Roles of the P1, P2, and P3 Residues in Determining Inhibitory Specificity of Kallistatin toward Human Tissue Kallikrein*

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 serpin with a unique P1 Phe, which confers an excellent inhibitory specificity toward tissue kallikrein. In this study, we investigated the P3-P2-P1 residues (residues 386–388) of human kallikrein in determining inhibitory specificity toward human tissue kallikrein by site-directed mutagenesis and molecular modeling. Human kallistatin mutants with 19 different amino acid substitutions at each P1, P2, or P3 residue were created and purified to compare their kallikrein binding activity. Complex formation assay showed that P1 Arg, P1 Phe (wild type), P1 Lys, P1 Tyr, P1 Met, and P1 Leu display significant binding activity with tissue kallikrein among the P1 variants. Kinetic analysis showed the inhibitory activities of the P1 mutants toward tissue kallikrein in the order of P1 Arg > P1 Phe > P1 Lys ≥ P1 Tyr > P1 Leu ≥ P1 Met. P1 Phe displays a better selectivity for human tissue kallikrein than P1 Arg, since P1 Arg also inhibits several other serine proteinases. Heparin distinguishes the inhibitory specificity of kallistatin toward kallikrein versus chymotrypsin. For the P2 and P3 variants, the mutants with hydrophobic and bulky amino acids at P2 and basic amino acids at P3 display better binding activity with tissue kallikrein. The inhibitory activities of these mutants toward tissue kallikrein are in the order of P2 Phe (wild type) > P2 Leu > P2 Trp > P2 Met and P3 Arg > P3 Lys (wild type). Molecular modeling of the reactive center loop of kallistatin bound to the reactive crevice of tissue kallikrein indicated that the P2 residue required a long and bulky hydrophobic side chain to reach and fill the hydrophobic S2 cleft generated by Tyr215 and Trp219 of tissue kallikrein. Basic amino acids at P3 could stabilize complex formation by forming electrostatic interaction with Asp221 and hydrogen bond with Gln174 of tissue kallikrein. Our results indicate that tissue kallikrein is a specific target proteinase for kallistatin.

Kallistatin is a serpin that inhibits tissue kallikrein by forming a covalent serpin-proteinase complex (1). The inhibitory activity of kallistatin toward tissue kallikrein is abolished upon heparin binding to kallistatin (1). An unexpected finding is that kallistatin contains phenyalanine at the P1 position (2), since tissue kallikrein is known to have primary specificity for arginine and methionine at the P1 residue (3). Whether tissue kallikrein or other serine proteinase(s) is the target enzyme of kallistatin in vivo has not been determined. Kallistatin acts as a multifunctional serpin that performs various functions at different tissues. In addition to acting as a tissue kallikrein inhibitor, kallistatin is a potent vasodilator and regulator of vascular remodeling independently of its interaction with tissue kallikrein (4–6).

Tissue kallikrein is a serine proteinase that specifically cleaves low molecular weight kininogen at Met-Lys and Arg-Ser to generate the vasoactive kinin peptide (3). The physiological functions of tissue kallikrein are principally mediated by kinin, which binds to bradykinin B1 and B2 receptors. Binding of kinin to their receptors activates second messengers and mediates a road spectrum of biological effects including blood pressure reduction, muscular contraction, vascular permeability, neutrophil chemotaxis and pain, inflammatory cascades, and vascular cell growth (7). Low levels of tissue kallikrein are associated with hypertension and diabetes (8). Elevated levels of tissue kallikrein, however, are correlated with the pathogenesis of asthma, arthritis, and inflammatory articular diseases (9–11). Tissue kallikrein is also identified in colon and breast cancer cells and may be involved in malignant transformation by stimulating proliferation of tumor cells and increasing vascular permeability (12–15). Development of a specific and selective inhibitor to tissue kallikrein would be critical for studies of various biological functions of tissue kallikrein and potential therapies for certain inflammatory disorders and carcinomas.

In previous studies, the substrate and inhibitor specificity of tissue kallikrein was investigated using short synthetic peptides derived from the cleavage sequences of kininogen (16–21). Furthermore, several recent reports used extending synthetic peptides derived from the reactive center sequence of kallistatin to analyze the enzymatic specificity of tissue kallikrein (22–24). These studies showed that the P2 residue with a bulky and hydrophobic side chain, particularly Phe, is a critical determinant for tissue kallikrein specificity. Crystallographic analysis of porcine tissue kallikrein suggested that the hydrophobic phenyl side chain of Phe at P2 could be optimally accommodated into a hydrophobic crevice between Tyr215 and Trp219 of tissue kallikrein (25, 26). However, the knowledge on P3 specificity of tissue kallikrein is limited. Although some reports suggest that the P3 residue does not have much effect on tissue kallikrein specificity, the role of the P3 site has not been established (19, 20, 27). A previous study has used various serpin mutants to investigate the inhibitory specificity toward a number of serine proteinases (28). With the heparin-regulated activity and its specific inhibitory activity toward tissue kallikrein.
kallikrein, kallistatin is an ideal model to study tissue kallikrein specificity by structural and functional analysis.

In this study, we used kallistatin as a model to explore the inhibitory specificity of the P1, P2, and P3 residues toward human tissue kallikrein. We have created 57 kallistatin mutants with various amino acid substitutions at the P1, P2, or P3 position (residue 388, 387, or 386) and compared their inhibitory specificity toward human tissue kallikrein. The interaction of the P2 and P3 residues with the reactive site of human tissue kallikrein was further assessed by molecular modeling of kallistatin-tissue kallikrein complex. This study presents a comprehensive picture for the P1, P2, and P3 specificity of human tissue kallikrein. These results provide useful insights for future development of specific and potent tissue kallikrein inhibitors and for discovery of other physiological targets of kallistatin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Escherichia coli strain TOP10, the pTrc-His B expression vector, was purchased from Invitrogen (San Diego, CA); the restriction enzymes, T4 kinase, calf intestinal alkaline phosphatase, Klenow fragment, and isopropylthio-β-D-galactoside were from Life Technologies, Inc.; Taq polymerase was from PerkinElmer Life Sciences; nickel-nitrilotriacetic acid-agarose was from Qiagen (Santa Clarita, CA); the POROS® HE/1 column was from PerSeptive Biosystems (Cambridge, MA); heparin was from The Upjohn Co.; d-Val-Leu-Arg-methylcoumarinamide (MCA) and phenylmethanesulfonylfluoride were from Enzyme Systems Products (Livermore, CA); human tissue kallikrein was purified as described previously (29); anti-kallistatin monoclonal antibody was generated as described previously (30).

**Construction of the Human Kallistatin P1, P2, and P3 Variants**—A prokaryotic expression vector, pTrc His, was used to produce recombinant kallistatins. A hexahistidine sequence was added to the expression vector at the amino terminus of the recombinant human kallikrein so that the protein purification could be achieved by a metal affinity chromatography. The P1, P2, and P3 variants were constructed as described previously (31). Briefly, degenerate 5' oligonucleotides, 5'-CGATCAATTGCTCCTGGCAGA-3', 5'-CGATCAAATTCNNNTCTGCCCAGA-3', and 5'-CGATCCTGCAGA-3', containing degenerate codons at residue 388, 387, and 386, respectively, were used to generate pools of P1, P2, and P3 mutant fragments by polymerase chain reaction. The degenerate nucleotides are denoted by underlines. The mutant fragments were then cloned into the expression vector, pTrc-KS (31). The mutants with different P2 and P3 residues were identified by DNA sequencing.

**Expression and Purification of the Kallistatin Variants**—The method for the expression of the kallistatin variants were the same as described before (31) except that volume was scaled down to 25 ml of cell culture. After centrifugation at 4,000 × g for 5 min at 4 °C, cells were suspended in 1 ml of buffer containing 20 mM sodium phosphate, 0.5 mM NaCl, pH 7.8, 10 mM imidazole, 1 mM phenylmethanesulfonylfluoride, 2 mM benzamidine, 50 μl/g liter soybean trypsin inhibitor, 1 μM of leupeptin, 10 mM β-mercaptoethanol, and 0.5% Triton X-100. Cell lysates were prepared by adding 1 mg/ml lysosyme and incubated on ice for 30 min. The DNA and RNA were digested by adding 5 μg/ml of DNase I and 10 μg/ml of RNase A and incubated on ice for another 30 min. Cell lysates were centrifuged for 10 min at 15,000 × g and the supernatant was collected. To purify recombinant kallistatins, aliquots of 50% slurry of nickel-nitrilotriacetic acid-agarose (Qiagen) were added into each tube and mixed gently for 30 min at 4 °C. The supernatants were removed after centrifugation at 15,000 × g for 10 s. The resins were washed four times using 500 μl of wash buffer (20 mM sodium phosphate, pH 6.8, 500 mM NaCl, 20 mM imidazole, and 0.2% Triton X-100) and pelleted. The recombinant proteins were eluted from the resin by 60 μl of elution buffer (20 mM sodium phosphate, pH 8.0, 160 mM imidazole). The concentration of each kallistatin variant was determined by enzyme-linked immunosorbent assay (30).

**Tissue Kallikrein-binding Assay**—The binding assay for the wild-type and mutant kallistatins toward tissue kallikrein was performed according to the method described previously (1). Briefly, about 0.5 μg of recombinant kallistatins were incubated with 20,000 cpm of 125I-labeled human tissue kallikrein in 20 μl of 20 mM sodium phosphate buffer, pH 6.8, 500 mM NaCl, 20 mM imidazole, and 0.2% Triton X-100 and pelleted. The recombinant proteins were eluted from the resin by 60 μl of elution buffer (20 mM sodium phosphate, pH 8.0, 160 mM imidazole). The concentration of each kallistatin variant was determined by enzyme-linked immunosorbent assay (30).

**Analysis of Complex Formation by SDS-PAGE**—The ability of native and recombinant kallistatins to form SDS-stable complexes with tissue kallikrein was assessed by incubating 2 μl concentrations of different kallistatins with 1 μl of 125I-labeled human tissue kallikrein in 20 μl of 20 mM sodium phosphate buffer, pH 8.0, at 37 °C for 90 min. The binding reaction was stopped by adding 3× SDS-sample buffer and boiling for 5 min. The samples were resolved in 10% SDS-PAGE and analyzed by autoradiography. The relative intensity of the complexes formed by recombinant kallistatins and tissue kallikrein was calculated by densitometric analysis of the autoradiogram using NIH Image, version 1.47. The mutants with stronger binding activity were then expressed in a large quantity and purified to apparent homogeneity for further characterization.

**Expression and Purification of the Kallistatin Variants**—The mutants displaying better tissue kallikrein binding activity were expressed in 1 liter of cell culture, and then soluble cell lysates were isolated and purified by nickel affinity and heparin affinity chromatography as described previously (31). Protein purity was assessed by Coomassie Blue staining following SDS-PAGE. Kallistatin concentrations of the mutants were determined by a specific enzyme-linked immunosorbent assay.

**Analysis of Complex Formation by SDS-PAGE**—The ability of native and recombinant kallistatins to form SDS-stable complexes with tissue kallikrein was assessed by incubating 2 μl concentrations of different kallistatins with 1 μl of the serine protease in 50 μl Tris-HCl, pH 8.0, and 0.1 mM NaCl, at 37 °C for 2 h. Reactions were quenched by adding SDS sample buffer containing 100 mM dithiothreitol.
P1, P2, and P3 Specificity of Kallistatin

A

**P2 Variants**

B

**P3 Variants**
itol and boiled for 2 min, and samples were analyzed by SDS-PAGE. For monitoring the complex formation of kallistatin with chymotrypsin, a time course of the reaction (2, 5, 10, 15, and 60 min) was performed under the same condition as mentioned above in the absence or presence of 120 units/ml heparin.

**Determination of Stoichiometry of Inhibition**—The stoichiometry of inhibition (SI) values for the inhibition of human tissue kallikrein or chymotrypsin were determined as described previously (31). Different concentrations of the recombinant kallistatins were incubated with 25 nM tissue kallikrein or 50 nM chymotrypsin in 50 mM Tris-HCl, pH 8.0, 0.1 mM NaCl, and 0.1% bovine serum albumin. The reaction was carried out at 37°C for a period of time sufficient to ensure that complex formation was complete (24 h for tissue kallikrein and 3 h for chymotrypsin). The residual amidolytic activity was measured by adding 20 μl of the reaction mixture into 30 μl of substrate (Val-Leu-Arg-MCA for tissue kallikrein and Ala-Ala-Pro-Phe-MCA for chymotrypsin) in 2 ml of 50 mM Tris, pH 8.0, and 0.1 NaCl. The rate of substrate hydrolysis was monitored at 380-nm excitation and 460-nm emission. The inhibition stoichiometry was obtained from the abscissa intercept of a linear regression fit of the residual enzymatic activity versus the molar ratio of inhibitor to enzyme.

**Kinetic Assays**—The association rate constants of the P1, P2, and P3 mutants to human tissue kallikrein were determined under pseudo-first order condition as described previously (31). The association rate constants (kₐ) were calculated as described previously (31). The amounts of kallistatins were employed at least a 10-fold molar excess over serine proteinases. At least five inhibitor concentrations were examined for each reaction. The effects of heparin on the association rate were also demonstrated by preincubation of the recombinant kallistatins with 20 units/ml heparin at 37°C for 5 min followed by kinetic assays. Serine proteinases, their respective substrates, and reaction buffers used in the assays were as follows: human tissue kallikrein (3 nM), rat tissue kallikrein (3 nM), and activated protein C (8 nM) with 30 μM Val-Leu-Arg-MCA in 20 mM sodium phosphate, pH 8.0; human plasma kallikrein (3.5 nM), trypsin (2 nM), and plasmin (10 units/ml) with 30 μM Pro-Phe-Arg-MCA in 20 mM sodium phosphate, pH 8.0, 0.1 mM NaCl; human thrombin (1 nM) with 30 μM Phe-Pro-Arg-MCA in 20 mM sodium phosphate, pH 8.0, 0.1 mM NaCl; bovine pancreatic chymotrypsin (2 nM) with 30 μM N-succinyl-Ala-Ala-Pro-Phe-MCA in 20 mM sodium phosphate, pH 8.0, 0.1 mM NaCl; human neutrophil cathepsin G (70 μM) with 1.0 mM Glu-Pro-Arg-p-nitroanilide in 50 mM Tris-HCl, pH 7.5, 0.1% bovine serum albumin (BSA); and human neutrophil elastase (20 μM) with 1.0 mM p-nitroanilide in 50 mM Tris-HCl, pH 7.5, 0.1% BSA.

**Molecular Modeling of Kallistatin**—The atomic coordinates of the intact serpins, α-antitrypsin (2psi.pdb), ovalbumin (1ova.pdb), anti-thrombin (1ant.pdb), and cleaved formed of protein C inhibitor (1p1p.pdb) were obtained from the Brookhaven Protein Data Bank. A molecular model was created using the homology modeling module, Composer, in the SYBYL (version 6.5; Tripos, Inc.). The topologically equivalent residues across these serpins were determined first based on sequence homology. A structural alignment of the serpins was then performed with the equivalent residues as a starting point. This alignment determined the structurally conserved regions (SCRs) as well as the average framework of the SCRs. SCRs of kallikrein were generated by using fragments of the homologos to construct the backbone of the SCRs and a rule-based procedure to generate the side chains. The structurally variable regions were constructed by using fragments from known structures that are compatible with the rest of the model and then using sequence information to postulate the best single fragment to use in the final model. Since the reactive center loop is a highly variable region among serpins, there is no good template for modeling the reactive center loop of kallikrein. We chose the atomic coordinates of antithrombin to model the reactive center loop of kallistatin, P16–P5’, because the reactive loop of antithrombin has the same number of residues as kallistatin, and the hinge region of the reactive loop was partially inserted into the A β-sheet as predicted for serpins (32). The kallistatin model was then refined by side chain torsion relieving and energy minimization. The backbone of the whole model was constrained and then energy minimized by steepest descent until the maximum derivative was less than 5 kcal/(mol·Å) using a steepest-descent algorithm. Finally, conjugate gradient minimization continued until the maximum derivative was less than 0.1 kcal/(mol·Å).

**Molecular Modeling of Human Tissue Kallikrein**—The atomic coordinates of the x-ray structure of human tissue kallikrein were generously provided by Dr. Katz (26). The coordinates of the “kallikrein loop,” consisting of 14 residues from 95 [102] to 98 [115], are not available in this molecular model. The “kallikrein loop” was created by homology modeling using the program Composer, in the SYBYL (version 6.5; Tripos). The coordinates of the kallikrein loop from a theoretical molecular model of prostate-specific antigen (1pfa.pdb), obtained from the Protein Data Bank, were used as a template to model that of human tissue kallikrein. The method for generating the kallikrein loop of prostate-specific antigen was described in previous studies (33). The final model of tissue kallikrein was then refined by side chain torsion relieving and energy minimization. The backbone of the whole model was constrained and then energy-minimized by steepest descent until the maximum derivative was less than 50 kcal/(mol·Å). The constraint was then removed, and additional minimization was performed until the maximum derivative was less than 0.1 kcal/(mol·Å) using a steepest-descent algorithm. Finally, conjugate gradient minimization continued until the maximum derivative was less than 0.1 kcal/(mol·Å).

**Molecular Modeling of a Kallistatin-Tissue Kallikrein Complex**—The structures of porcine tissue kallikrein-bovine pancreatic trypsin inhibitor complex (1ka1.pdb) were used as templates to model human tissue kallikrein-kallistatin complex. Previous studies pointed out that the structures of the backbone atoms between residues P2 and P2’ of the serine proteinase inhibitors are highly conserved (34, 35). The P2-P2’ residues in bound bovine pancreatic trypsin inhibitor were, therefore, selected and changed the corresponding sequence of kallistatin. After flanking residues were deleted, the four-residue peptide was used to model the docking conformation of the reactive site. The reactive center loop of kallistatin was modeled based on the crystal structure of anti-
thrombin, whose P1 Arg residue pointed inward in an orientation not appropriate for interaction with the S1 site of serine proteinase. Therefore, the atomic coordinates of backbone and side chain structure of the computational mutated P2-P2’ peptide from bovine pancreatic trypsin inhibitor were used to force the corresponding region in the reactive loop of the kallistatin model to adopt the canonical structure of the peptide. The structure of the reactive center loop was then torsion relieved and energy minimized by the method mentioned above. After the model of human tissue kallikrein was superimposed onto the structure of porcine tissue kallikrein and energy-minimized, the reactive center loop of kallistatin was fit manually into the reactive crevice of tissue kallikrein using the docked bovine pancreatic trypsin inhibitor peptide as starting guideline. Torsion angles and steric clashes of the residues in the complex were adjusted to low energy rotamers to maximize steric fit. The complex was then energy-minimized as described previously.

**Simulated Mutagenesis of Kallistatin**—To evaluate the local interactions of mutated residues of kallistatin in a complex, the P2 or the P3 residue was computationally changed to the desired amino acids. The side chain of the mutated residue was adjusted to a low energy rotamer, and the complex was then minimized for 200 iterations of steepest descent.

**RESULTS AND DISCUSSION**

In the present study, we used intact recombinant kallistatin to investigate its reactive center loop, P1, P2, and P3 residues (residues 388, 387, and 386), toward specificity of tissue kallikrein by site-directed mutagenesis, protein engineering, and molecular modeling. The reactive center loop of a serpin can adopt an inhibitory conformation that fits optimally into the reactive pocket of its target serine proteinase. Several recent studies have used extended synthetic peptides imitating the reactive center sequences of serpins, instead of short peptide, to study the specificity of tissue kallikrein (22, 23). The limited proteolytic nature of tissue kallikrein as compared with other variants (Fig. 2A). In contrast, tissue kallikrein had the best substrate specificity for P3 Lys and the catalytic reaction may occur with any P1 amino acids that are not expelled by the S1 site.

For the P2 residue of kallistatin, the binding assay and densitometric analysis showed that the hydrophobic amino acids, Phe (wild type), Leu, Trp and Met (variants), at P2 displayed relatively stronger binding activity with tissue kallikrein as compared with other variants (Fig. 2A). In contrast, the hydrophilic and small amino acids at P2 had weaker binding activity with tissue kallikrein, varying from 2 to 80% compared with Phe, Leu, Trp, and Met. Using small synthetic peptides, Fiedler (20) also showed that Phe, Leu and Trp at P2 had better specificity for porcine tissue kallikrein. These results are consistent with the hypothesis that the hydrophobic S2 crevice created by the ring structures of Tyr<sup>91</sup>Ser<sup>117</sup> and Thr<sup>215</sup>Thr<sup>230</sup> prefer hydrophobic and bulky amino acids at P2. However, our results binding assay indicated that the other hydrophobic bulky amino acids Val and Ile displayed only about 50 and 30% of the binding activity of other hydrophobic amino acids, indicating that other structural factors also affect the specificity of the P2 residue.

For the P3 residue of kallikrein, basic amino acid Lys (wild type) and Arg exhibited the highest binding activity with tissue kallikrein among the P3 variants (Fig. 2B). Ala, Glu, Gly, His, Leu, Met, Ser, and Thr had approximately 75–80% of the binding activity of wild-type P3 Lys. Acidic amino acid residues Asp and Glu at P3 were not favorable for tissue kallikrein binding, with only 20 and 30% of the binding activity of P3 Lys. Cys, Asn, Ile, Phe, and Pro also showed low binding activity with approximately 5–25% of the binding activity of P3 Lys. It is difficult to explain various binding activities of other amino acids at P3 according to their properties. The variant P3 Pro, which has the same P3 residue as kininogen, unexpectedly displayed only 10% binding activity of P3 Lys. Consistent with our results, a previous study (24) showed that human tissue kallikrein had the best substrate specificity for P3 Lys and the

**TABLE I**

Inhibition stoichiometry for the interaction of kallistatin variants with human tissue kallikrein

<table>
<thead>
<tr>
<th>Plasma kallistatin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Wild-type kallistatin</th>
<th>P1 Arg</th>
<th>P1 Lys</th>
<th>P1 Tyr</th>
<th>P1 Met</th>
<th>P1 Leu</th>
<th>P2 Leu</th>
<th>P2 Met</th>
<th>P2 Trp</th>
<th>P3 Arg</th>
<th>P3 Pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 value</td>
<td>4.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.5</td>
<td>4.4</td>
<td>3.7</td>
<td>3.4</td>
<td>4.0</td>
<td>4.6</td>
<td>4.6</td>
<td>7.1</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

<sup>a</sup>The P3-P2-P1 residues of kallistatin are Lys-Phe-Phe.

<sup>b</sup>According to Chen et al. (31).
worst for P3 Pro among nine synthetic peptides derived from kallistatin. The basic amino acids at P3 are clearly the best for tissue kallikrein binding.

In the tissue kallikrein-binding assay, the results may not reflect accurate relative activity to wild-type kallistatin, since for some potent inhibitors, such as the wild type, P3 Arg, P2 Leu, P2 Trp, and P2 Met, their reactions with tissue kallikrein may have completed under the experimental conditions. Nevertheless, the inhibitory potency of these variants toward tissue kallikrein can be compared and estimated by the intensity of the complexes. To further characterize the kallistatin variants with higher binding activity with tissue kallikrein, P1 Arg, P1 Met, P1 Lys, P1 Tyr, P2 Leu, P2 Trp, P2 Met, P3 Arg, and wild type (P3 Lys-P2 Phe-P1 Phe) were selected and purified to apparent homogeneity in a large scale. P3 Pro was also selected for the following study, since the native substrate of tissue kallikrein contains Pro at P3.

Analysis of Complex Formation of Selected Kallistatin Variants with Human Tissue Kallikrein by SDS-PAGE—Fig. 3 showed the results of the reaction of selected kallistatin variants with human tissue kallikrein by SDS-PAGE analysis. Wild-type kallistatin and the selected variants, P1 Arg, P1 Leu, P1 Lys, P1 Met, P1 Tyr, P2 Leu, P2 Met, P2 Trp, and P3 Arg, formed 82-kDa SDS- and heat-stable complexes with human tissue kallikrein (Fig. 3A, lanes 3, 5, 7, 9, 11, and 13; Fig. 3B, lanes 4, 6, 8, 10, and 12). These recombinant kallistatins were also cleaved by tissue kallikrein and shown as lower molecular mass. P3 Pro, however, failed to form a detectable complex with tissue kallikrein and was detected as the cleaved form (Fig. 3B, lane 14). The results indicate that P1 Arg, P1 Leu, P1 Lys, P1 Met, P1 Tyr, P2 Leu, P2 Met, P2 Trp, and P3 Arg, like wild type, act as an inhibitor and a substrate for human tissue kallikrein, whereas P3 Pro acts more like a substrate.

SI—Table I summarizes the SI values of kallistatin variants with human tissue kallikrein. The SI values of selected variants with human tissue kallikrein varied from 3.4 to 4.6, except P3 Arg with a high value of 7.1. The data were consistent with the results of complex formation by SDS-PAGE analysis, indicating a role of these recombinant kallistatins as a substrate in addition to an inhibitor. The SI value of P3 Pro could not be determined under the experimental conditions, since it acts as a substrate for tissue kallikrein. According to our estimation, its SI would be over 50.

Inhibitory Activity of the Selected P1 Variants toward Different Serine Proteinases—Table II summarizes the $k_a$ of the P1 variants for different serine proteinases, including human and rat tissue kallikreins, human plasma kallikrein, thrombin, activated protein C, cathepsin G, neutrophil elastase, plasmin, trypsin, and bovine pancreatic chymotrypsin. The inhibitory activity of the kallistatin variants toward human tissue kallikrein are as the order of P1 Arg, P1 Phe, P1 Lys, P1 Tyr, P1

![Fig. 4. Time course of complex formation assay of chymotrypsin and wild-type kallistatin.](http://www.jbc.org/)
Met, and P1 Leu with $k_a$ values of $3.95 \times 10^4$, $1.6 \times 10^4$, $1.1 \times 10^4$, $8.3 \times 10^3$, $3.1 \times 10^3$, and $2.2 \times 10^3$ m$^{-1}$ s$^{-1}$, respectively. Although kallistatin P1 Arg has the best inhibitory activity toward human tissue kallikrein, it also inhibits nonspecifically several other serine proteinases including rat tissue kallikrein, thrombin, activated protein C, plasmin, trypsin, and chymotrypsin with $k_a$ values of $9.53 \times 10^4$, $2.32 \times 10^3$, $7.67 \times 10^2$, $1.87 \times 10^4$, $2.52 \times 10^3$, and $4.50 \times 10^2$ m$^{-1}$ s$^{-1}$, respectively. The inhibitory pattern of P1 Lys was the same as that of P1 Arg except that the $k_a$ values of P1 Lys with the serine proteinases were lower than those of P1 Arg variant.

The wild-type kallistatin (P1 Phe) has a better balance between the inhibitory potency and selectivity toward human tissue kallikrein than the P1 Arg variant, although P1 Phe also showed significant inhibitory activity, with a $k_a$ value of $6.5 \times 10^3$ m$^{-1}$ s$^{-1}$ toward bovine chymotrypsin. The SI value of wild-type kallistatin with chymotrypsin was around 16. Additionally, in a complex formation assay, wild-type kallistatin was digested by chymotrypsin to different extent of cleaved forms with lower molecular mass without a detectable complex on SDS-PAGE (Fig. 4). These results indicate that kallistatins act more like a substrate for chymotrypsin although they have a high $k_a$ value with chymotrypsin. Therefore, kallistatin is not an efficient inhibitor for chymotrypsin in terms of stoichiometric inhibition. The mutants, P1 Tyr, P1 Met, and P1 Leu, like wild-type kallistatin, exhibited inhibitory specificity toward chymotrypsin ($k_a$ was not determined), and they are also very selective for human tissue kallikrein, but their $k_a$ values are much lower than that of P1 Phe. The high selectivity of kallistatin for tissue kallikrein inhibition suggests that tissue kallikrein is likely to be the physiological target for kallistatin.

Effects of Heparin on the Inhibitory Activity of the P1 Variants—The effects of heparin on the inhibitory activity of kallistatin P1 variants toward different serine proteinases were summarized in Table II. Preincubation of wild-type kallistatin or the P1 variants with heparin completely abolished the inhibitory activity toward human tissue kallikrein. Heparin also entirely eliminated the inhibitory activity of P1 Arg and P1 Lys toward rat tissue kallikrein and slightly reduced the inhibitory activity toward plasma kallikrein. In contrast, heparin enhanced the inhibitory activity of wild-type kallistatin and the P1 variants toward chymotrypsin. The interaction of wild-type kallistatin and chymotrypsin increased 10-fold ($7.9 \times 10^3$ m$^{-1}$ s$^{-1}$) by heparin. In addition, heparin increased the $k_a$ values for P1 Arg and P1 Lys with thrombin about 5–10-fold and with activated protein C about 40–50-fold. Heparin has been known to enhance the inhibitory activity of most heparin-binding serpins toward their target proteinases (36, 37). The mechanisms have been explained by a ternary complex and an allosteric model (37–40). Unlike other heparin-binding serpins, the inhibitory activity of kallistatin toward tissue kallikrein is distinctively abolished by heparin. However, this unique inhibitory mechanism of heparin has not been well determined. Differential effects of heparin on the inhibitory activity of kallistatin toward tissue kallikrein versus chymotrypsin suggest that the specificity of kallistatin could be regulated by a heparin-like molecule in vivo.

Inhibitory Activity of the Selected P2 and P3 Variants toward Human Tissue Kallikrein—The $k_a$ of the P2 and P3 variants for human tissue kallikrein are summarized in Table III. The association rates of the P2 variants are in the following order: P2 Phe (wild type) > P2 Leu > P2 Trp > P2 Met. A similar order was reported in a previous study (24) comparing substrate specificity of human tissue kallikrein for Phe, Leu, and Met at P2 using synthetic peptides mimicking the reactive sequence of kallistatin. Similarly, Fiedler (20) showed that the P2 specificity of porcine tissue kallikrein was in the order of Phe > Leu > Trp > Met by comparing 10 synthetic dipeptides. Combining these data with our results, we conclude that tissue kallikrein has better specificity for P2 Phe, Leu, and Trp among 20 amino acids and that Phe is the most favorable amino acid. Kallistatin and protein C inhibitor are the only serpins known to inhibit human tissue kallikrein, and both serpins contain Phe at P2 (1, 41). Tissue kallikrein has primary specificity for Arg at P1, but among serpins with P1 Arg, only protein C inhibitor displays inhibitory activity toward human tissue kallikrein. This indicates that Phe at P2 plays a determinant role for the specificity of tissue kallikrein. The $k_a$ of the P3 Arg variant with tissue kallikrein was 2-fold higher than that of wild-type P3 Lys (Table I). Arg at P3 is apparently the most favorable amino acid for tissue kallikrein. The P3 Pro variant, with the same P3 residue as kininogen, showed a low associ-
tion rate constant less than $10^{-1} \text{s}^{-1}$. This result is consistent with a previous study (24), which showed that Pro at P3 was the worst substrate for human tissue kallikrein using synthetic peptide derived from kallistatin. Contrary to our results, Pro at P3 was considered to be accepted by tissue kallikrein according to the structure of porcine pancreatic kallikrein (25) and specificity study (20) using synthetic peptides derived from kinogen substrate. The synthetic peptide Pro-Phe-Arg derived from kinogen is, therefore, widely used as a substrate for tissue kallikrein. We speculate that P3 Pro in a short synthetic peptide and in an extended loop conformation could be presented differently in the reactive pocket of tissue kallikrein. Additionally, the P3 residue in different loop conformations would generate different residue contact with tissue kallikrein. This may explain differences in P3 specificity of tissue kallikrein using amino acid sequences based on kallistatin and kinogen.

**Modeling of Kallistatin-Tissue Kallikrein Complex**—Human tissue kallikrein was recently crystallized, but the structural coordinates of the “kallikrein loop” spanning from residue Asp95 to Glu115 could not be traced due to a disorder of this region (26). Human tissue kallikrein has a large sequence insertion in this loop as compared with porcine pancreatic kallikrein and other serine proteinases (33). In addition, the kallikrein loop of human tissue kallikrein is in an intact form (42, 43). The corresponding loop structures of other serine proteinases and kallikrein members are either not available or are in the cleaved form. A theoretical model of prostate-specific antigen was modeled with an intact kallikrein loop, and therefore it was used as a template to model human tissue kallikrein. To model the interaction of kallistatin with tissue kallikrein in a reasonable structural conformation, the P1′-P1′ carbonyl bond was adjusted to be perpendicular to the attacking reactive site Ser195 of tissue kallikrein, and the side chain of the P1 Phe was positioned toward Asp189 in the S1 pocket. A previous study suggested that the phenolic ring of P2 Phe in the substrate could optimally fit into the hydrophobic S2 pocket created by the ring structures of Tyr99 and Trp215 (25). Therefore, the side chain of P2 Phe was manually inserted into the S2 cleft by using a rotamer library. As the kallistatin model was manipulated to fit into the reactive crevice of tissue kallikrein, no steric clash was found between kallistatin and the surface loop surrounding the reactive crevice (Fig. 5).

**Correlation between Structure and Activity in Kallistatin-Tissue Kallikrein Complexes**—Molecular modeling of porcine pancreatic kallikrein and human tissue kallikrein suggests that tissue kallikrein prefers hydrophobic bulky amino acid, particularly Phe, at P2 (25, 26). In this study, we have shown that tissue kallikrein has significant specificity for the hydrophobic amino acids Phe, Leu, Trp, and Met but not for Val and Ile at P2. In the model of human tissue kallikrein, the ring structures of Tyr99 and Trp215 form a wedge-shaped hydrophobic cleft. The benzoic ring of P2 Phe can be accommodated almost parallel to the indole ring of Trp215 by the cleft and fills the hydrophobic gap. This optimal insertion generates strong hydrophobic interactions that stabilize the asso-

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**Fig. 6. Interactions of the P2 residue of kallistatin with the S2 site of human tissue kallikrein.** Different hydrophobic bulky amino acids occupying the P2 position (residue 387) are modeled to demonstrate their interactions with the S2 cleft created by Tyr99 and Trp215. The P2 residues used in the complex models are labeled at the bottom of each panel. Red residues represent tissue kallikrein, and cyan residues represent kallistatin.
cation of kallistatin with tissue kallikrein. Phe at the P2 position in our model was replaced by Leu, Trp, Met, Val, and Ile to examine how the S2 site interacts with these bulky hydrophobic residues (Fig. 6). Previous studies (20, 24) and our data showed that P2 Leu is the second best amino acid for tissue kallikrein binding. Molecular modeling demonstrated that both hydrophobic C61 and C62 groups of P2 Leu can fit into the S2 site, but they could not fill the hydrophobic cleft to produce intense hydrophobic interactions as compared with P2 Phe. Trp has a bulky double ring structure, which could be inserted into the S2 site after a suitable rotamer was adopted. However, the bulky double-ring structure is expelled by the hydrophilic active site. From the model, it is clear that a hydrophobic or small P2 residue would not fit into the hydrophobic S2 cleft and would leave an energetically unfavorable empty hydrophobic cavity at the S2 site. To generate intense hydrophobic interactions with the hydrophobic S2 cleft of tissue kallikrein, Ile has a relatively long side chain that would just reach the S2 site, but the C8 group is not as bulky as two C8 groups of Leu to have enough hydrophobic interactions with the hydrophobic cleft of tissue kallikrein. Moreover, like Val, the branched C1 group is expelled by the hydrophilic active site. From the model, it is clear that a hydrophilic or small P2 residue would not fit into the hydrophobic S2 cleft and would leave an energetically unfavorable empty hydrophobic cavity at the S2 site. To generate intense hydrophobic interactions with the hydrophobic S2 cleft, a P2 residue requires an extended side chain with a bulky hydrophobic group at the end to enter and fill the S2 site. However, a branched hydrophobic C1 group would be deleterious to the hydrophilic active pocket of tissue kallikrein.

The P3 residue is surrounded by loops 4 (residues 95 [103] to 98L [116]), 6 (residues 172 [189] to 177 [194]), and 8 (residues 216 [231] to 226 [242]) of tissue kallikrein according to our model. The residues Asp98J [114] and Glu98K [115] in loop 4 (kallikrein loop), His172 [189] and Gln174 [191] in loop 6, and Thr215 [230] and Tyr217[232] in loop 8 may have potential interactions with the P3 residue. According to our results, tissue kallikrein prefers basic amino acids such as Arg and Lys at P3. These results support a molecular model in which the acidic residue Asp98J [114] and Glu98K [115] may have electrostatic interactions with P3 Arg or P3 Lys. Moreover, the kallikrein loop (loop 4) is a very flexible structure, which allows the loop to easily adopt an optimal conformation to interact with the P3 residue. In our complex model, the basic residue at P3 is projected in close vicinity to Asp98J [114] in kallikrein (Fig. 7). Therefore, the basic residue at P3 may stabilize complex formation by generating a salt bridge with Asp98J [114]. In agreement with this model, our data also show that the acidic residues Asp and Glu at P3 have deleterious effects on tissue kallikrein binding, suggesting that the acidic residues could be expelled by Asp98J [114] and Glu98K [115] of tissue kallikrein. In addition to the electrostatic interaction with Asp98J [114], the amino group of Lys or Arg at P3 is able to form a hydrogen bond with the Oε1 group of Gln174 [191] to stabilize the binding. P3 Pro in the context of the reactive sequence of kallistatin is not favorable for tissue kallikrein binding. In our model, P3 Pro does not have any unfavorable contact with the residues surrounding it, but changing Lys to Pro bends the reactive center backbone. For long synthetic peptides or reactive loops to fit into the reactive site of tissue kallikrein, the conformation is the primary concern. We speculate that the distorted backbone damages the optimal geometry of the reactive center loop and thus impairs the interaction with the reactive site of tissue kallikrein. A short peptide substrate like Pro-Phe-Arg has a more flexible conformation, and therefore P3 Pro can be easily accommodated by the reactive site although Pro does not have a specific interaction with tissue kallikrein. The interactions of the P3 residue with Asp98J [114], Glu98K [115], His172 [189], Gln174 [191], Trp215 [230], and Tyr217 [232] of tissue kallikrein rely on the conformations of the amino acid at P3. By adjusting to suitable rotamers, we notice that different amino acids may form different hydrogen bonds with these residues or may have hydrophobic attractions to Trp215 [230] and Tyr217 [232]. On the other hand, different levels of steric hindrance and residue expulsion could occur during the interaction. Therefore, the binding activity is mainly determined by the balance among these effects. At this stage, we could not estimate the net energy of these effects on different P3 residues to explain various binding activities of other P3 variants toward tissue kallikrein. Nevertheless, the kallistatin-kallikrein complex model demonstrates the basic residue at P3 conferring the most potent binding activity with tissue kallikrein.

FIG. 7. Interactions of the P3 Lys of kallistatin with human tissue kallikrein. The residues of tissue kallikrein that may potentially interact with the P3 residue of kallistatin are shown as labeled. The potential salt bridge between P3 Lys and Asp98J and hydrogen bond between P3 Lys and Gln174 are shown as dotted lines. Red residues represent tissue kallikrein, and the cyan residue represents kallistatin.

In summary, our present study shows that P1 Phe in native kallistatin is most selective for human tissue kallikrein, while Arg at the P1 position displays a broad inhibitory spectrum toward other serine proteinases. Our results indicate that tissue kallikrein is potentially a physiological target for kallistatin, and heparin-like molecules may play a pivotal role in regulating the inhibitory specificity of kallistatin in vivo. These results also show that P2 Phe and P3 Arg/Lys confer the best specificity for tissue kallikrein. The model of kallistatin-tissue kallikrein complex demonstrates that the P2 residue requires an extended side chain with a bulky hydrophobic end to reach and fill the hydrophobic S2 cleft to stabilize the interaction. A branched hydrophobic C1 group in Val or Ile at the P2 position would be expelled by the hydrophilic active pocket of tissue.
kallikrein. The basic amino acids at P3 could stabilize tissue kallikrein binding by forming a salt bridge with Asp\(^{98}\) and a hydrogen bond with Gln\(^{174}\). Our present study and molecular model provide useful insights in designing kallikrein inhibitors for research and therapeutic applications and in predicting interactions of other residues between kallistatin and tissue kallikrein.

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Roles of the P1, P2, and P3 Residues in Determining Inhibitory Specificity of Kallistatin toward Human Tissue Kallikrein

Vincent C. Chen, Lee Chao and Julie Chao

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