Zinc ions promote the interaction between heparin and heparin cofactor II

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Abstract **The effects of bivalent cations on heparin binding, structure, and thrombin inhibition rates of heparin cofactor II were examined. Zn^{2+} – and to a lesser extent Cu^{2+} and Ni^{2+} – enhanced the interaction between heparin cofactor II and heparin as demonstrated by heparin affinity chromatography and surface plasmon resonance experiments. Metal chelate chromatography and increased intrinsic protein fluorescence in the presence of Zn^{2+} indicated that heparin cofactor II has metal ion-binding properties. The results are compatible with the hypothesis that Zn^{2+} induces a conformational change in heparin cofactor II that favors its interaction with heparin.**

Key words: Serpin; Heparin cofactor II; Zinc; Heparin; Thrombin

1. Introduction

Antithrombin III (ATIII) and heparin cofactor II (HCII) are members of different branches of the serpin family [1,2], which potently inactivate thrombin in the presence of heparin [3]. Despite this shared function the inhibitors are distinguished by several structural and functional aspects. Thrombin inhibition by HCII is not markedly potentiated by the high affinity heparin pentasaccharide sequence [4] that strongly modulates ATIII/thrombin interaction. In addition, thrombin inhibition by HCII is accelerated by compounds [5] which are not effective with ATIII. Further differences include the reactive center sequences which play an essential role in determining the reaction rates, and – in part – the target specificity of serpins. Furthermore, HCII has an N-terminus enriched in acidic amino acids that can provide a secondary contact site between the inhibitor and thrombin [6,7].

Heparin and heparin-like glycosaminoglycans (GAGs) represent important activators of thrombin inhibition by HCII, thus it is of considerable interest to investigate the interactions between the inhibitor and GAGs. The affinity of HCII for heparin seems to be modest as indicated by the low salt concentrations required for elution of the native inhibitor from matrix-bound heparin under the commonly applied conditions. However, various observations indicate that the intrinsic heparin affinity of HCII may be markedly higher [8–10]. Determinants of heparin binding by HCII have been identified in the helix A and D regions [3,11]. The intrinsic fluorescence of HCII is barely changed upon heparin binding, while dermatan sulfate addition results in a maximum decrease of the fluorescence intensity of 13% with a K_d of ~25 μM [12].

The interactions of heparin and other GAGs with proteins may be complex, due to the structural diversity of either of the interaction partners. GAGs may be modified through variations involving saccharide composition, size, extent and stereochemistry of modifications [13,14], resulting in selective binding of ligands. Heparin, for instance, binds Zn^{2+}, while dermatan sulfate does not [15,16], and several reports have documented that cations may affect binding of GAGs to proteins [17,18], sometimes involving structural and/or functional changes of the polypeptides.

2. Materials and methods

2.1. Materials

HiTrap Heparin HP (contains porcine heparin), and HiTrap Chelating HP columns were obtained from Amersham Biosciences. Chromozym TH and human ATIII (5.5 U/mg) were from Roche Diagnostics. Human α-thrombin (3030 NIH units/mg) and heparin from porcine intestinal mucosa (175 USP units/mg) were from Sigma. The streptavidin-modified sensor chip SA was purchased from BIAcore. Biotinylated heparin from porcine intestinal mucosa (5–8 mol% biotin) used for immobilization on chips was from Calbiochem (cat. no. 375054).

2.2. Protein purification

Human HCII was purified from outdated citrated plasma as described [19,20] followed by heparin affinity chromatography in the presence of 50 μM $ZnCl_2$. HCII purity was estimated by Coomassie brilliant blue staining after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Protein concentrations were determined by measuring the absorbance at 280 nm [21].

2.3. Chromatography

The relative affinity of HCII for heparin was determined by fast-protein liquid chromatography (Pharmacia Biotech). Dialyzed samples were loaded onto a 1 ml HiTrap heparin column equilibrated in 20 mM Tris–HCl, pH 7.4 with or without bivalent cations and eluted with a linear NaCl gradient (40 ml, 0–1 M) in the absence or presence of bivalent cations. The NaCl concentration was determined by on-line conductivity monitoring. Fractions of 1.5 ml were collected and assayed for HCII by Western blotting [22].

1 ml HiTrap chelating columns were charged with 5 ml of a $ZnCl_2$ solution (10 mM) in deionized water. After washing with deionized water, the column was equilibrated with buffer A (20 mM sodium phosphate pH 7.4, 0.5 M NaCl). Purified HCII (20 μg) was loaded and after washing with buffer A eluted with a linear imidazole gra-

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Abbreviations: ATIII, antithrombin III; GAG(s), glycosaminoglycan(s); HCII, heparin cofactor II; RU, response units; SPR, surface plasmon resonance

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dient (30 ml, 0–150 mM). In order to monitor the imidazole concentration, 0.55 M instead of 0.5 M NaCl was included in the imidazole-containing gradient buffer. Control experiments confirmed that HCII was not detached by the increased NaCl concentration from the column. HCII concentrations in column fractions were determined by a sandwich-type enzyme-linked immunosorbent assay (ELISA) [22].

2.4. Surface plasmon resonance (SPR) analysis

SPR experiments were carried out at 25°C with the aid of a BIAcore 3000 instrument (BIAcore), monitored with the BIAcore 3000 control software (version 3.1.1), and analyzed with the BIAevaluation software, version 3.1. Chips were pretreated with three 10 μl injections (10 μl/min) of 50 mM NaOH, 1 M NaCl, and then biotinylated heparin (60 μl, 20 μg/ml) in HBS-T buffer (10 mM HEPES, 150 mM NaCl, 0.005% Tween 20 (v/v), pH 7.4) containing 3 mM EDTA was injected (10 μl/min), followed by 10 μl of 10 mM HEPES, 2 M NaCl, 0.005% Tween 20 (v/v), 3 mM EDTA, pH 7.4 (10 μl/min). Binding of HCII was performed in Chelex-100 pretreated buffer (100 μl HBS-T; flow rate: 20 μl/min) in the absence or presence of metal ions. Buffer flow was maintained for 5 min after injection to allow sample dissociation. Chips were regenerated with 50 mM HEPES, 2 M NaCl, pH 9.5 (10 μl). Response unit (RU) values from lanes not exposed to biotinylated heparin were subtracted to account for non-specific HCII binding to the streptavidin surface.

2.5. Fluorescence spectroscopy

Fluorescence measurements were conducted as two-dimensional fluorescence spectroscopy (λ_{ex}: 240–350 nm) [23]. Excitation–emission spectra were collected at 20°C and processed to determine the emission spectra (λ_{em}: 240–500 nm) at the maximum excitation wavelength (280 nm). Aliquots of a $ZnCl_2$ stock solution were added to the HCII sample (11 μM) to determine the relative fluorescence intensity changes. The equilibrium constant was calculated by non-linear least square computer fit analysis from dilution-corrected data using the equation, $\Delta I/I_0 = (\Delta I/2I_0)\times([HCII]_0+[Zn^{2+}]+K_d-\{([HCII]_0+[Zn^{2+}]+K_d)^2-4\times[HCII_0]\,[Zn^{2+}]\}^{1/2})/[HCII_0]$ [24], assuming a stoichiometry of 1.

2.6. Determination of inhibition rate constants

The stoichiometry of inhibition (SI) and second-order rate constants for thrombin inhibition in the absence or presence of heparin were determined at room temperature in 20 mM Tris–HCl, 150 mM NaCl, 1 g/l PEG-8000, pH 7.4 [25,26] essentially as described, with a 10-fold molar excess of the inhibitors (100 nM) and Chromozym TH as substrate.

3. Results

3.1. Heparin affinity of HCII in the presence of bivalent cations

To determine whether bivalent cations affect the relative heparin affinity of HCII, the effects of metal ions on the elution of the purified inhibitor from HiTrap heparin columns were investigated. Without added bivalent cations HCII eluted with a peak at 220 mM NaCl from the matrix (Table 1), however, inclusion of 50 μM Zn^{2+} caused a shift of the peak fraction to 390 mM NaCl. Increasing the Zn^{2+} concentration further had no significant effect. Ni^{2+} and Cu^{2+} ions also increased the heparin affinity of HCII to some extent, however, the other cations examined did not change the HCII elution profile, even if used at a concentration of 1 mM, like Ca^{2+} or Mg^{2+}, nor did these ions extinguish the Zn^{2+} effect. The elution properties of thrombin and ATIII remained unchanged in the presence of Zn^{2+}.

Table 1
Effect of bivalent cations on the elution of HCII from HiTrap heparin column

Protein	Cation	Elution of peak fractions (mM NaCl)
HCII	–	220
	50 μM Zn^{2+}	390
	50 μM Cu^{2+}	270
	50 μM Ni^{2+}	280
	50 μM Mn^{2+}	220
	1000 μM Ca^{2+}	220
	1000 μM Mg^{2+}	220
	50 μM Zn^{2+}, 1000 μM Ca^{2+}	390
	50 μM Zn^{2+}, 1000 μM Mg^{2+}	390
ATIII	–	760
	50 μM Zn^{2+}	760
Thrombin	–	470
	50 μM Zn^{2+}	470

3.2. Effect of bivalent metal ions on HCII binding to a heparin-modified sensor chip

SPR provides a sensitive tool to analyze the interaction of proteins with immobilized ligands, the signals of which reflect the amounts of polypeptide bound. Addition of purified HCII to a heparin-modified sensor chip caused a signal that increased with increasing concentrations (0.04–3 μM) of protein applied (Fig. 1A). In the presence of 50 μM Zn^{2+}, the signal was about two- to three-fold higher (depending on the HCII concentration) than in its absence (Fig. 1B) and depended on the Zn^{2+} concentration (Fig. 1C), thus indicating that Zn^{2+} increased the binding capacity of the chip for HCII. Zn^{2+} alone did not cause a change in RU, and the addition of competitor heparin (30 μg/ml) to the analyte led to almost complete inhibition of HCII binding (not shown), indicating that the response reflected specific interactions between HCII and the immobilized heparin. With the exception of Cu^{2+} and Ni^{2+} (Fig. 1D), other bivalent metal ions did not markedly influence the sensorgram (not shown).

3.3. HCII is a Zn^{2+}-binding protein

The metal ions may bind either heparin or HCII or both molecules, to mediate the enhanced interaction. To investigate whether HCII binds Zn^{2+}, the purified inhibitor (20 μg) was fractionated on zinc chelating Sepharose. A portion of the protein was detected in the through-flow, while the bound part was eluted at ~25–35 mM imidazole (Fig. 2). HCII was also detached in the presence of 5 mM EDTA (not shown), indicating that the protein was not bound through interactions unrelated to metal ion complex formation.

3.4. Zn^{2+} enhances the intrinsic fluorescence of HCII

In order to probe the effects of Zn^{2+} on the structure of HCII, the intrinsic fluorescence emission of the inhibitor was investigated (Fig. 3). On excitation at 280 nm, the emission maximum (334 nm) was essentially unchanged upon addition of Zn^{2+}, however, the fluorescence intensity was enhanced in a concentration-dependent manner in the presence of Zn^{2+} with a K_d value of ~123 μM.

3.5. Rates of thrombin inactivation

In order to determine the effects of Zn^{2+} on thrombin inhibition by HCII the second-order rate inhibition constants k_2 were determined. Fig. 4 shows that in the presence of 50 μM Zn^{2+} the rate constants were ~1.5-fold lower than in its absence over the whole range of heparin concentrations tested with a maximum at 10 U/ml heparin in either case. The lowering of the rate constants was dependent on the Zn^{2+} concentration, ranging (at 2 U/ml heparin) from 1.5×10^8 M^{-1} min^{-1} in the absence of Zn^{2+} to 0.9×10^8 M^{-1} min^{-1} at 100 μM Zn^{2+}. Addition of the metal ions caused a change of the

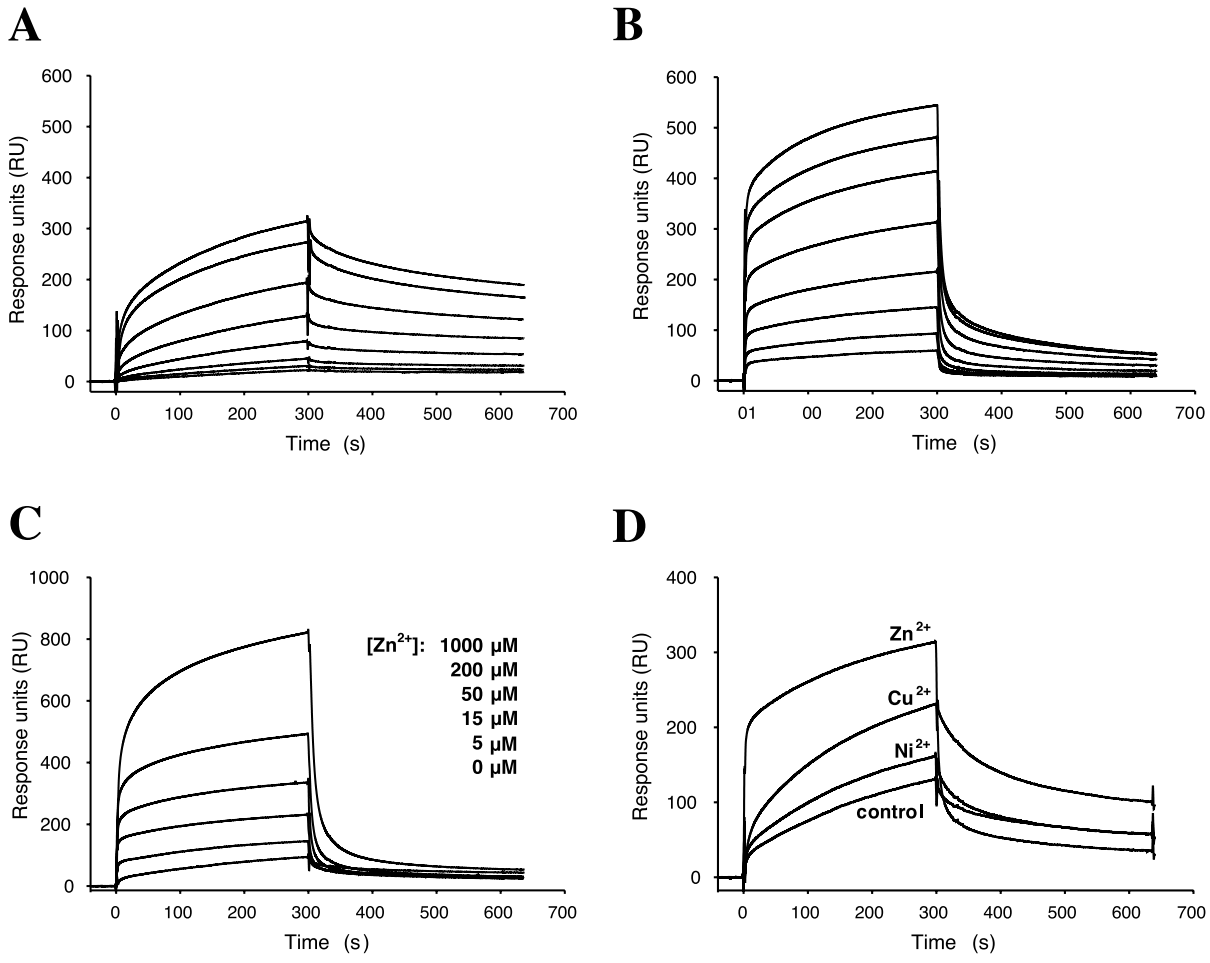

Fig. 1. SPR sensorgrams of association and dissociation between HCII and immobilized heparin. Analyte solutions containing eight different HCII concentrations (0.04, 0.08, 0.16, 0.33, 0.75, 1.5, 2.3, and 3 μM) were perfused without (A) or with (B) 50 μM Zn^{2+}. The binding curves of HCII (0.75 μM) in the presence of various concentrations of Zn^{2+} are shown in C. The effects of different bivalent cations (50 μM each) on the biosensor response are given in D.

SI values from ~1.8 to ~3.9 (not shown), suggesting that the lowered inhibition rates in the presence of Zn^{2+} were due to a shift of the partitioning of the serpin from inhibitory activity towards substrate-like properties. A modest decrease of the k_2 values from 2.2×10^5 M^{-1} min^{-1} in the absence of Zn^{2+} to 1.4×10^5 M^{-1} min^{-1} (100 μM Zn^{2+}) accompanied by an increase of the SI values from ~7 to ~13 was observed in the absence of heparin. Thrombin inactivation by ATIII was unaffected by the metal ions regardless of the presence of heparin, as found previously [27].

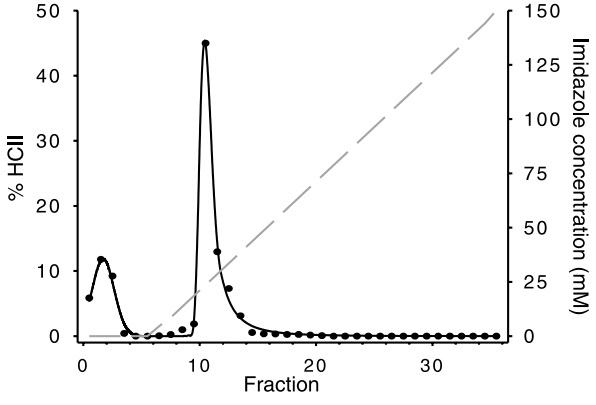

Fig. 2. Fractionation of HCII by metal chelate chromatography with a linear imidazole gradient. Column fractions (1 ml) were probed for the presence of HCII with a sandwich-type ELISA.

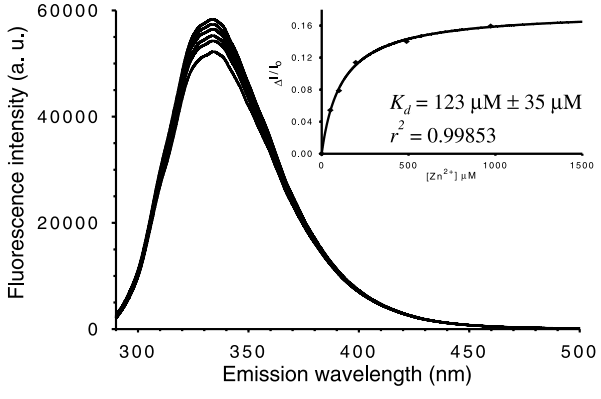

Fig. 3. Intrinsic fluorescence enhancement of HCII titrated with Zn^{2+} (0, 50, 100, 200, 500 and 1000 μM). The K_d value was calculated by non-linear regression analysis. a.u. = absolute units.

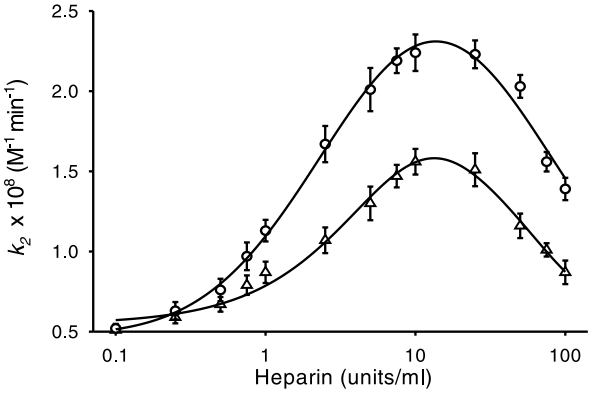

Fig. 4. Second-order rate constants of heparin-dependent thrombin inhibition by HCII in the absence (○) or presence (△) of 50 μM Zn^{2+}.

4. Discussion

The data presented demonstrate that Zn^{2+} and to a lesser extent Cu^{2+} and Ni^{2+} promote the interaction between HCII and heparin. Since HCII binds to Zn^{2+} as indicated by metal chelate chromatography and the metal ion-mediated changes of its intrinsic fluorescence, the results suggest that HCII may exist in at least two different conformations and that the metal ions alter the inhibitor's structure in a way that facilitates heparin binding. Additionally, Zn^{2+} could make binding sites in heparin more accessible to HCII.

There are several motifs that could mediate the interaction of human HCII with Zn^{2+}. The sequence HEQVHSILH (positions 155–163) – variants of which are present in some of the HCII orthologues [28,29] – shares similarity with Zn^{2+} coordinating structures in a variety of proteins [27,30–32]. The X-ray structure of native HCII [7] depicts a Ca^{2+} ion coordinated by His290, His292, and water molecules, however, we were unable to demonstrate an effect of Ca^{2+} on the intrinsic fluorescence of HCII. Less recognized is the fact that clusters of acidic amino acids may bind Zn^{2+} [33,34]. Protein domains with a large net charge tend to be flexible with low levels of ordered secondary structure, but may adopt more rigid conformations in the presence of ligands [35]. The N-terminus of HCII (positions 49–75), which includes a tryptophan residue, is strongly enriched in acidic amino acids; in total ~65% of these residues (including two nearly quantitatively sulfated tyrosine residues [22]) carry a negative charge and in HCII crystals, the acidic tail is unstructured [7].

While our results demonstrate an enhancing effect of Zn^{2+} and some other bivalent cations on heparin/HCII interaction, the physiological significance for this observation remains unclear. The free Zn^{2+} concentration in plasma is low [36,37], however, a number of proteoglycans bind Zn^{2+} [16,38], and some of these – like decorin and biglycan – have been proposed to serve as Zn^{2+} storage pools potentially providing metal ions for interaction with other proteins [16]. Zn^{2+} also participates in contact activation [39] and binding of Zn^{2+} to high molecular weight kininogen and factor XII may be accompanied by conformational changes [32,40]. Thus, the possibility that Zn^{2+} modulates the interaction between HCII and heparin, heparan sulfate proteoglycans, or cell surface proteins deserves further evaluation. Proteins like β-amyloid precursor protein [41], the prion protein PrP [42] and others show enhanced binding to heparin or heparin-like GAGs in the presence of Zn^{2+} and other bivalent cations. Thus these or other proteins could compete in a metal ion-modulated manner for the same targets.

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