetics of C1-INH with C1s using three different surfaces, *i.e.*, C1s, C1-INH and heparin indicates that the C1-INH binding to heparin increases its affinity toward C1s. Our SPR data clearly demonstrate that depending on the experimental layout and surface used the kinetic values obtained for the interaction of C-INH with C1s vary (Table 1). Published SPR data are based on simple direct or single capture type of interactions. A direct binding of C1-INH and other complement proteins to GAGs has been assessed by SPR before [16,19,20,30]. Our data for the direct binding kinetics of C1-INH and C1s with heparin are in agreement with those published by Caldwell et al. [16] and Rossi et al. [19]. The K_D value for the C1-INH interaction with heparin was determined as 2.87×10^{-7} M (Table 1) which is within less than a 2-fold agreement with that (1.67×10^{-7} M) published by Rossi et al. [19], and within a 4-fold agreement with the K_D (0.7×10^{-7} M) earlier reported by Caldwell et al. [16] . The K_D value determined for the interaction of C1s with heparin (4.88×10^{-7} M, Table 1) is within ~2.2-fold agreement with that recently published by Caldwell et al. [19]. In the earlier studies the potentiation of C1-INH by GAGs was demonstrated mainly by functional assays [16, 30]. The double capture SPR approach enabled us to evaluate the potentiation of C1-INH by heparin towards C1s using real time kinetics. The results from using different surfaces strongly suggest that heparin increases the association rate between C1-INH and C1s (Table 1). A 5-fold increase of the association rate between C1s and C1-INH captured on the immobilized antibody was observed due to presence of heparin ($1.75\times10^{4}\ M^{-1}\ s^{-1}$ for C1s injected after heparin versus $3.46\times10^{3}\ M^{-1}\ s^{-1}$ for C1s only).

Table 2
Percentage of the secondary structure elements determined by CDPro CONTIN.

Sample	L/P[a]	H(r)[a]	H(d)	S(r)	S(d)	Turn	Unrd	Total
C1INH	0	16.7	12.8	15.3	8.4	20.9	25.9	100.0
C1INH/$Hp^{0.6}$	0.6	16.3	13.3	14.0	9.5	21.9	25.1	100.1
C1INH/Hp^{1}	1.0	16.7	13.4	15.4	8.6	20.7	25.3	100.1
C1INH/Hp^{2}	2.0	15.9	12.9	15.7	8.9	20.5	26.1	100.0
C1INH/Hp^{4}	4.0	12.4	11.9	14.9	9.6	22.1	29.1	100.0
C1INH/Hp^{6}	6.0	14.6	13.2	14.5	9.6	22.1	26.0	100.0

[a] L/P, ligand-to-protein molar ratio; H(r) and H(d), α-helix regular and distorted; S(r) and S(d), β-strand regular and distorted; Unrd, unordered.

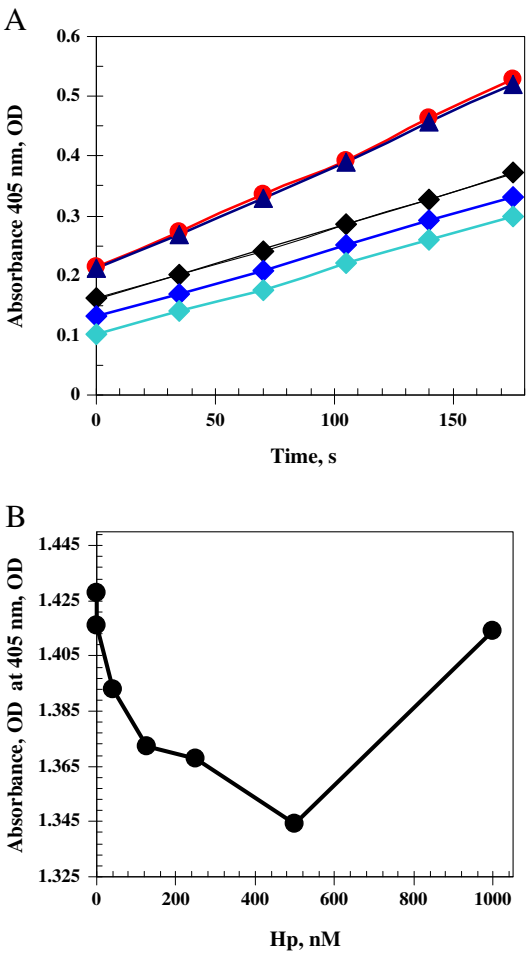

Fig. 5. Effect of heparin on the C1-INH and C1s using chromogenic assay for evaluation of C1-INH activity. (A) Continuous kinetics: C1s (0.4 nM), alone (red circles) and with 20 nM heparin (navy triangles); mixture of C1s (0.4 nM) with C1-INH (0.16 nM) alone (black rhombs), and with 20 nM and 40 nM of heparin (blue and cyan rhombs, respectively). (B) End-point kinetics (10 min) for the mixture of C1s (0.4 nM) with C1-INH (0.16 nM) with various amounts of heparin (0, 0.4 nM, 40 nM, 125 nM, 250 nM, 0.5 μM, and 1 μM).

An almost 11-fold increase was observed for the interactions between C1-INH and immobilized C1s when C1-INH was mixed with heparin ($1.09\times10^4\ M^{-1}\ s^{-1}$ for C1-INH/Heparin mixture versus $1.02\times10^3\ M^{-1}\ s^{-1}$ for C1-INH alone).

Since both C1-INH and C1s are heparin binding proteins [20,19], it was of particular interest to make sure that the binding kinetics observed for the interaction of C1s to C1-INH on heparin surface was not due to the binding of C1s to heparin. We determined the binding kinetics of C1s to heparin surface using similar conditions as those used for the interaction of C1-INH with heparin. Our data confirmed that the kinetic profile and constants for bindings of C1s to heparin (K_D 4.88×10^{-7} M) and the C1s to C1-INH on heparin surface (K_D 7.17×10^{-8} M) are completely different (Fig. 3B,C; Table 1).

We also conducted another experiment in which we first captured C1s on heparin surface then injected C1-INH at various concentrations to further evaluate whether the enhancement of the interactions between C1s to C1-INH is due to the heparin-induced activation of C1-INH, and not C1s. There was no measurable binding of C1-INH to C1s captured on heparin surface (data not shown). This experiment unambiguously indicate that the interaction between C1-INH and C1s is enhanced only when the C1-INH is first bound to heparin, thus supporting a mechanism according to which heparin first binds to C1-INH and neutralizes its surface thus accelerating its interactions with C1s.

Our SPR data for the potentiation of C1-INH by heparin are comparable with that evaluated by cell-bound C1 activity assay [16], i.e., a 6-fold augmentation of C1-INH by heparin was reported by Caldwell et al. [16].

Heparin possesses sulfate and carboxyl groups that bind and neutralize the positively charged patches on the protein surface. Heparin may mediate the inhibition of C1s by C1-INH through the following pathways: *(i)* Heparin interacts with the basic (Lys or Arg) patch near the serpin reactive center loop (RCL) (Fig. 6A and B) that results in charge neutralization, *(ii)* Heparin binds to C1s allosterically thus optimizing the active site region for interaction with C1-INH, and *(iii)* Heparin-bound C1-INH makes productive interactions with basic residues on the C1s, so-called "*sandwich mechanism*". As first proposed by Beinrohr et al. [31] heparin binds to C1-INH with low affinity and neutralizes surface charge at a specific region which results in attraction of C1s to this surface. Our comprehensive binding analysis of C1-INH with C1s with and without heparin using SPR supports the "*sandwich mechanism*".

4.2. CD assessment

As a method sensitive to conformational changes, the CD spectroscopy has been explored numerously for the evaluation of protein conformational changes due to interactions with heparin. However, the estimation of the secondary structure elements from CD data and other optical spectroscopic studies is empirical [32, 33]. It depends on the selected set of the reference proteins, on the calculation algorithm used, experimental conditions (buffer, pH, temperature), as well as on certain protein parameters, such as glycosylation, other posttranslational modifications, aggregation, etc. [33,34]. As the crystal structure of native human plasma C1-INH is not resolved yet because of the protein size, heavy glycosylation and heterogeneity, the evaluation of C1-INH secondary structure by CD provides a valuable insight. Whereas the percentage of the structure elements estimated from the CD data is not absolute, CD is a validated tool for the comparison of the conformational changes if the same experimental conditions, the same set of the reference proteins and the same algorithm for calculations are used. Comparison of the C1-INH and C1-INH/heparin CD spectra in the 180–240 nm range where the electronic transitions of the peptide bond occur provided an evidence that no remarkable changes in the C1-INH secondary structure occurred in the presence of heparin (Table 2).

It is known that heparin and other GAGs exhibit their characteristic CD in the same far-UV region that is used to estimate the protein secondary structure features [35–37]. However, in the concentrations used for C1-INH interactions (up to 27 μM of heparin) heparin does not show any optical activity that may overlap with the protein CD (Fig. 4, thin red trace). Despite the fact that heparin did bind to C1-INH and significantly enhanced the interactions of C1-INH with C1s our far-UV CD assessment did not visualize any remarkable conformational changes (Table 2, Fig. 4). There could be several reasons for that. Whereas heparin was often reported as a negatively charged template that per binding with a protein may induce its conformational changes that could be documented by CD [38,39], it is not always the case. For example, the heparin modulation of human plasma kallikrein activity was not accompanied by any visible change in the protein secondary structure [40]. Even the most extensively studied binding of heparin and ATIII, which is associated with a drastic 3-order increase of the ATIII potency explained via protein conformational changes, was actually supported by modest conformational alterations observed by CD [36,41]. It is reasonable to assume that even minor alterations in the protein/heparin CD spectra can be associated with quite dramatic changes in the protein potency. In the meantime, if by binding to C1-INH the heparin's action is mainly a

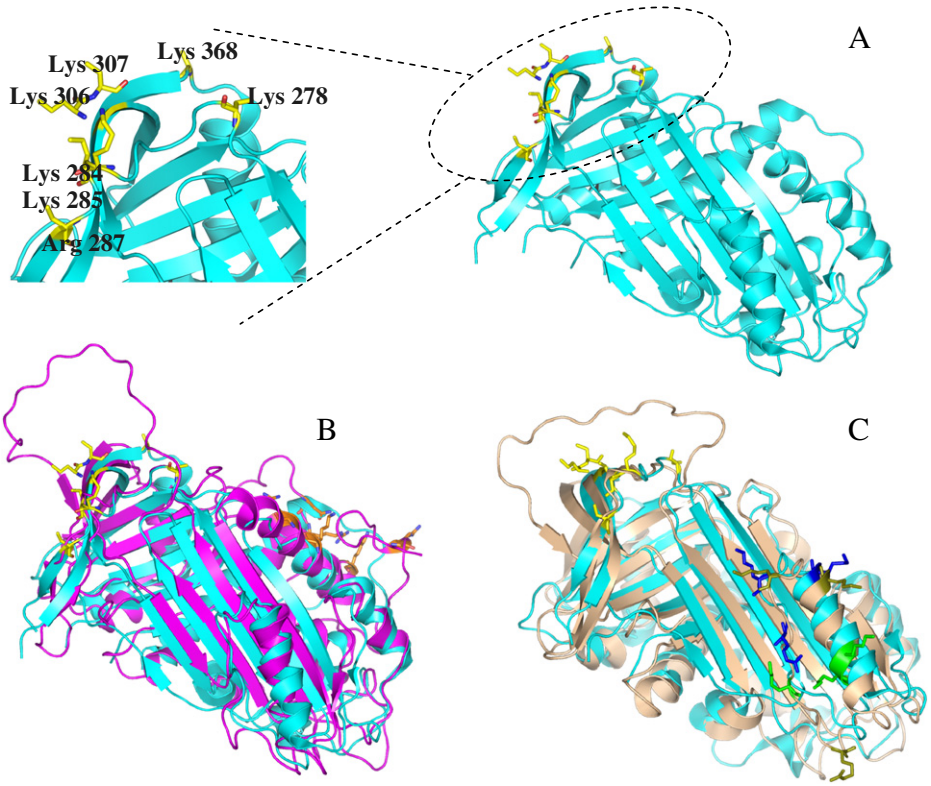

Fig. 6. Comparison of the crystal structures and heparin binding sites for C1-INH, ATIII and α_1-PI. (A) Crystal structure of the serpin domain of recombinant latent C1-INH (PDB 2OAY); Inset shows the positively charged amino acid residues of the heparin binding site [24]; (B) Cartoon representation of aligned structures of C1-INH (cyan) and ATIII (magenta, PDB ID: 3KCG); Amino acid side chains of heparin binding site are shown in stick representation, colored yellow for C1-INH and orange for ATIII; (C) Aligned structures of C1-INH (cyan) and α_1-PI (light pink, PDB ID: 1HP7). Amino acid side chains for heparin binding sites are shown in yellow for C1-INH and green for α_1-PI as identified in Ref. [23]; the residues in blue correspond to Lys 246, Lys 251 and Arg 254 of C1-INH that may serve as an alternative binding site.

neutralization of the surface charges of certain Lys and Arg residues, the conformational changes, indeed, can be minor.

4.3. Comparative evaluation of serpin structure

Structural aspects of the interactions of GAGs with proteins, including serpins, have been extensively studied [42]. However, ATIII is the only serpin which was co-crystallized with heparin pentasaccharide to a high structural resolution [43]. This structure showed a notably close contact interface between ATIII and heparin and is considered the prototype for heparin interaction with serpins such as heparin cofactor II, protease nexin 1, and plasminogen activator inhibitor [43]. However, other serpins do not have the same degree of specificity [44].

The presence of a different heparin binding site for serpins has also been postulated in recent studies with C1-INH [25]. Knowing that serpin domain of C1-INH has a similar core structure with that of α_1-PI and ATIII, we compared the location and structural characteristics of the heparin binding sites of these serpins and analyzed the data in the context of differences measured in the heparin binding affinity by SPR studies.

Overlapping the three dimensional structures of C1-INH and ATIII (Fig. 6B, cyan and magenta respectively) showed that, as expected, the two serpins have highly similar structure, reflected in the highly significant score and similar protein backbone trajectory. In contrast, the heparin binding site of ATIII (orange) is in a different part of the molecule from the putative heparin binding site of C1-INH (yellow). Similar trend was observed for overlapping C1-INH (Fig. 6C, cyan) and α_1-PI (Fig. 6C, tan). The locations of the heparin binding site earlier proposed for C1-INH (Fig. 6A-C, yellow) [25] and for α_1-PI (Fig. 6C, green) [45] do not coincide and are located in different parts of the serpin surface (Fig. 6C). However, the proposed binding sites for these two serpins show similar characteristics that distinguishes them from ATIII. Specifically, the putative binding sites, as proposed, are comprised of only a few residues and the site can be more accurately described as a "positive patch" rather than a defined "pocket" or cavity.

C1-INH is only one of these three serpins whose crystal structure is only obtained for the serpin domain of the recombinant human C1-INH in the latent form [25]. It should be mentioned that the putative heparin binding site of C1-INH may not be freely accessible to the heparin molecule if the RCL of apo-C1-INH would have a similar trajectory to that of the ATIII RCL. For instance, a positive patch located in the region of Lys 234, Lys 246 and Arg 254 residues of C1-INH that could play a role in binding heparin in a similar fashion. However, there is no experimental data yet to support this patch of C1-INH as an alternative heparin binding site, as well as earlier proposed heparin binding sites for C1-INH and α_1-PI. Thus, all the heparin binding sites proposed for C1-INH and α_1-PI remain to be identified experimentally.

In summary, the current study shows for the first time the application of SPR to study the mechanism of the increase in C1-INH activity by heparin. We have developed a novel double capture method to study the binding of C1s in presence of heparin. Our three different SPR experimental designs consistently confirmed the marked acceleration in the C1-INH activity and binding to C1s by heparin (from 5- to 11-fold increase of the association rate). The far-UV CD data indicate that heparin binding does not induce any significant conformational changes in the C1-INH structure, thus supporting a binding at the protein exterior with insignificant alterations in the protein secondary structure. Comparison of available crystal structures of ATIII and α_1-PI with that of C1-INH indicates different heparin binding sites for these structurally similar serpins. According to our structural analysis, the region around residues Lys 234, Lys 246 and Arg 254 may be considered as an alternative heparin binding site, which appears to have less steric

constraints than the earlier proposed site in the vicinity of the RCL. Further proteomic studies are feasible to determine the actual C1-INH binding site for heparin, as well as using heparin of various sizes and carbohydrate analysis to reveal the heparin binding site(s) for C1-INH.

Acknowledgements

The authors are thankful to Dr. Michael Murphy (GE Healthcare) for the consultation and discussion of the SPR results and to Dr. Abdu Alayash (Center for Biologics Evaluation and Research, FDA) for critical reading of the manuscript and helpful comments. MR is grateful to the Oak Ridge Institute for Science and Education (ORISE) for fellowship.

References

[1] B. Gooptu, D.A. Lomas, Conformational pathology of the serpins: themes, variations, and therapeutic strategies, Annu. Rev. Biochem. 78 (2009) 147–176.
[2] G.A. Silverman, J.C. Whisstock, S.P. Bottomley, J.A. Huntington, D. Kaiserman, C.J. Luke, S.C. Pak, J.-M. Reichhart, P.I. Bird, Serpins flex their muscle. I. Putting the clamps on proteolysis in diverse biological systems, J. Biol. Chem. 285 (2010) 24299–24305.
[3] A.E. Davis III, F. Lu, P. Mejia, C1 inhibitor, a multi-functional serine protease Inhibitor, Thromb. Haemost. 104 (2010) 886–893.
[4] D. Wouters, I. Wagenaar-Bos, M. van Ham, S. Zeerleder, C1 inhibitor: just a serine protease inhibitor? New and old considerations on therapeutic applications of C1 inhibitor, Expert. Opin. Biol. Ther. 8 (2008) 1225–1240.
[5] F.S. Rosen, A.E. Davis III, Deficiency of C1 inhibitor, Best Pract. Res. Clin. Gastroenterol. 19 (2005) 251–261.
[6] M.M. Frank, Complement disorders and hereditary angioedema, J. Allergy Clin. Immunol. 125 (2010) S262–S271.
[7] A.E. Davis III, P. Mejia, F. Lu, Biological activities of C1 inhibitor, Mol. Immunol. 45 (2008) 4057–4063.
[8] B.L. Zuraw, Clinical practice. Hereditary angioedema, N. Engl. J. Med. 359 (2008) 1027–1036.
[9] G.B. Caughman, R.J. Boackle, J. Vesely, A postulated mechanism for heparin's potentiation of C1 inhibitor function, Mol. Immunol. 19 (1982) 287–295.
[10] R.J. Boackle, G.B. Caughman, J. Vesely, Potentiation of factor H by heparin: a rate-limiting mechanism for inhibition of the alternative complement pathway, Mol. Immunol. 20 (1983) 1157–1164.
[11] W.A. Wuillemin, H. te Velthuis, Y.T. Lubbers, C.P. de Ruig, E. Eldering, C.E. Hack, Potentiation of C1 inhibitor by glycosaminoglycans: dextran sulfate species are effective inhibitors of *in vitro* complement activation in plasma, J. Immunol. 159 (1997) 1953–1960.
[12] R. Rent, R. Eisenstein, H. Gewurz, Complement activation by interaction of polyanions and polycations. I. Heparin-protamine induced consumption of complement, J. Immunol. 114 (1975) 120–124.
[13] B.A. Fiedel, R. Rent, R. Myhrman, H. Gewurz, Complement activation by interaction of polyanions and polycations. II. Precipitation and role of IgG, C1q and C1-INH during heparin-protamine-induced consumption of complement, Immunology 30 (1976) 161–169.
[14] R.B. Sim, G.J. Arlaud, M.G. Colomb, Kinetics of reaction of human C1-inhibitor with the human complement system proteases C1r and C1s, Biochim. Biophys. Acta 612 (1980) 433–449.
[15] M. Lennick, S.A. Brew, K.C. Ingham, Kinetics of interaction of C1 inhibitor with complement C1s, Biochemistry 25 (1986) 3890–3898.
[16] E.E. Caldwell, A.M. Andreasen, M.A. Blietz, J.N. Serrahn, V. VanderNoot, Y. Park, G. Yu, R.J. Linhardt, J.M. Weiler, Heparin binding and augmentation of C1 inhibitor activity, Arch. Biochem. Biophys. 361 (1999) 215–222.
[17] I. Björk, S.T. Olson, J.D. Shore, Molecular mechanisms of the accelerating effect of heparin on the reactions between antithrombin and clotting proteinases, in: D.A. Lane, U. Lindahl (Eds.), Heparin: Chemical and Biological Properties, Clinical Applications, Edward Arnold, London, 1989, pp. 229–255.
[18] S.T. Olson, I. Björk, R. Sheffer, P.A. Craig, J.D. Shore, I. Choay, Role of the antithrombin-binding pentasaccharide in heparin acceleration of antithrombin-proteinase reactions. Resolution of the antithrombin conformational change contribution to heparin rate enhancement, J. Biol. Chem. 267 (1992) 12528–12538.
[19] V. Rossi, I. Bally, S. Ancelet, Y. Xu, V. Frémeaux-Bacchi, R.R. Vivès, R. Sadir, N. Thielens, G.J. Arlaud, Functional characterization of the recombinant human C1 inhibitor serpin domain: insights into heparin binding, J. Immunol. 184 (2010) 4982–4989.
[20] H. Yu, E.M. Munoz, R.E. Edens, R.J. Linhardt, Kinetic studies on the interactions of heparin and complement proteins using surface plasmon resonance, Biochim. Biophys. Acta 1726 (2005) 168–176.
[21] T. Nillson, B. Wiman, Purification and characterization of human C1-esterase Inhibitor, Biochim. Biophys. Acta 705 (1982) 271–276.
[22] D.G. Myszka, T.A. Morton, CLAMP: a biosensor kinetic data analysis program, Trends Biochem. Sci. 23 (1998) 149–150.
[23] D.J. Johnson, J. Langdown, J.A. Huntington, Molecular basis of factor IXa recognition by heparin-activated antithrombin revealed by a 1.7 Å structure of the ternary complex, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 645–650.
[24] S. Kim, J. Woo, E.J. Seo, M. Yu, S. Ryu, A 2.1 Angstrom structure of an uncleaved alpha(1)-antitrypsin shows variability of the reactive center and other loops, J. Mol. Biol. 306 (2001) 109–119.
[25] L. Beinrohr, V. Harmat, J. Dobó, Z. Lorincz, P. Gál, P. Závodszky, C1 inhibitor serpin domain structure reveals the likely mechanism of heparin potentiation and conformational disease, J. Biol. Chem. 282 (2007) 21100–21109.
[26] H. Hasegawa, L. Holm, Advances and pitfalls of protein structural alignment, Curr. Opin. Struct. Biol. 19 (2009) 341–348.
[27] S. Munkvad, J. Jespersen, J. Gram, K. Overgaard, M. Ranby, Effects of methylamine and heparin on a rapid chromogenic assay of C1-esterase inhibitor in plasma, Clin. Chem. 36 (1990) 737–741.
[28] I.G. Wagenaar-Bos, C. Drouet, E. Aygören-Pursun, K. Bork, C. Bucher, A. Bygum, et al., Functional C1-inhibitor diagnostics in hereditary angioedema: assay evaluation and recommendations, J. Immunol. Meth. 338 (2008) 14–20.
[29] T.A. Murray-Rust, F.K. Kerr, A.R. Thomas, T. Wu, T. Yongqing, P.C. Ong, N.S. Quinsey, J.C. Whisstock, I.C. Wagenaar-Bos, C. Freeman, R.N. Pike, Modulation of the proteolytic activity of the complement protease C1s by polyanions: implications for polyanion-mediated acceleration of interaction between C1s and SERPING1, Biochem. J. 422 (2009) 295–303.
[30] I.G. Bos, G.J. van Mierlo, W.K. Bleeker, G.M. Rigter, H. te Velthuis, G. Dickneite, C.E. Hack, The potentiation of human C1-inhibitor by dextran sulphate is transient in vivo: studies in a rat model, Int. Immunopharmacol. 1 (2001) 1583–1595.
[31] L. Beinrohr, J. Dobó, P. Závodszky, P. Gál, C1, MBL-MASPs and C1-inhibitor: novel approaches for targeting complement-mediated inflammation, Trends Mol. Med. 14 (2008) 511–521.
[32] N. Sreerama, S.Y. Venyaminov, R.W. Woody, Estimation of protein secondary structure from circular dichroism spectra: Inclusion of denatured proteins with native proteins in the analysis, Anal. Biochem. 287 (2000) 243–251.
[33] N. Sreerama, R.W. Woody, Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set, Anal. Biochem. 287 (2000) 252–260.
[34] D.M. Rogers, J.D. Hirst, Calculations of protein circular dichroism from first principles, Chirality 16 (2004) 234–243.
[35] K. Matsuo, H. Namatame, M. Taniguchi, K. Gekko, Vacuum-ultraviolet circular dichroism analysis of glycosaminoglycans by synchrotron-radiation spectroscopy, Biosci. Biotechnol. Biochem. 73 (2009) 557–561.
[36] T.R. Rudd, E.A. Yates, M. Hricovíni, Spectroscopic and theoretical approaches for the determination of heparin saccharide structure and the study of protein–glycos–aminoglycan complexes in solution, Curr. Med. Chem. 16 (2009) 4750–4766.
[37] F.E. Stanley, A.M. Stalcup, The use of circular dichroism as a simple heparin-screening strategy, Anal. Bioanal. Chem. 399 (2011) 701–706.
[38] L.A. Kuhn, J.H. Griffin, C.L. Fisher, J.S. Greengard, B.N. Bouma, F. España, J.A. Tainer, Elucidating the structural chemistry of glycosaminoglycan recognition by protein C inhibitor, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 8506–8510.
[39] E. Wijelath, M. Namekata, J. Murray, M. Furuyashiki, S. Zhang, D. Coan, M. Wakao, R.B. Harris, Y. Suda, L. Wang, M. Sobel, Multiple mechanisms for exogenous heparin modulation of vascular endothelial growth factor activity, J. Cell. Biochem. 111 (2010) 461–468.
[40] A.J. Gozzo, V.A. Nunes, I. Cruz-Silva, A.K. Carmona, H.B. Nader, A. Faljoni- Alario, M.U. Sampaio, M.S. Araújo, Heparin modulation of human plasma kallikrein on different substrates and inhibitors, Biol. Chem. 387 (2006) 1129–1138.
[41] A.L. Stone, D. Beeler, G. Oosta, R.D. Rosenberg, Circular dichroism spectroscopy of heparin-antithrombin interactions, Proc. Natl. Acad. Sci. U. S. A. 79 (1982) 7190–7194.
[42] N.S. Gandhi, R.L. Mancera, The structure of glycosaminoglycans and their interactions with proteins, Chem. Biol. Drug Des. 72 (2008) 455–482.
[43] W. Li, D.J. Johnson, C.T. Esmon, J.A. Huntington, Structure of the antithrombin–thrombin–heparin ternary complex reveals the antithrombotic mechanism of heparin, Nat. Struct. Mol. Biol. 11 (2004) 857–862.
[44] W. Li, J.A. Huntington, The heparin binding site of protein C inhibitor is protease-dependent, J. Biol. Chem. 283 (2008) 36039–36045.
[45] V.K. Gupta, L.R. Gowda, Alpha-1-proteinase inhibitor is a heparin binding serpin: molecular interactions with the Lys rich cluster of helix-F domain, Biochimie 90 (2008) 749–761.