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Glycosaminoglycan-binding properties and kinetic characterization of human heparin cofactor II expressed in *Escherichia coli*

Suryakala Sarilla^a, Sally Y. Habib^a, Douglas M. Tollefsen^b, David B. Friedman^c, Diana R. Arnett^d, and Ingrid M. Verhamme^{a,*}

^a Department of Pathology, Vanderbilt University School of Medicine, C3321A Medical Center North, Nashville, TN 37232, USA

^b Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA

^c Proteomics Laboratory, Mass Spectrometry Research Center, Vanderbilt University, Nashville, TN 37232, USA

^d Department of Molecular Physiology & Biophysics, Vanderbilt University School of Medicine, 2215 Garland Avenue, Nashville, TN 37232, USA

Abstract

Irreversible inactivation of α -thrombin (T) by the serpin, heparin cofactor II (HCII), is accelerated by ternary complex formation with the glycosaminoglycans (GAGs) heparin and dermatan sulfate (DS). Low expression of human HCII in *Escherichia coli* was optimized by silent mutation of 27 rare codons and five secondary Shine–Dalgarno sequences in the cDNA. The inhibitory activities of recombinant HCII, and native and deglycosylated plasma HCII, and their affinities for heparin and DS were compared. Recombinant and deglycosylated HCII bound heparin with dissociation constants (K_D) of 6 ± 1 and 7 ± 1 μ M, respectively, ~6-fold tighter than plasma HCII, with K_D 40 ± 4 μ M. Binding of recombinant and deglycosylated HCII to DS, both with K_D 4 ± 1 μ M, was ~4-fold tighter than for plasma HCII, with K_D 15 ± 4 μ M. Recombinant HCII, lacking N-glycosylation and tyrosine sulfation, inactivated α -thrombin with a 1:1 stoichiometry, similar to plasma HCII. Second-order rate constants for thrombin inactivation by recombinant and deglycosylated HCII were comparable, at optimal GAG concentrations that were lower than those for plasma HCII, consistent with its weaker GAG binding. This weaker binding may be attributed to interference of the Asn¹⁶⁹ N-glycan with the HCII heparin-binding site.

Keywords

Recombinant heparin cofactor II; Serpin; Thrombin; Serine protease inactivation

Thrombin, the central proteinase in the coagulation cascade, cleaves soluble fibrinogen to insoluble fibrin which polymerizes to form a blood clot. Thrombin is inactivated by circulating plasma heparin cofactor II (HCII), a member of the superfamily of serine

*Corresponding author. Fax: +1 615 343 7023. Ingrid.Verhamme@vanderbilt.edu (I.M. Verhamme).

¹Abbreviations used: serpin, serine proteinase inhibitor; HCII, heparin cofactor II; AT, antithrombin; RCL, reactive center loop; SDS–PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; T, human α -thrombin; GAG, glycosaminoglycan; IPTG, isopropyl-1-thio- β -D-galactopyranoside; TNS, 2-(p-toluidinylnaphthalene-6-sulfonic acid; DS, dermatan sulfate; CBS31.39, CH₃SO₂-D-Leu-Gly-Arg-p-nitroanilide; Chromozym TH, Tos-Gly-Pro-Arg-p-nitroanilide; pNA, p-nitroaniline; Hepes, N-(2-hydroxyethyl)-N'-2-piperazine-ethanesulfonic acid; PEG, polyethylene glycol 8000.

proteinase inhibitors (serpins) [1–3]. Serpins possess a reactive center loop (RCL) that is cleaved by their target proteases, with formation of a stabilized, covalent serpin–enzyme complex in which the protease active site is distorted. The thrombin–HCII reaction is accelerated by binding of the sulfated glycosaminoglycans (GAGs), heparin and dermatan sulfate (DS) [4–6], and various other polyanions [7–10] in a ternary complex, and plays an important role in prevention of arterial thrombosis [11]. The thrombin–HCII mechanism utilizes an allosteric interaction of a unique acidic region in the HCII N-terminus with thrombin exosite I [1,12,13], as well as high M_r GAG template formation between thrombin exosite II and the GAG-binding site on helix D of HCII [14,15]. The Glu⁵³-Asp⁷⁵ sequence in the HCII N-terminus contains two hirudin-like repeats that are absent in antithrombin (AT) [1,13,16,17], and the crystal structure of the HCII-S195A-thrombin Michaelis complex shows direct contact of HCII residues 56–72 with exosite I [18]. This sequence becomes available for allosteric thrombin interaction upon GAG binding of HCII in ternary complexes with thrombin [18,19]. The template mechanism is characterized by bell-shaped logarithmic dependencies of proteinase inactivation rates on the GAG concentration at fixed inhibitory serpin concentrations, and has been demonstrated for GAG-accelerated thrombin inactivation by the serpins AT [20], HCII [4,7,14,21], plasminogen activator inhibitor-1 (PAI-1) [22], and protease nexin-1 [23].

Mutants of HCII are valuable tools for studying its inhibitory mechanism and other functional properties. Previous studies have utilized partially purified *Escherichia coli* lysates of recombinant human HCII, mutated in the reactive site and the GAG-binding site, respectively, for qualitative characterization of the bifurcated inhibitory and substrate HCII partitioning mechanism [24], and identification of the Arg and Lys residues involved in the partially overlapping binding sites for heparin and DS [21,25–27]. N-terminal deletion mutants were created to probe the role of the HCII Glu⁵³-Asp⁷⁵ sequence in the interaction with thrombin exosite I [19]. Expression of glycosylated and sulfated human HCII in insect cells [28,29] and Chinese hamster ovary cells [1] has proven useful for studying the biological roles of these posttranslational modifications, and their possible effects on GAG binding. In most of these studies, HCII was detected by immunoblotting and complex formation with radioiodine-labeled thrombin. Quantitative binding and kinetic studies of the thrombin–HCII mechanism require substantial amounts of HCII, and one of the goals of this work was to optimize heterologous expression in *E. coli* for fast, quantitative production of wild-type and mutant HCII.

Numerous serpins have been expressed in *E. coli* with varying degrees of success [30]. Low HCII expression levels are in part due to the presence of 27 rare codons [31,32] in the reported HCII sequence [33], as shown in Fig. 1. Of these, 13 are rare Arg codons, with one AGG AGG tandem repeat, and several rare codons in close proximity of one another. AGGAGG is also present in the polypurine sequence (AAGGAGG), known as the Shine–Dalgarno (SD) sequence [34], which is part of the ribosome-binding site (RBS). Four additional secondary SD-like polypurine sequences were identified in the HCII cDNA. Substantial improvement of HCII expression was achieved by substitution of the rare codons with their nonrare counterparts with the highest codon usage in abundantly expressed *E. coli* genes during the exponential growth phase [35], and silent mutation of the secondary SD-like sequences. The expressed serpin, with an additional Met residue at the N-terminus [36], lacks Tyr sulfation in positions 60 and 74, and *N*-glycosylation at positions Asn³⁰, Asn¹⁶⁹, and Asn³⁶⁸. This study quantitates the dissociation constants (K_D) for binding of representative template-forming heparin and DS to HCII expressed in *E. coli*, in comparison with native and deglycosylated HCII from human plasma, and compares the kinetic profiles of heparin- and DS-accelerated thrombin inactivation by these three HCII forms in a ternary complex model. Our findings indicate that, although recombinant and deglycosylated HCII bind GAGs 4- to 6-fold tighter than plasma HCII, their inhibitory efficiencies at optimal

GAG concentrations are essentially comparable, justifying the use of *E. coli* recombinant HCII for kinetic studies that dissect the encounter, loop insertion, and complex stabilization steps of the thrombin–HCII interaction mechanism.

Materials and methods

Proteins and materials

The following materials were purchased: oligonucleotide primers (Midland Certified Reagents); Tartoff-Hobbs modified Terrific Broth (TB), MagicMedia, and *pfu* DNA polymerase (Invitrogen); Luria-Bertani (LB) broth Miller (Fisher Scientific); the restriction enzymes *Nco*I and *Sna*BI, and DNA markers (New England Biolabs, Beverly, MA); *E. coli* BL21-CodonPlus (DE3)-RIPL competent cells, DH5 α cells, XL1-Blue supercompetent cells, and QuikChange mutagenesis kits (Stratagene); Wizard *plus* SV Minipreps DNA extraction kits, and Modified Trypsin-Gold (Promega); heparin-Sepharose, Mono S and Mono Q HR 5/50 GL 5 ml prepacked FPLC columns (GE Healthcare); EDTA-free complete protease inhibitor cocktail, and chromogenic substrate Chromozym TH (Roche Applied Science); heparin sodium salt M_r 12,000 from porcine intestinal mucosa, and PNGase F from *Elizabethkingia meningoseptica* (Sigma–Aldrich); DS M_r 30,000 from porcine intestinal mucosa (Calbiochem); chromogenic substrate CBS 31.39 (Stago); and the fluorescence label TNS (Molecular Probes). The expression vector pET-3d containing the human HCII cDNA was created as published previously [37]. Human prothrombin [38] and HCII [14] were purified from plasma. α -Thrombin, prepared as described [38], was $\geq 90\%$ active as determined by active site titration [39]. Protein concentrations were determined by absorbance at 280 nm with the absorption coefficients and molecular weights (M_r) of 1.83 (mg/ml) $^{-1}$ cm $^{-1}$ and 36,700, for thrombin [40]; 0.59 and 65,600 for plasma HCII [3,5]; and 0.73 and 54,960 for recombinant HCII [41,42]. Active HCII concentrations were determined by stoichiometric titration with thrombin in DS-accelerated reactions. Plasma HCII was deglycosylated by incubation of 2 mg/ml HCII with 15 units of PNGase F in 50 mM Hepes, 0.1 M NaCl, 5 mM CaCl $_2$, 1 mg/ml PEG 8000 buffer, pH 7.4 (reaction buffer) for 24 h at room temperature, under nondenaturing conditions, and glycan fragments were removed by desalting on a 1-ml G25 column. DS was treated with nitrous acid to remove traces of heparin [12].

Site-directed mutagenesis and expression of recombinant human HCII

Silent mutation of 27 rare codons and 5 secondary SD-like sequences in the HCII cDNA was performed at the positions listed in Tables 1 and 2, using the Stratagene QuikChange site-directed mutagenesis kit. Human HCII cDNA, cloned into the pET3d - derived vector, using 5' *Nco*I and 3' *Sna*BI restriction sites, was used to transform DH5 α *E. coli* cells. Mini DNA preps were made from 5 ml overnight cultures, incubated at 37 °C. The DNA was digested with the restriction enzymes, and DNA quality and restriction sites were verified by 0.7% agarose gel electrophoresis. The HCII constructs were amplified by PCR using the forward and reverse sets of the primers listed in Tables 1 and 2. The reaction (50 μ l), containing 50 ng pET-3d HCII, 125 ng each of forward and reverse primer, and 1 μ l of dNTP mix, was initiated with *Pfu-Turbo* DNA polymerase (2.5 U/ μ l). Cycling parameters were 95 °C, 60 s (denaturation); 55 °C, 60 s (annealing); 68 °C, 15 min (extension); 16 cycles, followed by cooling at 4 °C. Amplification was verified by 0.7% agarose gel electrophoresis. Parental, methylated DNA in the amplification mixture was digested immediately with the restriction enzyme *Dpn*I (10 U/ μ l) for 1 h at 37 °C, and the mutagenized pET-3d plasmid was used to transform XL1-Blue supercompetent cells by the heat-shock method [43]. Transformed cells were grown on agar plates containing 50 μ g/ml ampicillin for 16 h at 37 °C. LB broth (10 ml) containing ampicillin was inoculated with selected colonies, and incubated overnight at 37 °C for plasmid isolation, using the SV

Minipreps DNA isolation kit. The plasmids were sequenced to verify the accuracy of each mutation. XL1-Blue glycerol stocks of correctly mutated clones were stored at -80°C .

BL21-CodonPlus (DE3)-RIPL competent cells were transformed with modified pET-3dHCII by heat shock. Transformed cells were plated onto agar plates containing 50 $\mu\text{g/ml}$ ampicillin, and colonies ($>20/\text{plate}$) were grown for 16 h at 37°C . LB media (5 ml) containing 50 $\mu\text{g/ml}$ ampicillin was inoculated with several colonies and incubated overnight at 37°C for preparation of glycerol stocks. Starter cultures (100 ml) of TB, MagicMedia, and LB media containing 50 $\mu\text{g/ml}$ ampicillin, inoculated with transformed BL21-Codon-Plus (DE3)-RIPL cells, were grown overnight at 37°C . Ten milliliters of this starter culture was used to inoculate 1-L media flasks, with a total prep volume of 10 L, and the cultures were grown under constant shaking at 37°C . Cultures in LB and TB media, grown to A_{600} 0.6, were induced with 0.3 mM IPTG, after a 1 h cool down to 25°C , and grown overnight under constant shaking at 25°C . MagicMedia, utilizing the T7/lacUV5 promoter mechanism, required no IPTG induction, and cultures were grown overnight at 25°C . The cell pellets were harvested by centrifugation and stored at -80°C until further use.

Purification of recombinant human HCII

Cells (100 g pellets) were resuspended in 200 ml of 50 mM Hepes, 50 mM NaCl, 0.1 mM EDTA, and 0.02% NaN_3 , pH 7.5 (start buffer). Protease inhibitor cocktail (2 tablets), 6 mg of lysozyme powder, and 1 mM PMSF (final) were added to the buffer prior to resuspension of the cell pellet. Cells were sonicated in an ice bath, for 5 cycles of 45 s, with 10 min intervals, and cell debris was removed by centrifugation. The crude lysate was loaded onto a heparin-Sepharose column (2.5×23 cm) in start buffer at room temperature, and the column was washed until the absorbance at 280 nm (A_{280}) was ≤ 0.08 . Protein was eluted using a 250×250 ml linear gradient of 0.05–0.50 M NaCl in start buffer, and 5.0 ml fractions were collected. HCII activity was measured spectrophotometrically by reacting 10–20 nM α -thrombin with 100 μl fraction sample in 1 ml 50 mM Hepes, 0.1 M NaCl, 5 mM CaCl_2 , 1 mg/ml PEG 8000 buffer, pH 7.4 (reaction buffer), in the presence of 1.5 μM heparin (M_r 12,000), and 100 μM of competing chromogenic substrate CBS31.39, and continuously monitoring the inhibition of pNA production at 405 nm. Active fractions were pooled, dialyzed against Mono Q and S HR 5/50 start buffer, 20 mM potassium phosphate buffer, pH 7.4, and applied to the Mono Q FPLC column equilibrated in start buffer. The column was washed until $A_{280} \leq 0.05$, and eluted with 35 column volumes of 0.05–0.50 M NaCl gradient in start buffer, in 1.5 ml fractions. Active HCII fractions were pooled, dialyzed against start buffer, and applied to the Mono S FPLC column. After washing with 5 column volumes of start buffer, elution was performed with a 30 column volume 0.05–0.50 M NaCl gradient in start buffer, in 1.5 ml fractions. Active fractions were analyzed by 4–20% SDS–PAGE. Purified HCII was concentrated, dialyzed against 50 mM Hepes, 0.1 M NaCl, pH 7.4, storage buffer, and quick-frozen at -80°C .

Mass spectrometry of native and recombinant HCII

Plasma and recombinant HCII were resolved by SDS–PAGE and visualized by Coomassie Blue R250 staining. Gel-excised protein bands of interest were equilibrated in 100 mM NH_4HCO_3 and then reduced with dithiothreitol (3 mM in 100 mM NH_4HCO_3 , 37°C for 15 min) and alkylated with iodoacetamide (6 mM in 100 mM NH_4HCO_3 , 15 min). Gel slices were dehydrated with acetonitrile and then rehydrated with 15 μl of 25mM NH_4HCO_3 containing 0.01 $\mu\text{g}/\mu\text{l}$ Modified Trypsin-Gold protease, and digestion was carried out for >2 h at 37°C . Peptides were extracted with 60% acetonitrile, 0.1% trifluoroacetic acid, dried by vacuum centrifugation, and reconstituted in 10 μl of 60% acetonitrile, 0.1% trifluoroacetic acid. Aliquots (0.4 μl) were applied to target plates and overlaid with 0.4 μl of α -cyano-4-hydroxycinnamic acid matrix (5 mg/ml, supplemented with 1 mg/ml ammonium citrate).

Matrix-assisted laser desorption/ionization, time-of-flight (MALDI-TOF) MS, and tandem TOF/TOF MS/MS was carried out using a Voyager 4700 mass spectrometer (Applied Biosystems), operated in positive-ion reflectron mode. Each MALDI-TOF mass spectrum (representing a peptide mass map of the digested protein) was calibrated to within 10 ppm using trypsin autolytic peptides present in the sample (m/z 842.50, 1045.56, and 2211.10). Peptide masses were then manually compared to the list of predicted peptides for HCII from each digested protein (either isolated from plasma or expressed/purified from *E. coli*), as well as to each other.

Fluorescence equilibrium binding of GAGs to recombinant, native, and deglycosylated HCII

Binding of heparin and DS to native, deglycosylated, and recombinant HCII (500, 480, and 405 nM, respectively) was quantitated in reaction buffer, pH 7.4, at 25 °C, by measuring the change in fluorescence of the reversible probe TNS (12 μ M final concentration) in the HCII·TNS complex upon GAG binding to HCII [44,45]. Titrations were performed with an SLM 8100 spectrofluorometer in the ratio mode as described previously [46], at 330 and 428 nm excitation and emission wavelengths, with 8 nm band passes. Blank titrations with TNS and GAGs were performed in parallel to correct for background fluorescence. Data were expressed as the fractional change in the initial fluorescence $((F_{\text{obs}} - F_0)/F_0 = \Delta F/F_0)$ as a function of total titrant concentration, and were fit by the quadratic binding equation for binding of a single ligand [47]. The fitted parameters were the maximum fluorescence intensity changes $\Delta F_{\text{max,HCII,GAG}}/F_0$ and the dissociation constants $K_{\text{HCII,GAG}}$ for heparin and DS binding to HCII.

Kinetics of GAG-accelerated thrombin inactivation by recombinant, native, and deglycosylated HCII

Kinetic experiments and stoichiometric titrations were performed at 25 °C in pH 7.4 reaction buffer. The stoichiometries of thrombin reacting with native, deglycosylated, and recombinant HCII were determined in the absence of GAGs, and in the presence of 5 μ M heparin or DS. Incubations were performed in PEG 20,000-coated 1 ml Eppendorf tubes containing 0.5–1 μ M active-site titrated thrombin and increasing HCII concentrations. Reactions were incubated overnight, and thrombin activity was assayed with the chromogenic substrate CBS31.39 (100 μ M) at 405 nm with a Shimadzu 2401 spectrophotometer. Residual rates for thrombin–HCII incubations were compared to thrombin rates in the absence of HCII, and the stoichiometries were determined from the equivalence points at which complete thrombin neutralization was observed. The second-order rate constants of thrombin inactivation by the three HCII forms were measured from the loss of thrombin chromogenic substrate activity, under pseudo-first-order conditions (~ 500 nM $[\text{HCII}]_0$, 50 nM $[\text{T}]_0$), in the absence of GAGs. Residual thrombin activity was measured at discrete time intervals, using discontinuous sampling into cuvettes containing 150 μ M S2238 or 120 μ M Chromozym TH, and expressed as the fractional residual thrombin activity, $[\text{T}]_t/[\text{T}]_0$. The first-order time courses of the fractional residual thrombin activity were analyzed by nonlinear least-squares fitting of

$$[\text{T}]_t/[\text{T}]_0 = \exp^{-kt}, \quad (1)$$

with the first-order rate constant $k = k'[\text{HCII}]$, the product of the second-order rate constant k' for the HCII–T reaction and the HCII concentration. Thrombin inactivation by HCII in the presence of GAGs was monitored by continuous competitive chromogenic substrate hydrolysis, under first-order conditions of chromogenic substrate and inhibitor, as described

previously [14,48–50]. K_m and k_{cat} for hydrolysis of Chromozym TH and CBS31.39 were 11 μM and 167 s^{-1} , and 240 μM , and 49 s^{-1} , respectively [50]. Thrombin was added to a mixture of HCII, GAG, and chromogenic substrate at time zero, and pNA formation was monitored continuously at 405 nm. Time courses were analyzed by a nonlinear least squares fit by

$$[pNA]_t = A \cdot (1 - \exp^{-k_{obs}t}) + X, \quad (2)$$

with $[pNA]_t$ the concentration of pNA formed during the reaction, A the amplitude of the progress curve, and the observed pseudo-first-order rate constant $k_{obs} = k/(1 + [S]_0/K_m)$. K_m and $[S]_0$ are the Michaelis constant and concentration of chromogenic substrate, and k is the first-order rate constant without chromogenic substrate competition as described above. X is a slow, second exponential to account for the combined effects of the uncatalyzed reaction and slow reacting thrombin forms [51].

GAG and HCII dependencies of k_{obs} were established as described previously [14,50], and analyzed by Eq. (3), using the mass conservation equations for thrombin, HCII and GAG:

$$k_{obs} = \frac{k_{lim}[HCII]_{free}/(1 + [S]_0/K_m)}{K_{complex}(1 + K_{T,GAG}/[GAG]_{free}) + [HCII]_{free}/(1 + [S]_0/K_m)} \quad (3)$$

The fixed parameters were K_m and $[S]_0$, the Michaelis constant and total concentration of the chromogenic substrate; and $K_{HCII,GAG}$, the dissociation constant for GAG binding to HCII, appearing in the mass conservation equation for HCII, and determined independently by equilibrium binding. The fitted parameters were k_{lim} , the limiting first-order rate constant for GAG-accelerated thrombin inactivation; $K_{T,GAG}$ for binding of GAG to thrombin; and $K_{complex}$ the apparent Michaelis constant for assembly of the ternary T-GAG-HCII complex. $[GAG]_{free}$ and $[HCII]_{free}$ were calculated from the mass balances. Least-squares fitting of binding and kinetic data were performed with SCIENTIST Software (MicroMath). All reported estimates of error represent ± 2 SD.

Results

Site-directed mutagenesis and expression of recombinant human HCII

Of the 27 rare codons, 11 were clustered in positions Arg192-Arg193; Ile134-Pro136; Arg218-Arg222; Arg369-Arg371; and Arg473-Pro477-Arg479. Initial silent mutation of these clusters, and positions Pro9 and Arg361, combined with the use of the BL21-CodonPlus (DE3)-RIPL cells, containing extra copies of the *E. coli* argU, ileY, leuW, and proL tRNA genes, improved expression levels in TB and LB media, with higher expression at 25 °C compared to 30 and 37 °C. Expression in MagicMedia, utilizing auto-induction, was very low, and the use of this medium was discontinued. Silent mutation of the remaining rare codons, and in the additional SD-like sequences AAGGAGG, AAGGAA, AGGAGG, and AAGGGG, respectively, spanning residues Lys¹¹-Gly¹³, Lys⁴²-Glu⁴³, Glu⁵⁵-Asp⁵⁷, and Lys³¹⁰-Gly³¹¹ (Tables 1 and 2) further substantially improved expression levels, with comparable yields in TB and LB media. The IPTG induction concentration for optimal expression in these systems was determined to be 0.3 mM.

Purification of recombinant human HCII

HCII eluted from the heparin-Sepharose, Mono Q and Mono S columns between 0.20 and 0.30, 0.10 and 0.20, and 0.08 and 0.20 M NaCl, respectively. Fig. 2A shows the SDS-PAGE

of recombinant HCII, expressed from the construct bearing 13 initial mutations, after heparin-Sepharose chromatography, and after partial purification on Mono Q FPLC. Yields of these preps were typically ~3 mg from 10 L starting media. Fig. 2B–D show the elution profiles and SDS–PAGE of HCII expressed from the final construct containing all silent mutations, after consecutive heparin-Sepharose, and Mono Q and Mono S FPLC purification. Shallow FPLC gradients were necessary to resolve the HCII peak. HCII yields of 10 L preps after Mono Q were up to 35 mg, with one major HCII peak, and several side peaks containing HCII as well as impurities and minor bands of cleaved HCII. These side pools were saved for rechromatography on Mono Q. Impurities and cleaved protein in the major HCII peak were removed by Mono S FPLC. Final yields after Mono S FPLC were typically 10–20 mg.

Mass spectrometry of native and recombinant HCII

The plasma and recombinant HCII spectra showed complete similarity and accounted for all major ions observed (Fig. 3), except for one peptide with mass/charge ratio m/z of 1120.63, identified as QFPILLDFK in plasma HCII, as predicted from the genomic sequence [52], and QFPILLDFR with m/z 1148.64 in recombinant HCII [33]. The recombinant HCII spectrum also showed a peptide with m/z ratio 1131.62, with expected loss of 17 Da due to the cyclization of the N-terminal Q to pyroglutamate on the QFPILLDFR peptide. Sequence confirmation was provided by tandem TOF/TOF mass spectrometry on these ions, where the fragmentation pattern was consistent with the expected amino acid sequence (data not shown). The QFPILLDFR peptide ionized much more efficiently due to the presence of arginine, and altered the fragmentation pattern in a predictable way. The K-R substitution at position 218 in human HCII has been reported as a polymorphism [33,53,54].

Fluorescence equilibrium binding of GAGs to recombinant, native, and deglycosylated HCII

GAG binding to HCII was measured by the fluorescence change of the reversible TNS-HCII complex, specifically reporting GAG binding to the serpin heparin-binding site [44,45]. The analysis for binding of heparin with M_r 12,000 gave apparent dissociation constants $K_{\text{HCII,H}}$ 6 ± 1 μM for recombinant HCII, 7 ± 1 μM for deglycosylated plasma HCII, and 40 ± 4 μM for native plasma HCII; and respective fluorescence amplitudes $\Delta F_{\text{max,HCI,H}}/F_o$ -60 ± 1 , -31 ± 1 , and $-47 \pm 2\%$ (Fig. 4A). Binding of DS with M_r 30,000 was characterized by $K_{\text{HCII,DS}}$ 4 ± 1 μM for recombinant HCII, 4 ± 1 μM for deglycosylated plasma HCII, and 15 ± 4 μM for native plasma HCII; and respective fluorescence amplitudes $\Delta F_{\text{max,HCI,DS}}/F_o$ -68 ± 4 , -32 ± 1 , and $-42 \pm 4\%$ (Fig. 4B), with one GAG-binding site assumed on HCII. Fluorescence amplitudes for heparin and DS binding were similar for each HCII form, with consistently larger amplitudes for GAG binding to recombinant HCII.

Kinetics of GAG-accelerated thrombin inactivation by recombinant, native, and deglycosylated HCII

Stoichiometric titration of recombinant HCII with thrombin demonstrated a 1:1 thrombin:HCII stoichiometry for the uncatalyzed and DS-catalyzed reactions, and a 1:1.3 stoichiometry for the heparin-catalyzed reaction, in good agreement with the results for native and deglycosylated HCII. Rates of uncatalyzed inactivation of thrombin by recombinant, native, and deglycosylated HCII were indistinguishable, with second-order rate constants of 460 ± 30 , 460 ± 20 , and 420 ± 20 $\text{M}^{-1} \text{s}^{-1}$, respectively, as shown in Fig. 5. The GAG and HCII dependencies of the first-order inactivation rate constant k_{obs} for recombinant, native, and deglycosylated plasma HCII are shown in Figs. 6A and B and 7A and B, respectively. Simultaneous analysis of the GAG and HCII concentration dependencies by Eq. (3) and the mass conservation equations for thrombin, HCII, and GAG gave similar second-order rate constants ranging from 9 to 11 $\mu\text{M}^{-1} \text{s}^{-1}$ for the heparin- and

DS-catalyzed reactions of recombinant and native HCII, and $13\text{--}16\ \mu\text{M}^{-1}\ \text{s}^{-1}$ for deglycosylated HCII; however, these differences were not significant. The equilibrium and rate constants for all interactions are given in Table 3. The K_D values for heparin and DS binding to recombinant, plasma, and deglycosylated HCII, obtained by equilibrium binding, and fitted from the kinetic data, were in good agreement.

Discussion

Quantitative expression of wild-type HCII in *E. coli* has to date been performed mainly by semilarge scale incubation [19], or high cell density fermentation [37]. In our initial studies, expression of wild-type HCII in BL21-CodonPlus (DE3)-RIPL cells at 37 °C, induced by 0.3 mM IPTG, did not significantly improve the yield, although these cells contain a plasmid encoding extra copies of rare *E. coli* tRNA genes. Variation of cell lines, media, expression temperature, duration of incubation, and of type and concentration of induction agent resulted only in marginal improvement in some cases, whereas other conditions caused complete arrest. Expression of HCII in *S. cerevisiae* W303-1a, using the p423-MET25 construct [55], although readily detectable by immunoblotting, was insufficient for production of mg quantities of HCII, mainly due to low codon usage of the Arg CGG, CGA, and CGC, Ser TCG, and Leu CTC codons in *S. cerevisiae* (0.4–0.9% fraction, GCG Codon-Frequency Program, Accelrys).

Heterologous expression of human HCII in *E. coli* is negatively affected by the presence of 27 rare codons, some of them clustered. The codon usage in *E. coli* is the lowest for the Arg codons AGG and AGA, 0.3% and 0.6%, respectively [56], and the abundance of their cognate tRNAs is similarly very low [57]. Other codons with low usage are Ile ATA (0.6%), Leu CTA (0.8%), Arg CGA (1.1%), and Pro CCC (1.6%). Consecutive AGG codons in various heterologous genes are known to cause ribosome stalling, resulting in frameshift, hopping, or chain termination, depending on the location of the AGG AGG sequence [58,59]. Similarly, other secondary SD-like sequences in the cDNA of certain proteins were reported to cause significant decrease or even arrest of expression [60,61]. Both in-frame and out-of-frame AGG clusters may result in inhibition, suggesting that reduced expression is caused by the SD-like sequence binding to 16S RNA [62]. Gene optimization was performed in two stages. Initial silent mutation of 13 rare codons including the tandem AGG repeat and several clustered sequences significantly improved expression, and a final optimization stage including the remaining rare codons and secondary SD-like sequences resulted in vastly improved yields, up to 35 mg/10 L media in the second purification step, and 10–20 mg/10 L after final purification. Optimal conditions of expression temperature (25 °C) and induction conditions (0.3 mM IPTG) were established. Yields in LB and enriched TB [63] were comparable, in contrast to Magic-Media utilizing auto-induction [64], in which no expression was observed. Cell densities ($\text{OD}_{600\text{nm}}$) after overnight IPTG induction in LB and TB at 25 °C were between 3.5 and 4, below those typically obtained in high-density fermentation at 37 °C. Optimized high-density methods with controlled pH and oxygen availability, utilizing colony selection, have been described [65]; however, high cell density may result in plasmid loss from *E. coli* [66]. Expression at 25 °C decreases protein misfolding and processing in inclusion bodies, sometimes encountered during 37 °C incubations [67]. In endogenous *E. coli* proteins, codon usage in the second triplet, following the N-terminal Met, is selective [68], and further improvement of HCII expression may be obtained by silent mutation of the GGG, the codon for HCII N-terminal Gly, to GGT.

Both recombinant and deglycosylated HCII exhibited similar, increased GAG-binding affinities compared to native plasma HCII, indicating that the presence of the Arg²¹⁸ polymorphism in the recombinant protein did not affect GAG binding. Steric hindrance and

charge effects of the *N*-glycan chain at Asn¹⁶⁹, in the vicinity of the HCII heparin-binding site, may be responsible for the reduced GAG affinity of plasma HCII. Isoforms of plasma HCII lacking Asn¹⁶⁹ *N*-glycosylation, similar to AT- β , lacking *N*-glycosylation at Asn¹³⁵, have not yet been reported. AT- β , representing 5–10% of the total circulating antithrombin, binds heparin 3.5-fold tighter than fully glycosylated AT [69], and exerts its antithrombotic function at the endothelial and subendothelial cell surface [70,71]. Native and deglycosylated HCII possess two sulfated Tyr residues at positions 60 and 73, increasing the net negative charge of the protein compared to *E. coli* recombinant HCII. Expression of HCII in CHO cells treated with tyrosine sulfation inhibitors reportedly resulted in production of nonsulfated, glycosylated HCII with increased affinity for heparin-Sepharose [52]. During purification we did not observe significant differences in chromatographic behavior of plasma HCII and HCII from *E. coli* lysates on heparin-Sepharose [14], possibly due to the presence of large concentrations of other heparin-binding proteins and compounds in the first purification step, and differences in heparin-Sepharose matrix-binding behavior may become apparent for the purified HCII forms. Whereas our fluorescence equilibrium binding results demonstrated similar GAG affinities of recombinant and deglycosylated HCII in a solution system, the resulting quenches in fluorescence intensity were much more pronounced for recombinant HCII, with ~2-fold larger amplitudes for recombinant HCII, compared to the Tyr-sulfated native and deglycosylated HCII. These results suggest that the increased affinity for GAGs may at least in part be due to the absence of *N*-glycosylation, and that the sulfated Tyr residues in HCII may affect the fluorescence of the TNS probe bound to the serpin, but not necessarily the affinity of GAGs in solution. Recombinant deletion of HCII residues 1–74, with both sulfated Tyr residues contained in the acidic region spanning residues 53–74, was shown to result in a 2-fold increase in affinity for the heparin-Sepharose matrix, and a drastic reduction of GAG-accelerated reactivity toward α -thrombin possessing an intact exosite I [19]. Binding of the hirudin-like acidic N-terminal region in HCII to thrombin exosite I is an essential part of the inactivation mechanism [18,19], and our kinetic studies with recombinant HCII proposed to investigate whether the sulfation status of both tyrosines in the acidic region is obligatory for proper interaction with thrombin exosite I. If this were the case, we would expect to observe substantial differences in the kinetics of GAG-accelerated thrombin inactivation by recombinant and deglycosylated HCII.

The HCII dependencies of the inactivation rate, measured at the respective optimal GAG concentrations, displayed hyperbolic saturation, and the logarithmic GAG dependencies were bell-shaped, consistent with a mechanism of ternary thrombin-GAG-HCII complex formation. The amplitudes of the GAG dependencies for recombinant HCII were lower due to the lower fixed HCII concentrations in these experiments. The HCII dependencies defined the limiting rates, k_{lim} , the ternary complex affinities, K_{complex} , and the second-order rate constants k' expressed as the ratio $k_{\text{lim}}/K_{\text{complex}}$, whereas the GAG dependencies defined the GAG affinities for thrombin ($K_{\text{T,GAG}}$), HCII ($K_{\text{HCII,GAG}}$), and also the ternary complex affinities, K_{complex} . The maxima of the logarithmic GAG concentration dependencies of the inactivation rate were shifted toward lower GAG concentrations for recombinant and deglycosylated HCII compared to native HCII, consistent with tighter GAG binding of these forms. Whereas the limiting rates for the recombinant HCII reactions were typically lower than those for deglycosylated and native HCII, the second-order rate constants k' were largely comparable, indicating equally efficient ternary complex formation and inhibitory behavior. The fitted dissociation constants for GAG binding to HCII in the kinetic profiles were generally higher than those measured independently by equilibrium binding, and were similarly lower for recombinant and deglycosylated HCII, compared to native HCII. The fits of the GAG profiles, spanning concentrations that differ by 3–4 orders of magnitude, with equal weighting of data, gave comparable values for GAG binding to thrombin, from 0.4 to 1 μM , validating the data analysis employing a linkage mechanism of ternary complex

formation [72]. The affinities of these GAGs in binary and ternary complexes with thrombin and HCII, although representative, are expected to vary with the type, degree of sulfation, and molecular weight of the GAG.

In conclusion, the present work demonstrates that the increased GAG affinity of *E. coli* recombinant HCII is largely caused by the lack of *N*-glycosylation. Tyr sulfation in the acidic N-terminal HCII sequence does not appear to play a major role in GAG binding, and is not required for efficient GAG-accelerated thrombin inactivation. This study reports for the first time a quantitative analysis of the binding and kinetic properties of recombinant human HCII expressed in *E. coli*, of importance in comparative interpretation of the kinetic behavior of HCII variants engineered as functional probes of the thrombin inactivation mechanism.

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1 10 20 CHO 40
 M G S K G P L D Q **L** E K G G E T A Q S A D **P** Q W E Q L N N K N L S M P L L P A D F
 50 SO4 70 SO4 80
 H K E N T V T N D W I P E G E E D D D Y L D L E K **I** F S E D D D Y I D I V D S L S V
 90 100 110 120
 S P T D S D V S A G N I L Q L F H G K S R I Q R L N I L N A K F A F N L Y **R** V L K D
 130 140 150 160
 Q V N T F D N I F **I** A **P** V G I S T A M G M I S L G L K G E T H E Q V H S I L H F K D F
 CHO 180 190 200
 V N A S S K Y E I T T I H N L F R K L T H R L F **R** **R** N F G Y T L R S V N D L Y I Q K
 210 220 230 240 250
 Q F P I L L D F **R** T K V **R** E Y Y F A E A Q **I** A D F S D P A F **I** S K T N N H I M K L T
 260 270 280 290
 K G L **I** K D A L E N **I** D P A T Q M M I L N C I Y F K G S W N K F P V E M T H N H
 300 310 320 330
 N F R L N E **R** E V V K V S M M Q T K G N F L A A N D Q E L D C D I L Q L E Y V G G
 340 350 360 CHO 370
 I S M **L** I V V P H K M S G M K T L E A Q L T **P** R V V E **R** W Q K S M T N **R** T **R** E V
 380 390 400 410
 L L P K F K L E K N Y N **L** V E S L K L M G I **R** M L F D K N G N M A G I S D Q **R** I A
 420 430 440 ◇ 450
 I D L F K H Q G T I T V N E E G T Q A T T V T T V G F M P L S T Q V R F T V D R **P** F
 460 470 480
 L F L I Y E H R T S C L L F M G **R** V A N **P** S **R** S

Fig. 1.

Amino acid sequence of human HCII. HCII expressed in *E. coli* possesses an N-terminal Met residue, not included in the sequence numbering. Rare codons are indicated in underlined boldface. Positions of Tyr sulfation (SO4) and Asn *N*-glycosylation (CHO) in plasma HCII are indicated in the sequence. The Leu–Ser scissile bond in the RCL is indicated by a diamond (◇).

Fig. 2. Purification of recombinant HCII. (A) SDS-PAGE of recombinant HCII (active fractions) after partial purification on heparin-Sepharose (left panel) and Mono Q FPLC (right panel) of HCII expressed from a construct with 13 initial mutations, with plasma HCII (lanes 1) as a standard. Arrows indicate the HCII bands. The electrophoretic mobility of recombinant HCII is slightly faster than that of plasma HCII due to the lack of glycosylation. (B) Elution profile on heparin-Sepharose of HCII expressed from the construct containing all silent mutations, and SDS-PAGE of the active fractions (lane 1, plasma HCII; lanes 2–6 are fractions 32, 35, 40, 45, 50). (C) Elution profile of recombinant HCII on a 5 ml Mono Q FPLC column, with SDS-PAGE of active fractions (lane 1, plasma HCII; lanes 2–10 are

every 5th fraction, from 20 to 60). (D) Elution profile of recombinant HCII on a 5 ml Mono S FPLC column, with SDS-PAGE of active fractions (lane 1, M_r markers; lane 2, plasma HCII; lanes 3–9 are every 5th fraction, from 10 to 40). A_{280} values of the fractions were measured in 1 cm cuvettes. The yield of this prep was 14 mg.

Fig. 3. MALDI-TOF peptide mass map of plasma and recombinant HCII. The ionization patterns of plasma HCII and recombinant HCII are shown in the top and bottom panels, respectively. Ionization of plasma HCII yields the QFPILLDFK peptide with m/z ratio 1120.63. The corresponding peptide in recombinant HCII is QFPILLDFR with m/z ratio of 1148.64, resulting from a polymorphism. An additional peptide is present, with m/z ratio 1131.62, and expected loss of 17 Da due to the cyclization of the N-terminal Q to pyroglutamate on the QFPILLDFR peptide.

Fig. 4.

Fluorescence equilibrium binding of heparin and DS to HCII. Fractional change in fluorescence ($\Delta F/F_0$) of 500 nM plasma HCII (●), 480 nM deglycosylated plasma HCII (○) and 405 nM recombinant HCII (■) in the presence of 12 μM TNS as a function of total heparin concentration ($[Heparin]_0$) (A) and total dermatan sulfate concentration ($[DS]_0$) (B). Solid lines represent least-squares fitting of the data by the quadratic equation for binding of a single ligand [47], with the fitted parameters given in the text. Binding experiments were performed and analyzed as described under Materials and methods.

Fig. 5. Inhibitory activity of recombinant, native, and deglycosylated HCII in the absence of GAGs. Thrombin activity, expressed as a fraction of the initial concentration ($[T]_t/[T]_0$), is shown as a function of time, for reactions of 402 nM recombinant HCII (■), 500 nM plasma HCII (●), and 538 nM deglycosylated HCII (○) with 50 nM thrombin, in the absence of GAGs. Solid lines represent the fit of the data by Eq. (3). Experiments were performed and analyzed as described under Materials and methods.

Fig. 6.

Heparin and DS concentration dependencies of k_{obs} . The first-order inactivation rate constant (k_{obs}), measured in the presence of 120 μM Chromozym TH, as a function of total heparin (A) or DS (B) concentration. Recombinant ($\blacksquare$), plasma ($\bullet$), and deglycosylated ($\circ$) HClI concentrations were 84, 107, and 103 nM, respectively, and thrombin concentrations ranged from 0.6 to 2.7 nM. Solid lines represent global least-squares fitting by Eq. (5), with the fitted parameters given in the text. Experiments were performed and analyzed as described under Materials and methods.

Fig. 7.

HCII concentration dependencies of k_{obs} . The first-order inactivation rate constant (k_{obs}), measured in the presence of 120 μM Chromozym TH, as a function of total recombinant (■), plasma (●), or deglycosylated (○) HCII concentration ($[\text{HCII}]_0$), in the presence of 1.66 μM heparin (A) or 3.7 μM DS (B). Thrombin concentrations ranged from 0.6 to 6.9 nM. Solid lines represent global least-squares fitting by Eq. (5), with the fitted parameters given in the text. Experiments were performed and analyzed as described under Materials and methods.

Table 1

Silent mutations of rare codons in the HCII cDNA.

Position	Primer sequence	Rare codon
9	5'GAT CAG CTG GAG AAA GGC GGG GAA ACT GCT CAG 3'	CTA
21	5' GCT CAG TCT GCA GAT CCG CAG TGG GAG CAG TTA 3'	CCC
66	5' CTG GAC CTG GAG AAG ATC TTC AGT GAA GAC GAC 3'	ATA
120	5' GCT TTC AAC CTC TAC CGT GTG CTG AAA GAC CAG 3'	CGA
134/136	5' TTC GAT AAC ATC TTC ATC GCA CCG GTC GGC ATT TCT ACT 3'	ATA, CCC
192/193	5' ACT CAT CGC CTC TTC CGC CGC AAT TTT GGG TAC 3'	AGG, AGG
218/222	5'ATC CTG CTT GAC TTC CGC ACT AAA GTA CGC GAG TAT TAC TTT GCT 3'	AGA, AGA
231	5' TTT GCT GAG GCC CAG ATC GCT GAC TTC TCA GAC CCT GCC TTC ATA TCA 3'	ATA
240	5' TCA GAC CCT GCC TTC ATC TCA AAA ACC AAC AAC 3'	ATA
255/262	5' GGC CTC ATC AAA GAT GCT CTG GAG AAT ATC GAC CCT GCT ACC CAG 3'	ATA, ATA
299	5' TTC CGG CTG AAT GAG CGT GAG GTA GTT AAG GTT 3'	AGA
337	5' GGG GGC ATC AGC ATG CTG ATT GTG GTC CCA CAC 3'	CTA
356	5' GAA GCG CAA CTG ACA CCG CGG GTG GTG GAG 3'	CCC
361/369/371	5' GTG GTG GAG CGC TGG CAA AAA AGC ATG ACA AAC CGC ACT CGC GAA GTG CTT CTG CCG 3'	AGA, AGA, CGA
386	5' GAG AAG AAC TAC AAT CTG GTG GAG TCC CTG AAG 3'	CTA
396	5' AAG TTG ATG GGG ATC CGT ATG CTG TTT GAC AAA 3'	AGG
412	5' GGC ATC TCA GAC CAA CGT ATC GCC ATC GAC CTG 3'	AGG
455	5' TTC ACT GTC GAC CGC CCG TTT CTT TTC CTC ATC 3'	CCC
473/477/479	5'C TTC ATG GGA CGT GTG GCC AAC CCG AGC CGT TCC TAG AGG TGG 3'	AGA, CCC, AGG

Forward primer sequences, with rare codons corrected at the positions in boldface. Original rare codon sequences are listed in the third column.

Table 2

Silent mutations of SD-like sequences in the HCII cDNA.

Position	Primer sequence	Silent mutation
9/12	5' GAT CAG CTG GAG AAA GGC GGG GAA ACT GCT CAG 3'	CTA, GGA
42	5' CCT GCC GAC TTC CAC AAA GAA AAC ACC GTC ACC 3'	AAG
55/56	5' TGG ATT CCA GAG GGG GAA GAA GAC GAC GAC TAT CTG 3'	GAG, GAG
192/193	5' ACT CAT CGC CTC TTC CGC CGC AAT TTT GGG TAC 3'	AGG, AGG
310	5' TCC ATG ATG CAG ACC AAA GGG AAC TTC CTC GCA 3'	AAG

Forward primer sequences, with silent mutations in the Shine–Dalgarno-like sequences introduced at the positions in boldface. Original sequences are listed in the third column.

Table 3

Equilibrium binding and kinetic parameters for GAG-accelerated thrombin inactivation by HCII.

	Recombinant HCII	Deglycosylated HCII	Native HCII
$K_{\text{HCII,H}}$	$6 \pm 1 \mu\text{M}^{\text{a}}$	$7 \pm 1 \mu\text{M}^{\text{a}}$	$40 \pm 4 \mu\text{M}^{\text{a}}$
	$5 \pm 2 \mu\text{M}^{\text{b}}$	$13 \pm 5 \mu\text{M}^{\text{b}}$	$60 \pm 10 \mu\text{M}^{\text{b}}$
$K_{\text{HCII,DS}}$	$4 \pm 1 \mu\text{M}^{\text{a}}$	$4 \pm 1 \mu\text{M}^{\text{a}}$	$15 \pm 4 \mu\text{M}^{\text{a}}$
	$8 \pm 5 \mu\text{M}^{\text{b}}$	$12 \pm 5 \mu\text{M}^{\text{b}}$	$40 \pm 10 \mu\text{M}^{\text{b}}$
$K_{\text{T,H}}$	$0.4 \pm 0.3 \mu\text{M}$	$0.8 \pm 0.2 \mu\text{M}$	$1.0 \pm 0.5 \mu\text{M}$
$K_{\text{T,DS}}$	$0.6 \pm 0.3 \mu\text{M}$	$1.0 \pm 0.4 \mu\text{M}$	$1.0 \pm 0.3 \mu\text{M}$
$K_{\text{TH,HCII}}$	$0.010 \pm 0.004 \mu\text{M}$	$0.018 \pm 0.003 \mu\text{M}$	$0.030 \pm 0.010 \mu\text{M}$
$K_{\text{TDS,HCII}}$	$0.020 \pm 0.006 \mu\text{M}$	$0.020 \pm 0.003 \mu\text{M}$	$0.022 \pm 0.002 \mu\text{M}$
$k_{\text{lim,H}}$	$0.11 \pm 0.01 \text{ s}^{-1}$	$0.24 \pm 0.01 \text{ s}^{-1}$	$0.28 \pm 0.04 \text{ s}^{-1}$
$k_{\text{lim,DS}}$	$0.17 \pm 0.03 \text{ s}^{-1}$	$0.32 \pm 0.03 \text{ s}^{-1}$	$0.24 \pm 0.04 \text{ s}^{-1}$
k'_{H}	$11 \pm 4 \mu\text{M}^{-1} \text{ s}^{-1}$	$13 \pm 4 \mu\text{M}^{-1} \text{ s}^{-1}$	$9 \pm 3 \mu\text{M}^{-1} \text{ s}^{-1}$
k'_{DS}	$9 \pm 3 \mu\text{M}^{-1} \text{ s}^{-1}$	$16 \pm 3 \mu\text{M}^{-1} \text{ s}^{-1}$	$11 \pm 2 \mu\text{M}^{-1} \text{ s}^{-1}$

Dissociation constants for heparin (H) and dermatan sulfate (DS) binding to native, deglycosylated, and recombinant HCII were determined by equilibrium binding^a and nonlinear least-squares fitting of the inactivation rates by Eq. (3) and the mass conservation equation for HCII^b. Dissociation constants for heparin and DS binding to thrombin ($K_{\text{T,H}}$, $K_{\text{T,DS}}$), and ternary complex formation ($K_{\text{TH,HCII}}$, $K_{\text{TDS,HCII}}$), and limiting rates ($k_{\text{lim,H}}$, $k_{\text{lim,DS}}$) for thrombin inactivation were determined by kinetic analysis of the HCII and GAG concentration dependencies by Eq. (3). Second-order rate constants k'_{H} and k'_{DS} were calculated as $k_{\text{lim,H}}/K_{\text{TH,HCII}}$ and $k_{\text{lim,DS}}/K_{\text{TDS,HCII}}$, respectively. Experiments were performed and analyzed as described under Materials and methods. Errors represent ± 2 SD.