

Reactive-site specificity of human kallistatin toward tissue kallikrein probed by site-directed mutagenesis

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Abstract

Kallistatin is a serine proteinase inhibitor that forms complexes with tissue kallikrein and inhibits its activity. In this study, we compared the inhibitory activity of recombinant human kallistatin and two mutants, Phe388Arg (P1) and Phe387Gly (P2), toward human tissue kallikrein. Recombinant kallistatins were expressed in *Escherichia coli* and purified to apparent homogeneity using metal-affinity and heparin-affinity chromatography. The complexes formed between recombinant kallistatins and tissue kallikrein were stable for at least 150 h. Wild-type kallistatin as well as both Phe388Arg and Phe387Gly mutants act as inhibitors and substrates to tissue kallikrein as analyzed by complex formation. Kinetic analyses showed that the inhibitory activity of Phe388Arg variant toward tissue kallikrein is two-fold higher than that of wild type (P1Phe), whereas Phe387Gly had only 7% of the inhibitory activity toward tissue kallikrein as compared to wild type. The Phe388Arg variant but not wild type inhibited plasma kallikrein's activity. These results indicate that P1Arg variant exhibits more potent inhibitory activity toward tissue kallikrein while wild type (P1Phe) is a more selective inhibitor of tissue kallikrein. The P2 phenylalanine is essential for retaining the hydrophobic environment for the interaction of kallistatin and kallikrein. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Kallistatin; Serine proteinase inhibitor; Tissue kallikrein; Site-directed mutagenesis

1. Introduction

Kallistatin is a serine proteinase inhibitor (serpin) that inhibits the activity of human tissue kallikrein by forming a covalent complex [1]. It is synthesized mainly in the liver and is rapidly secreted into the

circulation [2,3]. Kallistatin purified from human plasma is an acidic glycoprotein with a molecular mass of 58 kDa [1]. Among all mammalian serpins, kallistatin has a unique reactive center with Phe–Phe–Ser at P2–P1–P1' positions [1,2]. The amino acid sequence of mature human kallistatin shares 44–46% identity with other serpins such as human α 1-antichymotrypsin, α 1-antitrypsin and protein C inhibitor [2]. Kallistatin is a heparin-binding serpin and the inhibitory activity of kallistatin toward tissue kallikrein is regulated by heparin. Kallistatin binds strongly to tissue kallikrein but only weakly to elastase or chymotrypsin [4]. Whether tissue kallikrein or other unidentified serine proteinase(s) is the target

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enzyme of kallistatin in vivo has not been established.

Human tissue kallikrein is a serine proteinase responsible for the generation of vasoactive kinin peptide by cleavage of kininogen at two specific peptide bonds, Met–Lys and Arg–Ser [5]. In addition to kallistatin, α 1-antitrypsin and protein C inhibitor have been identified as potential endogenous tissue kallikrein inhibitors. α 1-Antitrypsin was first discovered to inhibit tissue kallikrein but the progress of binding activity was slow ($k_a = 7.7 \text{ M}^{-1} \text{ s}^{-1}$) [6]. Protein C inhibitor was subsequently shown to display inhibitory activity toward tissue kallikrein; however, it also inhibits a wide spectrum of other serine proteinases [7]. Kallistatin was identified as a specific tissue kallikrein inhibitor possessing a comparable association rate with that of protein C inhibitor [1]. It is colocalized with tissue kallikrein in various tissues, including the kidney, adrenal gland, pancreas, stomach and aorta, indicating anatomical basis of the interaction between kallistatin and tissue kallikrein in these tissues [2,8,9]. The bioavailability and clearance rate of tissue kallikrein have also been found to be modulated by kallistatin in rat circulation [10]. These findings indicated that kallistatin may regulate tissue kallikrein's activity in vivo.

Kallistatin belongs to the serpin superfamily which shares a conserved tertiary structure containing a protruding reactive-center loop, extending from P15 to P5' at the carboxyl-terminus [11]. The reactive-center loop of a serpin is highly susceptible to proteolysis since the P1 residue in the reactive site mimics the substrate of its target proteinase and thus determines the specificity of serpins. The association of the P1 residue of a serpin with an active site of its target serine proteinase initially forms a tetrahedral or an acyl-enzyme intermediate [12,13]. Following the cleavage between P1 and P1', a conformational rearrangement is initiated by insertion of a portion of the cleaved reactive center loop into β -sheet A and results in a stable conformation [14].

The inhibition of tissue kallikrein by kallistatin is an unusual event since human tissue kallikrein displays primary specificity for arginine and methionine at the P1 position of its native substrate kininogen while the P1 residue of kallistatin is phenylalanine. The hydrophobic residue P2Phe of kallistatin may serve as a determinant for the inhibitory activity of

kallistatin toward tissue kallikrein. This notion is consistent with previous studies showing that tissue kallikrein prefers a hydrophobic and bulky amino acid at the P2 position of a native or synthetic substrate [15]. In order to analyze the roles of the P1Phe and P2Phe residues in the inhibitory activity of kallistatin toward tissue kallikrein, we created mutants at both key positions by site-directed mutagenesis. Wild-type kallistatin and two variants containing mutations at P1 or P2 position of the reactive-center loop were purified and characterized with respect to their interactions with tissue kallikrein by binding and inhibitory assays. One variant, Phe388Arg which contained arginine instead of phenylalanine at P1, was created to compare the inhibitory specificity with the wild-type kallistatin (P1Phe) toward tissue kallikrein versus plasma kallikrein. The Phe387Gly variant, which had the hydrophobic P2Phe residue replaced by a small, hydrophilic Gly residue, was constructed to evaluate the significance of the hydrophobic P2 residue.

2. Materials and methods

2.1. Materials

Escherichia coli strain TOP10, the pTrc-His B expression vector were purchased from Invitrogen (San Diego, CA, USA); the restriction enzymes, T4 kinase, calf intestinal alkaline phosphatase, Klenow fragment and isopropylthio- β -galactoside (IPTG) were from BRL (Gaithersburg, MD, USA); Taq polymerase was from Perkin Elmer-Cetus (Norwalk, CT, USA); the ECL Western blotting kit was from Amersham (Buckinghamshire, UK); nickel-nitrilotriacetic acid (Ni-NTA) agarose was from Qiagen (Santa Clarita, CA, USA); POROS HE/1 column was from PerSeptive biosystems (Cambridge, MA, USA); D-Val-Leu-Arg-MCA (methylcoumarinamide), D-Pro-Phe-Arg-MCA and phenylmethylsulfonyl fluoride (PMSF) were from Enzyme System Products (Livermore, CA, USA). Human tissue kallikrein was purified as described previously [16] and human plasma kallikrein was purchased from Enzyme Research Laboratories (South Bend, IN, USA). Anti-kallistatin monoclonal antibody was generated as described previously [4].

2.2. Construction of the recombinant kallistatin expression system

The cDNA with an additional nucleotide, T, at the 5' end encoding the mature kallistatin without signal sequence, was synthesized by the polymerase chain reaction (PCR) according to the method described previously [2]. The expression vector pTrc-His B, under the direction of the *Trc* promoter and regulation of *lac* operator, was linearized by *NheI/EcoRI* and filled in by Klenow fragment. The kallistatin cDNA was dephosphorylated by calf intestinal alkaline phosphatase and cloned into the expression vector. The orientation and fidelity of the kallistatin cDNA sequence were confirmed by sequencing. The pTrc-His expression system with its own ATG translation initiation codon fuses six histidine residues to the N-terminus of the recombinant kallistatin. The hexahistidine tag was constructed for protein purification by a metal-affinity chromatography. The expression construct was denoted pTrc-KS and the expressed recombinant kallistatin was denoted rKS.

2.3. Site-directed mutagenesis of kallistatin variant

The strategy of site-directed mutagenesis for two mutants of kallistatin, Phe388Arg and Phe387Gly, was based on a previously described method [17]. All the mutant fragments were synthesized by PCR using oligonucleotides containing the desired mutations in the primers. For Phe388Arg and Phe387Gly, the 134-bp mutant fragments were generated by PCR using pTrc-KS as a template and the oligonucleotide, 5'-CTATGGTTTCGTGGGGT-3', as the 3'-end primer. Two oligonucleotides, 5'-CGATCAAATTC-CGCTCTGCCCAGA-3' and 5'-CGATCAAAGGT-TTCTCTGCCCAGA-3', were used as the 5'-end primers to prime the reactions, respectively. Both the 5'-end primers included desired mismatches (marked by underlines) and *NruI* sites at their 5' ends. The PCR fragments were phosphorylated by T4 kinase digested with *SalI* and then ligated into *NruI/SalI*-digested pTrc-KS. The mutants Phe388Arg and Phe387Gly contained mutations at the P1 and P2 positions, respectively. The constructs were verified without any mutations other than at the desired site by DNA sequencing.

2.4. Western blot analysis

The recombinant kallistatins were identified with an ECL Western blotting kit according to the manufacturer's protocol using a specific monoclonal antibody against kallistatin. Briefly, aliquots of *E. coli* crude extracts and chromatographic fractions or purified recombinant proteins were resolved on 10% SDS-polyacrylamide gels under reducing conditions. The resolved samples were then electrotransferred from gels onto Immobilon-P membranes. The membranes were then blocked at room temperature for 1 h with BLOTTO [18]. After blocking, the membranes were incubated at room temperature for 1 h with mouse anti-kallistatin monoclonal antibody, G4C10 [4], at a 1:200 dilution in BLOTTO. Then the membranes were washed 3× for 10 min each with Tris-buffered saline, pH 7.4 and incubated for 30 min with a 1:2000 dilution of horse radish peroxidase labeled anti-mouse IgG in Tris-buffered saline, pH 7.4. After five washes of 10-min duration each, the membranes were immersed in detection solution (prepared by mixing equal volumes of detection solution 1 and 2 supplied in the ECL kit) for 1 min. The membranes were exposed to Kodak X-omat film for a few seconds to 10 min at room temperature.

2.5. Expression and characterization of wild type and mutant recombinant kallistatin

E. coli strain TOP10 transformed with DNA constructs for expression of the recombinant kallistatins were grown to a density of 0.6 OD_{600 nm} at 37°C in 1 l of 2×YT medium containing 50 µg/ml of ampicillin. The protein expression was induced by the addition of 1 mM of isopropylthio-β-galactoside (IPTG) at 30°C and the cells were grown for 5 h. The cells were then harvested by centrifugation at 4000×g for 30 min at 4°C. The cells were suspended in 20 ml of buffer containing 20 mM sodium phosphate, 0.5 M NaCl, pH 7.8, 10 mM imidazole, 1 mM PMSF, 2 mM benzamidine, 50 µg/l soybean trypsin inhibitor, 1 µM of leupeptin and 0.5% Triton x-100. The cells were disrupted by passing through a French press cell (Amicon) at 110 MPa three times. The homogenate was then centrifuged at 20 000×g for 1 h to remove insoluble cell debris. Aliquots of the supernatant were removed for analyses.

2.6. Purification of wild-type and mutant recombinant kallistatins

The soluble fraction of the cell extracts was loaded onto a nickel-affinity column (Ni-NTA agarose, Qia-gen), which was equilibrated with a binding buffer containing 20 mM sodium phosphate, pH 7.8, 0.5 M NaCl and 10 mM imidazole. The column was washed by a wash buffer containing 20 mM sodium phosphate, pH 6.8, 500 mM NaCl, 20 mM imidazole and 0.2% Triton X-100. After the spectrophotometric reading at OD₂₈₀ declined to the base line, the bound recombinant proteins were eluted by elution buffer containing 20 mM sodium phosphate, pH 8.0, 500 mM NaCl and 150 mM imidazole. The kallistatin-positive fractions were combined and dialyzed against 20 mM sodium phosphate, pH 7.0, at 4°C. The processed proteins were then further purified by a heparin-affinity chromatography (HE/1 perfusion chromatography) equilibrated with 20 mM sodium phosphate in a BioCAD *SPRINT* system. The recombinant proteins were eluted with a linear gradient of 0–500 mM NaCl in 20 mM sodium phosphate buffer, pH 7.0. Kallistatin-positive fractions were concentrated and buffer-exchanged by Centriprep-10 concentrators (Amicon) with several changes of 20 mM sodium phosphate buffer, pH 8.0 and then stored at –20°C for protein assays.

2.7. Enzyme-linked immunosorbent assay (ELISA) of recombinant kallistatin

The concentrations of recombinant kallistatins were determined by an ELISA using a rabbit anti-kallistatin polyclonal antibody as previously described [4].

2.8. Determination of stoichiometry of inhibition

The stoichiometry of inhibition (SI) values for the inhibition of human tissue kallikrein were determined by incubating different concentrations of native kallistatin or recombinant kallistatins, 0, 15, 20, 25, 50, 75 and 100 nM, with 25 nM tissue kallikrein in 50 mM Tris–HCl, pH 8.0, 0.1 M 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 (about 24 h). The

residual amidolytic activity was measured by adding 20 µl of the reaction mixture into 30 µM D-Val-Leu-Arg-MCA in 2 ml of 50 mM Tris, pH 8.0 and 0.1 NaCl and monitoring the rate of substrate hydrolysis 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.

2.9. Assessment of the stability of complexes between tissue kallikrein and kallistatins

The stability of tissue kallikrein–kallistatin complexes was investigated by incubating 100 nM tissue kallikrein with 400 nM native kallistatin or recombinant kallistatins in 50 mM Tris–HCl, pH 8.0, 0.1 M NaCl and 0.1% bovine serum albumin, at 37°C for over 180 h. At different times, the residual enzymatic activity was measured by the method mentioned before.

2.10. Analysis of complex formation by SDS–PAGE

The ability of plasma and recombinant kallistatins to form SDS-stable complexes with tissue kallikrein was assessed by incubating 2 µM of different kallistatins with 1 µM tissue kallikrein in 50 mM Tris–HCl, pH 8.0 and 0.1 M NaCl, at 37°C for certain time periods as follows: plasma and wild-type recombinant kallistatin, 10 min; Phe388Arg, 2 and 10 min; Phe387Gly, 10 min and 4 h. Reactions were quenched by adding SDS sample buffer containing DTT, boiled for 2 min and analyzed by SDS–PAGE on a 10% polyacrylamide gel.

2.11. Kinetic assays

Association rate constants of the recombinant kallistatins and human tissue kallikrein were determined under pseudo-first-order condition. The inhibitory reactions of recombinant kallistatins toward human tissue kallikrein were performed at 37°C in 1 ml of reaction buffer containing 20 mM sodium phosphate, pH 8.0, 100 mM NaCl and 0.1% bovine serum albumin. An excess of inhibitors, wild-type and variant kallistatins, were incubated with 3 nM human tissue kallikrein at various enzyme/inhibitor molar ratios.

At certain incubation intervals, 100 μl of the reaction mixture was removed and added immediately into 30 μM of D-Val-Leu-Arg-MCA in 2 ml of buffer containing 50 mM Tris-HCl buffer, pH 8.0. The residual enzymatic activity of tissue kallikrein was measured at 25°C by monitoring the release of amino-methyl coumarin at the wave length of 380 nm excitation and 460 nm emission in a Perkin Elmer LS-50 luminescence spectrometer. The residual enzymatic activity was proportional to the initial velocity, which was obtained through the slope of the absorbance plotted against time. The apparent rate constant, k_{obs} , was calculated using the equation $\ln([E_t]/[E_0]) = -k_{\text{obs}}t$. $[E_0]$ represents the initial enzymatic activity of tissue kallikrein without the addition of kallistatin. $[E_t]$ represents the remaining enzymatic activities of tissue kallikrein at different intervals of incubation with kallistatin. The association rate constant was calculated using the equation $k_{\text{ass}} = k_{\text{obs}}/[I]$, where $[I]$ represents the concentration of the inhibitor in the assay mixture. For comparison of the specificity of the wild-type and Phe388Arg kallistatins toward both human tissue and plasma kallikreins, the inhibitory assay on human plasma kallikrein was performed using the same approach described above. The substrate used for plasma kallikrein was 30 μM of D-Pro-Phe-Arg-MCA.

3. Results

3.1. Expression and purification of recombinant kallistatins

Recombinant kallistatins were expressed in an *E. coli* expression system as an intracellular protein. Immunoreactive kallistatin levels were measured by ELISA. The expression level of kallistatin ranged from 2 to 10 mg l^{-1} of culture that contains about 10 g of wet cell pellet. The wild type and variant kallistatins in the supernatant of cell extracts could be identified as a single band of 46 kDa by Western blotting using a monoclonal antibody (G4C10) against kallistatin. The corresponding band was not detected in the cell extracts from transformants harboring vector DNA alone (data not shown). The results confirmed the specificity of the monoclonal antibody to histidine-tagged recombinant kallistatins.

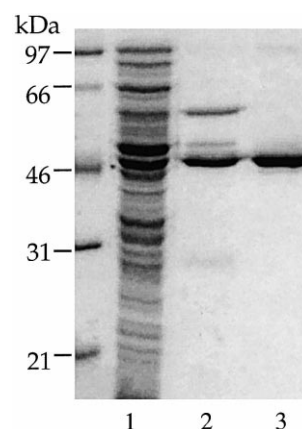


Fig. 1. SDS-PAGE of recombinant kallistatins. The purified recombinant kallistatins migrated as single bands with a molecular mass around 45 kDa stained with Coomassie blue. Lane 1, soluble cell extracts; lane 2, sample from eluate of nickel-affinity chromatography; lane 3, sample further purified by heparin-affinity chromatography.

After the cell extracts were processed by a nickel-affinity column, there were still some contaminating proteins in the eluted fractions even though a stringent wash with a buffer at lower pH was applied (Fig. 1, lane 2). Therefore, the samples were further purified on a heparin-affinity column. With a NaCl gradient, the recombinant kallistatin was eluted between 300 and 350 mM NaCl. Purified recombinant kallistatin migrated as a single band at approximately 46 kDa on SDS-PAGE (Fig. 1, lane 3). A lack of glycosylation in the prokaryotic expression system resulted in recombinant kallistatin having a lower molecular mass as compared to the 58 kDa native kallistatin. All of the purified recombinant kallistatins were further confirmed by Western blot analysis with a molecular mass of 46 kDa (Fig. 2).

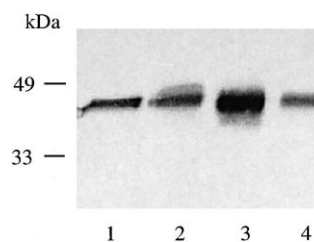


Fig. 2. Western blot analysis of recombinant kallistatins. The purified recombinant kallistatins were probed by a specific monoclonal anti-kallistatin antibody. Lane 1 is the crude protein extract from *E. coli* transformants. Lane 2, wild-type kallistatins; lane 3, Phe388Arg and lane 4, Phe387Gly.

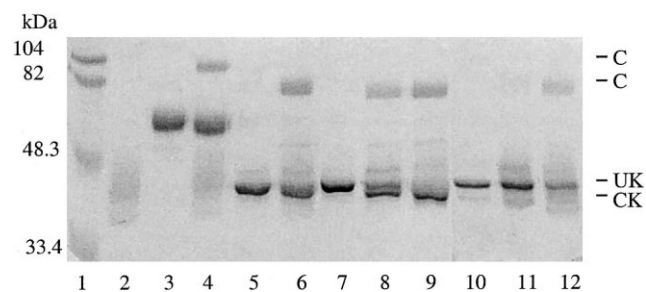


Fig. 3. Complex formation of tissue kallikrein with recombinant kallistatins. The reactions of various recombinant kallistatin (2 μM) with human tissue kallikrein (1 μM), carried out at 37°C, were analyzed by SDS-PAGE. Lane 1, molecular mass standard; lane 2, human tissue kallikrein; lanes 3, native kallistatin alone; lane 4, native kallistatin incubated with human tissue kallikrein for 10 min; lane 5, recombinant wild-type kallistatin alone; lane 6, recombinant wild-type kallistatin incubated with human tissue kallikrein for 10 min; lane 7, Phe388Arg alone; lane 8, Phe388Arg incubated with human tissue kallikrein for 2 min; lane 9, Phe388Arg incubated with human tissue kallikrein for 10 min; lane 10, Phe387Gly alone; lane 11, Phe387Gly incubated with human tissue kallikrein for 10 min and lane 12, Phe387Gly incubated with human tissue kallikrein for 4 h. C stands for complex form, UK for uncleaved form and CK for cleaved form.

The yield of the purified recombinant kallistatins varied among the mutants from 1 to 3 mg l⁻¹ of culture.

3.2. Analysis of complex formation by SDS-PAGE

The inhibition of tissue kallikrein by kallistatin was accompanied by formation of a SDS- and heat-stable high molecular weight complex of 85 kDa. Fig. 3 shows the results of complex formation of kallistatins with tissue kallikrein identified by SDS-PAGE and stained with Coomassie blue. Complex formation was visualized following a 10 min incubation of tissue kallikrein with wild-type kallistatin (lane 4) or native kallistatin (lane 6). Upon a longer incubation wild-type kallistatin was completely converted into complexed and cleaved forms (data not shown). At 2 min incubation of Phe388Arg with tissue kallikrein, a high molecular weight complex as well as a cleaved form in a size smaller than 46 kDa were detected (lane 8). After 10 min incubation almost all of Phe388Arg was converted to either a cleaved or complexed form (lane 9). These results indicated that wild-type and Phe388Arg kallistatins act as inhibitors and substrates for tissue kallikrein. By contrast, Phe387Gly (P2) had a drastically slower

rate of complex formation than wild-type kallistatin and the Phe388Arg variant since a prominent complex was detected only after a 4 h incubation with tissue kallikrein (lane 12). These results indicate that P2 substitution with a small and hydrophilic residue reduces the binding activity of kallistatin to tissue kallikrein and suggest that phenylalanine at the P2 position is a critical determinant for tissue kallikrein to accommodate a hydrophobic and bulky residue.

3.3. Stoichiometry of inhibition (SI)

The stoichiometry of inhibition indicates the number of kallistatin molecule required to inhibit one molecule of tissue kallikrein. Fig. 4 shows the determination of SI values of the plasma and recombinant kallistatins. The SI values of the plasma, wild-type, Phe388Arg and Phe387Gly kallistatins range from 4 to 5 (Table 1). For a typical serpin-proteinase reaction, the SI value is usually near 1. The high SI value of the recombinant kallistatin is in agreement with the analysis of complex formation by SDS-PAGE, which shows that a certain population of the recombinant kallistatin is cleaved by human tissue kallikrein.

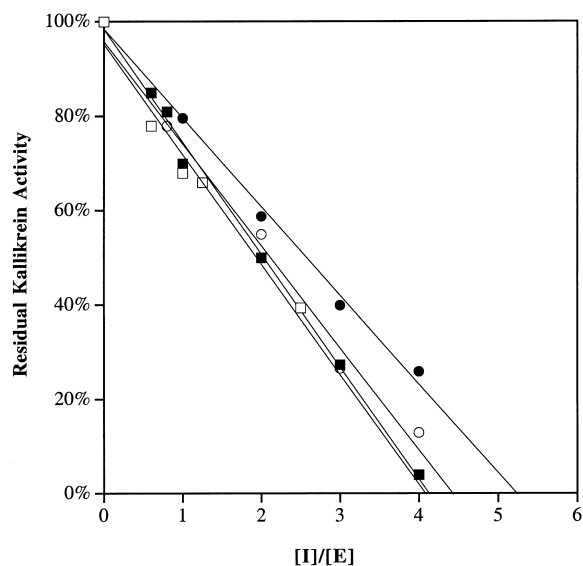


Fig. 4. Stoichiometry of inhibition of native and recombinant kallistatins with human tissue kallikrein. Human tissue kallikrein (25 nM) was incubated with different concentrations of native kallistatin (\square), recombinant kallistatin (\blacksquare), Phe388Arg (\circ) and Phe387Gly (\bullet) (15, 20, 25, 50, 75 and 100 nM). Residual enzyme activity was measured after an incubation of 24 h.

3.4. Assessment of the stability of complexes between tissue kallikrein and kallistatins

To assess the stability of the kallistatin–kallikrein complex, kallistatin was incubated with tissue kallikrein at an inhibitor/enzyme molar ratio of 4/1, since the result of stoichiometry of inhibition indicates that full inhibition of one tissue kallikrein molecule requires four kallistatin molecules. The results show that tissue kallikrein was completely inhibited by plasma and recombinant kallistatins for over 180 h (Fig. 5). According to our kinetic results, the reactions for plasma, wild-type and P1Arg kallistatins should be completed in less than 2 h and all of the tissue kallikrein should be in complexed form with kallistatin and the rest of the kallistatin should be in the cleaved form. Therefore, we are certain that free and active kallistatins are not available to re-complex with tissue kallikrein. At the same ratio Phe388Arg and Phe387Gly inhibited 75–70% of tissue kallikrein activity (Fig. 5). The partial inhibition of tissue kallikrein by the kallistatin mutants P1Arg and P2Gly is caused by high SI values of both mutants with tissue kallikrein (Table 1). Under our experimental conditions, the molar ratio of serpin over proteinase was not sufficient for mutants P1Arg and P2Gly to fully inhibit tissue kallikrein. However, the results showed that partial inhibition of tissue kallikrein was still stably maintained for at least 72 h followed by a slow restoration of tissue kallikrein activity. The complexes formed with the mutants are less stable than the ones formed with plasma and wild-type kallistatins may be explained by degradation of the complexes by free tissue kallikrein.

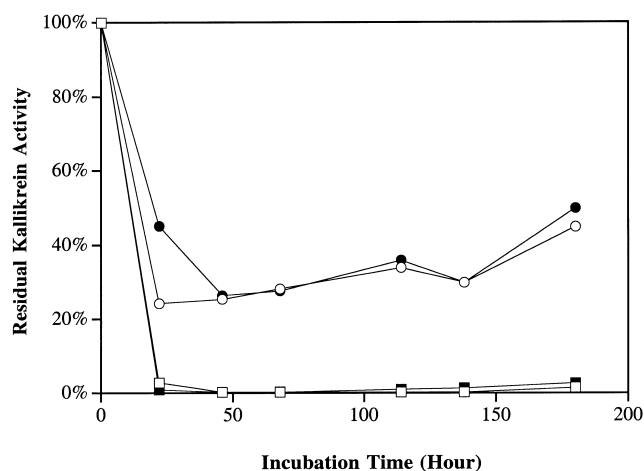


Fig. 5. Assessment of the stability of kallistatin–kallikrein complex. Stability of the complexes between tissue kallikrein and kallistatins was investigated by incubating 100 nM tissue kallikrein with 400 nM native kallistatin (□), recombinant kallistatin (■), Phe388Arg (○) or Phe387Gly (●). The residual enzymatic activity was measured at different time points for over 180 h.

3.5. Inhibitory activities of wild-type and mutant kallistatins toward tissue kallikrein

Kinetic analysis of the inhibition of human tissue kallikrein by recombinant kallistatins were performed under pseudo-first-order conditions using an excess of recombinant kallistatins at various molar ratios to tissue kallikrein. For the wild type and variant Phe388Arg (P1 mutation), the concentrations at 0.15, 0.2, 0.25, 0.3 and 0.35 μM were used and the concentrations of 0.4, 0.6, 0.8, 1.0 and 1.2 μM were used for Phe387Gly (P2 mutation). The recombinant kallistatins inhibited the activity of tissue kallikrein in a concentration- and time-dependent (0 to 15 min) manner. The pseudo-first-order constants (k_{obs}), which are proportional to the concentrations of recombinant kallistatins, were calculated from slopes

Table 1

Association rate constants (k_{ass}) and SI values for inhibition of human tissue kallikrein and plasma kallikrein by kallistatins

	Tissue kallikrein		Plasma kallikrein
	k_{ass} ($\text{M}^{-1} \text{s}^{-1}$)	SI value	k_{ass} ($\text{M}^{-1} \text{s}^{-1}$)
Native kallistatin	$(1.42 \pm 0.13) \times 10^4$	4.1	–
Recombinant kallistatin	$(1.64 \pm 0.30) \times 10^4$	4.1	–
Phe388Arg	$(3.95 \pm 0.96) \times 10^4$	4.4	$(9.60 \pm 0.84) \times 10^3$
Phe387Gly	$(1.79 \pm 0.42) \times 10^3$	5.2	ND

–: no inhibitory activity

ND: not determined

of $\ln([E_t]/[E_0])$ versus time by linear regression. The association rate constants (k_{ass}) were determined from the slope of k_{obs} versus the concentration of kallistatin plot. The k_{ass} is $1.42 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for native kallistatin, $1.64 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for recombinant kallistatin, $3.95 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for Phe388Arg and $1.79 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for the Phe387Gly (Table 1). The association rate constants of native kallistatin and recombinant wild-type kallistatin were similar, indicating that the addition of a hexahistidine sequence at the amino terminus did not affect the inhibitory activity of the recombinant kallistatin. The kinetic data show that arginine substitution for phenylalanine at P1 increases by two-fold the inhibitory activity of kallistatin toward tissue kallikrein. The replacement of the hydrophobic and bulky phenylalanine with a hydrophilic and less bulky amino acid (glycine) at P2 dramatically decreases the inhibitory activity of kallistatin, suggesting that phenylalanine is critical in maintaining a hydrophobic environment for the recognition by tissue kallikrein.

3.6. Comparison of the specificity of the wild-type and Phe388Arg kallistatins toward human tissue and plasma kallikreins

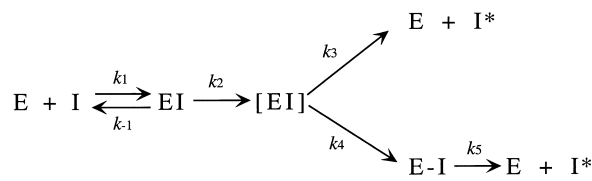
Table 1 shows the association rate constants of the wild type and Phe388Arg toward plasma kallikrein measured under pseudo-first-order conditions. The wild-type kallistatin did not show any inhibitory activity toward plasma kallikrein, while Phe388Arg, with an arginine at the P1 position, significantly inhibited the enzymatic activity of plasma kallikrein with a k_{ass} of $9.6 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (Table 1). The results show that a substitution of the phenylalanine with an arginine at the P1 position dramatically changes the inhibitory specificity of kallistatin toward plasma and tissue kallikrein. The result clearly demonstrates that P1 phenylalanine in the kallistatin molecule confers its selectivity toward human tissue kallikrein.

4. Discussion

Kallistatin has a unique P1 residue, phenylalanine, among all the mammalian serpins. It is intriguing to find that native kallistatin with a P1Phe has a superior inhibitory activity toward tissue kallikrein since

human tissue kallikrein cleaves its native kininogen substrate at Arg–Ser and Met–Lys bonds by limited proteolysis. Furthermore, unlike other heparin-binding serpins whose inhibitory activities are enhanced upon binding to heparin, the inhibitory activity of kallistatin toward tissue kallikrein is reduced by heparin [1,19–21]. Therefore, kallistatin has become an ideal model for studying the mechanisms for tissue kallikrein inhibition. In the present study, we developed a simplified scheme for the expression and purification of recombinant kallistatins that enables the analysis of the P1 and P2 specificity of kallistatin by site-directed mutagenesis. Our results showed functional significance of specific residues occupying positions P1 and P2. The interactions of tissue kallikrein with purified wild type and two kallistatin variants, Phe388Arg and Phe387Gly, were evaluated by monitoring the formation of SDS-stable complexes and kinetic analyses. Although the Phe388Arg variant showed a higher inhibitory activity toward tissue kallikrein than wild-type kallistatin, it is not a selective inhibitor for tissue kallikrein as it also inhibits plasma kallikrein activity. By contrast, wild-type kallistatin with phenylalanine at P1 is selective for human tissue kallikrein. The bulky phenylalanine residue at the P2 position appears to be critical for fitting into a hydrophobic S2 site of human tissue kallikrein. These data provided insight regarding the critical residues in the reactive-center loop of kallistatin that are vital in determining its inhibitory specificity toward tissue kallikrein.

The progress of the interaction between serine proteinase and serpin has been proposed as shown in Scheme 1 [22]. The initial binding of a serpin (I) with a serine proteinase (E) first forms a reversible Michaelis complex (EI). Following that, the complex proceeds to a tetrahedral transition which subsequently leads to a covalent acyl intermediate ([EI]) formed by a cleaved serpin and the proteinase. The cleaved serpin in this intermediate subsequently initiates a conformational change to trap the proteinase



Scheme 1.

in a stable covalent complex (E–I), or the proteinase can release the serpin as in a normal substrate reaction to yield an inactive cleaved serpin (I*) and an active enzyme (E). The stable covalent complex (E–I) eventually turns over to produce free enzyme and cleaved serpin. The tendency of a serpin to act as a substrate is estimated by its stoichiometry of inhibition (SI), the number of inhibitor molecules depleted before a complex formed. The SI value is determined by $1+(k_3/k_4)$ and for a typical serpin, k_4 is very large relative to k_3 which gives a SI close to 1. Kallistatin has a SI around 4, indicating that the k_3 of kallistatin is three times higher than k_4 . It has been suggested that the rate of insertion of the reactive-center loop into β -sheet A may determine the favorable path for the covalent intermediate [EI]. If the insertion rate is faster than the deacylation rate of [EI], k_4 will dominate whereas if the deacylation rate is faster then k_3 will dominate. The tissue kallikrein–kallistatin intermediate seems to have a high deacylation rate (k_3) and thus kallistatin behaves not only as an inhibitor but also a substrate toward tissue kallikrein. Our results also showed that the tissue kallikrein–kallistatin complex is stable for more than 150 h without significant dissociation, indicating a very low k_5 . Even though kallistatin also acts as a substrate for tissue kallikrein, once a stable complex is formed, kallistatin can effectively inhibit tissue kallikrein for several days at a molar ratio of 4 to 1.

The question whether kallistatin or other serpins is the native inhibitor of tissue kallikrein remains open. Kallistatin and protein C inhibitor are the only members exhibiting significant inhibitory activity toward tissue kallikrein among serpin family. Compared to other serpins for their target proteinases, kallistatin appears not the most favorable for tissue kallikrein inhibition because of the SI value of 4 and the considerably lower association rate than the rates for interactions of some serpins in the presence of heparin and for other heparin-independent serpins with their target enzymes. However, the rate constant and SI value determined *in vitro* may not always reflect a serpin's reactivity under physiological conditions. Previous *in vivo* studies have suggested that tissue kallikrein could be regulated by kallistatin. First of all, kallistatin is co-localized with tissue kallikrein in various tissues [2,8,9]. Second, kallistatin may reduce the clearance rate of human tissue kallikrein in rat

circulation to regulate the bio-availability of tissue kallikrein [10]. Third, in a hypotensive tissue kallikrein-transgenic mouse model, intramuscular injection of plasmid DNA containing the rat tissue kallikrein-binding protein (RKBP) gene, a functional analogue of kallistatin in rat, results in reversal of the blood pressure of the hypotensive transgenic mice [23]. Based on these findings, kallistatin may potentially play a role in regulating the physical functions of tissue kallikrein.

The results of the present study show that the P1 mutant Phe388Arg has a better inhibitory activity than that of wild-type kallistatin toward tissue kallikrein. In addition, this mutant but not wild-type kallistatin can also inhibit plasma kallikrein. Tissue kallikrein is known to have a strong preference for arginine at P1 as indicated by studies using synthetic peptides derived from the sequence of kininogen [24–26]. However, arginine is a very common cleavage residue for several proteinases. An inhibitor with P1Arg may also inhibit other serine proteinases. It has been shown that serpins with arginine at P1, such as protein C inhibitor, antithrombin III, C1 inhibitor and plasminogen activator inhibitors, have relatively broad inhibitory spectra for serine proteinases [27–29]. Kallistatin with a P1Phe residue is likely more selective for tissue kallikrein compared with the mutant with P1Arg substitution, though the inhibitory activity is slightly compromised.

Although tissue kallikrein has significant specificity for Arg at P1, only protein C inhibitor among serpins with P1Arg commits the inhibitory activity toward tissue kallikrein. This finding suggests that other residues in addition to P1 may also determine the inhibitory specificity toward tissue kallikrein. Studies using synthetic substrates have shown that human tissue kallikrein has a higher affinity toward substrates with phenylalanine at the P2 position [15]. Furthermore, analysis of the three-dimensional structure of tissue kallikrein by X-ray crystallography suggests that a hydrophobic S2 site created by Tyr99 and Trp215 may accommodate a hydrophobic and bulky P2 residue, such as phenylalanine [30]. In agreement with these studies, our results show that Gly substitution for P2Phe of kallistatin is deleterious in its inhibitory activity toward tissue kallikrein. The small amino acid residue may leave the hydrophobic S2 site of tissue kallikrein an empty hydro-

phobic space that is unfavorable for a stable binding and thus the inhibitory activity of kallistatin is significantly reduced. It should be noted that both kallistatin and protein C inhibitor, the only serpins able to inhibit tissue kallikrein, have a phenylalanine at the P2 position. Collectively, these findings support the hypothesis that the P2 residue with a bulky hydrophobic side chain is more efficient in interacting with tissue kallikrein than the one with a short hydrophilic side chain.

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