A Positively Charged Loop on the Surface of Kallistatin Functions to Enhance Tissue Kallikrein Inhibition by Acting as a Secondary Binding Site for Kallikrein*

Received for publication, June 28, 2000, and in revised form, September 12, 2000
Published, JBC Papers in Press, September 15, 2000, DOI 10.1074/jbc.M005691200

Vincent C. Chen, Lee Chao, and Julie Chao‡

From the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425

Kallistatin is a serine proteinase inhibitor (serpin) that specifically inhibits tissue kallikrein. The inhibitory activity of kallistatin is abolished upon heparin binding. The loop between the H helix and C2 sheet of kallistatin containing clusters of basic amino acid residues has been identified as a heparin-binding site. In this study, we investigated the role of the basic residues in this region in tissue kallikrein inhibition. Kallistatin mutants containing double Ala substitutions for these basic residues displayed a 70–80% reduction of association rate constants, indicating the importance of these basic residues in tissue kallikrein inhibition. A synthetic peptide derived from the sequence between the H helix and C2 sheet of kallistatin was shown to suppress the kallistatin-kallikrein interaction through competition for tissue kallikrein binding. To further evaluate the function of this loop, we used α1-antitrypsin, a non-heparin-binding serpin and slow tissue kallikrein inhibitor as a scaffold to engineer kallikrein inhibitors. An α1-antitrypsin chimera harboring the P3-P2' residues and a sequence homologous to the positively charged region between the H helix and C2 sheet of kallistatin acquired heparin-suppressed inhibitory activity toward tissue kallikrein and exhibited an inhibitory activity 20-fold higher than that of the other chimera, which contained only kallistatin's P3-P2' sequence, and 2300-fold higher than that of wild-type α1-antitrypsin. The α1-antitrypsin chimera with inhibitory characteristics similar to those of kallistatin demonstrates that the loop between the H helix and C2 sheet of kallistatin is crucial in tissue kallikrein inhibition, and this functional loop can be used as a module to enhance the inhibitory activity of a serpin toward tissue kallikrein. In conclusion, our results indicate that a positively charged loop between the H helix and C2 sheet of a serpin can accelerate the association of a serpin with tissue kallikrein by acting as a secondary binding site.

Serine proteinase inhibitors (serpins) comprise a superfamily of single-chain proteins that share a highly conserved tertiary structure. Most of the family members act as inhibitors for serine proteinases and regulate a variety of physiological processes by inhibiting their target proteinases (1). The inhibitory activity of a serpin is determined primarily by its protruding reactive center loop that docks into the reactive cleft of its target proteinase and traps the enzyme in a covalent complex (2). For some serpins, a secondary binding site remote from the reactive center loop is required for proteinase inhibition (3–8). The secondary binding sites interact with complementary sites and often promote the interaction between a serpin and its target proteinase. Another functional structure affecting the inhibitory specificity of a serpin is the heparin-binding site. For most of the heparin-binding serpins, heparin binding accelerates the association of serpins with their target proteinases (9–13). The heparin-accelerated inhibition of proteinases has been explained by a ternary complex and an allosteric model (9, 10, 14–17). For development of specific and potent inhibitors of serine proteinases, it is essential to identify the structural elements related to the inhibitory activity and understand the orchestration of these structural elements on the inhibition.

Kallistatin is a serpin that specifically inhibits human tissue kallikrein by forming a covalent enzyme-inhibitor complex (18). The high selectivity of kallistatin toward tissue kallikrein is attributed to its unique Phe-Phe pair at the P2-P1 residues in the reactive center (19). Kallistatin is also one of the heparin-binding serpins (18), which include antithrombin, protease nexin I, heparin cofactor II, plasminogen activator inhibitor, and protein C inhibitor (PCI). Unlike most heparin-binding serpins whose inhibitory activities are accelerated by heparin, the inhibitory activity of kallistatin toward tissue kallikrein is abolished upon heparin binding (18). The heparin-suppressed effect is also observed in tissue kallikrein inhibition by PCI (20). Heparin-binding sites of serpins are composed of clusters of basic residues that bind to the acidic groups of heparin (21). These sites are distributed mainly in the D helix, whereas that of PCI is in the H helix (10, 11, 22–25). Compared with other serpins, kallistatin has a distinct heparin-binding site localized in the region between the H helix and the C2 sheet (26). The heparin-binding sites of kallistatin and PCI are close to each other in primary structure. However, the major heparin-binding residues K312:K313 of kallistatin are located at the N terminus of the C2 sheet, whereas the major heparin-binding residues R269:K270 of PCI are located in the H helix. Additionally, amino acid sequence alignment of serpins shows that kallistatin has a unique insertion of 3–4 residues in the loop between the H helix and C2 sheet. A molecular model of kallistatin indicates that these extra residues may bulge the loop toward the reactive center loop (19). The basic amino acid residues K312:K313 in this loop are thus positioned in close proximity to the reactive center loop.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425. Tel.: 843-792-9927; Fax: 843-792-4850; E-mail: chaoj@musc.edu.

The abbreviations used are: PCI, protein C inhibitor; PCR, polymerase chain reaction.

* This work was supported by National Institutes of Health Grant HL 44083. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This paper is available online at http://www.jbc.org

Vol. 275, No. 51, Issue of December 22, pp. 40371–40377, 2000
Printed in U.S.A.
vicinity to the reactive site (Fig. 1). We therefore hypothesize that the positively charged region between the H helix and C2 sheet of kallistatin could have a direct involvement in the interaction with tissue kallikrein in addition to heparin binding.

In the present study, we investigated the role of the basic residues in the region between the H helix and C2 sheet of kallistatin in tissue kallikrein inhibition by three approaches. First, we evaluated the association rates of tissue kallikrein with kallistatin variants containing double Ala substitutions for these basic residues in this loop. Second, we used a synthetic peptide derived from the sequence between the H helix and C2 sheet of kallistatin to assess its capability to compete with kallistatin for tissue kallikrein binding. Finally, we created chimeric \( \alpha_1 \)-antitrypsin, which harbors the P3-P2 sequence and a sequence homologous to the region between the H helix and C2 sheet of kallistatin, to evaluate the effect of this positively charged loop on tissue kallikrein inhibition. \( \alpha_1 \)-Antitrypsin was used as a scaffold because it has neither heparin binding activity nor significant inhibitory activity toward tissue kallikrein. This study provides evidence that the positively charged loop between the H helix and C2 sheet of kallistatin acts as a secondary binding site to accelerate the interaction of kallistatin and tissue kallikrein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Escherichia coli strain TOP10 and the pTrc-His B expression vector were purchased from Invitrogen (San Diego, CA), restriction enzymes and isopropyl-1-thio-\( \beta \)-D-galactopyranoside were from Life Technologies, Inc. (Gaithersburg, MD), Taq polymerase was from PerkinElmer Life Sciences (Norwalk, CT), nickel-nitrilotriacetic acid-agarose was from Qiagen (Santa Clarita, CA), POROS® HE/1 and QE columns were from PerSeptive Biosystems (Cambridge, MA), heparin (150 units/mg) was from The Upjohn Co. (Kalamazoo, MI), and D-Val-Leu-Arg-methylcoumarinamide was from Enzyme System Products (Livermore, CA). Human tissue kallikrein was purified as described previously (27), and synthetic peptides were synthesized and provided by Dr. L. Juliano (Department de Biofisica, Escola Paulista de Medicina, Sao Paulo, Brazil).

**Construction, Expression, and Purification of Kallistatin Mutants Containing Mutations in the Region between the H Helix and C2 Sheet**—The kallistatin variants K307A/R308A and K312A/K313A were designed to contain Ala substitutions for the basic amino acid residues in the region between the H helix and C2 sheet. K307A/R308A and K312A/K313A were created, expressed, and purified by the method described previously (26).

**FIG. 1. Molecular model of human kallistatin.** The Phe\(^{388}\) (P1), Phe\(^{387}\) (P2), and Lys\(^{386}\) (P3) residues in the reactive center loop and the basic residues Arg\(^{306}\), Lys\(^{307}\), Arg\(^{308}\), Lys\(^{312}\), and Lys\(^{313}\) in the positively charged region between the H helix and C2 sheet are shown.

**FIG. 2. Effects of kallistatin synthetic peptides on the binding between tissue kallikrein and kallistatin.** The tissue kallikrein binding assays for kallistatin were performed in the presence of different concentrations of synthetic peptides. A, reactions of kallistatin competing with 6.5, 12.5, 25, 50, 100, 200, and 400 m\( \mu \)M HC2 peptide for tissue kallikrein binding. B, reactions of kallistatin with tissue kallikrein in the presence of 100, 200, 300, and 400 m\( \mu \)M C4 peptide. The synthetic peptides and the concentrations added in each tissue kallikrein binding assay are indicated on each lane. The reaction conditions were as described under “Experimental Procedures.” Cx, tissue kallikrein-kallistatin complex. HK, free \( ^{125} \)I-labeled human tissue kallikrein.

**TABLE I**

| Comparison of the association rate constants (\( k_a \)) of kallistatin P1Arg and the mutants containing mutations in the heparin-binding site for tissue kallikrein | \( k_a \) (M\(^{-2}\) s\(^{-1}\)) |
|---|---|
| Kallistatin (P1Arg) | 3.9 \( \times \) 10\(^4\) |
| K307A/R308A | 1.1 \( \times \) 10\(^4\) |
| K312A/K313A | 8.0 \( \times \) 10\(^3\) |

*According to Chen et al. (29).*

---

---
Dues) and the T7 promoter as primers and the full-length nucleic acids are underlined. The mutated residues in both α1-antitrypsin chimeras are shown in bold.

Design of Synthetic Peptides—A synthetic peptide, HC2, was designed according to the sequence between the H helix and C2 sheet spanning amino acids 300–319, RNWNLKRRNYFVKLEILHPK. The basic residues are underlined. A negative control C4 peptide derived from the surface region around the C4 sheet was synthesized, spanning amino acids 222–242, FFSSRTPTKDFYDENTTV. All synthetic peptides were dissolved in 2% Me2SO for use.

Effect of Kallistatin Synthetic Peptides on the Binding between Tissue Kallikrein and Kallistatin—125I-labeled human tissue kallikrein (1 × 10^6 cpm/20 μl) was preincubated with different concentrations of synthetic peptide at 37 °C for 10 min. The concentrations of the HC2 peptide in the assay were 12.5, 25, 50, 100, 200, and 400 μM; and the concentrations of the control peptide, C4, were 100, 200, 300, and 400 μM. The reaction mixtures were then incubated with 0.5 μg of kallistatin for 1 h. The reaction mixtures were resolved in 10% SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography.

Construction of α1-Antitrypsin Expression System—The α1-antitrypsin cDNA was synthesized from total RNA of human hepatocytes by reverse transcription-PCR with the primers 5'-ATGCCTGCTCTCTGTCG-3' and 5'-TTATTTTTGGGGTGGGATT-3'. After gel purification and phosphorylation of the PCR product, the full-length α1-antitrypsin cDNA was cloned into pBluescript. A previous report showed that deletion of 5–10 amino acids from the N terminus of α1-antitrypsin increased recombinant protein expression without affecting the inhibitory activity of α1-antitrypsin (28). Therefore, the α1-antitrypsin cDNA with an 8-codon deletion from the N terminus was synthesized by PCR using synthetic oligonucleotide 5'-AGGTGCTAGGCCAAGAGCAGATACT-3' (harboring a NheI site as indicated by the underlined residues) and the T7 promoter as primers and the full-length α1-antitrypsin cDNA as template. After the cDNA was cut with NheI and EcoRI, it was cloned into prokaryotic expression vector pTrc His linearized by the same restriction enzymes. The pTrc His vector adds a hexa-histidine sequence at the N terminus of a recombinant protein for purification by metal-affinity chromatography. The addition of a hexa-histidine sequence for purification was applied in many studies that showed no effect on the inhibitory activity of serpins (29, 30).

Construction, Expression, and Purification of α1-Antitrypsin Chimeras—Two α1-antitrypsin chimeras, denoted as α1-AT/KS and α1-AT/KS/H, were created by site-directed mutagenesis using a sequence overlap-extension PCR method. α1-AT/KS was constructed with the P3-P2 loop of kallistatin, and α1-AT/KS/H was constructed with not only the P3-P2' sequence but also with a sequence homologous to the sequence between the H helix and C2 sheet of kallikrein using α1-antitrypsin as a scaffold. To create α1-AT/KS, the α1-antitrypsin cDNA in pBluescript was used as a template, with oligonucleotides 5'-GAGG-GCCAAATCTTTCCTGACCCCCGGAG-3' and 5'-TCGGGGGGTGGTG-CAGAGAAGATTTGGCCCT-3' as internal primers (mutant bases are indicated by the underline) and oligonucleotides 5'-AGGTTGCTAGCCAGAGACGATACT-3' and the T7 promoter primer as outside primers in the PCR. The mutant cDNA synthesized by PCR is digested by NheI and EcoRI, it was cloned into prokaryotic expression vector pTrc His linearized by the same restriction enzymes. The pTrc His vector adds a hexa-histidine sequence at the N terminus of a recombinant protein for purification by metal-affinity chromatography. The addition of a hexa-histidine sequence for purification was applied in many studies that showed no effect on the inhibitory activity of serpins (29, 30).

Effect of Heparin on the Tissue Kallikrein Binding Activity of Wild-type α1-Antitrypsin and the α1-Antitrypsin Chimeras in the Absence or Presence of Heparin—The tissue kallikrein binding assay for the α1-antitrypsin chimeras, α1-AT/KS and α1-AT/KS/H, was performed after preincubation of α1-AT/KS and α1-AT/KS/H with different concentrations of heparin (0, 10, 30, and 50 units/ml). The reaction conditions were as described under “Experimental Procedures.” The tissue kallikrein activity of α1-antitrypsin chimeras and the concentrations of heparin used in the tissue-kallikrein assay are indicated for each lane. Cx, tissue kallikrein-kallistatin complex. HK, free 125I-labeled human tissue kallikrein.
K307A/R308A and K312A/K313A were markedly reduced to
of the
ka
remaining tissue kallikrein activity was measured by D-Val-Leu-Arg-
preincubated with 0, 5, 10, 20, 30, and 50 units/ml heparin in 20 mM
sodium phosphate buffer, pH 8.0, at 37°C for 10 min. The mixtures
were then incubated with 6 nM tissue kallikrein for another 90 min. The
remaining tissue kallikrein activity was measured by n-Val-Leu-Arg-
methylcoumarinamide hydrolysis.

rate constants of α1-antitrypsin chimeras (α1-AT/KS and α1-AT/KS/H)
and kallistatin mutants (K307A/K308A and K312A/K313A) were deter-
mined according to the method described previously (29).

RESULTS

Kinetic Analysis of the Inhibitory Activity of Kallistatin Mu-
nants K307A/R308A, K312A/K313A, and Kallistatin P1Arg
toward Human Tissue Kallikrein—We have localized the hepari-
phin-binding site of kallistatin in the region between the H helix
and C2 sheet by showing that both K307A/R308A and
K312A/K313A kallistatin mutants have reduced heparin binding
capacity and less sensitivity to the heparin-suppressed
effect on tissue kallikrein inhibition (26). Kallistatin mutants
K307A/R308A and K312A/K313A containing double Ala sub-
stitutions for the basic amino acid residues in the region be-
tween the H helix and C2 sheet were created using kallistatin
P1Arg as a scaffold. To investigate the importance of these
basic residues in the inhibitory activity of kallistatin toward
tissue kallikrein, we measured the association rate constants
(kₐ) of the kallistatin mutants and kallistatin P1Arg with
tissue kallikrein. As shown in Table I, the kₐ of the mutants
K307A/R308A and K312A/K313A were markedly reduced to
1.1 × 10⁴ and 8.0 × 10³ M⁻¹ s⁻¹, which were only 35% and 21% of
the kₐ (3.9 × 10⁴ M⁻¹ s⁻¹) for kallistatin P1Arg (29). Kinetic
analysis of the inhibitory activity of these mutants indicates
that the basic amino acid residues in the region between the H helix
and C2 sheet are crucial for the inhibitory activity of
kallistatin toward tissue kallikrein.

Effect of Kallistatin Synthetic Peptides on the Binding be-
tween Tissue Kallikrein and Kallistatin—To determine
whether the positively charged loop between the H helix and
C2 sheet of kallikrein can bind to tissue kallikrein, a synthetic
peptide HC2 derived from this region was used to compete with
kallikrein for tissue kallikrein binding in a kallistatin-kal-
likrein binding assay. The results showed that the HC2 peptide
interfered with the complex formation of tissue kallikrein and
kallikrein (Fig. 2). The HC2 peptide at 6.25, 12.5, 25, 50, 100,
200, and 400 μM competed with 0.5 μM kallikrein in a dose-de-
pendent manner for tissue kallikrein binding and reduced the
intensity of the band around 85 kDa representing the tissue
ekallikrein-kallikrein complex (Fig. 2A). The control peptide
C4, derived from the surface region around the C4 sheet, did
not significantly affect the formation of the kallistatin-kal-
likrein complex even at concentration as high as 400 μM (Fig.
2B). These results suggest that the region covering the H helix
and C2 sheet in kallistatin can bind to tissue kallikrein.

Construction of α1-Antitrypsin Chimeras—To further investi-
gate the role of the positively charged loop between the H helix and C2 sheet of kallistatin in tissue kallikrein inhibition,
we engineered serpin chimeras using α1-antitrypsin as a scaf-
fold. α1-Antitrypsin has no known heparin binding activity,
and it is a very slow progressive inhibitor of tissue kallikrein
with a kₐ of 7.7 M⁻¹ s⁻¹, which is more than 3 orders of
magnitude lower than that of kallistatin (31). Many studies
have shown that substitution of the reactive center residues of
a serpin changes its inhibitory specificity (32, 33). We first
constructed a α1-antitrypsin chimera α1-AT/KS containing
kallistatin’s reactive center sequence by replacing the P3-P2’
sequence of α1-antitrypsin (IPMSI) with that of kallistatin
(KFFSA) (Fig. 3). Using α1-AT/KS as a backbone, the other
chimera, α1-AT/KS/H, was engineered with a positively
charged sequence homologous to the region between the H helix
and C2 sheet of kallistatin. The region between the H helix
and C2 sheet in α1-antitrypsin contains fewer basic resi-
dues (only R305:R306) as compared with kallistatin, which
contains R306:K307:R308 and K312:K313 in this region. In
addition, α1-antitrypsin harbors many acidic residues, such as
Asp301, Glu303, and Asp304, in this region that may affect the
binding of α1-antitrypsin to tissue kallikrein, and the chimeras
constructed a LKKRFYRR sequence homologous to the he-
parin-binding sequence of kallistatin to replace EDRR in this
region (29). Kinetic analysis of α1-antitrypsin chimeras, however,
still contained contaminating proteins after processed by a nickel-affi-
inity column. The samples were further purified by anion-
exchange chromatography. The chimeras α1-AT/KS and α1-
AT/KS/H were eluted by 170–190 and 100–130 mM NaCl,
respectively. The recombinant proteins were able to reach 95%
purity.

Effects of Heparin on Tissue Kallikrein Binding Activity of
the α1-Antitrypsin Chimeras, α1-Antitrypsin, and Kallis-
tatin—To determine whether the α1-antitrypsin chimeras, α1-

| Table II |

| Comparison of the association rate constants (kₐ) of kallistatin α1-antitrypsin, and the chimeras |
|----------------|
| kₐ (M⁻¹ s⁻¹)   |
| α1-Antitrypsin  | 7.7a   |
| α1-AT/KS       | 8.7 × 10² |
| α1-AT/KS/H     | 1.8 × 10⁴ |
| Kallistatin    | 1.9 × 10⁴ |

*According to Geiger et al. (31).  
According to Chen et al. (29).
Effects of Heparin on the Inhibitory Activity of the α1-Antitrypsin Chimeras toward Tissue Kallikrein—The inhibition of tissue kallikrein by α1-antitrypsin chimeras in the presence of different concentrations of heparin was quantified by measuring residual amidolytic activities of tissue kallikrein after reaction with α1-antitrypsin chimeras. The results are shown in Fig. 6. After incubation for 3.5 h in the absence of heparin, the enzymatic activity of tissue kallikrein was completely inhibited by α1-AT/KS/H, and about 86% of the enzymatic activity was inhibited by α1-AT/KS. Preincubation with heparin at 10 units/ml suppressed the inhibitory activity of α1-AT/KS/H and restored >90% of tissue kallikrein activity. However, α1-AT/KS was not significantly affected by heparin, even at a high concentration of 50 units/ml, because it still inhibited approximately 80% of tissue kallikrein activity. The results indicate that the insertion of the homologous heparin-binding site of kallistatin between the H helix and C2 sheet confers heparin-suppressed tissue kallikrein inhibition to α1-antitrypsin chimeras.

Comparison of Association Rate Constants (ka) of Kallistatin, Wild-Type α1-Antitrypsin, and the α1-Antitrypsin Chimeras with Human Tissue Kallikrein—The ka values were determined under pseudo-first-order conditions using at least a 50-fold molar excess of inhibitors over tissue kallikrein, and the results are summarized in Table II. Wild-type α1-antitrypsin is a slow inhibitor for tissue kallikrein with a low ka of 7.7 s⁻¹ M⁻¹ (31). α1-AT/KS, with the P3-P2' sequence of kallistatin, showed a boosted ka of 8.7 × 10² M⁻¹ s⁻¹, which is 100-fold higher than that of wild-type α1-antitrypsin toward tissue kallikrein. By incorporation of the basic residues in the region between the H helix and C2 sheet, the inhibitory activity of α1-AT/KS/H was further enhanced to 1.8 × 10⁴ M⁻¹ s⁻¹, which is approximately 20-fold higher than that of α1-AT/KS and 2300-fold higher than that of wild-type α1-antitrypsin. The ka of α1-AT/KS/H is promoted to a value comparable with that of kallistatin, 1.9 × 10⁴ M⁻¹ s⁻¹ (29). These results indicate that the positively charged loop between the H helix and C2 sheet in kallistatin accelerates its association with human tissue kallikrein.

DISCUSSION

A positively charged region between the H helix and C2 sheet of kallistatin has been identified previously as a heparin-binding site that is responsible for the heparin-suppressive kallikrein inhibition (26). In the present study, we demonstrate that this positively charged loop can facilitate the inhibitory activity toward tissue kallikrein by a direct interaction with...
tissue kallikrein. This is the first report successfully converting a non-heparin-binding serpin to a heparin-regulated serpin by insertion of a heparin-binding motif. Additionally, we are able to engineer a potent tissue kallikrein inhibitor, whose inhibitory activity increases 2300-fold to a level comparable with that of kallistatin, by transferring the P3-P2′ sequence and a positively charged sequence homologous to the region between the H helix and C2 sheet (homologous heparin-binding sequence) of kallistatin into α1-antitrypsin. We have provided several lines of evidence to show that clusters of positively charged residues in the region between the H helix and C2 sheet are critical for enhancement of the inhibitory activity of kallistatin toward human tissue kallikrein. First, substitution of the basic amino acid residues with alanine in this positively charged loop resulted in a 70–80% reduction of the inhibitory activity of kallistatin toward tissue kallikrein. Second, a synthetic peptide corresponding to the sequence between the H helix and C2 sheet of kallistatin competed with kallistatin for tissue kallikrein binding. Finally, insertion of a sequence homologous to this positively charged loop of kallistatin into the region between the H helix and C2 sheet of α1-antitrypsin resulted in a 20-fold increase of the association rate with tissue kallikrein.

The reactive center loop of a serpin is a primary determinant for the inhibitory specificity toward serine proteinases. Previous studies have showed that swapping of the reactive site among serpins is insufficient to transfer the complete inhibitory property of one serpin to another, suggesting requirement of other structural elements to fully restore the inhibitory activity (33). Therefore, an efficient association between a serpin and a proteinase may rely on additional interactions to stabilize complex formation other than the reactive sites. Several lines of evidence indicated that interaction between serpins and serine proteinases, such as plasminogen activator inhibitor and plasminogen activator, heparin cofactor II and thrombin, and antithrombin III and urokinase, requires a secondary binding to promote the association (3–8). Consistent with these previous studies, our results showed that substitution of the reactive center of α1-antitrypsin with that of kallistatin increased the inhibitory activity of α1-antitrypsin toward tissue kallikrein 100-fold but did not completely transfer the inhibitory activity of kallistatin to α1-antitrypsin. The inhibitory activity was further increased >20-fold to a level comparable with that of kallistatin by incorporating clusters of basic residues between the H helix and C2 sheet, indicating a critical role of this positively charged loop in tissue kallikrein inhibition. The notion that this loop may promote the inhibition by acting as a secondary binding element is further supported by the results of a peptide competition assay. The synthetic peptide derived from the sequence between the H helix and C2 sheet of kallistatin competes for tissue kallikrein binding, suggesting a direct interaction of this region with tissue kallikrein.

The loop between the H helix and C2 sheet of kallistatin is bulged in close vicinity to the reactive center loop in the tertiary structure (26). When the reactive center loop of kallistatin docks into the reactive crevice of tissue kallikrein, the bulged...
loop between the H helix and C2 sheet of kallistatin would be in an accessible position to interact with tissue kallikrein. Tissue kallikrein is an acidic protein with a pI of 3–4, and it is highly negatively charged under neutral conditions (34). In a molecular model of human tissue kallikrein, several acidic amino acid residues were found to be distributed in loops surrounding the reactive crevice that may constitute cation-binding exosites (Fig. 7) (35). Accordingly, we speculate that the high density of positively charged residues in the loop between the H helix and C2 sheet of kallistatin may interact with these acidic amino residues in the loop surrounding the reactive crevice of tissue kallikrein. Therefore, the basic residues between the H helix and C2 sheet in a serpin could enhance tissue kallikrein inhibition by two mechanisms. First, these basic residues may direct the reactive center loop of a serpin in a suitable orientation to interact with the reactive crevice of tissue kallikrein by a secondary binding with exosites in the loop around the catalytic center. Second, these basic residues could stabilize the serpin-kallikrein complex formation by electrostatic interactions with cation-binding exosites of tissue kallikrein. The molecular model of the kallistatin-kallikrein complex predicts that Arg\textsuperscript{308}/Lys\textsuperscript{313} and Lys\textsuperscript{307}/Lys\textsuperscript{312} may have potential electrostatic interactions with Glu\textsuperscript{107} of tissue kallikrein, respectively (Fig. 8).\textsuperscript{2} Electrostatic interactions between these acidic residues of kallikrein and kallistatin need to be further investigated to support the hypothesis.

The association of kallistatin with tissue kallikrein can be blocked by heparin (18). The mechanisms by which heparin suppresses the association of kallikrein and kallistatin are not clear. Our study suggests that binding of heparin to the positively charged loop between the H helix and C2 sheet may interfere with the secondary binding of kallistatin with tissue kallikrein. In addition, heparin with negatively charged groups may expel the acidic charged amino acids of tissue kallikrein from association with kallistatin. Moreover, binding of heparin to this region may also generate steric hindrance that blocks the docking of kallistatin’s reactive loop into tissue kallikrein’s reactive center. This notion is supported by molecular modeling of a three-dimensional structure of kallistatin, which shows that the basic residues in this loop are in close vicinity to the reactive center loop (Fig. 1) (19).

Our previous study showed that kallistatin mutants K307A/R308A and K312A/K313A displayed reduced heparin binding capacity and increased resistance to heparin inhibition upon interaction with tissue kallikrein (26). In agreement with these results, the α1-antitrypsin chimera α1-AT/KS/H, which contains an insertion of the homologous heparin-binding site of kallistatin, acquires a heparin-suppressed inhibitory activity toward tissue kallikrein. The findings further confirm the conclusion that the region between the H helix and C2 sheet in kallistatin is a major heparin-binding site responsible for heparin-suppressed protease inhibition. In addition, the experiments using chimera α1-AT/KS/H demonstrated that the heparin-binding site can be used as a functional unit to convert a non-heparin-binding serpin into a heparin-regulated serpin. The same concept and approach could also be applied on heparin-binding sites in other serpins, such as the D helix in antithrombin, plasminogen activator, and heparin cofactor, to convert a non-heparin-binding serpin to a serpin with heparin-regulated activity similar to the donor.

In conclusion, we identified dual roles of the positively charged loop between the H helix and C2 sheet of kallistatin in binding to heparin and in acting as a secondary binding site for tissue kallikrein to accelerate tissue kallikrein inhibition. The success of engineering an α1-antitrypsin chimera with heparin-regulated activity and enhanced inhibitory activity toward tissue kallikrein provides evidence supporting the importance of the basic residues in the region between the H helix and C2 sheet of kallistatin in tissue kallikrein inhibition. Moreover, the studies using chimera demonstrate that the heparin-binding site or the secondary binding site of kallistatin can be used as a functional module to design inhibitors for tissue kallikrein. These findings provide useful insights for future development of inhibitors for serine proteinases.

REFERENCES

1. Potempa, J., Korzus, E., and Travis, J. (1994) J. Biol. Chem. 269, 15957–15960
2. Plootnick, M. I., Mayne, L., Schechter, N. M., and Rubin, H. (1996) Biochemistry 35, 7586–7590
3. Le Bonniec, B. F., Guinto, E. R., and Stone, S. R. (1995) Biochemistry 34, 12241–12248
4. Rogers, S. J., Pratt, C. W., Whinha, H. C., and Church, F. C. (1992) J. Biol. Chem. 267, 3615–3617
5. Madison, E. L., Goldsmith, E. J., Gething, M. J., Sambrook, J. F., and Gerard, H. D. (1990) J. Biol. Chem. 265, 21423–21426
6. Ehrlich, H. J., Gebbink, R. K., Keijer, J., Linders, M., Preissner, K. T., and Pannekoek, H. (1990) J. Biol. Chem. 265, 13029–13035
7. Lawrence, D. A., Strandberg, L., Ericson, J., and Ny, T. (1990) J. Biol. Chem. 265, 20283–20301
8. Donovan, P. M., Vaughan, P. J., and Cunningham, D. D. (1994) J. Biol. Chem. 269, 17199–17205

2 The chymotrypsinogen nomenclature is used to number amino acid residues of human tissue kallikrein (35). The residue numbering of human tissue kallikrein is indicated between brackets whenever appropriate.
A Positively Charged Loop on the Surface of Kallistatin Functions to Enhance Tissue Kallikrein Inhibition by Acting as a Secondary Binding Site for Kallikrein

Vincent C. Chen, Lee Chao and Julie Chao

*J. Biol. Chem.* 2000, 275:40371-40377.
doi: 10.1074/jbc.M005691200 originally published online September 15, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M005691200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 32 references, 20 of which can be accessed free at http://www.jbc.org/content/275/51/40371.full.html#ref-list-1